

Manual of Fish Sclerochronology

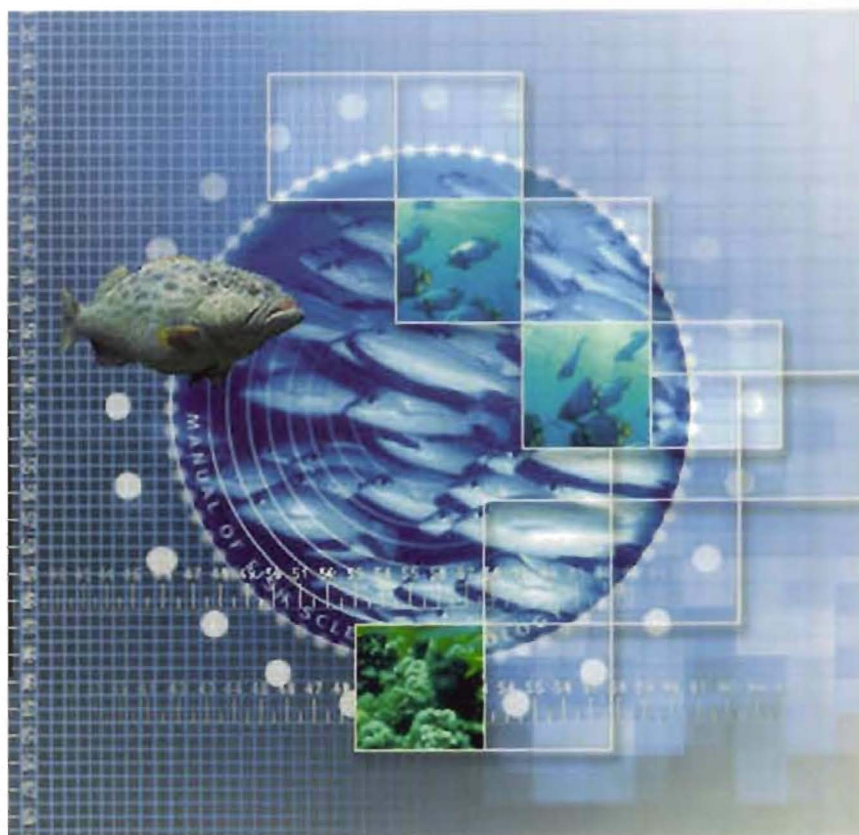
Editors

Jacques Panfili

Hélène de Pontual

Hervé Troadec

Peter J. Wright



Manual of fish sclerochronology

Editors

J. Panfili, H. de Pontual, H. Troadec†,
P.J. Wright

In homage to Hervé Troadec,
researcher at the LASAA, tragically killed
in an air crash on 29 August, 2001.



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Preface

Knowledge of the age of individual members of populations of animals greatly improves analyses of temporal variation in structure and abundance. The age structure of the population can be described, and rates of key processes, such as growth, recruitment, and mortality, can be quantified. This is particularly important when considering the dynamics of exploited populations and managing fisheries resources. In addition to the total increase in mortality rate caused by fishing, it is imperative to determine how the increased mortality is distributed among age groups in the population. Age-structured population analyses are therefore incorporated in the classical fish stock assessment models; i.e., the mathematical tools that worldwide form the preferred basis of scientific management advice on fishery resources.

Over the years, the recognised importance of age determination has made it an increasingly active field in fisheries science. Ever since scientists and technologists studying age and growth of fish first planned to meet at Smolenice, Czechoslovakia, in 1968 and then gathered five years later at Reading, England, at the First International Symposium on Age and Growth of Fish, communications and science transfer have been an important component of this field of fisheries science. Over the past three decades, regular international conferences and workshops have been convened, specific laboratory manuals prepared, and general reviews published, some of which present excellent overviews of the history and development and current standing of various techniques.

Despite the major research effort focused on interpreting age for an extended period of time, age determination of fish and, indeed, other organisms is still not a simple matter. Within international advisory bodies, notably ICES, a need is often recognised and expressed for revising or testing ageing protocols for many species. Maintaining consistency within and among interpreters and laboratories is a continuous and seemingly never-ending process. In many species, very unfortunate controversies over ageing methodology have arisen and lasted for many years, usually because several techniques for the same species have given different results and none of these procedures have been validated. Indeed, for many species, no valid age-interpretation techniques have been developed. Unfortunately, some consider that for some species "ageing is still more an art than a science". The final goal of all age-interpretation work should be to establish methods that are accurate and precise, yet practical; i.e., that can be used routinely to age sufficiently large samples.

In Europe, the European Union financially supported the concerted action "The European Fish Ageing Network" (EFAN) (1996-2000). During the four years, 99 scientists in Europe participated in the project, representing 35 different universities and research institutes from 16 European countries. EFAN aimed to develop, conduct, and coordinate collaborative research and training and thereby ensure that age determination becomes a reliable element of the assessments underlying the scientific management of fisheries and environmental resources. EFAN operated through a series of five "cells" that contain various topics: Methodologies and Procedures, Information Processing, Information Exchange and Training, Validation of Ageing, and Research and Application.

In spite of all previous efforts and increasing interest in the field, there has been no all-inclusive text covering the diverse sub-disciplines that have developed and the various calcified structures used - scales, skeletal hard structures, and otoliths. The editors and the authors, who have all been active members of EFAN, have assembled here a comprehensive manual that is one of the most detailed reviews of the subject done to date. These authors are well-known authorities in the field, have published widely, and have accumulated a wealth of practical experience in the science and technology of age and growth determination of fish. The thoroughness and diversity of the manual are apparent at a glance, particularly its combined treatment of the theoretical and the practical.

The manual addresses a broad range of topics, including the interpretation of various types of calcified structures, and details the validation of interpretation techniques, using direct, semi-direct, and indirect procedures. It includes an extensive review of the use of the data, particularly growth and growth analysis, ecological applications, and demographics. An insightful section considers computer-assisted aspects of the technology; every student who has ever delved into the technology has wished for its ultimate automation. The review of the rapidly developing and powerful field of chemical analysis will be particularly valuable to those interested in both the general and the particular. Preparation and examination techniques are detailed from a practical point of view. Useful decision trees and quick and easy reference material for everything from structure removal, storage, and archiving to reducing handling and preparation contamination are provided. For the first time, a comprehensive glossary is presented that includes terminology for all types of calcified structures. Reference material is extensive but naturally is not all-inclusive, given the mass of published literature that has accumulated.

The breadth and depth of this outstanding contribution will make it an important reference manual for years to come. The comprehensive scientific, technical, and practical information assembled here ensures that it will be a well-leafed reference manual within easy reach and equally valued on the laboratory bench and office desk of professionals researching, routinely applying, or just interested in this fundamental, important fisheries science and technology.

The reviewers

John M. Casselman
Glenora Fisheries Station
41 Fish Hatchery Lane
R.R. 4, Picton
Ontario K0K 2T0, Canada

Erlend Moksness
Institute of Marine Research
Flødevigen Marine
Research Station
N-4817 His, Norway

Foreword

Sclerochronology, the study of calcified structures to reconstruct the past history of living organisms, is central to fish biology and fisheries management. Whilst there have been many publications, symposia and reviews in this field these have all been limited in scope. This manual aims to provide an overview of the current theoretical and practical aspects of sclerochronological studies. Such a review is timely since there have been tremendous research and technological developments in this field during the past decades. By providing information on the nature of calcified structures, the uses of these structures for fish research and the methodology for preparation and examination, the manual attempts to provide a thorough guide to researchers, technicians and students new to the field as well as those interested in expanding their range of expertise.

Originally, the manual was conceived by the LASAA (Laboratoire de sclérochronologie des animaux aquatiques), a joint laboratory between the French research institutes Ifremer (Institut français de recherche pour l'exploitation de la mer) and IRD (Institut de recherche pour le développement). It was first presented in 1996 at the European Fish Ageing Network (EFAN) preparatory meeting in Ghent, Belgium. EFAN, a FAIR Programme Concerted Action sponsored by the European Commission, was established to promote exchanges, collaborative research and training in sclerochronological studies. By means of this network it was possible to expand the authorship and with it the aims and scope of the manual. This expansion and production of the project was further facilitated by a FAIR accompanying measure to EFAN, ManAgeFish, which additionally enabled the development of the manual's multimedia version and meetings between authors. Work on the manual began in 1999 and the book was reviewed during author meetings in 2000 (University of Balears, Palma de Mallorca, Spain) and 2001 (Muséum national d'histoire naturelle, Paris, France).

The manual is presented on two media, a book and a multimedia DVD-version. Each chapter is written by a group of leading workers in their field. Whilst the authors are from European institutes, their experience includes both temperate and tropical regimes as well as marine and freshwater habitats. As a consequence the manual is extensively illustrated with examples from around the world and the techniques described should be relevant to fish from all regions, and therefore to researchers working in any region or ecosystem.

Whilst the multimedia version derives from the book it greatly benefits from the addition of videos and the interactivity permitted by digital media. This DVD version is thus supplemented by (1) a set of

42 videos which illustrates the main technical procedures, (2) interactive and animated images and (3) a navigation mode based on aims and constraints (decision tree) which presents an alternative to a classical sequential reading.

The number of bibliographic references in the field of sclerochronology is enormous and as such the manual does not attempt to present an exhaustive bibliography. However, there is an extensive list of references from earlier reviews, journals and some grey literature, in order to help guide the reader. Further, a list of acronyms, an index and a glossary can be found at the end of the book, which is particularly aimed at those who are unfamiliar with the field.

The work of the editors has been greatly aided by a technical review of the structure and English by Hugh Allen (AMC, Cordoba, Spain) at the beginning of 2001. The scientific content of the manual has been reviewed by Professors John Casselman of the Canadian Ontario Ministry of Natural Resources and Erlend Moksness from the Norwegian Institute of Marine Research during the spring 2001. After accreditation by the editors, the English version has been translated in French by the French authors of the book.

The editors

Chapter I

Introduction

A. General introduction

H. de Pontual, J. Panfili, P.J. Wright, H. Troadec

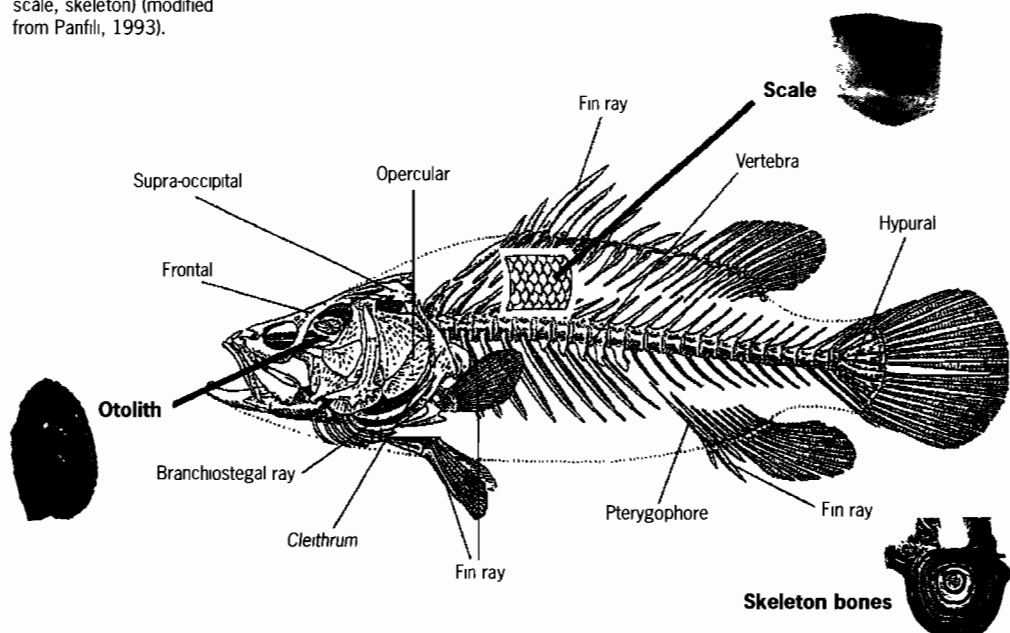
Etymologically, the term “sclerochronology” is derived from the Greek roots *sklêros* “hard”, *khronos* “time” and *logos* “reason”. Literally, this science aims to reconstruct the past history of living organisms from the study of their calcified structures (CS). Its scope covers problems of age estimation as well as the estimation of the time and duration of life history events. Its methods are based on the study of various types of signals that provide temporal references, whether structural, chemical and/or optical.

Data on the age and growth of fishes are essential for the understanding of vital traits of species and populations (e.g. lifespan, age at recruitment, age at sexual maturity, reproduction periods, migrations, mortality) and the study of population demographic structure and its dynamics (e.g. in age based stock assessment). The ecological and paleoecological applications of such data include the study of adaptive responses of populations to environmental pressures, whether natural (climatic variations) or anthropogenic (e.g. fishing, pollution, coastal zone development). Given the current poor state of many aquatic resources, the demand for reliable sclerochronological data for decision-making related to fishery management and sustainable exploitation of aquatic resources is growing.

As with the CS of some invertebrates (e.g. cephalopods, molluscs or hermatypic corals), it has long been observed that those of fish show roughly periodic structural patterns (fig. I.A.1) that are related to variations in growth rate induced by both environmental (biotic or abiotic) and endogenous factors such as ontogenic events (see Bagenal, 1974; Summerfelt & Hall, 1987). Sclerochronology has a great deal in common with dendrochronology, i.e. tree-ring science. The latter has developed, in a more easily studied environment (terrestrial environment and fixed site), an advanced methodology for the analysis of tree ring-climate relationships.

Calcified structures have the potential to grow throughout the life of the fish and act as a permanent record whose definition varies from one structure to another in relation to its specific biomineralisation processes and functional role. Three major types of mineralised parts have proved to be informative, resulting in the division of sclerochronology into three sub-disciplines (fig. I.A.1): scalimetry, which deals with scales, otolithometry, otoliths and skeletochronology, bones.

Figure I.A.1
Various calcified structures that can be utilised for sclerochronological studies, and the three main types of structure (otolith, scale, skeleton) (modified from Panfili, 1993).



What does each CS record?

As permanent recorders, CS potentially constitute more or less precise individual biological archives which need to be decoded to extract the relevant and useful information. Visual signals present various kinds of periodicity which depend on both the CS and the scale of observation. For instance, while seasonal patterns may be observed on every CS, daily increments are only observable on otoliths at high magnification. Bones and scales act to different extents as reservoirs of calcium and phosphorus salts and thus undergo resorption and remodelling processes. Such properties have to be taken into account when interpreting the information provided by these structures. Disruptions in growth patterns are frequently observed and correspond to life events

whose identity is sometimes anything but obvious. Moreover, information acquisition and interpretation are often complicated by the great plasticity of CS observable at various scales (individuals, populations, species). However, at the individual level, the interpretation of increments in CS can lead to a true chronological record of life events and growth.

What are the methods employed to reveal the information required?

Data provided by CS analysis result from an acquisition process in which methodology plays a pivotal role. Many methods of extraction, preparation and observation have been developed in the course of the 20th century, the choice of which depends fundamentally on the CS to be studied as well as on the level of information required (type and temporal precision).

Are the data obtained accurate and precise?

Data quality is a key issue in all sclerochronological studies. Age estimates that are neither accurate (i.e. close to the true value, which is essentially not known at least for wild fish) nor precise (i.e. presenting large disagreements between repeated measurements) would be of poor value for subsequent use.

Validation studies that aim to verify the presumed periodicity of a given signal are essential basis for sclerochronological studies. They are the only way to test the technology and the resulting accuracy of age estimation. It is also essential to assess data precision, i.e. variability between repeated interpretations (readings) of a given CS (either between or within readers) for revealing the most appropriate schemes for reading and interpretation. Recourse to computer vision techniques assists the reader at various levels of CS processing. Such techniques increase reading precision, provide unequalled measurement capability and allow interpretation data to be conserved.

Are the chemical patterns recorded informative and what do they reflect?

The idea that CS possess chemical tags or “chemo-prints” was recognised in the 1960s, opening another research field which is rapidly expanding thanks to the development of ever more sophisticated and sensitive analytical tools. The range of applications is potentially enormous, which explains the amount of research effort recently devoted to the field (more than 400 articles have been published during the past decade). The discipline also has to face questions of data quality,

which is particularly difficult to assess, as well as those of interpretation of complex chemical signals that are under both environmental and physiological control.

How does this work?

Despite a rather long history and development, sclerochronology is far from being an exact science and, in many cases the decoding schemes for structures and chemistry remain incomplete and debatable, especially in some species. On-going experimental work in the laboratory and mesocosms provide strong sources of support for the validation of hypotheses drawn from field data. Ultimately, however, better understanding of the biomineralisation processes and regulation mechanisms is essential to the full interpretation of signals in CS.

The literature on fish CS is enormous: for instance the ASFA (Aquatic Sciences and Fisheries Abstracts) database contains more than 2 300 references on otoliths from 1978 to 2000. Although some specific topics have been dealt with in book reviews or symposium proceedings (e.g. Bagenal, 1974; Prince & Pulos, 1983; Casselman, 1987; Summerfelt & Hall, 1987; Baglinière *et al.*, 1992; Smith, 1992; Stevenson & Campana, 1992; Secor *et al.*, 1995a; Fossum *et al.*, 2000), none of these offers an overview of the current standing of methods, practices and applications of sclerochronology for new workers in the field or other interested end-users. This book is intended as an attempt to fill this gap.

The first part of the book deals with the basis of sclerochronological studies by providing descriptions of CS, their increments and the regulation of the incremental deposition process. The rationale behind sclerochronological studies and the uses of CS are mentioned in order to guide the reader for future analysis. A detailed description of the validation process is provided, as this is one of the most important steps in sclerochronology. Some uses of the data are then described in order to outline the most important applications of CS in research and fisheries assessment. As most laboratories involved in sclerochronology are now equipped with image analysis systems with capabilities ranging from fully interactive to automated data digitalisation, the basic principles of image processing of CS are also described. The book then provides a review of otolith microchemistry, which is a rapidly developing research field in sclerochronology. Finally, detailed descriptions of the numerous techniques for preparation and observation of CS are provided in order to help the reader to progress in this field.

B. Historical

F.J. Meunier, J. Panfili

Estimates of the age of individual living organisms are needed in many types of studies. The methods developed to collect age information may be very old, as in the case of dendrochronology, i.e. estimating the age of trees from their annual growth rings. In fact, knowledge in this field has existed for many centuries, as was noted by Leonard de Vinci and by Montaigne (1580-1581) in accounts of his travels in Italy: "... the Artist... told me that all the trees show as many rings as they have years... The part of the trunk oriented towards the north is narrower, shows the tighter rings...; so whatever the wood that we show him, he prides oneself on being able to age the tree...". Meanwhile, the Swedish monk Hederström (1759) was the first to propose in an 18th century treatise that vertebral rings in fish could be counted to estimate their age. However, the first serious attempts for estimating the age of Vertebrates were not made until the end of the 19th century. Normally, the ages of animals were estimated on the basis of repeated features in a calcified organ or a hard tissue, for which reason the various methods were summarized by the term "sclerochronology". Within this field, more precise words were developed to characterize specialized approaches (scalimetry, otolithometry, skeletochronology).

Studies of cyclic patterns recorded in the CS of fishes are very numerous, and they cannot all be included here. However, a certain number of references concerning the theoretical aspects of this field of research are useful, as they highlight advances in our understanding of the growth of hard parts and their ability to record time (Bagenal, 1974; Bagenal & Tesch, 1978; Summerfelt & Hall, 1987; Baglinière *et al.*, 1992). These studies can be traced back to the beginning of the 20th century but the ideas and underlying background for this science were laid down even earlier.

We thus initially deal with works which gradually developed into the methodologies usually used today for bony fishes (Osteichthyans). We then consider the history of more recent work which has permitted the development of similar approaches in cartilaginous fishes (Chondrichthyans).

1. Osteichthyans

1.1. Scalimetry

Leeuwenhoek (1696), and then Réaumur (1716), suggested that concentric peaks (i.e. *circuli*) on the surface of a fish scale corresponded to

the various stages of growth. Kuntzman (1824), later challenged the validity of using the *circuli* as indicators of annual growth. Several authors concentrated on the relationship between adjacent *circuli* and plates (Agassiz, 1833-1844; Peters, 1841). Steenstrup (1861), followed by Baudelot (1873), showed that new *circuli* were added to the periphery of the scale throughout growth. Hoffbauer (1898, 1900) observed in carp that the growth of the scale was more significant in periods of food abundance and that, when food was scarce, the scale *circuli* were narrower. He then proposed the use of these structures as a means of obtaining the average age of fish and suggested practical applications based on carp.

In spite of some reservations (Brown, 1903; Tims, 1906), ichthyologists at the turn of the 20th century developed a method of individual age estimation in fishes starting from the variations in the arrangements of the circular peaks and other ornamentations on the surface of elasmoid scales (Walter, 1901; Thomson, 1904; Hoffbauer, 1905; Dahl, 1907; Esdaile, 1912, *inter alia*).

Johnston (1905, 1907, 1908, 1910) discovered and described the marks of reproduction ("spawning marks") on the scales of salmon and provided the foundations of the technique of back-calculation to follow the annual growth of fish. Dahl (1911) analysed the causes of the false marks and with Lea (1911), he gave the first practical applications of scale measurement (calculation of successive sizes in individual fish).

Conscious that the use of the scale in the age estimation rested on assumptions rather than on well established facts, Masterman (1913) published a critical study on salmon in which he demonstrated the necessity of proving the cogency of his method, by combining morphological, experimental and statistical observations. In fact, what he raised was the significant question of the validation of interpretative assumptions. In the experimental domain it was Winge (1915), on Gadidae, who developed the use of marked and recaptured animals, although Johnston (1907) had already practised this technique on salmon. Following these pioneer works, many practical studies based on scales were published in specialized reviews of ichthyology and fisheries.

The growth of the scale surface layer is indefinite and is closely linked, in certain areas and during clearly-defined seasons, with variations in the external medium. It is known that, during poor seasons, the interval separating the *circuli* is narrower and/or that new *circuli* are formed that do not match the first (Ombredane & Baglinière, 1992); this narrowing or discordance is very clear under transmitted light and was first named *annuli*. From the discovery of these *annuli* was born the measurement of scales: the age estimation of fish and estimates of individual growth (Dahl, 1907, 1911; Graham, 1929; Vibert & Lagler, 1961; Daget & Le Guen, 1975, *inter alia*).

Although empirical in its early stages at the beginning of the 20th century, scalimetry was capable of offering very satisfactory results on many occasions, in particular for the species of interest for fisheries. However, Walter (1901), Brown (1903), Dahl (1911) and others, showed that serious causes of error could appear with the occurrence of false marks or false *annuli*, because scales may also record various significant biological events and stresses (physiological wounds, shocks, poor summer conditions), whether periodical or not. Moreover, in certain species, the *annuli* are difficult to observe, making age estimation very delicate or impossible. Other species lack scales. Ichthyologists then turned to other CS, otoliths and/or bones, which also record the events that affect growth.

1.2. Otolithometry

Otolithometry is another method of age estimation that is very widely used in Teleost species (Stevenson & Campana, 1992). This branch of science was also developed at the end of the 19th century, starting with the work of Reibisch (1899). The otolith, a calcium carbonate concretion of the inner ear, with theoretically indefinite growth, is not strictly an element of the skeleton, but its mineral-bearing nature gives it similar properties as far as sclerochronology is concerned. Originally, as with scales, otoliths have been used to study seasonal and annual growth cycles, and there is now an abundant literature on the subject. Their observation as whole parts or after preparation is capable of providing accurate age estimations (Vibert & Lagler, 1961, *inter alia*). However, the discovery by Pannella (1971) of daily increments inside the otolith structure has opened up new fields of investigation. Specialists were enabled to reconstruct the specific stages in the life history of individuals, most of the time for larvae and juveniles. Otolith microstructure is thus a very sensitive recorder that covers a wide time scale. Nevertheless, the techniques used to reach the finest scale can be expensive and time-consuming. More recently, at the beginning of the 1980s, it was discovered that otoliths also incorporated chemical elements from their environment (biotic and abiotic) via complex physiological processes. Chemical analysis of otolith contents offered new methods of research and areas of interest, particularly in ecology and the reconstruction of life history traits. Here also the techniques are expensive and still under development. The number of studies of otolith works is already large, and symposia devoted solely to otolith research and its applications have already occurred during the past ten years (Secor *et al.*, 1995a; Fossum *et al.*, 2000). Recently, otoliths have been increasingly used for age estimation of fish and they are also valuable in other fields than the study of age and growth (Fossum *et al.*, 2000).

1.3. Skeletochronology

If the development of fish skeletochronology began a few years after the first steps in scale measurement, the first applications appeared, in fact, in the middle of the 18th century, with the work of Hederström (1759). This author, generally ignored, was shocked by the fabulous longevities usually ascribed at that time to common fish like pike (more than 200 years, see also Casselman, 1974). Having observed that the vertebrae of several species showed rings, he suggested that they could offer an index of age. The enumeration of these rings on the vertebrae of several species (pike, cod, perch, eel, bream) gave him, for the age of these animals, values close to those accepted today. On the basis of his results, Hederström created the foundations of stock management techniques, certainly awkward, but probably the first in the field of aquaculture. Published in Swedish this work remained unknown for many years and it was necessary to wait until 1904 to find studies based on bones, in connection with scales and otoliths (Heincke, 1904, 1908; Cunningham, 1905). An endorsement must be made on the work of Clerk (1927), the first modern author to develop skeletochronology. He made a comparative study of the "growth periodicity of bones" in Osteichthyans, Amphibians and Mammals. He insisted much on the fact that growth is a complex phenomenon under the control of external factors (such as the influence of the environment and the climate) as well as internal factors. He recognized that cyclic structures appear in the periostic bone and showed that certain bones, through the abundance of their periostic formations, are more suitable than others for the analysis of the cyclic growth on the one hand, and that the processes of rebuilding involves the destruction of these structures on the other.

The range of the tools for skeletochronological studies grew rapidly, from hard spines to soft fin rays, endoskeletal parts of pectoral fins, *cleithra*, supra-occipital, opercular, vertebrae. Menon (1950) drew up an exhaustive list species by species. Authors usually employ either whole parts or slices, exploiting the differences in transparency of the osseous layers deposited during the seasons: opaque zones during the growing season, translucent *annuli* during slow growth. When the *annulus* is reduced to a narrow line of annual growth cessation, a simple histological method earlier developed for Tetrapods (Klevezal & Kleinenberg, 1967; Smirina, 1974; Pascal & Castanet, 1978, *inter alia*), based on staining with haematoxyline, facilitates the observation of these chromophilic rings (Meunier *et al.*, 1979; Boët, 1981; Meunier & Pascal, 1981). The use of bones for age estimation is nevertheless limited to Osteichthyans species, in which scalimetry and otolithometry give doubtful results. Access to the skeleton is often more difficult than the simple removal of scales, or even of otoliths.

2. Chondrichthyans

The first works on sclerochronology for Chondrichthyans are much more recent (by at least a half century) than those relating to Osteichthyans. Sharks and skates lacking both an osseous skeleton and otoliths (reduced only to fine calcium carbonate granules), it was necessary to use other tissues to record skeletal growth marks. Generally speaking, specialists use the vertebral bodies, of which the cartilaginous structure is fairly well known (Ridewood, 1921; Moss, 1977; Hoening & Walsh, 1982) or spines of the dorsal fins, when these exist (Squaliformes and Heterodontiformes), and whose morphogenesis has also been studied on several occasions (Markert, 1896; Goodrich, 1907; Peyer, 1957; Holden & Meadows, 1962; Maisey, 1979). Growth marks were also described on the teeth (Tanaka, 1990) but these did not give rise to skeletochronological applications.

Since the work of Ridewood (1921), it has been known that in many Chondrichthyans, the vertebral bodies present concentric rings of calcareous cartilage separated by rings from slightly or uncalcified cartilage, giving the vertebrae different properties of transparency to light. These rings are more or less visible at the surface of the vertebral cones. This regular alternation of calcified and less calcified sectors probably corresponds to more or less regular cyclic phenomena. Haskell (1949) was the first to clearly formulate the assumption of a close connection between these vertebral rings and the growth of the animals, and who proposed to use vertebral sections to estimate age. The first effective practical use for the age estimation for a skate was published by Ishiyama (1951) and for sharks by Parker & Stott (1965). These first studies were followed by new applications, particularly in the past two decades.

A second approach to age estimation in Chondrichthyans is based on the study of the spines of the dorsal fins. However, this concerns only taxa which possess these spines (i.e. Heterodontiformes and certain Squaliformes). The spines of the dorsal fins have a structure similar to that of the teeth and cutaneous denticles. They consist of dentine organised around a long cavity and covered with enamel or emailoid (Markert, 1896; Maisey, 1979). Unlike teeth and the cutaneous denticles, which are subject to replacement, these spines are capable of growing indefinitely, with rhythmic discontinuities. If those are synchronized with a seasonal rate/rhythm they can be used for age estimation (Kaganovskaia, 1933; Bonham *et al.*, 1949; Holden & Meadows, 1962).

With the development of skeletochronological studies for Chondrichthyans, the same difficulty in automating reading techniques as for Osteichthyans arises. Some studies on vertebral bodies have been

performed. Densitometric analyses of quantitative variations in minerals, either direct by X-ray spectrometry, or indirect, on the basis of radiographs, are the most promising methods. However, this implies that the growth target criteria of the skeletal structures are sufficiently well established to be used as a support for automation (Cailliet & Tanaka, 1990).

Chapter II

Types of calcified structures

Calcified structures have different ontogenic origins and properties. Therefore, in order to appreciate the potential uses and limitations of otoliths, scales and skeletal tissue for sclerochronological studies it is important to understand the nature and formation of increments within these different CS. In this chapter, we describe the function, morphology and structure for the different CS. Further, we review the current understanding of accretion processes and how these are influenced by internal and external factors.

A. Otoliths

P. J. Wright, J. Panfili, B. Morales-Nin, A. J. Geffen

1. Description and function

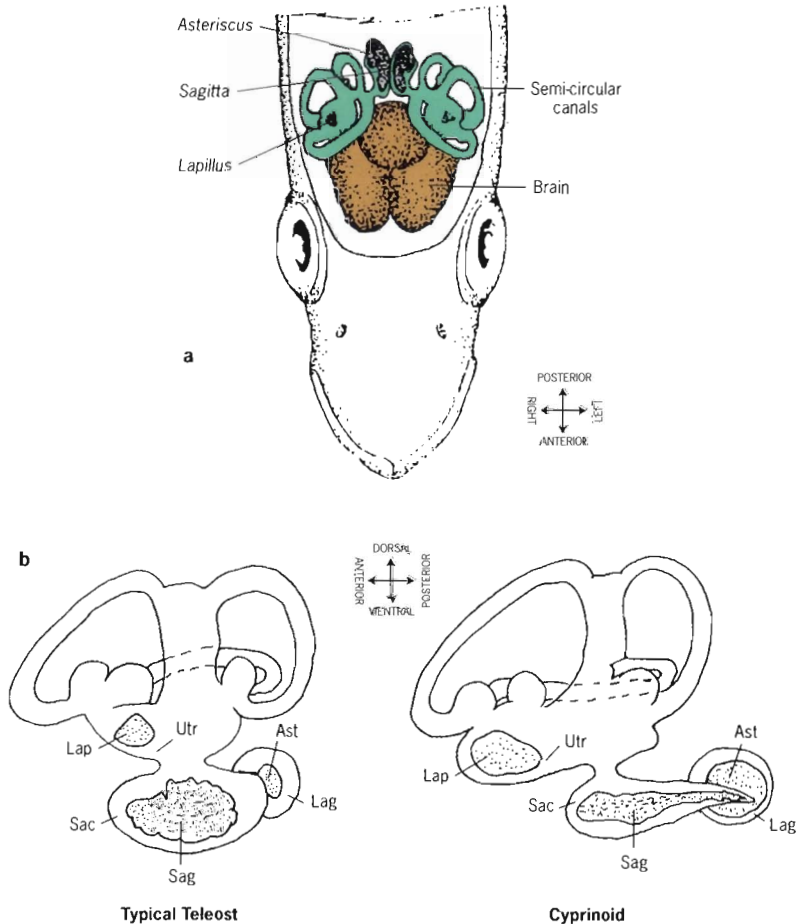
The inner ear, which is found in all jawed Vertebrates, functions both as an auditory system that detects sound waves and a vestibular system which detects linear and angular accelerations, enabling the organisms to maintain balance. In fish, the inner ear is a paired structure embedded in the cranium on either side of the head close to the midbrain. Each ear is a complicated structure of canals, sacs and ducts filled with endolymph, a fluid with special viscous properties (fig. II.A.1). The gross anatomy of these labyrinths and the structure of the labyrinthine mechanoreceptor organs are known from many fish species (Lowenstein, 1971). Teleosts have three semi-circular canals arranged orthogonally to each other, which detect angular accelerations. The canals open into a series of expanded interconnected chambers or otic sacs that contain a sensory tissue, the *macula*, that detects both linear accelerations and sound.

In Osteichthyan species there are three such otic sacs, each containing a calcareous structure, an otolith, that acts as a mechanoreceptor stimulating the kinocilia ("hair" cells) of the *macula*. The three otic sacs are the *sacculus*, *utricle* and *lagena*, which contain the *sagitta*, *lapillus* and *asteriscus* otoliths, respectively (fig. II.A.1). Each otolith is fixed over the *macula* by an otolithic membrane, into which sensory cilia project. According to Dunkelberger *et al.* (1980), the otolithic membrane consists of two zones: a structured gelatinous zone that covers the sensory region of the *macula*, which usually exhibits a reticulated or honeycomb architecture, and the sub-cupular zone, which consists of very loose networks of fibres covering sensory and non-sensory regions of the *macula*. The gelatinous zone extends from the otolith surface to the tips of the sensory hairs and its primary function is probably that of

mechanoreception. The lumen of the entire system is filled with endolymph. In species of Ostariophysi the swimbladder is used to enhance auditory stimulation of the inner ear (Popper & Fay, 1993).

Figure II.A.1
Position of the otoliths within the inner ear of Teleost fish (modified from Secor *et al.*, 1992).
a) Dorsal view of the vestibular apparatus in a typical Teleost species. The top of the cranium is cut away (frontal section).
b) Otoliths within the labyrinth systems of typical Teleost and Ostariophysean fishes.

Ast = asteriscus;
Lag = lagena (vestibule);
Lap = lapillus;
Sac = sacculus (vestibule);
Sag = sagitta;
Utr = utriculus (vestibule).



1.1. Description

The otoliths of the three otic sacs differ in size and shape (fig. II.A.2). Differences in otolith shape tend to reflect phylogeny and development, although there is considerable inter- and intra-specific variation (fig. II.A.3). Inter-specific differences in shape appear to be due to both genetic and environmental influences (Lombarte & Lleonart, 1993; Nolf, 1995; Totres *et al.*, 2000). Due to their inter-specific variation in shape, otoliths have been found to be useful in taxonomy (Hechr, 1979), as well as permitting the study of food webs from partially digested remains (Surer & Morel, 1996; Olsson & North, 1997; Watanabe & Saito, 1998; Alonso *et al.*, 1999, *inter alia*). Similarly, otoliths from archaeological and paleontological finds have also been

used in the reconstruction of paleoenvironments and paleofauna (Nolf, 1995). Otolith morphometrics have also been used in species identification and to study geographical variations in populations and stocks of fish (Messieh *et al.*, 1989; Castonguay *et al.*, 1991; Campana & Caselman, 1993; Friedland & Reddin, 1994).

Figure II.A.2
The three otoliths
of *Vinciguerria nimbaria*
(Photichthyidae). S, sagitta;
L, lapillus; A, asteriscus.
Scale bar = 300 μ m (from
Tomás & Panfili, 2000).

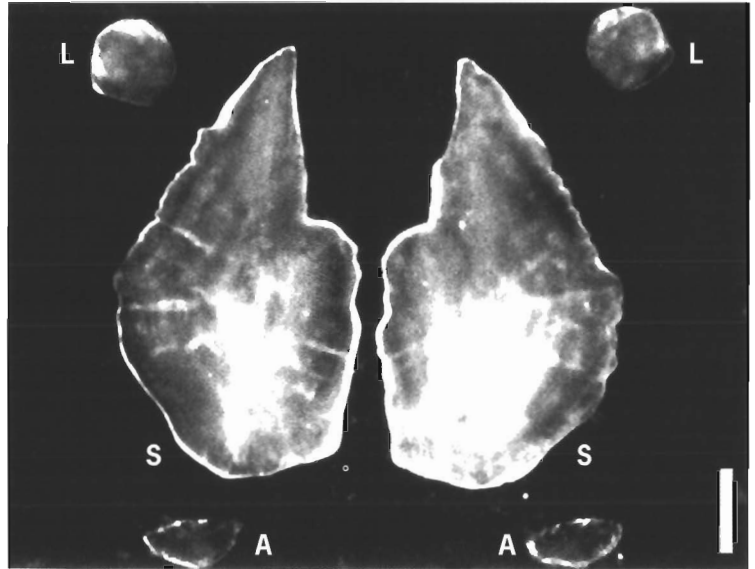
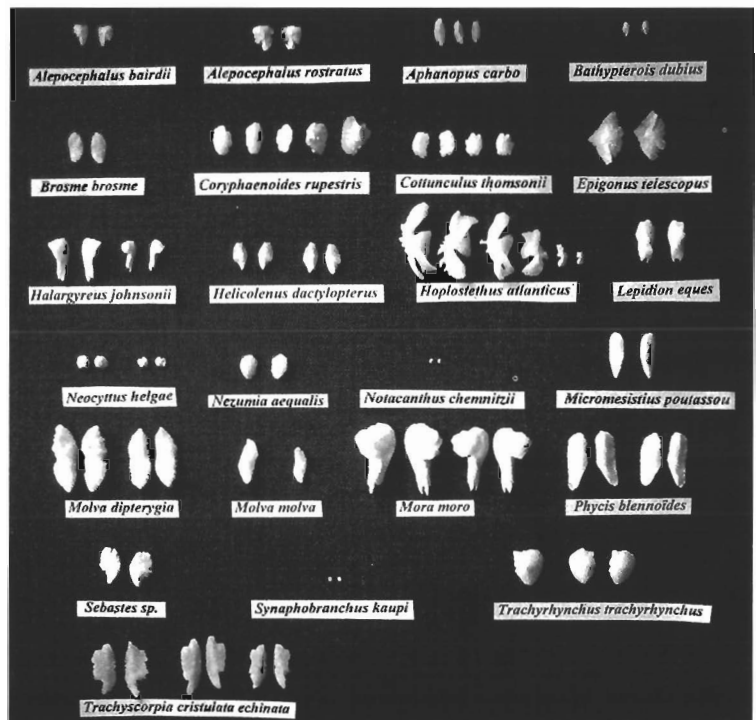


Figure II.A.3
Examples of various otolith
shapes from deep water
species (photo V. Allain).
Scale bar = 10 mm.

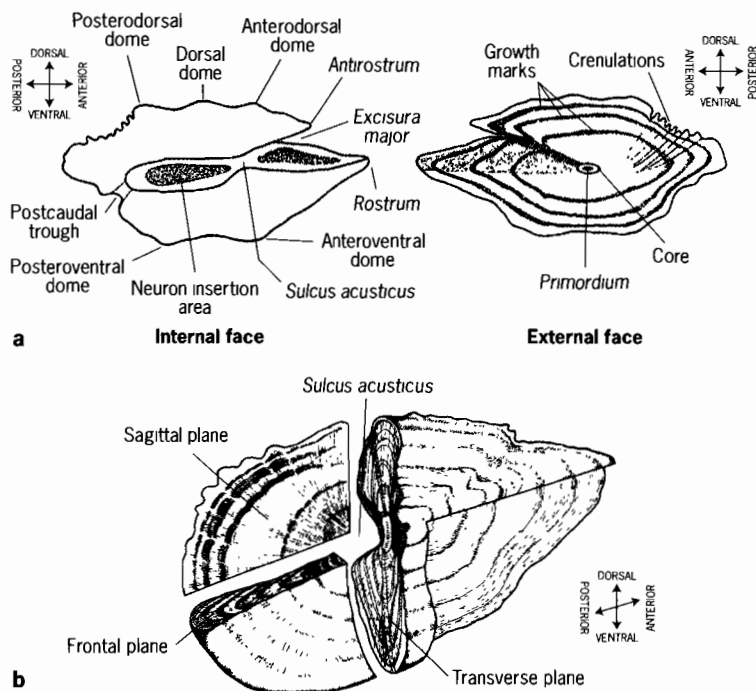


In most species the *sagitta* is the largest otolith and is most often used in age estimation. However, the *asteriscus* is the largest otolith in Ostariophysean species (Adams, 1940). Most studies of otolith formation have focused on the *sagitta* and *sacculus*. In the literature, the term “otolith” is often used to describe any one of the three pairs, generally the *sagitta*, but it is important to define this in any study.

Otoliths are generally laterally compressed and left-right symmetrical, except in flatfish and catfish. Details of the terminology utilised in describing otolith morphology are given in figure II.A.4a. An otolith has three planes of orientation, following those of the fish; sagittal, frontal and transverse (fig. II.A.4b). This orientation must be defined carefully in describing any otolith preparation, and reference should always be made to the standard terminology (e.g. transverse, sagittal, frontal sections). The proximal face of the *sagitta* has a groove, the *sulcus acusticus* (fig. II.A.4 a, b), which allows contact with a sensory epithelium (*macula*) of the *sacculus* (Dunkelberger *et al.*, 1980; Fay, 1980; Platt & Popper, 1981). A typical *sagitta* is elliptical on its sagittal plane, is compressed in its internal-external axis, with a convex proximal face and a concave distal face, and a main axis of growth oriented in the antero-posterior direction (fig. II.A.4). However, in several epipelagic and pelagic fish such as tunas, Istioforids, dolphin-fish, Cyprinidae and deep water species dorsal and ventral sides of the otoliths are asymmetrical, displaying a butterfly shape.

Figure II.A.4
A cross section through a typical *sagitta* illustrating the component parts (modified from Pannella, 1980).

a) Internal and external faces of a typical *sagitta*.
b) The three planes of orientation of a typical *sagitta*.



Otoliths are formed extracellularly from the crystallisation of the aragonite form of calcium carbonate onto an organic matrix template composed largely of a keratin-like protein, otolin, which is rich in aspartate and glutamate residues (Degens *et al.*, 1969; Watabe *et al.*, 1982; Morales-Nin, 1987a). The otolith grows or accretes by the addition of concentric layers of proteins and calcium carbonate, resulting in a structure somewhat comparable to that of an onion (chap. III.C).

1.2. Function

Fish labyrinths are involved in the maintenance of equilibrium and have nervous cells that are sensitive to pressure, gravity, angular movement and sound vibration (Grassé, 1958; Lowenstein, 1971; Blacker, 1974). The *pars superior* of the labyrinth (semi-circular canals and *utricle*) deals with postural information whilst the *pars inferior* (*sacculus* and *lagena*) is the sound receptor (fig. II.A.1). Teleost otoliths are similar to, but larger than, the otoconia of other Vertebrates. The otoliths are involved in mechanoreception, acting as electromechanical sound and displacement transducers that convert shear forces into electrical impulses by distorting the kinocilia of the nervous end-organ or *macula* in the fish inner ear (Popper & Hoxter, 1981). Relative motion between the sensory epithelium and the otolith bends the ciliary bundles and stimulates the eighth cranial nerve. The otoliths add mass to the gelatinous layer of the three otic sacs, increasing their sensitivity to gravitational and other linear acceleration forces (Ross & Pote, 1984). The *sulcus acusticus* of the otolith has a direct relationship with the *macula* of the vestibular epithelium, which is directly connected with the auditory nerve (Grassé, 1958). The receptor systems are rather different for Ostariophysean species, in which the internal ear is in contact with the swimbladder through a complex of bones known as the Weber complex (Grassé, 1958).

It has been hypothesised that sound reaches the fish ear via two different pathways. Because the fish's body is approximately the same density of water, it moves with the water in response to an impinging sound field. The otolith, however, is denser than the rest of the body, and so moves with a different amplitude and phase from the sensory *macula* and the body. Thus, the sound source directly stimulates the inner ear. In addition, because the swimbladder contains gas less dense than the body, the walls of the swimbladder vibrate. This produces indirect stimulation through otolith displacement (Popper & Lu, 2000).

2. Periodic increments

Otoliths exhibit a range of incremental structures that are often formed regularly over time scales ranging from sub-diurnal to annual. Unlike skeletal calcium, which may be mobilized for homeostasis

(Simkiss, 1974), otoliths do not appear to be subject to mineral resorption except under extreme stress (Mugiya & Uchimura, 1989). Consequently, otoliths appear to be highly suitable for age estimation. Fish age estimation depends on visible changes in otolith growth. The growth patterns of most interest are at four levels of resolution:

- primary increments, permitting a resolution of days;
- seasonal zones, permitting a resolution of several months or a growth season;
- annual increments, permitting a resolution of years;
- discontinuities in the otolith (ultra)structure, which correspond to various stresses that were not necessarily regular during the life history of the individual.

The mechanisms which produce these visible patterns are slightly different, although at the operational level they are the result of variations in the relative calcium and protein content of the increments or zones (Dannevig, 1956; Morales-Nin, 1987a).

2.1. Primary increments

Primary increments are formed from the successive deposition of a mineral-rich and a matrix-rich, mineral-deficient layer around a core (Watabe *et al.*, 1982; Morales-Nin, 1987a; Mugiya, 1987; Zhang & Runham, 1992a). Various names have been given to the two layers that form these primary increments. A review of otolith terminology presented at the first international symposium on otolith research proposed the terms L- and D-zones (Kalish *et al.*, 1995), for the mineral- and matrix-rich layers respectively. These terms refer to the bipartite appearance of the increments, L- and D-zones appearing light and dark respectively when viewed under transmitted light (fig. II.A.5a, b). The difference in the chemical composition of the two zones also leads to their different appearance under scanning electron microscopy following acid etching. The L-zone is rich in calcium carbonate and appears elevated in SEM whereas the D-zone is richer in protein and poorer in calcium and appears like a ridge in SEM (fig. II.A.5c, d, e). This terminology will therefore be used in the following review.

Pannella (1971; 1974) first discovered primary increments in otoliths and suggested that they were deposited daily. The large literature on daily increments has led many researchers to infer that primary increments can be assumed to be formed daily. However, such an assumption is invalid for a number of reasons. Otolith increment deposition may not be daily or be easily discernible in all species (Geffen, 1982; McGurk, 1984; Al-Hossaini & Pitcher, 1988; Morales-Nin, 1992). Inter-observer comparisons have shown that otolith structures are often interpreted differently by different readers (Campana & Mokness, 1991). Primary increments may not be deposited daily until some time after hatching (Geffen, 1987).

Sub-daily increments and discontinuities in the increment record may also occur (Campana & Neilson, 1985). The daily deposition of increments generally appears to cease in the adult and/or juvenile life history stages of long-lived fish (Pannella, 1971, 1980). In some cases this apparent cessation in the daily periodicity might be related to the formation of very narrow growth increments below the detection limit of the light microscope (Morales-Nin, 1988; Morales-Nin & Ralston, 1990). However, ultrastructural investigations have also demonstrated that primary increments are not deposited daily in some species (Volk *et al.*, 1995). Clearly then, the interpretation of microstructural growth patterns in wild fish requires an understanding of the physiological process and regulation of otolith accretion and of the environmental factors that influence them (Campana & Neilson, 1985). For otolith primary increments to be of use in age estimation, the processes involved in their regulation must either be synchronized to cyclical environmental events or possess an endogenous circadian rhythm, entrained to a diel environmental cycle (Geffen, 1987). In addition, increment formation must be independent of

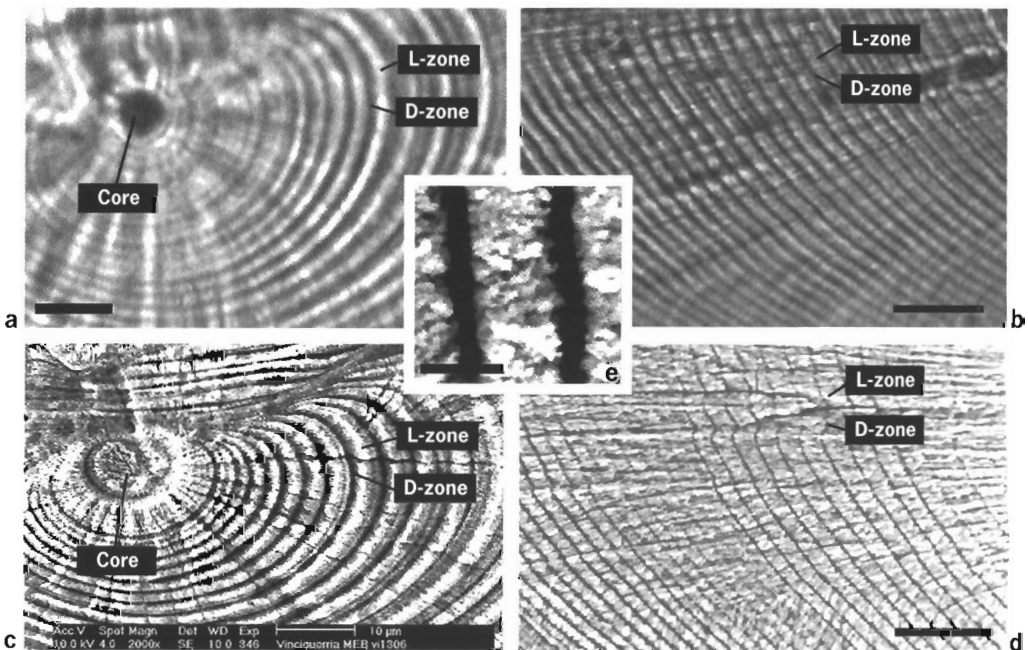


Figure II.A.5 - A transverse thin section of the otolith of *Vinciguerria nimbaria* (Photichthyidae). The primary increments composed of L- and D-zones are clearly visible.

a) Detail of the core area under transmission light microscopy. Scale bar = 10 µm (photo J. Panfili).

b) Detail of the adult growth area under transmission light microscopy. Scale bar = 10 µm (photo J. Tomás).

c) Detail of the core area after acid etching under SEM. Scale bar = 10 µm (photo L. Marec & E. Dabas).

d) Detail of the adult growth area after acid etching under SEM. Scale bar = 10 µm (photo J. Tomás).

e) Detail of the primary increments after acid etching, under SEM. The L-zone is rich in aragonite crystals whereas the D-zone corresponds to deep grooves. One primary increment is equal to 1 L-zone + 1 D-zone. The figure shows one complete L-zone and 2 complete D-zones. Scale bar = 1 µm (photo L. Marec & E. Dabas).

somatic growth. Experiments have shown that otoliths continue to accrete even when somatic growth has naturally ceased (Brothers, 1981; Wright *et al.*, 1990; Mugiya & Tanaka, 1992) or has been artificially restricted (Mosegaard *et al.*, 1988). This continuity may be related to differences between the growth of sensory systems such as the inner ear and other parts of the body.

Primary increments are only visible at high magnification (light microscopy or electron microscopy). They vary in size from less than 1 μm to 12 μm (Pannella, 1974). The width of the D-zone is always less than 1 μm (around 200 to 500 nm) whereas the width of the L-zone is more variable (from around 0.4 μm to 10 μm). However, because otoliths do not grow uniformly the increment width will also depend on the *radius* along which it is measured and how the otolith is sectioned (chap. III.C). Sub-daily increments may be laid down particularly during periods of fast growth. These structures can generally be differentiated from daily increments because they tend to be less well-defined and distinct than daily increments (Campana, 1992).

2.1.1. *Primordium*

The otolith develops from one or more partially calcified *primordia* exocytosed by epithelium cells in the inner ear (Mann *et al.*, 1983). These cores have been termed primordial granules and they are the primary or initial components of the *primordium*. In *sagittae* the granules may be composed of vaterite, whereas the rest of the *primordium* is typically aragonite. Examples of these different types of *primordium* are shown in figure II.A.6. The *primordium* can be either circular, elongated or multiple, depending on the species. In the case of multiple *primordia* these coalesce to form the core of the otolith (fig. II.A.6). The term “*nucleus*” has also been used to describe the core region of the otolith, although this term is not recommended since it has also been used to describe a much larger central area of the otolith (see glossary).

2.1.2. First ring

Otoliths develop in the later part of the egg stage in fish. For some time after formation, the otoliths grow continuously and mostly without obvious incremental accretion. The time at which incremental deposition begins differs from species to species. This point in time is often (conveniently) marked by a distinctive feature, usually a prominent check (fig. II.A.6d, e). There is considerable confusion about the terminology used to name this feature, as well as about its biological significance and timing. This first increment may not have the same physiological basis in all species. However, there are practical advantages in standardising counting procedures using this as a reference point (Neilson & Geen, 1982).

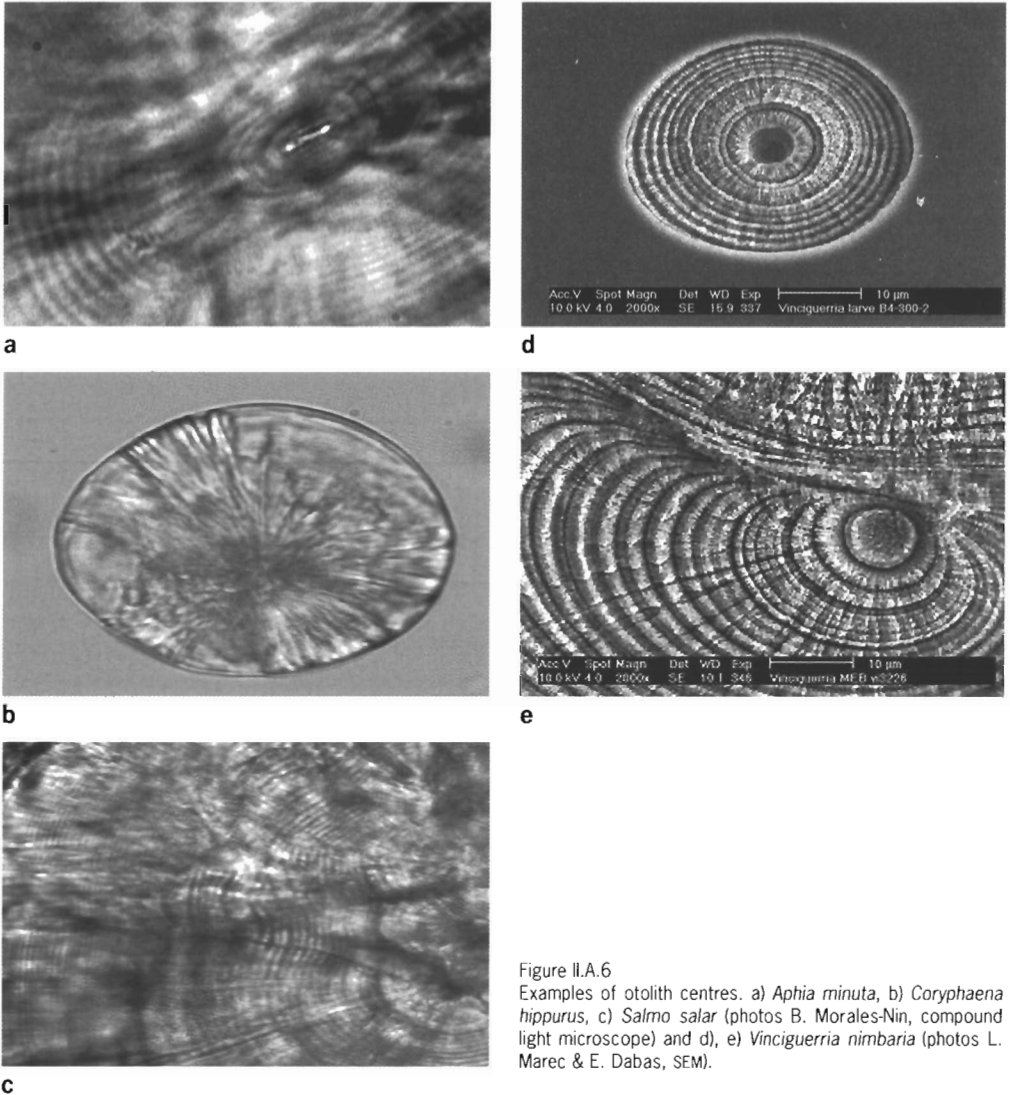


Figure II.A.6

Examples of otolith centres. a) *Aphia minuta*, b) *Coryphaena hippurus*, c) *Salmo salar* (photos B. Morales-Nin, compound light microscope) and d), e) *Vinciguerria nimbaria* (photos L. Marec & E. Dabas, SEM).

In several species increments have been noted which are most likely to have been formed before hatching (fig. II.A.7). It has not been demonstrated conclusively whether or not these increments are true structures or merely optical artefacts. The opacity of pre-hatch increments is rather different from post-hatch primary increments (fig. II.A.7). These structures are most apparent in young larvae with small otoliths. Hatching is not really a developmental stage in fish larvae, since a single batch of siblings may hatch at different stages of morphological development. In many species the first increment or otolith check may

be formed on the day of hatching, and may be properly termed a hatch ring. However, in other species, the first increment may be formed in association with a particular developmental stage, irrespective of whether the embryo has hatched or not. For example, in *Solea solea* the increment that is formed when the mouth opens is more prominent than any preceding increments, and this increment is used as the reference point for counting (Lagardère, 1989). In *Clupea harengus*, the first prominent increment is formed well after hatching and towards the end of the yolk-sac stage (Geffen, 1982). In both species the timing of this increment varies, and depends on developmental rates. In juvenile otoliths of the Siluriforme species *Hoplosternum littorale* and *Megalechis thoracata*, the hatch check is well differentiated (Ponton *et al.*, 2001) (fig. II.A.7). In these species the hatched larvae are well developed and the otoliths appear to exhibit some increments before the hatch check (fig. II.A.7). In a number of other species, the transition to exogenous feeding is represented by a prominent increment. Examples of first increments cited in the literature are presented in table II.A.1.

Figure II.A.7
Examples of otolith hatch check in *Megalechis thoracata*. The black arrows indicate the hatch check and the white arrow the multiple *primordia* cores. The hatch check has been localised after a validation experiment. Some increments are also present inside the check and then before birth. Scale bar = 50 μ m (photo D. Ponton).

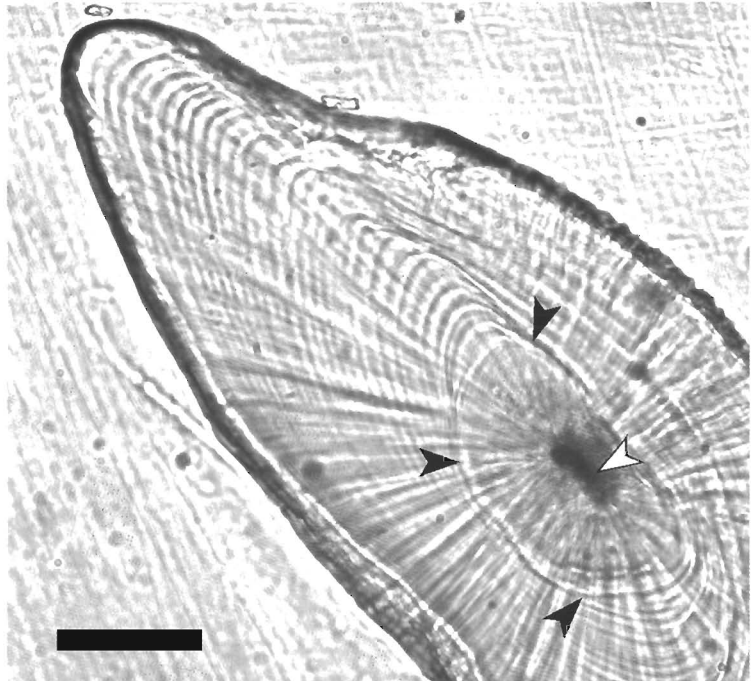


Table II.A.1. - References on otolith structures associated with life history events. Otolith structures refer to those found in *sagittae* except those denoted with an asterisk, which were *lapilli* (from Wright *et al.*, 1998).

Species	Term used by author	Alternative terminology	Related life history event (timing)	Reference
<i>Annodytes marinus</i>	Yolk-sac absorption check Secondary growth centre	Accessory <i>primordia</i>	Yolk-sac absorption Metamorphosis & settlement	(Wright, 1993) (Wright, 1993)
<i>Anguilla anguilla</i>	First ring Deep groove Transition ring	First check Transition ring	End of yolk-sac phase First ingestion End of metamorphosis	(Lecomte-Finiger, 1992) (Lecomte-Finiger, 1992) (Lecomte-Finiger, 1992)
<i>Chromis atripectoralis</i> *	Check	Settlement check	Time of settlement	(Thorrold & Milicich, 1990)
<i>Clupea harengus</i>	First heavy ring Hatch check Hatch check	First check First check First check	End of yolk-sac phase End of yolk-sac phase Between hatch and yolk-sac absorption	(Geffen, 1982) (Moksness, 1992) (Hoei, 1997)
<i>Engraulis encrasicolus</i>	Check ring	First check	End of yolk-sac phase	(Palomera <i>et al.</i> , 1988)
<i>Gadus morhua</i>	Nuclear check Yolk-sac check Hatch check Check	First check First check First check First check	Hatching End of yolk-sac Time of hatching Hatching	(Bolz & Lough, 1983) (Bolz & Lough, 1983) (Campana, 1989) (Geffen & Nash, 1995)
<i>Halichoeres tenuispinus</i>	Hatch check	First check	Hatching	(Kishiro & Nakazono, 1991)
<i>Hoplosternum littorale</i> *	Hatch check	First check	Hatching	(Ponton <i>et al.</i> , 2001)
<i>Melanogrammus aeglefinus</i> *	Nuclear check Yolk-sac check Hatch check (two)	First check	Hatching End of yolk-sac phase Hatching	(Bolz & Lough, 1983) (Bolz & Lough, 1983) (Campana, 1989)
<i>Merluccius merluccius</i>	Accessory <i>primordia</i>	Accessory <i>primordia</i>	Metamorphosis & settlement	(Morales-Nin & Aldebert, 1997)
<i>Microstomus pacificus</i>	Accessory <i>primordia</i>	Accessory <i>primordia</i>	Metamorphosis (eye migration to settlement)	(Toole <i>et al.</i> , 1993)
Myctophidae (55 species)	Accessory <i>primordia</i>	Accessory <i>primordia</i>	Transforming larvae	(Linkowski, 1991)
<i>Oncorhynchus keta</i>	Hatching check Seawater transfer ring	First check Transition ring	Hatching Seawater transfer	(Volk <i>et al.</i> , 1984) (Volk <i>et al.</i> , 1984)
<i>Oncorhynchus gorbuscha</i>	Transition zone Emergence check	Transition zone Emergence check	Seawater transfer Emergence from bottom	(Volk <i>et al.</i> , 1995) (Mortensen & Carls, 1995)
<i>Oncorhynchus nerka</i>	Hatch ring First feeding check	First check	Hatching Critical period	(Marshall & Parker, 1982) (Marshall & Parker, 1982)
<i>Oreochromis niloticus</i>	Hatching check	First check	One day after hatching	(Zhang & Runham, 1992a)
<i>Pleuronectes americanus</i>	Accessory growth centres Secondary growth centre	Accessory <i>primordia</i> Accessory <i>primordia</i>	During and after metamorphosis Changing habitat Metamorphosis	(Jearld <i>et al.</i> , 1993) (Sogard, 1991)
<i>Pleuronectes platessa</i>	Accessory <i>primordia</i> Accessory <i>primordia</i>	Accessory <i>primordia</i> Accessory <i>primordia</i>	Metamorphosis Settlement	(Al-Hossaini <i>et al.</i> , 1989) (Karakiri & Westernhagen, 1989)

<i>Pollachius virens</i>	Hatch check	First check	After hatching	(Campana, 1989)
<i>Pomacentrus coelestis</i> *	Check	Settlement check	Time of settlement	(Thorrold & Milicich, 1990)
<i>Scophthalmus maximus</i>	First heavy ring	First check	End of yolk-sac phase	(Geffen, 1982)
<i>Sebastes jordani</i>	Extrusion check	First check	Parturition	(Ralston <i>et al.</i> , 1996)
<i>Solea solea</i>	Hatch check	First check	Hatching	(Lagardère, 1989)
	Mouth opening check		First feeding	(Lagardère, 1989)
	Yolk-sac exhaustion check		Starved larvae	(Lagardère, 1989)
<i>Theragra chalcogramma</i>	Check	Check	Physiological changes	(Nishimura, 1993)
<i>Trachyrhynchus trachyrhynchus</i>	Accessory <i>primordia</i>	Accessory <i>primordia</i>	Migration	(Massuti <i>et al.</i> , 1995)
<i>Vinciguerria nimbaria</i>	Hatch check	First check	Hatching	(Tomás & Panfili, 2000)

Salmonid otoliths display a number of prominent increments, each related to a different developmental event. The earliest increments which surround the entire set of *primordia* coincide with vascularisation of the yolk sac and the development of red blood cells. There is a prominent increment which marks hatching, and another prominent increment which marks emergence from the substratum. Under hatchery conditions, checks which correspond to first feeding have also been observed. For ecological studies, age estimations are based on counts from the emergence mark.

2.2. Accessory growth centres

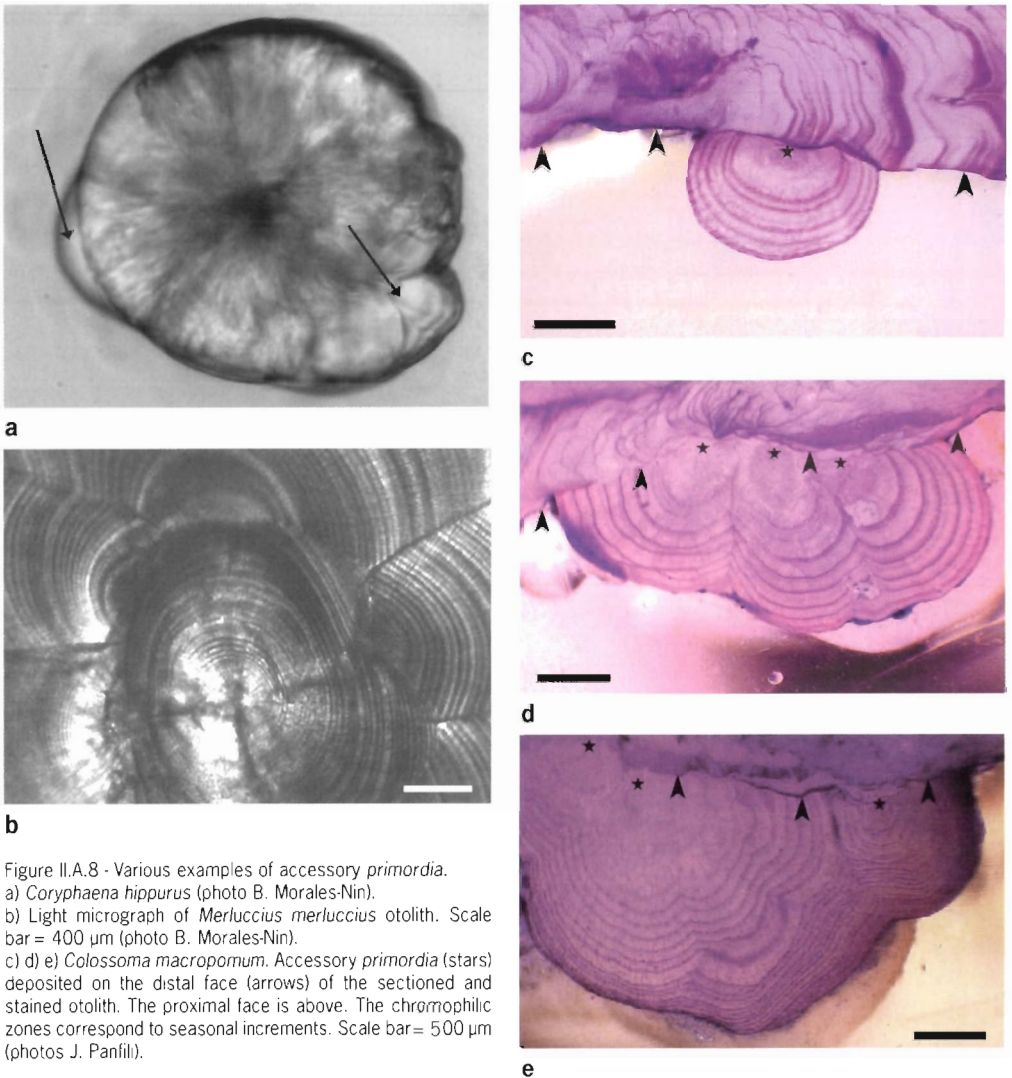
During the larval phase most otoliths continue to accrete around the *primordium*. However, in the otoliths of many species additional planes of growth are formed at later developmental stages and from these new series of increments emanate. These new planes in otolith growth result from the development of accessory growth centres. Accessory growth centres are particularly common in the largest otolith (*sagitta*) of most species and the *lapillus* of Cyprinids or *asteriscus* of Ostario-physeans. Accessory growth centres are often referred to as accessory *primordia*. However, the term accessory growth centre is preferred, in order to avoid confusion with *primordia* which contain multiple primordial granules. Figure II.A.8a, b shows examples of accessory *primordia* in two marine species.

Since otolith shape influences sensitivity to sound frequencies (Popper & Hoxter, 1981), the formation of accessory growth centres may be related to a transition in physiology, habitat or behaviour. These structures are found in many species that undergo a marked habitat change at the transition from the larval to the juvenile stage. Examples of these structures in juvenile fish are shown in table II.A.1. Accessory

primordia can also be found on the otoliths of adults. For example, they are very common on the distal face of the *asteriscus* of *Colossoma macropomum*, where they appear as autonomous structures which grow with the otolith, also showing seasonal increments (fig. II.A.8c, d, e).

2.3. Seasonal and annual increments

Seasonal increments, also termed seasonal zones, marks, rings or *annuli* (see glossary), are often distinguishable on otoliths. They are often visible in tropical as well as temperate species. These zones can be visible in both whole (untreated) otoliths and/or after some form of preparation (chap. VIII). The two main types of seasonal zones have



different opacities. Under transmitted light the opaque zone is dark and the translucent zone is bright, and under reflected light the opaque zone is bright and the translucent zone is dark (fig. II.A.9). In addition to their macroscopic appearance the two seasonal zones differ with respect to the width of primary increments, the thickness and size of aragonite crystals (Morales-Nin, 1987a), the frequency of growth discontinuities and organic layers (Mugiya *et al.*, 1985), the ratio of calcium carbonate to protein matrix (Casselman, 1974, 1982, 1987; Mugiya, 1984), and elemental ratios (Casselman, 1982, 1983; Kalish, 1989, 1991a). It is the combination of these factors that leads to differences in the optical densities of the two zones. Seasonal zones can reach a few hundred microns in width and are therefore visible to the naked eye or at low magnifications (10x – 40x). The difference in the organic matrix content of the two zones can be underlined after burning which turns the organic matrix a rich opaque brown, or after staining, which colours the chromophilic organic zones (fig. II.A.10 and chap. VIII).

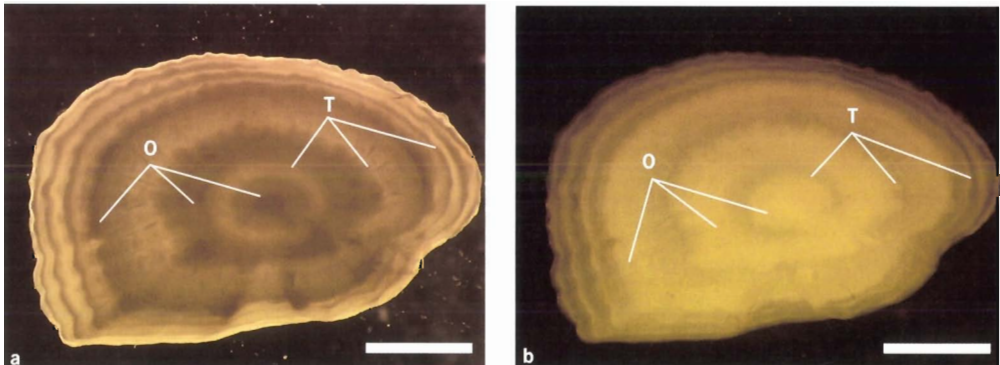
Annual increments, also termed annual marks, rings or *annuli*, are often interpreted when taking into account the succession of several seasonal increments. Most temperate and many tropical species exhibit annual increments, usually comprising opaque and translucent zones. However, in some tropical species biannual growth increments have been reported, probably related to multi-annual changes in environmental and hydrological factors (Yosef & Casselman, 1995).

Figure II.A.9

Whole otolith of a plaice, *Pleuronectes platessa* (47 cm TL), showing opaque (O) and translucent (T) zones viewed under transmitted light (a) and under reflected light against dark background (b). The opaque zones are dark in transmitted light and bright in reflected light, and inverse for translucent zones. Scale bar = 2 mm (photos J. Panfili).

2.4. Lunar-related structures

A common feature of the otoliths of juvenile and adult fish is a pattern of thick increments separated by numerous less prominent increments. This pattern is usually repeating and has been hypothesised to reflect lunar cycles. The best examples of lunar patterns are seen in the otoliths of juvenile flatfish, but they have been described in a wide range of species. Presumed lunar patterns have been described in



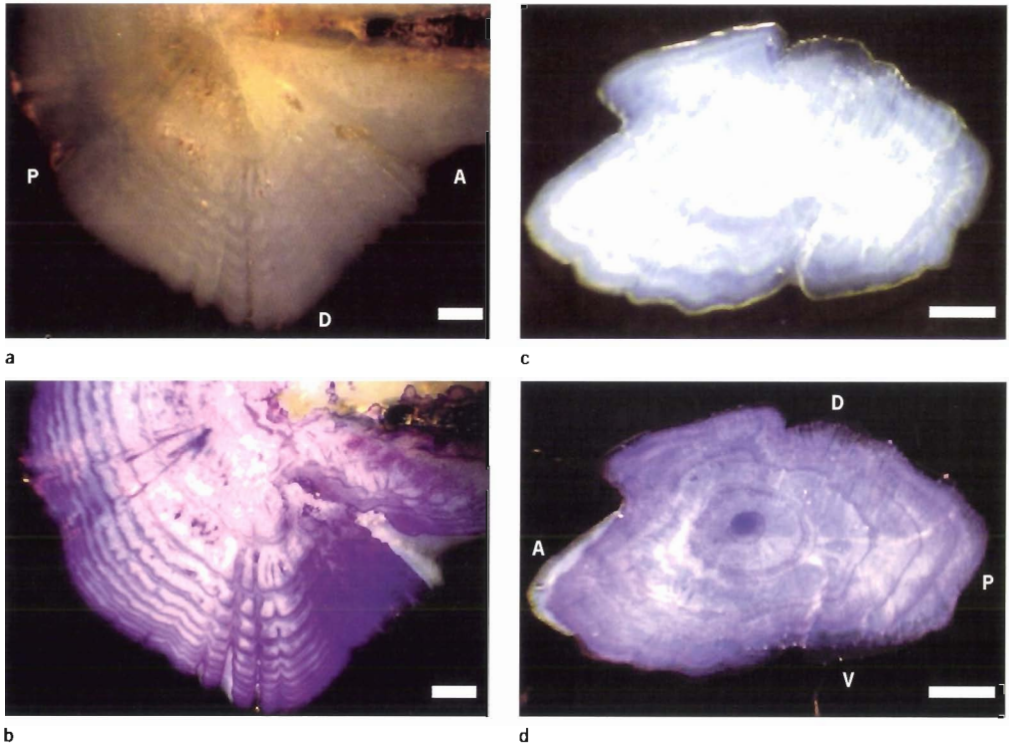


Figure II.A.10 - Enhancement of the otolith zones after staining. A, anterior; D, dorsal; P, posterior; V, ventral (photos J. Panfili).
 a) Sagittal section of an otolith (asteriscus) of *Colossoma macropomum* (Serrasalmidae, 63 cm SL). Central and dorsal areas observed in reflected light against a dark background. Scale bar = 1 mm.
 b) Sagittal section of the *Colossoma macropomum* otolith (a) after acid etching (EDTA) and staining with toluidine blue. Central and dorsal areas observed in reflected light against a dark background. The growth zones are enhanced. Scale bar = 1 mm.
 c) Sagittal section of an otolith of *Anguilla anguilla* (Anguillidae, 55 cm TL) observed in reflected light against a dark background. Scale bar = 500 μ m.
 d) Sagittal section of the *Anguilla anguilla* otolith (c) observed in reflected light against a dark background, after acid etching and staining with toluidine blue. Scale bar = 500 μ m.

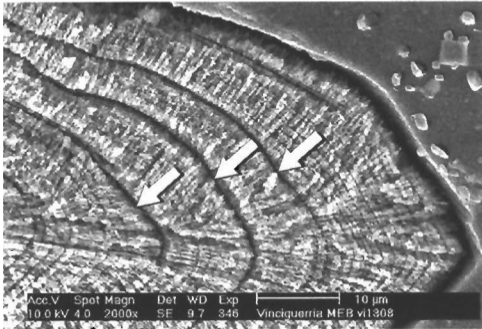
bathypelagic fish otoliths as well, bringing into question the actual cause of the patterns and what cyclical (physiological or behavioural) processes they may reflect.

The terminology used to refer to the presumed lunar pattern varies (Pannella, 1980). "Lunar pattern" usually refers to sets or sequences of increments, each beginning with a prominent, high-contrast, increment which is most often termed a "check" (discontinuity; see 2.5) regardless of the cause of formation. The check is followed by a set of lower-contrast increments. The widths of the L-zone of the check and subsequent increments are usually uniform. Several authors have made use of these repeating features for age determination, on the assumption that each sequence represents a 14-day lunar cycle. Fewer have attempted to discover the source of the environmental signal that

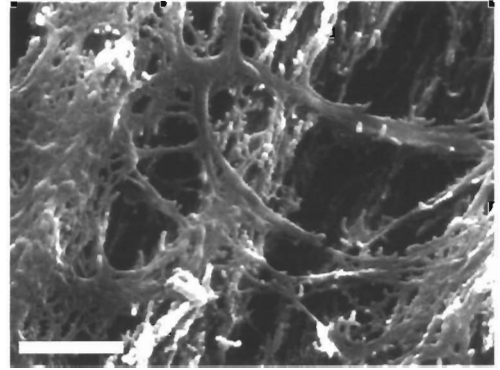
imprints the distinctive pattern. Campana (1987) described alternating patterns of high and low contrast increments, each containing either approximately seven or 14 increments. The formation of these patterns corresponded well to variations in tidal height and the lunar cycle. Geffen & Nash (1995) showed that the pattern in *Pleuronectes platessa* contained seven increments, and that the discontinuity which separated the normal increments coincided with dates half-way between spring and neap tides. Linkowski (1996) has also described a clear lunar pattern in the primary increment growth of four species of the genus *Hygophum* in the North Atlantic.

2.5. Structural discontinuities

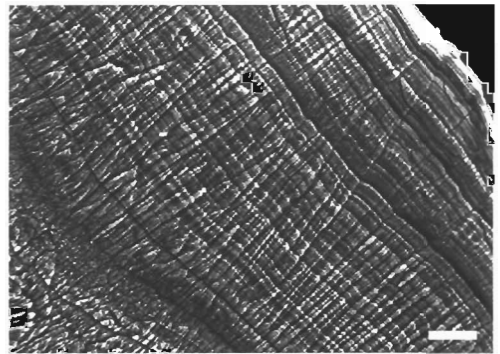
Structural discontinuities, also known as checks, are breaks within the regular arrangement of the primary increments (Pannella, 1980; Campana & Neilson, 1985; Gauldie, 1987; Morales-Nin, 1987a; Gauldie & Nelson, 1988). These may interrupt the succession of seasonal or daily increments in a cyclic or acyclic way. They can be distinguished under high or sometimes low magnification, usually after some preparation. The discontinuity affects the growth pattern or the direction of growth. They generally appear after acid etching as deep grooves in the otolith surface and under the microscope they appear as wide D-zones (fig. II.A.11a). The organic matrix is usually abundant in these discontinuities (fig. II.A.11b) (Morales-Nin, 1986b), which may be why they are often stainable after acid etching by certain histological dyes (Pannella, 1980). A discontinuity preceded by increments of decreasing width might correspond to a seasonal ring (fig. II.A.11c). Discontinuities are typical of all species and are probably induced by disturbances or stresses suffered by individuals in their biotope. Pannella (1980) proposed a classification for discontinuities (checks) according to their structures and presumed causes, although without much justification. However, research has demonstrated that certain discontinuities are related to developmental events, such as the change from pelagic to demersal life, or settlement in coral reef species. For example, rhythmic growth patterns and checks in *Merluccius capensis*, *M. paradoxus* and *Genypterus capensis* were found to be related to activity patterns and different life strategies (Morales-Nin, 1987b). As a discontinuity represents an interruption in growth of unknown duration, its interpretation can be ambiguous and may pose problems in making estimates of daily age (Campana & Neilson, 1985). When the duration of formation of a discontinuity is known, for example with a winter stress check, such structures can be useful for estimating annual age.



a



b



c

Figure II.A.11 - Discontinuities found within otoliths.

a) Discontinuities (arrows) in the regular arrangement of otolith primary increments of *Vinciguerria nimbaria* (Photichthyidae) (photo J. Panfili).

b) Scanning electron micrograph of a demineralised otolith of *Dicentrarchus labrax* showing the organic matrix and the transversal groups of fibres corresponding to discontinuities. Scale bar = 1 µm (photo B. Morales-Nin).

c) Scanning electron micrograph of a *Dicentrarchus labrax* demineralised otolith showing the decreasing increment width and the structural discontinuity corresponding to a seasonal increment. Scale bar = 10 µm (photo B. Morales-Nin).

2.6. Secondary growth zones

In many species the estimation of annual increments is made difficult by the presence of non-periodic “secondary” zones. This term applies to a range of non-seasonal zones characterised by different opacities and thickness. The two major types of secondary zones are false and split rings. False rings appear as translucent zones within an opaque zone. They are particularly common in the first year of otolith growth and in many cases are easily confused with the first annual increment (fig. II.A.12). For instance, depending on the spawning period, age-0 *Trachurus mediterraneus* presents four types of otoliths that differ in the presence, number or appearance of false rings (Karlou Riga, 2000). Split rings appear as double structures, almost as though they were composed of two unusually thin translucent bands separated by a very thin opaque band. In some species the annual increment is composed of multiple rings, with a narrow well-defined translucent zone followed by some very opaque material (fig. II.A.13).

Problems in distinguishing between secondary and true seasonal zonations is a major cause of age-reading errors. There are currently no objective criteria for identifying secondary structures, despite extensive reviews of this problem. Little is known about the causes of these secondary structures, although a number of factors including temperature, food intake and developmental transitions have been implicated in their formation.

Secondary growth structures are also present at the primary increment level in species with peculiar life histories, such as Myctophids and related species which start their life in the upper layers of the water column and later make diel migrations between deep water during the day and the surface at night. Secondary related daily increments have been described in three species of tropical Myctophids (Gartner, 1991) and in *Vinciguerria nimbaria* (Tomás & Panfili, 2000). These secondary increments appear to be sub-daily increments. Due to the possible variation in incremental structures discussed above, it is important to describe all structures recorded carefully according to the standard terminology (see glossary).

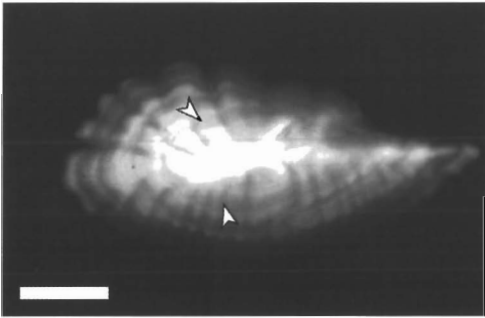
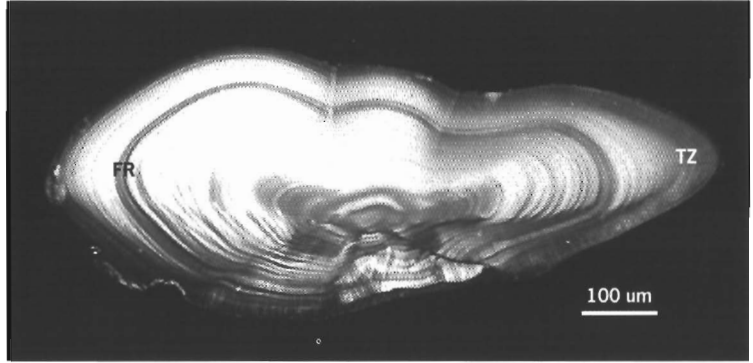
3. Regulation of incremental deposition

3.1. Exogenous influences on primary increment periodicity

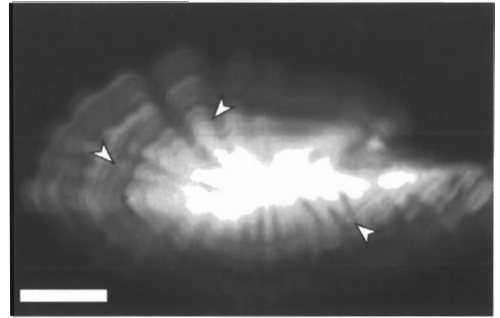
Several studies have examined the relationship between increment formation and specific environmental factors and a number of possible synchronising factors have been proposed. Pannella (1980) suggested that increment periodicity may be related to the number of peaks in feeding activity. Feeding frequency has been reported as influencing increment periodicity in some species, for example, *Oncorhynchus tshawytscha* (Neilson & Geen, 1982) and *Pleuronectes platessa* (Al-Hossaini & Pitcher, 1988), but not in others such as *Lepomis macrochirus* (Taubert & Coble, 1977), *Oncorhynchus nerka* (Marshall & Parker, 1982), *Platichthys stellatus* (Campana, 1983) and *Salmo salar* (Wright *et al.*, 1991). Moreover, starved fish often continue to deposit daily increments (Taubert & Coble, 1977; Marshall & Parker, 1982; Campana, 1983; Wright *et al.*, 1990). There thus appears to be little evidence to support Pannella's hypothesis of a relationship between increment periodicity and peaks in feeding activity.

Otolith growth is sensitive to temperature in a number of species (Brothers, 1981; Mosegaard *et al.*, 1988) and Brothers suggested that temperature fluctuations are a major influence on increment formation in temperate stream fish. Thermally-induced marks on otoliths demonstrate how strong and sudden temperature variations may disrupt otolith growth (Volk *et al.*, 1994). Gauldie & Nelson (1990a) proposed a carbonic anhydrase-regulated system for otolith formation.

Figure II.A.12
False ring (FR) in the sagitta
of a 1-year old whiting,
Merlangius merlangus.
A translucent zone (TZ)
of an annulus is also shown
for comparison.
Scale bar = 0.1 mm
(photo P.J. Wright).



a



b

Figure II.A.13
False rings.
Scale bar = 1 mm
(photos B. Morales-Nin).
a) Sagittal otolith
of *Trachurus trachurus*
capensis showing
the multiple zones
in the nuclear area.
b) Multiple growth zones
in a *Trachurus trachurus*
capensis otolith.

Such a chemical system would have temperature as its main external controlling factor. However, the role of the organic matrix in otolith formation is not well understood, and this might also be related to various external cues (chap. II.3.4).

Light-dark cycles appear to be necessary for daily increment formation in larval *Lepomis macrochir* (Taubert & Coble, 1977) and *Fundulus heteroclitus* (Radtke & Dean, 1982). Campana & Neilson (1985) suggested that such dependence on light-dark transitions may be mediated by age, as light-dark cycles appear to be essential for daily increment deposition in the larval but not the juvenile stages of *Porichthys notatus* (Campana, 1984). Many deep-sea fish exhibit microscopic increments with rhythmic groupings and a similar structure and thickness to those found in shallow-water fish, despite the absence of light and feeding daily rhythms (Gauldie, 1987; Lombarte & Morales-Nin, 1989; Gauldie, 1990; Morales-Nin *et al.*, 1996b). In these species, small variations in tidal currents along the slope or vertical migrations of planktophagous prey may act as a daily *Zeitgeber*.

In a review of environmental manipulation experiments Campana & Neilson (1985) suggested that the endogenous circadian rhythm controlling otolith accretion was entrained to photoperiod, but could be masked by sub-daily temperature cycles or feeding patterns. If increment periodicity is controlled by an endogenous circadian rhythm then increment deposition would be expected to continue in the absence of entraining stimuli, although the absence of an entraining stimulus would be expected to eventually lead to a divergence from a daily deposition rate. Several studies have shown a continued daily increment deposition rate in the absence of one potential entraining stimulus such as light-dark transition. Constant daily rates of increment formation have been reported in juvenile fish held under constant light (Campana, 1984), darkness (Radtke & Dean, 1982) and in the absence of cyclical variations in light, temperature or feeding frequency (Wright *et al.*, 1991). However, environmental manipulation experiments do not provide unambiguous experimental evidence of an endogenously regulated cycle of increment formation, since fish may have an endogenous feeding rhythm. Moreover, no study has demonstrated a divergence from a single increment per day, as might be predicted when there is no entraining stimulus, although this may reflect the short (<30 days) duration of these experiments.

Support for light-dark transitions as a sign for entrainment has come from ultrastructural and radio-labelling experiments. Tanaka *et al.* (1981) demonstrated that in *Tilapia nilotica*, the order of formation of the L- and D-zones was dependent on photoperiod, as a reversal of the light-dark cycle was found to induce a reversal in the order of the two layers. Using radiolabelled calcium (^{45}Ca) to study *in vivo* otolith calcification in *Carassius auratus*, Mugiya and coworkers (1981) found an apparent diel cycle in calcification that was associated with photoperiod. However, these experiments were flawed because no consideration was given to the possible effects of isotopic equilibration on ^{45}Ca incorporation. Nevertheless, later *in vivo* experiments, involving juvenile *Salmo salar* subjected to an isotopic equilibration period, did demonstrate that otolith calcification was entrained to dark-light transitions (Wright *et al.*, 1992). Radiolabelling experiments have also demonstrated diel cycles of both calcification and organic matrix formation, associated with photoperiod, within isolated *sacullae* (Mugiya, 1987).

3.2. Exogenous regulation of annual increment periodicity

At present the regulation of annual increment formation in otoliths is not well understood, although it is commonly assumed that seasonal zones are related to seasonality in somatic growth and environmental factors. One view is that seasonal variation in otolith formation is related to cyclical physiological changes in the fish such as the onset

of reproductive activity or the accelerated somatic growth that occurs in spring (Johnson, 1983; Fowler, 1990). An alternative suggestion is that the physiology of otolith formation is independent of other somatic and reproductive processes taking place within the fish, and is an independent physiological response to environmental variation (Loubens, 1978; Fowler & Doherty, 1992). Evidence for and against these hypotheses generally takes the form of correlations in the timing of the different processes and is usually too weak and insufficient to allow either hypothesis to be rejected. The formation of the zones in relation to reproductive activity is controversial, given that in several species the opaque zone coincides with the time of year when fish are reproductively active, while in others the formation of regular translucent zones has been related to maturity and spawning. However, zone formation is frequently seen in the juvenile stages of many fish species (Johnson, 1983; Fowler, 1990). Furthermore, it is difficult to envisage how reproductive activity could directly affect otolith composition since, although reproducing females have elevated plasma calcium concentrations, this takes the form of protein-bound calcium which will not affect calcium ion levels in the endolymph (Kalish, 1991a). As yet no experimental studies have been performed in order to distinguish between calcium and organic components in plasma and otolith formation.

The season of formation of opaque and translucent zones may change during development and in relation to geographical distribution. In *Gadus morhua* from the North Sea, for example, the opaque zone forms earlier towards the southern extremes of this species' range and becomes progressively later further north. Within each stock younger fish begin to lay down the opaque zone up to four months before older fish. Spawning occurs when the translucent zone is well into the process of formation. The temporal delay in opaque-zone formation increases with age (Williams & Bedford, 1974). Vianet *et al.* (1989) provide other examples of geographical differences in four Pleuronectiform species from Europe, in which the translucent zones are formed during the summer in the Mediterranean but in winter in Northern European waters. The time of translucent-zone formation in *Sebastes entomelas* from the U.S. Pacific coast has been found to vary with sex, geographical area and year (Pearson, 1996). In this species a link between temperature and translucent zone formation is apparent although other temperature-related factors, such as food availability or nutrient content of the prey, may also be important. Further evidence of temperature-related zone formation was found for several species of Acanthurids from eastern Australia (Choat & Axe, 1996). Recapture of tetracycline-marked fish showed that the formation of opaque zones corresponds to the rise in water temperature in the summer.

3.3. Influences on accretion rate

Temperature can enhance otolith accretion beyond the point at which somatic growth is adversely affected (Mosegaard *et al.*, 1988), although high temperatures can also have a negative effect on increment width (Gutiérrez & Morales-Nin, 1986; May & Jenkins, 1992; Ralston & Howard, 1995). Experiments have shown that otoliths continue to accrete even when somatic growth has ceased naturally (Wright, 1990; Mugiya & Tanaka, 1992) or has been artificially restricted (Mosegaard *et al.*, 1988). This continuity results in slow-growing individuals having relatively large otoliths. In order to explain this phenomenon, Secor & Dean (1989) suggested that otolith accretion may be determined by the interaction of two components: the daily periodicity of increment formation, which continues even during periods of no somatic growth, and an amplitudinal component that varies with somatic growth. However, a number of experimental studies have found that the increase in otolith accretion rate with temperature is much more similar to the increasing trend in metabolic rate than to the optimum curve of somatic growth rate (Mosegaard *et al.*, 1987; Mosegaard *et al.*, 1988; Hoff & Fuiman, 1993). Moreover, studies by Wright (1991a) and Yamamoto *et al.* (1998) have shown that individual differences in increment width correlated with resting metabolic rate rather than somatic growth. Mosegaard *et al.* (1988) suggested an isometric relationship between resting metabolic rate and otolith growth based on the relationship between changes in the otolith weight of groups of fish and the temperature at which they were held, and extrapolated from general temperature-resting metabolism relationships for fish. However, recent measurement of individual changes in oxygen consumption and increment size indicates that otolith accretion responds more conservatively to a change in temperature than in resting metabolic rate (Wright *et al.*, 2001). The specific dynamic action potential, the metabolic response associated with food intake, also appears to have an influence on otolith accretion rate (Fallon-Cousins, 1999). The process governing accretion rate thus appears to be related to components of the metabolic rate. Given the influence of these components on otolith accretion rate, periods of starvation would only be expected to lead to a gradual decline in increment widths. Evidence for such a response has been found in a number of experimental studies (Neilson & Geen, 1985; Eckmann & Rey, 1987; Molony & Choat, 1990; Umezawa & Tsukamoto, 1991; Bradford & Geen, 1992; Zhang & Runham, 1992a; Molony, 1996).

3.4. Physiological regulation of otolith formation at the sacculus level

Wilbur (1980) suggested that biomineralisation systems had three properties in common:

- all systems involve the transport of ions and provide supersaturated concentrations of ions (i.e. which exceed the solubility product) at the mineralising surface. This enables the formation of crystalline *nuclei* and the growth of crystals;
- a sufficiently alkaline pH must be maintained so that, once begun, mineralisation can continue;
- crystal formation is often intimately associated with organic material.

The observation that otolith increments are composed of a mineral-rich and a mineral-deficient zone (L- and D-zones, respectively) suggests that one or more of the above properties must vary. The periodic deposition of mineral-rich zones may therefore (i) be related to a diurnal physico-chemical limitation in crystallisation (involving either a lowering of the calcium and hydrogen carbonate ion concentration at the otolith surface or a decline in endolymph pH), or (ii) involve the organic matrix. An analogy with the mineralisation of mollusc shells suggests that either the insoluble organic matrix deposited in the mineral deficient layer (D zone) acts as a barrier to crystallisation or crystal growth-inhibiting compounds within the soluble organic matrix are secreted on to the mineral-deficient layer (Wheeler *et al.*, 1981; Wilbur & Saleuddin, 1983). These possible forms of regulation have been considered for otoliths during the past three decades.

The otolith is precipitated from the fluid of the endolymphatic sac of the inner ear. Otolith calcium carbonate is in the form of twinned aragonite, although abnormal crystalline otoliths are composed of calcite (Morales-Nin, 1985) or vaterite (Gauldie, 1986). Calcium reaches the endolymph primarily from the blood plasma (Kalish, 1989, 1990, 1991a; Wright *et al.*, 1992). Otolith calcification is rate-limited by the number of nucleation sites provided by the insoluble matrix (Crenshaw, 1982; Mann *et al.*, 1983) as well as by physico-chemical conditions at the otolith surface. The rate of insoluble matrix production will therefore be the ultimate determinant of the rate of otolith calcification (Saitoh & Yamada, 1989; Wright, 1990). This matrix is also a significant factor controlling the shape of the otolith (Degens *et al.*, 1969; Dunkelberger *et al.*, 1980; Mugiya, 1987; Gauldie, 1991, 1993; Zhang & Runham, 1992b; Payan *et al.*, 1999). As in mollusc shell, otoliths possess a soluble proteinaceous matrix that is capable of regulating the rate of mineral deposition (Wright, 1991b). Variations in the rate of production of this protein may therefore regulate the rate of mineralisation. The less soluble otolith matrix is composed of a collagen-type protein (Degens *et al.*, 1969). The matrix is denser in the early development phase and its amino acid composition changes with age (Morales-Nin, 1986a,b).

Investigations of isolated *sacullae* have indicated that active, regulated ionic transport occurs through the epithelia (fig. II.A.14b). Endolymph calcium ion concentration is influenced by intracellular active transport that is sensitive to plasma calcium concentration. Similarly, proton secretion through the *sacculus* is driven by an energy-dependent (Na-ATPase) mechanism that is sensitive to plasma pH (Payan *et al.*, 1999). Changes in plasma ion concentration would therefore be expected to have a direct effect on that of the endolymph. However, the precise mechanism by which plasma calcium and pH induce changes in the physico-chemical conditions at the otolith surface is not clear. This is because the sensory kinocilia bathed by the endolymph are sensitive to changes in Ca^{2+} concentrations well below the solubility product needed for calcification (Mugiya, 1987; Wright *et al.*, 1992). The seasonal variation in free Ca^{2+} ions in the endolymph of rainbow trout ranges from 65.4% of total calcium levels during fast growth to 79.1% during slow growth (Mugiya, 1966), which probably represents the range over which Ca^{2+} can vary without causing physiological malfunction of the neural mechanisms of the *macula* (Gauldie, 1990). It is thus necessary to explain how ion levels are elevated at the otolith surface above the background concentrations found in the endolymph. Proximo-distal gradients of ion concentration have been detected in the endolymph, a condition which will favour the biomineralisation process (fig. II.A.14b) (Payan *et al.*, 1999). Calcareous spherules have been observed in close association with the otolith surface of a number of fish species and these may be involved in elevating ion concentration at the otolith surface (Dale, 1976; Wright, 1990). These spherules are formed and secreted from the otolithic membrane and are transported to the surface of the otolith within the fibrous sub-cupular meshwork (Dale, 1976; Wright, 1990). Diurnal rhythmicity in otolith calcification may be mediated by a diel variation in plasma chemistry, as Mugiya (1984) and Wright *et al.* (1992) found a parallel diel decline in otolith calcification and total and free plasma calcium concentration. Mugiya (1984) also found a seasonal reversal in the rhythm of otolith calcification associated with a reversal in the diurnal plasma calcium cycle. However, similar cycles in plasma and endolymph composition in *Pleuronectes platessa* (Edeyer *et al.*, 2000) were not associated with changes in the ionic gradients within the endolymph (Payan *et al.*, 1999). Nevertheless, Wright *et al.* (1992) found that an induced depression in plasma calcium led to a net loss of calcium from the mineralising otolith increment, which indicates that calcium ion concentration at the otolith surface is sensitive to plasma concentration. While there may be a periodical ionic limitation to otolith calcification, however, this alone cannot explain reports of a diel variation in matrix secretion (Mugiya, 1987; Wright *et al.*, 1990) or the formation

of matrix-rich layers (Watabe *et al.*, 1982; Morales-Nin, 1987a; Mugiya, 1987). The distribution of matrix and mineral in the otolith appears to occur in two phases. The first is associated with the twinning plane of the basic aragonite crystal (Gauldie & Xhie, 1995). Twinning is a complex process (Bloss, 1971) which stabilises crystal polymorphism and increases the growth rate of the crystal (Smith, 1974; Davey *et al.*, 1993). The second phase of the matrix-mineral association appears in the form of the dense band of fibres that corresponds in size and orientation to the D-zone of the primary increment (Dunkelberger *et al.*, 1980; Morales-Nin, 1987a). This observation is consistent with the diel variation in insoluble matrix protein indicated by radio-labelling experiments (Mugiya, 1987). The two phases of the protein matrix may play different roles, the first being to

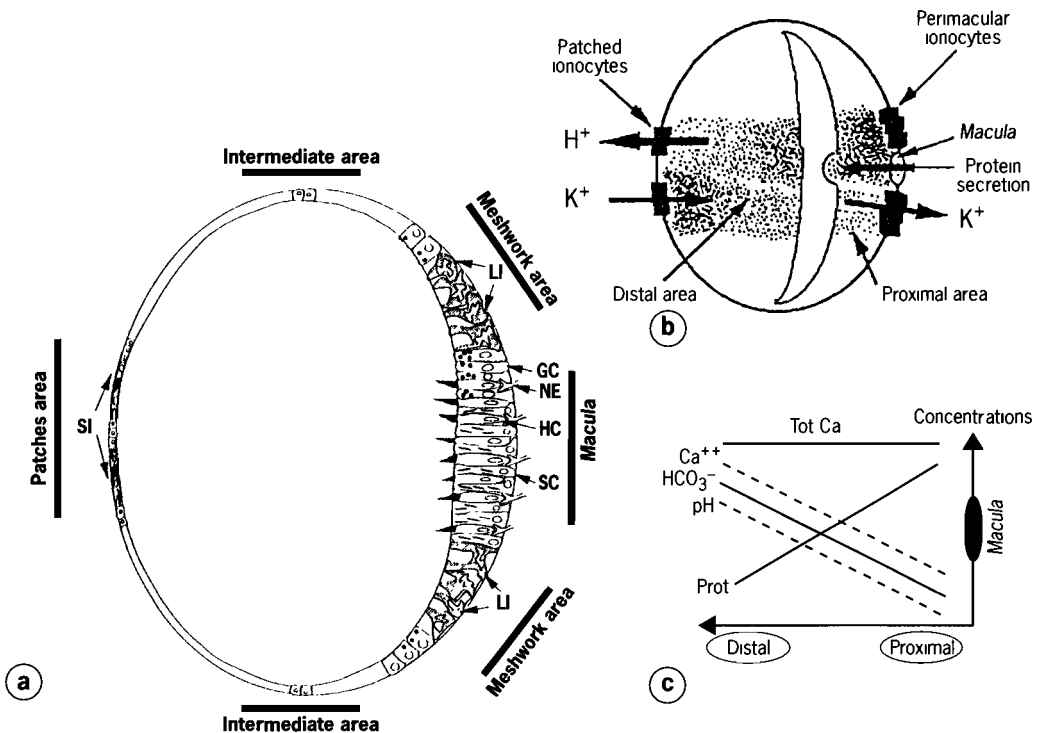


Figure II.A.14 - Schematic representation of the saccular epithelium (transverse section of a sacculus) of the inner ear of a Teleost and the hypothetical model of elemental transport across the epithelium (modified from Pisam *et al.*, 1998; Payan *et al.*, 1999). a) Map of the cell distribution within the saccular epithelium. The macula consists of hair cells (HC), which are in contact with nerve endings (NE), supporting cells (SC) and, at its periphery, granular cells (GC). It is surrounded by a "meshwork area" containing large ionocytes (LI). The "patches area" contains small ionocytes (SI).

b) Hypothetical model of elemental transport across the saccular epithelium. Note that overall movement of H^+ results in net excretion of H^+ .

c) Schematic representation of chemical concentrations in the proximal and distal zones. The Y-axis shows the concentrations while the X-axis shows the proximal-distal otolith axis. Proteins (Prot), total calcium (TotCa) and HCO_3^- concentration were directly measured, whereas Ca^{2+} and pH concentrations were estimated.

provide a template for crystal growth and the second to stabilize the otherwise soluble (Wright *et al.*, 1992) and thermo-dynamically unstable aragonite morph (Mann *et al.*, 1983; Gauldie & Xhie, 1995). It is therefore necessary to consider the regulation of both ion concentrations and matrix production in the periodic deposition of L- and D-zones. Given the correlation between otolith calcification and plasma ion concentration, the concentration of certain ions in the plasma may have a direct effect on cellular secretions of matrix or may covary with some other signalling factor. In addition, the calcification neurosecretory activity in the *macula* has a daily cycle which is related to the deposition of daily increments (Gauldie & Nelson, 1990b; Edeyer *et al.*, 2000). A number of recent studies have identified the function of the different regions within the saccular epithelium and the importance of these regions to otolith growth (Payan *et al.*, 1997; Pizam *et al.*, 1998; Payan *et al.*, 1999) (fig. II.A.14). The secretory cells are mostly located in the macular area. Within the endolymph, proteins are more concentrated in the proximal region, while total CO₂ is higher in the distal region (fig. II.A.14c).

In summary, the evidence to date indicates that the formation of the calcium carbonate-rich L-zone is influenced by intracellular active transport of calcium ions (Mugiya, 1986) and protons through the *sacculus* (Payan *et al.*, 1997; Payan *et al.*, 1999) which in turn are sensitive to plasma calcium concentration and pH (Wright *et al.*, 1992; Payan *et al.*, 1997). Secretion of the proteinaceous matrix varies diurnally with a peak during the formation of D-zone (Mugiya, 1987; Edeyer *et al.*, 2000). Production of the protein matrix template and the soluble protein inhibitor must also have a role in limiting the rate of mineral accretion during the formation of the L-zone.

3.5. Hormonal regulation of otolith formation

The mechanisms that determine periodic mineralisation are probably under endocrine control (Campana & Neilson, 1985; Mugiya, 1987), either directly or indirectly via metabolic influences (Geffen, 1983; Mosegaard *et al.*, 1988). Growth hormone (STH) may also be involved, since hypophysectomy has been found to produce a reduction in otolith growth (Mugiya, 1990) and otolith demineralisation (Simkiss, 1974), and otolith mineralisation in hypophysectomised fish can be restored by injections of pituitary extract (Simkiss, 1974). Such hormonal regulation could influence both ion transport and matrix production in the *sacculus*. Wright *et al.* (1992) suggested that as plasma calcium concentration is regulated by hyper- and hypo-calcemic hormones, diel changes in the plasma concentration of these hormones may be indirectly responsible for the periodic decline in otolith calcification. Moreover, carbonate crystallisation in molluscs involves neu-

ral control (Zylstra *et al.*, 1978). Neural control of calcium concentration in the *sacculus* provides a physiological explanation for the direct tracking of seasonal and daily total calcium levels of the blood plasma by the endolymph (Mugiya & Yoshida, 1995).

Entrainment to light-dark cycles suggests the involvement of the pineal-hypophysial complex. Endocrine secretion displays a circadian periodicity in many animals and through the intermediary of metabolic rate, ultimately controls most physiological processes (Simpson, 1978). Endocrinological studies have demonstrated diurnal variations in the levels of several hormones in the plasma of Teleosts (Matty, 1985). These include thyroxine (T₄) (Eales *et al.*, 1981) a hormone known to influence skeletal growth and calcification in rainbow trout (La Roche *et al.*, 1966).

B. Scales

F.J. Meunier

1. Description, diversity and function

Fish scales display a high degree of polymorphism (Goodrich, 1907; Van Oosten, 1957; Bertin, 1958; Whitear, 1986). Forming in the upper part of the dermis (Fig. II.B.1a), they are mineralised elements that belong to the superficial skeleton. This book essentially deals with the Teleosts, so we shall only discuss the scales of this taxa. The teleostean scales belong to the elasmoid type like the scales of the Amiidae, the Coelacanthidae and the Dipnoi (Goodrich, 1907; Kerr, 1952; 1955; Castanet *et al.*, 1975; Meunier, 1980; 1984a; Meunier & Zylberberg, 1998). Some other “primitive” Osteichthyan taxa, such as the gars and the bichirs, have ganoid scales, whose structure differs from that of elasmoid scales (Goodrich, 1907; Kerr, 1952; Francillon-Vieillot *et al.*, 1990; Zylberberg *et al.*, 1992). These are thick and juxtaposed scales with a surface of polystratified hypermineralised substance, the ganoïne, which has an epidermal origin (Meunier *et al.*, 1987; Sire *et al.*, 1987). The bony basal plate of these ganoid scales shows growth marks, probably with an annual cycle, but they have never been used for ageing studies except in paleo-ichthyological work (Thomson & McCune, 1984). The elasmoid scales are usually regarded as being of dermal origin (Zylberberg *et al.*, 1992) whereas ganoid scales are of epidermo-mesodermal origin (Sire *et al.*, 1987).

Two forms of elasmoid scales have been described (fig. II.B.2): ctenoid and cycloid, according to whether or not they possess more or less minute spines on their posterior margin (Goodrich, 1907; Bertin, 1958). The general structure of these scales, cycloid and ctenoid, is the same. The spines of ctenoid scales show various morphologies (Roberts, 1993) but this has no consequences for the cyclical growth marks. Whatever the type of elasmoid scales (cycloid or ctenoid), the superficial layer in most species displays concentric ridges which are frequently crossed by radial groves, the *radii*.

Elasmoid scales can be divided into two main regions (fig. II.B.1b): the anterior (or covered area) and the posterior (or covering area). They are deeply inserted into the dermis and enclosed in a scale-pocket (Sire, 1988). The scale-pocket, which is associated exclusively with elasmoid scales, is the dermal space which houses the scale. It is clearly delimited from the subjacent *stratum compactum* by the “scale-pocket lining” a bilayered sheet of fibroblast-like cells (Whitear *et al.*, 1980). Elasmoid scales are imbricate, thin lamellar plates (Bertin, 1944) that consist of two layers (Bertin, 1958; Zylberberg *et al.*, 1992)

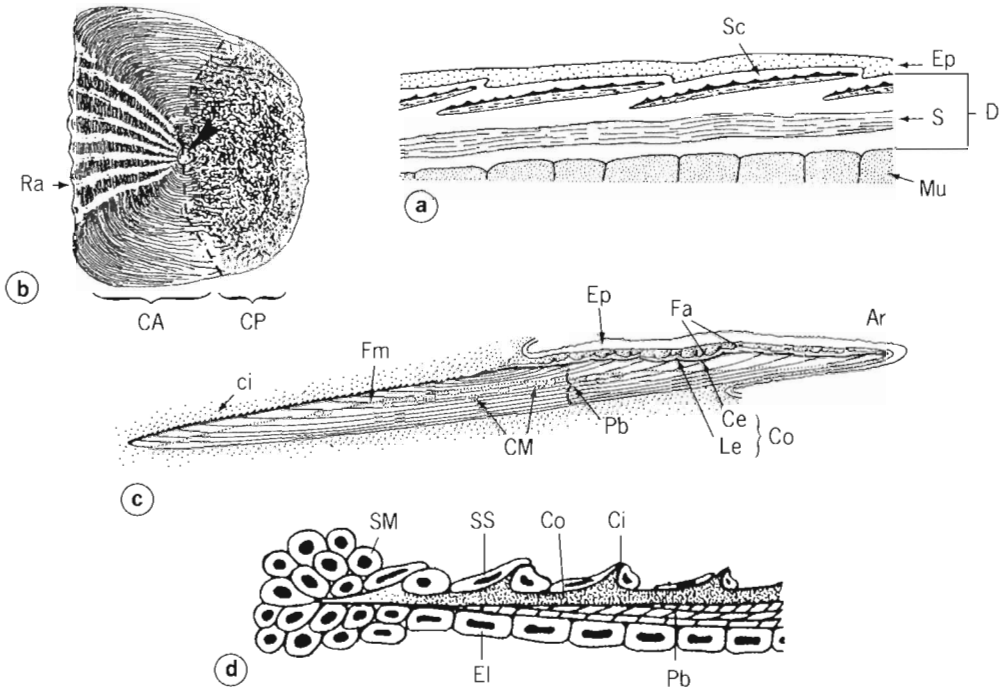


Figure II.B.1 - a) Antero-posterior section in the tegument of a Teleost fish showing the imbricate scales obliquely inserted in the dermis. Ep = epidermis; D = dermis; Mu = muscles; S = *stratum compactum*; Sc = scale. b) Superficial view of a typical elasmoid scale (Cichlidae); CA = anterior area; CP = posterior area; Ra = *radius*. c) Diagram of a longitudinal section of an elasmoid scale (adapted from Sire, 1985). Ar = posterior; Ce = external layer; Ci = *circulus*; Co = bony layer; CM = Mandl's corpuscles; Ep = epidermis; Fa = attach fibers; Fm = mineralising front; Le = limiting layer; Pb = basal plate. d) Cross section in the margin of an elasmoid scale showing the scleroblasts of the episquama (upper), the hyposquama (lower) and of the margin (left). Ci = *circulus*; Co = bony layer; EL = elasmoblast; Pb = basal plate; SM = marginal scleroblast; SS = superficial scleroblast (photos F.J. Meunier).

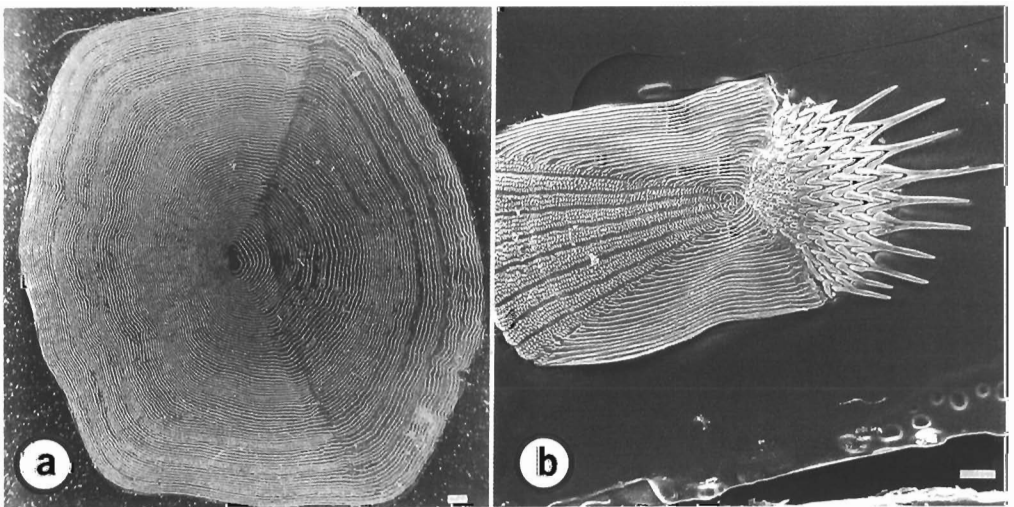
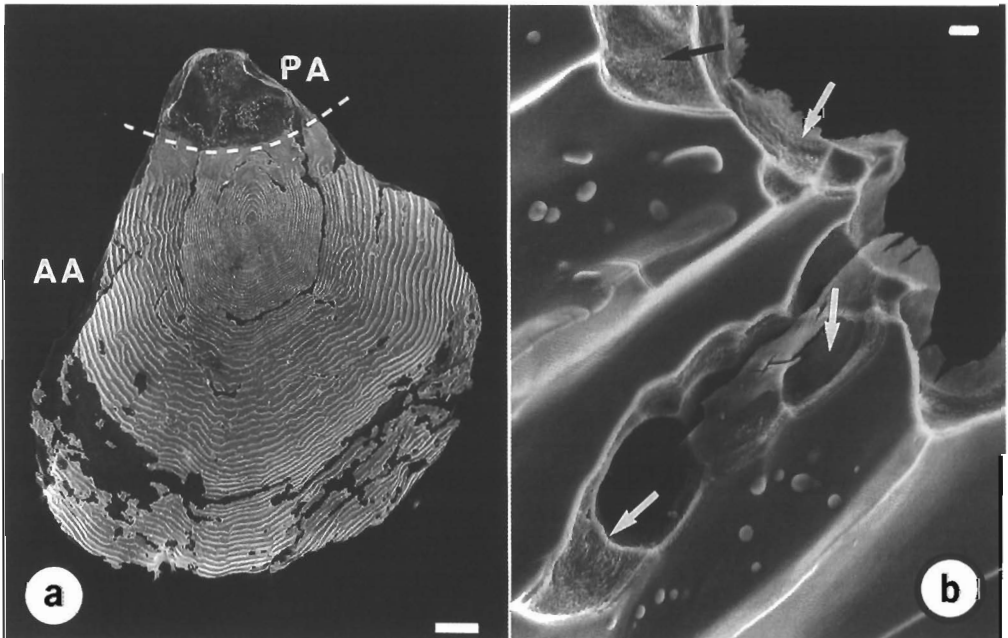


Figure II.B.2 - Superficial side of elasmoid scales (SEM). a) Cycloid scale of *Coregonus lavaretus* (Salmonidae, Salmoniformes). Scale bar = 500 μ m. b) Ctenoid scale of *Microchirus azevia* (Soleidae, Pleuronectiforme). (the anterior part of the scales is on the left). Scale bar = 250 μ m (photos F.J. Meunier).

never been used as an indication of external cyclical phenomena. Moreover, they do not obscure previously deposited *circuli* and do not interfere with the ability to interpret age.

Cyclical growth marks have been described only on the surface of the scales and they can be studied using classical light microscopy techniques or, if necessary, with SEM. Cyclical events such as seasonal metabolic slowing, spawning maturation, etc., induce morphological modifications of the ornamentations, especially the ridges or *circuli* which become much narrower and, then form an *annulus*. Frequently, when the scales grow again during the spring, the new *circuli* are more or less discordant on the ones which had been deposited during the previous year. In other circumstances, the *annulus* may be strengthened by a marginal process of erosion. This is the case in migratory Salmonids (Crichton, 1935; Van Someren, 1937; Richard & Baglinière, 1990) (fig. II.B.5) and menhaden (June & Roithmayr, 1960), for example. So far, however, no study has demonstrated that cyclical climatic variations can also have repercussions on the histological organisation of the basal plate. The older the fish, the more numerous are the *strata* in the basal plates of the scales. However, studying the number of *strata* in the basal plate failed to age coelacanth (Sire, personal communication), whereas the growth marks of the superficial layer gave an interpretation of age (Hureau & Ozouf, 1977).

Figure II.B.5
Surface of scales of a male salmon showing erosion areas. a) The whole scale. Scale bar = 500 μ m. b) Detail of the erosion cavities. Scale bar = 5 μ m. AA = anterior area; PA = posterior area (photos A. Kacem).



In Elasmoid scales, the external layer and the limiting layer are regularly mineralised, whereas mineralisation of the basal plate is incomplete (Meunier, 1984a,b; Zylberberg *et al.*, 1992). At the location of the *radii* the superficial layer lacks mineralisation (Sire & Meunier, 1981; Meunier, 1984b; Zylberberg *et al.*, 1992). The specific organisation of the collagenous component of the organic matrix (plywood-like structure) results in an original mineralisation route: Mandl's corpuscles (Baudelot, 1873; Schönbornner *et al.*, 1981; Zylberberg *et al.*, 1992). The diameter of the fibrils is significantly greater than that of

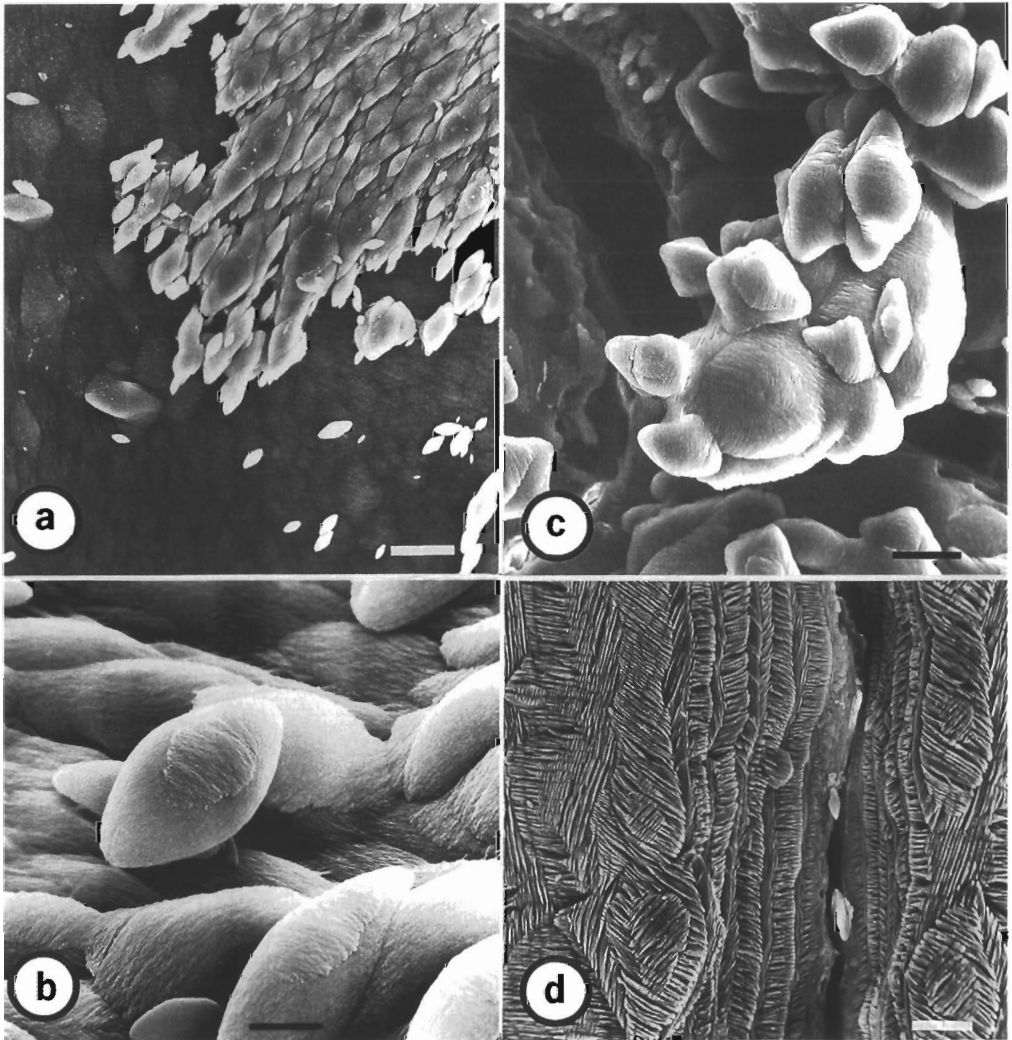


Figure II.B.4 - Mineralising front of the basal plate (SEM). a) Scale of *Esox lucius*; the surface of mineralising front is smooth. Scale bar = 50 µm. b) Detail of Mandl's corpuscles in a scale of *Astronotus ocellatus*. Scale same as (c). c) Fusing Mandl's corpuscles in a scale of *Ophicephalus striatus*. Scale bar = 50 µm. d) Detail of the mineralising front of the basal plate in a scale of *Hoplias aimara*. The mineralising front is rough (numerous cracks) because of the presence of TC fibres. Scale same as (c) (photos F.J. Meunier).

bone collagenous ones; this peculiarity seems to induce the specific mineralising process with the Mandl's corpuscles, which have a characteristic geometrical shape (fig. II.B.4a,b). They develop ahead of the mineralising front of the basal plate, then fuse with each other (fig. II.B.4c) before merging with the mineralising front which appears smooth (fig. II.B.4a,c) when uncalcified organic matrix is removed (Sire & Meunier, 1981; Zylberberg *et al.*, 1992). When they are present, TC fibres also occur in the mineralisation process of the basal plate (Zylberberg & Nicolas, 1982; Zylberberg *et al.*, 1992, *inter alia*); in such cases the mineralising front is rough (fig. II.B.4d) (see also Meunier, 1997).

Being mineralised, the scales provide an internal reservoir of minerals, especially of calcium (Simkiss, 1974; Takagi *et al.*, 1989) which can be depleted under specific physiological circumstances (Mugiya & Watabe, 1977; Persson, 1997, *inter alia*) such as in adult salmon during the transition to maturity (Persson *et al.*, 1998; Kacem & Meunier, 2000).

Among the function of elasmoid scales, there are essentially protection of the body and hydrodynamic functions (see Burdak, 1979). An important peculiarity of elasmoid scales is their ability to regenerate. Teleost fish lose a certain proportion of their scales in the course of their life (McCart, 1967; Fouda, 1979; Shackleton, 1988). When a scale is lost, the scale-pocket is usually undamaged and it remains as an empty space in which a new scale rapidly regenerates (Sire, 1988; Bereiter-Hahn & Zylberberg, 1993). The regenerated scale resembles its predecessor but its superficial ornamentations differ from that of the ontogenetic scale, while the previous growth marks disappear (Neave, 1940; Sire, 1987) so that the new ones are unusable for age estimation. The number of regenerated scales grows with age, which may make it difficult to count the growth marks. For this reason, it is necessary to sample an adequate number of scales in order to age fish by means of scalimetry.

2. Growth of scales and growth marks

Two processes are involved in the growth of scales: 1) the surface of the external layer increases owing to the activity of marginal scleroblasts (fig. II.B.1d) which are osteoblast-like cells; 2) the basal plate thickens with the deposit of new collagenous *strata* synthesized by specific scleroblasts (forming the hyposquama), the elasmoblasts which underlie the deep surface of the scale.

It is generally believed that the external layer does not thicken during the growth of fish, at least in the anterior area of the scale (Zylberberg *et al.*, 1992). However in some cases, this layer does show thickening, which appears as thin incremental lines (Meunier, 1997) but they have

(fig. II.B.1c); a thin ornamented superficial layer, “the external layer”, which overlies a thick lamellar, partially mineralised basal plate with a characteristic plywood-like structure called isopedin, which is regarded as a derived bony tissue (Meunier, 1987-1988). In the posterior region of the scale which is overlaid by the epidermis, a limiting layer overlies the external layer (Schönbörner *et al.*, 1979). In some cases, specific collagenous fibrils inserted in the external layer of the posterior area cross the limiting layer and fasten on to the basal membrane of the epidermis (fig. II.B.1c); these are called anchoring fibres and they are believed to strengthen the cohesion between the scale and the epidermis during swimming (Zylberberg & Meunier, 1981; Sire, 1985). Depending on the species, the collagenous fibres of the basal plate are organised in two main spatial networks either as a double-twisted “plywood” structure (fig. II.B.3a), usually in the taxa of the lower Teleosts, or as an orthogonal “plywood” (fig. II.B.3b) which characterises derived groups (Meunier & Castanet, 1982; Meunier, 1987-1988). Generally speaking, the diameter of these fibres, which lie parallel to each other in a given *stratum*, is greater (50 nm to 150 nm) than the diameter of the collagenous fibres of typical bone (20-40 nm) (Meunier, 1987-1988). Another type of collagenous fibrils, the TC fibres which are orthogonal to the *strata* of the basal plate, has been described in several taxa, specially in Cyprinid and Characiforme fishes (Zylberberg & Nicolas, 1982; Zylberberg & Meunier, 1996; Meunier, 1997).

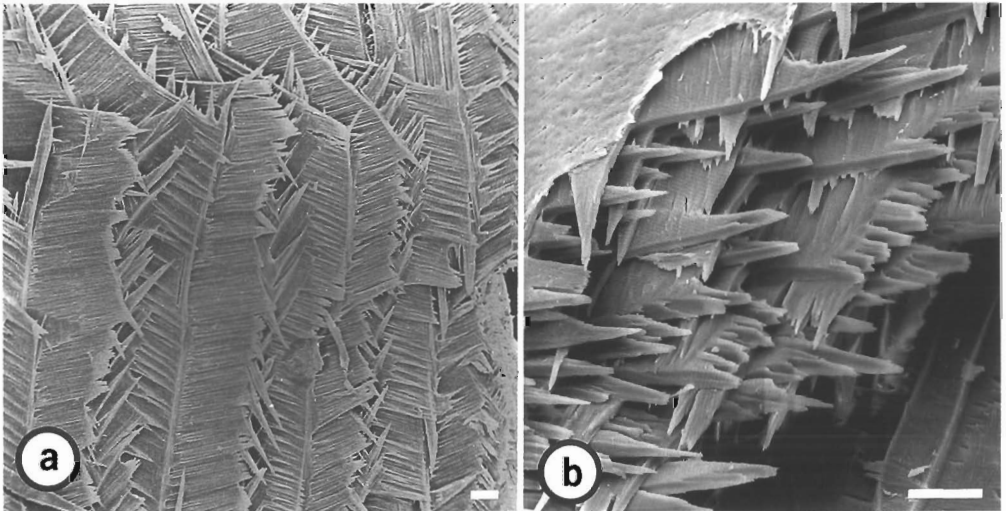


Figure II.B.3 - Freeze-fractured scales (SEM): a) *Leporinus friderici* (Anostomidae, Characiformes). Scale bar = 100 μ m. b) *Macrourus berglax* (Macrouridae, Gadiformes). Scale bar = 25 μ m (photos F.J. Meunier).

On the other hand, growth marks are obvious in the bony basal plate of ganoid scales. They show the same histological characteristics as the growth marks of the bony skeleton, i.e. a wide area of rapid growth and a narrow area of slow growth which may be hypermineralised as in bichir scales (Meunier, 1980). For this they will be discussed along with annual bone marks.

3. Regulation of growth marks

In our opinion, the various factors involved in the regulation of scale growth marks are the same as those governing skeleton growth marks. They may both show annual growth rhythms but, to the best of our knowledge, no accurate infra-annual cycle (lunar or daily growth marks) has been described, unlike in the case of the otoliths (see before). We therefore consider the regulation of scale growth marks in the following chapter, which is devoted to skeleton growth marks (see chap. II.C.3).

C. Skeleton

F.J. Meunier

1. Morphology of bones and their structural organisation

In Osteichthyans, as in Tetrapods, the term “bone” can refer to various concepts:

- an anatomical organ such as a vertebra, *operculum*, or spiny fin ray;
- a tissue, i.e. the bone tissue which makes up all bone;
- chemical constituents, i.e. the organic macromolecules and mineral crystallites characteristic of bone tissues. To avoid any confusion, Petersen (1930) defined four successive levels of integration of bone corresponding to four different modes of investigation (tab. II.C.1, see also Francillon-Vieillot *et al.*, 1990). It is essentially the first and second levels of integration which are involved in studies of age estimation. But for a better understanding of the histophysiology of bone in relation to the various cyclical and non-cyclical constraints that determine bone structure, we also briefly describe the third and fourth levels of integration.

Table II.C.1. - Levels of integration of bone (modified from Petersen, 1930; Francillon-Vieillot *et al.*, 1990).

Order of structure	Approximate scale	Structures characteristic of the order	Examples of techniques used	Related biological problems
First order	1m-1mm	Anatomical level of integration Bone morphology, vascular orientation, <i>trabeculae</i>	Dissection, X-ray, sawing; Observation: naked eye, binocular microscopy	Comparative anatomy and morphology of the skeleton; Overall growth
Second order	1 mm-100 µm	Histological level of integration Orientation, size, number of <i>trabeculae</i> , vascular canals; structure of extracellular matrix	Histology, histochemistry, micro X-ray, labelling <i>in vivo</i> , Optical microscopy, SEM	Classification of tissues, growth and remodelling processes; Bone histophysiology
Third order	100 µm-1 µm	Cytological level of integration Details of cells, extracellular matrix: orientation, amount, organisation	Cells; High power of light microscopy, polarizing microscopy, SEM, TEM	Cytology, cytochemistry of bone cells; phosphocalcic metabolism at cellular level. Ultrastructure of bone matrix
Fourth order	1 µm-10 nm	Molecular level of integration chemical and biophysical organisation of organic and mineral components	TEM, X-ray diffraction, microprobe, biochemical and molecular techniques	Ultrastructural relationships between cell organelles and extracellular matrix; biochemistry of bone matrix and bone mineralisation

1.1. Constituents of bony tissue

In Osteichthyans, as in Mammals, bony tissues are made up of an organic matrix, mineral constituents and various types of cell (Francillon-Vieillot *et al.*, 1990; Meunier & François, 1992a). The organic matrix is a network of more or less oriented collagenous fibrils embedded in various complex molecules such as the proteoglycans (Glimcher, 1998). The mineral constituent consists essentially of hydroxyapatite which produces crystallites that deposit directly on the collagenous fibrils. The mineralisation of bony tissues in Osteichthyans is an inotropic process, i.e. there is an interaction between collagen fibrils and mineral crystalites (see Ørving, 1968 and chap. II.C.1.1.3).

1.1.1. Cells

The bony cells (scleroblasts of Klaatsch, 1890) are of three types: 1) the osteoblasts, which are localised on the bony surfaces and which deposit the organic and mineral constituents of bone; 2) the osteocytes, which are embedded in the bony substance and which play a trophic function in bony tissue; 3) the osteoclasts, whose main role is the destruction of bone (Francillon-Vieillot *et al.*, 1990; Sire *et al.*, 1990; Ricqlès *et al.*, 1991; Meunier & François, 1992a).

The osteoblasts first synthesise the proteic macromolecules, essentially collagen, in front of the bone surface. They are then progressively surrounded by the organic matrix, subsequently forming osteocytes. They also contribute to the mineralisation of the organic matrix after it is deposited. When osteoblasts are relatively numerous on the external surface of a bone, they form a sort of thick membrane, the periost. When they lie on the surface of vascular cavities they form the endost (Francillon-Vieillot *et al.*, 1990).

The osteocytes are usually more or less “star-shaped” cells with numerous branched cytoplasmic extensions in the extracellular matrix (fig. II.C.1a) (Stephan, 1900). However, a specific characteristic of many Teleosts is the lack of osteocytes in the bones, especially in the more highly evolved groups such as the Acanthomorpha (perch, wrasse, etc.) (Kölliker, 1859; Blanc, 1953; Moss, 1961a,b, 1965; Meunier, 1983; Hughes *et al.*, 1994). It is likely that more than half of the 23 600 known Teleost species (Nelson, 1994) have acellular bone (anosteocytic bone of Weiss & Watabe, 1979). It is generally believed that the lack of osteocytes in acellular bone is a result of the withdrawal of the osteoblasts ahead of the organic matrix front as soon as they have synthesized bony substance (Moss, 1963; Meunier, 1987). Nevertheless, in some species primary bone (see chap. II.C.1.3.2) is crossed by cytoplasmic extensions originating from superficial osteoblasts (fig. II.C.2) (Moss, 1965; Meunier, 1983, 1987; Meunier

& Huyseune, 1992; Hughes *et al.*, 1994). These cytoplasmic extensions may be more or less ramified in the bony tissues. As far as skeletochronology is concerned, the cellular nature of bony tissue, i.e. cellular bone versus acellular bone, is of no great importance for the seasonal rhythmic growth of bones, and many acellular bone species

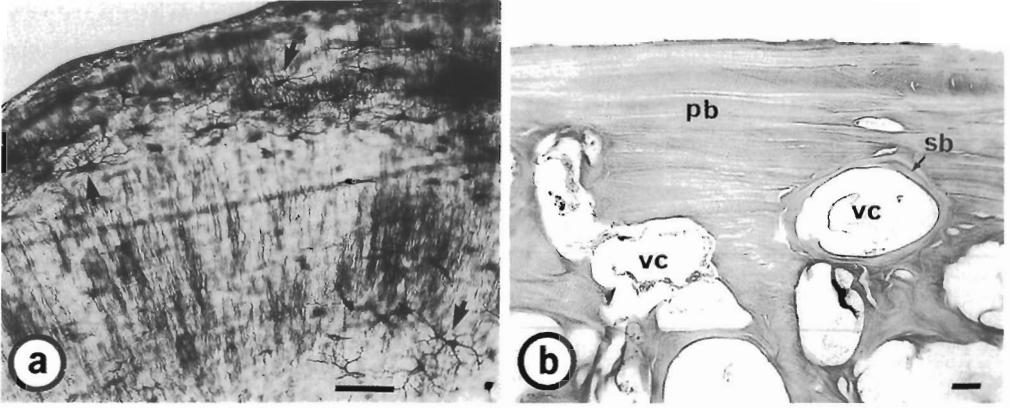


Figure II.C.1. a) Cellular bone (ground section, transmitted natural light) in the frontal of *Arius proops* (Siluriformes) showing typical star-shaped osteocytes (arrows) with numerous cytoplasmic processes; Scale bar = 50 µm. b) Acellular bone in the supraoccipital (cross section, Masson's trichrome staining) of *Trachurus trachurus* (Carangidae); pb = primary bone, sb = secondary bone, vc = vascular cavity or canal. Scale bar = 100 µm (photos F.J. Meunier).

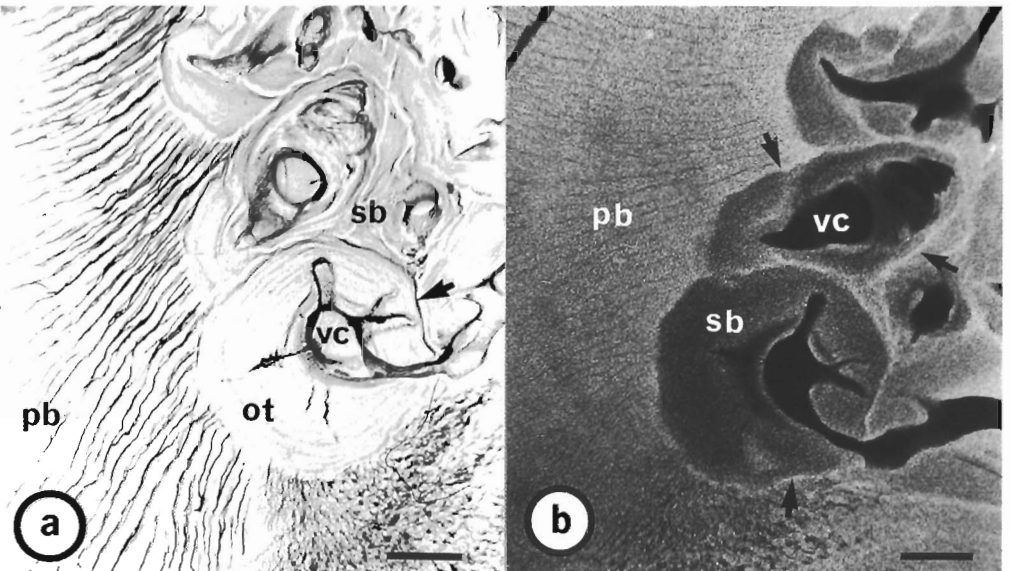


Figure II.C.2 - a) Acellular bone (ground section, transmitted natural light) in a dorsal fin ray of *Lethrinus nebulosus* (Perciformes, Lethrinidae) showing numerous osteoblastic canaliculi in the primary bone. Secondary bone is limited by cementing reversal lines (arrow) and is devoid of canaliculi. b) Microradiography of the same section showing the hypermineralised cementing lines (arrows). Secondary bone is less mineralised than primary bone; ot = osteon; pb = primary bone, sb = secondary bone, vc = vascular cavity or canal. Scale bar = 100 µm (photos F.J. Meunier).

which show growth marks are aged on the basis of the study of their bones (Casselman, 1974; Meunier *et al.*, 1979; Johnson & Saloman, 1984, *inter alia*).

The osteoclasts, whose existence was denied for several decades (Blanc, 1953; Moss, 1963) have been now generally recognised and accepted (López, 1973; Meunier, 1983; Glowacki *et al.*, 1986) in both cellular and acellular bone. But unlike mammalian bone, in which they are typically multicellular, they are frequently unicellular in fish bone (Sire *et al.*, 1990). The osteoclasts destroy bony tissues, forming a depression on the bone surface: Howship's *lacuna*. When several Howship's *lacunae* merge, the osteoclasts may create fairly wide cavities. After some time, these cavities may be more or less filled with new tissue known as "secondary" bone (see chap. II.C.1.3.2). This process of erosion and reconstruction is called bone remodelling (Enlow, 1963). If the remodelling of bone is very developed it may contribute to the disappearance of growth marks and, thus lead to underestimates of age. For this reason, it is essential to know how to recognise the process of bone remodelling (see below) for age estimation studies by means of bone histology.

1.1.2. Organic matrix

The main part of the organic matrix is formed by the collagenous fibrils; in mammalian bone it is thought that collagenous fibrils represent 23 to 32% of the dry weight (Casselman, 1974), depending on the degree of mineralisation, the collagenous fibrils being lower when bone is hypermineralised (Herring, 1972). These fibrils are embedded in complex proteoglycans that play various poorly understood roles. The collagenous fibrils can be divided into two main categories: the intrinsic fibrils that constitute the frame of bone and the extrinsic fibrils which have an anchoring function for various structures (generally ligaments and tendons) in bone. Sharpey's fibres are extrinsic collagenous fibrils (Francillon-Vieillot *et al.*, 1990; Meunier & François, 1992a); they are made of closely-packed collagen fibres (= bundles) which insert more or less perpendicular into the collagenous frame of bone (fig. II.C.3a). These Sharpey's fibres when present warrant that bone is primary (see below).

The collagenous intrinsic fibrils make up a three-dimensional network, whose organisation is well known and which corresponds to specific biological constraints. Three spatial models have been described for collagenous arrangement in bone: "woven-fibre bone matrix", "parallel-fibre bone matrix" and "lamellar bone matrix" (Francillon-Vieillot *et al.*, 1990; Ricqlès *et al.*, 1991). First described in mammalian bone, these three spatial arrangements of collagenous matrix have been also recognised in fish bone (Meunier & François, 1992a; Meunier & Huisseune, 1992). Collagenous fibrils look like

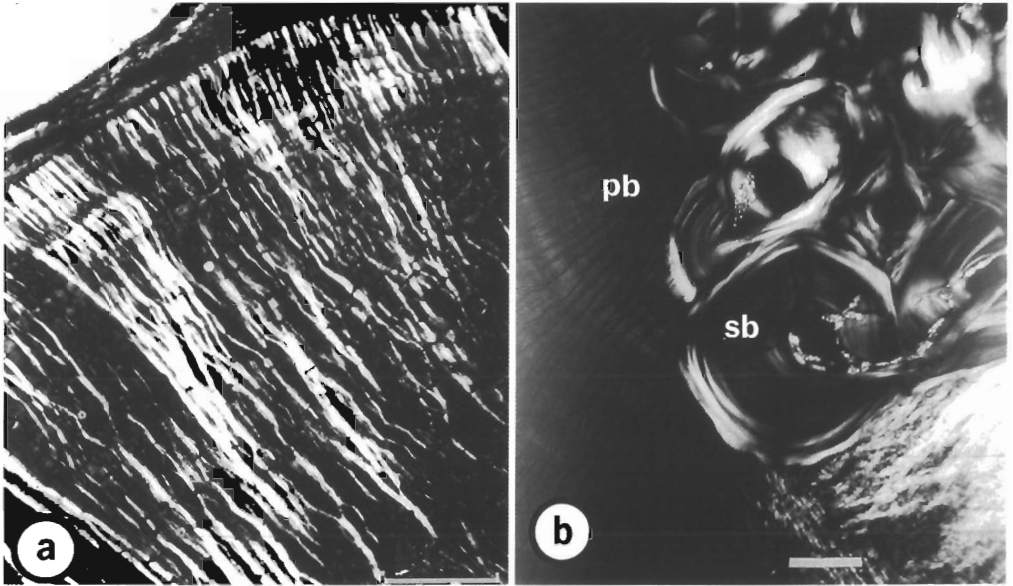


Figure II.C.3

a) Sharpey's fibers in the dorsal spiny ray (cross ground section, transmitted polarised light) of *Cyprinus carpio* (Ostariophysi, Cyprinidae). They are bright and orthogonal to the bone surface.

b) Acellular bone (ground section, transmitted polarised light) in a dorsal fin ray of *Lethrinus nebulosus* (Perciformes, Lethrinidae) showing lamellar secondary bone; pb = primary bone, sb = secondary bone. Scale bar = 150 μ m (photos F.J. Meunier).

paracrystalline structures and as such they deviate polarised light. This property is used to study the orientation of the collagenous fibres in bony tissues.

The woven-fibre bone is formed of loosely packed coarse collagen fibres of different sizes; they are distributed without any ordered spatial arrangement. Under polarised light, woven-fibre bone looks dark in cross-section. When present, the osteocytes (in cellular bone) are randomly distributed and they are rather round, star-shaped and elongated by a large number of ramified cytoplasmic processes that run in very fine tunnels called *canaliculi*. The poor spatial organisation of woven-fibre bone is interpreted as being characteristic of a rapid process of osteogenesis, as takes place in embryonic bone (Castanet *et al.*, 1992; Castanet *et al.*, 1993).

In parallel-fibre bone, also known as "pseudo-lamellar bone", the collagenous fibrils are closely packed and all have the same general orientation. They run approximately parallel to each other and parallel-fibre bone appears homogeneously dark or light under polarised light, according to the orientation of the histological section. The osteocytes are flattened and randomly distributed and their cytoplasmic elongations preferentially run in the same direction as the major axis of the cell. Parallel-fibre bone is regarded as being intermediate between typical woven and lamellar bone as far as its physiological significance is concerned (Castanet *et al.*, 1992, 1993).

Lamellar bone is the most highly organised. It is formed of successive thin layers (or *lamellae*) within which the closely-packed collagen fibrils lie in parallel but the direction of fibrils changes from one *lamella*

to the next, at a mean angle of close to 90° . This regular organisation gives rise to alternate dark and light layers under polarised light and corresponds to a plywood-like structure (Giraud-Guille, 1988). The osteocytes are generally more or less flattened and localised between two *lamellae*. Lamellar bone is generally regarded as being associated with a low rate of osteogenesis, at least in primary bone (Castanet *et al.*, 1993). A plywood-like structure is also well developed in the basal plate of elasmoid scales, where it forms isopedin with two main types of spatial arrangement (Meunier, 1987-1988). The first, the orthogonal "plywood", looks very similar to lamellar bone where the angle of the fibrils between two consecutive *lamellae* is 90° , but with a diameter of fibrils significantly higher and thicker *lamellae* in isopedin. In the second type, the twisted "plywood", there are two systems that fit together (odd and even), in each of which the fibrillary direction rotates more or less regularly with an angle clearly different from 90° (Giraud *et al.*, 1978; Meunier & Castanet, 1982). The rotation is sinister in the elasmoid scales of the Actinopterygii (Meunier & François, 1980; Meunier & Castanet, 1982; Meunier, 1987-1988) and dexter in the elasmoid scales of the Sarcopterygii (Giraud *et al.*, 1978; Meunier & François, 1980).

1.1.3. Mineral content

The mineralisation of bone is a complex phenomenon (Glimcher, 1998). In this treatise, we limit ourselves to a brief survey of the topic. The mineral content consists of calcium phosphate, which forms crystallites close to hydroxyapatite (Glimcher, 1998). However, fish bone mineral also contains a significant amount of carbonate as well as very small amounts of several other components such as fluoride, citrate and ions: Mg, Si, Sr, Mn, Ba, (Francillon-Vieillot *et al.*, 1990; Zylberberg *et al.*, 1992). While it used to be believed that an amorphous phase preceded crystallite deposition (Termine, 1972), it is now asserted that the "amorphous" hydroxyapatite is in fact made of minute crystallites (Glimcher, 1998). However, the high level of whitlockite in several Osteichthyan species shows that the crystallisation of calcium phosphate is not perfect (see also Baud, 1978; Tochon-Danguy & Schönborn, 1981).

The mineral deposits are always formed shortly after the organic matrix. The surface of the organic matrix, which is mineralising, forms the front of mineralisation. The delay between the synthesis of the organic matrix and its mineralisation may lengthen significantly, especially in the basal plate of scales (see above). Moreover, in these tissues, the diameters of the collagenous fibrils are larger than those of bone collagenous fibrils. This peculiarity seems to be responsible for the particular mineralising process involving the corpuscles of Mandl,

which are geometrical mineral concretions that develop ahead of the mineralising front of the basal plate before merging with it (Schönborner *et al.*, 1981).

The organic bone matrix controls the orientation of the hydroxyapatite crystallites which are usually oriented along the collagen fibrils. In such cases, mineralisation is regarded as being inotropic (Ørvig, 1968). The converse situation is spheritic mineralisation characterised by a radiating organisation of crystalites independent of the collagenous fibrils which leads to mineralised globules, but this is relatively rare (Francillon-Vieillot *et al.*, 1990).

There are two ways of measuring the quantity of mineral in bone: the rate of mineral and the degree of mineral. The rate of mineral, which is obtained through the incineration of dry bone, gives a global assessment of the mineral constituents in bone; values usually lie between 60 and 70% of the dry weight in Osteichthyans (Moss & Freilich, 1963; Casselman, 1974; Meunier, 1983; Casadevall *et al.*, 1990; Kacem *et al.*, 2000). The degree of mineralisation involves complex technical methods (Boivin & Baud, 1984) but provides fine data at the histological level, measuring local variations in mineral levels that may be of physiological significance. The degree of mineralisation of bone is expressed in terms of the mass of hydroxyapatite per unit volume i.e. g/cm^3 (Boivin & Baud, 1984). In Osteichthyans the few values available lie between 0.8 and 1.35 g/cm^3 (mean values, see Meunier, 1983). The degree of mineralisation seems to be higher in acellular than in cellular bone (Meunier, 1983; 1984a).

1.2. Spatial organisation of bony tissue

The description of cell types, of the organic matrix and its spatial organisation of collagenous fibrils, and of the mineral component allow the characterization of bony tissues (Francillon-Vieillot *et al.*, 1990; Ricqlès *et al.*, 1991; Meunier & François, 1992a). However, certain other accurate structural measurements may offer complementary data that will be useful in deciphering temporal and physiological information, which are hidden in the bony tissue. Such data include the cementing lines and the vascular network of bone.

1.2.1. Cementing lines

Close study of stained histological preparations can show a number of chromophylic lines in the extracellular matrix which may or may not be repetitive (fig. II.C.4). They are usually very narrow and can be stained with haematoxylin and Periodic Acid Schiff. They are bright under transmitted light or dark in reflected light on unstained ground sections. There are two kinds of cementing lines: the "resting lines" and the "reversal lines". The resting lines or arrested growth lines

(AGL) (in French: “lignes d’arrêt de croissance”; LAC) are concordant with the bone *lamellae* (fig. II.C.4a). In carp, the only fish in which their ultrastructure is known, they are approximately 2.5 μm thick and they consist of thin fibrils, 5 nm in diameter (Castanet, 1981).

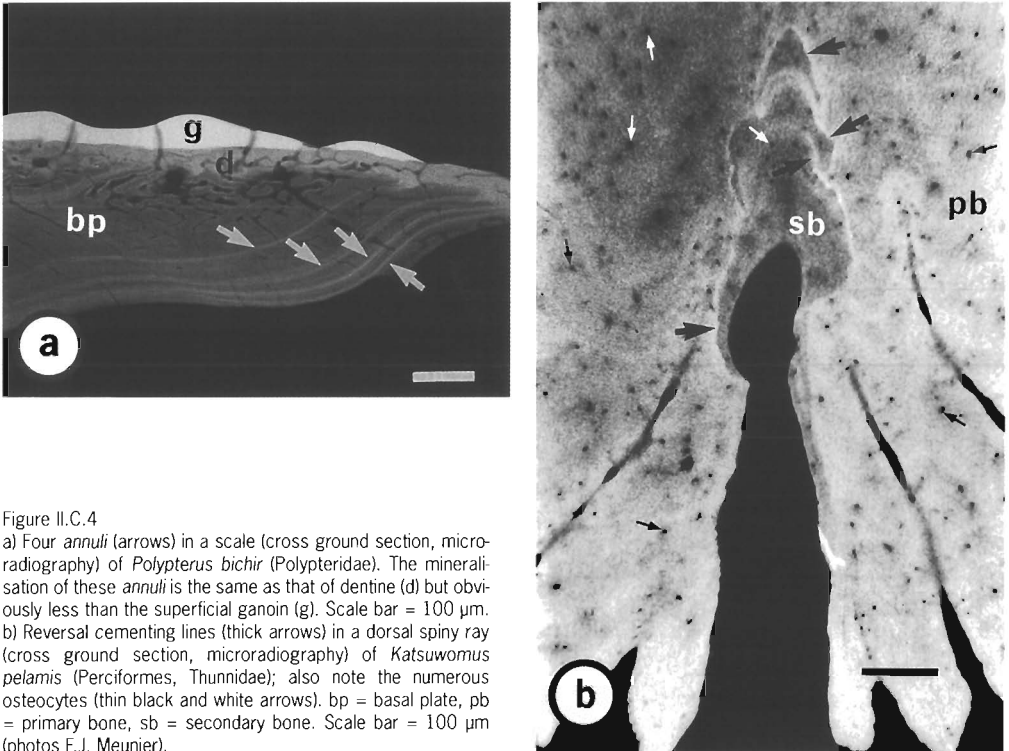


Figure II.C.4

a) Four annuli (arrows) in a scale (cross ground section, micro-radiography) of *Polypterus bichir* (Polypteridae). The mineralisation of these annuli is the same as that of dentine (d) but obviously less than the superficial ganoin (g). Scale bar = 100 μm . b) Reversal cementing lines (thick arrows) in a dorsal spiny ray (cross ground section, micro-radiography) of *Katsuwonus pelamis* (Perciformes, Thunnidae); also note the numerous osteocytes (thin black and white arrows). bp = basal plate, pb = primary bone, sb = secondary bone. Scale bar = 100 μm (photos F.J. Meunier).

The second type of cementing lines, the reversal lines (in French “lignes de résorption”) emphasize small discordances in successive bone deposits (fig. II.C.2b, 4b). The reversal lines represent a break in the appositional growth of bones (Castanet *et al.*, 1993). They are delicately crenellated and hypermineralised. They separate a deposit of secondary bone from another older one (see below) (Amprino & Engström, 1952). This reversal line acts as a cement or paste between the two bony areas. In bony fish these cementing lines are 1 μm thick and consist of a double edge of opaque material (Castanet, 1981). The crenellated aspect of the reversal lines is a result of the osteoclastic activity that precedes the deposit of secondary bone (Matrajt *et al.*, 1964).

1.2.2. Vascularisation of bony tissues

Bony tissue includes empty spaces that house blood vessels, adipose cells and fibrocytes. These spaces are called vascular canals or vascular cavities, depending on their shape (Francillon-Vieillor *et al.*, 1990;

Ricqlès *et al.*, 1991). One of the functions of the vascular canals is a trophic role for bone since they provide metabolites to the surrounding bony tissues. They form a vascular network which is more or less developed, depending on the taxon. When vascular areas are present, it is known as vascular bone (fig. II.C.5a); when bony tissue lacks vascular structures, it is called avascular bone (fig. II.C.5b) (Francillon-Vieillot *et al.*, 1990; Meunier & François, 1992a). In spongy bone, the total volume of the cavities is higher than bony substance volume (fig. II.C.5a). Generally speaking, the vascular canals are regarded as being more developed when the metabolic activity of the fish is high, as for example in the tunas (Amprino & Godina, 1956).

Compact bone is generally relatively poor in vascular areas whereas it is characterised by spongy bone. When present, this tends to be rather localized in the inner region of bones and is surrounded by a more or less thick compact bone (Meunier & François, 1992a).

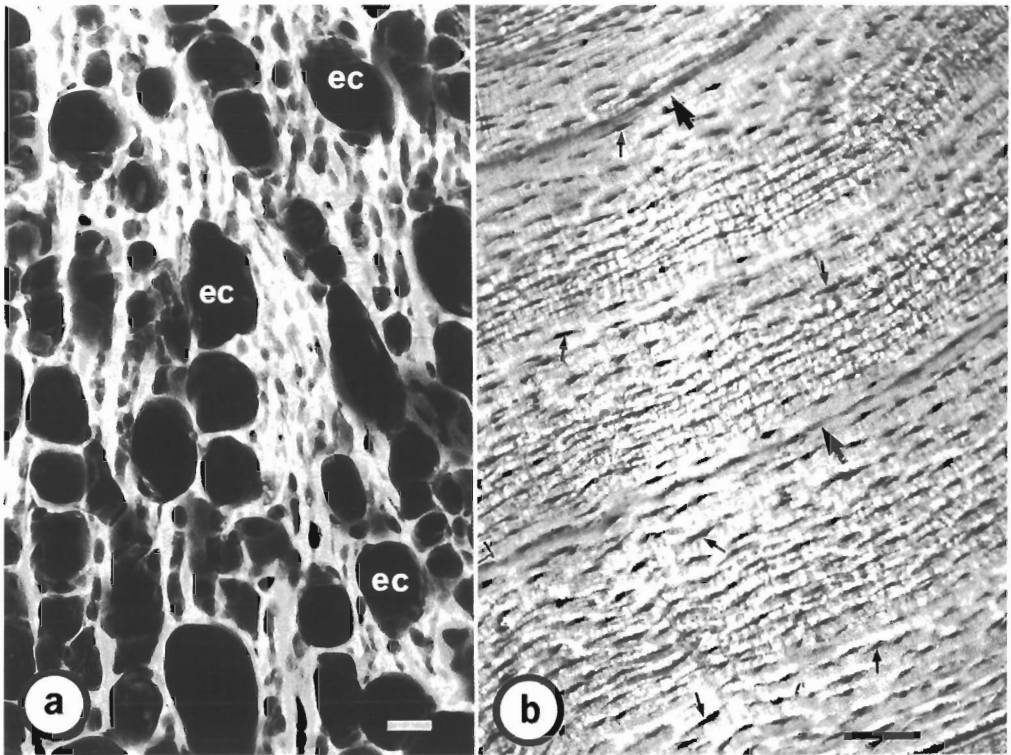


Figure II.C.5 - a) Spongy bone in the supra-occipital (cross ground section, microradiography) of *Pomadasys hasta* (Perciformes, Haemulidae) showing erosion cavities (ec); Scale bar = 50 μ m. b) Avascular bone in the dorsal spiny ray (cross ground section, transmitted natural light) of *Cyprinus carpio* (Ostariophysi, Cyprinidae) showing two annuli (thick arrows) and flattened osteocytes (thin arrows) characteristic of pseudo-lamellar bone. Scale bar = 50 μ m (photos F.J. Meunier).

In elasmoid scales, vascular canals are scarce. However, transverse holes ("canalicules perforants" of Baudelot, 1873) have been described in the posterior area of scales which completely cross in certain taxa: for example *Cyprinus carpio* and *Mugil cephalus*, where they are 30–50 µm in diameter (Baudelot, 1873), *Latimeria* (Meunier & Castanet, 1982). These holes house, among other structures, vessels and nerve fibres (Baudelot, 1873); they are true vascular canals but they do not play a role in locating superficial growth marks.

In any bone it is possible to characterise different modes of spatial organisation of the vascular canals. We can take as example the spiny ray of the catfish, *Hoplosternum littorale* (Callichthyidae) which grows in length and in thickness. We can define three types of vascular canals (Francillon-Vieillot *et al.*, 1990):

- "radial vascular canals", which link the inner part of the bone (eventually the medullar cavity) to its outer marginal region;
- "longitudinal" vascular canals, which lie parallel to the proximo-distal axis of the ray;
- "circular" vascular canals, more or less concentric and parallel to the surface of the bone.

It is clear that in any given bone we can describe the complex organisation of the vascular network with the association of these different primary vascular types (see for example fig. II.C.6.a, b).

There are two types of vascular canals: the first are called primary vascular canals, i.e. they form at the same time as the periostic deposit of bone. The osteoblasts secrete bony components around the vessels of the periost so that they are directly incorporated into the bony tissue.

It is generally believed that the more numerous the primary vascular canals or cavities, the more active is the global metabolism of the animal (Ricqlès *et al.*, 1991). The second type consists of the secondary vascular canals, which result from the resorption activity of the osteoclasts. These cells act as trepans, forming tunnel-shape cavities that are invaded by vessels. On the walls of these secondary vascular canals, endosteal osteoblasts often deposit a new bony substance which makes up the secondary bone and which is separated from the older bone by a reversal cementing line (see previous paragraph). When these secondary vascular canals are regular and more or less parallel to the great axis of a bone, they are called secondary osteons (fig. II.C.2).

1.3. Histophysiological significance of bony tissue

A comparative study of bone in the various classes of Vertebrates (including fossil taxa) shows that the basal constituents (cells, organic matrix, mineral crystallites) all appeared at the same point in geological time as bony tissue differentiation, i.e. during the Ordovician (Ricqlès *et al.*, 1991; Janvier, 1996). Moreover, there do not exist any

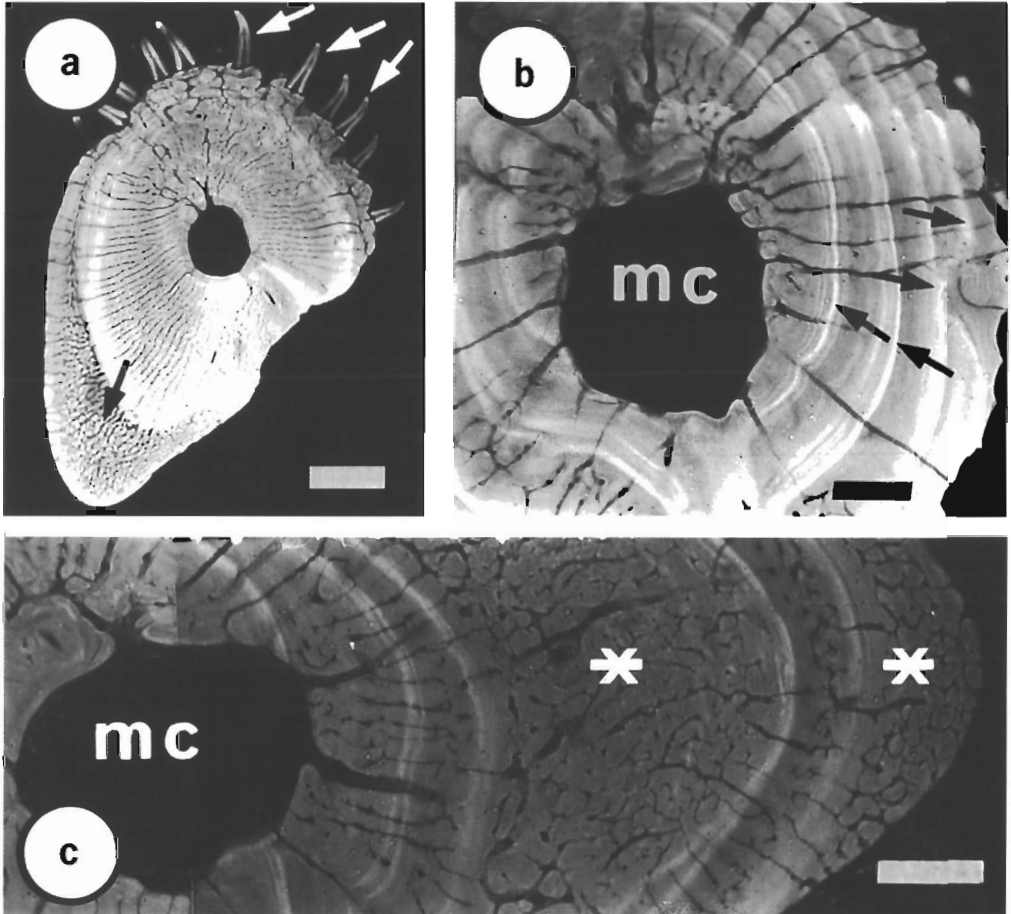


Figure II.C.6 - *Hoplosternum littorale*. Cross section of pectoral spiny rays (microradiography) (photos F.J. Meunier). a) an experimental bred specimen; showing the superficial odontods (white arrows) and one area of hypervascularised bone (black arrow) corresponding to the first spawning season. Scale bar = 1 mm. b) One wild female showing four hypermineralised annuli and the poorly vascularised bone; Scale bar = 1 mm. c) One wild male showing two hypervascularised zones (asterisk). Scale bar = 1 mm. mc = medullar cavity.

evolutionary trends in bone structure that are directly related to the evolution of vertebrate taxa, but rather to the constraints linked to physiological specialization. The classification of bony types which follows from the various association between the presence or absence of osteocytes, arrangements of collagenous fibrils, the presence or absence of vascular areas etc., has no evolutionary significance. On the contrary, it has various types of physiological significance which are expressed in Osteichthyans as well as in Reptiles and Mammals (Francillon-Vieillot *et al.*, 1990; Ricqlès *et al.*, 1991) and which are related to epigenetic constraints such as seasonal growth, metabolic intensity and breeding.

1.3.1. Bone growth

Bone growth is an appositional process that results from the activity of the osteoblasts, which are located in the periosteal membrane (see above). Periost produces primary bone which last for the life of the fish or can be partially destroyed, to be replaced by new bone, which is then considered as secondary bone (but see below). The structure of primary growing bony tissue depends on the rate of osteogenesis; i.e. whether it is young or adult, on the one hand, or a fish with a high or a low metabolic rate, on the other. Whatever the factors that influence the rate may be, the osteoblasts are under the influence of seasonal rhythms.

The measurement of periosteal osteogenesis is based on vital labelling techniques (Meunier & Boivin, 1974; 1978; Beamish & McFarlane, 1983; Babaluk & Craig, 1990; Boujard & Meunier, 1991; Trébaol *et al.*, 1991). There are very few studies of the rate of periosteal growth. Depending on the length of the fish, its age and physiological stage, periosteal osseous apposition ranges between 0.1 to 20 $\mu\text{m}/\text{day}$ (Simmons *et al.*, 1970; Casselman, 1974; Boujard & Meunier, 1991; Meunier & François, 1992b, *inter alia*).

1.3.2. Bone remodelling

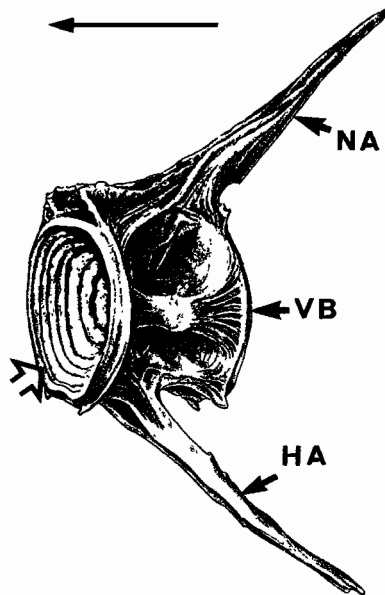
In chapter II.C.1.1, which dealt with the study of elementary components of bony tissues in Osteichthyans, we saw that it was necessary to discriminate primary from secondary bony tissue. Primary bone is new bone that is deposited by periostic cell, i.e. osteoblast, activity, without previous bone destruction. These cells are also responsible for increases in bone thickness by adding new peripheral layers. On the contrary, secondary bone, which is deposited by endosteal osteoblasts, follows an erosive process that is the result of osteoclast activity (fig. II.C.3b, 5a). These osteoclast cells, frequently unicellular in fish bone (see above, Sire *et al.*, 1990), induce vascular cavities and/or vascular canals which may be progressively filled by new bony tissue. This secondary bone is always separated from the primary one by a specific cementing line named "reversal cementing line" which has the same histological properties as the resting lines, except for their irregular appearance (see before). This phenomenon is called bone remodelling and even if primary and secondary bony tissues show the same basic constituents they are of quite different physiological significance (Francillon-Vieillot *et al.*, 1990; Meunier & François, 1992b).

Remodelling is not very developed in most Osteichthyan taxa, so bony tissues can usually be regarded as relatively permanent throughout the life of individual fish. On the other hand, tuna and related species show abundant remodelling in their skeleton, at least in the axial skeleton (Amprino & Godina, 1956; Poplin *et al.*, 1976), superficial bones such

as fin rays being relatively spared (Meunier, unpubl. observations). Age estimation can possibly be done by studying otoliths (Stéquert *et al.*, 1995; Itoh & Tsuji, 1996, *inter alia*). Moreover, body growth in fish is regarded as unceasing (Dutta, 1994; Goss, 1994). The bony skeleton grows throughout life, even though after first reproduction growth slows down significantly (Brown, 1957). The skeleton records, via specific structures, i.e. skeletal growth marks, the rhythm of growth as well as the events which affect it. Therefore, certain skeletal elements can often be used as a record of age as soon as the bony tissues show repetitive structures, the growth marks, and if these structures are synchronised by a cyclical seasonal factor.

Certain Teleost families (Carangidae, Drepanidae) or individual species (belonging to the Gadidae, for example) may develop hyperostosis, i.e. abnormally swollen bones (Desse *et al.*, 1981; Driesch, 1994, *inter alia*). In these bones, growth (primary bone apposition) seems to have been stimulated and remodelling is generally very abundant (Desse *et al.*, 1981; Meunier & Desse, 1994; Meunier & Zylberberg, 1998). So they are not at all suitable for ageing studies.

Figure II.C.7
A caudal vertebra of a Teleostean fish showing seven growth marks (annuli) on the centrum surface (hollow arrow). The arrow points the head of the fish. (HA = haemal arch, NA = neural arch; VB = vertebral body). (After Laerm, 1976).



2. Periodic increments or “the skeleton as a flight-like recorder”

Flat bone does not need elaborate special processing for skeletochronological study (fig. II.C.7, 8a), unlike “long” bones like spiny rays, which must be processed using histological techniques (fig. II.C.6, 8b, 9). Normally, mild cleaning and observation in a clearing

medium (see chap. VIII) are sufficient for easy discrimination of growth marks on flat bone (Casselman, 1974; Boujard *et al.*, 1991; Lecomte *et al.*, 1993, *inter alia*). On the other hand, in long bones the material is observed either on a polished surface after the undemineralised bone has been sectioned (Deelder & Willemse, 1973; Beamish & McFarlane, 1987), on ground sections (Sneed, 1951; Marzolf, 1955; Van der Waal & Schoonbee, 1975; Olatunde, 1979; Loubens & Panfili, 1992, *inter alia*) or on stained frozen sections on demineralised material (see chap. VIII). The main advantage of these technical constraints are that they allow the possibility of accurate decipherment of the spatio-temporal events which have occurred during the lifetime of the fish (Castanet *et al.*, 1992, 1993).

Generally speaking, the growth marks shown by flat bones (opercular, vertebrae, *cleithra*, etc.), are the “zones” and “annuli” (tab. II.C.2) and their use for ageing is very similar to scalimetry. Authors who have made use of skeletochronology have paid very little attention to the significance of the structural support of the cyclical growth marks.

Figure II.C.8

a) *Mylopus rhomboidalis*. Opercular (reflected light on black background). Scale bar = 1 cm.
b) *Cyprinus carpio*. Cross ground section of dorsal spiny ray (transmitted light). Scale bar = 1 mm.
(photos F.J. Meunier).

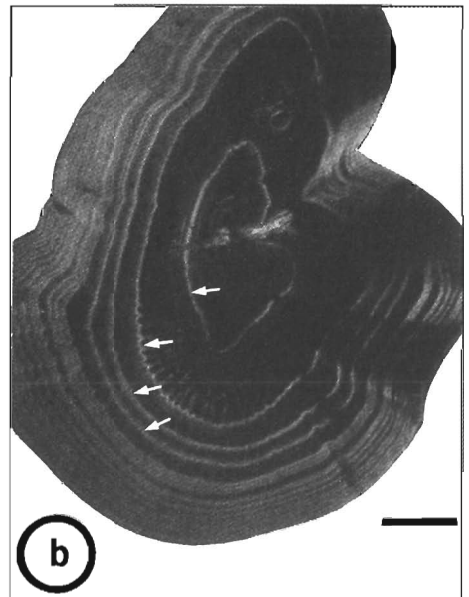
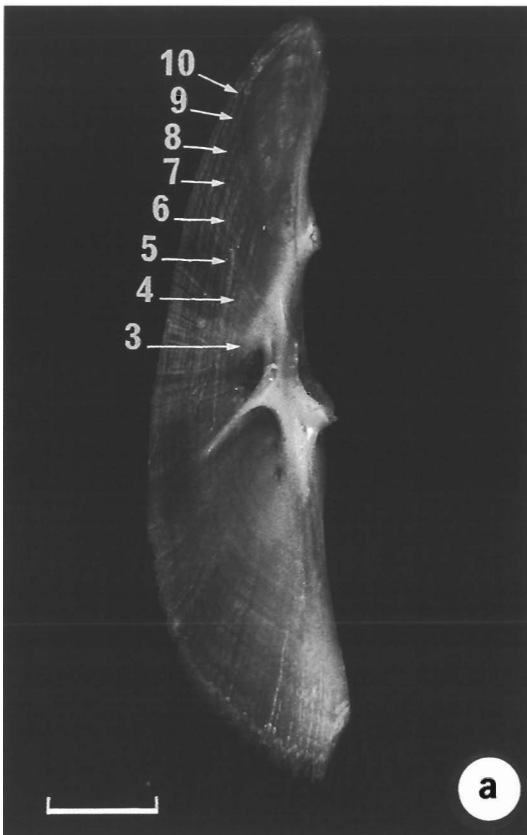
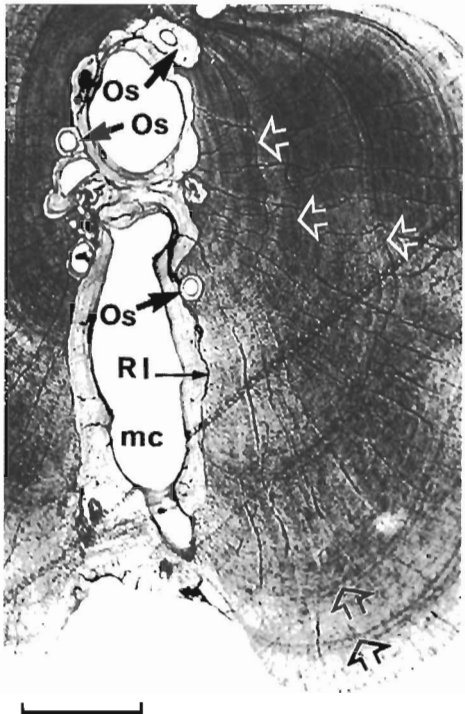


Figure II.C.9
Five AGL (arrested growth lines) (hollow arrows).
Cross section of a spiny ray (haematoxyline) of *Plagioscion squamosissimus* (Sciaenidae). Bone is acellular and show, around the medullar cavity (mc), secondary bone with some secondary osteons (Os), separated from primary bone by a reversal cementing line (RI).
Scale bar = 200 μ m (photo F.J. Meunier).



However, knowledge of their histological structure is essential to the understanding of the relationships between the skeleton and the genetic and epigenetic factors of growth (Castanet *et al.*, 1977; Castanet *et al.*, 1993).

Table II.C.2 - Formation of the growth marks (modified from Castanet *et al.*, 1993).

High metabolism period			Low metabolism period	
Temperate: spring-summer			Temperate: winter	
Tropical: wet season			Tropical: dry season	
	Young -----	Adult	Young -----	Adult
Fast-growing bone	Woven bone	Pseudo-lamellar bone	Pseudo-lamellar bone	Lamellar bone
Intermediary growing bone	Woven bone	Pseudo-lamellar bone	Annulus with Lamellar bone AGL	
Slow growing bone	Pseudo-lamellar bone	Lamellar bone	AGL	AGL

2.1. Growth marks

Growth marks that are useful for ageing are only found in primary bone. This is why the integrity of this type of bone is essential for age estimation studies, because remodelling destroys this particular information (Castanet *et al.*, 1992; Castanet *et al.*, 1993). For example, it is practically impossible to estimate the age of tuna by their vertebrae because these undergo an important remodelling process that destroys

primary bony areas. Moreover, growth marks have specific properties, under light microscopy, which are used to distinguish them in age estimation studies. There are three categories of growth marks in bone: “zones”, “*annuli*” and “arrested growth lines” (AGL).

The “zone” is the thickest growth mark. It appears dark under transmitted light and bright in reflected light (fig. II.C.8b). When bone is vascularised, vascular canals and cavities are more numerous in zone marks than in *annuli*. In fast-growing young animals, opaque layers, i.e. fast-growing layers (zones), are made of woven fibre bone with isodiametric osteocytes randomly distributed. In adults, the bone growth decreases because of the competition of reproductive metabolism, the woven fibre bone is replaced by parallel-fibre bone or, eventually by lamellar bone, both with more or less flattened cell *lacunae* (fig. II.C.5b) (Castanet *et al.*, 1992; Castanet *et al.*, 1993).

Annuli are clearly less thick than zones. Generally speaking, in bony fishes the *annulus* consists of one or more bony *lamellae* which are weakly hypermineralised in comparison with the mineralisation of the fast-growing bone of the zone (Meunier, 1988; Castanet *et al.*, 1993, *inter alia*) (fig. II.C.4a, 6). Moreover, the density of osteocytes is slightly lower in the *annulus* (Castanet *et al.*, 1977; Castanet *et al.*, 1993). *Annuli* or slow-growing layers correspond to slow osteogenesis. So, they are normally narrower than the adjacent bones (i.e. zones) and they are made of lamellar bone. They always appear more translucent than the zones, i.e. white in transmitted light and dark in reflected light.

The “arrested growth lines” are cementing lines which are concordant with the bony layers (see above). In any species the *annulus* is lined on its peripheral side by an AGL, sometimes by two (or more) AGL. AGL are rest lines (Castanet, 1981; Ricqlès *et al.*, 1991), i.e. they mark a temporary cessation of local osteogenesis (Castanet *et al.*, 1992; Castanet *et al.*, 1993). They generally are more translucent and more refringent than other marks. So they appear as the brightest structures when bone is observed with polarised light. They also are most chromophilic (with Haematoxylin, PAS stains) and frequently hypermineralised. In adults, they may frequently be the only sign of the cold season.

The combination of a fast-growing zone and an *annulus* represents the growth of one year. The zone corresponds to a period of fast growth, i.e. bone deposited during the period of high metabolism. Contrary, the *annulus* marks the slowing growth of skeleton when the metabolic activity is falling. It is now well known that bone growth marks are more or less distinct according the growth dynamic of the various skeletal elements (Castanet *et al.*, 1992; Castanet *et al.*, 1993). So the selected bones will be those which have the highest number of marks and the clearest ones. Moreover, there will be little or no remodelling (fig. II.C.10b,e).

Figure II.C.10

a-b) *Arius proops* (modified from Meunier *et al.*, 1997).

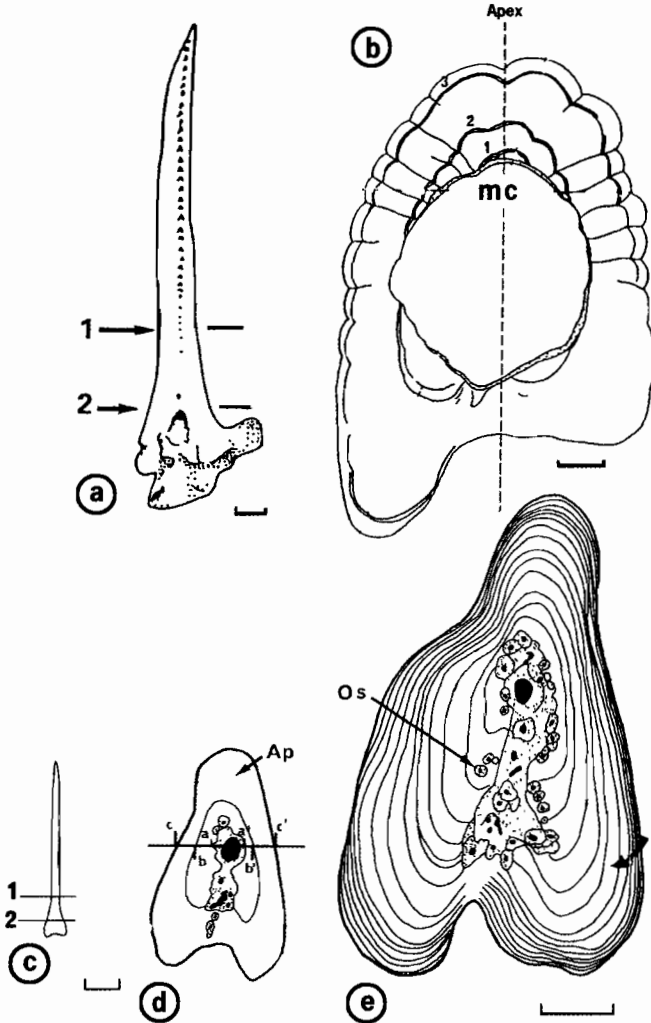
a) Pectoral spiny ray 1-2: localisation of the suitable level for the sections. Scale bar = 5 mm. b) Drawing of a ground section showing three *annuli*, the medular cavity (mc). Resorption has destroyed the main part of the first *annulus*. Scale bar = 1 mm

c-e) *Lethrinus nebulosus* (modified from Meunier *et al.*, 1979). c) Dorsal spiny ray 1-2 localisation of the suitable level for the sections. Scale bar = 10 mm

d) Drawing of a ground section showing one *annulus*

e) Drawing of a ground section showing fourteen *annuli*, an obvious narrowing of the six last ones after the first spawning. Resorption has partly destroyed the first *annulus*.

(d,e; scale bar = 500 μ m). Ap = apex, Os = osteon. Dotted area = secondary bone.



2.2. Determinism of the structure and spatial organisation of growth marks

Osteichthyans (as Chondrichthyans) are poikilothermic fishes and, as so, they grow all along their life even if growth rate decreases when they age. Moreover, longevity of fishes is highly variable according to the taxa, some of them reaching more than fifty years, even more than a century (Das, 1994, *inter alia*).

The growth rate of bone is time-dependent and it expresses different histological characteristics (tab. II.C.2) (Castanet *et al.*, 1992; Castanet *et al.*, 1993). The external periodic and aperiodic phenomena which lead to bone growth variations interact with general individual

growth processes and locally with those of the different skeletal elements. According to bone morphogenesis, the histological structure and the spatial aspect of growth marks changes from place to place in the same bone and between different bones of the same individual. This fact is very important for the choice of suitable bones for ageing studies.

Similarly, according to the general evolution of the growth rate throughout life, the structure of growth marks and their sequence will change from birth to death. Until sexual maturity, when body growth rate is high, *annuli* or AGL are well separated by wide zones of fast-growing tissues. They will become closer and closer during adulthood and very close to each other with ageing (fig. II.C.8b, 10e) (Castanet *et al.*, 1992, 1993). Of course, when skeletal growth definitively stops, no more growth marks are recorded locally. This has practical consequences for age determination if growth stops relatively early in life. Finally, individual and inter-populational skeletal growth variations also lead to differences in the histological expression and spatial organisation of bone growth marks (Castanet *et al.*, 1992, 1993).

In fish, as in Tetrapods (for example, see Castanet & Naulleau, 1985, for the viper), the variations of the histological characteristics between the cyclical growth marks are linked to various internal factors (e.g. tissue characteristics, hormonal environment, sexual maturity, genetical programme) and external factors (e.g. seasonal alternances, food resources, biotope changes after settlement migration). The rhythm of bone deposition and the alternating bony types deposited have an internal determinism that orders the skeletal growth cycle. However, the external factors take place as synchronizer if they are themselves submitted to cyclical rhythms (Castanet *et al.*, 1992, 1993). They check the width of the various deposits and, also, the time of their change, by modulating the effects of the endogenous rhythm of growth. Moreover, any cyclical anomaly of an external factor, can in turn generate more or less fine structural differences in the bony structure and, so, creates "false growth marks" or "supernumerary growth marks". If certain supernumerary growth marks are purely accidental (e.g. after a food scarcity, a dryness, a marked disease) others can result from normal acyclical events of life: hatching and/or yolk reduction ("birth mark") (Lecomte *et al.*, 1985, 1989), migration during recruitment (supernumerary acyclical growth marks) (Meunier *et al.*, 1979), breeding ("breeding marks"), annual adult migrations (Compean Jiménez & Bard, 1980). These supernumerary marks appear as an *annulus* or AGL, the histological characteristic of which are similar to that of normal *annulus* and AGL. Such supernumerary growth marks, as birth marks, are also known in Chondrichthyans (Branstetter & Stiles, 1987; Seki *et al.*, 1998; Wintner & Cliff, 1999).

2.3. Some examples

The carp (*Cyprinus carpio*) is a sedentary fish of the temperate regions, whose skeleton consists of weakly vascularised cellular bone, especially in the spiny rays of the dorsal and anal fins (Stephan, 1900; Meunier & Pascal, 1981). Several bones show annual growth marks: the mandible of the lower jaw (Castanet *et al.*, 1970), the vertebrae (Castanet *et al.*, 1977), the operculae (English, 1952; Sigler, 1958) and the dorsal and anal spiny rays (Castanet *et al.*, 1970; Meunier & Pascal, 1981). The spiny rays are most suitable for estimating the age of these animals. During the winter, there is no bony growth on the spiny ray and before the return of metabolic activity an *annulus* is deposited. This consists of one or two bony *lamellae* and at least one resting line. Appositional growth then begins and continues during spring, summer and early autumn with the deposition of a succession of regular bony *lamellae* (Meunier & Pascal, 1981). The *annulus* is weakly hypermineralised (Castanet, 1981) and the Ca/P ratio of the mineral is the same as that of fast-growing bone (Meunier, unpublished data).

Numerous catfish species have pectoral and/or dorsal spiny rays. These often show growth marks (fig. II.C.10b), fast-growing zones and slow growing zones or *annuli* and they are generally used (Marzolf, 1955; Boët, 1981; Lecomte *et al.*, 1989, *inter alia*) because of the lack of scales in these fish, where the otoliths do not give good results. An interesting case is that of the Atipa (*Hoplosternum littorale*), a South American catfish (Callichthyidae) which lives in coastal swamps. This fish builds a nest with grass and bubbles for spawning. At the same time the male develops secondary sex characteristics on the pectoral spiny rays, whose epithelium thickens (Winemiller, 1987) while typical hypervascularised bone is deposited on the external margin of the ray (Boujard & Meunier, 1991). When spawning is complete, bony growth falls drastically and the periost deposits avascular pseudolamellar bone. During the following breeding season a new area of hypervascularised bone forms, and so on. By counting the number of hypervascularised clusters the age of the male can thus be estimated. In the female the only growth marks are alternating zones and hypermineralised *annuli* (fig. II.C.6b).

Another example is fish with acellular bone, such as the pike, *Esox lucius*. This fish, which occupies a similar biotope to the carp, shows fine annual growth marks on the *cleithra*. Casselman (1974) described on ground sections hypermineralised *annuli* whose Ca/P ratio is the same as that of bone deposited during active growth periods. However, these bones being essentially flat, the study of growth marks is easy since it is sufficient to observe the *cleithra in toto* in a clearing medium.

Lethrinus nebulosus is a tropical fish that lives in lagoons in New Caledonia. The skeleton of this fish, a Perciforme, consists of acellular bone and it shows fine growth marks: fast-growing zones and resting lines

on the dorsal spiny rays (first dorsal fin) and on the vertebrae (Meunier *et al.*, 1979). The bony tissue of the latter shows important and irregular resorbed areas so they are not at all suitable for skeletochronology, unlike the rays. This fish is very long-lived (at least 25-27 years) and one or two resting lines may have disappeared, but comparative study with the otoliths has confirmed the utility of the spiny rays in estimating the age of these fish (Meunier *et al.*, 1979).

Similar alternations of fast-growing zones and *annuli* are very marked in other acellular boned fishes, such as the *illicia* of monkfish, *Lophius* spp., (Peronnet *et al.*, 1992; Yoneda *et al.*, 1997) or the dorsal spine of the grey trigger fish, *Balistes* spp., (Johnson & Saloman, 1984), or cellular bone fish: pectoral spiny rays of the sturgeons, *Acipenser* spp., (Magnin, 1962; Brennan & Cailliet, 1989). In the latter species the *annuli* are also hypermineralised (Meunier, unpublished data). The Thunnidae, which are Perciformes, are remarkable because they have cellular bone (fig. II.C.4b) (Stephan, 1900; Amprino & Godina, 1956). For example, the skipjack (*Katsuwonus pelamis*) displays cyclical growth marks on its spiny rays in the first dorsal fin (Batts, 1972) and on the vertebrae. On the rays, the *annuli* are relatively wide and they appear to be hypermineralised although they do not show resting cementing lines. However remodelling, which is related to the high metabolic rate of the fish, may destroy some growth marks in some cases, resulting in a underestimates of age (Cayré & Diouf, 1980).

3. Regulation of the incremental deposition

Despite the extraordinary diverse biology of fishes, bony tissues provide an accurate recording of mean biological events for animals including the annual cycles. At the present time no subannual growth cycles (lunar and/or daily cycles) have been recognized in bony growth marks in fish (but see "supernumerary growth marks") contrary to the otoliths. *Annuli* have a very similar structures whatever the taxa which are very different in terms of both phylogeny and biotopes (e.g. carp and tuna).

3.1. Growth mark formation and seasonality

In fact, we may ask if yearly variations of climate directly affect the histological structure of bone so as to deposit the growth marks or if their exist relays and more precisely biological relays, between these external factors (e.g. temperature, rain) and the registering property of bone.

Fishes are poikilothermic species. When they live in temperate climates they are exposed to seasonality of an external factor which induces a yearly cyclical biological rhythms, especially for body growth, i.e.

skeleton growth inducing deposition of bone growth marks. Other cyclical biological functions such as reproduction can also induce cyclical growth marks, generally AGL. In many studies of fishes living in temperate countries, a set of growth marks (i.e. zone + *annulus* or/and AGL) are laid down each year as validated with vital labelling (see chap. IV) in both Osteichthyans (Casselman, 1974; Meunier & Pascal, 1981) and in Chondrichthyans (Holden & Vince, 1973; Beamish & McFarlane, 1983; Smith, 1984; Officer *et al.*, 1997, *inter alia*).

In tropical climates, there is less contrast in seasonality compared to temperate ones. Nevertheless, some physico-chemical parameters of the environment may show a weak yearly cyclical variation that may be enough to induce and synchronize cyclical growth marks. In tropical Africa as in tropical South America, dry and wet seasons play respectively the same role as winter and spring-summer in temperate areas. The histological expression of these alternated dry and wet seasons is well marked in fish bones (Quick & Bruton, 1984; Lecomte *et al.*, 1985, 1986, 1989).

For the Neocaledonian *Lethrinus nebulosus*, whose physico-chemical parameters are apparently stable throughout the year, temperature is the only climatic factor which fluctuates significantly in the course of the year, albeit with a very low amplitude: 20.5°C to 26.5°C (Meunier *et al.*, 1979). This low amplitude of temperature seems enough to synchronize a growth cycle of the fish and to induce fine growth marks: fast-growing zones and resting lines on the dorsal spiny rays (Meunier *et al.*, 1979).

Seasonal but non-annual increments have been found in fish from some tropical regions. It is important to draw attention on specific growth cycles in certain fishes. Monthly sampling for several consecutive years in French Guiana have shown that some fish species, *Arius couma*, *A. proops* (two Siluriformes), *Leporinus friderici* (Anostomidae) and *Myleus rhomboidalis* (Serrasalminidae) deposit in their skeleton (spiny rays for the Siluriformes, opercles for the two others) two "zones" and two apparent "*annuli*" per year (Lecomte *et al.*, 1985, 1989, 1993; Boujard *et al.*, 1991; Meunier *et al.*, 1997). The two fast growth periods alternating with two slow growth periods correspond respectively to rainy and dry seasons. Similar double growth cycles are also known in tropical Africa for freshwater fishes (Garrod, 1959; Okedi, 1969; Bruton & Allanson, 1974; Blake & Blake, 1978; Robben & Thys van den Audenaerde, 1984) as for sea fishes (Poinsard & Troadec, 1966; Warburton, 1978). Another case of *annulus* formed twice a year ("*biannuli*") is *Oreochromis niloticus* in Lake Awassa (Ethiopia) which have two primary breeding seasons separated by about six months (Yosef & Casselman, 1995).

3.2. Calcium metabolism

The hypermineralisation of *annuli* is common (e.g. found in carp, catfishes, skipjack tuna) but with exceptions like *Lethrinus nebulosus* for example which lacks hypermineralised growth marks (Meunier *et al.*, 1979). When present, this hypermineralisation can not be attributed to variation in plasma calcium concentrations. Effectively, in the case of adult carp, for example, there is an annual hypercalcemia in plasma but only during June-July (Meunier & Pascal, 1981), i.e. at the time where the growth of the skeleton is active. The difference of mineralisation between fast-growing “zones” and slow growing “*annuli*” must rather originate from physico-chemical differences of the organic matrix. Moreover, these differences in the degree of bone mineralisation last the life of animals; there is not standard mineralisation in fish bone.

3.3. Feeding

Feeding is directly under the control of seasonality in temperate countries. Effectively, food is scarce or lacking during winter season. Accordingly, food intake and consequently body growth significantly decreases. In tropical countries feeding can also be an important link between seasonality and body growth. During the rainy season rivers overflow and fishes invade the flooded forest where food is abundant and rich. For example in French Guiana, the coumarou, *Mylopus rhomboidalis*, which show obvious growth marks (Lecomte *et al.*, 1993), feeds during the wet season on seeds which have high nutritional value and during the dry season on leaves of podostemonaceae (coumarou grass) with lower nutritional value (Boujard *et al.*, 1990). Another Guyanese species, *Leporinus friderici* which is an omnivorous fish, essentially feeds on seeds, fruits and terrestrial insects that they find under the overflow forest during the wet season and seems starving in the river deeps during the dry season (Boujard *et al.*, 1990). Such observations have also been noticed at the whole Amazonian scale (Lowe-McConnell, 1964, 1979; Goulding, 1980; Junk, 1985, and others).

3.4. Reproduction

Spawning is another vital function that is tightly related to seasonality. Its effect on growth can be in synergy with low metabolic time (i.e. during winter) or shifted forward to the high metabolic season. In the second case, spawning can lead to the formation of a “false growth mark” inside the “zone”.

Following sexual maturity, an important component of the metabolic activity is used for breeding so that remaining for body growth decreases, as do the bony deposits. Consequently, the successive *annuli* and/or AGL become significantly narrower (Meunier *et al.*, 1979).

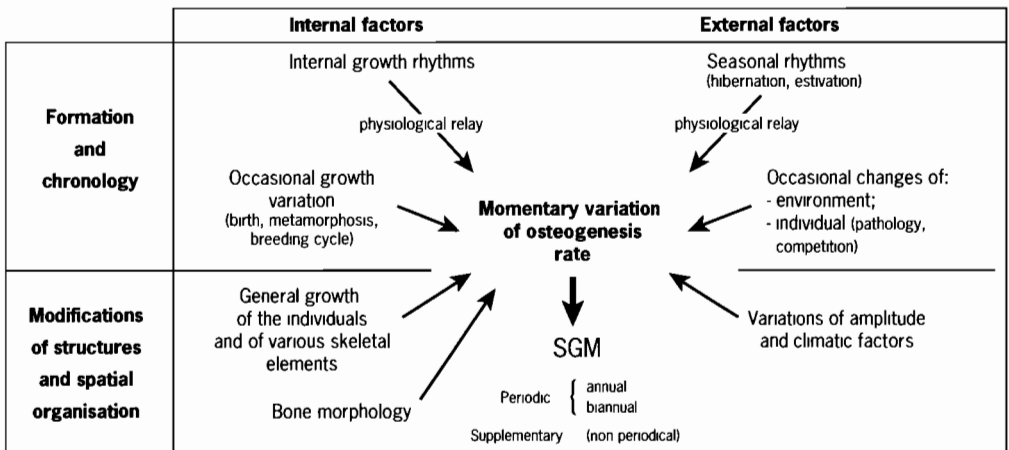
When the fish become older (specially in long life fishes) the “zones” can become so tight than the counting of *annuli* can be very difficult and unreliable: see for example the sturgeon.

We have seen before (see chap. II.C.2.2) that reproduction can be responsible of the spawning marks on scales especially in Salmonids (Johnston, 1905, 1907, 1908, 1910; Dahl, 1907, 1911, *inter alia*). This is certainly linked with calcium metabolism because of the high need of this cation during spawning (see Persson, 1997, *inter alia*). Calcium is partly supplied by scales that is why their integrity is affected (Crichton, 1935; Van Someren, 1937; Persson, 1997; Kacem *et al.*, 2000).

4. Conclusion

It appears that bony tissues can accurately adapt their structure according to the variations of the environment through physiological relays. Many events during the life of a fish that will be finely registered in the bony structure including more or less seasonal cycles, changes in biotopes and/or life mode because of migrations and first sexual maturation (tab. II.C.3). Consequently, the histological study of growth marks in primary bones can be a suitable method for individual age estimation in fish especially when otolithometry and scalimetry fail age estimation. However, the use of skeletochronology requires as precise a knowledge as possible of the biology of the animals, since skeletal growth is sensitive to many internal and external factors. As for scalimetry and otolithometry, validation (vital labelling, monthly sampling, see chapter IV) is a necessary step to test the annual growth rhythm.

Tableau II.C.3 - The internal and external factors and the spatio-temporal organisation of the skeletal growth marks (SGM) (from Castanet *et al.*, 1993).



When skeletochronology is appropriately applied and if the skeletal structure is suitable, it can provide many practical applications to the study of population dynamics. They can also have other applications in paleobiology and archeology. Paleophysiological hypothesis (Casteels, 1974 on vertebrae) and/or paleoclimatic hypothesis (Burdak, 1979 with scales) can be proposed in any Osteichthyans owing to the study of cyclical growth marks on appropriate bones (or scales or even otoliths) and with comparisons with extant species (Van Neer, 1993a). The skeletochronological analysis of bones of the archeological sites, if there are abundant material, always compared with the living can allow valuable data on the food of prehistoric peoples and on their fishing techniques: size of fishes, seasonality of fishing, fishing apparatus (Desse & Desse, 1983; Van Neer, 1993b; Van Neer *et al.*, 1999).

Chapter III

Sclerochronological studies

Contrary to generally accepted views, sclerochronology, and particularly age estimation in fish, is not an easy science. It involves a series of process and data-processing sequences that are often complex and time-consuming. Before beginning such studies it is necessary to know in advance the constraints on available time and costs, and especially the final objectives and expected results. This chapter will help to choose from among the multitude of relevant techniques and research methods:

- We distinguish between studies devoted to age estimation and those dedicated to the knowledge of life history features of individuals (age at maturity, lifespan, metamorphosis, recruitment, migrations, etc.);
- With regard to methodology, we discuss the difficulty of observing growth marks in three-dimensional space represented by growing CS. The complex shape and growth patterns in CS present particular difficulties in initial preparation stages.

A. Age estimation

B. Morales-Nin, J. Panfili

Age estimation involves several stages, from the choice of the CS to be aged and the level of precision required, to more technical problems such as preparation and the observation of growth marks. The incremental growth pattern also has to be selected and its temporal meaning determined. Moreover, the reader needs to gain experience in interpretation in order to obtain a high level of consistency in his interpretation criteria and to transmit them to, or calibrate them with, other experts. Once this process has been completed, it should be refreshed at intervals in order to be consistent over time.

Age estimation is a dynamic process which can be summarised in seven separate steps:

- selection of one or several CS, depending on their presence or absence, the problems of resorption and the synchronicity of CS growth in the population (chap. II);
 - recognition of growth patterns, including the periodicity of the growth marks under study and the range of the time scale covered (chap. II);
 - choice of the method of preparation, depending on the technical problems of preparation, the readability of the marks using the method employed and the degree of precision expected (chap. VIII);
 - confirmation of the consistency and intercalibration (multiple interpretation by one and/or several readers) (chap. IV);
-

- validation of the accuracy of the interpretation (i.e. time separating the growth marks that are being interpreted) (chap. IV);
- deployment of experience and knowledge in interpreting the growth marks;
- application of the results obtained (chap. V and VII).

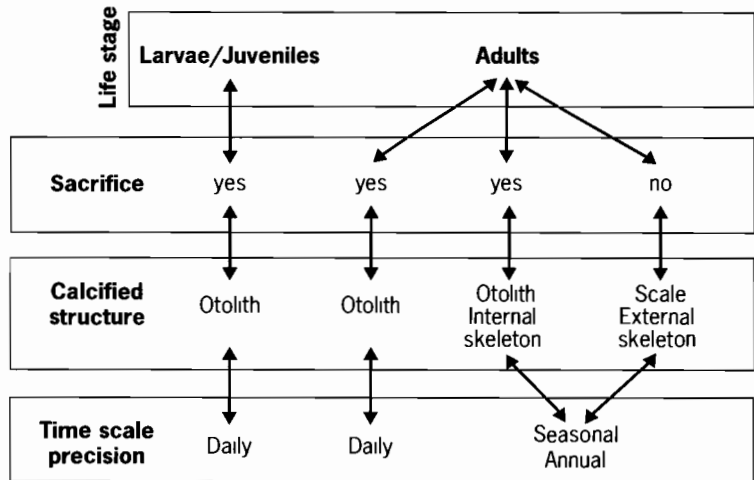
1. Criteria for the choice of calcified structures

Depending on the CS selected, it may be necessary to kill the fish to extract them (e.g. internal bones and otoliths). The choice of CS will thus depend initially on whether or not the fish can be killed (fig. III.A.1). Moreover, sampling at the quayside does not permit the fish to be mutilated and spoiled for sale, and some precautions must be taken when extracting otoliths (chap. VIII.B.3.1.4). Secondly, the availability of the CS will determine the choice of tissue. For example, in species without scales (e.g. Siluriformes) or with small scales that are easily lost (small pelagic), or in species that lack otoliths (e.g. Elasmobranchs), other structures will have to be used. In large pelagic fishes such as tunas, swordfish and Istiophorids, which have minute otoliths compared to the size of the fish, the vertebrae are frequently used. These are general rules with many exceptions. Other bones, such as fin rays, spines, *cleithra* and opercular, are more rarely utilised. However, they can be useful for Siluriformes, although our knowledge of their otoliths is still very limited. Bony spines from the dorsal fin are also employed in some large pelagic species and in tropical fish species such as Balistidae.

The degree of accuracy required regarding age estimation and the life phase to be studied are important in determining the choice of one structure over another (Fig. III.A.1). As daily increments are only found in otoliths, they are the obvious structure to be used when ageing is to be done at this level. For larvae and juvenile specimens they are the only choice. Otoliths have another important characteristic that makes them very appropriate, in that they do not act as calcium stores, unlike scales and other bony parts (Simkiss, 1974). They are therefore not resorbed except under extreme stress (chap. II.A), and thus maintain a more complete sequence of growth structures.

Of the three types of otoliths, the *sagitta* is the most frequently employed except in Ostariophysean, in which the *asteriscus* is larger. In some studies of larvae and juvenile fish, or even adults, the *lapillus* has been utilised, due to its smaller size and simpler preparation (the daily increments can be directly observed on whole or slightly polished *lapilli*). However, the onset of formation is not simultaneous in the three otoliths, with *sagitta* and *lapillus* coming first. They can thus not be employed indiscriminately (Secor *et al.*, 1992).

Figure III.A.1
Choice of a calcified structure depending on the accuracy of the time scale required, the life stage studied and the possibility of individual sacrifice for sclerochronological study.



2. Selection of growth increments

The selection criteria for growth increments in CS are:

- their distinctiveness and ease of systematic identification;
- the constancy of their periodicity;
- their synchronicity in any given population.

The degree of precision required in the study and the life stages to be considered are additional types of information required for the selection of an appropriate growth increment: daily, seasonal or annual. Larvae fish lack seasonal and annual growth increments, leaving daily increments of otoliths as the only choice. This choice might also be the only one available for species with a short lifespan (less than one or two years). Seasonal increments are employed when their time-scale is suitable for the population under study. Seasonal increments are found in all CS from bones to otoliths. Most age estimation studies are based on seasonal increments, particularly annual increments, which allow fish from the tropics to polar regions to be aged. Contrary to generally held, but incorrect, ideas, the changing of the seasons in the tropics, even on a very small scale, does lead to seasonal deposition and rhythms on CS, making them suitable for estimating the age of tropical species, even if this is more difficult in equatorial regions. In adult fish and/or long-lived species the presence of growth discontinuities and the complications inherent in counting large numbers of daily increments make the method less suitable. We cannot recommend the use of daily increments to estimate age in years, unless for special reasons. The time required to obtain the thin otolith sections that are necessary for daily increment reading in adults also limits the feasibility of this method for large thick otoliths.

Methods of preparing CS are generally more simple when seasonal growth marks rather than daily ones are being studied, ranging as such procedures do from observing the whole CS to preparing transverse embedded sections in resin (e.g. thin slices of otoliths). The time required for a single preparation is very variable. For very small otoliths, daily increments can be observed directly without preparation but it is often necessary to employ complicated and time-consuming methods to observe these increments (e.g. grinding and polishing, thin slices, SEM, etc.). The user must therefore be careful to evaluate these parameters (level of preparation, duration of preparation, etc.) before starting a study. He must obviously synthesise the previous preparations used by previous workers, and which can be found in the literature.

The identification of seasonal increments, or even daily ones, is never easy, due to the presence of false or split rings and discontinuities, that correspond to non-seasonal events (migration, spawning). Identification is based on their distinctiveness, their continuity around the CS, their thickness and their relative width (chap. III.C). These should decrease in width from the central parts to the edge of the CS, in line with the fall in rates of growth with age (chap. V).

Another relevant criterion for selecting an appropriate growth structure is the number of fish to be aged. This also involves the costs of sample collection and CS preparation, including the time required to prepare the samples and to interpret them. The use of daily increments in juveniles and adults is time-consuming (see above and chap. VIII) if the complete increment sequence from the core to the edge is required. For instance, the time required for a skilled person to mount, grind and polish an otolith to obtain a thin slice is one to two hours. Preparing large numbers of thin slices may therefore limit the usefulness of the method. Another fact to be considered is that in some species and at certain periods in their life cycle (e.g. migration), the daily increment widths may be below the detection limits of the compound microscopes (Campana *et al.*, 1987; Morales-Nin, 1988).

Therefore, before starting a new study the CS and the incremental growth pattern to be selected should be carefully considered. Table III.A.1 summarises all these considerations. The presence of secondary growth centres or other structures that might interrupt the growth pattern should also be taken into account in the evaluation of the suitability of a given structure for ageing purposes.

Table III.A.1 - Some considerations regarding the choice of calcified structure and the growth marks for a sclerochronological study.

	Otolith	Scale	Bone
Preparation	+/- long	none	+/- long
Growth marks (time scale)	- primary (<i>daily</i>) - opaque (<i>seasonal</i>) - translucent (<i>seasonal</i>) - discontinuity (<i>irregular or seasonal</i>)	- <i>circuli</i> - discontinuity (<i>seasonal</i>)	- opaque (<i>seasonal</i>) - translucent (<i>seasonal</i>) - discontinuity (<i>seasonal</i>)
Advantage	- no resorption - sometimes no preparation	- no sacrifice - no preparation	- sometimes no sacrifice
Disadvantage	- sacrifice - sometimes long preparation and interpretation times	- regeneration - resorption and remodelling	- resorption and remodelling (sometimes strong) - sometimes long preparation time required

3. The process of age estimation

The first step in the age estimation process is to read the selected calcified structure using an incremental growth pattern appropriate for the objectives of the study; the next is to interpret the age and provide some applications (chap. V).

Some inevitable interpretation criteria for the populations and/or the species must first be defined; for example, the location of the hatching check, the first increment, the transition zones on the CS, the nature of the edge, etc. The biological information available for the species should be used to define these criteria empirically. They can then be compared in order to establish an "alphabet" (identification of incremental growth patterns) and to determine the "grammatical rules" involved (the interpretation criteria based on existing knowledge) to attribute ages (Sych, 1974).

The consistency of the age estimation process then needs to be determined. This means the ability to identify the same structures consistently. The repeatability of the age estimation procedure (internal bias) must be determined and the ages calibrated (external bias) with other experts (chap. IV.D). The experience of the reader is very relevant to the success of limiting the bias. Even experienced readers, however, might show low levels of accuracy when starting work on a new species. Once a satisfactory level of expertise in age estimation has been reached, steps should be taken to prevent the methodology from deteriorating or changing over time, in order to maintain adequate quality of the ageing procedures.

Finally, the ages must be validated, in the sense that the accuracy of the ageing has to be established. The temporal meaning of the interpreted structures must be determined in order to evaluate the closeness of the estimated age to the accurate age of the fish (chap. IV).

3.1. Specific age estimation

Age estimation on the basis of daily increments yields a direct and precise value of age. It is calculated from the number of validated daily increments (number of D-zones or L-zones) plus the interval between hatching and formation of the first increment (chap. II.A.2.1).

In contrast, estimating specific ages on the basis of seasonal increments requires some additional calculation: it can be calculated as the number of months or years. Once all the increments on a CS have been identified, the following information should also be taken into consideration in assigning the correct age:

- date of capture;
- individual date of birth (exact, or average or standard for the population);
- main periods of seasonal increment formation;
- nature of the CS edge.

The true date of birth of an individual, as determined from studies of reproduction and/or analysis of daily increments, is not always known. In such cases, for convenience and ease in collating age data with other statistics collected on a calendar year basis, the convention is to accept 1st January as the date of birth for the entire the population. Validation studies (chap. IV) allow the precise period of seasonal increment formation to be determined. Figure III.A.2 shows examples of the method whereby age can be calculated in terms of number of months and years for fish caught at different times of the year. The conversion of an increment count to an age estimate involves considering the relationship between the date of increment formation and the date of capture and nominal birth date.

3.2. Age group and assignation to age class

The individual age group and age class are used in population dynamics and population life history studies, in both of which a single age in years is required. Once all the increments on a CS have been identified, a reader can establish the age of the fish by simply counting the number of seasonal increments on an annual basis. As in the case of specific age estimation, other information should also be taken into account:

- date of capture;
- the peak spawning period for a given population (precise or average or standard population date of birth);
- main periods of seasonal increment formation (see validation studies, chap. IV);
- nature of the CS edge.

In order to be able to establish from the CS the age group/class to which a fish belongs it is necessary to count the number of annual increments from the centre towards the outer edge of the CS. If the date of capture is known, it is possible to calculate the year in which

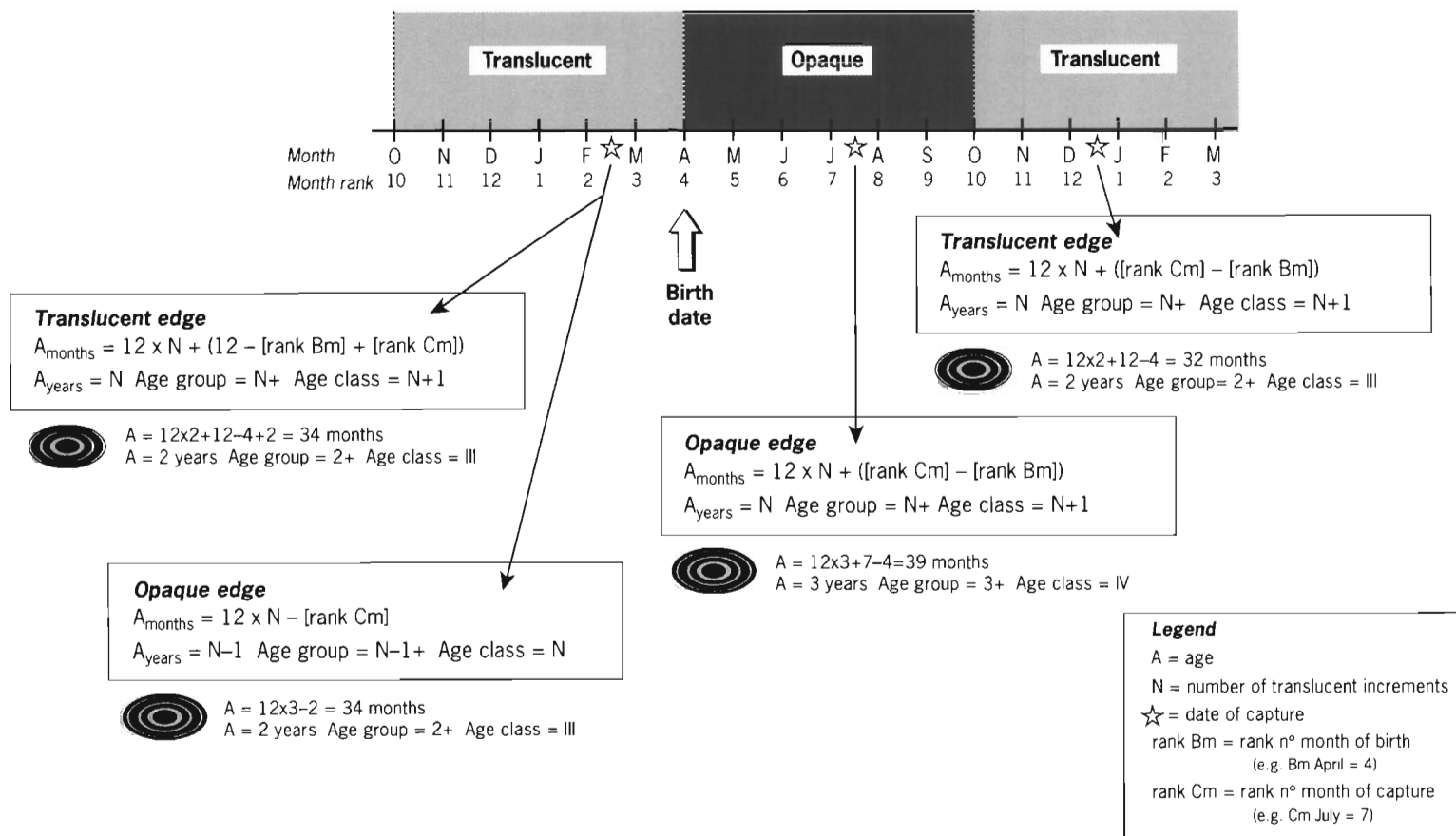


Figure III.A.2 - Theoretical calculation of age (months, years) and assignment of age group and age class from a calcified structure. The birth date for all individuals of the population corresponds to April 1st, which is also the beginning of the formation of the opaque increment on the CS. Validation has shown that each year both a translucent and an opaque increment are laid down. The age is calculated by counting the number of translucent increments, but taking into consideration the date of birth and the date of capture. As a rule, a translucent marginal increment is not counted as an annual increment. Some examples of individuals captured at different times of the year are shown. On any given date of capture (e.g. February), the nature of the edge of the CS may differ (opaque or translucent), but the age is the same.

the fish was spawned. Arabic numerals are traditionally used to reflect an age group and Roman numerals the age class. In the example given in figure III.A.2, the translucent increments are counted for ageing. As a rule, if the marginal increment is translucent it is not counted because it is not fully formed. The age group or age class to which a fish will be assigned depends on the year in which it was spawned and on the date of capture. The age group is the number of calendar years after the birth date. The age class corresponds to the number of years since birth. The most recently spawned cohort (the “young-of-the-year”), consisting of individuals between 0 and 12 months of age, constitutes age group 0+ and age class I. Individuals spawned during the previous year make up age group 1+ and age class II. The year class is the calendar year in which a fish was born. In figure III.A.2, for the fish captured in July 2000, the year class is 1997 and it was born in April 1997.

For the purposes of stock assessment the true birthday is generally assumed to be unknown as spawning time may vary slightly from year to year or, in the case of some species e.g. *Merlangius merlangus*, the spawning season may extend over four or five months. Thus, in order to ease the task of collating data from a variety of sources most fish species in the northern hemisphere are given a nominal birth date of 1st January. Normally, an annual increment is only counted when the translucent zone is complete, usually about March or April. However, because of the 1st January rule the marginal increment, even if it is an incomplete translucent zone, is counted as the annual increment in the period January to March. On 1st January of each year, the fish in each cohort thus become one age group and one age class older.

B. Life history events

A.J. Geffen, H. de Pontual, P.J. Wright, H. Mosegaard

1. Age at maturity

Reproduction places a severe energy demand on fish, and after the onset of maturity there may be a reduction in somatic growth in favour of storage for reproductive activity. This affects the growth of calcified structures and the influence of reproductive activity is often recorded permanently in scales and otoliths. So-called spawning checks (Williams & Bedford, 1974) have been reported in many species, but no published experimental studies have established the precise relationship between reproduction and the features that have been observed.

In theory, the process of gametogenesis utilises both energy and calcium reserves that could otherwise be used for the growth of calcified tissues. Oogenesis in particular involves the production of vitellogenin, which is a calcium-binding protein. Kalish (1991a) observed significant changes in the level of free Ca in the blood plasma and endolymph associated with increases in the gonadosomatic index. Thus, in female fish, there is a decline in the amount of Ca available for otolith growth. Narrow translucent zones in the otoliths of female fish have been assumed to be hypomineralised, the result of reduced calcium availability during gonad maturation (chap. VII.E.2.2). However, since translucent formation is mineral-rich and protein-poor and since feeding activity in a number of species is also reduced during the final stages of gonad maturation and spawning, this may influence the rate of protein synthesis available for otoliths as well as for other tissues and result in the observed translucent structures. This is clearly in line with early findings in Arcto-Norwegian cod (*Gadus morhua*) (Rollefsen, 1933) of a correspondence between age of maturation and the onset of narrow annual otolith zones with a relatively wider translucent zone.

Because the pattern of calcified tissue growth may change significantly after maturation, age at first maturity can often be determined directly from the juvenile (sub-adult) area of the tissue. Fish that mature late may display numerous regular *annuli*, and age at first maturity can be easily determined by means of conventional methods. In other species, particularly those that mature at less than one year of age, it is necessary to use otoliths, and to determine the age at first maturity from primary increment counts. Diadromous species may present a further complication because the effects of migration and sexual maturation are not always synchronised. Species with spawning

migrations present less of a problem, and the transition between fresh, estuarine, or ocean waters may accentuate spawning checks (or *vice versa*). However, feeding migrations may induce checks or changes in the growth pattern of calcified tissues that may obscure the pattern induced by reproductive activity.

2. Lifespan (longevity)

The ability to estimate the age of fish from CS may directly support the determination of lifespan or longevity in individual fish populations and species. Short-lived species, especially those that live for a year or less, can only be aged on the basis of counts of otolith primary increments (chap. III.A). The accurate determination of lifespan may be complicated by the effects of reproductive activity in such species (see above, chap. III.B.1).

There is a choice of method for other species, since annual growth zones can be observed in many different tissues. As far as estimates of age are concerned, estimates of longevity can often be verified by comparing different CS (chap. IV.D). However, the apparent advantage of being able to compare age information using different methods and structures can lead to controversy. In *Sebastes* spp., there are unresolved contradictions in the maximum ages recorded in various CS. Similarly, the analysis of macro- and microincrements in *Hoplostethus atlanticus* otoliths produces different ages. Longevity has been estimated to be up to 70 years on the basis of *annuli*, but as low as 40 years when microincrement counts are used. When different structures such as otoliths, scales or bones are being compared, it should also be noted that different CS have different allometric relationships to fish growth (Casselman, 1990), which may influence the ability of the CS to reflect annual variations at great age. It has thus been shown that in very old fish, scales may underestimate age relative to that estimated by otoliths.

Estimates of longevity can sometimes be validated by radiometric ageing techniques (chap. VII.E.3). These chemical chronometers do not provide precise estimates of age because of current instrumentation limitations, and so far they have only been applied successfully to long-lived species.

3. Metamorphosis and settlement

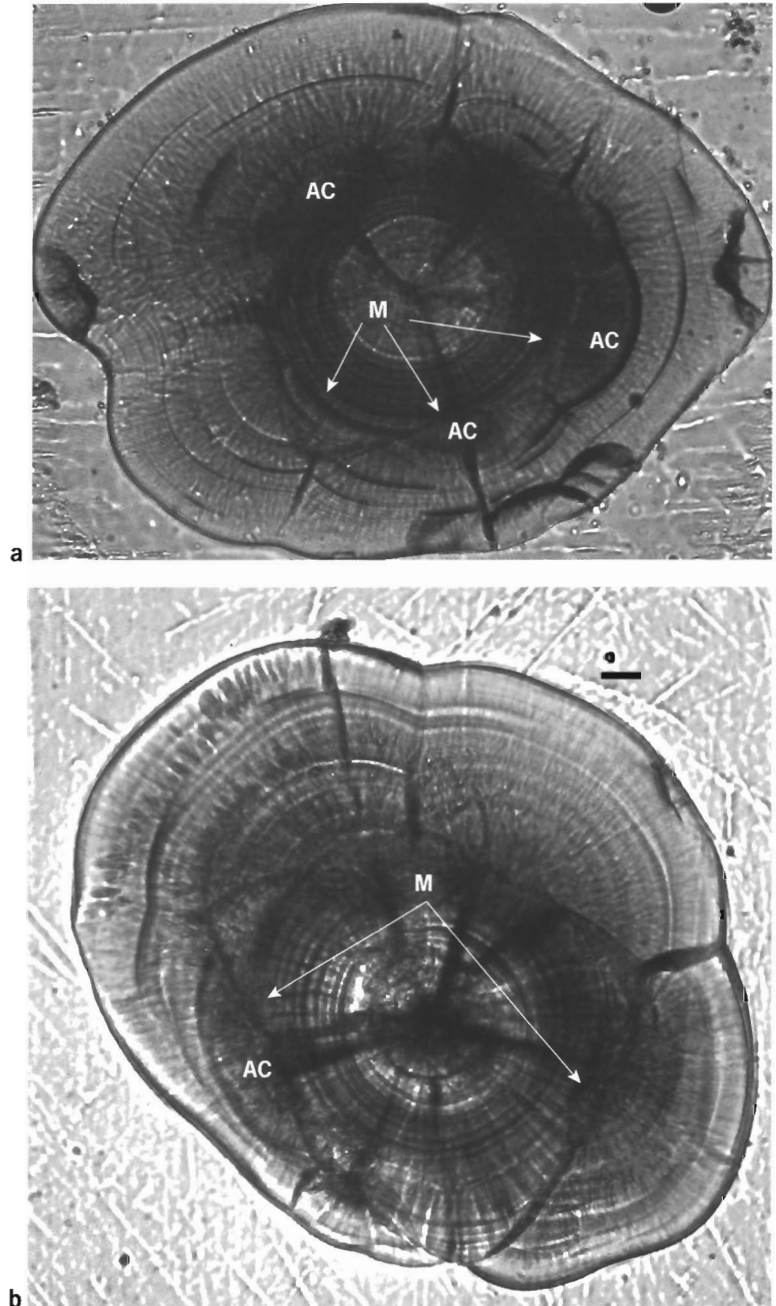
In most fish species metamorphosis is a transition that marks the end of the larval stage. At the completion of metamorphosis, fish are regarded (and often defined) as resembling the adult form of the species. The difference between newly metamorphosed fish and adults

is sometimes considered to be only a matter of size and sexual maturity. In reality, metamorphosis is a transition phase, which varies widely from species to species. The key discriminating features of metamorphosis, in the context of sclerochronological studies, are the termination of cutaneous respiration and the completion of ossification. Thus, by the end of metamorphosis, fish have complete skeletal structures and scales, while the exchange of ions with the environment is limited to transport across the gills and through the digestive tract. Because complete ossification defines the endpoint of metamorphosis, only the otoliths can be used to study events and processes that occur before or during this stage. Metamorphosis is associated with physiological, morphological, behavioural, and often habitat changes. In some species these changes are dramatic and occur over a short period of time, in others the changes are gradual. Any of these changes can result in changes in otolith formation, leading to differences in otolith shape or in the pattern of increments. Eels in particular undergo dramatic changes in both the appearance and composition of otoliths, that can be linked directly to physiological changes during metamorphosis (Otake *et al.*, 1994, 1997; Antunes & Tesch, 1997).

The most frequently observed change in otolith shape is the result of accessory growth centres, which usually form at the end of metamorphosis. These have been described in several Gadiformes and flatfish (fig. III.B.1). A close link between the progress of metamorphosis and the appearance of accessory growth centres has been described in plaice (*Pleuronectes platessa*) (Modin *et al.*, 1996). In the lesser sandeel (*Ammodytes marinus* Raitt) the completion of metamorphosis is associated with the formation of the post-rostral secondary growth centre (see glossary; Wright 1993). In the Pacific Dover sole (Toole *et al.*, 1993) *Microstomus pacificus* the accessory growth centres seem to form throughout a lengthy period of metamorphosis. Changes in the optical density and spacing of increments may also accompany metamorphosis. These changes may be gradual and encompass several increments, as observed in herring (*Clupea harengus*), or may be more abrupt as in some coral reef species (Victor & Brothers, 1982, see also "settlement", below).

These results permit detailed studies of the duration of the larval phase, useful in investigations of drift, metapopulation dynamics, and spawning behaviour. In some cases, the formation of daily increments has been validated before, during and after metamorphosis, thus enabling detailed studies of early life history transitions to be made. In other cases, where validation has not included the period of metamorphosis, it is often possible at least to be able to determine the age at the start of metamorphosis, and the date of its completion (fig. III.B.1).

Figure III.B.1
Otoliths of juvenile
Pleuronectes platessa,
indicating the initial high
contrast increment (M) and
accessory growth centres
(AC) formed at the end
of metamorphosis.
The minimum time since
settlement is estimated from
the first increment
completely surrounding the
accessory growth centres.
Scale bar = 10 μ m
(photos A.J. Geffen).
a) 30 mm fish,
approximately four weeks
after settlement.
b) 25 mm fish,
approximately two weeks
after settlement.



The physiological changes associated with metamorphosis may also be studied by means of otolith microchemistry. "Ontogenetic" variations in element composition can be observed in data collected along transects from the otolith core to the edge. Changes in the Sr/Ca ratio in particular have been described (chap. VII.E.2). In future, it may be possible to compare the rate of change in elemental ratios to growth after metamorphosis, as a means of measuring the physiological condition of individuals in the past.

Most marine and many freshwater fish species produce planktonic larvae. When the adult habit is demersal, there is usually a transition from the planktonic larval to a demersal juvenile stage, and this transition is termed "settlement". Both the timing of settlement and the duration of the preceding larval stage are of interest in recruitment, life history and ecological studies. Settlement marks can often be identified on otoliths, and are the result of either physiological changes at metamorphosis, changes in behaviour and diet caused by the moving out of the plankton, or both.

There are numerous examples of these applications in the literature, the most common probably being the use of metamorphosis or settlement checks to define the end of the larval period in order to estimate larval duration and drift. Studies of coral reef species often rely on otolith checks to fix dates of settlement, identify settlement cohorts, and determine larval drift (Heath, 1992; Kingsford & Atkinson, 1994; Schultz & Cowen, 1994). In temperate species too, otolith features associated with metamorphosis and settlement are used to study sub-cohort dynamics (Al-Hossaini *et al.*, 1989), mortality patterns (Van der Veer *et al.*, 2000), larval stage duration (Suthers & Sundby, 1993) and drift (Desaunay *et al.*, 1996).

4. Migration

Many fish move between different water masses in the course of their seasonal migrations. Many species also exhibit ontogenetic shifts in habitat that consist of a long-term or permanent move into new waters. Questions of species and population biology often require information about the timing of these migrations. These questions can be addressed through examination of changes in the appearance of increments in otoliths and scales.

Changes in the pattern of scale *circuli* are used to identify anadromous migrations in Salmonids, and these also provide information about relative rates of growth in freshwater and marine environments. Similarly, the pattern of otolith increments is often used to identify the timing of catadromous migrations in eels. Similar analyses can also be used to discriminate between anadromous and resident individuals in waters in which two populations with different behaviour mix.

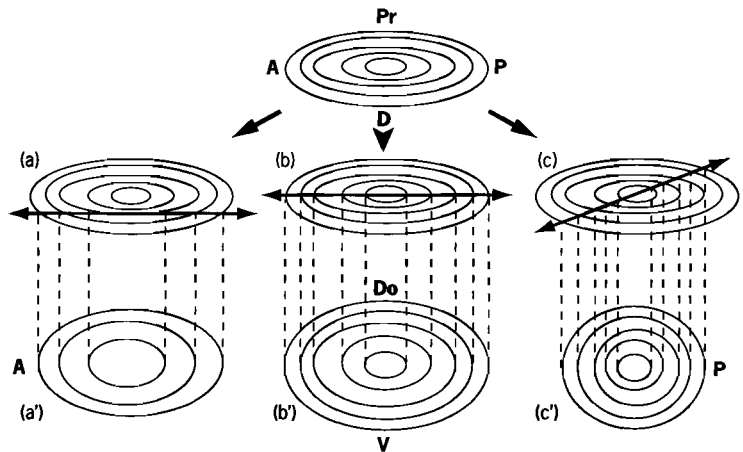
The composition of scales and otoliths also changes when fish move into new environments, and this can be exploited to obtain detailed data about fish age at migration and the duration of the transition between different environments. Oxygen isotope ratios and elemental ratios (Sr/Ca) show rapid changes in response to salinity and are thus the most useful means of tracking diadromous migrations (chap. VII.E.1.1).

C. Influence of shape and structure on the interpretation

J. Panfili, B. Morales-Nin

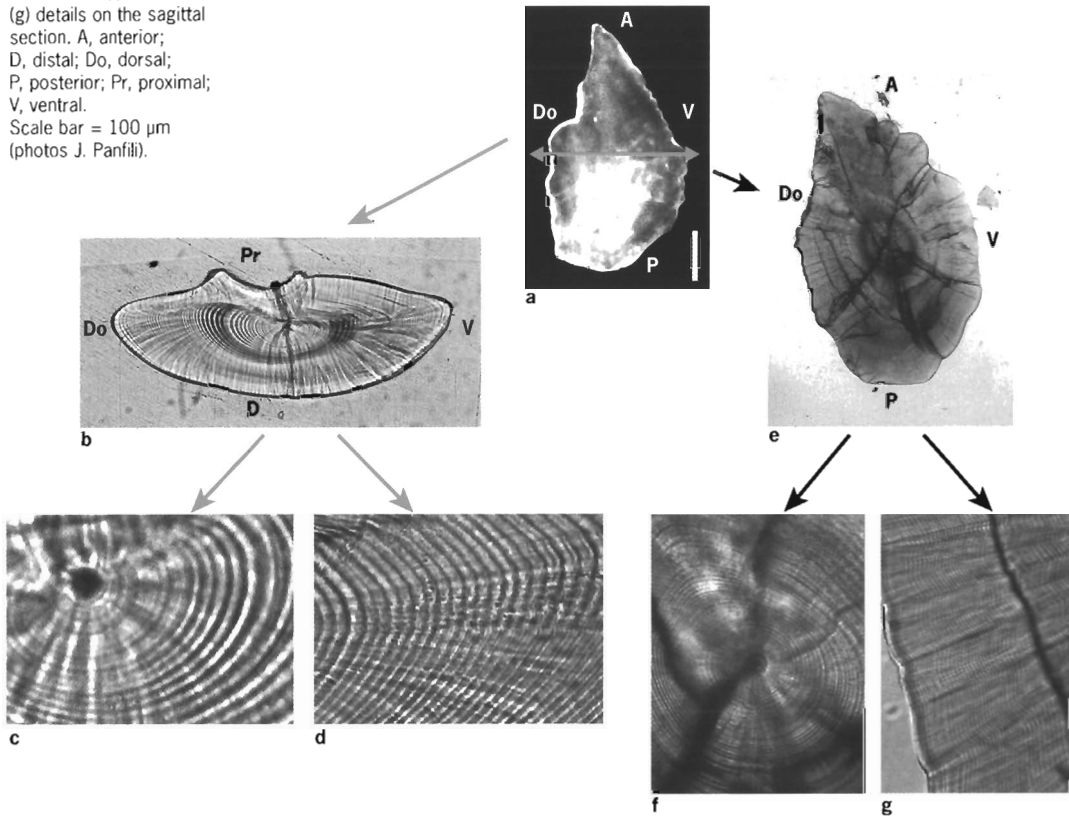
CS growth patterns, except for those of scales, involve three-dimensional bodies whose dynamics of shape and size are determined by a combination of endogenous factors (genetic, controlling shape) and exogenous factors (environment, through physiology, controlling the size) (chap. II). Their function and time-keeping properties produce internal growth structures with different spatial dispositions. Even in the observation of whole or thick preparations, the focal plane of the binocular or compound microscope will reduce the amount of 3D information integrated in increment identification to a 2D view. When thin slices are prepared the problem is even more pronounced, resulting in the rejection of many preparations. Depending on the sectioning plane of the preparation and the degree of eccentricity of the core, growth increments will have different appearances (Williams & Bedford, 1974). A failure to section close to the core will result in errors in age interpretation and in discrepancies in the aspect of the growth increments between samples (fig. III.C.1). The more complex the preparation technique the more difficult are observations of the pattern of increments. In otoliths, for example, surface preparation techniques such as staining and SEM can give erroneous images of the real internal growth structures, depending on the level of sectioning employed (Panfili & Ximénès, 1992). At this level, errors can occur when measurements are being made.

Figure III.C.1
Diagram of a theoretical otolith (onion model) with growth zones and the effect of different sectioning planes. (a), (b), (c), lateral views of the same otolith. The double arrow indicates the plane of section. (a'), (b'), (c'), sagittal observation of the section and location of the growth zones. The images offered by the three sectioning planes are quite different. A, anterior; D, distal; Do, dorsal; P, posterior; Pr, proximal; V, ventral. (from Panfili & Ximénès, 1992, by permission of the publisher Academic Press).



The most obvious consequences of shape and structure for observations of the growth pattern are found in the otoliths, because of the extraordinary variety of shapes that can be found in different species. It is worth remembering here that shape can be used for species identification (Torres *et al.*, 2000) and in paleontology (Nolf, 1995) (chap. II). The problem would be somewhat reduced in less complexly shaped otoliths, as are found in hake, Perciformes or other highly evolved species (fig. III.C.2), but can be very notable in many species whose otoliths are bulky or irregular in shape. Otoliths tend to become more complicated and ornate with age, with curvilinear growth produced by preferential growth on the internal side. It can therefore be very difficult to obtain a single plane of sectioning though the core of the otolith to its edge.

Figure III.C.2
Example of the effect of the 3D shape on a 2D observation for a typical Teleost otolith (*Vinciguerrina nimbaria*). The growth patterns are presented on thin sections for a transverse plane (b) and a sagittal plane (e). (c) and (d) show details on the transverse section and (f) and (g) details on the sagittal section. A, anterior; D, distal; Do, dorsal; P, posterior; Pr, proximal; V, ventral. Scale bar = 100 μ m (photos J. Panfili).



Because the otolith grows at different rates depending on its axis of orientation, the increments in growth would display differences in density depending on the otolith area. This is relevant, for instance, with sub-daily increments and split rings, depending on the time-scale being studied (fig. III.C.3). In the fastest growth area, usually the antero-posterior axis, the sub-daily increments are wider and appear more clearly than in the dorso-ventral axis. The same is true for split rings that might obscure the seasonal increments. Three-dimensional structures such as the accessory centrum laid down around the *nucleus* in flatfish and Gadoids would appear differently, depending on

Figure III.C.3
Split microincrements (arrows) on the transverse section of the otolith of *Vinciguerria nimbaria*. Growth is compressed in the proximo-distal axis. Not all microincrements are visible on the proximal face. D, distal; Do, dorsal; P, proximal. Scale bar = 20 μm (photo J. Panfili).

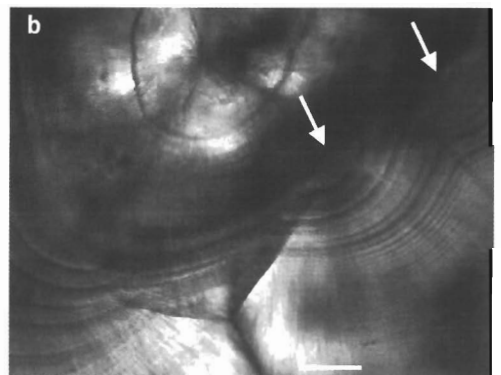
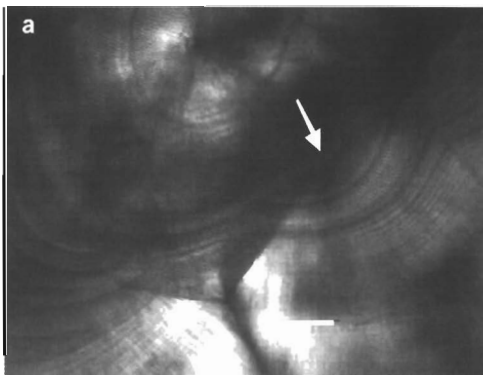
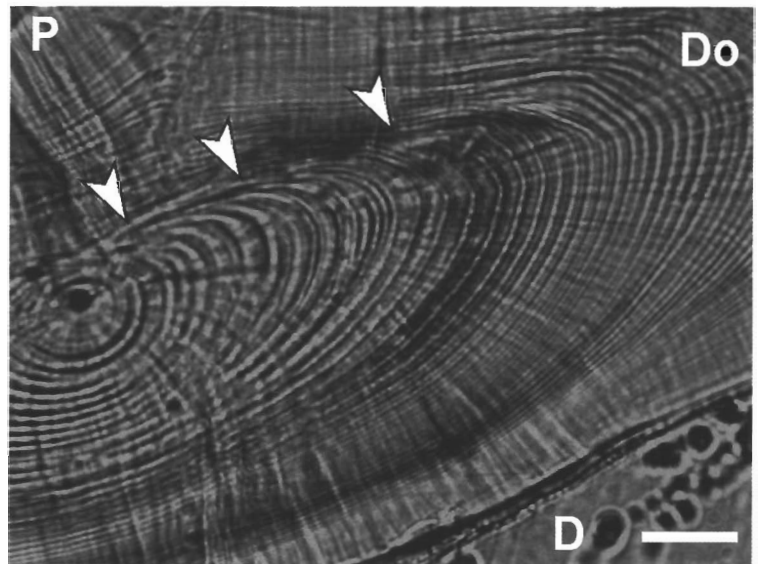


Figure III.C.4 - Differences in the observation due to changes in the section in an otolith of *Merluccius merluccius*. Central area showing core and accessory *primordia* (arrows). a) observation before and b) after further polishing. Scale bar = 60 μm (photos B. Morales-Nin).

the plane of sectioning (fig. III.C.4). Furthermore, the increments laid down after the accessory centres may not match those in the nuclear area (fig. III.B.1).

The observation of the patterns of increments in the internal structure of the otolith is more complex because of the different scales of observation, which range from daily to annual. Observation depends on the method of preparation but also on the magnification employed. The correspondence between daily increments observed under compound microscopy and under SEM, for example, is known precisely (chap. II.A), but that between daily microincrements and seasonal increments is still a matter of controversy.

The problem of reducing a 3D observation to 2D is similar, but less pronounced, when preparing skeletal bones such as the spines (fig. III.C.5). The basal (ventral) part of the spine retains the most growth increments but is often affected by bone remodelling whereas the outer (apical) edge may miss several or all increments. The spinal section should be strictly transverse to the longitudinal axis in order to avoid distortions in the incremental patterns. Vertebrae are generally observed whole, but frontal sections may be necessary in order to reveal increments in the vertebral bodies (Loubens & Panfili, 2000).

In theory, incremental patterns should be homologous in all axes, but due to different growth rates that cause particular three-dimensional distortions they may become heterogeneous. Standardisation is thus necessary when sections are being prepared (chap. VIII.C.2.3) and when growth increments are being read. The need for a standard preparation is obvious in the case of complex otoliths and spines. Although the continuity of the pattern of increments around the CS is a matter of interpretation, a standard reading axis should be selected. In the case of counts of daily increments, a single reading axis may not be feasible due to breaks in the visibility of the growth patterns. Counting paths need not be linear, and another reading axis close to the main one should be selected after a single distinct increment (Campana, 1992) (fig. III.C.6). Even after sectioning, any remaining 3D information on the CS preparation may still obscure growth patterns under light microscopy, due to problems of parallax and focal plane selection (Campana, 1992), causing subjective errors in the interpretation. These ambiguities are reduced following surface preparation and observation techniques such as SEM or staining (chap. VIII.D.2 and VIII.C.2.8).

Selection of a suitable measurement axis requires the same orientation of the axis and clarity of increment criteria as those associated with increment counts (Campana, 1992). Because 3D growth is extracted from a 2D image, the measurements should consistently follow the linear or curvilinear growth patterns along the axis selected. This is now possible with the aid of image analysis systems (chap. VI).

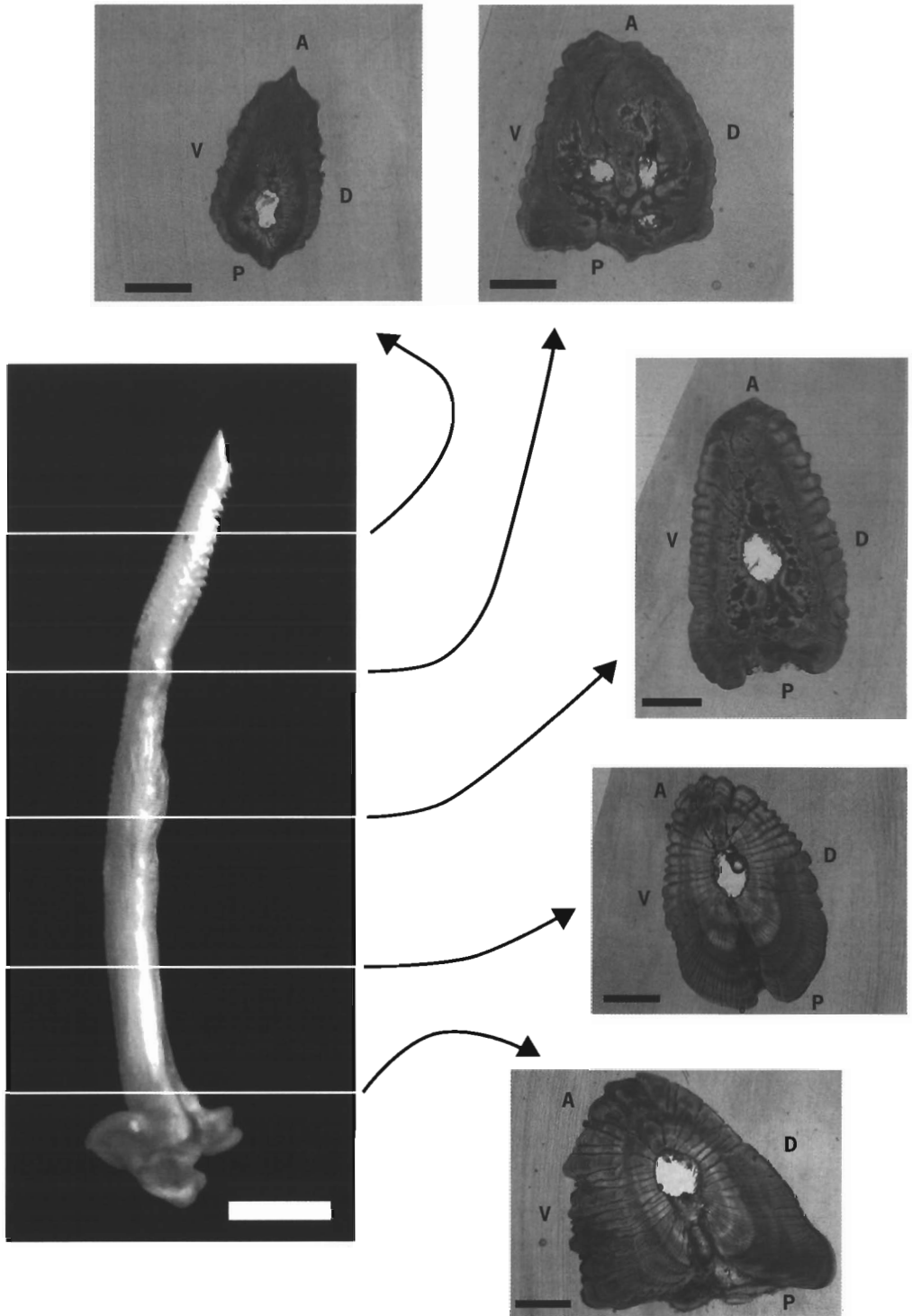
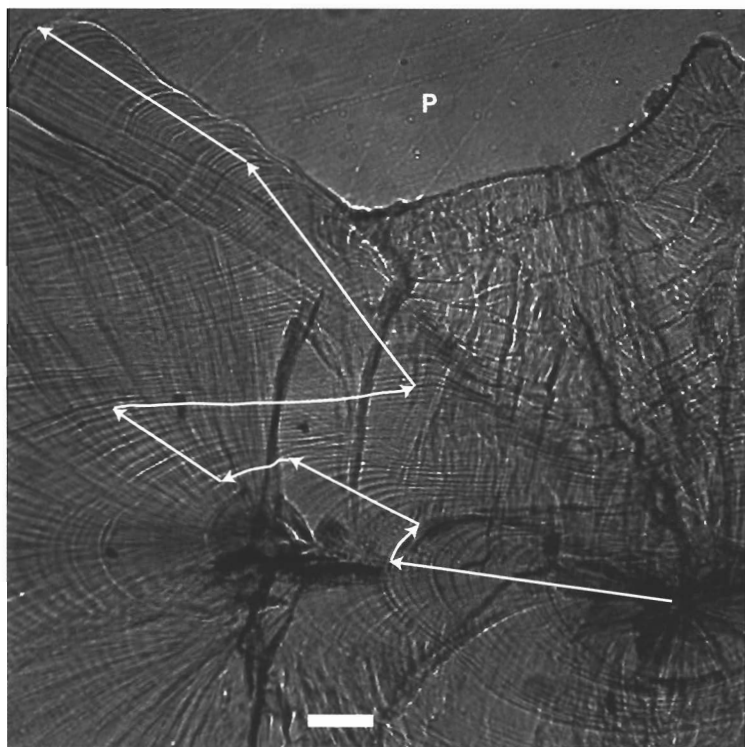


Figure III.C.5 - Various levels of section for the pectoral spine of *Pangassius hypophthalmus*. The pattern of the increments is very different between all the levels of section: the information is lost towards the outer apical part. Standardisation of the section level is thus essential in any sclerochronological study. A, anterior; D, dorsal; P, posterior; V, ventral. Scale bar = 200 μm for the sections and scale bar = 15 mm for the whole spine (photos J. Panfili).

Figure III.C.6
Changes in reading axis
for daily increments
on transverse thin section
in otoliths of *Oreochromis
niloticus*. The reading is
from the core to the edge
along the *sulcus acusticus*
on the proximal side (P).
The arrows indicate
the reading paths, which
changes when
microincrements
are unreadable or unclear.
The reading cannot be made
directly from the core
to the edge on a linear path
because growth is reduced
in the proximal side during
the first days of life.
Scale bar = 20 μm
(photo A. Malam Massou).



Chapter IV

Validation and verification methods

It is impossible to know the absolute age (true age) of a fish taken at random in its natural environment. Many fishery analyses are based on accurate estimates of the age structure of the catches. The recognition that ageing errors are important has stimulated interest in the validation of age estimation methods. Validation means proving that a technique is accurate, and accuracy can be demonstrated or estimated. Estimates of accuracy are less valuable, but in some cases only an estimate is possible (Archibald *et al.*, 1983). Beamish & McFarlane (1983) pointed out the importance of validating ages and, in their review of the literature, emphasised that few studies validated their own methodology. Since then, however, many validation studies have been performed. Theoretically, a validation should be made of every population of any given species, since there may be important differences between them: for example, this is the case for migratory species such as salmon and eels. Ideally, validation would entail monitoring individuals from hatching to adult stages through metamorphosis (Geffen, 1992). Although validating an age estimation technique for the entire age range of the population is very difficult, a combination of methods might offer greater certainty in the accuracy of age estimates. Validation should be an obligatory step in all sclerochronological studies, especially in species in which no age estimation studies have been performed. Two aspects must be determined: (1) that the increments are laid down with a periodicity that can be related to a regular time scale (i.e. accuracy) and (2) that the ageing structure has a consistent interpretable pattern of increments (i.e. precision). The methodologies available for validating the frequency of formation of increments can be grouped into the following four main categories which constitute the basis of this chapter:

- direct validation, which takes into account a precise temporal reference mark on a CS relative to the other growth marks; this is an individual-based technique that often uses marking and/or rearing;
- semi-direct validation, which requires the observation of the time series of growth marks on a large number of individuals; this is a population-based technique;
- indirect validation, which requires the comparison of the individual age estimates with statistical age estimated from length frequency distributions as well as other age data; this is also a population-based technique;
- corroboration, which simply involves multiple interpretations (obtained after one or more readings of one or more CS).

A. Direct validation

P.J. Wright, J. Panfili, A. Folkvord, H. Mosegaard, F.J. Meunier

Direct validation leads to knowledge of the age on the basis of a CS of a single individual. It often refers to a precise temporal reference growth mark compared to other marks. Direct validation procedures include a series of observations of marked individuals released in their natural environment and/or reared fish under controlled or semi-controlled conditions (Brothers *et al.*, 1976; Neilson & Geen, 1981; Gefen, 1992). The introduction of datable marks on the CS of fish before rearing and recapture is a common preliminary stage. These marks may be chemically or intrinsically induced.

1. Marking

In this section, we consider the whole process of “marking” from the capture of individuals in their natural environment, through marking (fish and/or CS), release in the natural (or artificial) environment, to recapture at a given time. As the interval between release and recapture is known, the duration of the growth periods can be calculated. As CS marking usually cannot be detected externally it is necessary to put an external mark or tag on the fish.

1.1. Marking fish

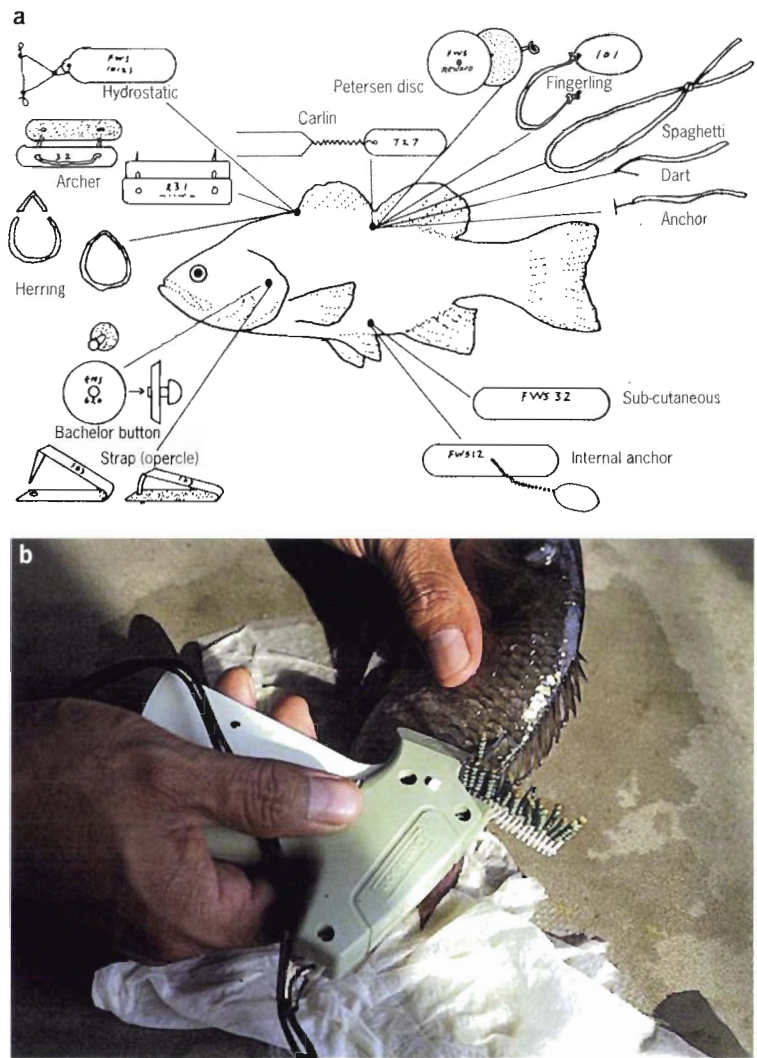
The marking and tagging of individuals is an extremely widespread process in population dynamic studies. In this section, we summarise the main approaches to fish marking. Further details of marking and tagging can be found in Parker *et al.* (1990). Clearly, individual marking and the type of mark used depend on the size of the fish, being unsuitable for larvae. Marking is essential for age validation studies in which it is necessary:

- to locate individuals or groups of fish which have marked calcified structures;
- to locate individual fish from whom a calcified structure has been removed. This is only possible after scale extraction (Beamish & McFarlane, 1983; Matlock *et al.*, 1987; Beall & Davaine, 1988) or sometimes after extracting part of a fin ray (Rochard & Jatteau, 1991). The increments in such structures are then compared between the dates of initial capture (marking) and recapture.

A wide range of external tags is available for individual marking (McFarlane *et al.*, 1990). Some examples are shown in figure IV.A.1a. Fixing the tags is made easier by the use of suitable guns (fig. IV.A.1b). The introduction of small internal fish tags, such as the

coded visible implants that are implanted under the skin in the cranial region, and coded (micro)wire tags, may be preferable to external tags because the former do not affect the hydrodynamic performance of the fish. Electronic pit-tags (passive integrated transponder) injected under the skin allow information on the individual to be read with minimal handling. These tags minimise the biological impacts of marking, such as causing changes in behaviour, and provide clear and unbiased data. For further information on fish tags, see international distributors of external tags such as Hallprint Tags (Australia), Biomark (USA), FishEagle International (UK), Northwest Marine Technology Inc. (USA). Newly developed electronic tags are now available which mark the fish externally and record several types of data

Figure IV.A.1
External marking of fish.
a) Principal types of external tags and anatomical sites for attachment on fish (from McFarlane et al., 1990).
b) External marking with anchor T-tag for an African tilapia (*Sarotherodon melanotheron*).
The numbered tag is put under the skin in the muscle using a special tagging gun (photo J. Panfili).



from the natural environment (e.g. water temperature, pressure, solar radiance) and sometimes aspects of fish physiology (e.g. internal temperature). Details of these data storage tags can be obtained from Star-Oddi (Iceland) and Lotek (Canada).

Batch-marking techniques usually fall into one of two categories; tattoo-ink marking and cold-branding marking. Tattooing is done by injecting a dye under the skin or by means of a jet-spray inoculator to apply coloured dots to selected areas on individual fish (generally on the belly if this is less pigmented). As an example, a suitable method for small fish is subcutaneous injection of Alcian blue dye at a dilution of 1% in a solution of physiological serum. Fluorescent elastomere may also be suitable for small fish and provide more discernible marks (suppliers include Northwest Marine Technology Inc., USA). Jet-spray inoculators are only suitable for large juvenile fish (> 10 cm). The cold-branding technique allows numbered marks to be made on the skin. The method involves immersing numbered tools in liquid nitrogen and then applying these directly to the skin. These techniques may allow individual coding but they tend to be only suitable for short-duration experiments as the dye or brands may disappear as the skin is renewed. However, they can be visible for several months after marking.

1.2. Marking calcified structures

Unless fish have been reared from hatching, it is necessary to place a mark on the CS to act as a temporal reference. The various methods used to mark calcified structures can be categorised as follows:

- fluorescent dyes;
- temperature-induced marks;
- light cycle-induced marks;
- radioisotope marking with radioactive strontium, ^{85}Sr ;
- elemental marking with Sr or lanthanides.

However, methods 2-5 are only suitable for otoliths.

Environmentally induced marks, resulting from either a change in habitat or feeding events, can also be used as a reference point.

1.2.1. Fluorescent dyes

Chemical marking with fluorescent dyes has long been the main method used for direct validation experiments. The method relies on the incorporation of a specific chemical compound into the mineralising surface. Since the 1960s, several markers have been used in Osteichthyan species, the earliest of which were tetracyclines (De Bont, 1967; Weber & Rigway, 1967; Casselman, 1974; Meunier, 1974; Meunier & Boivin, 1974; McFarlane & Beamish, 1987). Since then, fluorescein or calcein (Meunier, 1974; Meunier & Boivin, 1978; Wil-

son *et al.*, 1987; Tsukamoto, 1988; Beckman *et al.*, 1990), orange xylenol (Meunier, 1974), and alizarin (Meunier & Boivin, 1978; Tsukamoto, 1988) have been used. These substances have a broad spectrum and are suitable for CS in all Vertebrate species.

These compounds all have the capacity to emit a specific coloured fluorescence under ultraviolet light, and are thus located *a posteriori*. Tetracycline emits yellow fluorescence, fluorescein yellow-green, and both alizarin and orange xylenol emit red. The coloration of the marks depends on the wavelength of the light source and the optical filters used (tab. IV.A.1).

Table IV.A.1 - Various fluorochromes and their excitation and emission characteristics under compound microscopy (reflected light).

Fluorochrome	Excitation wavelength (mean in nm)	Fluorescence wavelength (mean in nm)	Excitation rays	Excitation colour	Filter type (reflected light)
Tetracycline	390	560	blue/violet	yellow	D
Alizarin	556	596	green	red	N2
Fluorescein	490	525	blue	yellow-green	A
Xylenol orange	470	530-650	blue	orange-red	I2/3

Tetracycline (TC, $C_{22}H_{24}N_{12}O_8$) and its derivatives (oxytetracycline, OTC, tetracycline hydrochloride, TCHC, tetracycline dihydrochloride, TCDHC), are broad-spectrum antibiotics, and they generally produce excellent results in all species. However, during the last decade, the use of tetracyclines has been restricted in some countries. In many countries including the USA, Canada and Japan, they may not be used in the field, or be released into natural or semi-natural waters. This is because these antibiotics have a very low rate of natural degradation and some microorganisms become resistant to tetracycline (Coyne *et al.*, 1994; Kerry *et al.*, 1994; Smith, 1995; Vaughan & Smith, 1996). Researchers ought therefore to check their national legislation on environmental protection before using tetracycline.

For fluorescein (FC, $C_{20}H_{12}O_5$), the results tend to be more variable, from slightly poorer marking (Tsukamoto, 1988), to better than other fluorescent markers (Thomas *et al.*, 1995). Since the restrictions on the use of tetracycline, two dyes of the alizarin family that efficiently produce fluorescent marks in fish otoliths, alizarin complexone (AC, 1,2-dihydroxyanthraquinone-3-yl-methylamine-N, N-diacetic acid, $C_{19}H_{15}NO_8 \cdot 2H_2O$) and alizarin Red S (AR, 1,2-dihydroxyanthraquinone sodium sulphonate, $C_{14}H_7O_7NaS$), have come into widespread use.

The fluorescent dyes are available in different forms. Tetracycline is found in two forms:

- a powder in various concentrations, from pure chemical tetracycline to active tetracycline with an excipient, as used by veterinarians (e.g. Acti-Tetra B®);
- various concentrations of a stable solution (e.g. Terramycine®). Fluorescein (calcein) is available as a powder or in solution. Alizarin is only available as a powder, and solutions must be prepared.

These dyes are applied in various ways; by injection, whole-body immersion, or incorporation in food (see following). There is some variation in the results of marking between the markers and their method of application (Thomas *et al.*, 1995) but all calcified structures can be labelled at the same time (fig. IV.A.2). Further methods applying internal markers have also been proposed.

1.2.1.1. Injection

Intraperitoneal or intramuscular injection is the most widespread technique used to mark juvenile and adult fish. Intraperitoneal injection is recommended (fig. IV.A.3). Some stable solutions of TC exist for veterinary use (e.g. Terramycine®). Injections of excessively high concentrations of TC can cause the death of the fish (Meunier & Boivin, 1978; Beamish & McFarlane, 1987). Meunier & Boivin (1978) estimate that concentrations of 50-100 mg of TC per kg of body weight do not affect later growth. McFarlane & Beamish (1987) recommend injections of 25-35 mg TC per kg. On the basis of several studies it appears that concentrations between 25 and 100 mg TC.kg⁻¹, and especially of 50 mg.kg⁻¹ live fish, are generally acceptable (Meunier & Pascal, 1981; Babaluk & Campbell, 1987; Babaluk & Craig, 1990; Bumguardner, 1991; Hall, 1991; Murphy & Taylor, 1991). However, the rate of injection depends upon growth rate, longevity, and the structure being labelled (Casselman, 1983). Multiple marking can be achieved by injecting fish at intervals of a few weeks or months.

Before injecting fish with fluorescent dye, it is preferable to anaesthetise them. Several products that are widely used in aquaculture are available for this purpose. Phenoxyethanol (C₈H₁₀O₂) at a concentration around 3‰ can be recommended. The concentration must be tested before experimental use, since susceptibility to this anaesthetic differs between species and sizes.

1.2.1.2. Whole-body immersion

This is especially useful for young fish when it is impossible to inject dye. Individual larvae or juveniles are bathed in solutions of a specific concentration for a given time. There are many references on this subject, which have used a wide range of dye concentrations and durations (tab. IV.A.2).

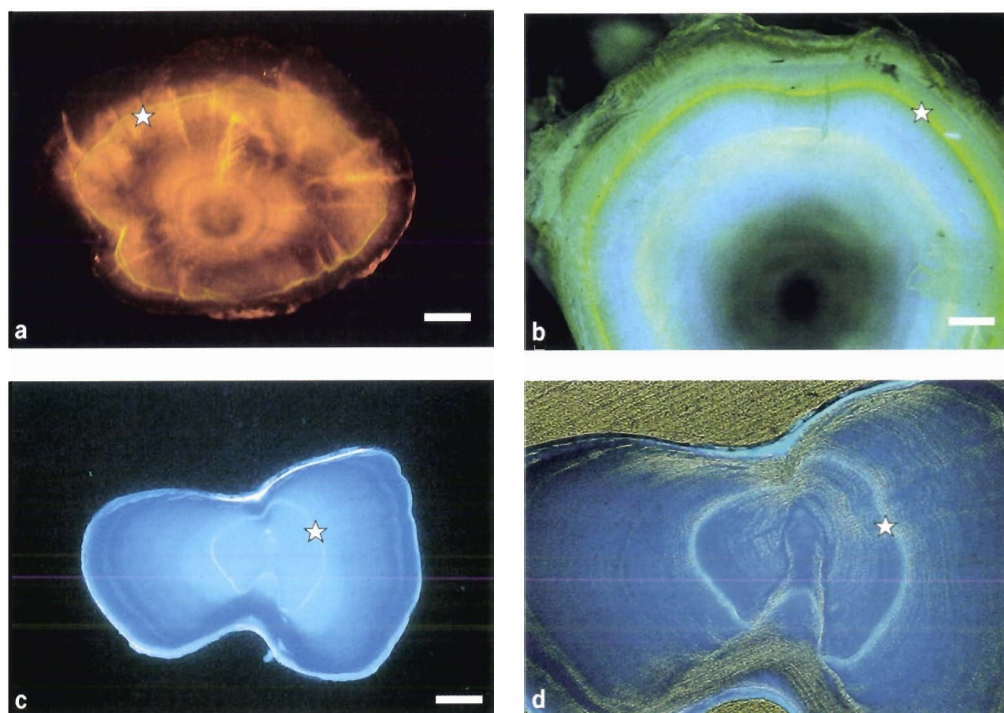


Figure IV.A.2 - Tetracycline marks (☆) on different calcified structures. All the preparations are observed under epifluorescent uv light microscopy (photos J. Panfili).

a) Whole otolith of eel (*Anguilla anguilla*) observed under reflected light. Scale bar = 200 μ m.

b) Whole vertebrae of pike-perch (*Stizostedion lucioperca*) under reflected light. Scale bar = 1 mm.

c) Slice of the dorsal ray of pike-perch (*Stizostedion lucioperca*) under reflected light. Scale bar = 400 μ m.

d) Detail of the dorsal ray (c) of pike-perch (*Stizostedion lucioperca*) under transmitted light.

Figure IV.A.3

Internal marking with tetracycline injection for an African tilapia (*Sarotherodon melanotheron*). The fish was anaesthetised in advance. The solution is injected intraperitoneally at a concentration of 50 mg tetracycline per kg of live fish. This fish had been previously tagged with an anchor T-tag (fig. IV.A.1) (photo J. Panfili).



Table IV.A.2. - Concentrations of fluorescent dyes and duration of immersion marking for eggs and/or larvae.

Substance	Concentration (mg.l ⁻¹)	Solution	Duration	Quantity of fish	Reference
Tetracycline	100-500	NaCl 1%	1-2 h		(Hettler, 1984)
Tetracycline	200-300	no	24-48 h	eggs	(Tsukamoto, 1985)
	200-300		3-24 h	larvae	
Tetracycline	100-600	no	12 h	eggs, larvae, juveniles	(Dabrowski & Tsukamoto, 1986)
Oxytetracycline	50	no	12h.d ⁻¹ (4 d)	87000-126000	(Lorson & Mudrak, 1987)
Tetracycline	200-300	no	24 h		(Tsukamoto, 1988)
Tetracycline	400	NaCl 15%	24 h	200	(Siegfried & Weinstein, 1989)
Oxytetracycline	400-500	no	24 h	15.l ⁻¹	(Tzeng & Yu, 1989)
Alizarin compl.	50 -200	seawater	24 h	10 million	(Tsukamoto <i>et al.</i> , 1989a)
Tetracycline	10000	hyperosmotic	3 mn 30 s	500 kg	(Alcobendas <i>et al.</i> , 1991)
fluorescein	20000			1.25 million	
Oxytetracycline	350	no	3 h	1.2-7 million	(Secor <i>et al.</i> , 1991)
Alizarin compl./ alizarin red S	100-200 100	34 ppt seawater	24 h	1.18 million	(Blom <i>et al.</i> , 1994)
Alizarin compl.	100	no	14 h	1000	(Ahrenholz <i>et al.</i> , 1995)
Oxytetracycline	10000	hyperosmotic	1-10 mn	100-40000	(Rojas-Beltran <i>et al.</i> , 1995)
Alizarin compl.	250	25 ppt seawat	15 h	114	(Szedlmayer & Howe, 1995)
Oxytetracycline	350-400	no	6-8 h	600000	(Reinert <i>et al.</i> , 1998)

To mark a large number of larvae, some authors recommend increasing the densities of fish (Secor *et al.*, 1991), or accelerating processing time while plunging the larvae into a hyperosmotic solution (sodium chloride 5%) for a few minutes then in 1% TC (Alcobendas *et al.*, 1991). Other studies show that embryos can be marked in the egg (Tsukamoto, 1985; Ruhlé & Grieder, 1989; Muth & Bestgen, 1991). For larvae, multiple marking (incorporation of several fluorochromes) is possible by subjecting them to successive baths at intervals of a few days (Tsukamoto, 1988; Hendricks *et al.*, 1991). It is important to bear in mind that too high a concentration of TC can kill larvae (Nagiec *et al.*, 1988).

The immersion technique is quite simple: fish are put in a tank with an air supply, the appropriate concentration of fluorescent dye is added to the water and the process is continued for a given duration and then the bath is emptied and filled with freshwater (tab. IV.A.2). This procedure can be adapted to the species and the material used. The most important factor affecting treatment success seems to be the oxygenation of the water, as bathed fish can be particularly sensitive to a lack of oxygen. The procedure given below is generally suitable for egg and larval stages (Blom *et al.*, 1994):

- the quantity of dye needed to produce a final concentration (bath solution) of alizarin AC or AR of 100 mg.l^{-1} is weighed and added to a beaker (50 mg.l^{-1} of AC has also been shown to be sufficient in several cases);
- the dye is dissolved in 1N potassium hydroxide until its colour changes from brown-reddish to a darker bluish-red colour (at a pH of around 7.5-8.0 for AC and AR);
- the solution is diluted with distilled water to produce a working solution of 50 to 100 ml before further dilution in saline;
- a further dilution in saline is made while adding the working solution to the unit used for marking to achieve a bath solution of the desired strength (see above);
- it is important to check that the pH is below 9 in the bath solution, in order to avoid mortality. If necessary acid (e.g. hydrochloric acid) should be added to lower the pH;
- since the solution will be stagnant during the marking process, it is essential to maintain the oxygen saturation above 80% by aeration;
- marking may last for 12-24 h, after which the fish eggs and larvae should be removed from the bath or the solution replaced with clean salt water.

1.2.1.3. Incorporation into food

This technique, suggested by Weber & Rigway (1967), has not found many followers. However, Nordeide *et al.* (1992) showed that this method could allow a large number of individuals to be marked with food to which tetracycline has been added at a concentration of $10 \text{ g OTC. kg dry feed}^{-1}$. Oral administration of fluorescent markers seems to be successful at concentrations of 25 mg.kg^{-1} and 50 mg.kg^{-1} food weight for calcein and tetracycline, and at 50 mg.kg^{-1} for alizarin complexone (Thomas *et al.*, 1995). These authors have demonstrated that the marking quality of alizarin complexone is poorer than that of calcein and tetracycline.

1.2.1.4. Fluorescent dyes

While fluorescent dyes are suitable for various temporal scales of validation, such as the seasonal deposition of all types of CS and the daily deposition of otoliths, they necessitate the use of ultraviolet light. The growth of the CS will to some extent determine the success of marking. In cod and herring, for example, otolith growth prior to hatching is relatively high, and this is therefore a suitable period for marking. Marks induced at the egg stage are easily detectable throughout the larval stage and well into the juvenile stage (Blom *et al.*, 1994). In the case of herring, the demersal eggs are commonly incubated on glass slides and sheets of plastic. This makes them especially suitable for marking at this stage since small volumes of bath solution can be used

without the problem of retrieving the eggs from the solution. This is important, since some of the dyes, especially alizarin complexone, are expensive.

As mentioned above, multiple markings can be done and used as group marks on sub-populations with different characteristics (Tsukamoto *et al.*, 1989b). It is important to allow sufficient time between markings to prevent separate marks from merging (fig. IV.A.4). During the larval stage the rate of growth during the period prior to marking will also affect marking success. For example, in 32 day-old herring larvae marking success varied between 10 and 55% in groups fed maintenance rations for two weeks before marking, while success was nearly 100% in groups fed to excess during the same period (Folkvord *et al.*, 2000). In cases in which marking success was low, the otolith accretion rate was also generally very low, with daily increment widths of less than 0.5 μm .

For validation studies of annual growth cycles, it is necessary to maintain the fish for more than 12 months, either in the wild or in experimental ponds. The longevity of the fluorescent mark is good (especially for tetracycline and fluorescein) in internal organs such as otoliths or vertebrae but is of shorter duration for the scales because of the deleterious effect of sunlight on these photosensitive dyes. Several authors have described experiments that continued for several years. For example, tetracycline-marked elvers in the Rhine showed fluorescent marks five years after immersion (Meunier, 1994). Beamish & McFarlane (2000) have also observed tetracycline marks on the otoliths of *Anoplopoma fimbria* more than 20 years after they had been injected.

1.2.2. Temperature-induced marks in otoliths

Temperature has been shown to have an immediate affect on both otolith accretion rate and microstructural composition (Mosegaard *et al.*, 1987; Berghahn & Karakiri, 1990; Bergstedt *et al.*, 1990; Volk *et al.*, 1990; Munk & Smoker, 1993). Optically opaque or translucent otolith formation is believed to reflect the composition of the organic content and aragonite crystal structure. Temperature fluctuations will therefore leave discernible traces on the microstructure. In a laboratory experiment, manipulated temperature fluctuations imprinted a coded sequence of checks according to the expected otolith formation rate at the temperatures used (fluctuations between a baseline level of 10°C and peaks of 14°C; fig. IV.A.5). Temperature variations have been used to create "bar codes" in commercial salmon stock with large numbers of individuals (Volk *et al.*, 1990, 1995). Brothers (1990) and Volk *et al.* (1999) have produced extensive reviews on otolith thermal marking techniques.

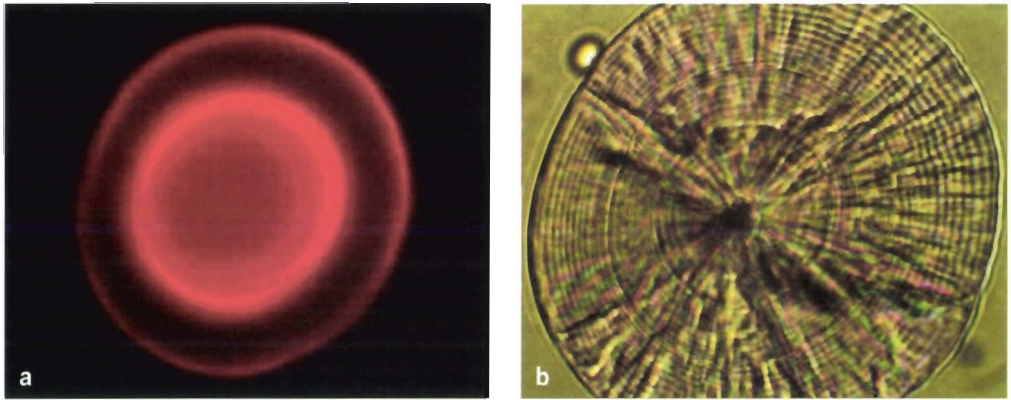


Figure IV.A.4 - Example of double alizarin complexone marks on a larval herring (*Clupea harengus*) sagitta visible under fluorescent light (a) and the same otolith viewed under transmitted light (b). Scale bar = 20 μm (photos A. Folkvord).

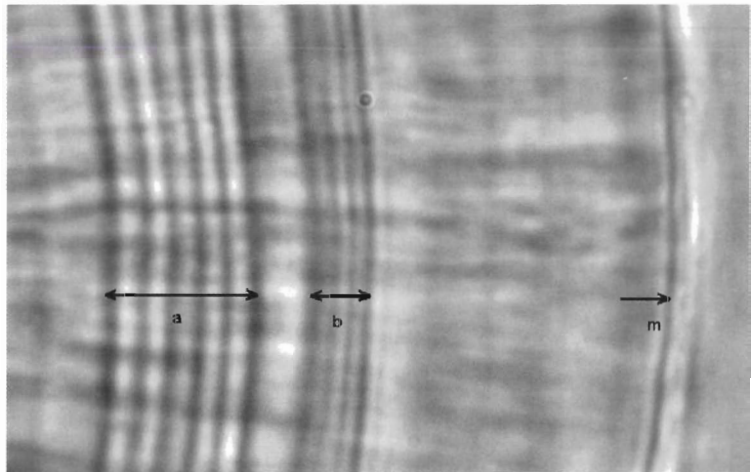


Figure IV.A.5 - Example of temperature-manipulated increment formation in brown trout (*Salmo trutta*) alevin sagitta, manipulated at 135 degree-days after hatch. Basal conditions were 11.5°C in darkness (photo H. Mosegaard).

a) Six manipulation periods with 2 h at 14.5°C and 6 h at 11.5°C.

b) Four manipulation periods with 2 h at 14.5°C and 2 h at 11.5°C. Lights were on during the warm phases of each manipulation. m = otolith margin.

1.2.3. Light cycle-induced marks in otoliths

Otolith calcification is entrained to light-dark transitions (Wright *et al.*, 1992). In some species a 6L:6D photoperiod can mask the diel periodicity of increment formation, leading to the formation of an indistinct zone of increment formation. An example of 6L: 6D photoperiod marks in juvenile salmon is shown in figure IV.A.6. This marking approach can be improved by heating the environmental water to 3°C above ambient temperature during the light phase of sub-daily light cycles (fig. IV.A.7).

Figure IV.A.6
Example of a mark induced
by a 3-day period of 6L: 6D
photoperiod in juvenile
Salmo salar
(photo P.J. Wright).

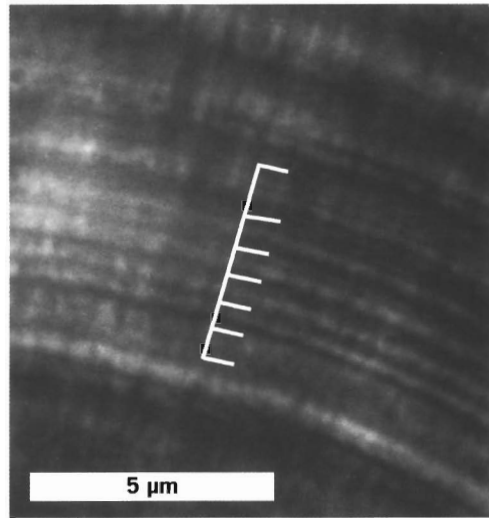
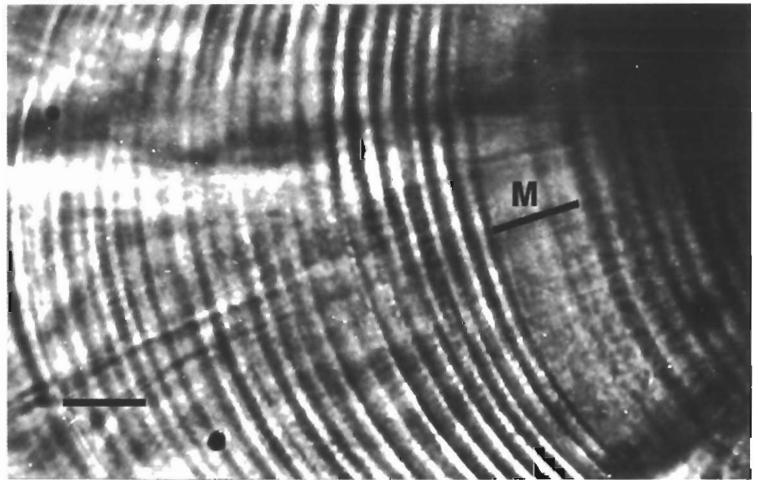


Figure IV.A.7
Example of mark (M)
induced by combined 6L:
6D photoperiod with 3°C
temperature rise during the
light phase in *Gasterosteus
aculeatus*.
Scale bar = 10 μm
(photo P.J. Wright).



1.2.4. Elemental otolith marking (^{85}Sr , Sr and lanthanides)

Otolith marking using ^{85}Sr is not very common, partly due to safety regulations imposed on work using radioisotopes. In a study of the dispersal of larval whitefish, Lehtonen *et al.* (1992) marked newly hatched larvae with ^{85}Sr in a 1400 k Bq.l⁻¹ solution of ^{85}Sr . The radioactive label in the fish larvae could be detected in whole larvae for up to 60 days after marking. The extent to which otolith composition contributed to the retention of the mark is unclear, and further studies would have to be carried out to evaluate marking procedures which would leave detectable radioactive Sr in the otoliths.

Larval and juvenile otoliths have been marked by altering the elemental composition of the rearing water. Two strategies that differ in principle can be employed. First, the addition of a relatively common element such as strontium (Sr) has been used to alter the Ca/Sr ratio in the otoliths. Strontium chloride has been used to mark salmon fry with good success (Schroder *et al.*, 1995). A 24 h exposure to SrCl bath solutions in concentrations of 120 ppm and higher yielded at least a five-fold higher Sr concentration in the experimental otoliths than in controls. The Sr marks could be detected after 21 months with Wavelength Dispersive Spectrometry or Backscattered Scanning Electron Microscopy (chap. VII). In a second approach, marking has also been achieved by immersing fish in solutions containing elevated levels of rare earth metals (lanthanides). These elements are normally very rare in fish otoliths, and marking with different proportions of these elements can produce several unique group marks in different sub-populations. Ennevor & Beames (1993) used different concentrations and procedures for marking coho salmon (*Oncorhynchus kisutch*) fry and smolts with lanthanum and cerium, and the elements could be detected up to 10.5 months after treatment. However, the mode of incorporation of these rare elements into the otoliths is uncertain, and sensitive analytical techniques need to be employed to detect their presence (chap. VII).

2. Breeding and rearing

Generally speaking, methods that release fish to the wild, or in which fish are maintained under semi-artificial conditions which provide for good growth and natural behaviour (i.e. mesocosms), are preferable for validation studies (Geffen, 1992). The best approach for age validation is to release marked fish into the wild, as has been done with Japanese red sea bream (Tsukamoto *et al.*, 1989a), eel from the Rhine river (Meunier, 1994) and Pacific salmon (Volk *et al.*, 1990, 1999). However, because of the large numbers of fry that need to be marked to collect sufficient returns, releasing fish is generally not feasible (Geffen, 1992). For this reason, validation generally requires fish to be reared from eggs or wild-caught fish. Caution should be taken with the rearing conditions as these should be as close as possible to the natural environment of the species (fig. IV.A.8). Field enclosures or ponds (fig. IV.A.8c,d), have been employed in several studies (Liew, 1974; Geffen, 1982; Simoneaux & Warlen, 1987).

The conditions under which validation studies are conducted are also very important, since factors such as feeding level, feeding periodicity, container size and temperature all affect the rate of growth of fish and may also influence increment periodicity (Geffen, 1982; Neilson & Geen, 1982; Radtke & Dean, 1982; McGurk, 1984; Al-Hossaini &

Pitcher, 1988). In some laboratory studies, an increment periodicity lower than one per day has been related to the inability to maintain the high larval growth rates observed in the wild (Al-Hossaini & Pitcher, 1988). Rearing conditions in the laboratory often produce unnatural stresses, leading to the formation of sub-daily increments and interruptions or checks in growth (Pannella, 1980; Morales-Nin, 1987a). There also tends to be a less clear distinction between L- and D-zones in fish reared under constant temperature and light levels than in fish reared in field enclosures (Geffen, 1982; Campana, 1984).



Figure IV.A.8 - Various types of aquaculture systems usable for validation experiments: from artificial (a) to semi-natural environments (f). The experiment should be as similar as possible to the natural environment of the fish. a) artificial ponds for freshwater aquaculture (Thammasat University, Thailand); b) concrete tanks with freshwater in ambient weather conditions (Centre de recherches océanologiques, Lajo, Ivory Coast); c) cages submerged in a brackish water lagoon (Centre de recherches océanologiques, Lajo, Ivory Coast); d) cages in the open sea (Research Institute for Marine Fisheries, Sulawesi, Indonesia); e) wide concrete marine tanks open to the lagoon (Albion Fisheries Research Center, Mauritius); f) semi-natural ponds for freshwater aquaculture (Centre national de recherche agronomique, Ivory Coast) (photos J. Panfili).

For these reasons, validation studies should ideally be carried under both laboratory and field conditions or in the field alone (Geffen, 1982; Gjosaeter *et al.*, 1984; Campana & Neilson, 1985).

3. Data treatment

Statistical treatment of data from a validation experiment is relatively simple. In general, the relationship between the number of days elapsed after tagging and/or breeding and the number of increments on the CS analysed is calculated from a linear regression, as follows:

(IV.A.1) number of increments = $a \cdot \text{number of days of growth} + b$
 where a is the slope and b is the intercept of the regression.

The next step to analysis involves testing whether the slope differs significantly from a slope equal to 1 ($a = 1$) and an intercept equal to 0 ($b = 0$). Significance is usually determined using a Student *t*-test. Several results can be obtained:

- If $a = 1$ and $b = 0$, the counted increments are related to the number of days of the experiment. The validation is accomplished;
- If $a = 1$ and $b \neq 0$, the rate of deposition corresponds to the days but it does not start from the first day of the experiment;
- If $a \neq 1$ and $b \neq 0$, there is not direct relation between the number of increments and the number of days and the deposition is not daily. However, in the case where a change in the increment deposition rate is apparent, separate tests can be made in each age interval to check if the deposition rate is daily during one of the time intervals. This is especially important in cases where increment deposition rate may be initially lower than 1 per day, but subsequently increases. Including all the early increments-at-age in such a case will lead to underestimation of the increment deposition rate at subsequent ages/stages where the deposition rate could be daily.

The above approach is often the only statistical justification given for daily increment deposition. However, this approach of taking non-rejection of the null hypothesis as positive evidence that the null model is correct, ignores the potential of Type II error, that is concluding increments are formed daily when in fact they are not. The probability of avoiding Type II error can be measured by calculating the statistical power. By calculating power for the regression of increment count on time, one can determine the likelihood that a significant difference in slope would be detected if one really existed and thus evaluate the conclusion that increments are formed daily. Methods for determining statistical power of regressions are now widely available in statistical packages. Rice *et al.* (1987) also provide a useful review of this issue.

B. Semi-direct validation

J. Panfili, B. Morales-Nin

One of the most used validation methods, semi-direct validation, consists of observing the evolution of CS marginal zones over time. This is a powerful method which requires regular observations throughout the time period concerned, according to the cycle of growth increments (daily, seasonal or other) under study, and the observation of a large number of individuals. The method consists of observing the edges of the CS, selecting a given mark, and following its formation through time in a single population. The result is an average and/or percentage of observations at population level. The selected mark must be sufficiently precise to permit the detection of its formation at the extreme edge of the CS, which may be difficult. Semi-direct validation is usually used to validate seasonal deposition, and two types of studies are possible, one of which uses qualitative data and the other, quantitative data.

1. Qualitative and/or quantitative data?

1.1. Qualitative data

This process consists of evaluating the presence or absence of the mark at the edge of the CS, and expressing the results in percentages for the population under study. The evolution of this percentage through time is then studied: figure IV.B.1 shows the result for one growth cycle per year (fig. IV.B.1a) and two cycles per year (fig. IV.B.1b). The second case is less common in a temperate environment but it has been observed for tropical fishes (Yosef & Casselman, 1995). In North America, a system of edge codes, first presented in Casselman (1987) and elaborated in Casselman (1996), is now widely used.

1.2. Quantitative data

This method consists of measuring the distances separating the latest marks at the edge of the CS. The axis of measurement and the description of the marks being utilised must be rigorously standardised. The absolute marginal distance (AMD) is the distance that separates the last mark from the edge (fig. IV.B.2). The relative marginal distance (RMD) is the ratio of the absolute marginal distance to the distance separating the two last marks ($D_{i,i-1}$) (fig. IV.B.2):

$$(VI.B.1) \quad RMD = \frac{AMD}{D_{i,i-1}}$$

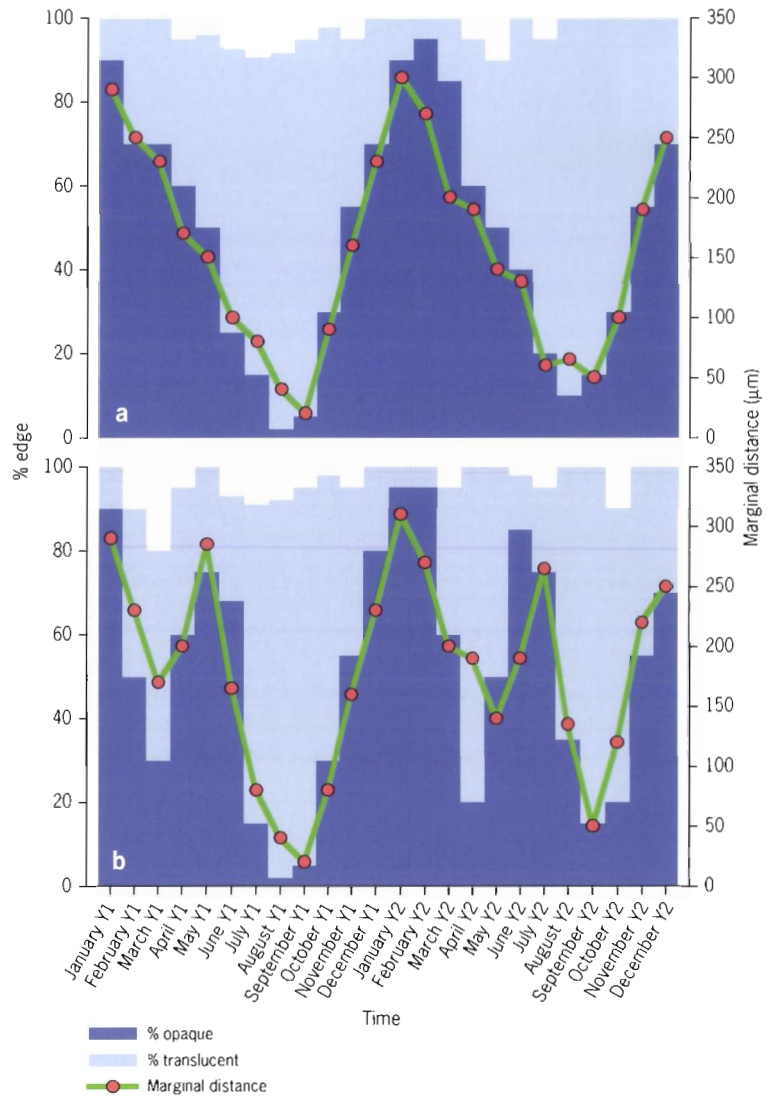


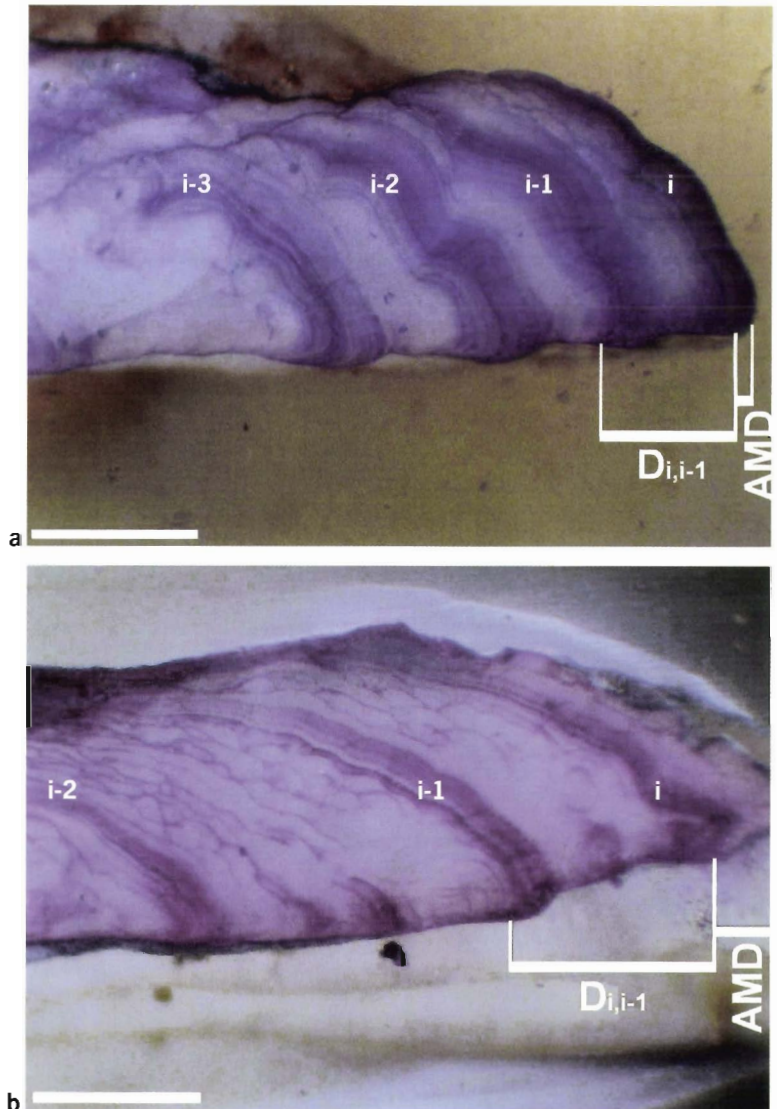
Figure IV.B.1 - Theoretical graph of the evolution over time (two consecutive years) of the percentage of opaque edges, the percentage of translucent edges and the margin width on a CS. These examples are totally artificial and they are not taken from observations in the field. The nature of the edge, opaque or translucent, is determined macroscopically. The margin width corresponds to the distance between the beginning of the last opaque zone and the edge.

a) Theoretical graph of the appearance of one cycle per year. The opaque zone is laid down at the end of each year (maximum percentage) while the translucent zone is completely deposited in August. For some CS, the evaluation of the nature of the edge is not possible and the sum of percentages differs from 100% (e.g. July Y1). The margin width follows the evolution of percentages over time.

b) Theoretical graph of the appearance of two cycles per year. The opaque zones are laid down around May and at the end of each year (maximum percentage) while the translucent zones are deposited in April and August every year. An inter-annual variation is observed with a slight shift of the zone deposition every year: the first opaque zone is totally deposited in March in year 1 and in April in year 2. For some CS, the evaluation of the nature of the edge is not possible and the sum of percentages differs from 100% (e.g. March Y1). The margin width follows the evolution of percentages over time.

The use of AMD is recommended when working separately on different age (or increment) classes. It takes directly into account the differences in growth rates between individuals. The RMD, which is sometimes expressed as a percentage, compensates for the effects of the reduction in growth with age: it is less sensitive to the variations in growth rate since measurements are relative (fig. IV.B.3). It is often more difficult to follow a given cycle with the AMD than the RMD. In general, quantitative data regarding the margin cannot be measured for 0-year-old individuals (without increments), unless there is a

Figure IV.B.2
Edge aspect of transverse stained sections of otoliths of *Colossoma macropomum* (Serrasalmidae). AMD, absolute marginal distance; $D_{i,i-1}$, distance between the last zone i and the previous one $i-1$. The relative marginal distance is then $RMD = AMD / D_{i,i-1}$. Scale bar = 500 μm (photos J. Panfili).
a) Stained zone on the edge of a 455 mm (SL) individual caught in September in Bolivian waters.
b) Unstained zone on the edge of a 457 mm (SL) individual caught in March in Bolivian waters.

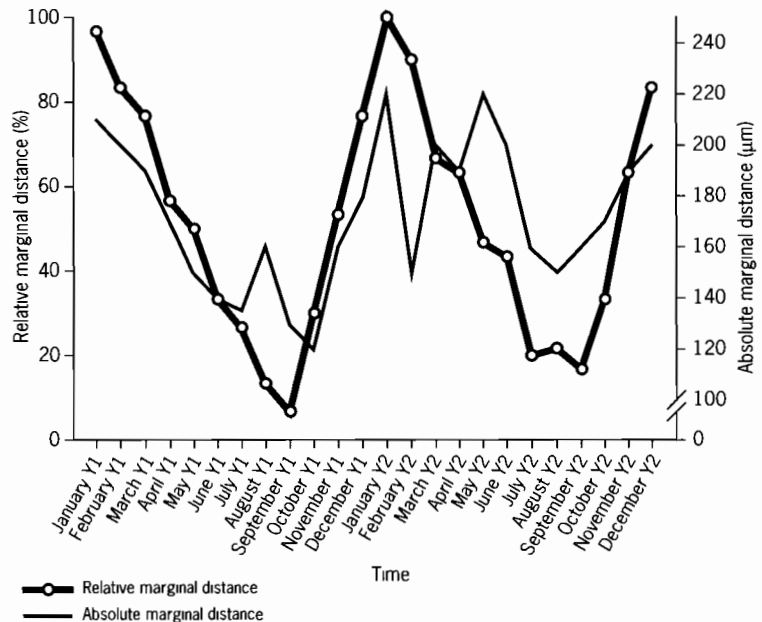


demonstration of equivalence between the period of birth and that of the formation of the considered increment. Otherwise, the quantitative method is only used with fish more than one year old.

1.3. Examples of semi-direct validation studies

We give here an example of the observation of margin of the transverse stained section of the otolith of *Colossoma macropomum* (Serrasalmidae) from the Mamoré basin (Bolivian Amazonia). For qualitative data, the otolith edge is either stained (fig. IV.B.2a) or left unstained (fig. IV.B.2b). It can be difficult to interpret the edge because of the accumulation of stain at the resin-otolith interface. For quantitative data, the measurement axis and where to measure the increment (start, middle, end) need to be clearly defined. In the example, the beginning of the stained zone is taken as the starting point. The dimensions of the stained zones may vary because a stained zone consists of several chromophilic bands (fig. IV.B.2a). The semi-direct validation applied to the *C. macropomum* otolith has shown that stained zones are laid down annually and correspond to the dry season in Bolivian Amazonian freshwaters (Loubens & Panfili, 1997).

Figure IV.B.3
Theoretical graph of the evolution over time (two consecutive years) of the margin width of the last growth zone on a calcified structure. The relative margin width in percentage is the division of the distance between the beginning of the last zone and the edge (AMD) by the distance of the last complete zone ($D_{i,i+1}$). The zone is fully formed at the end of each year (maximum width). The signal is clearer for the relative distance than the absolute one because in the last case all age classes are mixed and the reduction of growth with age is not taken into account.



The marginal increment analysis has been used mainly to determine the timing of annual increment formation for all types of CS (scales, otoliths, vertebrae, spines) in temperate Teleost species (Beall & Davaine, 1988; Robert & Vianet, 1988; Crawford *et al.*, 1989;

Schramm, 1989; Vianet *et al.*, 1989; Mann & Beaumont, 1990; Fletcher, 1991; Hales & Hurley, 1991; Hyndes *et al.*, 1992; Tserpes & Tsimenides, 1995; Pearson, 1996; Machias *et al.*, 1998; Vilizzi & Walker, 1999, *inter alia*), tropical Teleosts (Loubens, 1978; Cayré & Diouf, 1983; Davis & Kirkwood, 1984; Sosa-Nishizaki *et al.*, 1989; Loubens & Panfili, 1992, 1997, 2000; Crabtree *et al.*, 1995; Yosef & Casselman, 1995; Fabre & Saint-Paul, 1998; Chung & Woo, 1999; Morales-Nin *et al.*, 1999), deep water species (Massutí *et al.*, 1994; Gordon & Swan, 1996; Morales-Nin *et al.*, 1996b) and sharks (Lessa *et al.*, 1999).

The semi-direct validation of daily increments has been much less developed but this method has also been employed (Tanaka *et al.*, 1981; Ré *et al.*, 1985; Geffen, 1987; Gartner, 1991). It has not often been used in this way because it is technically much more complicated, involving complex otolith preparation techniques (e.g. thin slices, SEM). Because of the low power of resolution of the compound microscope the SEM must often be used.

2. Problems of edge interpretation of otoliths

All semi-direct validation studies have mentioned the difficulty of evaluating the nature of the otolith edge: opaque v. translucent, stained v. unstained, etc. There may also remain a certain number of “uninterpretable” otoliths when we examine the edge. Three explanatory hypotheses can be put forward for the observation of whole otoliths:

- The otolith is thicker in the central area than at its edge, where the marks are less clear. Under transmitted light the edge can be highly refringent (fig. IV.B.4a). If this is the case, the whole otolith should be prepared (e.g. thick section) to enhance the observation of the edge.
- The interpretation of the nature of the edge refers to the observation of the previous marks (e.g. opaque and translucent) and the density of these marks varies according to the observed otolith area.
- The growth rate differs in the various otolith faces (e.g. anterior, posterior, dorsal, ventral), leading to thicker increments on the preferential axis of growth (fig. IV.B.4b). The observation is thus different regarding the otolith area, making it necessary to define interpretation criteria (increment continuity around the otolith, presence or absence on a particular area). In older fish, growth is often limited to the internal and dorso-ventral sides of the otolith and marginal increments are only visible in these areas.

These remarks also concern observations of otolith microincrements, as those deposited near the edge tend to be less visible (fig. IV.B.4c).

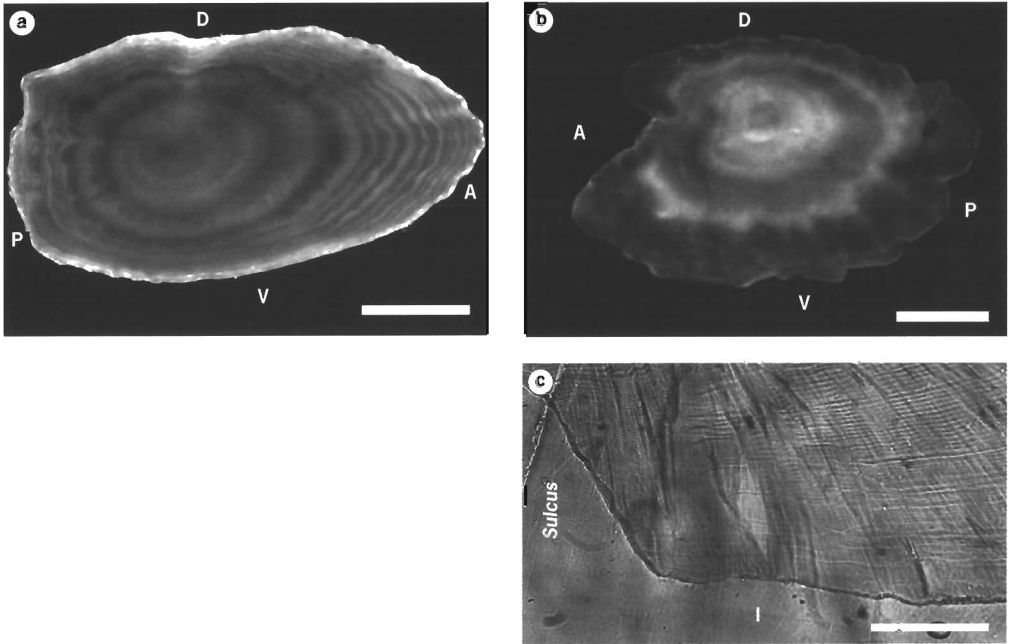


Figure IV.B.4 - Difficulties in interpreting the edge aspect of otoliths. A, anterior; D, dorsal; I, internal; P, posterior; V, ventral (photos J. Panfili).

a) Whole otolith of plaice (*Pleuronectes platessa*) under transmitted light. The edge of the otolith is particularly refringent and it is difficult to interpret it. Scale bar = 2 mm.

b) Whole otolith of the European eel (*Anguilla anguilla*) under reflected light. The aspect of the edge is opaque on the ventral and anterior face and more translucent on the posterior and dorsal face. This is due to two factors: (1) the reduced thickness of the otolith on the edge and (2) the differences of growth rates in each face. Scale bar = 500 μ m.

c) Thin transverse section of the otolith of the oxeye scad (*Selar boops*) under transmitted light. Edge interpretation is also difficult when looking at the microincrements which lose contrast near the edge. Scale bar = 20 μ m.

C. Indirect validation

B. Morales-Nin, J. Panfili

Indirect validation methods are based on corroborative information that supports age interpretation but does not validate the periodicity of the CS incremental growth patterns. The methods most frequently employed are based on length frequency information, which is routinely gathered in fishery studies. These methods were originally used in the 19th century and had a second peak in popularity when cheap computer hardware became available and fast software was developed. Other methods are based on age information obtained at seasonal or daily levels.

1. Comparison with length distributions

Length-based methods analyse the length composition of catch landings in order to identify the various age-groups present, based on the assumption that each age group has a normally distributed length composition and a different modal length. Petersen (1891) was the first to identify the modes in the distribution of lengths as corresponding to age groups. Age class 0 corresponds to the smallest mode present in the sample obtained after the spawning period, while subsequent modes correspond to age 1, etc. (fig. IV.C.1).

Since then, many methods have been developed to identify the age classes in a given length composition. These include: a) graphical methods, which identify the groups of points obtained by mathematical transformation of the length frequencies that correspond to an age class (Cassie, 1954; Bhattacharya, 1967); b) computational methods, which employ the maximum likelihood (Hasselblad, 1966), combined with previous information regarding the age classes present and their mean length (McDonald & Pitcher, 1979), or incorporating biological information (Schnute & Fournier, 1980, *inter alia*).

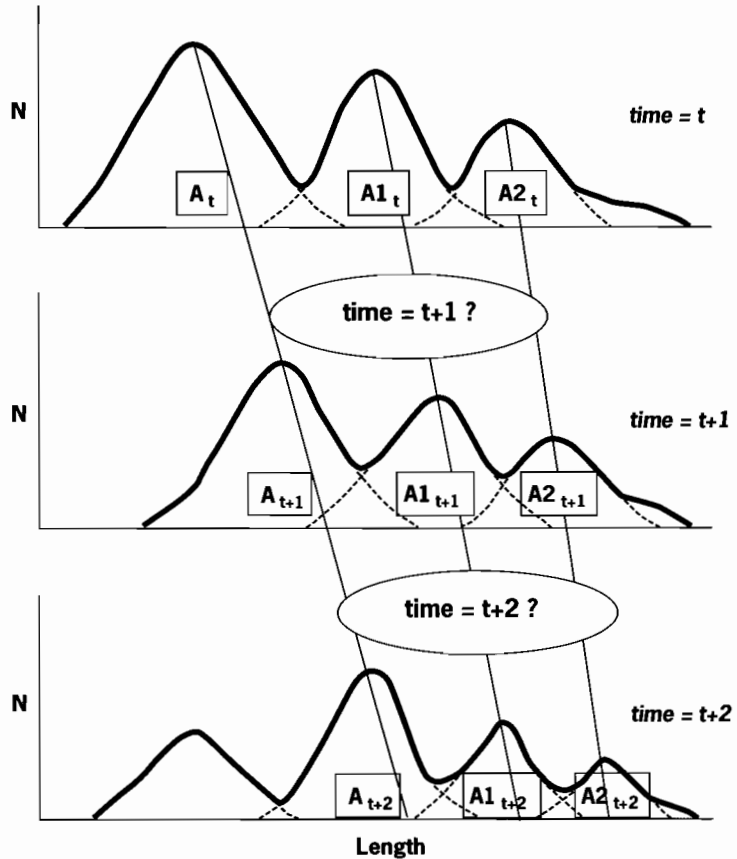
If age-length keys from CS readings, corresponding to the same samplings as the original length frequencies, are available, predicted length-at-age distributions from the CS can be compared with observed distributions by means of the chi-square test.

The usefulness of statistical methods for the desegregation of age groups by length frequencies is limited when:

- the species has a relatively long spawning period;
- there is variability in growth rates, which generally increases with age. Both of these conditions cause high overlapping of lengths between age groups and impede the identification of the age groups.

Figure IV.C.1

Basic principles involved in methods for length-frequency analysis. Hypothetical set of length-frequency samples captured at three different times (t , $t+1$, $t+2$). At time t , there are three age groups (A_t , $A1_t$, $A2_t$) with normal length-frequency distributions. The analysis hypothesises that at time $t+1$, the ages become A_{t+1} , $A1_{t+1}$ and $A2_{t+1}$ and that at time $t+2$, the ages become A_{t+2} , $A1_{t+2}$ and $A2_{t+2}$. Note that at time $t+2$, a new age group appears in the sampled population. The interconnecting lines follow the hypothetical modal progression of the lengths.



Thus, length-based methods may only be reliable for the first age groups or for species with a short lifespan.

Another approach is to calculate the von Bertalanffy growth parameters from the length frequencies (Pauly & David, 1981; Casselman, 1987) and compare them with the growth parameters obtained from age-length keys elaborated from CS readings.

2. Methods using age information

There are strong indications that the age-reading method is accurate if the age composition of exceptionally good or weak year classes can be followed over a long period of time. The strength or weakness of a year class would be lost rapidly if the age reading method was not accurate, because of the assignment of ages to wrong year classes (Eltink & Kuiter, 1989). However, reader bias, which is defined as the subjective assignment of ages caused by knowledge of the existence of strong or weak year classes, is likely to have an effect on the age reading results.

This reader bias can be excluded by age-reading a set of CS of an exceptionally strong year class collected in different years (an approximately equal number in each year). Analysis of these age readings will determine whether a CS set from an extremely strong year class can be used in CS reading workshops to estimate the precision, accuracy and relative/absolute bias in age reading (an example for horse mackerel is presented in ICES (1999), and to estimate the effect of age reading errors on the assessment.

There now exists considerable evidence that daily increment observations can assist in the interpretation of otolith macrostructure (see review in Arneri *et al.*, 1998). Counts of microscopic daily increments have been used to directly verify that one opaque and one translucent zone represent an *annulus* (Victor & Brothers, 1982). However, this verification approach is limited by:

- the problem of detecting fine microscopic increments in fish more than one year old;
- the need to validate the daily periodicity of increment structures;
- the difficulty of identifying the macrostructure (*annuli*) when the microstructure is being observed at high magnification. Due to these problems daily increments are normally used to validate only the first *annulus*.

An interesting alternative method is to follow a single cohort of juveniles recruited during the same year, with a number of samples collected during that period, count the increments in numbers of otoliths and compare the results with the number of days elapsed between samples. The linear regression between dates of captures and the mean age in days of the samples should have a slope equal to 1 to indicate daily increment deposition (Hoedt, 1992). This method requires that there should not have been any migration to or from the sampling area.

When juvenile fish are being aged by the daily increments in their otoliths, their dates of birth can be determined from their date of capture and their age in days. If there is a good match between the spawning period and the back-calculated date of birth the daily nature of the increments has been supported (Morales-Nin & Aldebert, 1997).

D. Verification

B. Morales-Nin, J. Panfili

Verification confirms the consistency of the interpretation of age, i.e. the repeatability and/or precision of a numerical interpretation that may be independent of age. For example, if two readers agree on the number of increments present in a CS, or if two different CS from the same animal are interpreted as showing the same number of increments, verification has been achieved (Wilson *et al.*, 1983). Indices of precision are easily generated, and these can provide useful information concerning sources of error in studies of ageing. Common applications include comparisons of age readers and of ageing methodologies (Secor & Dean, 1989). They can also be used to judge the relative difficulty of ageing different species, and to reject samples of questionable reliability (Campana & Jones, 1992). However, agreement between readings of different CS only means that increment formation corresponds to major life events, not that it necessarily has any temporal significance.

1. Different readings from one or several readers: consistency bias

When interpreting a CS, it is always necessary to read it more than once in order to reduce part of the subjectivity. In order to maintain the independence of readings, physical data (e.g. size, date of capture) should not be given to the reader(s). Several statistical indexes and tests are available for determining the degree of agreement between readings. One of the simplest methods is to compare the results of several readings from one or several readers for the same CS. The percentage agreement (PA) can then be calculated: this is equal to the ratio between the number of coincident readings and the total number of readings (in percent). However, PA depends on the lifespan of one species: a 95% PA to within one year between two age readers of Pacific cod would indicate poor precision, given the few year classes in the fishery. On the other hand, a 95% PA to within five years would be good precision for a spiny dogfish, given its 60-year lifespan

(Beamish & Fournier, 1981). These authors have therefore suggested using an Average Percent Error (APE) index:

$$(IV.D.1) \quad APE = 100 \% \cdot \frac{1}{R} \sum_{i=1}^R \frac{|X_{ij} - \bar{X}_j|}{\bar{X}_j}$$

where X_{ij} is the i^{th} age estimation of the j^{th} fish, $\bar{X}_j$ is the mean age of the j^{th} fish, and R is the number of times each fish is aged. When averaged across many fish, this becomes an index of mean APE. Chang (1982) suggested incorporating the standard deviation in the previous equation rather than the absolute deviation from the mean age. The resulting equation produces an estimate of the Coefficient of variation (CV), and does not assume that the standard deviation is proportional to the mean:

$$(IV.D.2) \quad CV = 100 \% \cdot \frac{\sqrt{\sum_{i=1}^R \frac{(X_{ij} - \bar{X}_j)^2}{R-1}}}{\bar{X}_j}$$

CV can be averaged across a number of fish to produce a mean. CV is statistically more robust than APE and is thus more flexible (Kimura & Lyons, 1991). There is no CV threshold value for accepting or rejecting the readings, because these depend on the species and the range of ages. Laine *et al.* (1991) suggested a maximum CV value of 5% as the limit for acceptable readings.

Considerable efforts are made by international committees to standardise the readings. Exchange programmes and reading workshops have been organised in this aim (Eltink *et al.*, 2000). Reading results can be analysed using a specially designed spreadsheet (Eltink, 1994; 1997).

For reading daily otolith increments we also recommend that the same reader should read the otolith twice, first from the *primordium* to the edge and then from the edge to the *primordium*, along the same growth axis (Campana, 1992). If no significant difference is found between these two readings for the whole sample after a paired t-test, the mean can be used to estimate the age (Campana & Jones, 1992). These authors also suggested using some form of weighting in order to take into account the credibility of individual readings.

2. Different readings from several calcified structures

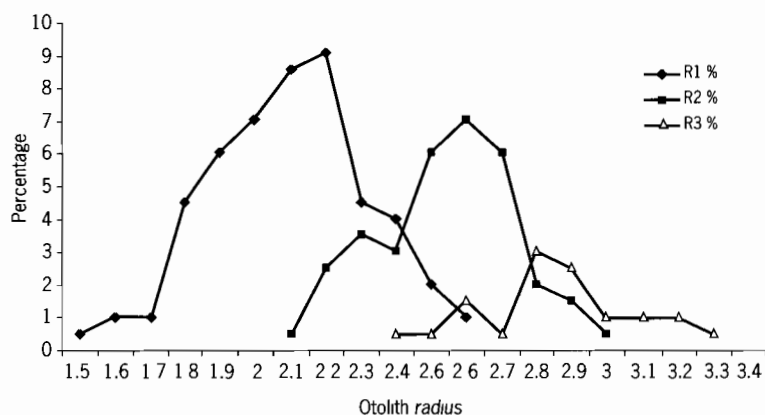
If the formation of the increments is due to specific major life history events, they should be present in all the CS of one or all members of the sample. Different readings from several CS should produce the same results. In order to compare these readings the same indexes as those described above (chap. IV.D.1), APE and/or CV, are used. Another test for comparisons of two CS readings (e.g. otoliths v. vertebrae) is to prepare a two-way contingency table and carry out a symmetry test (Hoenig *et al.*, 1995).

Although left and right otoliths are generally similar (except in flat-fish, chap. II.A), in order to avoid bias otoliths from the same side should be interpreted. If any problems of interpretation should arise, the other otolith of the pair can be used.

3. Trends in patterns of growth

Another verification criterion is the regularity of the increment formation. Increment widths should show a falling rate of CS growth with age. This linearly decreasing interval between increments forms the basis of age estimation (May, 1965). Furthermore, if increment formation is a response to a major environmental event, it should be synchronised throughout the population. The regularity of the growth patterns may thus be demonstrated by plotting the increment-to-centre distances for all CS (fig. IV.D.1) and testing the normality of the distributions by means of a Kolmogorov-Smirnov test.

Figure IV.D.1
First three ring distances, measured as the ring radius from the centre, in otoliths of *Salpa salpa*, showing a normal distribution and decrease in growth with age.



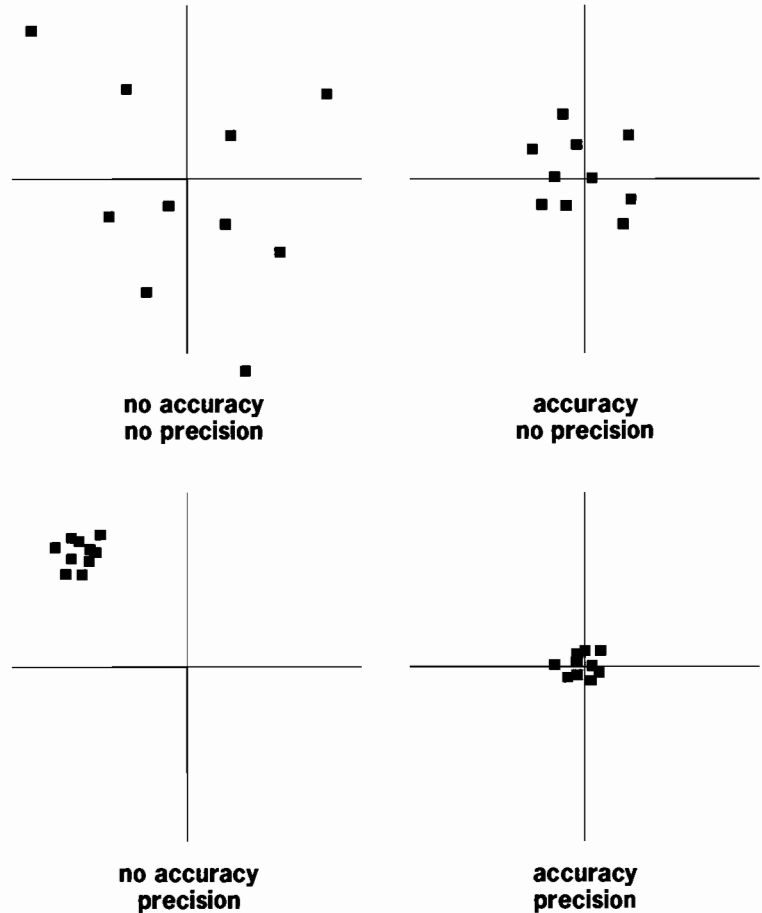
Another method of using trends in patterns of growth is to compare the length-at-age obtained from direct CS readings and the back-calculated length-at-age (chap. V.A.2). The observed length-at-age from

the CS interpretation must be calculated from fish sampled during the period of the measured (for back-calculation) increments (translucent, opaque, check, etc.). The observed length-at-age will otherwise correspond to a period of a complete year for a given age group, while the back-calculated length corresponds to a particular time of the year. This will give rise to differences between both mean lengths and standard deviations.

4. Accuracy and precision

Accuracy is the closeness of the estimate of a quantity (measured or computed value) to its true value (fig. IV.D.2). Precision is the closeness of repeated measurements of the same quantity (fig. IV.D.2). For a measurement technique that is free of bias, precision implies accuracy, but the two parameters are not identical. In any study of ages, it is necessary to determine both of these parameters and to take steps to

Figure IV.D.2
Accuracy versus precision
in sclerochronological
studies. The age estimation
results (black squares)
are plotted against the target
true age value (cross point
of the XY axis). Accuracy
is the closeness to the true
value whereas precision
is the closeness of repeated
measurements.



improve them. However, accurate estimates need not be precise, and *vice versa* (Campana & Moksness, 1991). Thus a mean age can be accurate while the individual observations that led to its estimation were not precise. Conversely, as is often the case in ageing studies, age estimates can be precise (either within or among readers) but not necessarily accurate (Campana & Jones, 1992).

Chapter V

Some uses of individual age data

Growth and mortality rates are of fundamental importance in fish population dynamics, and normally age information is needed to estimate these rates precisely. Rather than attempting to provide an exhaustive review of examples in this manual, some specific examples on the use of age data will be provided. In the first part of the chapter, general definitions of growth measures that require input of age data are presented. Various body parts of an organism may grow at different rates during development, and this is briefly summarized in the context of allometry and relative condition. A major advantage of the incremental structure of the CS is that it also enables the estimation of fish size at different earlier points of time during its lifespan thus providing a growth sequence or trajectory on an individual basis by means of back-calculation procedures. The background and assumptions underlying back-calculation of fish size based on CS are given. The second part of the chapter deals with examples of ecological applications where the use of individual age data is required. The use of age-based data is considered to be essential for the understanding of important processes regulating the recruitment of marine fish populations. Potentially higher mortality rates can be associated with individuals of small- or low rank size, sub-optimal growth and birth date and/or mismatching environment, and the mechanism may be documented by analysis of CS from individuals sampled over the period of selective mortality (e.g. Meekan & Fortier, 1996).

Many exploited fish stocks in the developed world fisheries are assessed by age structured population models. Worldwide fisheries laboratories routinely utilise CS to determine fish age by reading seasonally formed growth structures. Collecting, reading and processing CS like otoliths, scales and bone structures amounts to a substantial part of the total expenditure for fish stock assessment. In the last part of this chapter several examples are given of how age-based data are incorporated in fish stock assessments. Emphasis is put on presenting the benefits and requirements of using age-based models versus other types of models, and on how the added information provided by the age estimation can justify the increased effort.

A. Growth and growth analysis

A. Folkvord, H. Mosegaard

1. Models of growth

1.1. Measures of growth

Absolute growth refers to the total increase in body material or body dimensions, and absolute growth rate is defined as the absolute growth over a given time period (see below). An example is the daily increment width in larval fish otoliths, which represents the radial otolith growth over a 24-h period. If the absolute growth rate is constant over time or the increase is constant on an absolute scale, i.e. the increment widths are constant or the otolith size is steadily increasing in absolute terms, we have linear growth.

In the following we use S for the size of the calcified structure (CS, representing scales, otoliths etc.), and T for time. The size may refer to such measures as *radius*, length, area, or weight. We can define absolute growth rate as (AGR):

$$(V.1) \quad AGR = \frac{S_2 - S_1}{T_2 - T_1}$$

Measures of absolute growth are sometimes of limited value since they are highly dependent on the size of the structure we are measuring. One way of dealing with the problem of different sizes is to calculate relative growth, which is absolute growth divided by initial size. Relative growth thus expresses the proportional increase in size. In a similar manner as for absolute growth rate, we can calculate relative growth rate (RGR) as (average) relative growth over a given time period:

$$(V.2) \quad RGR = \frac{S_2 - S_1}{S_1 \cdot (T_2 - T_1)}$$

When the relative growth rate is constant over a short period of time, we can express it as:

$$(V.3) \quad g = \frac{dS}{dt \cdot S}$$

where g is also called the instantaneous growth rate. Integrating over time we obtain the following expression of g (assuming it to be constant):

$$(V.4) \quad g = \frac{\ln S_2 - \ln S_1}{T_2 - T_1}$$

In cases where the growth rate is adequately described by the above relationship, we have a case of exponential growth, i.e. the proportional increase in size is constant per unit time. If a day is the time unit, the daily growth rate (*DGR*) can then be calculated as:

$$(V.5) \quad DGR = (e^g - 1)$$

where g is calculated as above on a daily basis (Ricker, 1975). *RGR*, g and *DGR* are given as daily proportions. We can multiply each of the growth measures by 100 to obtain the growth rate in percent per day. Specific growth rate, *SGR*, is often used for g or for $100 \cdot g$.

When the growth rate is very high on a unit time basis, the *SGR* and *DGR* will give increasingly divergent values. This is due to a "compound interest" situation where growth is added to the growth within a day. This effect may be illustrated by replacing *DGR* with g (*SGR*) in the equation below:

$$(V.6) \quad S_2 = S_1 \cdot (1 + DGR)^{T_2 - T_1}$$

The use of *SGR* instead of *DGR* in the equation will result in an underestimate of S_2 .

Most growth curves can be treated as linear over short periods of time. When the time duration increases, however, most growth curves become non-linear. An exponential growth curve may be appropriate for shorter periods, e.g. in the larval stage in herring (Fiksen & Folkvord, 1999), but in most cases organisms are unable to maintain constant exponential growth rates in the long term, due to variations in food supply and/or physiological constraints (size-related surface-volume constraints).

An important distinction has to be made when dealing with individual growth data between longitudinal and cross-sectional data (Chambers & Miller, 1995). Longitudinal data are of the type where several measures of the size of a single individual are available at various times, whereas with cross-sectional data, only the sizes at sampling (or harvest) are available (fig. V.A.1). In fisheries it is very common to use size-at-age data based on individual size at catch as the basis for constructing growth curves (cross-sectional data). This corresponds to using the size of individual fish at the intersection of the vertical line (time of sampling) with the respective individual growth curves at the lower left projection in figure V.A.1b. Obviously, the collection of these data points will not represent the actual growth rate of any of the individual fish studied, and any selective loss of large or small individuals over time will bias our growth estimates. On the other hand, if several measures of size-at-age of all the individuals from the lower right projection in figure V.A.1b are used, unbiased individual growth estimates can be obtained. The CS enables the

extraction of such longitudinal data from individual fish, and depending on the questions asked, the extra effort of ageing and constructing individual growth trajectories may be worth while (chap. V.B and V.C). The use of the cross-sectional data will still require the fish to be aged, but in this case only average population growth-at-age measures instead of individual growth measures will be obtained. In these cases comparisons of size-at-age between groups can be carried out using analysis of covariance (ANCOVA) based on the size-at-age data from respective groups. On the other hand, the statistical treatment of longitudinal data may solve for the serial correlation of increment widths of the CS (Chambers & Miller, 1995; Ralston & Howard, 1995; Jones, 2000). Both repeated measures ANOVA and time series models (e.g. ARIMA) are designed for this purpose, and a further discussion of these techniques is presented in the references above.

In many cases, when the selective mortality in the population is negligible, or can be corrected for, cross-sectional data may suffice. A possible way to account for size-selective mortality between samplings is to rank the fish of the initial sample according to size, and to exclude the proportion of the initial sample that corresponds to the selective mortality between the samples, before calculating the population growth rate (Folkvord, 1997). There is also the possibility of obtaining average individual growth measures using cross-sectional data. The size of a five-year-old harvested fish will provide a sufficiently accurate measure of average growth from birth, but no time-resolved individual growth information will be available in this case.

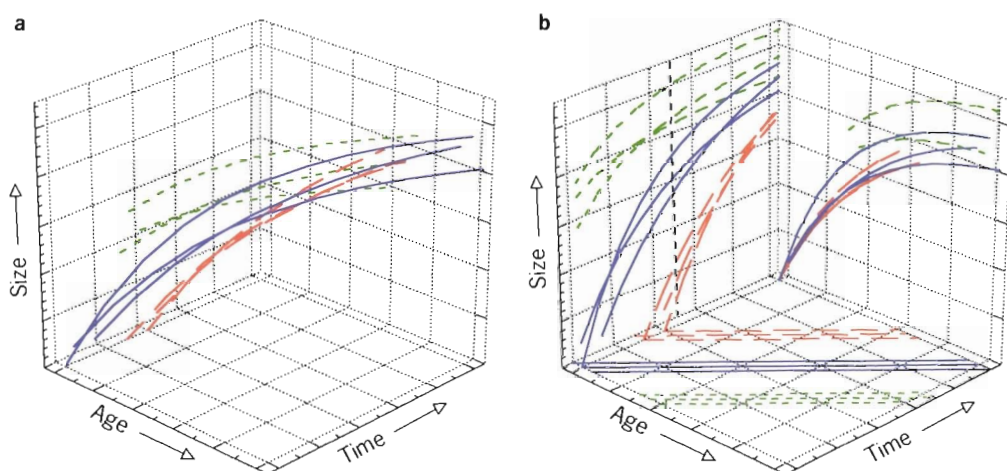


Figure V.A.1 - Three-dimensional representation of growth and size-at-age (modified from Chambers & Miller, 1995). Individuals from different cohorts are presented in different lines and colours. a) The growth of individuals over time and age. b) The projections of individual growth trajectories to age and size plane, time and size plane, and time and age plane respectively. The intersection between the dashed vertical line on size and time plane and the respective growth curves represent the typical cross-sectional size-at-age data obtained from a given sample.

1.2. Allometry and age-independent growth models

Different body parts grow at different relative rates. In the case of weight and length, the relationships between these measures are frequently used in various ways to assess the overall growth and condition of the fish (e.g. Ferron & Leggett, 1994). The relative size of CS (e.g. otoliths) in comparison with the size of the fish may also be influenced by the previous growth and environmental history of the fish (Casselman, 1990).

In the following, we use W as the measure of fish size (weight) and S as otolith (or scale) size. An allometric relationship between fish size and otolith size can be expressed as:

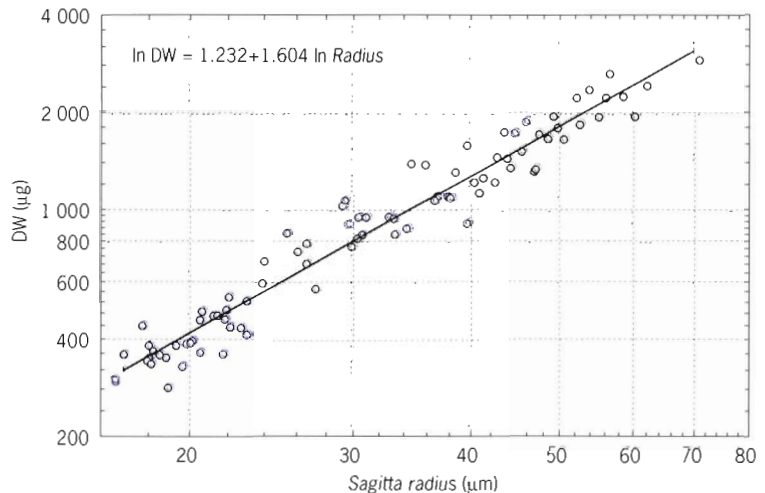
$$(V.7) \quad W = a \cdot S^b$$

or in log-transformed form:

$$(V.8) \quad \log W = \log a + b \log S$$

In fish-length - fish-weight relationships, b tends to lie between 2 and 4 and is often close to 3. In the case of otolith-size - somatic-size relationships, b will depend on which measures are employed. When a one-dimensional measure of otolith size (e.g. *radius* along the anterior-posterior axis) and a three-dimensional measure of fish size (e.g. dry weight) are used, a value of b close to 3 would indicate isometric growth. This implies that the relative growth rate is similar in all dimensions (axes) (chap. II.A). A b lower than 3 would indicate that otolith growth in the anterior-posterior axis is relatively higher than in the other parts (dimensions) of the fish (fig. V.A.2). When fish length is used as a one-dimensional measure of size, b should be closer to 1 when otolith size is also measured using a one-dimensional measure as above (e.g. Hare & Cowen, 1995).

Figure V.A.2
Allometric relation of otolith radius (*sagitta*) and larval dry weight (DW). Data from herring larvae (*Clupea harengus*) with otolith radius >18 μm and reared at 8°C (data from Folkvord et al., 2000). Note that the slope of the regression line is less than 3, indicating that the growth is not isometric.



It has been found that growth of different CS only transitorily scales isometrically to somatic growth (Casselman, 1990). Instead, the growth of bone and to even greater extent, of scales is positively allometric (relatively faster bone and scale growth than somatic growth at increasing growth rates), whereas otolith growth shows negative allometry (relatively slower otolith growth than somatic growth at increasing growth rates). The relationship for northern pike between growth rate of CS and growth rate of body size B can be expressed as a second-degree polynomial:

$$(V.9) \quad dCS/dt = a \cdot (dB/dt) + b \cdot (dB/dt)^2$$

where b is less than 1 for otolith growth but greater than 1 for the growth of scales and *cleithrum* (Casselman, 1990). This model explains some general observations on nutritional conditions as indicated by prey availability, which influence the relative sizes of calcified structures and the body; i.e. when more prey was available, northern pike had relatively larger *cleithra*. The relative size of CS is thus an indicator of growth, reflecting changes in growth rate and nutritional status. This simplified model, however, does not account for certain extreme cases such as the continued growth of otoliths concurrent with growth checks or even the resorption of scales when large fish stop growing (Mosegaard *et al.*, 1989; Casselman, 1990).

In many cases, relative growth rates in various dimensions will not remain constant over long periods of time, and the log-log plot between the variables will thus appear to be non-linear. A marked transition in length-weight relationships (or otolith-size - fish-size relations), may occur during important developmental changes such as metamorphosis in flatfish or smoltification in Salmonids, resulting in different relationships in the different stages or stanzas. In these cases it is normal to determine separate relations for each stage (Bagenal & Tesch, 1978).

In the same manner as one can use residuals from the log-length - log-weight regression directly as measures of relative condition, the residuals from the somatic-size - otolith-size regressions can be taken as measures of relative otolith size (Hare & Cowen, 1995). Morphological condition is a cumulative result of previous feeding and growth history and stage of fecundity, whereby individuals in good condition are characterised by relatively high weights at given lengths. As far as otoliths are concerned, relatively large otoliths tend to be associated with slower-growing rather than faster-growing fish at given lengths (Reznick *et al.*, 1989; Secor & Dean, 1989; Casselman, 1990; Hare & Cowen, 1995, fig. V.A.3). If CS size and somatic

size are closely related, the pattern of residuals of length-on-age and otolith *radius*-on-age should be positively correlated. This is indeed the case in the example shown in figure V.A.3c, and the higher the correlation between the residuals, the lower is the age-independent variability of somatic size and CS size. In the case of a perfect correlation, $r=1$, there is no age-independent variability, and knowing the somatic size would imply that the exact CS size was known, and *vice versa*. Similarly, the correlation of the age-on length residuals from figure V.A.3d with the residuals of the otolith *radius*-on-length residuals from figure V.A.3e will be positive if slow-growing individuals tend to have larger otoliths at a given length, as in figure V.A.3f. If relative otolith size is independent of growth then the correlation between the latter residuals will be 0, and this correlation has therefore been termed the "growth rate effect" (GRE) (Hare & Cowen, 1995). If fish growth may be described by a general differentiable growth model and CS scales allometrically to body size, then the relationship of calcified growth rate as a function of body growth rate, $dCS/dt = f(dL/dt)$, can be solved for a group of individuals following a similar pattern, with the allometric relationship, $CS = c + a \cdot L^b$, being independent of rate of body growth (Xiao, 1996). Three different growth models; i) von Bertalanffy, ii) logistic, and iii) Gompertz, all gave quite different types of relationships between somatic growth rate and otolith weight. Introducing variability in the parameters of each growth model showed the fragility of this approach when individual fish do not strictly follow the assumptions. Furthermore, in the same paper the author numerically investigated the common observation of a so-called growth rate effect on the otolith-size - fish-size relationship (e.g. Reznick *et al.*, 1989). Templeman & Squires (1956) made the observation that in haddock (*Melanogrammus aeglefinus*) the ratio of otolith length to total length decreased with fish size but for equal fish size increased with age. Xiao (1996) used these data on haddock in a simulation exercise of otolith size v. fish growth rate using the three earlier mentioned growth models ending up with some apparently quite unrealistic patterns in the relationship between otolith length and somatic growth rate for any of the models applied. This exercise demonstrates that even though otolith weight and length apparently may scale to fish length according to some age dependent relationship, individual otolith size - fish size growth trajectories may not easily be derived from population averages. The development of new integrated models reflecting the metabolically controlled otolith growth may in time yield more realistic patterns of somatic growth rate - otolith size relationship.

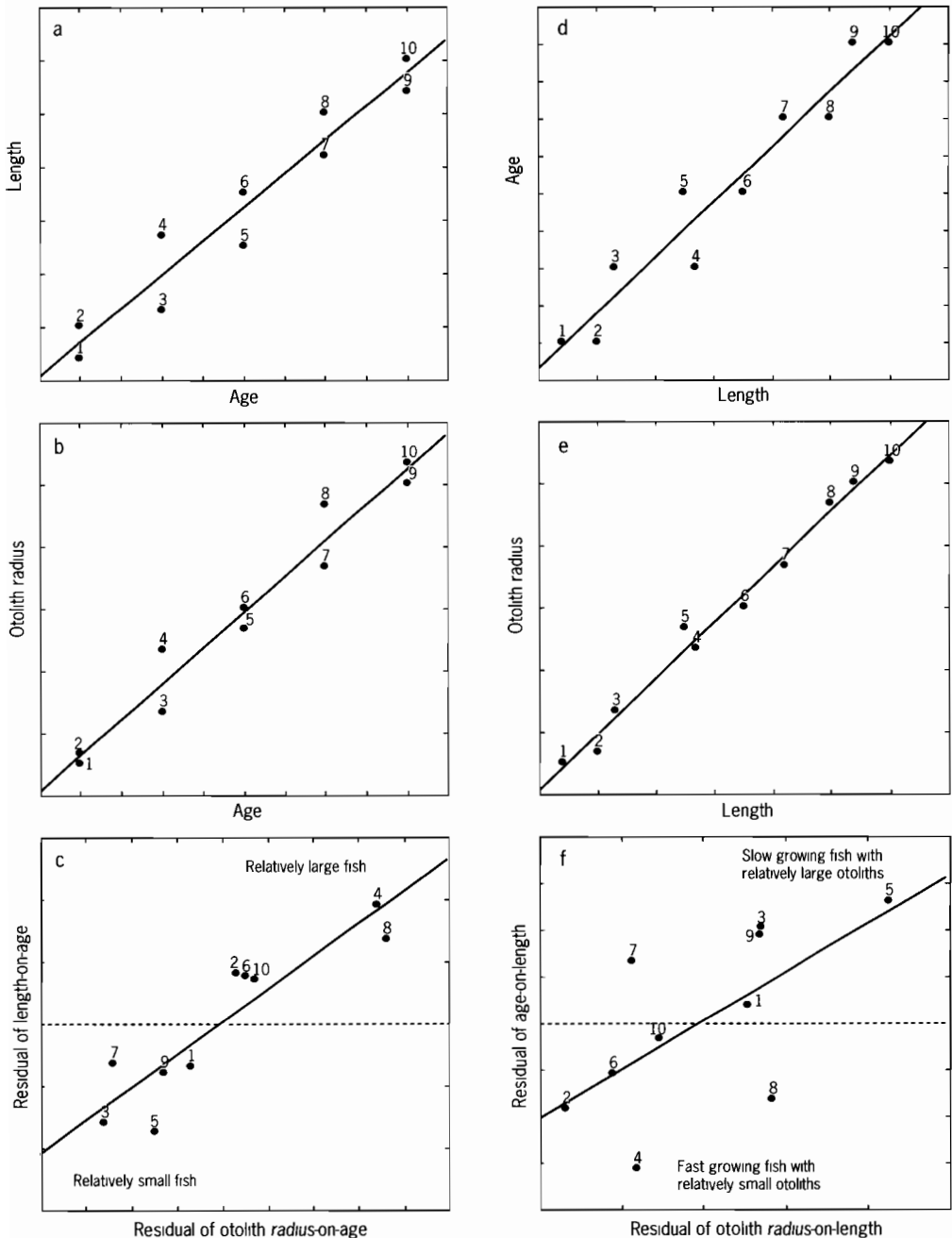


Figure V.A.3 - Schematic examples of growth of different body parts of a fish, a) length, b) otolith, and c) the corresponding correlation of length-on-age and otolith radius-on-age residuals from a) and b). A high correlation between the residuals indicates a low age independent variability of the otolith - fish length relationship, suggesting that cumulative otolith growth to a large extent reflects somatic growth and vice versa. The correlation between the residuals from the age-on-length relationship d) and otolith radius-on-length relationship e) represents a measure to what extent relatively fast-growing fish have relatively small otoliths (in the case of a positive correlation) at a given fish length (or in the case of a negative correlation to what extent the relatively slow-growing fish have relatively large otoliths). Positive correlations are indicative of a growth rate effect (GRE) where slow growing fish have relatively large otoliths and fast-growing fish have relatively small otoliths f) (modified from Hare & Cowen, 1995).

1.3. Age-based growth models

The most common growth model used in fisheries research is the von Bertalanffy growth function (VBGF). In its length-based form it is written as:

$$(V.10) \quad L_t = L_{\infty}(1 - e^{-K(t-t_0)})$$

where L_t is mean length at time t , and L_{∞} , K and t_0 , are the parameters to be determined. An implicit assumption of the VBGF is that the instantaneous growth rate:

$$(V.11) \quad \frac{dL}{dt} = a + bL$$

where b is negative.

This implies that the length growth rate decreases as size increases and approaches 0 as size approaches L_{∞} , the mean asymptotic length (Francis, 1995a). The parameter K is related to the curvature of the growth curve, where higher values of K indicate a more rapid change in the rate of length growth with increasing length. The last parameter, t_0 , represents the (theoretical) age at which the mean length would be 0. The growth curves of individual fish in fig. V.A.1 are all based on the VBGF. The differences in shape of the trajectories are due to different values of the three parameters in the respective equations. Although the VBGF can be derived from physiological principles, given certain assumptions, it is essentially an empirical model used for data-fitting. A simple way of estimating K and L_{∞} in the VBGF involves the use of a Ford-Walford plot, where L_{t+1} is plotted against L_t . To obtain the parameters using this method we rearrange the VBGF and set $e^{-K} = k$, and solve for $t+1$ to obtain:

$$(V.12) \quad L_{t+1} = L_{\infty}(1 - k^{(t-t_0)+1})$$

This can be rearranged to:

$$(V.13) \quad L_{t+1} = L_{\infty}(1 - k) + kL_t$$

which is of the form $y = a + bx$, where the parameters can be estimated by ordinary regression analysis. The slope is equal to k (or e^{-K}), which will give us the K parameter. The L_{∞} parameter can be found by determining the intersection between the regression line and the line of $Y = X$. These lines will intersect at the size where $L_t = L_{t+1}$, which will correspond to the asymptotic length. Alternatively, L_{∞} can be found by inserting k in the expression for the intercept, $L_{\infty}(1 - k)$.

The VBGF can also be written in a weight-based form as:

$$(V.14) \quad W_t = W_{\infty}(1 - e^{-K(t-t_0)})^3$$

where K and t_0 are as above and W_{∞} is the asymptotic weight (Campana & Jones, 1992). This growth function, in contrast to the length-based VBGF, has an inflection point at which the growth rate is at its highest.

Another frequently used growth function in fishes is the Gompertz growth function. This also has an inflection point in its growth curve, and like the weight-based VBGF it is asymmetrical around the inflection point. The Gompertz function can be formulated in several ways (Kaufmann, 1981), one of them being in an integral form:

$$(V.15) \quad W_t = W_{\infty} \cdot e^{(-e^{[-g(t-t_0)]})}$$

where W_{∞} is the asymptotic weight, g is a constant related to the change in size-specific change in growth rate.

The parameters of VBGF and other growth models can now be accurately estimated by non-linear repeated-measures methods (Jones, 2000). The inclusion of repeated-measures models will correctly deal with the interdependence of sequential data from the same individuals, and thus avoid the common error of running the estimation with incorrectly inflated degrees of freedom.

2. Back-calculation

One of the main advantages of using the incremental information stored in calcified structures in fish is the possibility of obtaining information about size at earlier ages at the individual level (longitudinal data).

2.1. Background and assumptions

Back-calculations of fish size have been made since 1910, when Lea and colleagues used growth patterns in herring scales to predict herring growth rates. Later applications have involved the use of otoliths, bones (e.g. *cleithra*, vertebrae) and rays, and the use of annual structures (macrostructure) and daily structures (primary increments or microstructure) (chap. II). Back-calculation is an important method of obtaining estimates of individual growth and previous size-at-age. In field-based studies it is the main alternative to mark-recapture studies, but it has the advantage that it can potentially be employed on virtually any harvested members of the population (stock), and not only to the limited number that have already been marked and then recaptured. The methodology has strong resemblance to dendrochronology (the study of tree-ring structures), which has been used to infer climate-induced variations in growth rates (e.g. Cook & Kairiukstis, 1990).

The back-calculation procedure can be defined as estimating fish size at an earlier time (or times) on the basis of a set of measurements of CS size and fish size made at a single point in time (usually at capture) (Francis, 1990). In the following we use L for fish size (e.g. length) and S for hard-part size (e.g. scales or otoliths). The size measures are indexed with i , representing an arbitrary age, and c , representing age

at capture. In this context a back-calculation formula (BCF) is one that enables L_t to be back-calculated from L_c , S_c and S_t .

In order to carry out a back-calculation correctly, three main assumptions have to be fulfilled:

- the size of the CS mark is the same as the size of the CS at the time the mark was formed (no resorption or degeneration);
- the assumed time of formation is correct;
- the BCF formula accurately relates body size to CS size for each fish.

Regarding the first assumption, the back-calculated fish lengths would be biased if the size of the mark in the CS had changed with time since deposition. In the case of otoliths it is generally believed that the structures do not change after deposition on to the otolith, partly because the otolith is regarded as an inert non-cellular structure (chap. II). On the other hand, scales may be liable to some resorption, especially in fish approaching their asymptotic length (Casselman, 1990), and for this reason the validity of scale-based back-calculations of growth in these fish may be uncertain. For the second assumption it is essential to know the age at the formation of the incremental marks in order to correctly assign the correct age to the back-calculated size. The proper validation of the incremental structures (chap. IV) is mandatory regardless of which CS is being used. Finally, it is essential to establish a relationship between CS size and somatic size that enables measured CS size to be translated into estimated somatic size. There is a large body of literature on how to proceed with back-calculation (see Francis, 1990 for review), and in the following sections we present a summary of some of the techniques employed.

2.2. Regression methods versus proportional methods

Earlier fish size can be calculated on the basis of a relationship between CS size and somatic size (some transformations could be involved in either variable, but that is not the main point here). The regression approach is based on a common relationship between somatic size and CS size, which can be described as:

$$(V.16) \quad \text{Regression BCF:} \quad L_t = b(S_t) \quad \text{where } b \text{ is a function.}$$

In its simplest form this would ignore the point at capture (S_c , L_c) which uniquely characterises the individual fish (fig. V.A.4a). In such a case all the back-calculated sizes would be derived from the same regression line. Alternatively, one might use the parallel lines (common slope) from each end-point (S_c , L_c), and determine earlier sizes from a series of parallel lines (fig. V.A.4b). The problem of such an oversimplified method is that it implies that the same slope between the CS-size - somatic-size relationship can be applied to all fish. A simplified regression approach is usually not to be recommended for back-calculations (Francis, 1990).

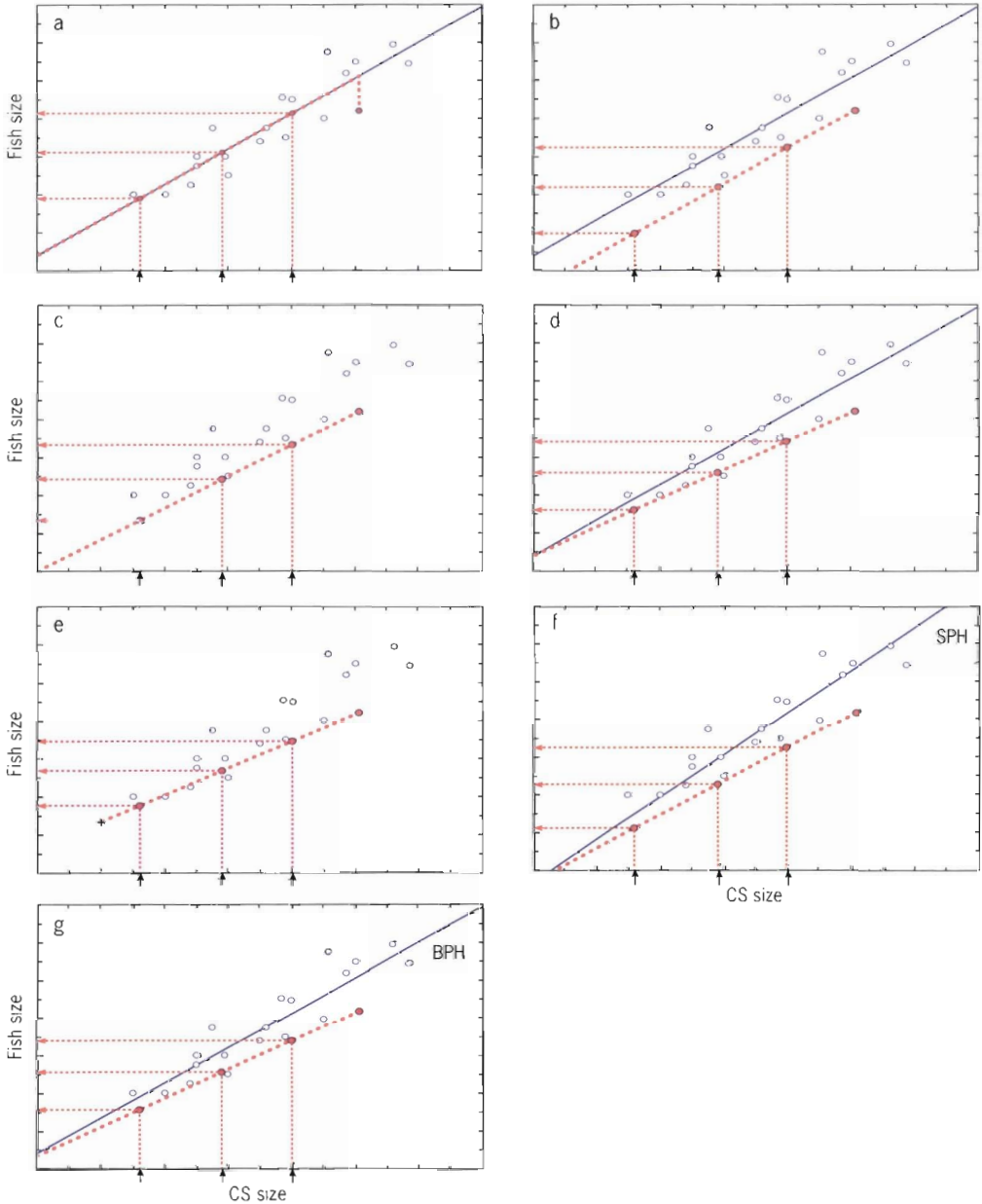


Figure V.A.4 - Back-calculation of fish size based on different back-calculation procedures. a) Regression approach assuming equal growth trajectories of all individuals, b) Regression approach assuming similar slope of CS size - fish size relation for all fish, c) Dahl-Lea procedure using direct proportionality (intercept at origin), d) Fraser-Lee procedure using set intercept on ordinate, e) Biological intercept method using a biologically determined starting point (marked +), f) linear SPH method, and g) linear BPH method. The same data set is used in all cases, and the growth is only calculated for one of the fish (solid points). The three arrows on the abscissa represent the CS mark sizes of the fish in study. The dashed line from the size at capture represents the back-calculated growth trajectory, whereas the vertical and horizontal dashed lines illustrated how CS sizes are translated to corresponding fish sizes. Differences in back-calculated sizes can be seen on the ordinate. The solid regression line represents the relationship between CS size (S) and somatic size (L) used to estimate the parameters in the BCFs. L on S regression is shown in a-b, d and g, while f is based on S on L regression.

In contrast, proportional methods define a series of lines originating from a common point; one for each individual fish. These lines will have different slopes and the point (S_c, L_c) would determine which line should be used for each specimen in the back-calculation. The underlying rationale for this approach is that fish with relatively large otoliths at a given somatic size at capture are most likely to have had relatively large otoliths at previous somatic sizes as well, while fish with relatively small otoliths relative to body size can be expected to have had relatively small otoliths at least at some time before capture. The principal difference between a regression approach and a proportional approach is visualised in figure V.A.4, and it can be noted that the improper use of the regression method in figure V.A.4a predicts an unrealistic reduction in fish size since the last increment formation, due to the relatively small otolith size at capture. The alternative of using the common slope from the regression to each point (L_c, S_c), would generate parallel lines to the overall regression lines, but in the example in figure V.A.4b, unrealistic fish sizes would be inferred at small CS sizes.

2.3. Starting points in proportional methods: statistical determination versus biological reference points

There are good reasons for assuming that proportional methods are preferable to single-slope regression methods in back-calculation. First of all there are no indications that one CS-size - somatic-size relationship will suffice for all individuals in a population. On the contrary, we frequently observe fish with different CS sizes at a given length, very much in the same way as we find fish with different relative conditions. Secondly, we have abundant documentation to the effect that fish scales (and/or otoliths) are formed within relatively narrow age and size windows. On a wider age and time perspective, this will manifest itself as a reasonably well-defined starting point for a CS-size - somatic-size relationship (i.e. the individual lines will originate from a common point rather than run in parallel). There are two principal ways of determining the common starting point of proportional methods in back-calculation. The point is either determined by regression estimation or is manually set on the basis of biological criteria (e.g. fish length at the onset of scale formation).

The most basic biologically based intercept determination is provided by the Dahl-Lea method, which assumes a constant proportionality between CS size and somatic size. This forces all lines to go through the origin, i.e. if fish size is 0, then CS-size is 0 (fig. V.A.4c):

$$(V.17) \quad \text{Dahl-Lea BCF:} \quad L_t = \frac{L_c}{S_c} S_t$$

A further extension of the biological intercept concept has intuitive appeal since it can be argued that the size at which a fish develops scales, for example, is relatively constant. In herring this takes place around metamorphosis, at lengths of 40-50 mm. This would correspond to the intercept in figure V.A.4d (i.e. the fish size when the CS size is 0). This is the reason for the intercept correction in the Fraser-Lee procedure, whether this is pre-determined on a biological basis or determined in the sample from regression. In such cases, the back-calculation formula (BCF) can be written as:

$$(V.18) \quad \text{Fraser-Lee BCF: } L_i = c + (L_c - c) \cdot (S_i / S_c)$$

where c is the pre-determined intercept (or determined by the regression $L = c + dS$). The rationale for the use of the determined intercept from the L on S regression in the BCF is presumably that L will be estimated from S . The mathematical validity of this reasoning has been questioned by Francis (1990), and a more stringent formulation is provided below.

One advantage of having the point set manually becomes apparent when there is non-linearity in the CS-size - somatic-size relationships in the early stages, that is not resolved by suitable transformations. In such cases the proportional back-calculation by regression methods will be dependent on the relative number of small and large individuals in the sample. The inclusion of many small individuals may create a biased regression line that does not reflect the overall linearity in the CS-size - somatic-size relationship in older, larger fish (Campana, 1990).

An extension of the Fraser-Lee intercept correction method is provided by Campana (1990) who developed the biological intercept method with an intercept correction that is not set at the ordinate, but rather at some point off the axes where both CS-size and somatic size are larger than 0. This is more appropriate in the case of otoliths, since these are already present in fish at the time of hatching, in contrast to scales which appear later in development. In practice, the biological intercept may also be determined by mean CS-size and somatic size at the start of proportionality. The BCF for the biological intercept method can be formulated as (Campana, 1990):

$$(V.19) \quad L_i = L_c + (L_c - L_o) \cdot (S_i - S_o) / (S_c - S_o)$$

where (L_o, S_o) represents the biological intercept (fig. V.A.4e). The formula can also be written as:

$$(V.20) \quad \frac{(L_i - L_o) - (L_c - L_o)}{(L_c - L_o)} = \frac{(S_i - S_o) - (S_c - S_o)}{(S_c - S_o)}$$

When $L_o = 0$ and $S_o = 0$, the BCF is identical to Dahl-Lea (direct proportionality through the origin); when only $S_o = 0$ it is identical to the Fraser-Lee BCF.

Although the biological intercept method often produces credible back-calculated sizes, it has been criticised for its lack of mathematical rigour and questionable statistical properties (Francis, 1995a). The method does not take into account the variability around hatching in CS-size - somatic-size relationships, although this is very small in absolute terms. Since the biological intercept is set more or less manually, it is difficult to compare the precisions of various back-calculation examples since it is not clear to what extent the result is dependent on the choice of a given starting point.

Although the utilisation of a realistic intercept is important for accurate back-calculated sizes at intermediate ages, the actual correlation of fish size versus otolith size at this initial age and the subsequent variation in proportionality between somatic and otolith growth are the two factors that determine the accuracy of back-calculated size at hatching. It has repeatedly been found that hatch stage variation in the otolith-size - fish-size relationship is high, with the result that otolith size may explain less than 25% of the variation in fish size. For this reason, strict adherence to (allometric) proportionality is essential for the back-calculation method.

An impression of the variation in proportionality between otolith size and fish size in small fish may be obtained by re-examining the results of a study on sockeye salmon *Oncorhynchus nerka* fry (Wilson & Larkin, 1982: approximate values from their figure 3). Fry between 0.5 and 1 g were marked individually and weighed initially and again after a 28 days growth period. The *sagitta* otoliths were measured along the same axis to the edge and counting back 28 increments. The assumption of allometric growth in the form of a non-linear power function between fish weight and otolith *radius* may be expressed in the following form:

$$(V.21) \quad W = k_j u S^v$$

where k_j is the individual scaling of the otolith-*radius* - fish-weight growth trajectory. A log-log plot of fish weight v. otolith *radius* for the two weighing events combined showed a linear trend and at first sight offered no contradiction to the assumption.

If k_j remains constant for an individual fish j , the correlation between individual residuals, $\log(k_j(t_1))$ v. $\log(k_j(t_2))$ at 1st and 2nd weighing event (t_1 and t_2), should be unity. A re-examination of the values presented by Wilson & Larkin (1982), however, showed an R-square of only about 0.32, indicating a high degree of crossover between otolith growth trajectories. This exercise shows one of the pitfalls of an unjustifiable proportionality assumption in back-calculation analysis.

2.4. Proportional methods: SPH versus BPH

When we consider proportional methods of back-calculation, we have basically two means of defining proportionality. The individual CS size of a fish may be proportional to the average CS size at any given fish size or the individual fish size can be proportional to the average fish size at any given CS size. In the former case we have what Francis (1990) has termed a scale-proportional hypothesis (SPH) whereas the latter case is representative of a body-proportional hypothesis (BPH). Using the same terminology as before (from Francis, 1990) for CS size and somatic size we can express the two relationships as $S = f(L)$, where average CS size is a function of observed somatic size, or alternatively, $L = g(S)$ where average somatic size is a function of observed CS size. In the linear case this is given as:

$$(V.22) \quad S = f(L) = a + bL$$

$$(V.23) \quad L = g(S) = c + dS$$

In the first case we use observed fish length to estimate average scale size at any given fish length. Since the relationships between average scale size and observed scale size should be constant over the entire size range of the individual fish, this proportionality can be expressed in general terms as:

$$(V.24) \quad f(L_1)/S_1 = f(L_2)/S_2 = \dots f(L_c)/S_c = \text{constant}$$

The constant between expected (average) scale size and observed (measured) scale size may for example be 0.9, i.e. the average scale size is 90% of the measured scale size of the individual fish at any given length (i.e. the scale size of the fish in question is about 10% larger than the average at any given fish size). The BCF for the scale proportional hypothesis can be written in a general form as:

$$(V.25) \quad f(L_i) = (S_i/S_c) \cdot f(L_c)$$

In the common linear case this translates to:

$$(V.26) \quad a + bL_i = (S_i/S_c) \cdot (a + bL_c)$$

With some minor rearrangement, the BCF for the SPH (scale proportional hypothesis) is then:

$$(V.27) \quad \text{SPH:} \quad L_i = -(a/b) + (L_c + a/b) \cdot (S_i/S_c)$$

where a and b are regression coefficients (intercept and slope) from the S on L regression (fig. V.A.4f).

Alternatively, if we employ a body proportional hypothesis (BPH), this implies that:

$$(V.28) \quad g(S_1)/L_1 = g(S_2)/L_2 = \dots g(S_c)/L_c = \text{constant}$$

If the constant in this case is 1.01, this implies that the average length (body size) is 1% greater than the observed body size at any given scale size (i.e. the body size of the fish in question is about 1% shorter than the average at any given scale size). The corresponding general BCF for the BPH is then:

$$(V.29) \quad L_i = [g(S_i) / g(S_c)] \cdot (L_c)$$

which in the linear case can be written as:

$$(V.30) \quad \text{BPH:} \quad L_i = [(c + dS_i) / (c + dS_c)] \cdot L_c$$

where c and d are regression coefficients (intercept and slope) from the L on S regression (fig. V.A.4g).

The Fraser-Lee procedure is not correctly based on a SPH or BPH (Francis, 1990). It lacks a clear mathematically stringent underlying hypothesis such as the SPH and BPH. It is also questionable whether it is correct to estimate average L for a given S in the way this is normally done. The estimated parameter c in the Fraser-Lee procedure (when estimated by regression), will always be larger than $-(a/b)$ in SPH (see above), and the corresponding back-calculated lengths will thus be smaller using the SPH. The Fraser-Lee procedure tends to produce similar results to the BPH since they are based on the same regression (S on L) (fig. V.A.4d,g).

There has been some controversy regarding which regression method should be employed for the estimation of the parameters to be used in the BCF. Francis (1990) has strongly advocated the use of ordinary regression due to the model description and the statistical properties that are desirable in connection with prediction. As an alternative, Ricker (1992) has recommended using the GM regression since it is difficult to assess which variable should be dependent and which independent in the ordinary regression. The central line obtained from the GM regression would thus represent a better fit to the data. The slope of the GM (Ricker) regression line can be obtained by:

$$(V.31) \quad v = \frac{b}{r} = \sqrt{\frac{b}{d}}$$

where v = estimated GM slope, b = estimated slope of the regression of Y on X , r = correlation coefficient between X and Y , and d = estimated slope of the regression of X on Y .

The type of regression to be used, however, should be dependent on the choice of proportionality hypothesis (see above), but unfortunately there are no firm rules for deciding which of the proportionality methods should be used. A comparison of back-calculated size from both SPH and BPH will provide an indication of the inherent imprecision of the back-calculation procedure itself. A high correlation

between CS size and somatic size for the target population will reduce the difference between SPH and BPH results. In all cases of back-calculation it is useful to compare the SD of back-calculated size-at-age with observed sizes at the same age. Inflated SDs of back-calculated sizes compared to the observed sizes may be an indication of problems with the back-calculation procedure.

2.5. Non-linear BCFs

As indicated by the general BCFs above, we are not restricted to linear relationships in the proportionality between CS size and somatic size. Several possible non-linear relationships can be used, and we present two alternatives below:

$$(V.32) \quad \text{Alt. 1:} \quad L = g(S) = \mu S^\nu$$

In this case fish size is a non-linear (power) function of CS size. The equation can be rearranged to a linear form by taking the natural logarithm on both sides of the equality sign:

$$(V.33) \quad \log L = \log \mu + \nu \log S$$

We determine ν by regression and apply the BCF:

$$(V.34) \quad \text{BCF:} \quad L_t = (S_t / S_c)^\nu \cdot L_c$$

When ν is determined from the regression of L on S , the BCF is consistent with a BPH. The back-calculation lines are linear and parallel on a log-log plot (common slope), and will converge towards $L_t = 0$ and $S_t = 0$ on the regular linear plot. If $\nu = 1$ the BCF is equal to the Dahl-Lea BCF.

In the second example of a non-linear BCF, fish size is expressed as polynomial function of CS size:

$$(V.35) \quad \text{Alt. 2:} \quad L = g(S) = c + dS + eS^2$$

The corresponding BCF can be rearranged to:

$$(V.36) \quad \text{BCF:} \quad L_t = L_c \cdot (c + dS_t + eS_t^2) / (c + dS_c + eS_c^2)$$

When c , d and e are fit by non-linear regression of L on S , the BCF is consistent with a BPH. If $e = 0$ then the BCF is identical to the common linear BPH. In the same manner as in the linear case, a BCF can be derived on the basis of the SPH by assuming that average scale size is a polynomial function of observed fish length. Bearing in mind the underlying assumptions regarding proportionality, a wide range of BCFs can be constructed on the basis of stringent mathematical principles. The advantage of this is that the statistical properties of the estimated parameters in the BCF will be known, and measures of the precision of the back-calculated sizes can be obtained.

2.6. Problems of back-calculation

The validity of the back-calculations is highly dependent on the fulfilment of the assumptions listed above. But even if the back-calculations have been carried out in a correct manner, the results themselves need to be interpreted with caution. As in other ecological studies, the inferences that can be made from back-calculations are related to the population (or part of the population) under study. Although accurate inferences can be made about individual growth trajectories, we are often interested in the growth pattern of the population as a whole. In these cases adequate sampling procedures are essential in order to ensure that we have obtained a random sub-set of the population or a random stratified sub-sample. Bearing in mind that there may be selective removal of members of the population through natural mortality and fishing, the population characteristics at any age will depend on the age of the fish used to assess these characteristics (e.g. average size at age 2).

It is quite common to observe that the average back-calculated size for a given age-group is smaller when calculated from older fish than from younger fish (Ricker, 1975). This effect is termed Lee's phenomenon, and it may be due to selective mortality, biased sampling, problems with the back-calculation, or a combination of these explanations.

One essential element in proportionality methods is the assumption of constant proportionality in an individual fish. Several authors have questioned this assumption, and have concluded that there is variability within an individual over time in the proportionality between CS size and somatic size (Zivkov, 1996). Experimental studies have also shown that the growth of the CS can be "uncoupled" from somatic growth for some time, although there still exists a strong overall correlation between CS size and somatic size (Mosegaard *et al.*, 1988; Secor & Dean, 1989). In the case of otoliths, these have been shown to continue to grow even though somatic growth has completely ceased during food restriction and starvation (Folkvord *et al.*, 2000). One could argue that even slightly starved individuals would be less likely to survive in the field, and that the problems of "decoupling" under natural conditions is less likely to occur than in the laboratory, because only reasonably healthy fish live long enough in the field to be sampled.

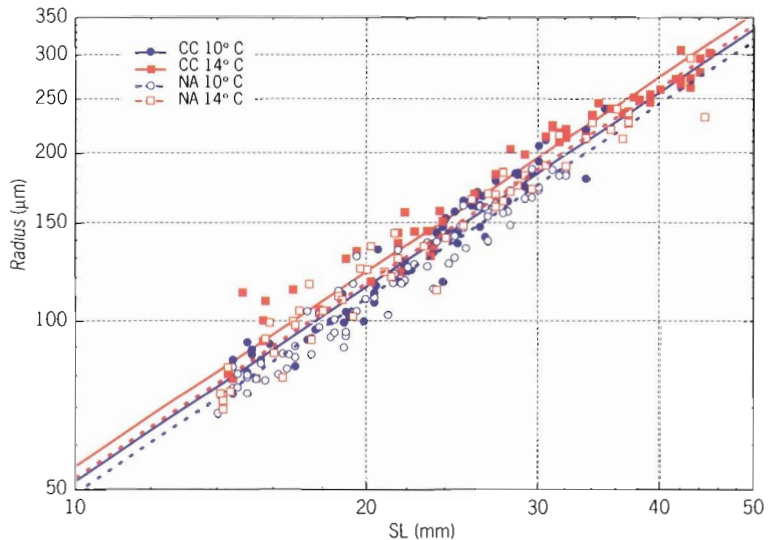
Also seasonal deviations from a general allometric relationship between CS and somatic growth may turn back-calculation into a non-trivial matter. In Northern pike the CS:body instantaneous growth rate ratios of both scale and *cleithrum* bone showed values above one during summer high growth and below one during winter low growth (Casselman, 1990).

The relationship between fish size and the CS size is also under environmental influence (e.g. temperature, Mosegaard *et al.*, 1988), and this adds uncertainty to the results of the back-calculation procedure. A temperature-dependent fish-size - CS-size relationship can generate a bias in the back-calculated sizes if a common relationship is used, and several examples of temperature-dependent relative otolith size are available (fig. V.A.5). In such cases the back-calculated fish sizes will to some extent reflect the ambient temperature history of the fish in addition to their previous size development. The problem of such a temperature relationship may be less if the fish in the population have been growing under similar ambient temperature conditions.

3. Recommendations

Growth analysis may appear to be a trivial problem, but the proper treatment of data is not always straightforward (Ricker, 1975; Kaufmann, 1981; Francis, 1995a). In the following, we offer a series of recommendations that are relevant to growth studies that involve the use of calcified structures (CS).

Figure V.A.5
Stock- and temperature-dependent allometric relations between fish length and otolith (*lapillus*) radius in juvenile cod (*Gadus morhua*). Offspring of CC (Norwegian coastal cod) and NA (Northeast Arctic cod) have been reared at constant temperatures (Otterlei *et al.*, *subm.*).



The data structure of the increment widths in CS should be analysed using multivariate statistical techniques (Chambers & Miller, 1995; Ralston & Howard, 1995; Jones, 2000). Both repeated measures ANOVA and MANOVA (multivariate ANOVA) can take into account the inherent autocorrelations, but MANOVA is generally preferred due to

its less rigorous assumptions regarding the underlying data structure (Chambers & Miller, 1995). Serial correlations of increment widths can also be effectively dealt with using time series models (e.g. ARIMA, Ralston & Howard, 1995). The effect of using univariate techniques without correcting for data dependence includes inflated degrees of freedom in the statistical tests, yielding artificially high precision and thus an increased likelihood of a type I error.

Special attention should be paid to scaling algorithms that combine several independent readings from the same otolith. This is particularly a problem with microstructure data, where many increments are counted and measured. In many instances this involves the combination of readings with varying increment numbers, and thus requires some splitting/joining of increments to achieve a common increment pattern (Methot, 1981). In cases where the sectors read are of different sizes, the use of relative increment widths (e.g. to the total *radius*) may provide the basis required for estimating a common pattern of otolith growth. From a statistical point of view, single readings of individual otoliths would yield a relatively simple error structure suitable for analysis. Computer-intensive techniques can be alternatives to statistical methods when the underlying assumptions of traditional statistical methods cannot be met. Bootstrapping can yield confidence intervals of increment widths and check sizes (e.g. Anderson, 1995).

A proper sampling strategy is also essential. A clear definition and documentation of the population units under investigation is required if we are to make valid comparisons between sub-populations or temporal comparisons within populations. Different patterns of growth obtained from two samples collected at different times and places may be due either to differences between sub-populations or to differences within a sub-population (caused by selective mortality or gear selectivity).

No single procedure can be regarded as better than all others in back-calculating fish sizes, and no specific procedure can therefore be recommended as such. However, the user should be aware of any underlying assumptions and limitations of the different back-calculation procedures, and should carefully check the pattern of back-calculated sizes against observed patterns of sizes at comparable ages. Francis (1990) recommended the use of more than one procedure during back-calculation, and this would be useful for several reasons. First of all, it would allow the possibility of determining which model agreed best with the actual data at hand. Secondly, the variability in the back-calculated sizes using different methods would give an indication of the variability in back-calculated sizes resulting from the choice of procedure.

An alternative means of working with longitudinal data obtained from CS is to use the incremental data from the CS directly, instead of “translating” them into fish size (Anderson, 1995; Hare & Cowen, 1995). Provided that there is a high correlation between fish size and size of the CS, the incremental CS data could be used as proxies or indices of fish size. The incremental data can then still be used to study size-dependent mortality (see chap. V.B).

B. Ecological applications

H. Mosegaard, A. Folkvord, P.J. Wright

1. Recruitment studies

The development of age estimation techniques to determine age at the annual level enabled Hjort (1914) to put forward his ground-breaking hypothesis of year-class fluctuations in fish stocks. The distribution of age-classes in *Clupea harengus* revealed that one year-class, the 1904 year-class, was clearly more abundant at an early stage, and remained the dominant year-class for nearly two decades. The underlying reasons for the abundance of this year-class were not evident, although its size was evident at an early age. The discovery of daily increments by Pannella (1971), opened up new possibilities of studying the dynamics of growth and survival within the first year of life in fish, thus enabling researchers to answer the fundamental question in fisheries biology of why there is variation in pre-recruit survival.

1.1. Documentation of selective mortality

One of the most powerful applications of individual age-structured data from hard parts (for example, and particularly, otoliths) is the combination of studies of growth and size-dependent mortality. Previously formed increment widths-at-age in an otolith (and also otolith size-at-age) will remain unchanged as the fish continues to grow over time. If the temporal significance of these increments has been established (see chap. IV), comparisons of increment widths at given ages of fish sampled at later stages will have the potential to reveal any changes in the increment width pattern (or otolith size distribution) among fish from the sampled population.

While many studies have investigated growth and survival rate, few have attempted to test a specific hypothesis. Anderson (1988) provided a general framework for considering the importance of feeding success and predation to mortality; the growth mortality hypothesis. Three mutually non-exclusive mechanisms have been proposed for the functional basis of the growth mortality hypothesis.

The "bigger is better" mechanism: if mortality is a decreasing function of size, then the larger individuals at any given age will have a lower probability of mortality than smaller individuals of the same age (i.e. Leggett & DeBlois, 1994).

The “growth rate” mechanism: if the probability of mortality is size-dependent, then higher growth rates will minimise the time over which individuals experience high mortality rates and thus, faster-growing larvae will have a lower probability of mortality than slower-growing larvae (Ware, 1975; Shepherd & Cushing, 1980).

The “stage duration” mechanism: if mortality rates are size- and growth-rate independent at any given stage, small percentage changes in instantaneous growth rate can have order-of-magnitude consequences for the number of survivors reaching a particular development stage if the later stage is subject to a lower mortality rate. Hence, individuals that develop faster and make the transition at earlier ages would have a lower probability of mortality than individuals that make the transition at greater ages (Chambers & Leggett, 1987; Houde, 1987).

It can be seen that mechanisms 1) and 2) are highly overlapping, since a larger size at any age is directly linked to an overall higher growth rate; mechanism 3) is also potentially related to the first two by the extent to which development rate is correlated with growth rate and size on an individual basis. These mechanisms may have a distinct definition in terms of the total or partial basis of selective mortality if they are specified in terms of each other.

One approach would be to show the partial effect of one mechanism on survival when the others are either held constant or individually accounted for. Verification of the “bigger is better” mechanism would require comparison of individuals with equal growth rate and age/size at stage transition. This is of course not possible with the “growth rate mechanism” since growth results in changing size, here the validity of the mechanism would demand indirect evidence through higher survival of initially smaller than of initially larger individuals during growth, until a certain size was reached.

To date, only the study by Hare & Cowen (1997) has attempted to test all three hypotheses. In order to test for size-dependent mortality (i.e. bigger is better) these authors examined whether the distribution of *radii*-at-increment, as a measure of size, changed with increasing age. An increase in the proportion of individuals with large *radii*-at-increment with age was seen as evidence for positive directional size-dependent selection. They and others (e.g. Hovenkamp, 1992) tested for growth-dependent mortality by examining whether the distribution of otolith growth between specific increments, as a measure of growth, increased with increasing age. An increase in the distance between specific increments with age was seen as evidence for positive directional growth-dependent selection. However, high correlations

between otolith size and otolith growth rate prevent a resolution of the partial importance of the "bigger is better" mechanism and the "growth rate" mechanism. Similar comparisons have been made of daily increment widths instead of several increments (Gallego *et al.*, 1999), although such an approach is subject to the technical limitations of accurately resolving and measuring daily increments.

The presence of otolith structures associated with hatching and larval metamorphosis (chap. II.A.2) enables comparisons of the stage duration to be made. However, testing for selection on the basis of stage duration is difficult from field sampling because there is normally no cross-sectional sample of fish before selection, to indicate size and age for transition, for comparison with a cross-sectional sample of fish that survived through to the transition. One approach to this problem is to compare the distribution of size and age at transition of experimentally reared fish with field samples of survivors (Hare & Cowen, 1997). However, this assumes that there is no selection and no difference in the studied trait in the reared group.

The proportion of fish with relatively small increment widths at a given age may decrease with time, indicating an effect of size-dependent mortality (i.e. higher loss of relatively smaller individuals in a cohort). In the example shown (fig. V.B.1), the mortality of the smaller (and slower growing) individuals is shown to occur between day 20 and 40. After day 40 none of the individuals represented with narrower increment widths at day 10 was left in the population. The timing of this size-dependent mortality would be difficult if not impossible to detect without analysis of the recorded growth pattern available in the CS. An example is provided from a mesocosm study with herring larvae, in which the initial feeding conditions were poor (mesocosm A, fig. V.B.2). The ultimate survivors became larger on average than the initial population at quite an early stage, and this suggests that the high mortality initially observed in this mesocosm was indeed size-dependent. Similarly, the proportion of fish with relatively slow otolith growth in the population may be shown to decline with time, indicating the existence of growth-dependent mortality. The interpretation of such analyses will depend on repeated samplings of the same population unit at intervals, the strength of the analyses lies in the continuous accretion of the CS providing data on the growth history between the samplings. If significant migration takes place to/from the population between samplings, however, such migrations cannot be distinguished from apparent size-dependent mortality during the same period.

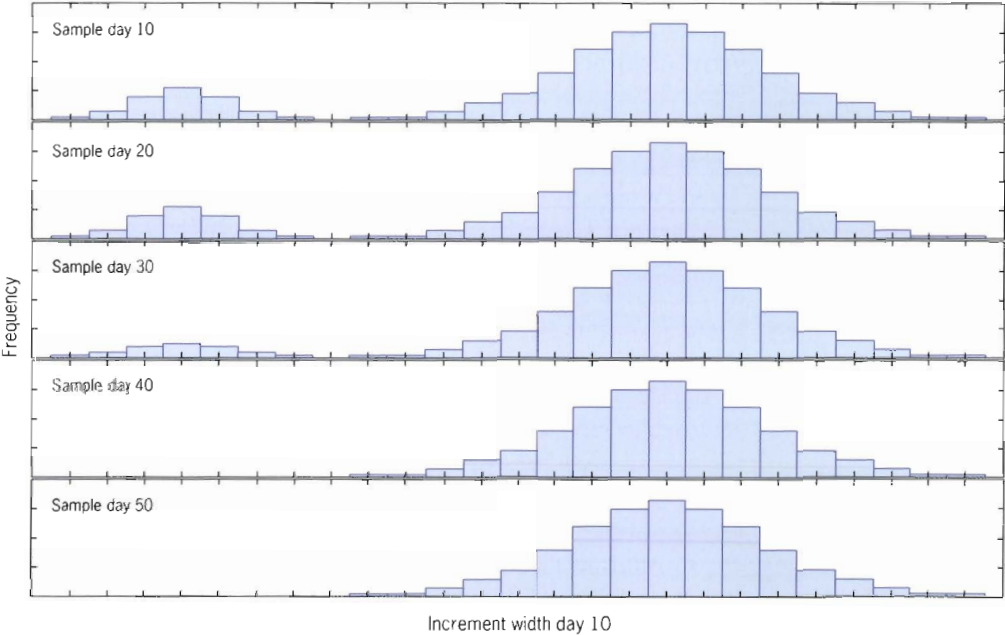


Figure V.B.1 - Schematic view of increment widths of a cohort of fish larvae (born on the same day) sampled just prior to midnight on different days. The upper panel represents the distribution of outer increment widths on the day of sampling, whereas the panels below represent the widths of the 10th, 20th, 30th and 40th increment from the edge respectively, thus corresponding to increments formed on day 10 (assuming daily increment formation).

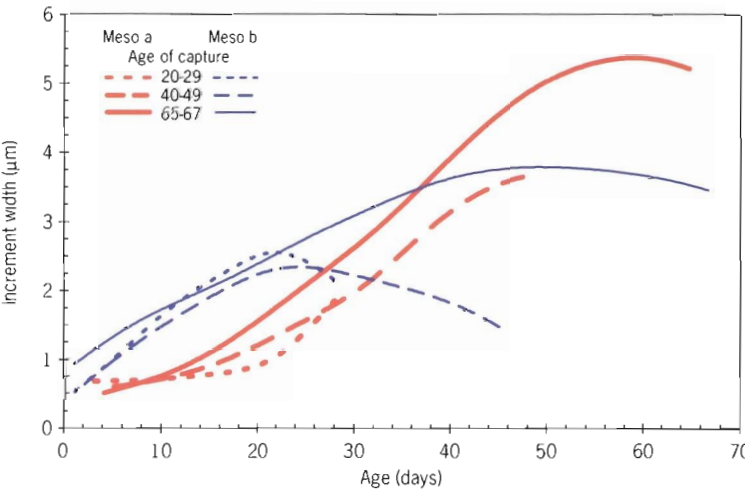


Figure V.B.2 - Increment width pattern (least-squares fit) of larval herring (*Clupea harengus*) reared in two mesocosms (thick lines mesocosm a, thin lines mesocosm b, modified from Folkvord, 1997). Larvae were sampled with two-chambered nets during the experiment (dotted lines, day 20-29, dashed lines day 40-49), and the survivors at the end of the experiment (solid lines, day 65-67) were sampled by draining the mesocosm.

1.2. Bigger is better

An example of an analysis that employed the method described above is provided by Meekan & Fortier (1996). Larval, pelagic, and settled juvenile cod were repeatedly sampled over a grid covering parts of the Scotian shelf in the Northwest Atlantic. On the basis of an annually invariant high linear correlation between *lapillus radius* and cod standard length ($R\text{-square}=0.97$) they used the biological intercept method (Campana & Jones, 1992) to back-calculate standard length at age. By using successive ten-day intervals of back-calculated growth from individual otoliths and a repeated-measures statistical approach, they were able to demonstrate different survival patterns of juvenile cod in two consecutive years. In one of the years the proportion of slow-growing juvenile cod decreased during the winter, and the juveniles that settled and eventually recruited to the stock consisted of fish that were relatively large at the pelagic juvenile stage. In a similar study, Campana (1996) presented a correlation between ultimate recruitment and growth of juvenile cod during the pelagic juvenile stage. These studies provide evidence that the ultimate survivors were not a random sub-sample of the initial population of cod larvae, and that in both cases there was a survival advantage associated with relatively faster growth.

1.2.1. Correlations of sizes at different ages and hierarchies

The maximisation of survival until recruitment has led to the development of quite different early life history strategies in different phylogenetic or ecological groups. It is believed that parental investment in the survival of eggs and embryos has led to evolutionary traits that optimise the size of individual offspring. The primary increment pattern recorded in the CS allows for analysis of further fitness-related life history transitions during subsequent growth.

In a study on Frazer River sockeye salmon, West & Larkin (1987) used otolith check formation to back-calculate distributions of individual size at emergence in a study of size-selective survival at later juvenile stages. They were able to show that individuals with a large otolith size at emergence had a much improved probability of survival. A reanalysis of the same material by Mosegaard (1990) showed that due to a low correlation between fish size and otolith size at the end of the yolk-sac stage selection for large fry size could not explain the change in the observed distribution of otolith sizes. With a low correlation, size-selective mortality has to produce a very high shift in the distribution of fry sizes to make a modest shift in otolith size distribution, as demonstrated in figure V.B.3.

Selective survival causing strong shifts in otolith size distribution may be explained by a coupling of large otolith size at emergence with individual competitive performance during the establishment of dominance hierarchies at first feeding as demonstrated experimentally in both brown trout, *Salmo trutta* (Titus & Mosegaard, 1991) and Atlantic salmon, *Salmo salar* (Metcalf *et al.*, 1992). A physiological explanation of the weak relationship between otolith size and fish size

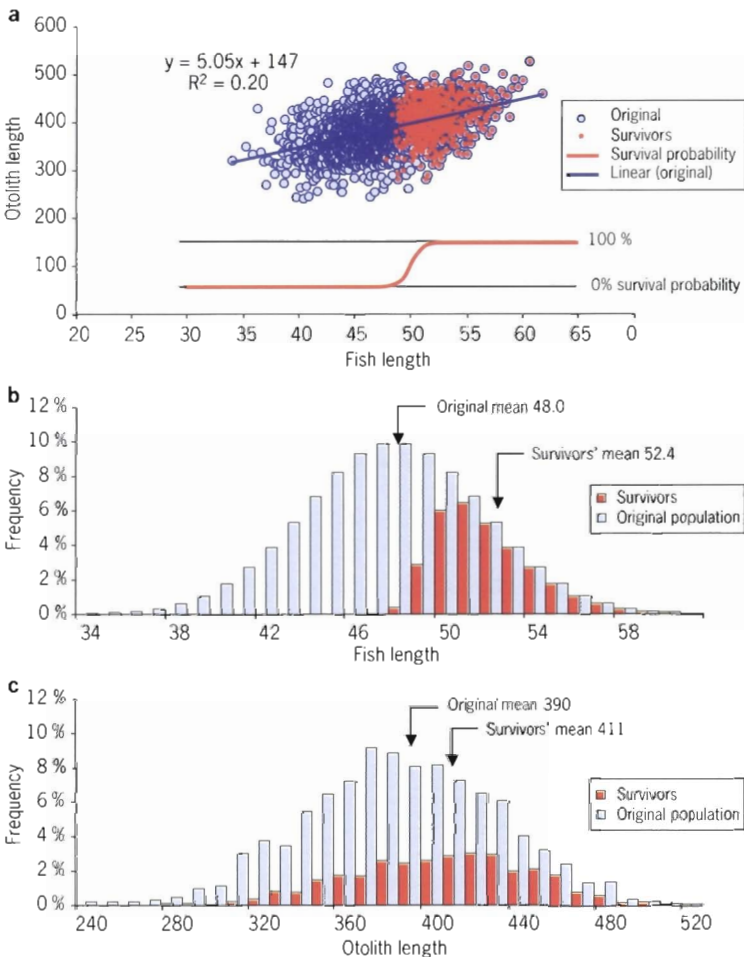


Figure V.B.3 - Simulated data demonstrating the shift in otolith size distribution by selection for large first-feeding salmon size (size scale is arbitrary).
a) Individual otolith length versus fish length. A random logistic survivorship function based on fish length was employed in the simulation (red curve separately in the plot); survival=1 if $1/(1+\text{EXP}(100 \cdot 2 \cdot L_{\text{alevin}})) > \text{random number } [0;1]$, where L_{alevin} indicated normally distributed alevin lengths, and otolith size a linear function of fish size with random normal error.
b) Fish length frequency distribution before (blue) and after selection (red).
c) Otolith length frequency distribution before (blue) and after selection (red).
With a low correlation between fish length and otolith size ($R\text{-square} < 0.25$) at first feeding, a strong selection for fish size will produce only a minor shift in the otolith size distribution.

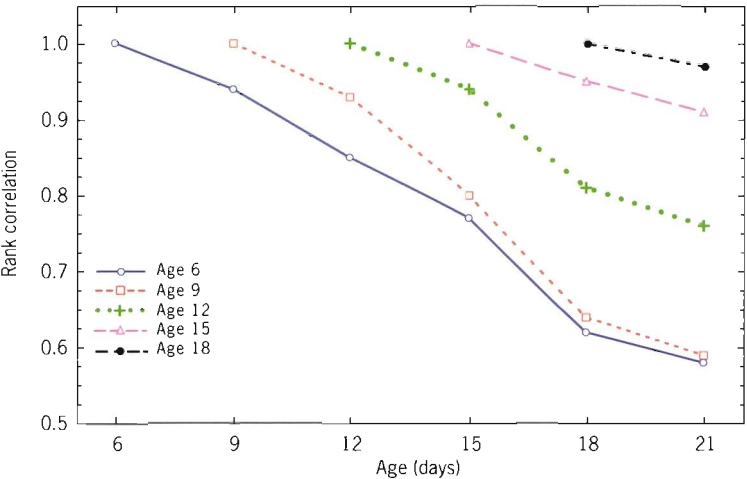
after yolk-sac resorption would rely on the metabolic control of otolith accretion rate (Mosegaard *et al.*, 1988; Wright, 1991a) and the increased energy costs reducing somatic growth in embryos with a high turnover rate and a post-emergent increase in growth potential (Titus & Mosegaard, 1991). The coupling of dominance status and otolith accretion rate with resting metabolism has further been supported by work on masu salmon, *Oncorhynchus masu* (Yamamoto *et al.*, 1998). Implications of individual characteristics at life history transitions for later survival have also been studied by otolith microstructure analysis in other systematic groups. Large hatch check size has been correlated with increased survival in cod (*Gadus morhua*) larvae in the Baltic (Grønkaer & Schytte, 1999).

The CS size-at-age data from individual fish can be used to study size-rank correlations within a cohort. In an example from field-caught larval menhaden, the rank correlation of otolith size was shown to decline from 100% to about 80% after six days, and further to about 60% after another 12 days of growth (fig. V.B.4). This shows that there is considerable “crossing over” in size rank within a short period of time. This may reflect medium-scale variations in environmental conditions and food availability experienced by cohort members or it may be due to genetic differences in growth capacity or to social interactions related to size-dependent hierarchies between individuals in the population (Imsland *et al.*, 1998).

2. Life history timing

Age-related growth sequences in the calcified structures of fish have the potential to date the occurrence of important life history events, when fish experience sudden changes in the environment and/or undergo major developmental changes.

Figure V.B.4
Rank correlations based on otolith radii sizes of Atlantic menhaden larvae (*Brevoortia tyrannus*). Correlations are calculated for otolith sizes at ages 6 to 18 for subsequent ages 6 to 21 at three-day intervals (data from Chambers & Miller, 1995).



2.1. Birth date distributions

A key argument proposed to explain variability in early survival rates concerns the synchrony between spawning and hatching times and favourable environmental conditions. Asynchrony between the timing of larval occurrence and favourable environmental conditions would be expected to lead to discrepancies between the "hatch date" composition of surviving recruits and the actual temporal pattern of hatching. Such a comparison of hatch date compositions is possible through the analysis of daily otolith increments (Campana & Jones, 1992). Birth date can be derived by subtracting the estimated duration of embryonic development from hatch date. This clearly requires field measurements of temperature and an experimentally derived relationship between embryonic development and temperature.

Campana & Jones (1992) provide a detailed account of hatch date analysis. Briefly, the technique compares the frequency distribution of hatch dates from at least two successive samples of fish collected at random from a population. The timing of the production of hatched larvae would normally be determined by means of frequent sampling throughout the hatching period. The frequency distribution of true hatch dates would then be compared with the back-calculated hatch dates of survivors of a given age, as estimated from subsequent sampling of the population. Statistical comparisons of hatch-date frequency distributions involve similar approaches to the analysis of growth-dependent mortality. The main problem with hatch-date analysis is that early-season larvae will have experienced greater cumulative mortality than those hatched late in the season and therefore will be underrepresented in the back-calculated hatch date distribution. Thus, in order to derive a true survivor hatch-date frequency distribution, survivors should ideally be collected over a period equal to the duration of the hatching period. In practice, most studies have attempted to overcome the problem of differential cumulative mortality by multiplying the numbers at a given age by the inverse of the survival rate between the age at capture and the age of the youngest fish in the sample. This survival rate should be based on an independent field estimate. As mortality tends to be inversely related to age, the need for differentiation of cumulative mortality declines with age. For this reason hatch-date analysis is more suitable for later life history stages such as juveniles, which tend to have a lower mortality rate than larvae.

Using the birth date technique it is possible to explore the fitness consequences of reproductive timing. Most studies of temperate species have found selection for late-hatched individuals between the time of hatching and larval metamorphosis (Crecco & Savoy, 1987; Rice *et al.*,

1987; Moksness & Fossum, 1991; Wright & Bailey, 1996). However, this form of selection may reflect the stage of development under consideration rather than life-time fitness since, in most temperate species, factors favouring the late production of offspring act early in life, whilst those favouring the early production of offspring act later in life (Schultz, 1993; Anderson, 1995; Miller, 1997). For example, conditions for early growth tend to improve for spring-spawned fish, thus favouring selection for late hatching. However, as early hatching maximises the time for growth this trait may be important where there is a selective advantage for an early date of particular life history events such as metamorphosis (Sinclair & Tremblay, 1984) or age at first maturity (Schultz, 1993). Given the importance of developmental stage in the interpretation of hatch-time effects on fitness, studies of hatch date should ideally include repeated samplings of a year-class throughout development, as in the study by Schultz (1993).

2.1.1. Approaches to testing for size-dependent mortality and hatch date

Differences among actual and back-calculated hatch date and size distributions have usually been tested for by means of the Kolmogorov-Smirnov test, which compares cumulative frequency distributions. However, several approaches have been developed to characterise how the pre- and post-selection samples differ. Two examples are given below.

The non-parametric technique of Schluter (1988) as modified by Anderson (1995) for cross-sectional data has been used to determine relative survival functions. This method uses cubic splines to estimate the form of selection acting on a quantitative trait. This spline-based method makes no assumptions of the underlying fitness function, assumes that the function is smoothly changing and allows calculation of the confidence intervals. The non-linear form of selection is calculated using a "before-selection" group and an "after-selection" group of otolith size measurements. A non-parametric spline-based regression is fitted to the before and after selection data, using maximum log-likelihood methods and confidence limits applied by bootstrapping, thus allowing for interpolation between non-sampled points. This approach allows estimation of relative fitness, whereas the absolute fitness function can only be found if population sizes or the relative sampling efficiency are known (Anderson, 1995). It should also be noted that otolith size is usually not the primary trait under selection but is only correlated with other traits of interest, e.g. body size, scope for growth or metabolic activity (West & Larkin, 1987; Titus & Mosegaard, 1991; Wright, 1991a).

Miller (1997) proposed an approach based on a residual analysis of patterns in pre-selective samples. In this approach an otolith-*radius* - age relationship from the field samples is used to derive the “before-selection” sample using a best-fit function. This function is then fitted to the otolith-*radius* - age data of the “after-selection” group and the residuals are plotted. Systematic deviation represents directional phenotypic selection, while stabilizing and disruptive phenotypic selection lead to an increasing or decreasing pattern in the residuals.

All these approaches are based on a cross-sectional approach to a single trait. However, any assessment of selection requires an ability to characterise the prior history of traits in the individuals that are selected. This task is simple for traits such as hatch date, since changes in the composition between collection times can only reflect selection. However, traits such as size, which change independently of selection, are far less easy to quantify because both size and growth rate can covary. Although body size may be back-calculated from otolith size there is currently no framework available to account for the combined effect of changes in growth and size, although longitudinal approaches based on the reconstruction of phenotypes have been suggested (see Miller, 1997).

2.2. Ontogenetic transitions

Hatch and first-feeding checks are often used indiscriminately for analyses of birth date distributions in species with small embryos and short yolk-sac development periods. However, in species with a protracted yolk-sac stage, such as Salmonids, the formation of otolith primary increments during this development permits separate estimation of dates for hatching and first feeding to be made.

Metamorphosis from the larval to the juvenile stage generally involves a marked morphological and developmental transition. Most larval *sagitta* and *lapillus* otoliths grow from a more or less spherical appearance at the yolk-sac stage to a gradually more disc-formed and somewhat asymmetrical shape at the late larval stage. The transition to the juvenile stage is in some taxa accompanied by the appearance of accessory growth centres in the *sagitta*, sandeels (Wright, 1993), plaice (Modin *et al.*, 1996), while the accretion rate of these accessory growth centre exceeds the concentric primary growth and thus markedly influences the shape of the otolith (chap. II.A and fig. III.B.1). Counting daily increments outwards from the centre of the otolith or in from the margin to the first accessory growth centre permits the estimation of age or date, respectively, for this life history event. In a number of demersal species, metamorphosis also coincides with the transition from a pelagic to a bottom-dwelling habit. Where

the pelagic and benthic habitats differ greatly the ontogenetically induced otolith growth transitions may be accompanied by environmentally influenced changes in patterns of otolith growth.

2.3. Environmental changes

Many fish species experience dramatic changes in their environment during their life cycle. Several examples are found among Salmonids, many species of which migrate from the freshwater environment where they were born to the saltwater environment where they feed until they eventually return to their native rivers to breed. The timing of the downstream migration can be traced in the otolith, from the marked changes that are reflected in its pattern of growth or chemical composition after the smolts have entered the sea. In chinook salmon (*Oncorhynchus tshawytscha*) juveniles display various migration strategies, and otolith microstructure characteristics specific to particular freshwater and ocean habitats enables the different juvenile life history types to be identified and the timing of their migration to the ocean studied (Zhang & Beamish, 2000). In Baltic juvenile brown trout (*Salmo trutta* L.) a migration from small freshwater streams to the brackish estuarine environment was indicated by the early accumulation of high Sr/Ca ratios in the otoliths of some individuals (Elfman *et al.*, 1999) supporting earlier direct observational studies of 0-group Sea-migration from this area (Titus & Mosegaard, 1991).

One of the first examples of dating migration is from American eel otoliths where the chronology of upstream migration from the sea could be traced by increasing Ca and decreasing Sr concentrations using WDA (Casselman, 1982).

Seasonal differences in food and temperature may also be reflected in the temporal record of primary otolith increments.

2.4. Spawning

Immature fish allocate their excess energy to somatic growth, while as they reach maturity they need to spend a growing proportion of their net energy surplus on gonadal development. The timing of first maturity is usually noted in CS as a change in growth pattern. Examples of changed otolith growth pattern are found in fishes from a wide range of climatic zones: Arcto-Norwegian cod, *Gadus morhua* (Rollefsen, 1933), capelin, *Mallotus villosus* (Hopkins *et al.*, 1986), plaice, *Pleuronectes platessa* L. (Rijnsdorp & Storbeck, 1991), orange roughy, *Hoplostethus atlanticus* (Francis & Horn, 1997). Another CS character connected with spawning is the resorption zone in scales often found in Salmonids, which is caused by hormonally induced changes in calcium metabolism during gonad maturation (Persson *et al.*, 1998).

3. Sampling selectivity

The differences in patterns of otolith size-at-age can also be used to study the effects of sampling avoidance and the time at which this becomes a problem. It is often found in studies of larval and juvenile fish that the fish eventually become large enough to avoid the nets used to catch younger larvae. If a subsequent non-biased sample of the population can be obtained using a different gear, a comparison of the increment growth pattern of fish sampled using the two gears will indicate the point in time at which avoidance begins to take place (fig. V.B.2). In this example, the larvae from mesocosm B were adequately sampled up to day 20 with the two-chambered net, but thereafter the fish caught using this net clearly represented the smaller fish in the population, compared with the survivors at the end of the experiment. Although the benefits of using the growth pattern of individual fish are evident in studies of this type, adherence to the assumptions made regarding the temporal meaning of the increments and the proper sampling of the population remains essential.

Differences in stage- or size-specific behaviour may also bias estimates of vital rates when using standard sampling from the fisheries. Juvenile and adult sandeels display burrowing behaviour that influences their availability to the fishery during nocturnal and seasonal periods of inactivity in the sediment. An example of this is the estimated growth of lesser sandeel (*Ammodytes marinus*) in the North Sea, (Pedersen *et al.*, 1999) where a decline in size-at-age was generally found towards the end of the growing season. In a site-specific study, unbiased samples of sandeels were obtained by digging out hibernating individuals from the sediment in February/March by means of dredging. Back-calculation analysis of the otolith size distribution at the initiation of the first translucent zone showed that different size fractions of the year-class were caught by the commercial fishery in different periods of the fishing season. By relating individual size to the width of this first annual growth structure the seasonal growth estimates could be corrected for sampling bias (Worsøe & Mosegaard, unpubl.).

C. Demographic structures in stock assessment models

B. Mesnil

Scientists dealing with the dynamics of many terrestrial or marine populations are often forced to take into account that these populations are age-structured (e.g. Charlesworth, 1994; Tuljapurkar & Caswell, 1996). The main reason for this is that several key parameters (growth, fecundity, and survival rates) that determine how the size or biomass of such populations change through time differ noticeably among age groups. In the case of fish (and shellfish), much scientific work is concerned with the dynamics of exploited populations, i.e. populations that are subject to both ecological and anthropogenic processes, with a view to providing advice about the sustainable use of aquatic resources. "Stock assessment" is the general term used for such investigations. As pointed out by Hilborn & Walters (1992), the aim of such studies is not only to assess the state of stocks and fisheries relative to historical states, biological reference points or management targets, but also to evaluate the consequences, for both fish stocks and fishermen, of alternative management scenarios.

This sub-chapter is not intended to provide a review of methods and models used in stock assessments. Rather, it attempts to point out the motives that underlie the development of some classes of age-structured models. In other words, the focus is on the "added value" of accounting for age structure in comparison with approaches that ignore it. This is put in the context of other chapters of the manual by calling attention to two aspects. One, which is treated in the discussion of each model, is the extent to which moving to a more elaborate class of model changes the requirements in terms of age determinations. The other aspect (section 2) refers to the processes that take place between reading the ages of a sample of fish and the stage at which the data (usually age compositions of catches or of some associated quantity) are used to estimate the parameters of age-structured models. It will be shown that the improvements afforded by more detailed models have a cost in terms of the quantity and quality of data required, thus justifying the recourse to the more efficient methods of obtaining age structures presented in this book.

1. Age-structured models for stock assessments and management advice

Most methods used for stock assessments involve a combination of a population dynamics model with models that relate observations to some attributes of the fish population (NRC, 1998). Fisheries scientists are also concerned with evaluating the effects of different fishing regimes on the future states of stocks and fisheries. For this reason, they also need models that translate changes in the control variables (catches and/or fishing effort), consistent with proposed management options, into appropriate changes in the population dynamics. The introduction of age structures into fisheries models follows from the recognition that the distinct age groups that make up a population (fig. V.C.1) provide different contributions to biomass production, reproduction or harvest through time or are not equally sensitive to management measures. This is in contrast to the simpler biomass dynamic or (surplus) production models (Schaefer, 1954) which describe stock dynamics only in terms of overall biomass.

Figure V.C.1
Typical structure of a population on an annual basis. Here, numbers at age are considered and one cohort (year-class 1992) is outlined diagonally. A similar structure applies to other variables such as catches, biomasses, mean weights at age, fecundities, natural and fishing mortalities.

Ages	Years						
	1992	1993	1994	1995	1996	1997	1998
0	N0,92						
1		N1,93					
2			N2,94				
3				N3,95			
4					N4,96		
5						N5,97	
6+							N6+,98

1.1. Single species, single fishery

The core age-structured models from which the extensions presented hereafter have evolved are the so-called analytic (or dynamic pool) models associated with the pioneering works of Beverton & Holt (1957) and Ricker (1958). These models treat the population as consisting of individual cohorts (broods born in a given year or season) and consider four basic processes at work in the evolution of populations through time: recruitment and growth, that increase the biomass of the population, and natural mortality and mortality due to fishing, that decrease it. The parameters associated with these processes may depend on age. This is obvious in the case of **recruitment**, which only concerns the youngest age group entering the fishery each year. Many stocks show considerable annual variations in the number of recruits, and it is important to account for this in assessments.

Growth is obviously a function of age, whether we are dealing with the discrete set of weights at age or with weight increments from one period to the next. The pattern of growth of most fish species is such that the growth rate is high at early ages and slows down as fish grow older, until some asymptotic value is reached. Growth is generally the first process analysed in any study of a fish stock and is the most immediate application of age data (chap. V.A). The reproductive potential of a population is also a matter of great interest for biologists, as it is an essential ingredient of sustainability criteria. In most species, **fecundity** (a combination of the number and quality of eggs) is dependent on the size and age of the fish. **Natural mortality** is inherently difficult to estimate, and even more so its variations with age. Therefore, it is often treated as constant in many applications but, as we will see later with multispecies models, it is important that the model structure should allow for age-specific parameters. Uncertainty regarding appropriate values for natural mortality is often the most critical issue in assessments and predictions. Finally, because the various age groups are not equally vulnerable to different types of fishing gear, fishing mortality is bound to vary with age for any given intensity of fishing. This is sometimes formulated as the “separable model”, in which **fishing mortality** $F_{a,y}$ at age a in year y is the product of an age-specific selectivity factor S_a (called “exploitation pattern” in Europe and “partial recruitment” in North America) and a year-specific factor f_y , which is related to the overall fishing intensity. Immigration and emigration are also processes that may affect the biomass of a population, with age-dependent parameters usually, but all models considered hereafter assume closed populations.

In recent decades analytic models have essentially been used to advise on TACs (Total Allowable Catches) and catch quotas, which are the prevailing management tool in North Atlantic fisheries, as in many others. In this case, the models are used in simulation mode, taking due account of the structure (abundance at age) of the initial population and, if possible, of the abundance of expected recruitments. In some cases, predictable changes in weight or fecundity at age are also taken into account. Each cohort is traced individually through time and the catch and biomass in each year are computed as the sums of contributions of each age group (fig. V.C.2). The typical outcome of these simulations is a table of predicted catches in the management year and of total or spawners' biomasses surviving in the stock for a range of fishing regimes (i.e. different overall fishing mortalities and exploitation patterns). Managers eventually select from among the predicted catches to set a TAC that is consistent with conservation needs and/or social and economic considerations. In heavily exploited

stocks, incoming recruits often contribute significantly to the expected catch. However, because recruitment is inherently difficult to predict, even with good pre-recruitment surveys, for more than a few years ahead, TAC predictions have generally been restricted to a very short time horizon (one or two years). The concern that this provides managers with insufficient foresight to guide their decisions has been addressed by broadening the scope of simulations in such a way that the medium-term consequences of management options can be shown explicitly. This necessarily involves stochastic (in the broad sense) variants of the analytic models. The purpose is also to quantify explicitly how uncertainties in the data (including age composition estimates) and in the parameters of the models translate into uncertainties and risks in the outcome of management decisions (e.g. Hilborn *et al.*, 1993; Smith *et al.*, 1993; Francis & Shotton, 1997).

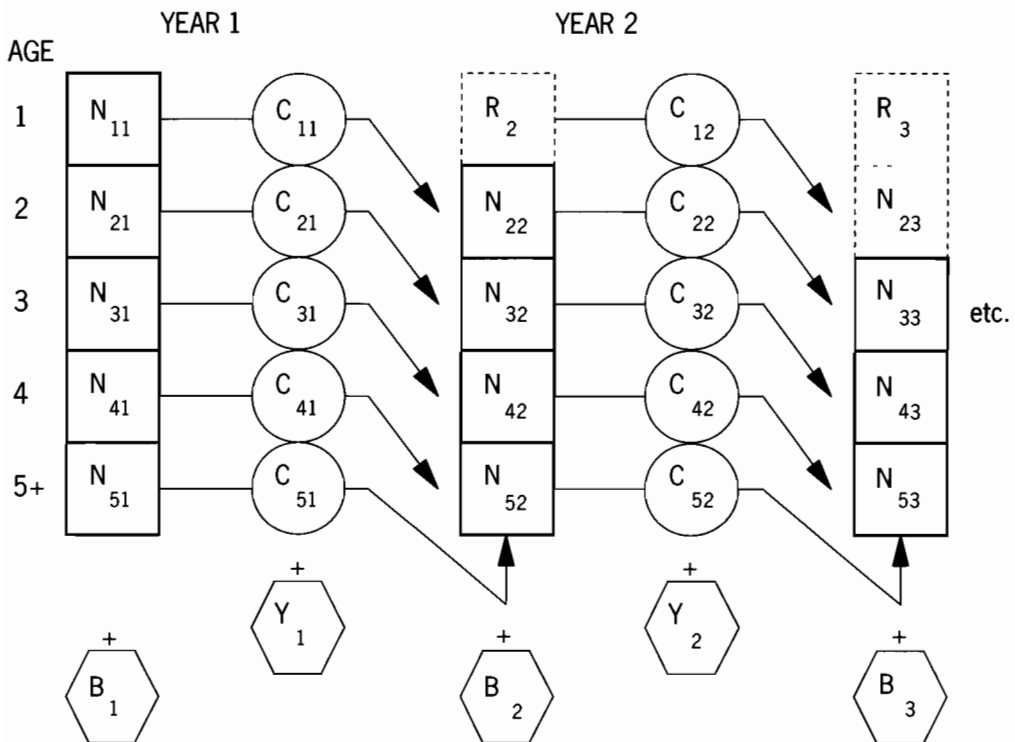


Figure V.C.2 - Outline of an age-structured simulation.

N: population number at start of years; recruitment R is the number at the youngest age.

As the simulation proceeds, incoming recruitments must be input, either manually or as the product of internal computations (stock-recruitment relationship).

B: stock biomass, sum of population numbers multiplied by mean weights at age.

C: catch in number calculated from supplied fishing mortalities at age consistent with the simulated management scenario.

Y: yield in weight, i.e. sum of catches in number multiplied by mean weights at age.

If we wish to put these models into use, we need to estimate their parameters. Favoured methods for estimating the parameters of analytic models belong to the class of age-structured stock analysis (ASA) techniques, in the generic sense proposed by Megrey (1989). These methods are also used to reconstruct the historical abundance and age structure of exploited fish stocks and to assess their current state. Megrey provides an excellent review of the numerous tools developed over the years, which include various forms of Virtual or Sequential Population Analysis (VPA or SPA) and of statistical catch-at-age analysis. In essence, all these techniques attempt to estimate population numbers at age and fishing mortalities, also by age, over the years, given the number of fish from each cohort that have been caught in successive years. Of particular interest is the estimation of numbers at age in the most recent year, since this is the initial structure from which simulations are started to forecast the development of the fishery in the short term, which is the main concern of managers and industry in TAC-based management systems. However, this is precisely where most techniques are in trouble: they provide reliable estimates for past states (provided catch-at-age data are accurate throughout the time series), but estimates of stock numbers and fishing mortalities at age are uncertain for the recent past, unless additional information can be used to redress this deficiency. In fact, most of the research deployed during the past decade around this methodology has focused on implementing statistically appropriate methods to incorporate additional data in order to “tune” or “calibrate” the estimation procedure, so that the indeterminacy in the system (i.e. more unknowns than there are equations for) can be reduced. In most instances, the ancillary information consists of time-series of abundance indices, preferably by age, obtained during scientific surveys or derived from catches per unit effort of specific fishing fleets. Age-disaggregated indices are all the more useful in this context, as they provide information for individual cells in the population-at-age matrix, but this implies yet more sampling and age readings. Another drawback of most ASA techniques, with implications in the context of age determination, is that they require continuity in the provision of catch-at-age data; any disruption in the time series creates almost insurmountable problems, unless very strong (but hard-to-validate) assumptions are made.

1.2. Single species, multiple fleets

It is seldom the case that a given stock is fished by a single, homogeneous type of vessel as is assumed in the basic model above. Most fisheries are operated by several types of vessel using a variety of mobile or set gears with different selection properties (mesh or hook sizes)

throughout the year or during particular seasons, and targeting different components of the stock (e.g. juveniles v. adults). These fleets may also be subject to different sets of regulations. Many fisheries conflicts arise because some fleets target young fish at the expense of others that depend entirely on the availability of older fish, due to their particular gears or fishing grounds (sequential fisheries). The manner in which fleets aiming at juvenile or mature fish, respectively, are balanced by management may have profound implications for the dynamics of the stock and for the sustainability of the whole fishery.

Dealing with distinct exploitation patterns in different segments of the fishery is something that biomass dynamic models are essentially unable to do, for which reason analytic models are needed. To be fair, length-based models are more suitable than age-structured models for dealing with changes in gear selectivity, which is basically a length-dependent process. However, they pose some problems in terms of spelling out the interim effects of such changes, which are what managers and fishermen are mainly concerned with. Multiple-fleet simulations are not particularly complex to set up if the initial population structure and the partial fishing mortalities at age generated by each fleet are available. These fishing mortalities can be adjusted to reflect changes in selectivity and/or multiplied by appropriate factors to reflect changes in nominal effort in the different fleets. It should be noted that for fisheries in which discarding takes place, the same procedure is used to treat landings and discards separately.

To the extent that time-series of catches at age are available for all fleets, estimating the population structure in any year is performed using exactly the same ASA methodology as in the simple model. This also provides the total fishing mortalities at age. If it is assumed that any age group is equally available to all fleets, then the partial fishing mortalities are easily derived on the basis of the ratio of the catch of each fleet to the total catch at each age in each year. In principle, there is no extra demand on sampling for ages.

1.3. Multiple species, technical interactions

An obvious weakness of the still prevailing single-species approaches to assessment and management, and one reason why they have been criticised, is that they fail to account for the fact that no species in any part of the sea can be regarded as being independent or isolated from others. Therefore, any management decision that is guided by one species is bound to have an impact on associated species to some extent, and may not be appropriate for these or for the aggregate. We will adopt the usual distinction between alternative multispecies

approaches, which put the emphasis either on biological interactions or on technical (or technological) interactions, bearing in mind that both kinds occur simultaneously in many fisheries. Because the latter are based on rather straightforward extensions of the above model, they will be dealt with first.

Technical interactions arise whenever the distributions of different species overlap, so that any fishing operation aimed at some desired species also results, intentionally or not, in catches of sympatric species. In other words, each amount of fishing effort deployed in an area generates fishing mortality on target species A, but inevitably also on the incidental (or by-catch) species B, C, etc. Likewise, the distribution of fishing mortalities across ages may be quite different for the various species concerned: the fishery may be taking adults of A together with juveniles of B, or *vice versa*. Thus, a fishing strategy or a management measure appropriate for species A can have unwanted side effects on the dynamics of the associated species, particularly when the latter are less productive. If we wish to evaluate whether some strategy or regulation is consistent with management objectives for the whole fishery, that is for all species, it is important that the methods employed should enable us to model the dynamics of all relevant species, and preferably with age-specific parameters for the same reasons as in the single-species case. Most applications that address technical interactions use so-called mixed-fisheries models (Mahon, 1985), in which the fishing fleets are subdivided into groups or "métiers" (Laurec *et al.*, 1991) that have consistent strategies towards particular subsets of the species assemblage.

Conceptually, age-based mixed-fisheries models are relatively easy to formulate and implement (CEC, 1987). Basically, they involve simulating the dynamics of all species simultaneously, the connections among species being handled through the fishing mortality parameters: a change in effort of each fleet is assumed to result in the same proportional change in fishing mortality on all species and ages caught by that fleet. For each species, the initial population at age and the reference exploitation patterns by fleet are estimated by separate single-species VPAs, i.e. using the same procedure as for the simpler models. There may be an additional demand for sampling and age data for some species that are not part of the regular sampling schemes because their low importance on the national or regional scale does not justify the cost, but that contribute a significant proportion of the income of some local fleets that would be affected by the proposed regulation. If the age data are not available or are too expensive to obtain, approaches that treat some species with age-structured models and others with biomass dynamic models are a possibility (Laurec *et al.*, 1991).

The apparent simplicity of technical interaction models hides at least two difficult issues. One is related to discarding. In many instances, the most critical interactions among species (or their ages) due to fishing take place at life stages that are eventually discarded by some fleets. If the fishing mortality parameters are estimated on the basis of landing data alone, the perception of the system and the assessment of the need for or effects of changing regulations can be seriously biased. Thus, including discards in such assessments is essential, and since fishermen are usually reluctant to report discards, this often entails major increases in the costs of data collection. However, compared to landings alone, there should be a marginal rise in the duty of age estimation (addition of all the sizes that are never landed). The other issue concerns predicting the response of fishermen to changes in abundance or regulations. Most applications assume that each vessel will maintain its current strategy, i.e. that it has a fixed matrix of catchability coefficients across species and ages. In reality, we can expect fishermen to adjust their fishing practices when the relative abundance or market values of species change, or when new regulations alter operating costs. However, understanding the behavioural, economic or social determinants of fishermen's choices in order to model and predict them in assessments is a challenging endeavour (Allen & McGlade, 1986).

1.4. Multiple species, biological interactions

There are several ways in which species interact naturally within ecosystems. Predator-prey interactions are perhaps the most obvious, being a major element in the natural mortality of the prey species. Also, the abundance and quality of prey influence predator growth. In addition, there may be competition for food or habitats.

It is clear that considerations of size and age structures do matter in predator-prey interactions. Whether they are predators or prey, not all individuals in a species' population are equal. Predation mortality occurs mostly on young stages, and the threat decreases as size and age increase. Fish of the same cohort can be prey when young and turn predator when older (e.g. in cases of cannibalism). Predators often switch from one set of prey to another as they grow. The quantitative effects of predation depend on the abundance of predators, and vary as weak or strong year-classes of predator species pass through the system. This is why the models mentioned below have been constructed from the onset in age-structured form. Thus, the term "predator" used throughout this section actually refers to a given age of a given predator species, and likewise for "prey". To the extent that most fishery applications only consider predator and prey species of commercial importance, for which age data are collected for single-species assessments in any case, there is not necessarily an extra cost associated with

the age-structured form. The “very expensive price tag” of biological interactions models (Hilborn & Walters, 1992) has mostly to do with the collection and analysis of stomach data.

As mentioned above, depending on the values used for natural mortality in assessments, the perception of historical states and, more critically, the implications of management options can be vastly different with regard to their medium- and long-term effects on various species. This is why the impact of predation, including cannibalism, upon natural mortality has received most attention in the fisheries context, and particularly within the International Council for the Exploration of the Sea (ICES) community (Daan & Sissenwine, 1991). In some instances, however, the effects of variations in the abundance of prey on the growth and reproduction of predators are also considered (e.g. Stefansson & Palsson, 1998).

The technicalities of biological interaction models are too complex to be explained here. Interested readers may refer to the overviews by Magnusson (1995) or Sparre (1991). Very briefly, estimating the fraction of natural mortality rate M at each age due to those predators included in the analysis involves suitability coefficients that are “measures of what the predator likes to eat as well as what it is able to eat” (Magnusson, 1995). In the ICES area, the method most often employed to estimate these parameters has been the Multispecies Virtual Population Analysis (MSVPA). Basically, MSVPA aims at estimating the size of each cohort through time, a process that must take into account not only what has been caught, but also what has been eaten, as reflected in the stomach contents of predators. Since, in any given year, the predation mortality on a cohort of a prey depends on the abundance of all cohorts of predators and prey, the equations must be solved simultaneously for all cohorts of all species. The main point here is that the relative species and age compositions of prey, appropriately weighted by the suitabilities, must match the composition observed in the stomachs of predators sampled. Since these suitabilities include a component of spatial overlap of the distributions of predators and prey, which depends on seasons, computations are actually made on a quarterly basis. This implies collecting stomachs for all species and ages (or size classes) of all predators, by quarter, over the entire area, and analysing them to derive the species and age (or size) composition of their prey. In the 1981 ICES project, over 54,000 stomachs were collected (Daan, 1987) and, even if these were pooled prior to analysis, the task of analysis was obviously formidable. This is why only two such large-scale sampling schemes have been feasible for the North Sea at an interval of ten years. Large numbers of otoliths (by species,

area and quarter) also had to be processed (note, however, that many of these were also required to derive the survey indices of abundance by age, which are a routine product).

1.5. Spatial models

All the models discussed so far assume that fish and/or fishing effort are randomly distributed over the fishery area, but there is a growing awareness that explicit spatial structures, for both fish and fishing activities, should be built into fisheries models. This is particularly an issue as there is a strong (although uncritical at times) movement to revive protected areas as a “precautionary” management tool (e.g. Guénette *et al.*, 1998 for a review). The traditional, “single-bucket” models are simply not adequate to evaluate properly the effects of such measures.

Although a variety of spatial models has been proposed over the years, many of them depend on the availability of particular data (from tagging, surveys, logbooks) and a general methodology has still not been adopted. There is little doubt, however, that the relevance of spatial models for management purposes is enhanced when they take age structures into account. In many stocks, the various age groups have clearly distinct spatial distributions (e.g. nursery grounds v. spawning grounds), and their patterns of movements also differ markedly (e.g. ontogenetic or spawning migrations). The corresponding parameters in models are thus likely to be age-dependent. The design of a protected area also needs to consider such questions as which species and ages are concerned (location, season, size of the reserve), how long they stay in the protected area (its size is again relevant), how fast they grow or mature while there, etc. Age structures are therefore important to take into consideration.

2. From age estimation to age-based models

2.1. Estimating the age composition of catches

Whatever kind of model is being considered, its application to real problems requires that its parameters be estimated from data. As pointed out above, the parameters of most age-based models are estimated by means of some variant of age-structured stock analysis techniques. The typical input required by these techniques is a matrix of the total catches taken at each age in each year by the fishery. Most variants in current use also try to take advantage of auxiliary information such as abundance indices at age obtained during research cruises or derived from catch rates at age from selected fleets. These data are just special cases of catches at age.

Two general strategies are utilised to estimate the age composition of catches. One is to sample fish randomly at landing sites, estimate the age of all sampled fish, and apply the proportions of the various ages observed in the sample directly to the total number caught (simple random sampling). The alternative is double sampling: in the first stage, fish are randomly sampled to record their length, while in the second, a sub-sample of the fish measured is taken and the age of those fish is determined. Sub-samples for age are usually stratified by size classes (a fixed or proportional number is taken at each size) and this leads to the age-length key (ALK; but in a different sense from Hilborn & Walters (1992) who call it the 'length-age key'). The ALK is a table, usually with ages in columns and lengths in rows, which gives the probability of a fish of being of age i given that it is of length j (fig. V.C.3). Once they have been turned into proportions, the cells in the ALK must sum to unity for each length class (across each row). When the vector of numbers caught at length is multiplied by the ALK matrix, a vector of numbers caught at age is obtained. Either the length composition (prior to applying the ALK) or the age composition (after ALK) can be raised to the overall catch. Variance formulae for the alternative strategies, together with considerations regarding the effects of sample size on precision and about the treatment of aging errors in stock assessments, are given in chapter VIII of Quinn & Deriso (1999). On purely statistical grounds (variance), the merits of simple v. double sampling may be equivocal (Kimura, 1977). However, in terms of efficiency, when costs are taken into account, the arguments generally turn in favour of double sampling because, as a rule, processing the samples for age estimation is far more expensive than sampling for length compositions (e.g. Lai, 1993). This is why the latter strategy and the ALK route are most frequently used.

There is a recurrent issue about the ALK that needs to be well understood. Whereas the distribution of lengths within ages is essentially determined by the growth pattern, which may not change significantly from year to year, the distribution of ages at a given length (the ALK) is also largely influenced by year-class strengths. Therefore, the ALK established for one year should not be applied to the length composition for another year. As illustrated in the case study below, it can be easily shown that this leads to serious misallocation of ages, and thus to errors in assessments. More precisely, as has been forcefully stated by Kimura (1977) and other experts, the samples used to build an ALK must be drawn from the same population as the one to which it is applied. This is why some research institutes rightly implement separate sampling schemes for age (and length) for different areas and

seasons for any given stock in a given year, on the grounds that the structure of the underlying populations do differ. At the very least, ALKs should be built anew each year. Note, however, that this condition would also apply to the alternative simple random sampling strategy.

2.2. Case study: age-length keys in practice

The main purpose of this example is to demonstrate, on the basis of real data (Celtic Sea whiting, *Merlangius merlangus*, data kindly provided by R. Bellail, Ifremer, Lorient, France), the consequences of applying an inappropriate age-length key to length compositions.

Table V.C.1 shows the age-length key (ALK in number of fish aged at each length) and, in the second column, the length composition of the catches of a particular fleet segment for 1992. The age composition of these catches obtained by applying the ALK, using the procedure explained in figure V.C.3, is shown in the bottom row. This example shows data pooled over the year whereas, in reality, the treatment is made on quarterly ALKs and length data, but this is immaterial for the purpose of this illustration. Using annual data may result in misallocation to ages for those age groups (typically the younger ones) that grow fast within the year, and some of this effect is apparent here: if caught early in the year, fish in the range 30–36 cm would all have been of ages 2 or 3, but rapidly growing or early-born age 1 fish also reach those sizes late in the year, and their presence is reflected in the age samples collected in the last quarter. When samples are simply pooled (without being weighted by the numbers caught in each season) to set up an annual ALK, the proportions of ages within length classes observed by the end of the year influence

Age					Age				
Length	1	2	3	etc.	Length	1	2	3	etc.
10	6				10	100			
11	8	2			11	80	20		
12	7	7			12	50	50		
13	3	9			13	25	75		
14		6	2		14		75	25	
15		1	9		15		10	90	
etc.					etc.				

Figure V.C.3 - Format of an age-length key (abbreviated).

Left: tally of numbers of otoliths or scales assigned to ages in processed samples;

Right: data transformed into proportions (percentages) within length classes.

For example, the catch in number at age 1 is computed as the sum of all fish caught at length 10, plus 80% of those at length 11, 50% of those at length 12 and 25% of those at length 13.

the allocation for the whole year, resulting in a degree of bias in the estimated age composition. The bias can be large when, as is the case here, the incoming year class is strong. The spread of lengths at each age and the overlap between adjacent ages are also amplified when annual data are used.

These problems can largely be avoided by employing the whole procedure on a seasonal basis (e.g. using quarterly length compositions and ALKs), which is a reason why this is usually done despite the additional cost. Another drawback of using seasonal ALKs is that it may be more difficult to obtain sufficient age samples for all sizes. Table V.C.1 indicates that large and old fish have been rather difficult to encounter, even for the whole year, although these fish are those for which the variance is largest, a factor that calls for larger samples in order to improve precision. However, it should be noted that in such a heavily fished stock, old fish make a very small contribution to the catch and to the stock, so that knowing their size precisely is not the main concern. Finally, it should be noted that about 1,700 fish were aged. The proportional sub-sampling scheme used may have resulted in some sizes and ages being much more sampled than others, but this scheme has better overall statistical properties than a fixed allocation scheme. This number is quite considerable, bearing in mind that it was for only one of several stocks that have to be monitored routinely by the scientific team involved.

Table V.C.2 shows the same items based on sampling in 1993, to which the considerations discussed above also apply. Comparing this with the previous table, it is interesting to see how the pattern between ages 1 and 2 in the ALK has dramatically changed as the strong 1991 year class proceeded from age 1 to age 2. The probability of drawing an age 1 fish in the "normal" length range for this age was much lower than in 1992, and this also applies to individual lengths. Within length class 30, for example, the proportions of ages 1 and 2 were about 6% and 80% respectively in 1993, compared to 18% and 53% in 1992. This illustrates the point that the structure of the ALK is not only influenced by the growth pattern, but also by the relative abundance of the various ages in the population from which it is sampled. Therefore, it is bound to change from year to year as strong or poor year classes pass through the population, or from one area to another if the age groups have different spatial distributions.

Table V.C.1 - Length composition, age-length key and resulting age composition for 1992.

Length composition		Age-length key							
L (cm)	Number caught '000	Age 1	2	3	4	5	6	7+	Total aged
24	16.4	5	0	0	0	0	0	0	5
25	75.7	7	1	0	0	0	0	0	8
26	296.0	13	6	0	0	0	0	0	19
27	622.5	22	15	1	0	0	0	0	38
28	973.4	19	29	5	0	0	0	0	53
29	1227.5	14	45	7	0	0	0	0	66
30	1419.5	14	42	22	1	0	0	0	79
31	1356.8	11	47	12	4	0	0	0	74
32	1298.1	8	56	13	0	1	0	0	78
33	1241.2	6	52	20	2	3	0	0	83
34	905.1	2	45	27	3	5	2	0	84
35	717.6	2	43	41	5	8	2	0	101
36	525.6	1	47	28	5	7	2	1	91
37	423.1	0	31	41	6	5	0	0	83
38	322.0	0	30	30	8	5	1	0	74
39	215.9	0	20	26	3	8	2	0	59
40	164.6	0	18	25	9	5	6	0	63
41	134.3	0	12	33	7	13	2	0	67
42	122.7	0	14	38	14	10	3	1	80
43	94.5	0	15	37	8	7	3	1	71
44	69.1	0	8	27	9	13	8	1	66
45	56.8	0	7	24	11	11	2	0	55
46	40.2	0	2	23	9	14	3	0	51
47	32.8	0	0	14	8	23	4	1	50
48	24.5	0	0	11	7	9	8	2	37
49	19.9	0	1	11	12	9	1	1	35
50	14.6	0	0	7	4	12	4	0	27
51	9.0	0	0	4	2	5	7	0	18
52	9.7	0	0	5	0	13	4	0	22
53	9.7	0	0	2	3	10	2	0	17
54	11.4	0	0	2	3	11	3	1	20
55	6.1	0	0	1	1	7	4	1	14
56	4.1	0	0	0	0	4	6	0	10
57	5.4	0	0	1	1	3	3	1	9
58	2.6	0	0	0	2	1	2	2	7
59	3.2	0	0	0	0	4	2	1	7
60	2.2	0	0	0	0	3	2	1	6
61	1.3	0	0	0	0	0	4	0	4
62	1.8	0	0	0	0	2	1	1	4
63	0.0	0	0	0	0	0	0	1	1
64+	0.4	0	0	0	0	1	1	0	2
Total	12477.4								
		Age composition							
		1972.6	6598.0	2892.0	416.9	452.8	129.3	15.9	

Table V.C.2 - Length composition, age-length key and resulting age composition for 1993.

Length composition		Age-length key							
L (cm)	Number caught '000	Age	1	2	3	4	5	6	7+ Total aged
24	4.0		0	1	0	0	0	0	1
25	17.0		1	2	0	0	0	0	3
26	75.1		0	7	0	0	0	0	7
27	178.6		2	12	1	0	0	0	15
28	379.6		4	22	0	0	0	0	26
29	751.7		8	42	4	0	0	0	54
30	847.3		3	43	8	0	0	0	54
31	995.5		4	42	15	0	0	0	61
32	970.6		1	33	31	1	0	0	66
33	1089.4		2	47	28	2	0	0	79
34	886.2		0	40	33	2	0	0	75
35	788.0		0	41	50	4	0	1	96
36	622.9		0	44	49	6	0	0	99
37	498.8		0	39	55	17	2	0	114
38	348.3		0	32	58	14	1	1	106
39	271.1		0	24	58	9	3	0	94
40	223.6		0	15	60	12	5	0	92
41	163.5		0	11	51	14	1	2	80
42	107.1		0	6	43	12	1	0	63
43	94.4		0	3	44	10	4	1	63
44	86.9		0	2	37	10	3	1	54
45	78.7		0	1	43	13	1	2	60
46	56.8		0	0	19	14	5	4	44
47	39.6		0	0	18	15	1	1	37
48	36.1		0	0	9	12	1	7	29
49	28.5		0	0	9	13	0	3	27
50	32.9		0	0	7	9	1	8	30
51	19.0		0	0	2	8	2	4	21
52	23.0		0	0	3	10	3	5	23
53	15.8		0	0	3	4	2	4	14
54	20.6		0	0	2	4	3	7	19
55	16.4		0	0	1	3	2	5	13
56	14.4		0	0	0	1	5	5	13
57	9.5		0	0	0	2	2	5	10
58	8.0		0	0	0	2	1	2	8
59	9.1		0	0	0	0	2	6	10
60	6.6		0	0	0	0	2	2	9
61	4.8		0	0	0	0	1	0	6
62	4.8		0	0	0	0	0	0	6
63	0.6		0	0	0	0	0	0	1
64+	0.6		0	0	0	0	0	0	1
Total	9825.2								1683
		Age composition							
		353.9	5142.0	3575.8	516.6	83.9	94.4	58.5	

Table V.C.3 first summarizes the age compositions obtained when the length compositions are processed correctly, with the ALK sampled in the same year. Although the examination of catches alone is not sufficient to judge the true abundance (it would need to be estimated by VPA based on total international catches through time), and bearing in mind that age 1 had not fully recruited to the fishery, there are indications that the 1991 year class was relatively strong in both years. The position is similar for the 1987 year class (aged 5 in 1992 and 6 in 1993) which makes a larger contribution than the adjacent ones.

Table V.C.3 - Age compositions for 1992 and 1993 derived using ALKs sampled in these years.

Age	Length 92 ALK 92	Length 93 ALK 93	Length 93 ALK 92
1	1972.6	353.9	989.2
2	6598.0	5142.0	4985.5
3	2892.0	3575.8	2665.0
4	416.9	516.6	439.5
5	452.8	83.9	543.7
6	129.3	94.4	178.2
7+	15.9	58.5	24.1

Final column: age composition estimated for 1993 based on the ALK set-up with 1992 samples.

The pattern is consistent and recent assessments confirm that these were among the good year classes in the decade. But, if we make the mistake of applying the 1992 ALK to the 1993 length composition (last column), this consistency breaks down. First, the contribution of the 1992 year class to the catch at age 1 is inflated by a factor of about three, at the expense of an underestimation of the catches made at ages 2 and 3. There also seems to be a large contribution of age 5, i.e. the 1988 year class rather than the strong 1987 year class, which is at odds with available evidence. In fact, this year class was the poorest in the time series. If such erroneous catch compositions had been input into any kind of catch-at-age analysis, they would have seriously distorted the estimations of stock abundance and fishing mortality for all cohorts. The particular mistake shown in this example (large catches spread over adjacent ages) would likely result in underestimation of fishing mortality for the strong cohort, but no general rule is applicable: the effects of aging errors upon assessment results would depend on the proximity and size of the age groups that are affected by the misallocation. Aging errors are one source of uncertainty and should be handled together with other sources, using appropriate methodology, to evaluate the overall quality of assessments.

3. Summary and critique

If all individuals in a stock were equivalent in terms of vital rates (growth, fecundity, natural and fishing mortality) or even abundance, there would be no need to suffer the pain of using relatively complex age-based models with the associated costs of data acquisition. Unfortunately, this is not the case, and fisheries biologists are often compelled to consider age structures in assessments. Other views have been expressed, however. In contexts where management advice is the only perspective of the assessments, models that depict reality accurately may eventually be less useful than models that support sound fisheries management over the years. In particular, the latter should be robust to uncertainties of all sorts and it is a well-known rule that the more detailed a model is, the more parameters it has, and the more it is sensitive to uncertainties. In some instances, simpler surplus-production models have proven to perform better than age-structured models, when age data are imprecise. However, this superiority has usually been established in contexts in which management is by TACs that are defined simply in terms of wholesale weight of fish. The conclusion may have to be revised when other management measures (mesh regulations, closed areas, etc.) are considered, or when the fisheries are highly mixed. In the light of current public concern about ecosystems and biodiversity, we may expect that advice based on assessments in which species are lumped together will increasingly be regarded as unacceptable if this implies ignoring the fate of some fragile species or stock components. A drawback of simple models is also that they cannot make use of extra information or knowledge which may be available.

Rather than opposing models that take full account of age structures to those that ignore them altogether, it may be more constructive to consider the potential of models of intermediate complexity (e.g. Conser, 1994; Jacobson *et al.*, 1994). Basically, these consider age structure only for those ages that exhibit high dynamics in their parameters, and lump all others into one compartment. Generally speaking, the former are the younger ages and the operational advantage is that they are also those for which age estimation is easier and cheaper. An extreme option, which may be justified when biomass production changes predominantly due to variations in recruitment, is to explicitly model only two stages in the population, the recruits and the recruited ages. These are fairly easy to identify, even from length compositions, particularly when the latter are recorded on a seasonal basis. More age groups in the juvenile phase can be differentiated if this is deemed to

be important to the problem at hand. In any case, having a superb model does not really help to improve management when enforcement is deficient.

Finally, the major problem with age-structured models used for stock assessments is not their complexity *per se*, which is modest compared to that of models used in other disciplines. The main obstacle is the cost of collecting and processing the data needed to estimate their parameters, compounded by the need to operate on a routine basis, as is required by managers. The treatment of material for age estimation often makes up a major part of that cost, given the amount of data that have to be handled, the skill and technology engaged, and the quality standards to be met. In addition, as emphasised in section 2, the whole task has to be redone from scratch every year. Given that relatively large sub-samples need to be taken for age estimation in order to achieve acceptable precision, and that many species and stocks have to be dealt with routinely, the amount of material (scales, otoliths, etc.) that have to be processed each year may be quite large. This calls for very efficient handling procedures that should be designed in the form of a production line (Morison *et al.*, 1998). Of course, quality should also be a major concern, requiring rigorously validated procedures for age estimation (chap. IV). Furthermore, in the context of assessments which imply the maintenance of time series, consistency through time is a critical element of quality and should be closely watched.

Such requirements mean that there is clearly a high price to be paid if we wish to reap the benefits of age-structured models, even in their simpler forms. The mere ability to continue using such models, even in their basic form for TAC advice, depends critically on the development of more efficient and automated procedures designed to contain costs while enhancing the precision and consistency of age estimations. This is all the more necessary if clients of advice continue to ask for more details, more species (whether commercial or not), more fleets, more areas, etc., to be included in assessments, while staff and budgets allocated to scientific institutes dwindle.

Chapter VI

Computer-assisted age estimation

H.Troadec, A. Benzinou

Age estimation using calcified structures is based on a repetitive process of interpretation of growth zones that associates visual perception mechanisms and biological knowledge. Faced with a multiplicity of sources of bias and the subjectivity of interpretation criteria, sclerochronologists have sought to introduce a certain objectivity into the basic data acquisition process, which is primarily visual, and in the logic of their interpretation. The appearance in the sixties of computers coupled to micro-densitometric systems inaugurated a relatively sparse series of studies that explored various methods of objectivation. The first aim of this chapter is to present the various ways of assisting this process of interpretation using a computer.

It is essential to be aware from the very beginning that the human "machine" excels in pattern recognition and that the results of attempts to automate the pattern recognition process very often fell well below the hopes raised by pattern recognition techniques. Their application to the calcified structure process did not deviate from this tendency and the first attempts at automation soon ran up against the complexity of the problem. Computers actually find it difficult to do what a young child is capable of doing. Most Computer-assisted age and growth estimation (CAAGE) systems share the common feature of being primarily based on image-processing tools that are based on mathematical concepts of signal theory. The second objective of this chapter is to offer the reader a better comprehension of these concepts and especially to analyse the effects of their application to biological material.

The wide range of CAAGE equipment currently in use in specialised laboratories is the reflection at the same time:

- of the complexity of calcified structures (perception of continuity, modulation of the signal, image quality, etc.);
- the diversity of the situations involved (macrostructures, microstructures, routine work, species, etc.);
- the multiplicity of the objectives (visual comfort, automation or assistance in counting, measurement capture or interpretation);
- the technical solutions available (image analysis, artificial intelligence, neural networks, 2D or 3D shapes, spectral analysis).

Our third objective is thus to help the reader to make the choice of a tool suitable for his needs.

A. What is a computer-assisted age and growth estimation (CAAGE) system?

An ideal CAAGE system would consist of a computer providing software and hardware capable of ensuring (1) assistance in the quantification of CS images, (2) assistance in the data interpretation process and (3) efficient management of data storage and exchange.

Among the CAAGE systems currently available, however, we primarily find systems based on an image analysis software core, which thus mainly offers help to the data quantification stage. Some of these systems provide specialised functions (back-calculation, curved profiles, memorising of the growth zone locations, introduction of biological constraints, etc.) but practically none of them offer any real assistance to interpretation, although occasional attempts to do so have been made. For this reason, we will concentrate here on visual data processing. A CAAGE system consists essentially of three units (fig. VI.1):

- a digital image source (camera, scanner, digitising tablet, SEM, microprobe, etc.);
- a central processing unit for image display and storage;
- a software unit that characterises the level of assistance offered by the CAAGE system by providing specialised functionality for the identification of basic features, morphometry and possibly, interpretation.

1. Visual data processing

The rapid evolution of numerical technologies makes it extremely difficult to describe the composition of an image analysis system in detail. Only a few years ago we could have praised the performance offered by specialised image-processing boards that offered frame-memory and real-time functions (array processor, morphological processors, etc.) but the increase in clock frequencies and storage capacities of microcomputers has made these almost obsolete today. For this reason, we focus our presentation on what we regard as the fundamental components of an image analysis system.

1.1. The digital image source

1.1.1. Digital image formation: 2D sampling

Starting from a simple representation of visible optical phenomena, the image gradually turned into the visualisation of radiation from the invisible part of the spectrum (infra-red, UV) and then of all kinds of measured physical quantities (radar, scanning electron microscopy, tomography, microchemical analysis, etc.). The term “digital” refers

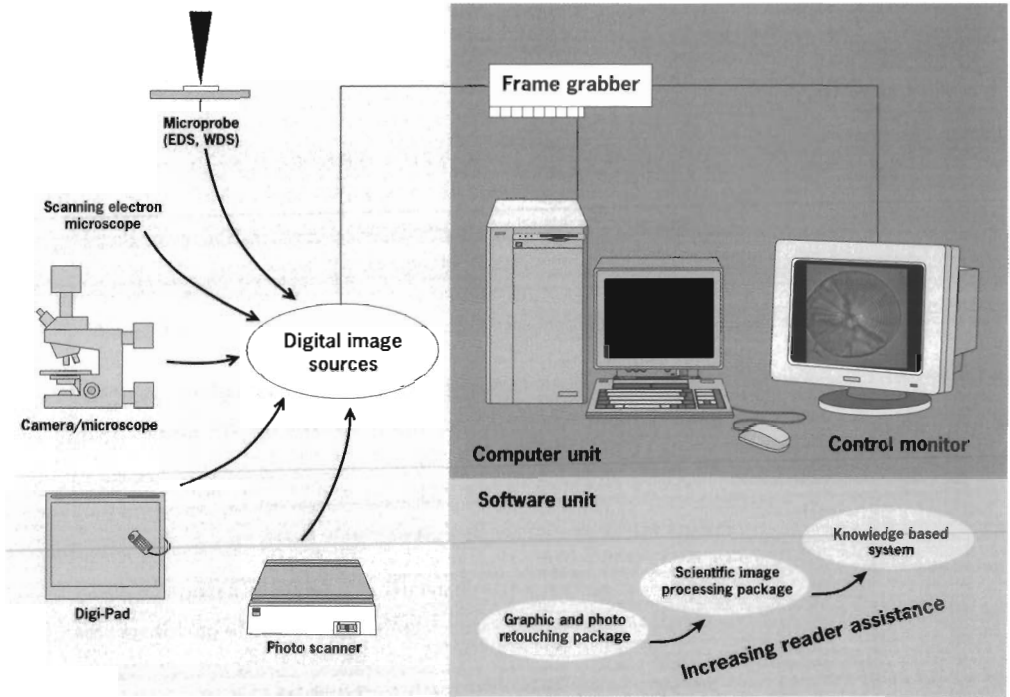


Figure VI.1 - Composition of a typical CAAGE (Computer-assisted age and growth estimation) system. The digital image sources range from a classical image camera to a chemical image produced by microprobes (e.g. in Casselman, 1983). The steady improvements in computer power and storage capacity enables us to handle larger images and utilise more sophisticated algorithms. The type of software unit will determine the degree of assistance provided to the reader by systems ranging from a simple interactive ring location to a fully automated interpretation process.

to the discrete nature of the information that constitutes the image as opposed to the continuous nature of analog signals. The real world being three-dimensional and dynamic, the image formed will obviously be no more than an instantaneous view of a projection of the scene as registered on the surface of the sensor.

The sensor determines the nature of the information represented by the image. For light information, the sensor is a device that is sensitive to light energy and that transforms the optical image into an electronic signal. The image sensors may be an analog video camera tube, but most modern cameras are based on solid-state semiconductor arrays known as "Charge-coupled devices" (CCDs). CCD sensor elements are photodiodes of a few microns in diameter that react to photonic excitation by producing electron charges that are collected throughout the array and converted to an output voltage. This collection process produces, an analog video signal for each row of CCD elements, implying that (1) the signal still needs to be sampled even when using a digital sensor, and (2) the horizontal resolution of the

digital image does not depend directly upon the number of CCD columns.

The video signal sampling is performed in three stages by an analog-to-digital converter (ADC) (fig. VI.2):

- **amplification**, which modulates the strength of the analog signal in order to obtain suitable levels at the input of the analog-to-digital converter device. At this stage, most digitisers allow the gain and offset of the signal to be modulated;
- **sampling**, which transforms the continuous analog video signal, coming from the camera, into a succession of discrete values. In this stage, the sampling frequency determines the spatial resolution of the image;
- **quantification**, which rounds off the analog samples to digital values so that they can be coded into a final number of bits. This determines the brightness resolution. Coding these measurements at a resolution of 8 bytes provides a range of 256 grey levels. The fact that the

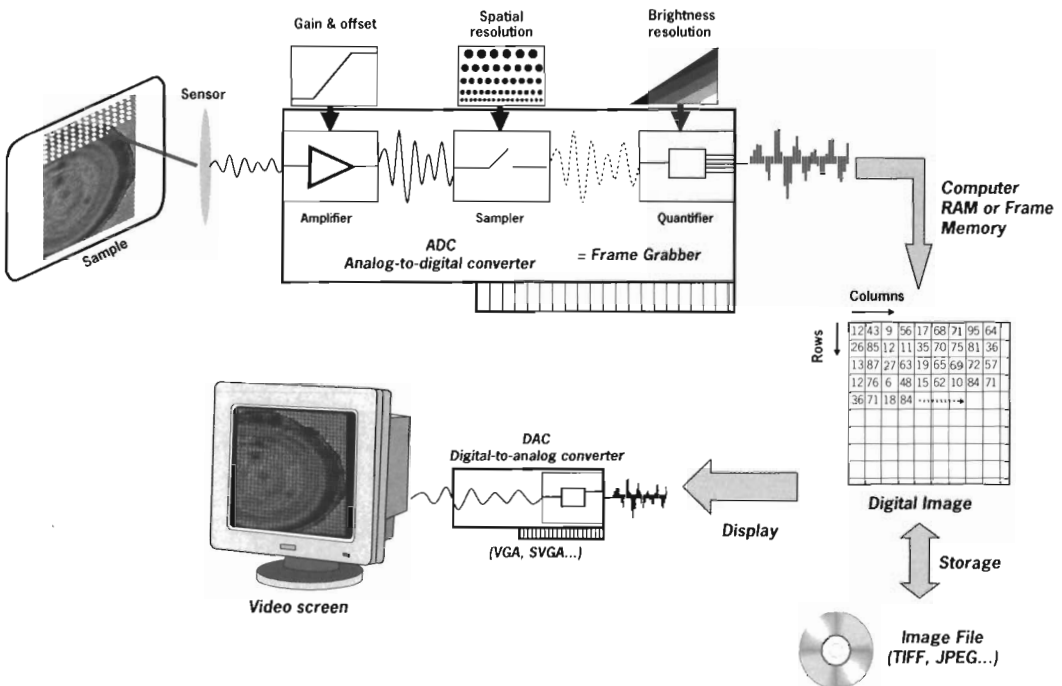


Figure VI.2 - Schematic presentation of an image digitalisation process. The electric signal generated by the sensor is amplified, sampled and quantified by the digitiser. These digitalisation steps will determine the dynamic, spatial and brightness resolution properties of the resultant digital image. The image is temporarily stored in the computer or the imaging board RAM memory, where it can be displayed on a video screen or written to hard disk or CD-ROM for long-term storage.

human eye cannot distinguish more than about sixty shades of grey underlines the importance of the image dynamic manipulation and of the contrast enhancement step. With standard sensors, the resolution may range from 512^2 pixels to 2048^2 pixels, representing a file size of between 262 Kbytes and 4.2 Mbytes. The effects of different spatial and brightness resolution are shown in figure VI.3.

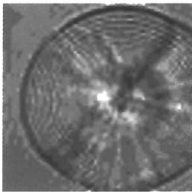
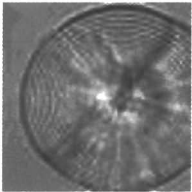
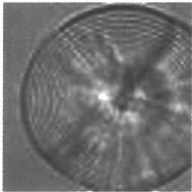
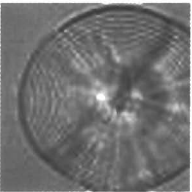
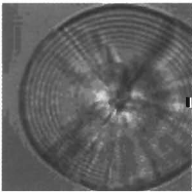
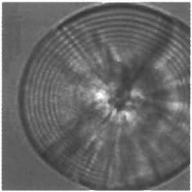
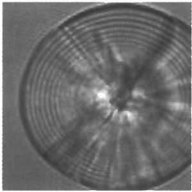
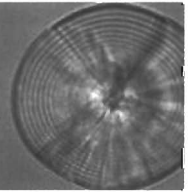
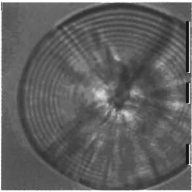
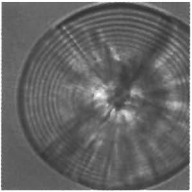
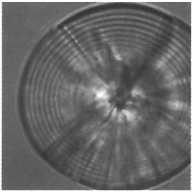
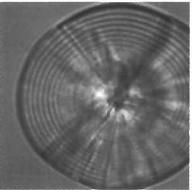
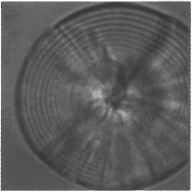
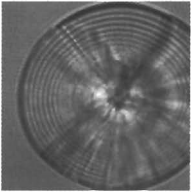
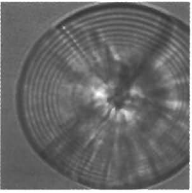
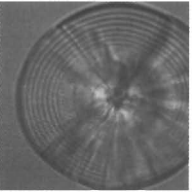
Grey levels Image size	8 (3 bits)	16 (4 bits)	32 (5 bits)	256 (8 bits)
64 x 64 pixels				
128 x 128 pixels				
256 x 256 pixels				
512 x 512 pixels				

Figure VI.3 - Effect of varying brightness and spatial resolution on digital image quality. Image resolution varies from 64^2 to 512^2 pixels and grey level depth from 3 to 8 bits.

A colour image is generated by covering a single sensor with red, green and blue filters in the case of a colour mono-CCD camera, or the three individual sensors for tri-CCD cameras.

Most current scientific digitisers provide square pixels, i.e. images with a 1 to 1 aspect ratio, but the electronics regulating the sampling frequency is liable to drift, and standard television images have an aspect ratio of 4:3. In either case, this means that the sampling within a video line will differ from the sampling between lines, resulting in a digital image with rectangular pixels. Since each is geometrically distorted ignoring this factor will bias the measurements. Most scientific image processing software enable correction factors to be introduced.

Each individual pixel of an image I can be located by its coordinates (x,y) while its grey level value is given by $I(x,y)$. The digitised image is stored in RAM and reconverted to an analog video signal by the DAC (Digital-to-analog converter) in order to be displayed on the screen (fig. VI.2).

Note that a 4:3 ratio sampling can be particularly pernicious as when displayed on your video screen, the DAC can geometrically modify your image. This can be easily checked by digitising a circular object and measuring it horizontally and vertically just by calculating the Euclidean distance from the (x,y) coordinates. If you notice a significant discrepancy, then you must take it into account in your measurements particularly when using it for shape features.

1.1.2. CS image acquisition

In accordance with the concept that "a good original image is better and avoids extensive further processing", we should try to utilise the best hardware available and to standardise and optimise the illumination conditions. CS consist of concentric layers that are more or less crystalline, oriented in various directions, and this feature a wide range of contrasts. Combined microscopy and video techniques such as polarisation microscopy, image integration and image mosaic building tools help to produce the images required.

1.1.2.1. Polarisation microscopy

The illumination system of a binocular microscope, and to a lesser extent that of a compound microscope, is subject to ambient disturbances from reflections or generated by the observed material itself (birefringence of certain crystals) or it may generate, by its very nature, a multiplicity of incidental rays (e.g. dark-field illumination). In all these cases, the use of a polarising filter enables certain light sources to be selected and, particularly in the case of transmitted dark-field illumination, also enables background noise to be drastically reduced (Macy, 1995; Welleman & Storbeck, 1995; Troadec *et al.*, 2000) (fig. VI.4).

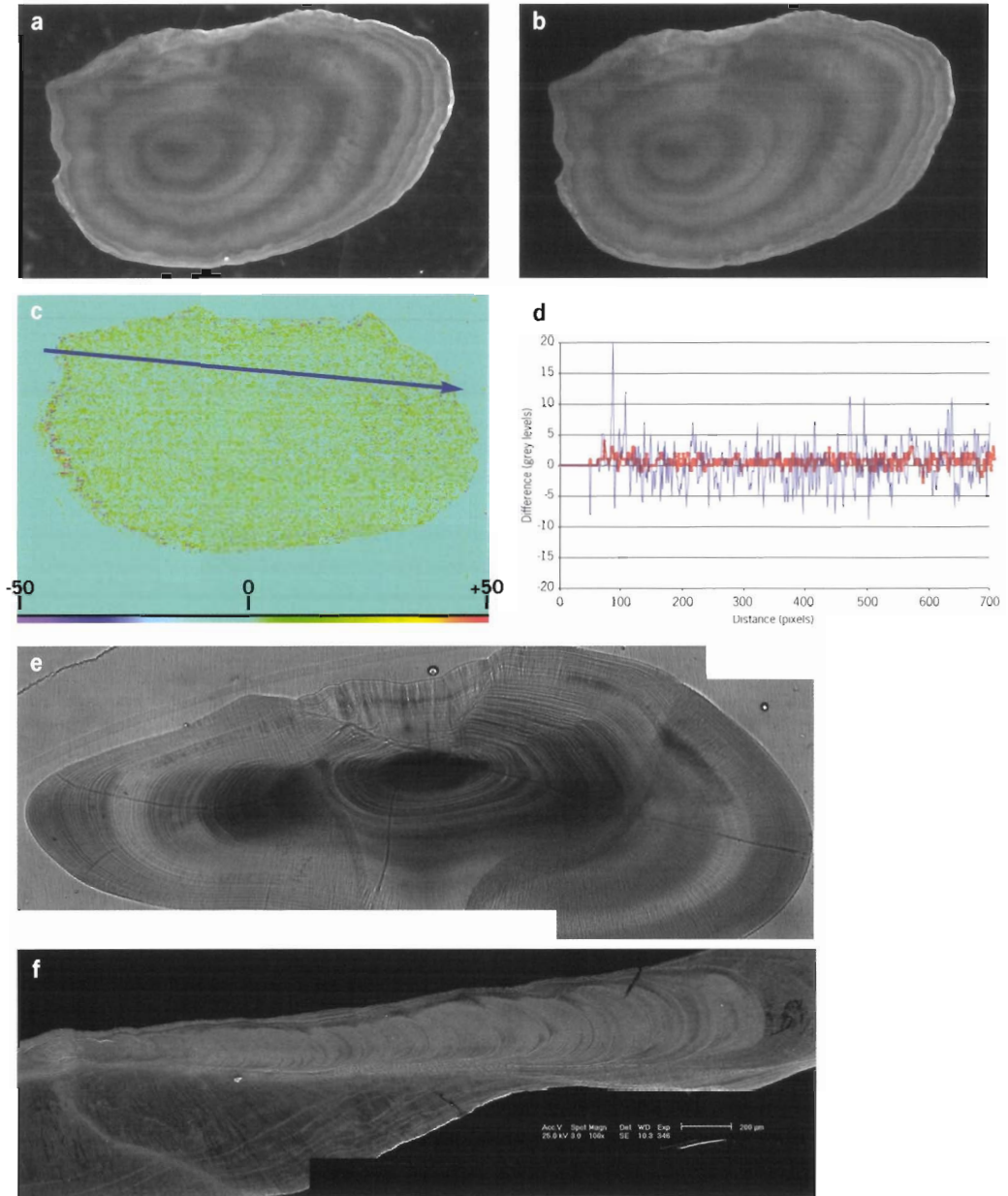


Figure VI.4 - CS digital image acquisition: plaice (*Pleuronectes platessa*) otolith image with (a) normal light and (b) polarised light. Effect of image averaging on noise distribution: (c) false colour representation of the difference between two consecutive images acquired with a standard CCIR camera. An intensity profile (d) shows that the difference in pixel value has a significant magnitude (blue line) on a single image acquisition compared with averaged images (red line, N=10). Construction of a CS image mosaic that allow the processing of the entire CS at high resolution on (e) whiting (*Merlangius merlangus*) otolith (4 images), (f) SEM image of an orange roughy (*Hoplostethus atlanticus*) otolith (3 images).

1.1.2.2. Image integration

The noise generated by the whole image acquisition process (variations in light intensity, electronic noise, etc.) can be observed as the variability of the response of a single pixel under constant illumination. Figure VI.4c presents a false colour image of the difference between two consecutive single images acquired on a standard CCIR camera. The intensity profile acquired in this image (fig. VI.4d) shows that a pixel value may vary by as much as ± 50 grey levels for the same scene. In order to reduce the effects of this scattering, several successive images can be added to eliminate this random noise. When working with still microscopic images, as in our case, frame averaging can be very efficient. It can be performed by software or even by hardware on some imaging boards (ex: Matrox Meteor, Scion AG-5, etc.). Hardware quality (camera, frame-grabber, etc.) is constantly improving the signal-to-noise ratio, but the intensity profile of a difference of averaged images (fig. VI.4d) shows that pixel value variability is significantly reduced by image averaging. Tuning the exposure time parameter that is available on some digital cameras is another possible way to integrate light information.

1.1.2.3. Image mosaics

The relationship between the growth zone dimension and that of the CS rarely allows the CS to be visualised as a whole through the microscope eyepiece. Moreover, even when the resolution allows it, centring each sample by means of the zoom is in practice very tough because of the need for a calibration value for each image. It should be remembered that certain types of sclerochronological processing, such as proportional back-calculation methods, require the whole CS. In such cases, field-by-field digitisation is often unavoidable in practice, and this later poses the problem of how to map the image set.

A satisfactory solution might consist of building up a panoramic view of the CS by means of an image mosaic. This can be done using a set of overlapping field images, either manually (Macy, 1995) or automatically, as proposed in some current software. These tools are now relatively reliable as long as the operator is careful not to make abrupt changes in focus or illumination. It is not always easy to cope with this restriction, particularly on CS that present growth zones which may not be visible in the same focal plan and with brighter marginal zones. Since such mosaic images can represent several tens of megabytes, processing them requires an adequate amount of RAM and their storage demands image formats that offer significant compression ratios (e.g. JPEG) (fig. VI.4).

1.2. Digital image processing

The image instantaneously displayed on the screen is a complete or partial representation:

- of a CS, resulting from a calcification process that has induced an alternation of proteinic and hypercalcified zones;
 - that is usually observed as a thin slice obtained by a cutting, grinding and polishing process (except the scales);
 - and that has been digitised through series of optical, electronic and digital processes which themselves generate various types of noise.
- The sources of degradation of the relevant information are numerous and the diversity of the biological material does not always allow us to extrapolate solutions that have been developed on a given species with a given process of preparation. Thus, Welleman & Storbeck (1995) consider that image enhancement is not necessary, whereas Macy (1995) uses a histogram equalisation and double iteration of a directional edge-detection filter. It is noteworthy that both use polarised light, though the former works on whole plaice (*Pleuronectes platessa*) otoliths with highly contrasted macrostructures and the latter on squid statoliths (*Loligo pealei*) with poorly contrasted microstructures that are severely disturbed by radiate crystalline material.

A digital image is not usually exploited in its rough form, but requires contrast reshaping and filtering of some components. Suitable digital filtering can greatly improve the "readability" of a CS, but any misuse such as a bad parameterisation, the ignorance of its intrinsic properties or even an abusive iteration process may have significant effects on the results, ranging from a simple growth-mark edge delocalisation to the introduction of additional marks. Much current photo-retouching software presents contrast enhancement filters that produce "good-looking" images but have a poor ability to parameterise their spatial resolution. It is important to realise that the application of filters to the image can ultimately affect quantitative measurements. Hereafter, we will only discuss filters whose spatial effects are clearly stated and controllable.

The development of an effective data processing sequence not only requires that we know the basic principles of the filters used, but also that we possess the knowledge of how to utilise a good filter at the right spot in the data-processing sequence. A classical digital image-processing sequence consists of three basic stages (fig. VI.A.5):

- pre-processing (optimisation of dynamics, contrast enhancement, noise reduction, edge detection);
- analysis (object measurement and symbolic information processing);
- interpretation (understanding the scene).

More detailed information can be obtained from the literature (González & Wintz, 1987; Coster & Chermant, 1989; Haralick & Shapiro, 1992; Cocquerez & Philipp, 1995; Davies, 2000).

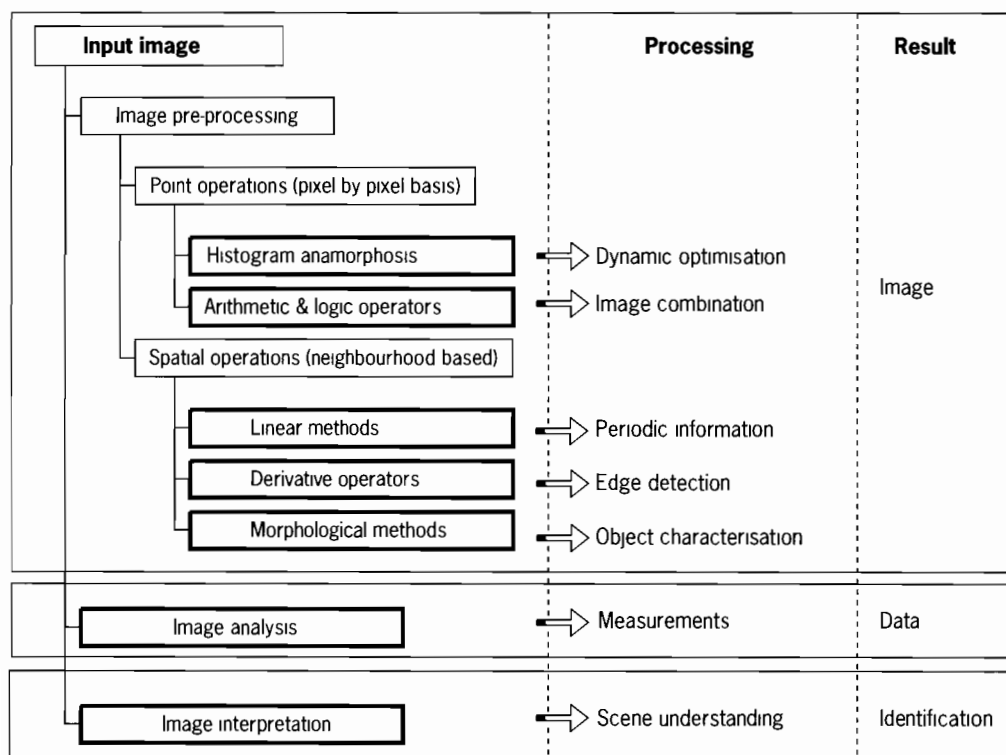


Figure VI 5
Classification of the main image-processing techniques presented as a tree structure in relation to the most important results produced by each class.

1.2.1. Contrast enhancement with point operations

One of the errors most commonly made by new users of image processing systems is "to believe only what they see". As mentioned above (see chap. VI.A.1.1.1) human perception of grey shades is far poorer than that of artificial sensors. Thus, depending on acquisition conditions, an image will be capable of presenting all the relevant information in a reduced but imperceptible dynamic range to the observer's eyes. A good example is given by the gradient operator (see chap. VI.A.1.2.1.3); when computing its magnitude we obtain an image with positive and negative values of small amplitude since it is a 1st-order derivative. If the dynamic of this image is not enhanced we will probably never notice that it contains all the available information about object edges. The concentration of information on a few grey levels will thus generate:

- less easy legibility for the observer, as a result of poor contrast;
- calculation inaccuracy due to rounding errors.

A well-contrasted image is an image whose distribution of grey levels, its so-called histogram, is stretched to fill the whole of the available dynamic range. We can modify this distribution by transforming the histogram by means of translations, stretching and local histogram compressions obtained by means of a linear or non-linear anamorphosis function. Figure VI.6 illustrates various histogram modifications and their effects on the legibility of images.

The ultimate objective is not always to balance histogram dynamics but may be to highlight certain image features or to extract area of interest from them. We can thus implement histogram modification functions that operate on simple thresholds or more complex transformations such as normalisation or equalisation. However, it is important to remember that the choice of transformation depends simultaneously on the type of image and the aim in view, whether aesthetic or functional, and that certain anamorphoses that enhance detail contrast may have the important fault of also amplifying noise. Images can also be combined pixel by pixel, through arithmetic (+, -, $\times$, $\div$, min, max) or logical (AND, OR, XOR, NOT) operations. With these operators, we can perform image averaging, superimpose two images, subtract a background pattern or even detect object motion from image to image. This is intensively used in image spatial filtering when subtracting frequency components is desirable, or with morphological filters so as to make logical tests.

1.2.2. Noise reduction with neighbourhood operations

Because they operate on a pixel by pixel basis, point operations do not allow the spatial modification of image details. Comparing a pixel value with its neighbours offers information on local brightness trends. Neighbourhood operations are based on transformations that take the surrounding environment of each pixel into account. The techniques most usually used in such cases are:

- linear filtering based on frequency analysis, acting via the modification of the frequency spectrum of the image or by the use of convolution operators;
- non-linear filtering based on morphological transformations or local statistics (median filter).

Such techniques, which are extremely abundant and varied, make up most of the algorithms proposed by the image processing software, so a description of them appears to be essential. The presentation below highlights only a few techniques but should provide a useful introduction to the range available.

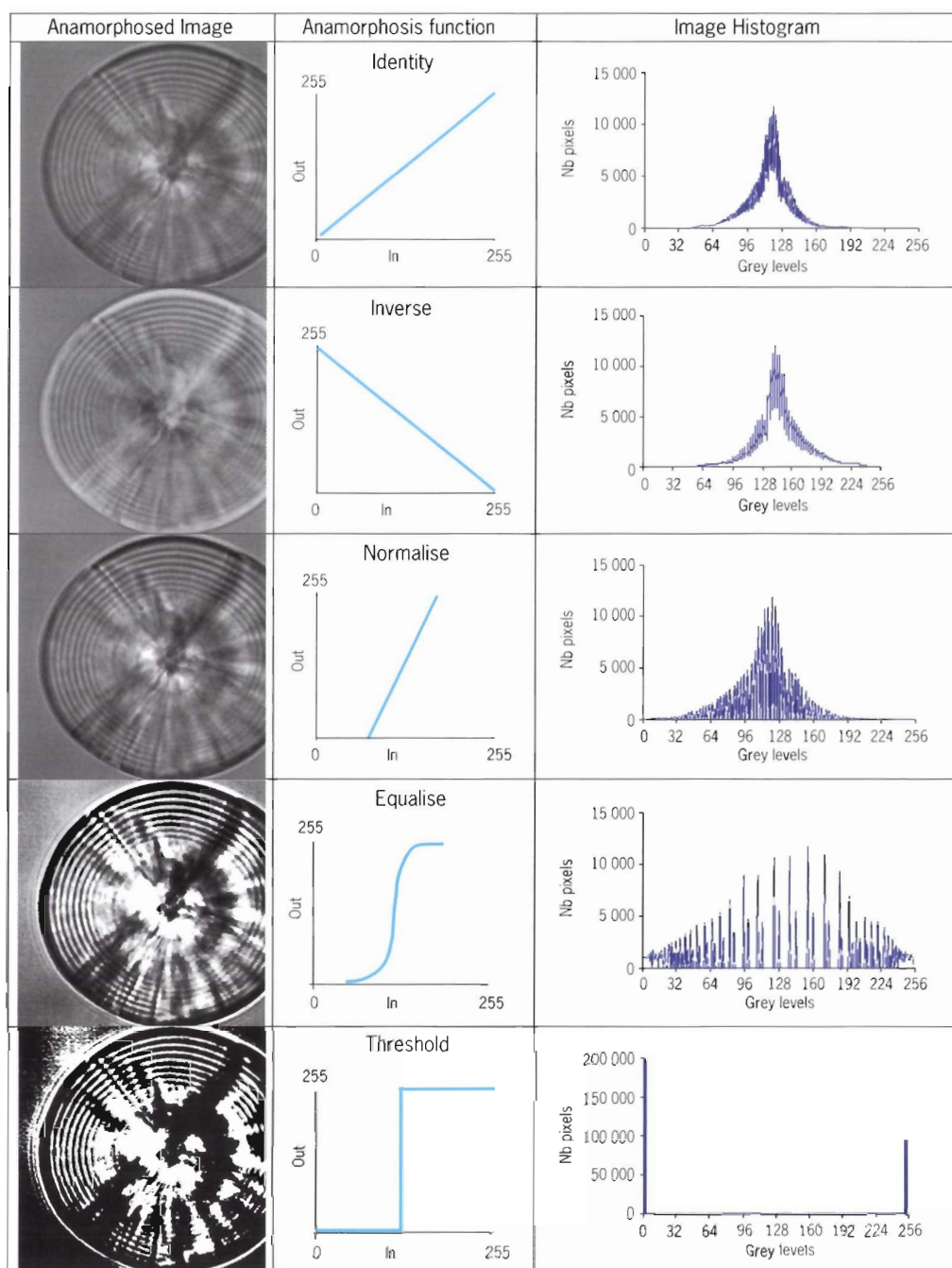


Figure VI.6 - Examples of image contrast enhancement by histogram transformation, i.e. on a pixel by pixel basis. Each column will present the anamorphosed image, the anamorphosis function and the resulting anamorphosed image histogram respectively. The computation of the anamorphosed value of a pixel is made by projecting it from the "In" axis to the "Out" axis through the anamorphosis function that acts as a mapping function.

1.2.2.1. CS image profile: a multi-component signal

When using neighbourhood operations we must be aware of how digital images are processed either in the spatial or frequency domain, and that both are representations of the same phenomenon. Moving from spatial to frequency representation is done by means of the Fourier transform. This converts the image into a sum of sine and cosine waves that represent its frequency content or spectrum. Each pixel value of the frequency spectrum corresponds to the weight of each frequency component in the overall signal. This transform, well known since its discovery by Jean-Baptiste Joseph Fourier in 1807, owes its success in digital signal processing to the development in 1965 of a fast computation algorithm: the FFT (Fast Fourier transform). Note that JPEG image compression format uses a relative of the Fourier transform, the Discrete cosine transform (DCT) using only cosine waves basis functions. The JPEG compression will consist essentially to eliminate the high frequencies, considering that they are not perceived by the human eye. On the other hand, these missing high frequencies may affect your forthcoming computer analysis.

With a spatial representation, the basic unit being the pixel, we can modify the image locally, while when a frequency representation is employed, the basic unit being the frequency, a filter will alter the whole image. The choice of representation depends upon the nature of the information required (structural or frequency) but the efficiency of a filter, designed and operating in one specific representation domain, can be evaluated in the other.

The alternation of bright and dark zones on a CS image can be considered, using a spatial representation or a frequency representation, as a set of concentric rings placed side by side (juxtaposed) or as a centred waveform respectively. In a frequency representation, a CS image profile can be modelled as a multi-component signal consisting of several basic frequency components that correspond for instance to periodic information, brightness and contrast trends, or to noise generated by the acquisition process. In the plaice otolith image profile shown in figure VI.7, it can be seen that the signal presents schematically:

- a low-frequency component corresponding to spatial variability in the CS illumination;
- a high-frequency component corresponding to electronic noise and to the fine texture of the CS;
- a "ring" component corresponding to the temporal periodicity of the growth zones observed;
- a growth component corresponding to the modulation of the growth zone width with time.

Basically, we retrieve each of these signal components, more or less clearly, in any CS signal. Neglecting any of these components, when it is strongly represented, lowers detection efficiency. Depending on our objectives and on the biological material available, we try to reduce image noise by suppressing the high-frequency components, or to homogenise image luminance by attenuating the low frequencies, or to improve the CS contrast by enhancing the “ring” component.

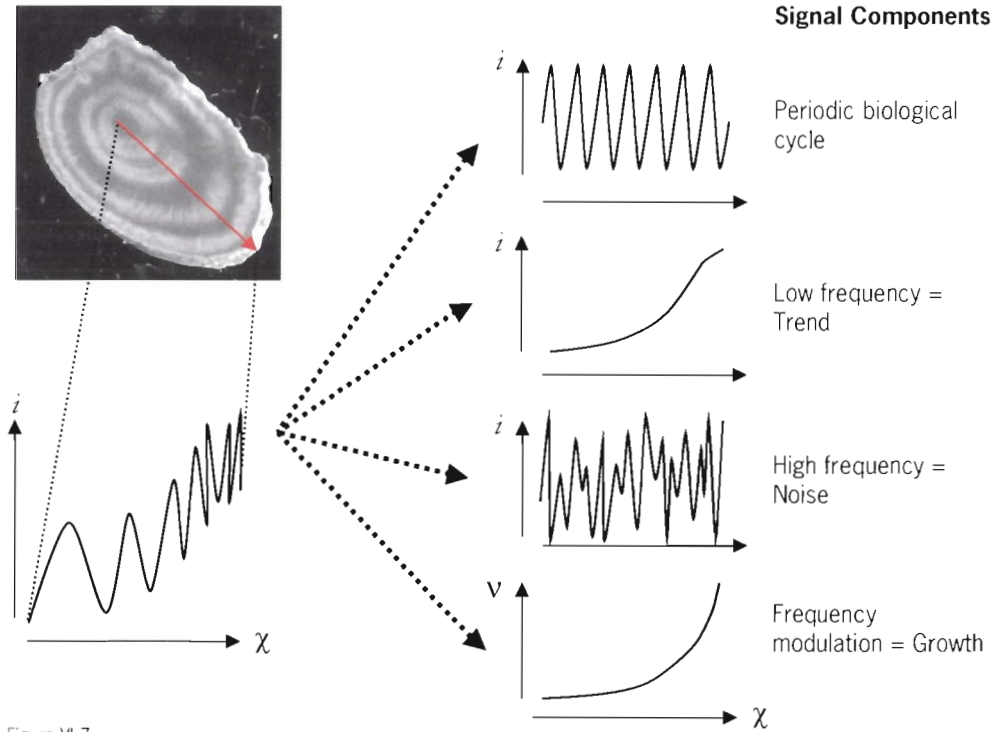


Figure VI.7
CS signal modelling
decomposition of a plaice
(*Pleuronectes platessa*)
image profile into its main
basic signal components.
The symbol i refers to
a light-intensity value (grey
level) and v to a frequency.

1.2.2.2. Spatial convolution: from moving average to Gaussian
Spatial filtering is mainly based on the operation of convolution of an image by a window of coefficients known as the convolution kernel. The process consists of moving this kernel across the image and to recompute each pixel value as the weighted sum of the adjoining pixels in a neighbourhood defined by the kernel size (fig. VI.8). Thus, depending on the distribution of these coefficients, we can select slow grey level transitions, corresponding to low frequencies, by using a low-pass set of coefficients, or conversely, select sharp grey level transitions, corresponding to high frequencies, by using a high-pass filter.

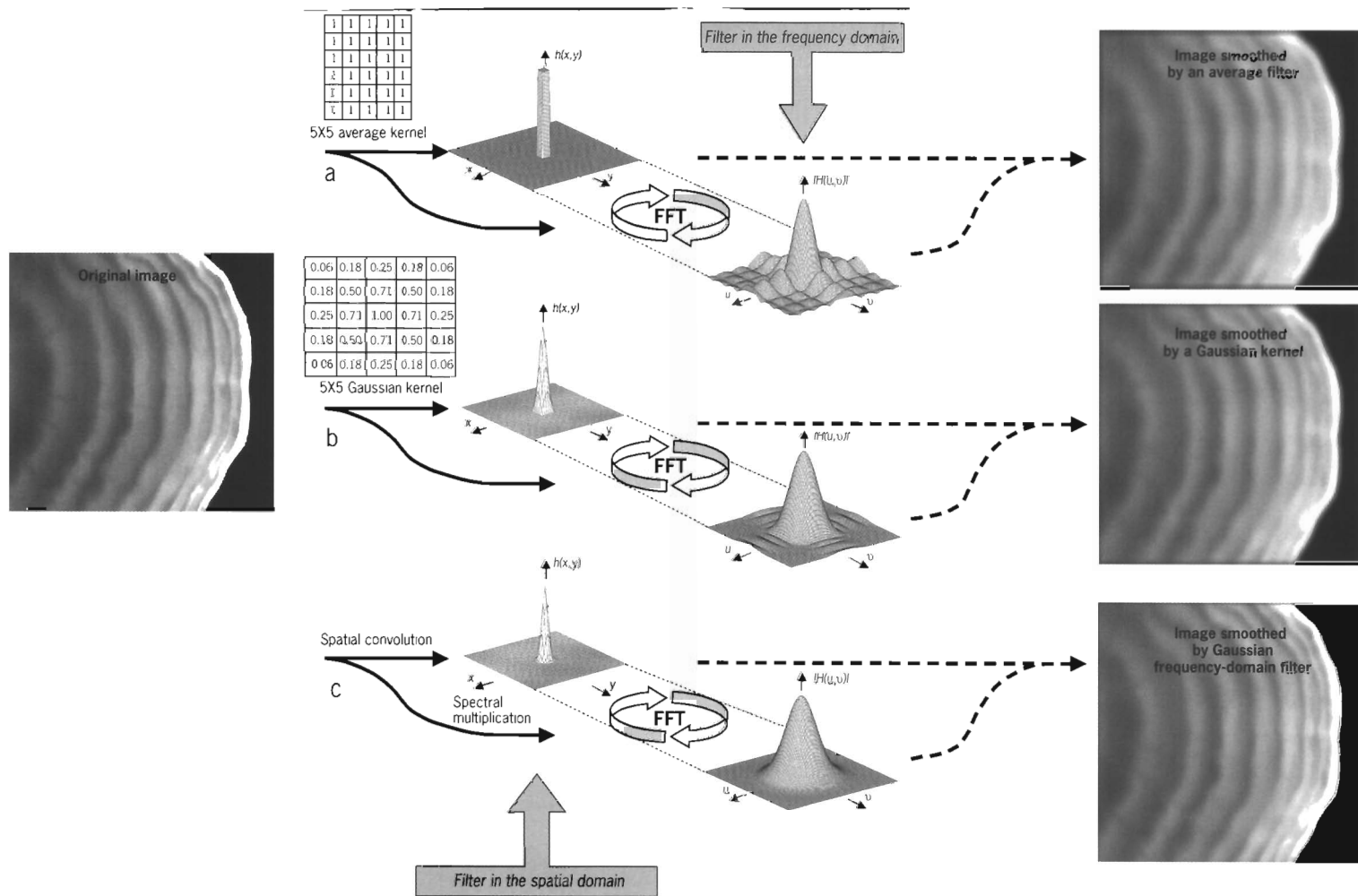


Figure VI.8 - Linear filters: smoothing filters designed in the spatial (convolution kernels) and in the frequency (impulse response) domain. a) The average filter is a good smoothing filter but in the frequency domain it displays lobes that makes it poorly selective regarding frequencies, resulting in a blurring of small ring contours. The Gaussian filter produces better results b) a Gaussian kernel is difficult to parameterise and is a truncated Gaussian function. c) A version of this Gaussian filter designed for use in the frequency domain will produce more accurate and selective results.

For a better understanding of this principle, let us observe how a moving average operates. This well known smoothing operation consists in replacing the value of a pixel by the average intensity of a given neighbourhood. The efficiency of such filtering, i.e. the increase in the signal-to-noise ratio, is highly proportional to the size of the convolution kernel. However, the more the kernel size increases, the more the transitions related to edges widen. This tends to delocate image contours which tend to become rather blurred (fig. VI.8a). Better preservation of the contour location necessarily implies a smaller kernel, but at the same time, the image will be smoothed very little. This observation faces us with two antagonistic filter properties, i.e. its efficiency in the spatial domain and in the frequency domain. The frequency response of the average filter presented in figure VI.8a shows clearly that this filter cannot separate one band of frequencies from another. Actually, the moving average is an excellent smoothing filter but a poor low-pass filter, which means that attenuating the noise simultaneously implies a loss of precision in the object location. We need to find a compromise: this is the Gaussian filter.

Altering the kernel coefficients enables us to balance the influence of certain pixels on the overall average and thus to be more selective in our choice of frequency components. Instead of having a gate-shaped impulse response like the average filter, the Gaussian filter has a bell shaped one. This filter offers the best compromise between signal-to-noise ratio and localisation accuracy. The theoretical basis of this tool, which is frequently used, have been widely discussed in the literature since the work of Marr, which was based on the study of the human visual system (Marr & Hildreth, 1980; Marr, 1982).

1.2.2.3. Spectral filtering: the Fourier transform

Spectral filtering is based on the modification of a signal in the frequency domain. A frequency representation of a spatial signal is obtained by using the Fourier transform. As we have already seen, spatial convolution operates in both the spatial and spectral domains. Among several reasons for employing spectral filtering are that:

- it is more efficient when the original problem can be easily expressed in terms of frequencies;
- the convolution of two signals is simply the product of their Fourier transform. This single property enables us to drastically speed up the convolution processing process;
- it allows the design of more efficient filters, with a wider support, that lower the truncation effects imposed in the spatial domain by the approximation of a filter shape on a small kernel.

How can we design a filter in the frequency domain? The naive approach consists in applying a gate function to the Fourier transform

of the image (fig. VI.8). This brutal way of limiting the number of coefficients of the frequency spectrum generates ringing and overshoot at edges and corners in the spatial domain, a phenomenon known as the Gibbs effect. Again in the frequency domain, we are faced with the necessity of compromising between frequency and spatial localisations. This remains a possibility by modifying the shape of the filter as described in figure VI.8 or by utilising recursive implementation of analog filters, which will avoid the Gibbs effect by simulating infinite support.

1.2.2.4. Linear filtering of CS images

The low-frequency (or trend) component in a CS signal (see chap. VI.A.1.2.2.1) resulting in spatial drift of the average luminance, may also be due to:

- heterogeneous lighting;
- spatial variability in the optical properties of the deposited material (narrowing of the growth zones with age, bringing closer the translucent zones);
- variability in sample thickness.

This phenomenon often appears as a brightening of the CS towards its edges, meaning that the luminance features of a growth zone, whether translucent or opaque, are not spatially constant. We can easily imagine the difficulty of applying a simple thresholding technique to these images since no value will be capable of separate the different growth zones adequately. Furthermore, linear filtering presupposes a signal stationarity, i.e., that the average value and standard deviation of a signal are spatially constant. In the same way, the efficiency of adaptive filters, i.e. their robustness to trends, are increased by the elimination of trends or at least by their attenuation. For that purpose, we employ “low-pass” filters to detect the low-frequency components and to remove them from the rough image. This could be done either by means of a Gaussian kernel, presenting the best compromise between spatial and frequency localisation (fig. VI.9), or using a morphological filter such as an “opening” (fig. VI.12).

1.2.3. Edge detection by derivative operations

We may now consider the image not as a sum of frequency components, but as a relief whose grey levels will represent altitudes. The image thus consists of a succession of peaks and valleys whose slopes are more or less steep. A highly contrasting object will present a steep slope of grey levels on its edge, whereas a weak slope characterises a homogeneous area of luminance. The detection of local discontinuities by means of slope computation by derivative operators enables object edges to be localised (fig. VI.10).

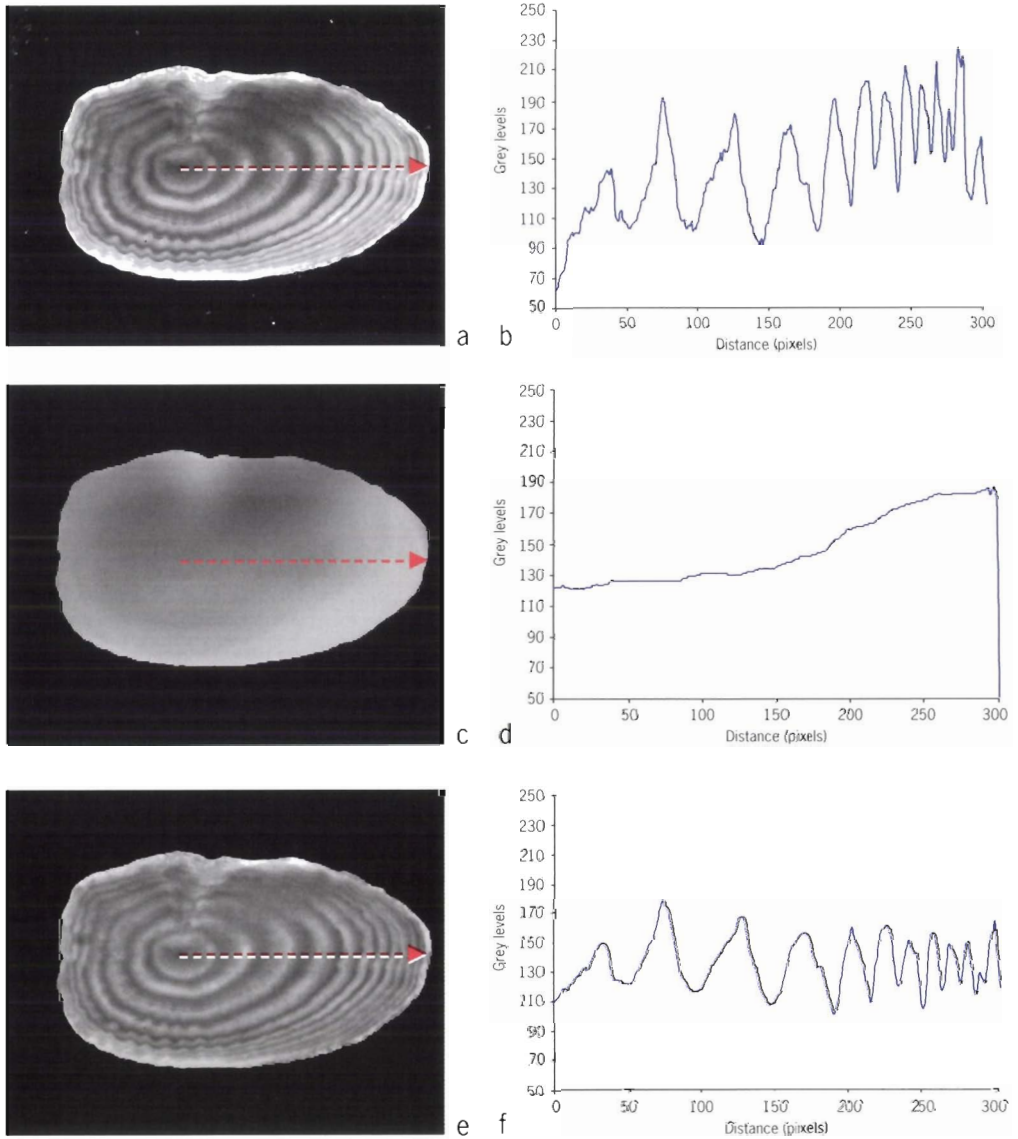


Figure VI.9
Elimination of the light trend
on a CS image and related
profile: a-b) rough place
otolith (*Pleuronectes
platessa*), c-d) image
of the estimated light trend,
e-f) detrended image.
Such processing facilitates
growth mark detections.

In the unidimensional case, the point of maximum slope of a grey level transition corresponds to the extreme values of its first-order derivative, i.e. its gradient. The human visual perception system applies to all what we see an enhancement of the second order derivative or Laplacian type.

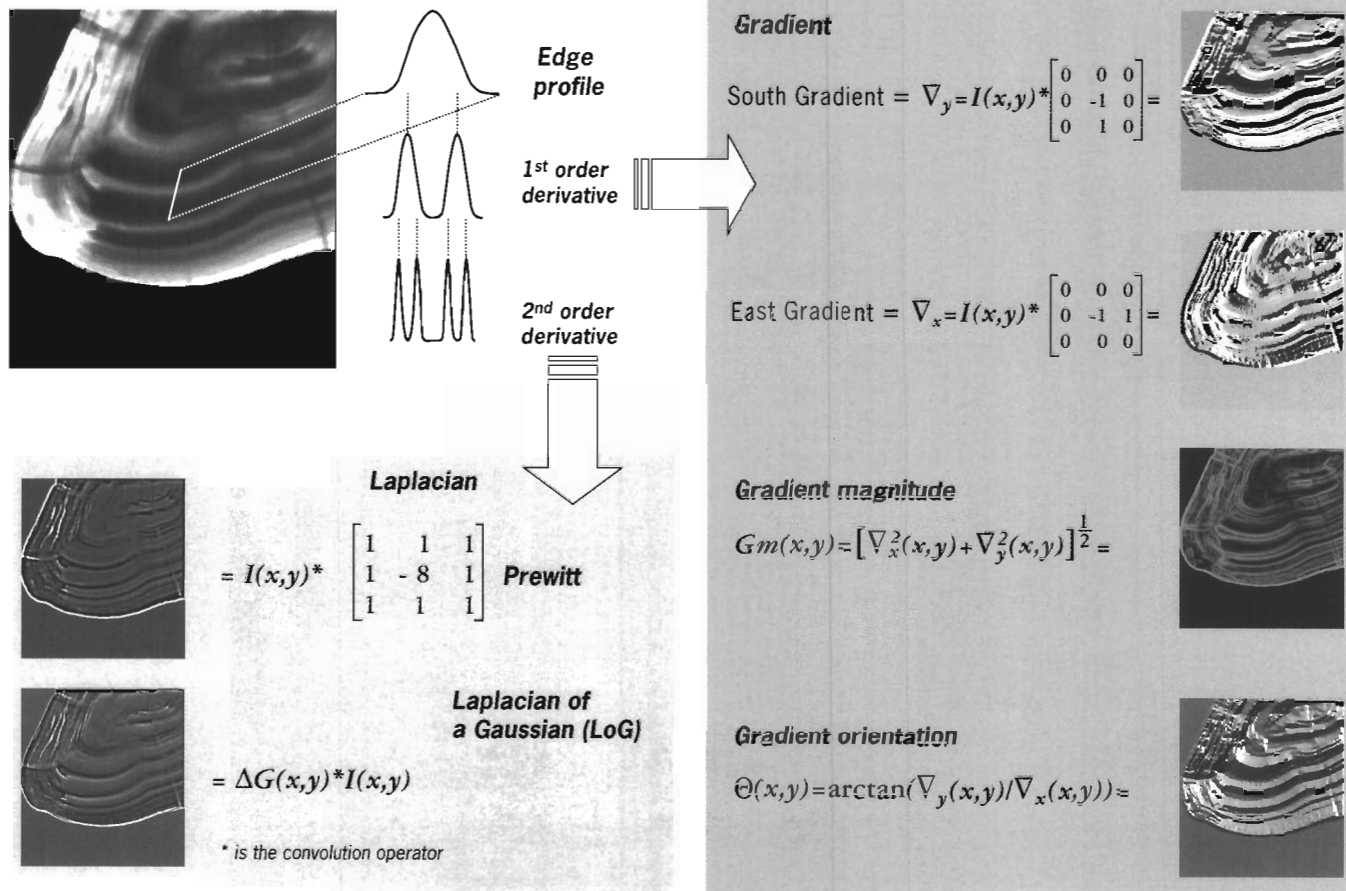


Figure VI.10 - Edge detection by derivative operators: processing of a section of a pollock (*Pollachius pollachius*) otolith by different derivative 1st and 2nd order operators (Visilog software, Noesis).

The 2D gradient can be approximated by means of the Robert, Prewitt or Sobel convolution kernels shown in figure VI.10. However, the use of small kernels makes it difficult to distinguish between local variations corresponding to edges and those corresponding to noise. The increase in the size of the computation kernel enables us to obtain an operator that is more robust to noise, this robustness being obtained at the expense of precision. In fact, all the derivative operators operate a smoothing function and once again impose on us the constraint of making a compromise between a good localisation of noisy edges and a poor noiseless localisation. The best compromise will be obtained by the operator of Marr & Hildreth (1980), the "Laplacian of a Gaussian" that allows the advantages of the Gaussian filter to be associated with those of the Laplacian. In the same way, the Canny-Deriche edge detector is a recursive algorithm for the determination of the gradient. In order to minimise the effects of noise, it smoothes the image before computing the gradient. A smoothing scale parameter allows us to accurately determine the smoothing intensity. If the value is large, the noise will be reduced but the edges will be less sharp and only the most significant edges will remain. It is important to select the right coefficient to lower the noise just sufficiently without defocusing the edges.

One way in which to sharpen a CS image is to anamorphose the image histogram. As mentioned in 1.2.1 there are various ways of doing so, but linear scaling and equalisation are the most frequently used methods. The latter is likely to be more effective, as it aims to balance the distribution of grey levels, but it will have a tendency to enhance noise. A more effective way to do this is to use deblurring algorithms. Blurred images are characterised by low contrast and particularly by smoothed edge transitions. Deblurring images enhances their contrast by reinforcing edge transitions. As blurring is an averaging, or integration operation, deblurring will be approximated by means of computing derivative operators. The Laplacian operator (2nd-order derivative) is a good approximation of a deblurring operator and is more efficient in sharpening edge transitions. An image $I(x,y)$ will be deblurred by (fig. VI.11):

$$(VI.1) \quad D(x,y) = I(x,y) - k \cdot \Delta I(x,y)$$

where Δ is the Laplacian operator and k a constant value.

1.2.4. Structural information detection by morphological operations

Contrary to the linear methods which consider an image as a sum of frequency components, mathematical morphology (Matheron, 1975; Serra, 1982, 1988) treats the image as a set of opaque objects in which superposition produces masking rather than summation. Saying that

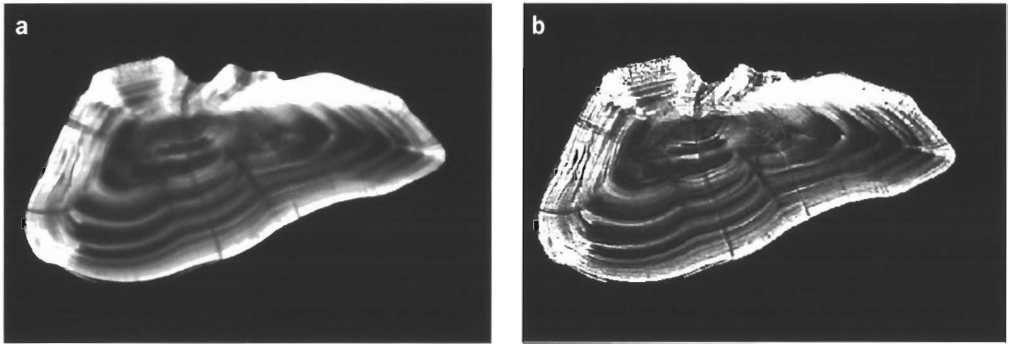


Figure VI.11
Image sharpening by
subtraction of a Laplacian
(second-order) derivative.
This is a good
approximation
of a deblurring filter
(Visilog software, Noesis).

object A hides object B is equivalent to saying that the contour of B is included in A. Thus, mathematical morphology will examine, by using set operators ($\cap$, $\cup$, $\subseteq$), the geometrical structure of an image by means of a reference object of known size and shape: the structuring element.

Erosion and dilation are the basic operations of mathematical morphology. Instead of re-computing a pixel value by means of a coefficient set as for the convolution, we test in each point of the image the inclusion or the intersection of the structuring element. In a binary image, coded into a series of 0 and 1, an erosion will consist of preserving the zones in which the structuring element is entirely included. A dilation, on the contrary, will consist of adding to an object all the points at which the structuring element intersects with it. An erosion will remove isolated points and small objects and will disconnect objects, whereas dilation fills the holes and connects the objects (fig. VI.12). These operations are also applicable to grey-scale images, in which erosion will be computed by the min function of pixels in the neighbouring region, defined by the structuring element, whereas a dilation will be the max function.

From these basic operators we can build operations that present similar but less destructive properties that better preserve the original shape. The opening of an image is a combination of an erosion followed by a dilation, while closing is a dilation followed by an erosion. All these transformations have the increasing property, i.e. that, if a O_1 object is included in another object O_2 , then the transformation of O_1 by a transformation T is included in $T(O_2)$. On the other hand the topological properties (holes, number of related components, etc.) are not preserved.

These basic processing operations will provide a very efficient CS growth ring detector: the Top-Hat transform. This consists of the subtraction of the original image to an opening, when looking for translucent rings, or of subtracting a closing from the original image in the case of opaque rings (fig. VI.12f).

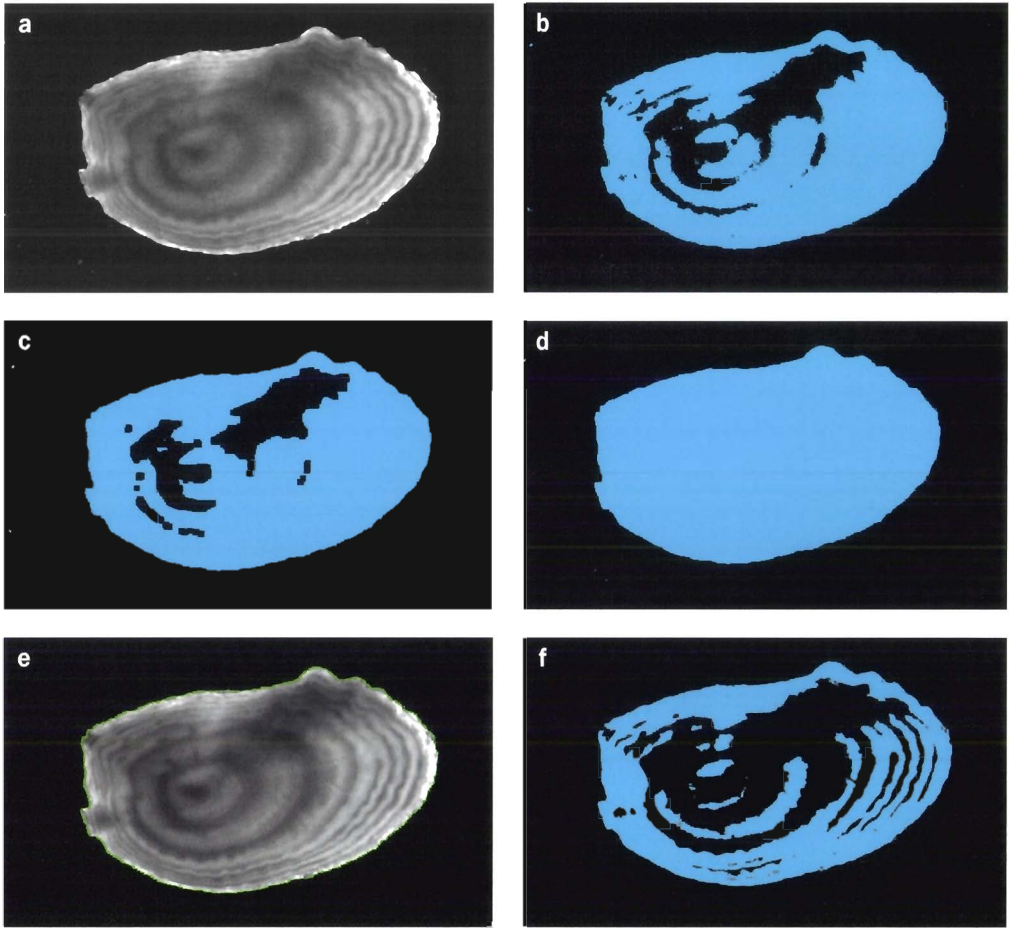


Figure VI.12 - Extraction of a plaice (*Pleuronectes platessa*) otolith contour (a) by morphological operators: a closing operator of a thresholded image (b) will close the contour and consists of dilating the image and then eroding it (c). Holes inside the object are filled by a hole-fill algorithm (d) based on geodesic dilation of the image complement. The outline of the biggest object will correspond to the otolith contour (e). The Top-Hat filter is an adaptive operator allowing the thresholding of objects of a definite size (f).

Other type of transformations will allow us to preserve the topological properties of the images. They consist, for binary images, of looking for particular neighbourhood configurations and removing the pixel corresponding to such configurations in the case of a thinning operation, or adding one in the case of a thickening operation.

1.2.5. The growth pattern: a limit to classical operators

One of the principal properties of the classical linear and non-linear digital filters is that they are shift invariant. This enables us to detect the same object wherever it is in the image. When the image represents a scene with a perspective, for instance a highway seen from a bridge, we cannot detect the objects in the foreground using the same

filter parameters as those for the background. With classical filters we need to enlarge the size range criteria and finally detect the foreground objects corresponding to noise. With CS, the growth pattern acts like a perspective, varying ring width and allowing us to relax our size criteria, and to gather noise.

When we have a rough idea of the growth pattern, a possible solution is to adapt the filter parameter to the perspective or growth pattern function. The dual solution consists of reshaping the signal, still on the base of the prior growth pattern knowledge, in order to subtract, or at least to attenuate the variation in the amplitude of peaks and valleys. In signal theory this is known as frequency demodulation (fig. VI.13).

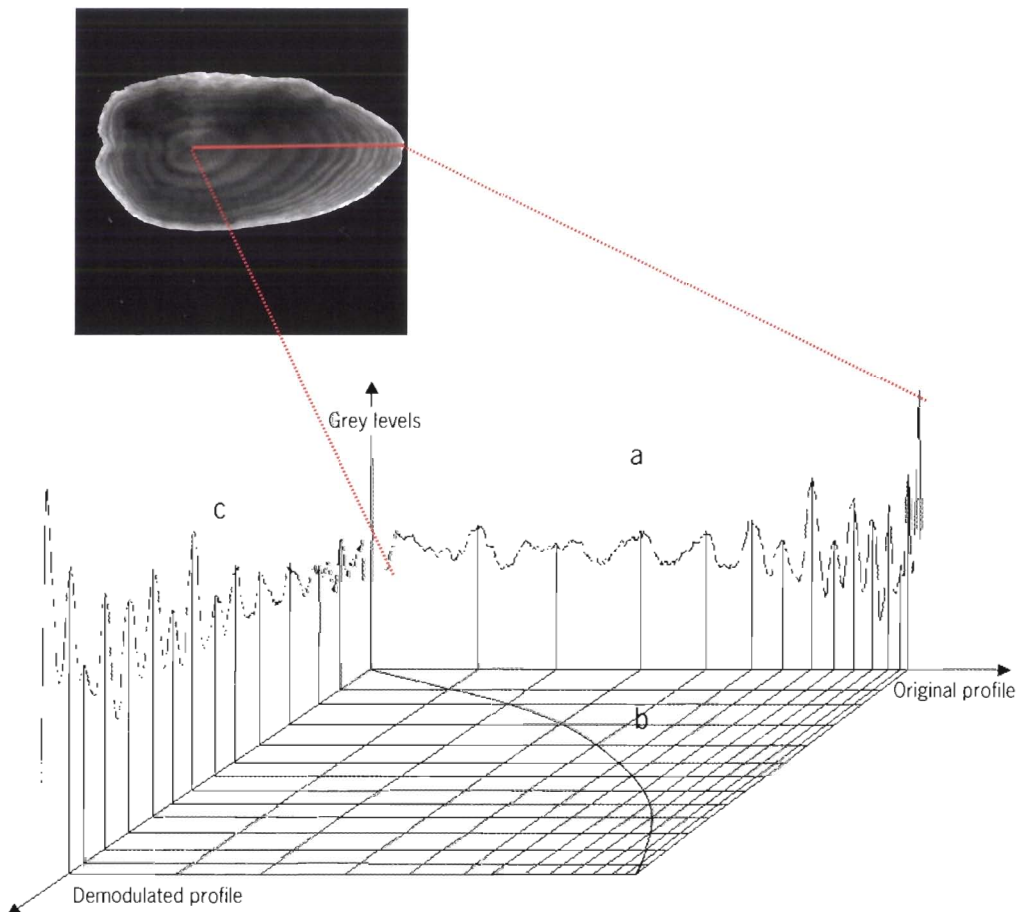


Figure VI.13 - Growth trend removal: graphic illustration of the demodulation algorithm on a plaice (*Pleuronectes platessa*) otolith image profile (a) using an *a priori* von Bertalanffy growth model (b). The demodulated (or anamorphosed) profile (c) presents rings of similar width, unlike the original.

This demodulation has been applied to otoliths of *Solea solea* larvae (Troade, 1991; Lagardère & Troade, 1997) with an exponential growth function, and to otoliths of adult *Pollachius virens* (Troade, 1991) and *Thunnus alalunga* dorsal fin ray sections (Troade & Antoine, pers. data) with a von Bertalanffy growth function. The aim was primarily to improve ring detection and at the same time to estimate the number of faint or missing rings due to bone alteration, for instance. The demodulation was followed by a Fourier transform and the frequencies with the highest energies were used to estimate the number of missing rings by extrapolation.

1.3. CS quantitative analysis: from 1D to 3D

While image processing can transform one image into another, quantitative analysis produces measurements starting out from an image. These measurements are usually calculated from binary images, but in some cases a grey-scale weighting can be introduced. Figure VI.14 presents and illustrates the basic parameters, our goal being merely to draw the reader's attention to the existence of various methods of processing the same parameter.

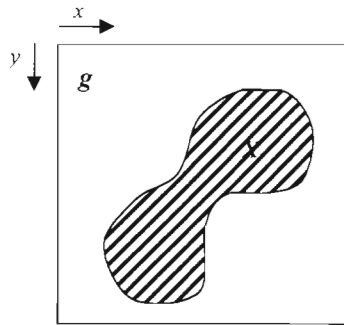
1.3.1. Growth zone counting: from 1D to 1.5 image profiles

Before image-processing systems came into use, sclerochronologists were already reducing their ring localisation to a single axis, specially when ring growth measurements were required. Except for some prospective work (Welleman & Storbeck, 1995; Troade *et al.*, 2000), the tools available today still use single profiles to detect growth zones on digital images. Why then are there such differences in their respective performances?

One of the main reasons is that the human reader detects the rings in 2D and locates them in 1D when most current ring detection software carries out the whole process with only 1D information. Nevertheless, if the growth zones do not vary too much in size, if their contrast is good enough and if the filter parameters can be suitably adjusted, we can expect to obtain a good detection rate of around 80%. In general, we can say that if we are able to count rings easily then automatic systems will be "not too bad".

When working on a single image profile, we definitely lose any ring continuity information and the signal will be very sensitive to local defaults that could be wrongly interpreted as peaks or valleys. In order to enhance the image profile signal and to approximate a perception of local continuity, some software now allows to integrate the data from several profiles and to combine them. This can be done in two ways (fig. VI.15):

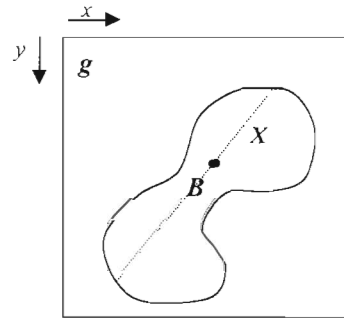
- rank by rank, implying that all pixels at the same distance from the origin are combined. This simple way is only applicable when we have



AREA

$$A(X) = \sum_i g(x_i, y_i)$$

with: $g(x_i, y_i) = 1$ if $i \in X$
 $g(x_i, y_i) = 0$ if $i \notin X$



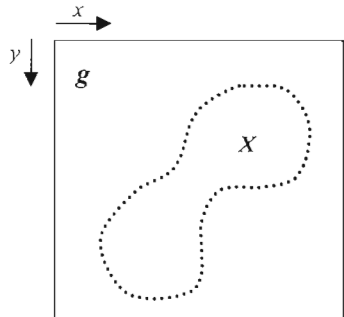
ORIENTATION = Direction of the major inertia axis estimated from second order moments

$$\theta = \frac{1}{2} \arctan \frac{M_{2xy}}{M_{2x} - M_{2y}}$$

with: $M_{2x} = \frac{1}{A(X)} \sum_i (x_i - M_{1x})^2$

$$M_{2y} = \frac{1}{A(X)} \sum_i (y_i - M_{1y})^2$$

$$M_{2xy} = \frac{1}{A(X)} \sum_i (x_i - M_{1x})(y_i - M_{1y})$$



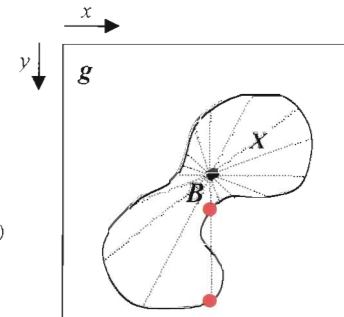
PERIMETER = Length of X boundary

1 - Sum of the boundary pixels in the different directions

$$L(X) = N_0 + N_{90} + N_{45} + N_{135}$$

2 - Crofton perimeter = sum of the boundary pixels corrected for diagonal terms (a = square pixel size)

$$L(X) = \frac{a}{4} \times \left[a \times (N_0 + N_{90}) + \frac{a}{\sqrt{2}} \times (N_{45} + N_{135}) \right]$$



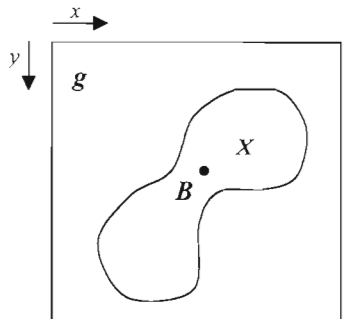
FOURIER DESCRIPTORS:

• With a polar representation (red points showing an ambiguity on a radial)

$$R(\theta) = A_0 + \sum_{n=1}^{\infty} A_n \cos(n\theta - \Phi_n)$$

• With elliptic descriptors (2 Fourier series corresponding to the projection of the shape on the x and y axis)

$$X_t = A_0 + \sum_{n=1}^N X_n, \quad Y_t = C_0 + \sum_{n=1}^N Y_n$$

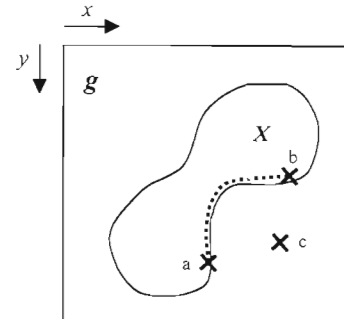


BARYCENTRE = Centre of gravity

$(X) = (M_{1x}, M_{1y})$ with:

$$M_{1x} = \frac{1}{A(X)} \sum_i x_i$$

$$M_{1y} = \frac{1}{A(X)} \sum_i y_i$$



GEODESIC DISTANCE

$dX(a, b) = \text{arc}(a, b)$ if a and $b \in X$

$dX(a, c) = +\infty$

Figure VI.14 - Image analysis: graphic illustration of some basic parameters used to measure and characterise objects on digital images.

a profile set that is precisely perpendicular to your growth zones and when the data points do not present too much curvature, so that data are not combined wrongly;

- after synchronisation of two landmarks, e.g. *nucleus* and edge. In this case, all the profiles are resized and data can be combined more safely.

Data from the different profiles can be combined by computing statistical estimators like the average (Cailliet *et al.*, 1996), but the median estimator, being more robust to aberrant data, is usually preferred (Troadec, 1991; Welleman & Storbeck, 1995).

Rings are assimilated in 1D profile to maxima or minima (peaks or valleys). Smoothing linear filters are used in order to select the main peaks or valleys, starting from a simple moving average algorithm (Cook & Guthrie, 1987; McGowan *et al.*, 1987; Szedlmayer *et al.*, 1991) and moving to more selective Fourier filters (Troadec, 1991; Cailliet *et al.*, 1996; Morales Nin *et al.*, 1998) or adaptive Top-Hat transforms (Troadec, 1991) (fig. VI.12f).

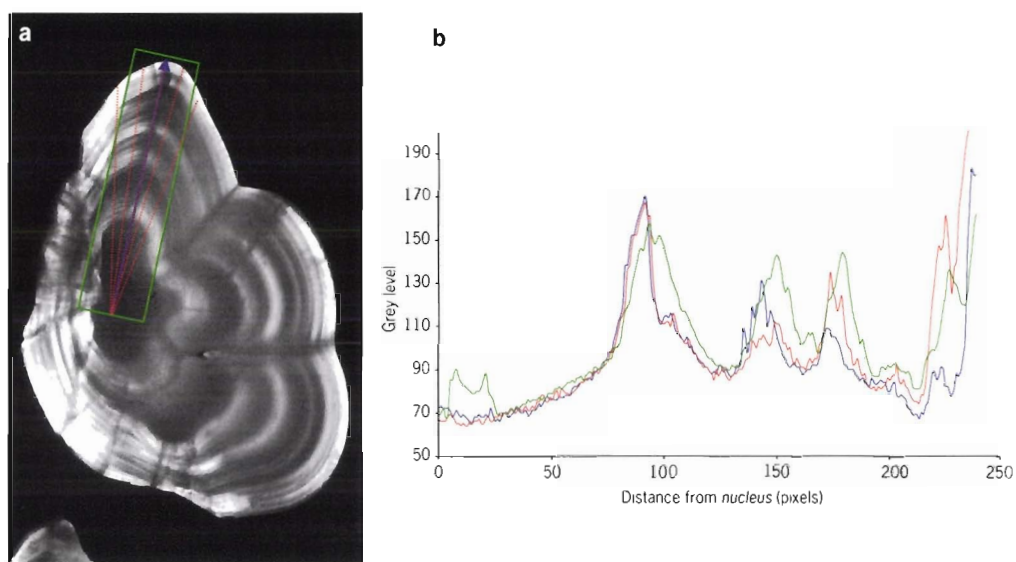


Figure VI.15 - Acquisition of image profiles: comparison of different computational methods on a pouting (*Trisopterus luscus*) otolith image (a): single profile (blue arrow), multiple-averaged parallel profiles (green box) and median of multiple synchronised radiate profiles (red dot lines). Graphic representation (b) of each method showing that single profiles will miss some rings while multiple profiles will tend to delocate or over-estimate ring width (TNPC software, Noesis).

When considering a peak (or a valley), we can locate the growth zone as being at:

- the grey value maximum in the peak (or a minimum for a valley);
- the geometric centre of the peak;
- the beginning of the peak;
- the end of the peak.

The extreme value method is the most widely used but it is essential to check whether the automatic ring location fits with your own perception. Actually, when we are faced with an homogeneous area we tend to locate the ring at the geometric centre of the peak, but if there is a luminance maximum, we will be attracted by it and locate the ring at this extreme. Generally speaking, the location of the beginning and end of the peaks is greatly influenced by the filter parameters.

All these methods that operate on a ring by ring basis can be considered as "local" compared to "global" methods that are based on a spectral estimation in which the number of rings is estimated on the whole signal on a global basis. These methods can be used in certain cases where the growth pattern does not vary excessively or where appropriate pre-processing has been performed in order to remove it (Troadec, 1991; Lagardère & Troadec, 1997).

1.3.2. 2D and 3D CS shapes

CS shapes can be used simply for analysis of the growth of surface or volume, as a specific signature or as an integrative feature of fish growth history characterising a stock and allowing the origin of individuals to be identified. This has been applied to scales, for example, in order to distinguish between North American and European *Salmo salar* (Pontual & Prouzet, 1987; Reddin & Pontual, 1992) and to distinguish between individuals of wild and hatchery origin (Ross & Picard, 1990), as well as to otoliths (Messieh *et al.*, 1989; Campana & Casselman, 1993; Friedland & Reddin, 1994) for stock discrimination. Shapes can be digitised with a digitising tablet but a camera is usually preferred in order to circumvent manual tracing errors. CS illumination must be optimised in order to limit segmentation (separation of the CS from the background) to a simple threshold that will provide a binary image.

Basically, two types of CS shape features are computed (fig. VI.14):

- simple shape factors like compactness (width/length), perimeter/length, perimeter/width, perimeter/sqrt(area), etc.
- Fourier coefficients, by which the outline is decomposed into a sum of sine and cosine waves with frequencies increasing in harmonics. The gross shape is described by low frequencies, while the addition of higher-order harmonics refines the CS shape description. The outline of the CS can be determined by an edge-following algorithm and the Fourier coefficients computed from a polar co-ordinate representation of the contour (Younker & Ehrlich, 1977) or from Cartesian co-ordinates with elliptical coefficients (Giardina & Kuhl, 1977). The latter approach is to be preferred as it circumvents the problem of identifying the centre point of the polar representation and can be applied, unlike the first approach, to complex shapes with pronounced convexities.

3D information is usually used by a reader faced with a new species. The choice of the best sectioning plan requires the observation of CS from different stages of growth, in different planes and following a series of grinding stages. This is done until the reader is able to reconstruct a mental tri-dimensional shape dynamic of the CS. While we wait for non-destructive methods such as computerised tomography to become available for CS work (Hamrin *et al.*, 1999) 3D representation is currently obtained by the alignment of serial cuts. These methods are still tedious and thus are not yet widely used in ageing laboratories.

The critical point about these techniques (fig. VI.16), is their ability to align the different elements of the set of slices. The individual steps involved are:

- embedding the CS in resin;
- cutting with a blade or a thread saw. This step determines the resolution on the z-axis;
- alignment made with software vis-à-vis a reference - the fiducial points - that can be obtained by drilling holes perpendicular to the grinding plane (Bailey *et al.*, 1995) or by a square embedded with the CS in the resin (Troadek & Fichou, pers. data);
- slice digitisation by a frame-grabber.

The image set can then be visualised as follows:

- a rough 3D representation as a volume image, pixels becoming 3D points called voxels. Certain software packages allow us to make distance measurements in 3D;
- a reconstructed view of the outer edge and/or of the ring edges after prior manual or automatic digitisation as described above for 2D shapes. The reconstruction software can then provide 3D shape features.

2. Cognitive information processing: Are you reading or interpreting?

Requiring a computer to simulate, either entirely or partially, an age estimation process implies the possession of a conceptual framework clearly based on a precise terminology. Putting his finger on the semantic confusion still maintained today by sclerochronologists in the undifferentiated use of the terms “reading” and “interpreting” Sych (1974) proposed to distinguish two components in an age estimation process:

- **reading**, which is the recognition of certain structures by means of a main alphabet or a key;
- **interpretation**, which is the comparison of structures with events in the life cycle.

This simple distinction, which seems obvious at first sight, made Sych the first to propose a logical and statistical framework for the analysis of calcified structures on the basis of rigorous decision trees, which

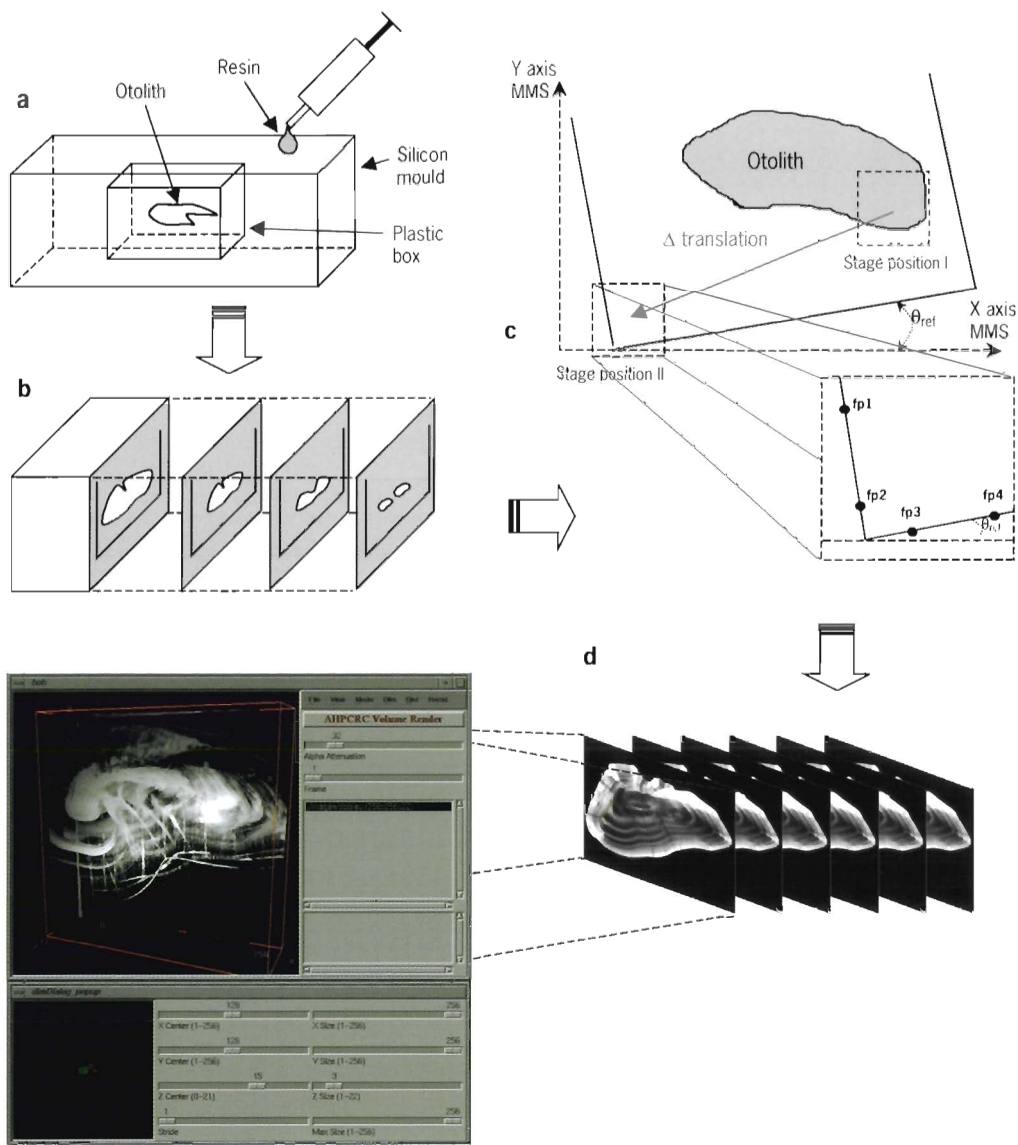


Figure VI.16 - CS 3D reconstruction: description of preparation process for reconstructing CS in 3D from serial cuts. a) Inclusion in a plastic box and embedding in a silicon mould, b) Serial cutting with a thread saw. c) Digitisation of the 1st CS slice on a microscope equipped with a motorised stage (MMS). Four fiducial points (fp) are acquired and the orientation is computed. Then, all the other slices are aligned in translation and rotation in reference to it. d) Visualisation of the image set with 3D volume rendering software (BOB software (Brick of Bytes), AHPCRC).

allow uncertainties regarding the decision rules to be managed. In line with a similar thought process, Casselman (1987) proposed that the data acquisition and interpretation steps should be clearly separated and suggested that "...the fallacy in past approaches has been that the two components - data extraction and data interpretation - could be

treated simultaneously...". Actually, the localisation of a growth ring on a calcified structure does not imply anything about its periodicity. It involves only a process of visual perception which can require more or less long training. This growth ring may subsequently be classified as having a given periodicity (annual, daily, false ring, etc.). This is the interpretation phase which requires knowledge that has been gained during validation experiments and in the process of previous interpretations. The former is based on purely visual identifying criteria (growth ring, *nucleus*, *primordia*, check, etc.), and can usually be fulfilled very quickly for a given CS and a given preparation method. The latter is based on more or less complex logical schemes, integrating information related, among other aspects, to the species concerned, the stock or population, and/or the catch zone, and combining instantaneous (or individual) and historical information (fig. VI.17).

Although they may be closely linked, these two processes require information of quite different natures (Troade, 1992). Dealing with them by computer involves two distinct scientific fields:

- pattern recognition for visual perception;
- the cognitive sciences for interpretation.

Although currently available CAAGE systems are almost exclusively based on image analysis systems, we can observe a growing interest in the interpretation stage, which probably constitutes the greatest source of variability (Morison *et al.*, 1998).

A first approach has been to use objective criteria in order to assign a growth zone a given periodicity. Pointing out that growth zones are deposited according to a relatively regular growth pattern, Small & Hirschhorn (1987) proposes a system based on fitting a growth model. A von Bertalanffy growth model is fitted for each calcified structure to a set of locations of digitised growth zones. The residual analysis provides criteria for determining the validity of the growth zones. This approach was then extended by the introduction of a constraint in the reading process through the demodulation of the image profile by a growth pattern (Troade, 1991; Lagardère & Troade, 1997).

Another approach consists of asking the computer to manage only the interpretation level of the age estimation process. Casselman (1987, 1996) developed a system for the interpretation of calcified structures, CSAIS (Calcified structure age interpretation system), and has developed and refined a software, CSAGES (Calcified structure age-growth data extraction software) (Casselman & Scott, 2000), that incorporates the system and uncouples the acquisition phase from data interpretation. The software is thus based on:

- a data acquisition module: CSAS (Calcified structure analysis software) that uses a digitizer integrated with a computer through a keypad;

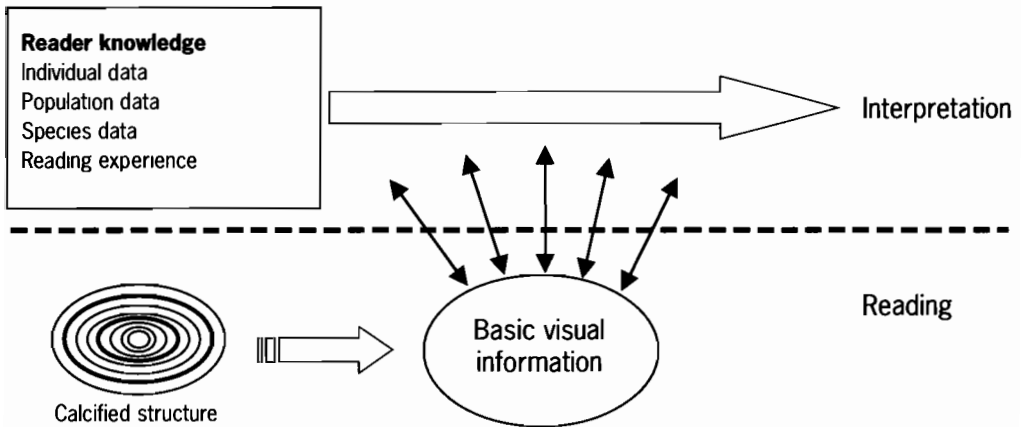


Figure VI.17
Conceptual scheme
presenting fish age
estimation as a two-level
process composed of (1)
a reading level based on
visual perception and (2)
an interpretation level
that combines data field
knowledge and reader
experience.

- an interpretation module: CSAGES (Calcified structure age-growth data extraction software, Version 5.2; Casselman & Scott, 2000) that deals with an important data file associated with calcified structures (environmental parameters, morphometric variables, conditions of data acquisition, etc.). The intention is to leave entirely to the human operator the task of visual perception, at which he is assumed to be more effective than present computer image analysis systems. At the same time, the operator provides a localisation and a fine classification of the checks or growth zones (*annulus*, *pseudo-annulus*, *partial annulus*) and a characterization of the calcified structure edge.

3. A little theory: visual perception and age estimation

In an article entitled "Why progress in machine vision is so slow?", Théo Pavlidis (1992) underlined, among other causes, the lack of interest in the basic functional principles of our visual system. The application of artificial vision techniques to the interpretation of calcified structures is no exception and feedback on visual perception concepts would enable us to better evaluate the complexity of the problem.

Various theories...

In the course of the past three decades, an ambition to reproduce computer visual perception mechanisms similar to those of human beings has developed. This task, so easily solved by any human observer, and which seems so simple and so obvious, is actually extremely complex, contrary to appearances, and poses enormous problems that are far from being solved today by cognitive psychology, physiology or exper-

imental neurophysiology. While we do not wish to be too sweeping, we may say that there are three theories (Benzinou, 2000):

- the theory of “direct perception” advanced by Gibson between 1950 and 1979, according to which the space-time structure of the sensory system implied in the vision, the so-called “optical network”, is sufficient to account for the process of perception. Any interpretation, construction or representation is then regarded as post-perceptive;
- the “Gestalt” theory, which associates two processes, a primary “bottom-up” process, which transforms and organises the retinal image in discrete units according to their geometrical and dynamic characteristics, and a secondary “top-down” process, which is non-visual and is directed by concepts, and which consists of associating, by means of progressive adjustments, these “objective components” with cognitive information pre-existent in memory (fig. VI.18a);
- lastly, the “constructivist” theory, for which perception is a kind of unconscious construction of significance, starting from incomplete information provided by the retinal image.

The contribution of neuroscience...

Visual perception thus appears to be a complex process for which the availability of an incomplete retinal image is by no means an obstacle. In order to be able to identify an object, the human visual system often has to ignore data, to seek meanings, gather elements, separate entities, continue contours, supplement forms and fill gaps.

The Gestalt point of view, according to which our visual system is a pattern construction machine, recently received the support of neuroscience by showing that certain retinal cells react selectively at a very early stage to discontinuities in luminous intensity. Pattern recognition involves cellular groupings in the geniculate body and visual cortex (cortical columns), some of which code for local alignments and contours in a given vicinity, and others that code for the polarities of contrast and discontinuities. Connections between these groupings enable us to make forms emerge in our perception (fig. VI.18b-d).

And what about calcified structures in this...

The perception of primary patterns in calcified structures involves identification processes of more or less closed concentric contours that present a range of contrasts and disruption in their continuity and in their optical density. All these parameters then vary according to the individual, the stage of development, the geographical area and the species involved. Perception also includes the processing of secondary patterns, such as scale *annuli* formed by the narrowing of *circuli*, or otolith *nuclei* whose contours include a set of *primordia*, or growth patterns made up of the varying width of growth zones.

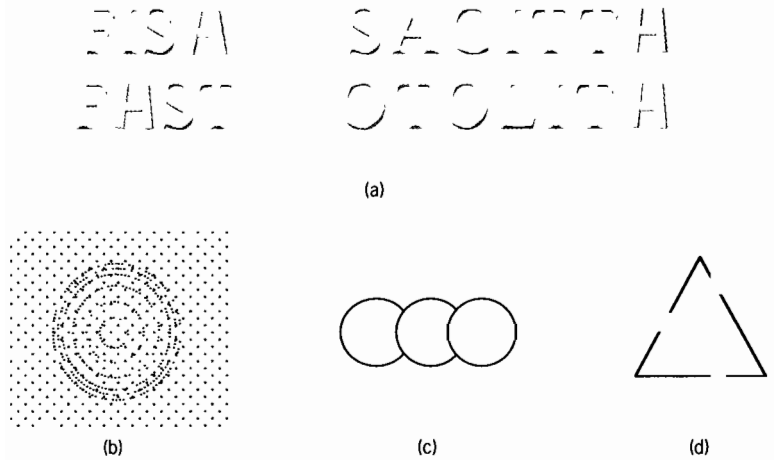


Figure VI.18 - Visual perception: a) Influence of context and knowledge on shape perception and completion. b) The perceptual system gathered the fragmented elements of each circle in only one unit although those are embedded in a noisy background. c) A figure made up of three complete discs interposed one behind the other emerges, rather than of only one in contact with two incomplete contiguous forms. d) Perceptive interpolation means that missing parts of an incomplete triangle are perceived as if they had also been present.

In conclusion we may say that age estimation on the basis of CS is a complex process involving information of different natures (visual, logic, biological) (Troade, 1992) and at different levels of perception, and that artificial vision techniques and concepts are multiple, not easily understandable by non-specialists and sometimes unsuitable for the purpose for which they are used (Benzinou, 2000). On this basis, we believe that only thorough interdisciplinary analysis, associating biological and signal processing concepts, will allow the development of efficient CAAGE systems.

B. How a computer can help in calcified structure analysis

Computer vision systems have gradually come to be regarded as a necessary component of the equipment of ageing laboratories, but this has not always been the case. This debate on the utility of the computers, heard many times during meetings and workshops, has opposed colleagues involved in various “branches” of sclerochronology, the most distant often being those involved in microstructure analysis and those specialised in the routine interpretation of otolith macrostructures. In fact, the arguments of the one party lost their relevance once they had been placed in the context of the work of the other. A CAAGE system now offers access to a wide range of tools that any age reader must be aware of. In addition to its own image acquisition, handling, processing and data storage functions, the computer is also an open door to other powerful methods of analysis such as signal processing technology or cognitive sciences (i.e., study of natural and artificial cognitive processes including artificial intelligence, cognitive psychology...), which themselves are currently continuously evolving.

1. What can you expect from a CAAGE?

When many virtues are attributed to the CAAGE systems: objectivity, accuracy, precision, productivity, memorising, most of them are quite simply properties of any computer assisted system. But what really happens when they are applied to age estimation problems? By emphasising the debatable concept of objectivity, have we hidden other properties that are just as essential to the analysis of calcified structures?

The systematic use of computers in sclerochronology started in the eighties in order to lighten the task of making repetitive measurements (microstructures analysis) and extracting shape factors. Assistance in morphometric data acquisition was, in practice, the first motivation of “sclerochronologists” in their recourse to CAAGE systems. Their other potentials for standardisation, precision or productivity gains or even data storage, which were only exploited in rather a scattered way, seem to have become essential only very slowly in laboratories working on routine stock assessments.

1.1. An aid to quantification

The first CAAGE systems were the outcome of the association of a computer and of a micro-densitometer (Ichiara, 1963; Van Utrecht & Schenckan, 1972) which allowed the recording and analysis of optical

profiles. Then, the appearance of cameras allowed two-dimensional information to be utilised (Mason, 1974), although the quantitative analysis continued to be more or less limited to image profiling. Of the quantitative information that is now available on calcified structures, we can distinguish:

- contrast information directly related to variations in optical density (zones of growth, checks, *circuli*, growth patterns);
- structural information relating to particular events (*nuclei*, *primordia*, accessory *primordia*), of particular structural arrangements (crossing-over, resorption marks, etc.) or reference positioning marks (reading axis);
- shape information.

If CAAGE systems offer very efficient assistance in the acquisition of contrast information, or assistance that is even essential for shape quantification, the situation is quite different for structural information, which is a more complex psycho-visual process, in which the human operator is still more powerful.

The ways in which a CAAGE system can help are:

- in producing a good-quality image with a high resolution and a high signal-to-noise ratio;
- in enabling us to improve image quality (contrast enhancement, noise reduction, etc.), tasks for which digital image processing offers many solutions (see chap. VI.A.1.2)

It also enables us to locate the growth zones on such images in a more or less automatic manner and thus to count them more easily. Growth zone localisation, associated with structural information such as the *nucleus*, offers us immediate access to the morphometric data of inter-increment distances as well as to other fine characterisation aspects of growth zones (intensity, contrast, frequency, etc.). The analysis of these data by spectral methods enables us to identify periodic phenomena (Vasil'kov, 1977, 1979; Geffen & Nash, 1995) and a prior knowledge of one of the signal components lets us introduce constraints into the age estimation process (Small & Hirschhorn, 1987; Lagardère & Troadec, 1997). Similarly, the analysis of temporal signatures associated with Bayesian methods allows incomplete growth histories to be exploited (Ogle *et al.*, 1996).

Image contrast enhancement also enables us to obtain a 2D contour of calcified structures and thus to quantify their shapes along with the automation of their measurements and their classification (Campana, 1987). This approach resulted in many studies of stock discrimination using fish scales and otoliths (Pontual & Prouzet, 1987; Campana & Casselman, 1993; Richards & Esteves, 1997) based on the external contours of the CS, a limitation which should disappear with the recent development of algorithms involving deformable models (Benzinou *et al.*, 1997; Troadec *et al.*, 2000).

1.2. Objectivity or standardisation?

Objectivity is the feature most frequently mentioned with respect to CAAGE systems (Fawel, 1974; Planes & Laval, 1990; Welleman & Storbeck, 1995; Cailliet *et al.*, 1996; Lagardère & Troadec, 1997). However, this is an ambiguous concept which very often hides a misreading of the deep mechanisms involved at the computer level in artificial vision (see VI.A.3.2.4), and which requires to be cleared up. By definition, according to the Cambridge International Dictionary of English, objectivity is "...not influenced by personal beliefs or feelings; based on real facts...".

On what does the objectivity of the computer in fact rest? Is it in the subjectivity of the programmer?

According to the algorithm selected, the computer will detect objectively, but more or less successfully, maxima or minima on an image profile, or perhaps growth zones if the algorithm was designed in 2D, and will finally detect any false rings if a clear definition is available. Most algorithms will then produce, in the presence of the same data, the same results, whether or not these are correct. The objectivity of the computer is thus structured by the conjunction of our capacity to correctly describe reality and the availability of suitable mathematical concepts.

In fact the "objectivity" of computers lies:

- in the reproductibility and the constancy of the results they produce, limiting drift but also the capacity to adapt that is often necessary with biological material;
- in our capacity to control the information introduced into the interpretation process, either by information masking (Morison *et al.*, 1998), or by the controlled introduction of information and constraints (Lagardère & Troadec, 1997).

Thus, the objectivity of the methods of computer vision is quite relative. In the face of discrepancies of diagnosis between human reader and computer, an expert will not have to abdicate his judgement and at the same time, the novice will still have carry out his training in the company of experienced human operators.

1.3. Accuracy and precision

The concept of accuracy has an absolute character and makes sense in sclerochronology only with reference to material of a known age or to validation experiments that permit estimates of growth mark periodicity to be made. The accuracy of a CAAGE system will depend straightforwardly (1) on image quality and (2) on the quality and availability of the identification criteria of growth zones as well as of their implementation in algorithms. Some studies of automated methods have thus noticed either a tendency to underestimate

(Troadec, 1991; Cailliet *et al.*, 1996) or to overestimate some age groups (Welleman & Storbeck, 1995; Troadec *et al.*, 2000), or better accuracy than that of the expert (Lagardère & Troadec, 1997). A doubling of image resolution does not provide an increase in performance (Troadec *et al.*, 2000). All depends in fact on the adequacy of the algorithm adopted; an underestimate of the growth zone number in old individuals is often related to overlooking the decrease in the width of the marginal growth zones in the course of time. On the contrary, overestimates maybe due to reduced implementation of the interpretation schemes, preventing the recourse to the usual criteria of ring validation (continuity, width/location of the growth pattern, etc.). Similarly, the capability of the human operator to focus his analysis on specific CS zones (Troadec *et al.*, 2000) or to adapt it to particular cases is still poorly simulated by current systems. However, we may notice the capability of CAAGE systems to reduce interpretation drifts (Morison *et al.*, 1998) by guiding the operators through a standardised process that is in itself an essential factor in improving the accuracy of the age estimation process. Incidentally, recourse to the computer will reduce, in a noticeable way, accidental errors such as retranscription or calibration errors (Fawel, 1974; Morison *et al.*, 1998).

Nevertheless, it should be recognised that even today, in spite of the continual improvement in their performances, CAAGE systems reach the expert accuracy only when CS present highly contrasted rings (Jearld, 1995) and remain generally on the low side of expert performance when they encounter interpretation difficulties.

Precision is regarded as the capacity of a system to produce repeatable measurements (see glossary). By its very nature, the use of a highly standardised procedure, coded in the form of computer program, will enable us to increase the precision of our estimates. The precision of a CAAGE system can be defined in the following terms:

- At the level of localisation and measurement of growth zones. Although poorly evaluated by users, the reproductibility of positioning, when carried out by a computer, is very high compared with that of a human operator.
- At the level of age estimation or the number of growth zones. While it enables us to apply the process in a rigorously identical way, it also supports the production of less variable estimates over time and between samples. In comparisons of the results produced by CAAGE systems with manual methods some authors (Cailliet *et al.*, 1996) concluded that they had a similar level of precision, while others noticed a slight advantage in favour of human operators (Szedlmayer *et al.*, 1991; Lagardère & Troadec, 1997).

1.4. Productivity

The productivity of a CAAGE system can be appreciated at various scales of the age estimation process, either at the level of a specific task such as the localisation of the growth zones, or at the level of the whole CS analysis process, or even at the level of the organisation of a stock assessment laboratory, by considering their capability for conserving, exchanging and revising interpretations. Does the CAAGE systems allow a real increase in productivity? And if so, how?

Time-saving is evoked by various authors (Cailliet *et al.*, 1996). Planes & Laval (1990) saved 50% on otolith microstructures, Watarai & Igarashi (1990, 1992) saved 30% on salmon scales, Szedlmayer *et al.* (1991) saved up to a ratio of 3.3, but none seem to have included the duration of ring detection control. Existing algorithms are essentially based on the processing of image profile. They allow entirely automated counting on very few species, i.e. reliable enough to avoid a systematic *a posteriori* control that can be a tedious task and time-consuming in the case of microstructures. In fact, all these authors were working in different contexts: macrostructures or microstructures, otoliths or scales, thin slice or whole samples, etc. This brings us to a question that will be addressed later: viz., what sort of study are we performing? Cailliet *et al.* (1996) consider that CAAGE systems do not allow us to treat samples prepared by means of fast methods (burning, whole sample) whereas Welleman & Storbeck (1995) and Troadec *et al.* (2000) evaluate fully automated algorithms on whole otoliths. Outwardly contradictory, these comparisons show that even though all are working within highly standardised contexts, the former worked on a deep-sea fish, *Sebastes rufus*, with high longevity and the latter on a flat fish, *Pleuronectes platessa*, with a short lifetime and a high rate of growth.

If digital technologies imply a high potential for gains in productivity, especially for routine readings and otolith microstructures analysis (batch digitalisation, "all day work"), they will be fully exploited only via the development of more reliable automation algorithms that will limit human intervention and operate night and day if necessary. However, such a development will also involve greater standardisation of sample preparation and interpretation procedures, phenomena which will be among the dominant tendencies in sclerochronology in the coming decade.

1.5. Data and knowledge preservation

Information storage, whether temporary or long-term, is one of the main advantages of CAAGE systems, from the simple capability of commenting about a CS on a video screen in a group (Morison *et al.*, 1998)

instead of having to follow one another on a microscope, to the direct storage of measurements (Cailliet *et al.*, 1996) or of interpreted images (EFAN, 1998).

This ability to store rough or "processed" information is essential to any long-term activity and/or an exchange framework, by facilitating the novice reader's training, by intensifying inter-reader exchanges, by improving the temporal stability of CS interpretations and finally by allowing revisions of such interpretations to be made. All these properties are essential, particularly to a quality insurance process.

However, currently available storage tools still present some defects:

- The digital image, after conventional digitalisation (CCIR camera), still does not provide the resolution of an average microscope. This limitation will tend to disappear with the improvement of frame-grabbing hardware. High-definition digital cameras (1024² pixels) already provide an image quality close to that obtained by the human eye in the eyepiece of the microscope.
- The availability of frozen images (Morison *et al.*, 1998) which do not allow further dynamic effects during observation, such as lighting variations, sample orientation or focusing adjustment. However, such operations are essential when three-dimensional information needs to be exploited throughout the thickness of the sample. Most of these limitations can be partially circumvented by the acquisition of image sequences or by the combination of data from different focal plans. This represents a huge amount of data, even if stored with a high compression ratio, and in any case means the loss of interactivity. Direct observation under the microscope is still often necessary.
- The longevity of image storage formats is not known, unlike that of conventional media. Formats are numerous, ranging from the well-known TIFF format to JPEG, which is among the most powerful in terms of compression ratio. An EFAN round table (EFAN, 1997) recommends for recourse to uncompressed formats for long-term storage and an ASCII format for associated data. In fact, it will be a question of defining exactly the nature of information to be stored and the expected duration of storage. The EFAN Cell "Information Processing" (EFAN, 1998, 2000) suggested a protocol using an image format (PaintShop-Pro, JascTM) which would enable graphic overlays to be managed to create a database of annotated images accessible via the Web by all the scientific community (<http://www.efan.no/tro/refdbin.htm>). This procedure has already been employed by some reading workshops (Anon., 1998). Storage solutions must adapt with use.

2. For what kind of study?

We have seen that the reasons for resorting to a CAAGE system are numerous and even though they still have some defects, the cost of these systems is no longer a real obstacle. Nevertheless, the various criteria involved will not carry the same weight but will depend on the objectives of the study. Do we need to automate growth zone counting, to memorise interpretations, to be able to exchange data with other readers? We usually distinguish age estimates related to one-off studies from those related to resource assessments, which are essentially recurrent (Morison *et al.*, 1998).

2.1. One-off studies

These studies aim to answer specific questions regarding the age and growth of given populations, or to use CS as recorders in order to analyse and put in temporal perspective certain parameters (salinity, environmental signature, etc.) or events (hatching, migration, reproduction, metamorphosis, etc.). The samples may be collected for several years and are then processed progressively or gathered for processing at the end of the study, very often by a single scientist.

In this type of study, the need for very long-term storage is less obvious but often concern microstructures analyses which require tools that allow good productivity. When experience of age estimation on the target species is non-existent, a validation study will be made, or the scientist will try to calibrate himself against other readers. Validation studies can be merged in the one-off studies as their goal is to answer a specific question, i.e. to define the periodicity of CS marks. In this case the storage of the results in an arbitrary form is fundamental.

2.2. Routine analysis

Routine analyses consist of establishing over a long period of time the evolution of the demographic structure of an exploited population. It is probably the most demanding type of activity in terms of the accuracy, precision and stability of inter- and intra-reader interpretative schemes. It obviously requires high productivity in order to deal with the large volume of samples involved. Generally speaking, routine analyses make use of several players: samplers, assistants, readers and assessors. These should have relatively massive recourse to CAAGE systems, but in fact the penetration of these systems has so far proved to be stronger among experts involved in one-off research studies than in routine laboratories. While they have most of the qualities required,

their performance is still regarded as being below that of human operators, particularly when they only deal with the counting of a few seasonal growth zones. Moreover, the introduction of such systems could lead to interpretation protocols being called into question and, in order to be effective, will require CAAGE software capable of integrating the whole analysis process (acquisition, interpretation, memorising). This will probably make up the framework of the routine ageing laboratories of tomorrow, but it still remains to be developed.

Chapter VII

Otolith microchemistry

H. de Pontual, A.J. Geffen

A. Introduction

Since the early 1970's there has been a growth in efforts to use the chemical composition of calcified structures to address a wide range of questions in fisheries science. This approach assumes that the chemical composition of hard tissues broadly reflects the physico-chemical characteristics of the environment to which fish are exposed.

Otoliths in particular have been referred to as continuous recorders of exposure to the environment (Campana *et al.*, 1997). Otoliths are metabolically inert, unlikely to be resorbed or reworked (Campana & Neilson, 1985) and grow throughout the life of the fish, so their composition probably has the highest potential of all calcified structures for carrying environmental information.

Recent years have seen a rapid development of the range of applications of otolith microchemistry (OMC), broadly divided into those that are concerned with fish ecology (reconstruction of the life histories of individual fish) and those that are concerned with populations (discrimination of fish populations, validation of age estimation). Otoliths are exceptionally pure in comparison with other biogenic carbonates. Otolith microchemistry is often interested in micro-scale variations in elemental concentrations and thus depends on several recent technical advances in analytical chemistry and geochemistry.

The decoding of otolith chemical information poses several problems: i) our poor knowledge of the mechanisms of element incorporation and their systems of regulation, ii) the difficulty of assessing the uncertainty (error probability) associated with the result of a particularly complex measurement procedure because all steps which lead to this result, starting from the fish capture, are potential sources of measurement error, and iii) the high costs of analyses. This has sometimes resulted in speculative interpretations and conflicting conclusions. However, advances in otolith microchemistry continue to develop, benefiting from a growing information base from on-going studies, from new technological developments, and from a growing understanding of the biomineralisation process.

The main goals of this chapter are to offer an insight into the state of the art of OMC, addressing those aspects of research that have applications in fish ecology and fisheries, analytical procedures and uncertainties in analytical processes. It largely refers to recent papers by Campana (1999) and Thresher (1999) that offer comprehensive overviews of current knowledge in fish otolith chemistry. Some basic chemical definitions and principles are reviewed in order to provide readers with the background essential for understanding microchemistry applications.

Otolith composition and its relationship with physiological and environmental conditions are described, followed by discussion of the various applications of microchemistry. The use of other calcified structures (scales, vertebrae, fin rays, etc.) is only briefly treated, as the methods and applications involved in recent years have concentrated primarily on the analysis of otoliths. The final section of this chapter presents some of the methodological issues that should be considered at the beginning of microchemistry studies.

B. Some basic chemical definitions and principles

Dalton's atomic theory of matter, which constitutes the base of modern chemistry, defines the *ATOM* as the simplest unit of matter capable of a separate existence (i.e. which cannot be subdivided in particles with the same characteristic properties), *ELEMENT* as substance with only one type of atoms, *COMPOUND* as substance containing more than one type of elements, and *MOLECULE* containing two or more atoms.

Although many subatomic particles are now known, for most chemical purposes the atom may be considered as consisting of a positively charged nucleus containing protons (positively charged) and neutrons (neutral), and surrounded by a cloud of negatively charged electrons. The atom (and hence the element and its position in the periodic table) is characterised by its atomic number (*Z*) which is defined as the number of protons in the nucleus and its mass number (*A*) which corresponds to the number of protons and neutrons (collectively called nucleons) in the nucleus.

Atoms with the same value of *Z* but different values of *A* (i.e. different numbers of neutrons) are termed *ISOTOPEs*. Isotopes of the same element have nearly identical chemical properties, yet they can have very different nuclear properties, possibly including radioactivity, magnetic characteristics and weight.

Variations in the relative abundance of the different isotopes of elements occur through different processes such as mass fractionations, radioactive decay and anthropogenic activities (e.g. processing of nuclear fuels, nuclear-weapons testing, etc.). Radioactive isotopes are unstable isotopes that spontaneously disintegrate over time to form other isotopes. Stable isotopes do not decay to other isotopes but may be produced by the decay of radioactive isotopes, and if so are termed

radiogenic. For instance, strontium has four naturally occurring stable isotopes: ^{84}Sr (0.56%), ^{86}Sr (9.86%), ^{87}Sr (7.0%), ^{88}Sr (82.58%). The numbers in parentheses refer to the naturally occurring relative abundance of these stable isotopes. ^{87}Sr can arise from either the primordial nucleosynthesis along with the other Sr stable isotopes, or from the decay of the radioactive metal ^{87}Rb .

Stable isotopes of light elements such as H, C, N, O and S are often measured in studies of trophic ecology, temperature effects and metabolism. There has also been recent interest in the isotope chemistry of heavy elements (e.g. Sr, U, Pb) as environmental indicators (Chesney *et al.*, 1998; Hobson, 1999).

Isotopic data is expressed as the relative difference of the sample from a standard, according to the following notation:

$$(VII.1) \quad \delta^h M = ((^h M / ^l M)_x / (^h M / ^l M)_{std} - 1) \cdot 1000$$

where $^h M$ and $^l M$ are the heavy and light isotopes of an element M, x is the sample and std is the standard. A delta (δ) value is reported in ‰ ('per mil') notation. A positive (negative) delta value indicates that the sample has a higher (lower) heavy to light isotope ratio than the standard. Several analytic standards are available for the different elements. For carbonates of low-temperature origin, $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ values are reported relative to either the PDB (Peedee belemnite) or the equivalent VPDB (Vienna-PDB) standard. Water oxygen isotopic values are usually reported relative to (Vienna) standard mean ocean water (SMOW or VSMOW) for marine studies, or standard light Antarctic precipitation (SLAP) for freshwater studies.

1. Stable isotope mass fractionation

Because of differences in atom bond strengths between heavy and light isotopes in molecules, isotopes fractionate between coexisting phases during physical or chemical processes.

Fractionation occurs via either kinetic or equilibrium processes the latter resulting in temperature-dependent isotopic fractionation. This is the basis of stable isotope thermometry.

Fractionation of isotopes between two phases A and B is expressed, for a given temperature, by the fractionation factor α :

$$(VII.2) \quad \alpha_{A-B} = R_A / R_B$$

where R is the isotopic ratio such as $^{13}\text{C}/^{12}\text{C}$, $^{18}\text{O}/^{16}\text{O}$, etc.

In terms of δ values, this expression becomes:

$$(VII.3) \quad \alpha_{A-B} = (1000 + \delta_A) / (1000 + \delta_B)$$

Values of α are generally very close to unity for most elements of interest, typically 1.00X, and it is common to discuss isotopic fractionations in terms of the value of X in ‰ (per mil fractionation).

Furthermore, the per mil fractionation is approximately equal to $10^3 \ln \alpha$ since $10^3 \ln(1.00X) \approx X$.

Additionally, when values of both δ_A and δ_B are less than about 10, the per mil fractionation, $10^3\ln\alpha$, is very well approximated by the Δ value, defined as:

$$(VII.4) \quad \Delta_{A-B} = \delta_A - \delta_B \approx 10^3\ln\alpha$$

A major reason for the interest in using the “ $10^3\ln\alpha$ ” expression is that, at biologically relevant temperatures ($< 200^\circ\text{C}$), $10^3\ln\alpha$ is nearly proportional to the inverse of temperature ($1/T$).

Interest in oxygen isotope records of marine carbonates as a tool for paleotemperature measurement rose in the early 1950s, and Epstein *et al.* (1951, 1953) provided the first paleotemperature equations based on laboratory-grown bivalves. Isotope fractionation is often referred to as being in equilibrium. In this context, equilibrium refers to the energy state rather than to a stable isotonic situation. The equilibrium energy state can be calculated directly from the strength of the atomic bonds, which differ for the different isotopes. Because temperature affects energy state in a predictable fashion, the isotope ratios of chemical processes, such as precipitation, are calculable. When the measured isotope ratios in carbonate conform to predictions (as illustrated in the above equations) the system is said to be in equilibrium.

2. Radioactive decay

Only a small proportion of isotopes are known to be indefinitely stable. All the others disintegrate spontaneously by processes broadly designated as radioactive decay. This process transmutes the so-called “parent” radioactive isotope into a more stable isotope, a so-called “daughter”, specific to that parent. The process continues until a stable nucleus is produced. For instance, the ^{238}U decay series undergoes 14 stages of decay reaction before the end product (^{206}Pb) is formed. Radioactive decay is a statistical process that depends only on the instability of each particular radioisotope. The decay activity over time can be expressed by the following exponential radioactive decay equation:

$$(VII.5) \quad A^t = A^0 \cdot e^{-\lambda t}$$

where A^0 is the initial activity of the parent isotope (in becquerels or disintegration.time $^{-1}$), A^t is the activity at time “t” and λ is the decay constant (in time $^{-1}$).

The rate of radioactive decay is typically expressed in terms of either the radioactive half-life, T, which is the time required for one-half of any given quantity of a radioisotope to decay, or the radioactive decay constant. They are related as follows:

$$(VII.6) \quad T = \ln 2/\lambda = 0.693/\lambda$$

If the initial activity A^0 of a closed system is known or can be estimated, the time elapsed since the system was formed can be estimated from equation VII.5. This is the basis for radiometric dating. When A^0 is not known, radiometric dating can be performed in some cases by using the so-called disequilibrium dating methods, which are based on the measurement of the activity ratio of two members of a decay series.

The principle is the following: once a parent isotope (P) has been incorporated into a system it begins to decay, a process that results over time in an increase in the activity (so-called ingrowth) of a shorter-lived daughter product (D). The activity of D, which depends on the balance between its own radioactive decay and its production by the decay of P, is given by the Bateman equation:

$$(VII.7) \quad A_D = A_P^0 [\lambda_D / (\lambda_D - \lambda_P)] (e^{-\lambda_P t} - e^{-\lambda_D t}) + A_D^0 e^{-\lambda_D t}$$

in which the second term is the residual daughter activity remaining from any daughter product that was present at $t=0$;

The ratio of the daughter activity to the parent activity can thus be derived from equation VII.5 and VII.7:

$$(VII.8) \quad A_D / A_P = [\lambda_D / (\lambda_D - \lambda_P)] (1 - e^{-(\lambda_D - \lambda_P)t}) + A_D^0 / A_P^0 e^{-(\lambda_D - \lambda_P)t}$$

If the system is not perturbed, a steady state in which the activity ratio remains constant will be reached, at a rate which depends on the daughter's and parent's half-lives. Two situations can occur:

The first one, known as "transient equilibrium" is the situation in which the half-lives of P and D are of the same order, but $T_P > T_D$. In that case, the activity ratio increases with time, approaching the value of $T_P / (T_P - T_D)$ asymptotically (fig. VII.1a).

The second situation called "secular equilibrium" occurs when the half-life of P is much longer than the half-life of D and the decrease of the activity of P is negligible over a practical time scale. In that case the Bateman equation reduces to equation VII.9 and the activity ratio simplifies to equation VII.10:

$$(VII.9) \quad A_D \approx A_P (1 - e^{-\lambda_D t}) + A_D^0 e^{-\lambda_D t}$$

$$(VII.10) \quad A_D / A_P = 1 - e^{-\lambda_D t} + A_D^0 / A_P^0 e^{-\lambda_D t}$$

In that case the activity ratio increases with time, approaching the value of 1 asymptotically at a rate which depends on the half-life of the daughter (fig. VII.1b).

In conclusion, in both situations, the measurement of the activity ratio can provide an estimation of the time elapsed since the parent isotope was incorporated, in this case into the carbonate sample. The respective half-lives of the parent and daughter govern the time-scale to which the method will be applicable. Due to the exponential form

of the decay law, the precision of the estimates, which depends on the analytical measurement errors of both parent and daughter activity, will decrease as the equilibrium state is approached (fig. VII.1). It is worth noting that the method is valid only if the system meets the criteria discussed below regarding otolith radiometric ageing.

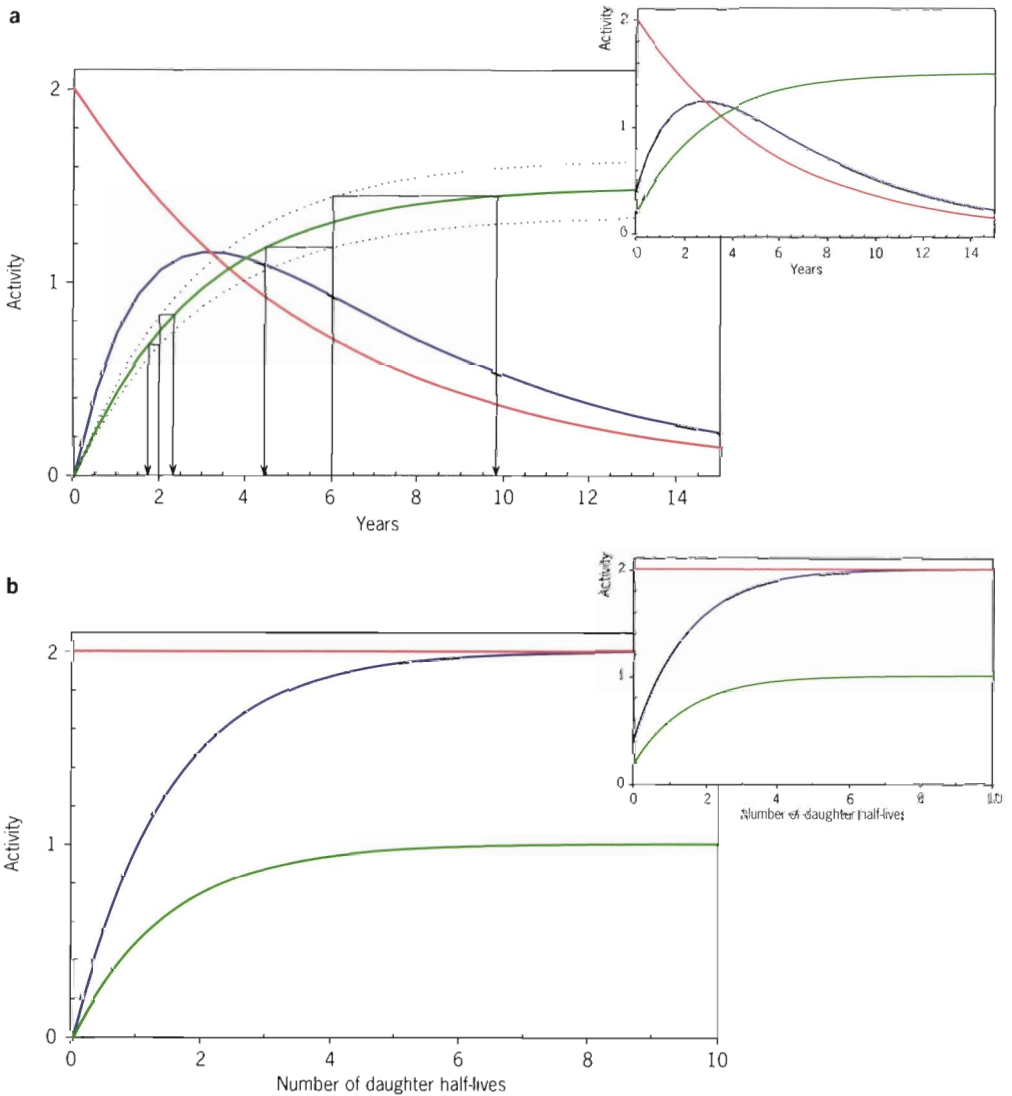


Figure VII.1 - Relationships of parent (red curve) and daughter (blue curve) activities and activity ratio (green curve) as a function of time. (.....) upper and lower estimates of activity ratio with a $\pm 10\%$ measurement error; (→) effect of the measurement error on the age estimate range.

a) Transient equilibrium situation. Modelled with $T_P = 5.76$ years and $T_D = 1.91$ years, the half-lives of ^{228}Ra and ^{228}Th respectively and A_P arbitrarily set to 2. Insert: initial activity ratio set to 0.2.

b) Secular equilibrium situation. A_P was arbitrarily set to 2. Insert: initial activity ratio set to 0.2.

C. What are otoliths made of?

Otoliths are composed of calcium carbonate (CaCO_3) crystallised on an organic matrix (Dannevig, 1956; Degens *et al.*, 1969). They are relatively pure compounds, as has been shown by a number of broad elemental assays which demonstrated that the total of inorganic impurities amounts to less than 1% of otolith weight (Edmonds *et al.*, 1992; Thresher *et al.*, 1994; Proctor *et al.*, 1995; Campana *et al.*, 1997). Recent studies have also suggested that the otolith is impregnated with the endolymph in which it bathes (Gauldie & Coote, 1997; Gauldie & Cremer, 1998; Milton & Chenery, 1998; Proctor & Thresher, 1998) indicating that the fluid component may be of major importance in determining the ultimate composition of the otolith (Thresher, 1999).

There are few studies of the fluid and organic components of otoliths, although these are critical to understanding otolith growth and composition.

In comparison with blood plasma, endolymph is rich in K^+ , relatively low in Na^+ , has a total Ca concentration of $\sim 1\text{--}2\text{ mM}$, and a relatively low protein content (fig. VII.2). The pH is more alkaline than that of the blood plasma, unlike what is observed in higher Vertebrates (Mugiya & Takahashi, 1985; Payan *et al.*, 1997, 1998). An important characteristic of the endolymph is that its principal components display increasing or decreasing proximodistal gradients of concentration that probably act as driving forces in the biomineralisation process and are able to generate a heterogeneous distribution of some elements (e.g. K) on the otolith surface (Payan *et al.*, 1999). Although the mechanisms are still poorly understood, it is now recognised that the endolymph proteins play a pivotal role in calcium carbonate precipitation and inhibition, as they also do for other processes of biomineralisation and organomineralisation (involving non-living organic matter) (see e.g. Trichet & Defarge, 1995).

The otolith protein matrix was first analysed and termed "otolin" by Degens *et al.* (1969). The total protein content of an otolith can be divided in two components: water-soluble proteins (WSPs) and water-insoluble proteins (WIPs). In tilapia, WSPs account for nearly half of otolith total proteins, are characterised by a high content of acidic amino acids and have a high capacity for calcium binding (Asano & Mugiya, 1993). They contain a glycoprotein that is probably involved in the regulation of accretion rate by acting as a calcification inhibitor (Wright, 1991b). The role of WIPs is presumably structural (Campana, 1999).

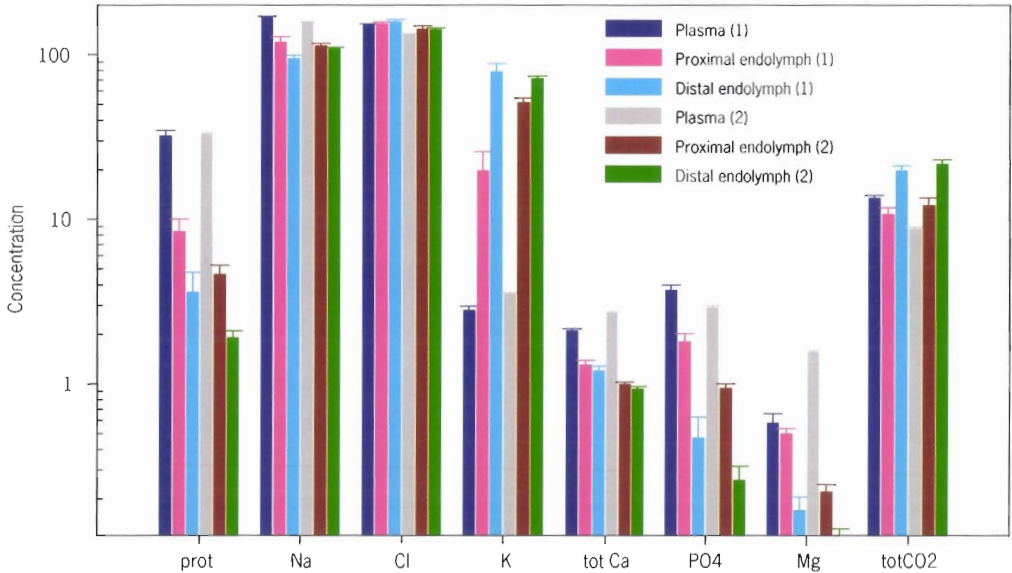


Figure VII.2
Ionic, total CO₂ and protein concentrations of plasma and saccular endolymph in trout (1) and turbot (2). Concentrations are given in mM for all constituents except for proteins, which are in g.l⁻¹. Data are from Payan *et al.* (1999).

The elemental composition of otoliths has been broadly assessed in recent years for a variety of species and environments. Campana (1999) reported a total of 31 elements that have been detected in otoliths, not including radioisotopes such as Th and Ra. As anticipated, and due to improvements in analytical detection limits, more elements have since been detected (e.g. Sc, Ti, V Geffen *et al.* unpublished and Tl, Rb Pontual *et al.*, 2000).

Figure VII.3 shows the distribution across the periodic table of elements detected in otoliths. The most abundant elements in the otoliths of marine species are found in groups 1-2 and 14-17 of the periodic table, which reflects to some extent the relative elemental abundance in seawater. Transition metals as well as lanthanides and actinides are less abundant.

The range of concentrations of otolith elements with respect to major habitat types is summarised in figure VII.4. The dominance of calcium carbonate means that Ca, C, and O are the major elements. Sr, Na, K, S, N, Cl and P have been reported in concentrations greater than 100 ppm (minor elements) whereas all other elements are present at trace or infra-traces levels and can only be assayed by means of very sensitive analytical techniques.

It is worth noting that the elemental composition depends on the otolith CaCO₃ polymorphs. Although aragonite is the normal crystal morph for *sagittae* and *lapilli*, it may be replaced, often partially, by vaterite (or more rarely calcite) in so-called abnormal "crystalline" otoliths (Carlström, 1963; Gaudie *et al.*, 1993). The mechanisms of replacement are not yet fully understood but clearly result in different

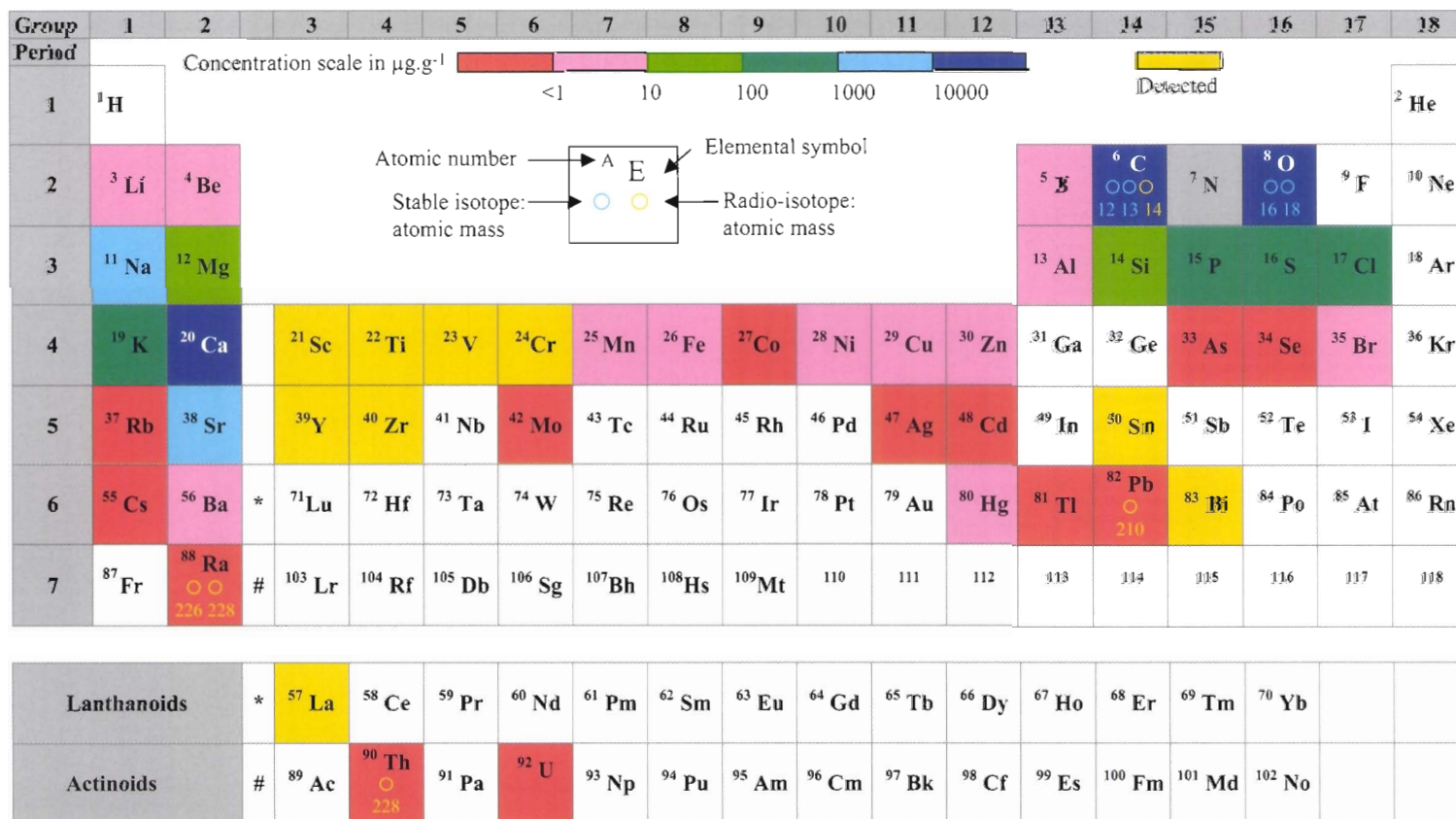


Figure VII.3 - Distribution across the periodic table of the elements detected in otoliths. Atomic mass is reported for isotopes used either in stable isotope chemistry or radiometric dating. The concentration ranges apply to results from marine fish. Nitrogen is present in significant quantities as part of the protein component, but the absolute concentration is not easily quantified.

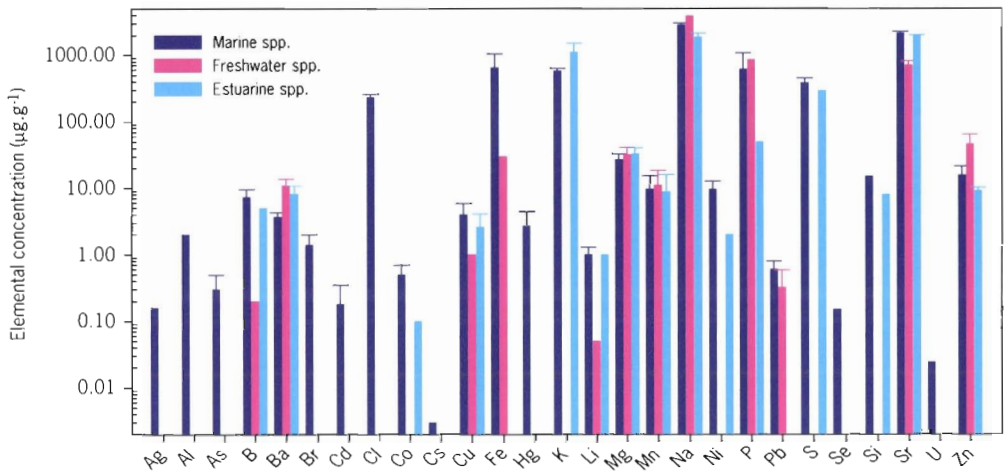
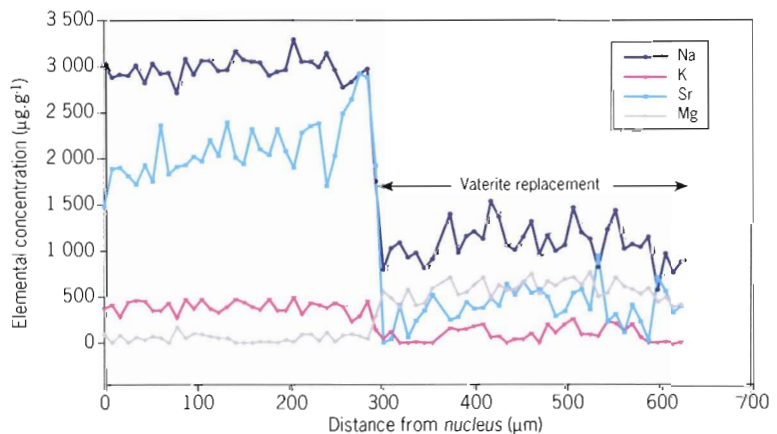


Figure VII.4 - Summary of published otolith minor and trace element composition (mean $\pm$ 1 SE) from three major habitat types. Bars without error bars indicate element concentrations that have been reported in only one study. Calcium (not shown) was reported as 38.02 ± 0.50 and $40.72 \pm 1.82\%$ of otolith weight for marine and freshwater species respectively. Data are from Campana (1999).

contents of minor elements and trace elements: for instance the vaterite portion of a turbot otolith has lower concentrations of Sr, Na and K and higher concentrations of Mg and Ca than the normal aragonite portion (fig. VII.5). Brown & Severin (1999) also reported decreases in Sr, Na and K in vaterite otoliths, but did not provide data on Mg concentration. Thus, it is important to ensure that otolith samples collected for analysis are 'normal' aragonite, and crystalline otoliths should be avoided for routine analyses. Micro-Raman spectroscopy can accurately determine the presence and location of changes in CaCO_3 polymorph (Gauldie *et al.*, 1997), and this information can help to interpret sudden (small spatial scale) changes in elemental composition.

Figure VII.5
Change in elemental concentration in a partially vaterite turbot otolith. Measurements were obtained using a Cameca SX 50 WDS fitted with five spectrometers (Pontual unpublished data).



D. Otolith elemental uptake: how and where?

Although this question has been paid relatively little direct attention until now, it is crucial for applications of OMC to assess what the process of otolith formation actually represents.

Unlike other biomineralisation processes, otolith formation is an acellular process and is completely dependent on the endolymph. The translation of environmental factors into otolith composition is a complex process which involves four nested compartments:

- the external medium, where variations in abiotic factors occur;
- the blood plasma which responds to the external medium but also exhibits endogenous variations;
- the endolymph, which modulates the various signals and regulates the formation of the otolith;
- the otolith itself, which integrates and records a response to all these signals.

Our current state of knowledge about each of these stages in otolith formation, and how each affects otolith composition, is summarised below.

1. Between the external medium (water and food) and the blood plasma

The inorganic plasma content is mainly derived from the surrounding waters, either by branchial uptake in freshwater fish or by assimilation through the intestinal epithelium in marine fish, which swallow considerable quantities of water as part of their osmoregulatory process (Simkiss, 1974). Uptake processes depend on a large number of factors, including chemical element properties (see e.g. Phillips & Rainbow, 1994). Schematically, uptake of major alkaline metal ions such as Ca, Na, Cl, and non-metallic ionic species such as chloride, sulphate and phosphate occurs through active transport pumps and is highly regulated. Trace metal uptake depends on the relative ambient ionic concentrations, and may be species- and environment-specific. The uptake process is regarded as passive, not requiring expenditure of energy. It depends on the affinity of water-soluble ions with organic ligands (e.g. metal-binding proteins) present in the membrane of the exchange surfaces.

Elemental uptake also depends on abiotic characteristics of the environment such as pH, salinity, dissolved oxygen, and temperature which determine the concentration of free ions available for uptake.

Along with uptake from surrounding waters, some elements, especially essential micronutrients and contaminants, are at least partly supplied by the diet, as has been shown by several studies of fish nutrition and ecotoxicology (see e.g. Bijvelds *et al.*, 1998; Cooley & Klaverkamp, 2000). At the present time, it is difficult to generalise about the incorporation pathways of all elements. Otolith Sr, in particular, has been shown to respond to assimilation from food and water in juvenile shad *Alosa sapidissima* (Limburg, 1995), juvenile tilapia *Oreochromis niloticus* (Farrell & Campana, 1996) and juvenile *Girella elevata* (Gallahar & Kingsford, 1996) but only from water in red drum *Sciaenops ocellatus* (Hoff & Fuiman, 1995).

2. From the blood plasma to the endolymph

Very few studies have examined the relationships between blood plasma and endolymph chemistry. Following pioneering works on otolith biomineralisation by Mugiya and co-workers (Mugiya, 1966; Mugiya & Takahashi, 1985), Kalish (1991a) examined the effects of physiology and environment on otolith chemistry by analysing seasonal variations in otolith composition (Ca, Sr, Na, K, S) in relation to variations in the chemistry of the blood plasma and the endolymph. Both ionic content and various metabolites such as proteins were analysed. In combination, these determine the proportion of free ions exchangeable through the saccular epithelium. Results indicated that the physiological (reproductive) status of the fish was the driving factor in the seasonal variation of the otolith Sr/Ca. Starvation slightly decreased plasma Ca concentration, and modified the acid-base equilibrium in the blood plasma as well as in the endolymph, but the ionic content of both fluids was similar in starved and feeding fish (Payan *et al.*, 1998). Edeyer *et al.* (2000) showed that plasma and endolymph composition varies during the daily cycle in *Scophthalmus maximus*, but ionic gradients within the endolymph (Payan *et al.*, 1999) are maintained. There are no published data on the content of trace elements in the endolymph, and the transport mechanisms for various elements through the saccular epithelium are still being debated. Given the complex cellular mapping of the saccular epithelium, including different types of ionocytes, (Takagi, 1997; Pisam *et al.*, 1998), it is likely that active and regulated ionic transports occur through this interface. Kalish (1991a) inferred from the correspondence between blood plasma and endolymph Sr/Ca that Ca and Sr may be transported into the endolymph by a paracellular route. Mugiya & Yoshida (1995), working on isolated *sacculi*, concluded that these elements were transported by a transcellular route in *Carassius aurata*.

3. From the endolymph to the otolith

The extent to which variations in endolymph composition ultimately affect the composition of the otolith has not been clearly established yet and the scarce data available on this subject only deal with calcium and a few minor ions. Although there are obvious relationships between the composition of endolymph and that of the otolith (Kalish, 1989, 1991a; Payan *et al.*, 1999), the correlation between the two media may be not as high as had been anticipated. Data from Kalish (1991a) indicate that seasonal variations in endolymph and otolith composition did not match closely for Na, K and Sr, although similar general trends have been observed for Sr. Payan *et al.* (1999) showed that the increase in the distal endolymph K concentration was reflected in the distal otolith K/Ca ratio. However the difference in Na/Ca between the proximal and distal sides of the otolith could not have been explained by the endolymph Na/Ca ratio.

Such studies pose various problems both due to the characteristics of the endolymph and the otolith and to methodological limitations. In the first place, the temporal resolution differs for the measurement of endolymph and otolith composition. Changes in the endolymph can be measured instantaneously, showing for example diurnal variations (Edeyer *et al.*, 2000). Changes in the otolith are integrated over a period which depends both on the diameter of the analytical probe (see below) and otolith growth rate. Secondly, methods of analysing endolymph composition have usually been restricted to measuring total ionic content, while it is actually the concentration of free ions (i.e. not linked to metal-binding proteins) that is the relevant information in terms of otolith element uptake. Lastly, methods of measuring chemical composition may also be limited by analytical interference for some elements of interest.

Element-specific incorporation processes have not been studied in detail. Elements are either incorporated within the crystal, adsorbed onto its surface or directly bonded to the organic matrix polymers. Incorporation within the crystal may occur by one of two mechanisms; substitution, which occurs for cations of similar size and charge (e.g. Sr can substitute for Ca), or co-precipitation, (e.g. magnesite MgCO_3). It is hypothesised that Li and Ba also share this mode of incorporation, whereas elements such as Na, Cl, Zn and K could be adsorbed onto the crystal surface in the interstitial space (Campana, 1999). Virtually nothing is known about the elements that could be bonded to the organic compounds, apart from the elements that make up the amino acids (C, H, O, N, P, S). In general, more information is available about the incorporation of cations than anions. In the past the incorporation of divalent cations into the mineralised portion of the otolith

has been assumed to be by substitution, whereas monovalent cations are thought to fit into inter-crystalline spaces. However, as the measurement of trace elements becomes more reliable, the existence of different mechanisms of incorporation will have to be acknowledged and reconciled with models of otolith composition. From this perspective, the current lack of data on the elements associated with the protein component of the otolith is especially problematic.

4. Elements that are likely to vary depending on environmental availability

This question is obviously of major concern in OMC. Campana (1999) identified elements that are likely to vary depending on environmental availability, on the basis of arguments which are briefly summarised here:

First, distribution coefficients D_e between water and otolith are useful indicators of elements that are highly physiologically regulated (i.e. discriminated for or against at one or more of the interfaces: gills, intestine, saccular epithelium, otolith). The distribution coefficient is $D_e = (\text{element}/\text{Ca})_{\text{otolith}} / (\text{element}/\text{Ca})_{\text{water}}$ with element and Ca referring to molar concentrations.

Very low distribution coefficients (< 0.05) are observed for elements such as Na, K and Cl, whereas D_{Sr} is about 0.14 and, for many trace elements, the distribution coefficient is greater than 0.25 and may even approach 1.

Secondly, comparisons of published data on water, blood plasma and otolith compositions (normalised to Ca) for freshwater and marine species lead to inconsistent relationships for Mg, Cu, P, and Na. The consistency was greater in the case of elements such as Sr, Zn, Pb, Mn, Ba and Fe, indicating that the relative environmental abundance of these latter elements may be well reflected in the otolith. Campana (1999) also noted that Li, Cd, Ni and other less abundant elements may well respond to environmental availability.

This classification remains somewhat speculative, and awaits further investigation. As noted by Thresher (1999) the distinction between minor ions that are physiologically regulated and “unregulated” trace elements may be problematic, given that some trace elements are known to be involved in fish metabolism as essential micronutrients, or are toxic (or both, depending on the concentration level). For instance, Zn is known to be an essential micronutrient that is involved in the formation of fish bone and cartilage, becoming toxic at high concentrations. Cadmium, which is also present in the environment, is a toxic metal thought to interfere with Zn metabolism. The intestinal assimilation of both elements has been shown to differ, possibly

because of the presence of carrier-mediated pathways for Zn uptake in the intestine (Baskin *et al.*, 1999). Regarding otolith Zn uptake, it is not clear, for instance, whether the oscillatory annual pattern of Zn concentrations observed in *Salvelinus alpinus* otoliths reflects changes in environmental bioavailability or fish metabolism (Halden *et al.*, 2000).

E. Applications of otolith microchemistry

1. Patterns of migration and environmental history

1.1. Salinity

1.1.1. Migration behaviour as revealed by Sr/Ca ratio

Seawater Sr and Sr/Ca exhibit a relatively small (2–3%) spatial variability with surface water slightly depleted relative to deep ocean and highest surface values found at high latitude and upwelling areas (Villiers, 1999). Although highly variable depending on geology, weathering and hydrographic conditions, freshwater Sr and Ca concentrations are much lower (about 60 µg/l and $1.5 \cdot 10^4$ µg/l respectively versus 8000 µg/l and $4.2 \cdot 10^5$ µg/l in seawater). The Sr/Ca variability (of about a factor 5) between fresh and salt water has supported the use of otolith Sr/Ca ratios in the investigation of the migration strategies of a number of species. Sr/Ca ratio can be measured using various techniques, some of which can provide high temporal resolution (fig. VII.5 and chap. VII.G).

Species that undertake diadromous migrations during their life cycle have been extensively studied. Anguiliformes (*Anguilla* spp.) exhibit a clear decrease in Sr/Ca ratio when they migrate from the marine environment to freshwater (Casselman, 1982; Otake *et al.*, 1994; Tzeng *et al.*, 1997). However, comparisons of the Sr chronology with metamorphic checks on the otolith have indicated that the initial decline in Sr/Ca is not related to the habitat as such but rather is associated with the leptocephalus-glass eel transformation (see below). Such chronologies have also provided information regarding the rate at which fish enter the estuarine nursery grounds.

Close relationships between otolith Sr concentration and ambient salinity have also been found in Salmonids and have been used to distinguish between anadromous and non-migratory individuals (Kalish, 1990; Halden *et al.*, 1995; Babaluk *et al.*, 1997). Some cases of residency have also been identified in striped bass *Morone saxatilis* populations previously thought to display anadromous migration behaviour (Secor *et al.*, 1995b; Secor & Piccoli, 1996).

Otolith Sr concentration has also been used to infer the down-estuary movements of juvenile *Alosa* spp. (Limburg, 1995, 1998), age- and size-specific coastal dependency of adult *Morone saxatilis* and spawning migrations of different species (Secor & Rooker, 2000).

Field studies have been confirmed by laboratory validations. For instance, Secor *et al.* (1995b) showed that Sr/Ca ratios in the otoliths of juvenile *M. saxatilis* were positively related to ambient salinity.

These authors also showed that the otoliths of fish exposed to various salinities also showed fluctuations in the Sr/Ca ratio that corresponded to the experimental salinity changes (Secor *et al.*, 1995b). Otolith Sr/Ca ratios reflect the ambient Sr/Ca ratio rather than salinity *per se* or absolute Sr water concentration. This may explain why studies that manipulated salinity over a narrow range of variation (e.g. Fowler *et al.*, 1995) failed to establish a clear relationship between otolith Sr/Ca and ambient salinity.

1.1.2. Sr isotope composition

It has been clearly established that the isotopic composition of dissolved Sr (measured by the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio) in stream water depends on local geology and is thus location-specific. A strong relationship between otolith $^{87}\text{Sr}/^{86}\text{Sr}$ and ambient water $^{87}\text{Sr}/^{86}\text{Sr}$ would be expected, assuming that the Sr isotopes do not fractionate through uptake and assimilation. This hypothesis was first validated on juvenile *Salmo salar* (Kennedy *et al.*, 1997). Moreover, Kennedy *et al.* (2000) recently showed that food contributes to the majority of salmon otolith Sr, suggesting that incorrect identification of the origin of individuals may result when comparisons are based on hatchery water source signature alone (Ingram & Weber, 1999).

Manipulating the temperature and salinity of rearing water of juvenile *Brevoortia patronus*, Chesney *et al.* (1998) found that otolith $^{87}\text{Sr}/^{86}\text{Sr}$ did reflect the salinity of the rearing water but was not affected by water temperature. However, they concluded from their experimental results that the utility of Sr isotope ratios as a precise indicator of salinity is limited to low salinity environments (<20) or over wide ranges of salinity.

1.1.3. Stable isotopes

Water masses are often characterised by differences in oxygen isotope ratios and fish movements between water masses can thus be traced by measuring the oxygen isotope ratios of the otoliths. Both temperature and water composition affect the otolith oxygen isotope ratio. In marine environments, the range of water isotope ratios is narrow, and most of the variation in otolith oxygen isotope ratio is due to temperature differences (fig. VII.6a). However, freshwaters vary considerably in oxygen isotope ratio because of the sources of these waters. The otolith oxygen isotope ratios of fish from freshwater environments, and from coastal waters affected by freshwater run-off, are significantly influenced by both temperature and the oxygen isotope ratio of the surrounding waters (fig. VII.6b). Accurate temperature predictions from these environments require concurrent samples of water to

measure the environmental isotope ratios. However, for migration studies, relative values of isotope ratios may be sufficient. For example, differences in isotope ratios can be used to distinguish between migrating and resident fish (Nelson *et al.*, 1989; Kalish, 1990; Northcote *et al.*, 1992).

In practice, otolith oxygen isotope ratios have to be measured from bulk samples (from dissolved whole otoliths or cores), so it is difficult to trace small-scale movements and migrations in individuals.

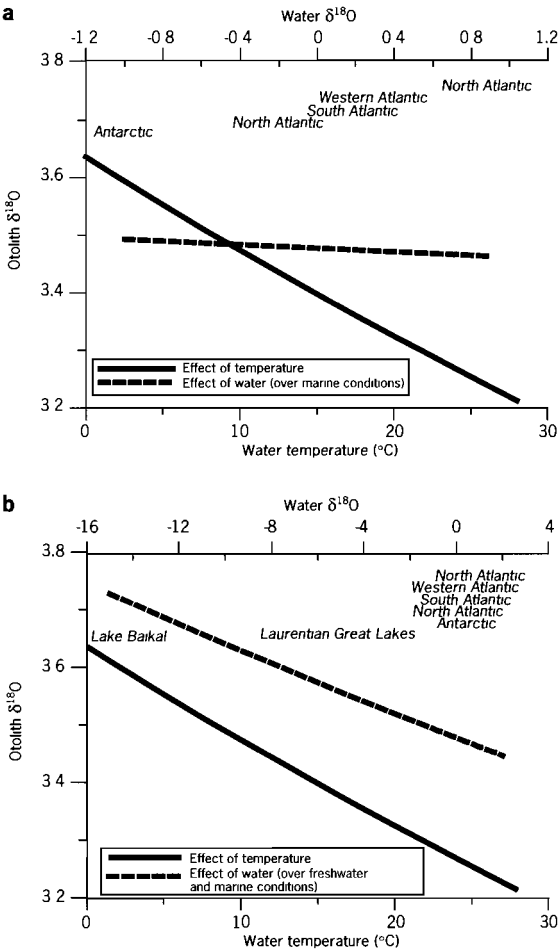


Figure VII.6 - Otolith oxygen isotope ratios (otolith $\delta^{18}\text{O}$) in relation to water temperature (solid line, lower axis) and in relation to water isotope ratio (water $\delta^{18}\text{O}$).
a) Across representative marine environments as indicated.
b) Across representative freshwater and marine environments as indicated.

Developments in the use of microdrills allow discrete samples of otolith material to be removed along tracks that follow growth zones. Up to 20 discrete samples can be taken within each *annulus* with the aid of computer-controlled micro-milling systems (Wurster *et al.*, 1999; Weidman & Millner, 2000).

1.2. Temperature

1.2.1. Sr/Ca ratios as indicators of individual thermal history?

Early geochemical studies predicted that increasing amounts of Sr should substitute for Ca in inorganic carbonate as temperature decreased (Kinsman & Holland, 1969, in Gallahar & Kingsford (1996), and Sr/Ca ratios have been measured in coral skeletons as a recording thermometer (e.g. Smith *et al.*, 1979). With regard to otoliths, numerous field and laboratory experiments have been performed, but these have provided conflicting conclusions. The relationship between Sr/Ca ratio and temperature has been reported as either negative, positive, non-existent or inconsistent (tab. VII.1).

Table VII.1. Some reported effects of temperature on Sr/Ca ratios (see also Secor & Rooker, 2000).

Species	Life stage	Experiment	T°C	Sr/Ca = f(T)	Comments	Reference
<i>Arripis trutta</i>	Juvenile	Lab-reared	13-22	→ ↗	No clear relationship	(Kalish, 1989)
<i>Fundulus heteroclitus</i>	Larval	Lab-reared	16-32	↗ ↘	Adjusted linear model	(Radtke & Morales-Nin, 1989)
<i>Clupea harengus</i>	Larval	Lab-reared	6-13	↗ ↘	Adjusted linear model	(Radtke <i>et al.</i> , 1990)
<i>Clupea harengus</i>	Juvenile	Lab-reared	2-18	↗ ↘	Adjusted hyperbolic model	(Townsend <i>et al.</i> , 1992)
<i>Gadus morhua</i>	Larval	Lab-reared	5-14	↗ ↘	Adjusted exponential model	(Townsend <i>et al.</i> , 1995)
<i>Haemulon plumieri</i>	Adult	Field	11-30	→ ↗	Inverse relationship between Sr/Ca and body growth rate	(Sadovy & Severin, 1992)
<i>Epinephelus guttatus</i>	Adult	Field	17-30	→ ↗	Inverse relationship between Sr/Ca and body growth rate	(Sadovy & Severin, 1994)
<i>Sciaenops ocellatus</i>	Larval Juvenile	Lab reared	21-34	↗ ↗	Adjusted model (Sr/K, Ca, Na); also investigated diet and salinity	(Hoff & Fuiman, 1995)
<i>Pagrus major</i>	Juvenile	Reared (sea farms)	21-29	↗ ↗	Also investigated Fe, Mn, Zn	(Arai <i>et al.</i> , 1995)
<i>Girella elevata</i>	Juvenile	Lab reared	19 & 28	→ ↗	Inconsistent effects of increasing and decreasing temperature; also investigated Sr-enriched water and diet	(Gallahar & Kingsford, 1996)
<i>Morone saxatilis</i>	Juvenile	Lab reared	15 & 25	→ ↗, ↗ ↘	Inconsistent effect of T with varying salinity	(Secor <i>et al.</i> , 1995b)

Kalish (1989) early expressed doubts about the universal temperature dependence of the Sr/Ca ratio in fish otoliths, suggesting that the endolymph Sr concentration may vary seasonally and with age in a given species rather than respond directly to temperature.

Townsend *et al.* (1992) argued that at extremely low temperatures fish become increasingly unable to physiologically control the penetration of Sr into the endolymph by the concentration of calcium-binding proteins in the plasma. These authors also noted that the slowing of growth and metabolism in winter provided support for the hypothesis of physiological interference processes on the incorporation of strontium. The latter hypothesis was supported by Sadovy & Severin (1992, 1994) who did not find any consistent temperature effect but related the Sr/Ca variation to that of body growth rate.

The apparent inconsistency of a general temperature effect has been "explained" by several authors as the result of the regulation processes of strontium incorporation (and more generally on elemental incorporation). In view of the potential dependence on multiple endogenous (species, life stage, age, somatic growth, sex, reproductive status) and exogenous factors (temperature, salinity, "stress", diet), and their interactions, it appears to be much more complex than previously thought.

1.2.2. Stable isotopes

The $\delta^{18}\text{O}$ of marine organisms has been widely used in the geological sciences to estimate temperature conditions during the life of organisms. The use of fossil and living coral and Foraminifera is a standard tool in determining climate variation over geological periods and the literature on the subject is enormous. Recent papers provide examples of the wide scope of these studies, including analyses of oscillations in El Niño events in the Pacific using reef-building corals (Wellington & Dunbar, 1995), global temperature changes, based on biogenic silica (Shemesh *et al.*, 1992) and estimation of warming in interglacial periods in the Aegean, based partly on fossil Foraminifera (Aksu *et al.*, 1995).

The application of these techniques depends on the assumption that the fractionation of the oxygen isotopes during the precipitation of biogenic carbonate is governed by predictable quantum energy equilibrium conditions determined solely by physico-chemical properties. Because the chemical bonds of the various isotopes have different strengths, temperature affects the ratio of these isotopes as they participate in chemical reactions, in this case as the isotopes of an element precipitate into calcium carbonate. The $\delta^{18}\text{O}$ increases as the temperature decreases, since the ^{18}O bonds are stronger and less thermal energy is available to release these isotopes in chemical reactions.

There are two important processes that may affect oxygen isotope ratios, and thus the temperature estimates, from biogenic carbonate. The first factor is the possibility of "vital effects" which encompass both species-specific differences in the temperature-isotope relationship and physiological processes that potentially affect the preferential participation of isotopes. Species differences have been noted. When they exist, departures from equilibrium are usually attributed to biochemical and metabolic factors that result in modification of the isotopic signatures preserved in calcium carbonate. Variations in growth rate, for example, may cause changes in precipitation kinetics and incorporation of respiratory carbon dioxide in addition to the reservoir of marine dissolved inorganic carbon which is normally utilised during calcification. Under these circumstances the isotopic record preserved by aragonite is no longer simply a function of temperature variation and will depend in part on physiological factors which may be organism-specific. It is therefore vital to test the assumption of equilibrium isotope partitioning, and especially to be able to test independently the relative influences of individual metabolism (or growth rate) and temperature. It is also likely that at a given temperature different carbonate materials such as aragonite and calcite may differ in their ^{18}O concentrations (Grossman & Ku, 1986).

The second factor that affects the isotope ratio of carbonates is the isotope ratio of the water (see fig. VII.6). Salinity and source of water are the major sources of variation in water oxygen isotope ratios. Generally, marine waters vary over a narrow range (-0.30 – 0.05), with the isotopic values of polar waters being more negative than those of mid-oceanic waters. Waters that are depleted in ^{18}O relative to SMOW (more negative), are called "isotopically lighter". However, freshwaters show greater variations, for example: the $\delta^{18}\text{O}$ value of Lake Baikal is -15.8 and the Laurentian Great Lakes vary between -6 and -9 .

The use of isotopic ratios to investigate aspects of the biology of extant fish populations has primarily been based on surveys of field-collected samples, often from a wide range of habitats (Devereux, 1967; Mulcahy *et al.*, 1979; Nelson *et al.*, 1989; Doering, 1991; Iacumin *et al.*, 1992; Meyer-Rochow *et al.*, 1992; Northcote *et al.*, 1992; Gauldie *et al.*, 1994).

To date, most experimental studies on fish have found a good relationship between temperature and oxygen isotope ratios. Carbon isotope ratios are more heavily influenced by growth than by ambient temperature (Radtke, 1984; Kalish, 1991b,c; Gauldie, 1996; Radtke *et al.*, 1996, 1998; Thorrold *et al.*, 1997; Gao & Beamish, 1999). However, few laboratory studies have controlled or monitored important factors that can introduce added variability, such as individual growth rate or the isotopic ratio of the water in the experimental

tanks. In a carefully controlled experiment, Thorrold *et al.* (1997) determined that oxygen isotope ratios were a reliable indicator of temperature in *Micropogonias undulatus*, and were relatively independent of individual growth rate.

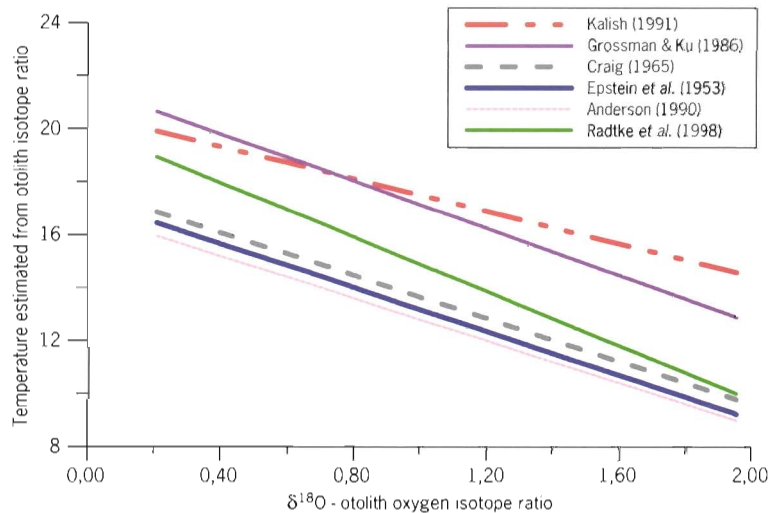
A large number of published equations relate water temperature and oxygen isotope ratios in carbonates (tab. VII.2 and fig. VII.7). These relationships are derived from different taxa and sometimes different polymorphs, and the primary difference between them is the fitting of polynomial v. linear models. There are also differences in intercept and in some cases also in the slope (fig. VII.7). Few studies have compared groups of species over a wide range of temperatures. Experimental work has only been carried out on two marine fish species (*Micropogonias undulatus*, (Thorrold *et al.*, 1997) and *Gadus morhua*, (Radtke *et al.*, 1996, 1998)) and one freshwater species (*Arripis trutta*, (Kalish, 1991c), and the temperature ranges in these studies do not overlap. It is therefore difficult to generalise about the exact relationship between temperature and the isotopic ratio of fish otoliths. It is more likely that the relationship is curvilinear, although the published polynomial fits are derived from Invertebrates and not Fish.

All the available experimental evidence indicates that a reliable relationship exists between the oxygen isotopic composition of otolith aragonite and environmental conditions experienced by individual fish, even though the determination of absolute temperature depends on the equation used (tab. VII.2). There are many applications in which relative changes in temperature are valuable, and the accuracy of the temperature estimates is therefore not at issue. However, where accurate temperature estimates are required, more experimental data is needed to better define the exact form of the relationship between otolith oxygen isotopic ratios and actual water temperatures.

Table VII.2 - Published equations relating water temperature and oxygen isotopes ratios in carbonates.

Taxa	CaCO ₃ polymorph	Temperature relationship	References
Molluscs	Calcite & aragonite	$T^{\circ}\text{C} = 16.5 - 4.3 (\delta^{18}\text{O}_{\text{carb}} - \delta^{18}\text{O}_{\text{wat}}) + 0.14 (\delta^{18}\text{O}_{\text{carb}} - \delta^{18}\text{O}_{\text{wat}})^2$	(Epstein <i>et al.</i> , 1953)
Not specified	Calcite	$T^{\circ}\text{C} = 16.9 - 4.2 (\delta^{18}\text{O}_{\text{carb}} - \delta^{18}\text{O}_{\text{wat}}) + 0.13 (\delta^{18}\text{O}_{\text{carb}} - \delta^{18}\text{O}_{\text{wat}})^2$	(Craig, 1965)
Foraminifera	Aragonite	$T^{\circ}\text{C} = 20.6 - 4.34 (\delta^{18}\text{O}_{\text{carb}} - \delta^{18}\text{O}_{\text{wat}})$	(Grossman & Ku, 1986)
Not specified	Calcite	$T^{\circ}\text{C} = 16.0 - 4.14 (\delta^{18}\text{O}_{\text{carb}} - \delta^{18}\text{O}_{\text{wat}}) + 0.13 (\delta^{18}\text{O}_{\text{carb}} - \delta^{18}\text{O}_{\text{wat}})^2$	(Anderson, 1990)
Fish	Aragonite	$T^{\circ}\text{C} = 20.52 - 3.067 (\delta^{18}\text{O}_{\text{carb}})$	(Kalish, 1991a)
Fish	Aragonite	$T^{\circ}\text{C} = 22.09 - 4.76 (\delta^{18}\text{O}_{\text{carb}} - \delta^{18}\text{O}_{\text{wat}})$	(Thorrold <i>et al.</i> , 1997)
Fish	Aragonite	$T^{\circ}\text{C} = 18.96 - 5.00 (\delta^{18}\text{O}_{\text{carb}} - \delta^{18}\text{O}_{\text{wat}})$	(Radtke <i>et al.</i> , 1998)

Figure VII.7
Comparison
of the temperature
estimates obtained from
otolith oxygen isotope ratios
by using different published
isotope-temperature
relationships shown
in table VII.2.



2. Metabolism and ontogenic events

A number of factors interact to affect the composition of fish otoliths. These factors complicate the interpretation of composition and have sometimes led to erroneous conclusions about the relationship between environmental conditions and otolith composition. Otolith composition reflects the combined effects of endogenous processes such as development and reproduction, and external conditions associated with habitat, behaviour and diet change. The following sections

discuss the influence of physiological processes on the availability and incorporation of elements into the otolith.

Metabolic rate is a major factor determining otolith growth, and for this reason may influence otolith composition through the control of the rate of incorporation of individual elements. The metabolism of an individual fish will also determine its exposure to the elements in the external environment by influencing food consumption and the exchange rate across the gills and epithelia. These factors may link metabolism and otolith composition in a general way, so that otolith concentrations of different elements will vary between individuals and also within individuals over seasonal cycles. Several studies of metal concentrations in fish otoliths have noted increased concentrations in the otoliths of faster growing fish (Papadopoulou *et al.*, 1978; Protasowicki & Kosior, 1988), which may indicate that higher metabolic rates are linked to higher rates of incorporation of some elements.

The best evidence for the influence of metabolism on otolith composition comes from studies of Sr/Ca ratios. Fluctuations in Sr/Ca were initially interpreted in terms of seasonal temperature variations, but evidence has accumulated that links Sr/Ca ratios more closely with individual metabolism. Inverse relationships have been reported between Sr/Ca and somatic growth rate (Sadovy & Severin, 1994; Friedland *et al.*, 1998), suggesting that more strontium may be incorporated into the otolith during periods of lower otolith protein synthesis and reduced accretion rate.

There is direct experimental evidence that metabolism is a factor in determining the $\delta^{13}\text{C}$ ratios in fish otoliths (Kalish, 1991b; Thorrold *et al.*, 1997). Unlike $\delta^{18}\text{O}$, $\delta^{13}\text{C}$ does not always show a close relationship to temperature and it is often closely related to individual fish size and growth rate. It has been suggested that some of the otolith carbonate may derive from metabolised carbon sources (entering the plasma as CO_2) and thus relate to fish metabolism rather than to external conditions.

Many of the changes observed in otolith composition have been characterised as ontogenetic variations. For the most part, it has yet to be determined whether variations in composition are directly linked to developmental events, and thus are strictly speaking ontogenetic, or whether the variation is a function of fish size or age. Cyclic variations in otolith composition have been observed in life history transects, and the amplitude of these variations is often lower in the outer sections of the otolith. This pattern is certainly a function of fish age; perhaps, for example, if adult fish move to minimise changes in temperature. Size-related changes, age-related changes, and stage- (development)-related changes are often closely coupled in fish, and it is therefore difficult to differentiate between purely ontogenetic patterns and

changes that occur with increasing fish size. Ontogenetic patterns should have a clear link to developmental processes and life history stages/events. Some variations in otolith composition will be both ontogenetic and size-related. For example, variation due to gonad maturation and spawning activity has both size and developmental components. The onset of sexual maturity is more likely to be size- than age-related, and signals the start of a distinct life history stage. Certain migration patterns are only size-related, and there is some debate as to whether these should be regarded as ontogenetic changes. In other cases, such as metamorphosis and settlement in flatfish, the developmental changes seem to be independent of fish size. There are often trends in the concentrations of different elements across the otolith, but to avoid confusion these changes should not be treated as ontogenetic unless they are clearly linked to developmental changes. Similarly, seasonal fluctuations should not be confused with ontogenetic changes, since these may be induced directly by external conditions rather than endogenous changes.

2.1. Metamorphosis

In some species of fish, the transition between the larval and juvenile stages is marked by dramatic changes in morphology, behaviour and habitat use. These changes can often be seen from changes in otolith composition and structure (chap. III.B.3). Although such variations in composition have not been explicitly investigated, several studies have ascribed variations in otolith composition to ontogenetic factors. For instance, the onset of metamorphosis from leptocephalus to glass eel has been shown to induce a rapid drop in otolith Sr/Ca ratios, coinciding with a rapid increase in increment width in *Anguilla japonica* (Arai *et al.*, 1997), *A. rostrata* and *A. anguilla* (Arai *et al.*, 2000). Such drastic changes are not believed to be related to habitat change. Otake *et al.* (1994, 1997) associated the decrease in otolith Sr/Ca ratio to that of the body Sr content, explaining it by the breakdown of the gelatinous extracellular matrix composed of sulphated glycosaminoglycans (GAG), which are known to have a high affinity for Sr. Pontual *et al.* (unpubl.) also identified changes in the composition of larval *Solea solea* otoliths, which may have been interpreted as the result of migration from coastal waters into estuaries, if similar changes were not also observed in the otoliths of individuals held under constant laboratory conditions. Fowler *et al.* (1995) described significant differences in composition between otolith cores and edges in fish held in constant laboratory conditions.

Changes in habitat associated with metamorphosis can certainly affect otolith composition. Shifts in diet may also have some effect, though this is probably of lesser magnitude. The effects of anatomical and

physiological changes during metamorphosis have yet to be addressed, whether experimentally or in the field. Processes such as calcification and development of the circulatory system can limit the elements and concentrations available for incorporation into the otolith. Development of enzyme systems in response to diet shifts may initiate competition for various metals that may have been incorporated into larval otoliths.

2.2. Reproduction

Two aspects of reproduction have the potential to affect the composition of fish otoliths through endogenous physiological processes. In female fish gametogenesis involves the translocation of material for yolk in the developing ova. The process of vitellogenesis requires calcium and there is an increase in the calcium-binding proteins produced (in particular vitellogenin). The effect on the otolith is to reduce the available concentrations of calcium and divert protein synthesis. The spawning checks observed in the otoliths of adult female fish may be the visible result of this process, although little work has been done to systematically determine the source of these features. In terms of otolith composition, fluctuations in the ratio of mineralised to organic components would be expected, and there may also be accompanying shifts in the incorporation rate of trace elements.

Both male and female fish lay down fat deposits that can be utilised as energy stores both for gametogenesis and for metabolic requirements during the reproductive season, especially if there is no feeding. Because many elements can be readily incorporated into fatty tissues, the mobilisation of these reserves can induce or accentuate seasonal fluctuations in these elements in the otolith.

Few authors have directly investigated the effects of reproductive activity on otolith composition, despite the impact this could have on the planning of surveys and the interpretation of population results (Thresher, 1999). Fluctuations in the major otolith elements, Ca, Sr, Na, and K, have been interpreted as indications of reproductive activity in several studies (Fuiman & Hoff, 1995). Kalish (1989) correlated changes in gonadosomatic index with blood plasma concentrations of calcium-binding proteins, Ca, and Sr in *Arripis trutta* and linked these to fluctuations in otolith Sr/Ca ratio. Experimental work on *Poecilia reticulata* (Thresher, 1999) suggested that increases in otolith Sr and Na were often associated with spawning (brood development) episodes. However, in these and other species the variability among individual females is high and it is difficult to generalise about the exact effects of reproductive activity on specific changes in otolith composition. Because these changes are a function of endogenous processes, it may be that individual variability is inherent and that cyclic

changes in some elements (Sr, Na) could provide more information about individuals (for example, about reproductive effort) than about populations.

3. Age estimation

Fish age estimation using otolith chemical composition does not compete with conventional age estimation methods, but has rather been developed with the aims either of validating the latter when they are not easily applied (e.g. long-lived species) or of producing an age range to help develop conventional ageing criteria. Three kind of methods have been investigated:

- radiometric methods based on natural radioisotopes disequilibria;
- nuclear weapons testing ^{14}C tracer;
- cyclical variations in otolith elemental composition.

3.1. $^{210}\text{Pb}/^{226}\text{Ra}$ and $^{228}\text{Th}/^{228}\text{Ra}$ disequilibria

The two nuclide pairs of interest in otolith radiometric dating are $^{210}\text{Pb}/^{226}\text{Ra}$ and $^{228}\text{Th}/^{228}\text{Ra}$, which are parts of the natural ^{238}U and ^{232}Th decay series respectively (tab. VII.3). Both pairs involve isotopes of radium that occur naturally in freshwater and seawater. Radium is a calcium analogue, following similar metabolic pathways, and thus can accumulate in calcified structures. ^{210}Pb and ^{228}Th are also present but in lower abundance. All have very low concentrations and hence activity, which causes problems for the precision of measurements.

Both pairs may be considered as simple daughter/parent decay pairs since the intervening isotopes in both cases are very short-lived (tab. VII.3).

Table VII.3 - Fish otolith radiometric dating: radionuclides of interest from the ^{238}U and ^{232}Th decay series with corresponding half-lives. Dashed lines indicate when intervening radionuclides are not shown.

^{238}U series	Half-life	^{232}Th series	Half-life
^{238}U	4.49 10^9 years	^{232}Th	1.41 10^{10} year
-----		^{228}Ra	5.76 years
^{226}Ra	1622 years	^{228}Ac	6.13 hours
^{222}Rn	3.82 days	^{228}Th	1.91 year
^{218}Po	3.05 minutes	-----	
^{214}Pb	26.8 minutes	^{208}Pb	Stable
^{214}Bi	19.7 minutes		
^{214}Po	164 microseconds		
^{210}Pb	21 years		

^{206}Pb	Stable		

The $^{210}\text{Pb}/^{226}\text{Ra}$ method, which follows the secular equilibrium scheme, should theoretically be applicable to age estimation over 100 years (five times the ^{210}Pb half-life). Actually, current levels of analytical precision makes it most suitable (i.e. with an acceptable uncertainty) within the range of 0-50 years (Campana, 1999). The limiting factor is now the measurement error of ^{210}Pb since the determination of ^{226}Ra activity in otoliths has recently been improved (Andrews *et al.*, 1999b).

^{228}Ra and ^{228}Th have similar half-lives so that their activity ratios vary with time according to the transient equilibrium scheme. According to Campana (1999) the applicable age-range is 0-8 years (see also fig. VII.1).

The main difficulty in radiometric dating is caused by the continuous growth of the otolith throughout the life of the fish. If whole otoliths are used, continuous deposition of new material must be taken into account and the radiometric equation reformulated using a model that accounts for the increase in mass over time. Such equations, which were first derived by Bennett *et al.* (1982) in their pioneering work on fish radiometric ageing, were subsequently corrected and adapted to various growth models (Campana *et al.*, 1993; Kimura & Kestelle, 1995). On the other hand, assuming that the core has roughly the same age as the fish (plus the time between collection and analysis), the Bateman equation can be directly applied when only core material is analysed. This method was first employed by Campana (1990) and should be preferred when it can be used.

Three assumptions are fundamental to radiometric ageing:

1. Once deposited, radionuclides do not migrate within the otolith and losses (gains) of any radionuclides of interest, including intervening radionuclides, only occur through the decay (in growth) process. In other words, the otolith acts as a closed chemical system.
2. The initial activity of daughter/parent pairs in the otolith is low, ideally close to zero and can be estimated.
3. Both parent and daughter nuclides are incorporated into the otolith at a rate that always maintains a constant ratio to the rate of mass increase of the otolith.

The question of "closure" is the most difficult to answer and has been strongly debated through theoretical arguments in several papers. Except for the work of Gauldie & Cremer (1998), which demonstrated the possibility of diffusion of ^{222}Rn out of otoliths of *Hoplostethus atlanticus*, there is a distinct lack of experimental evidence that would validate the assumption of closure for the radionuclides of interest in otoliths. In point of fact, a significant loss of ^{222}Rn during ^{226}Ra decay should result in lower ^{210}Pb activity and hence lower age estimates. However a number of works have resulted in old radiometric age estimates that roughly corroborate the estimates made using conventional

approaches. Certain methods of otolith storage and preparation have been shown to affect the stability of fine-scale spatio-temporal chemical distributions within the otolith (Milton & Chenery, 1998; Proctor & Thresher, 1998), and may have a bearing on radiometric ageing methods. The issue of internal migration only applies to studies using the core method.

The second assumption deals with the issue of allogenic (i.e. from an external source) uptake of the daughter nuclide or any intermediate product. This can generally be satisfied if otoliths of young fish can be used to estimate the initial daughter/parent activity ratio. If the core method is used the "reference fish" should be of the same age as the adult core and be available for appropriate year-classes and locations.

The third assumption is only necessary when whole otoliths and an otolith mass growth-in-time model are used. It requires the rate of incorporation of both parent and daughter nuclides to remain constant with respect to Ca throughout the lifespan. Violations of this assumption are likely to occur for species that undergo major changes in habitat during their life cycle (Fenton *et al.*, 1990; Andrews *et al.*, 1999a) or if the incorporation rate is an age-dependent process (Kastelle *et al.*, 2000). It also requires that the otolith mass growth rate is known or assumed, which yields a circular argument if a growth model is built up on the basis of *annulus*-based ages that have to be validated (West & Gauldie, 1994; Francis, 1995b; Kimura & Kastelle, 1995).

The $^{228}\text{Th}/^{228}\text{Ra}$ disequilibrium has been used to study the longevity of *Hirundichthys affinis*, for which neither scales nor otoliths can be used for conventional age estimation (Smith *et al.*, 1991; Campana *et al.*, 1993). Although the uncertainty associated with the Ra assay (since activity is extremely low) is a current limiting factor, this method could have widespread application in the study of short-lived species.

The $^{210}\text{Pb}/^{226}\text{Ra}$ disequilibrium has been used more extensively in order to confirm age estimates or the longevity of various species such as *Sebastes* spp. (Bennett *et al.*, 1982; Campana *et al.*, 1990; Kastelle *et al.*, 2000), *Anoplopoma fimbria* (Kastelle *et al.*, 1994), *Hoplostethus atlanticus* (Fenton *et al.*, 1992; Francis, 1995b; Smith *et al.*, 1995), *Allocyttus verrucosus* (Stewart *et al.*, 1995), *Coryphaenoides acrolepis* (Andrews *et al.*, 1999a), *Macruronus novaezelandiae* (Fenton *et al.*, 1990) and *Lutjanus* spp. (Milton *et al.*, 1995).

3.2. Atomic bomb radiocarbon chronometer

^{14}C is a cosmogenic radioisotope that is naturally produced by the disintegration of the nucleus of ^{14}N (stable) when it collides with cosmic rays. ^{14}C is unstable and decays back to ^{14}N with a half-life of 5 730 years, so that the well-known ^{14}C radiometric dating method (based on ^{14}C decay) has no practical use for age validation purpose (but see

Kalish, 1995). However, natural atmospheric radiocarbon levels have been modified by human activities since the beginning of the industrial revolution (fossil fuel combustion, nuclear power plants) and were particularly strongly affected by atmospheric nuclear weapon tests during the 1950s and 1960s. In less than ten years the atmospheric concentrations of ^{14}C doubled after remaining fairly constant for several thousand years. Since the end of the testing period, this concentration has been falling (fig. VII.8a) as the radiocarbon, in its oxidised form $^{14}\text{CO}_2$, exchanges with the other CO_2 reservoirs. As a result the upper layers of the world's oceans have undergone a significant input of radiocarbon which is clearly visible in ^{14}C time-series derived from measurements of either seawater-dissolved inorganic carbon (DIC) or by using proxies such as banded corals (e.g. Druffel & Linick, 1978) or bivalve shells (Weidman & Jones, 1993). Analyses of the spatial distribution of ^{14}C and its temporal 3D variations (fig. VII.8b,c) through mixing and advection provide invaluable information in various field of research (carbon cycle, global oceanic circulation, etc.). Kalish (1993) demonstrated that the bomb ^{14}C tracer was also incorporated into fish otoliths, and suggested using it as a date marker. Analyses were performed on otolith cores of New Zealand fish whose ages had previously been estimated by conventional age-reading methods. The reconstructed otolith ^{14}C time series showed a striking phase coherence with that of nearby corals (fig. VII.8d) and thus confirmed the *annulus*-based age estimate for those fish. External ^{14}C references are not required if a suitable collection of young fish from a given species and location can be used to establish a reference curve (Campana, 1997).

Looking at the chronology of upper ocean bomb radiocarbon contamination, otoliths of fish with presumed hatching dates between about 1960 and 1970 are most suitable for validation studies, although later years (1970 to the present) may be used if the decreasing rate of ^{14}C in the region of interest is sufficiently rapid (Kalish, 1995). Note that ^{14}C age estimates provide minimum ages since potential contamination of the core by adjacent younger material would increase the ^{14}C sample concentration if the fish had hatched during the period of increasing signal. This does not apply if the decreasing signal period is used. The synchrony of the bomb- ^{14}C contamination of the upper layers of the world's oceans has been well established, although there are substantial spatial variations in the magnitude of the phenomenon (fig. VII.8b). This is why this approach is most useful (or rather, less problematic) for species whose early life stages, at least, inhabit the surface mixed layer (Kalish, 1995). For species living below this habitat, it remains applicable at least to moderate depths (Kalish *et al.*, 1997), provided that a correction factor for ^{14}C -derived fish ages can

be estimated using existing data on the penetration rate of bomb radiocarbon, as this has proved to be highly variable, depending on location (fig. VII.8c). With regard to estuarine species, the bomb ^{14}C signal has been shown to lie intermediate in phase and magnitude between atmospheric and marine carbonate time series (Campana & Jones, 1998). This is due to more intense and rapid water-air exchanges in shallow, well mixed locations with strong riverine input.

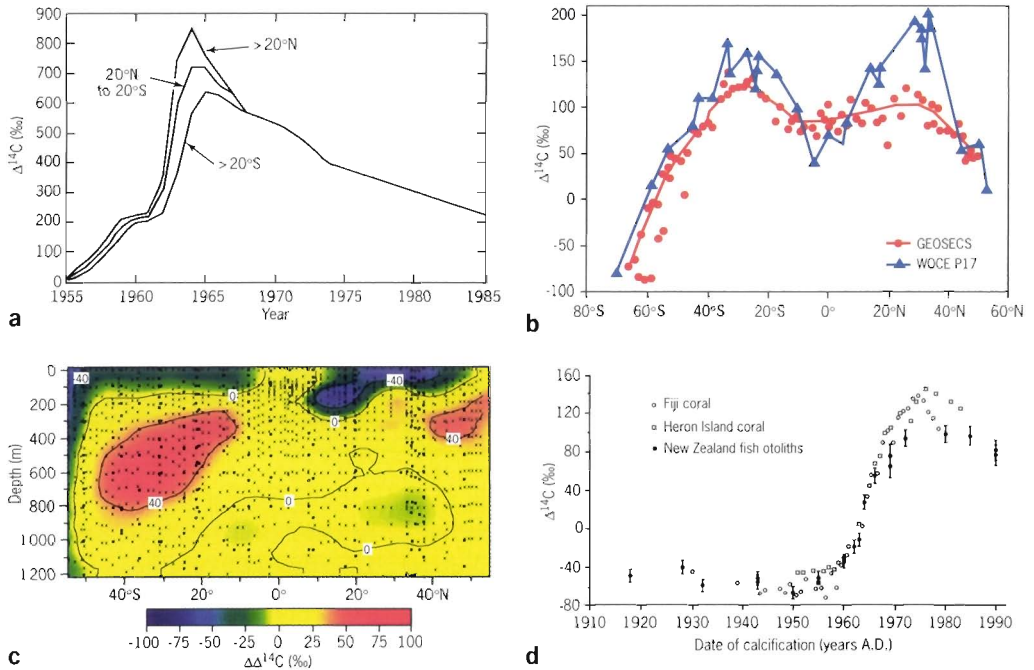


Figure VII.8 - The bomb ^{14}C chronometer.

a) Atmospheric history of $\Delta^{14}\text{C}$ in the northern (20°N) and southern (20°S) hemispheres showing the large change due to the atmospheric testing of nuclear weapons. From Broecker *et al.* (1985).

b) Surface values from Eastern Pacific GEOSECS (1973) section compared to WOCE section P17 (1992) data collected along 135°W . Values in the temperate zones of both hemispheres fell while those in tropical and equatorial latitudes rose. From Key (1997).

c) 3D changes in the $\Delta^{14}\text{C}$ (WOCE P17: black asterisks - Eastern Pacific GEOSECS: grey Xs) between 1973 and 1992. From Key (1997).

d) $\Delta^{14}\text{C}$ ($\pm 1\text{SD}$) of *Pagrus auratus* otolith aragonite from New Zealand over the period 1918 to 1990. $\Delta^{14}\text{C}$ measurements from hermatypic corals sampled in Fiji and Heron Island, Australia are presented for comparison. From Kalish (1993) with permission from Elsevier Science.

As pointed out by Campana (1999), in estuarine and deep-sea habitats the otolith radiocarbon signal may be obscured to some extent by that part of the carbon which is metabolically derived (one third according to Kalish (1991b) if the fish feed on prey whose ^{14}C content is different from that of the surrounding water.

Such studies require the use of accelerator mass spectrometry (AMS), a high-precision and very expensive technique. This is probably one of the reasons why applications in fish biology have remained relatively few despite its indisputable advantages compared, for instance, to the relatively poor precision of radiometric ageing.

Note that aside from fish validation studies, radiocarbon data may provide information that helps to elucidate questions in fish biology, such as the location of early-life habitats (Kalish, 1995). Moreover, by using known-age or validated-age fish, otolith ^{14}C time series can be valuable tools in other disciplines.

3.3. Other potential chronometers

Early studies suggested that variations in elements and element ratios could be used to verify fish age, if these variations were caused by cyclical seasonal factors such as temperature. Differences in Ca concentration, coinciding with optically different zones, were used to indicate seasonal cycles in eel otoliths (Casselman, 1982, 1987). Sr/Ca ratios were also suggested for this application (Radtke & Targett, 1984) but further studies have shown that there is too much uncertainty about the relationship between otolith Sr and environmental conditions to make this a useful chronometer. However, Thorrold & Shuttleworth (2000) recently observed cyclical variations in Sr/Ca ratios that closely matched the pattern of validated annual rings in *Pogonias cromis* otoliths.

Oscillations in the concentrations of other elements have also been observed. For instance, Halden *et al.* (2000) interestingly observed an oscillatory pattern of Zn in *Salvelinus alpinus* otoliths that closely matched their optical annual structures. Such signals may prove to be suitable chronometers in some situations, though their use should be carefully investigated first.

Weidman & Millner (2000) measured oscillations in $\delta^{18}\text{O}$ across cod otoliths by drilling discreet samples for IRMS analysis. The number of fluctuations observed corresponded well to conventional age determinations, suggesting that $\delta^{18}\text{O}$ transects could be used for age estimation in species that experience regular annual temperature fluctuations.

4. Stock-population discrimination

The major application that has driven most of the development in otolith microchemistry is stock discrimination. In numerous locations around the world spawning or feeding stocks of fish mix and for fisheries assessment and legal purposes it is necessary to identify and quantify the relative proportions of the individual components of the

stock. Even for a single fish stock, it is often desirable to be able to identify separate sub-populations that may contribute to the total fishery.

Stock discrimination using OMC does not imply genetic differences but is based on differences in elemental fingerprints that may be environmentally or physiologically induced in fish which spend at least part of their lives in different environments.

Stock discrimination studies have employed both analysis of the elemental composition of the whole otolith (bulk analyses, chap. VII.G.1.1.1) and analysis of specific areas of the otolith (surface analyses, chap. VII.G.1.1.2). Information from whole dissolved otoliths can often be collected more rapidly and with less risk of contamination, but in many cases the information required is contained in the inner layers of the otolith and can only be retrieved by surface techniques. Two lines of research are currently being pursued to further develop stock identification applications. One is the continued refinement of analytical tools and protocols to allow the efficient and accurate analysis of more elements; the second is the development of data analysis techniques that will allow the most efficient classification of stock components based on the smallest number of elements. It is likely that the selection of analytical technique and data analysis method will vary with the specific application, since in some locations differences may be caused by a few minor elements (Sr, Na), while in others the differences may be minor and determined primarily by the presence or absence of trace and ultra-trace elements. Thresher (1999; fig. 9) lists Na, Sr, Ba and Mg as the most frequently used elements for successful stock delineation. Otolith microchemistry has been employed in stock identification studies for species that occupy a range of different environments, both coastal and oceanic. The majority of studies have reported successful discriminations of groups of fish, but it is not known whether the technique can be universally applied or whether unsuccessful stock discriminations are simply under-reported. There were no detected differences indicative of geographical differences in the otolith composition of a southern ocean species (*Macruronus novaezelandiae*) taken from different spawning grounds (Kalish *et al.*, 1996). The rate of success in identifying stocks decreases when more specific stratified analyses are performed. Some location differences may disappear when the otolith differences are controlled for fish size (either statistically (Edmonds *et al.*, 1989), or by analysing different portions of the otolith (Dove *et al.*, 1996), for year-class effects (Edmonds *et al.*, 1995) or sex differences (Kalish *et al.*, 1996). Published work on stock identification represents the majority of the otolith microchemistry literature, and much of this work is reviewed

in Thresher (1999). Preliminary studies have been performed in many parts of the world, usually focussing on commercially important stocks with good prior knowledge of the components mix (Campana *et al.*, 1995), or using a pre-determined outlier population for comparison (Secor & Zdanowicz, 1998). Australian researchers have mounted large-scale studies of populations (Edmonds *et al.*, 1991, 1992, 1999), and have incorporated microchemistry into their standard stock assessment methods.

Otolith composition can also be used to identify changes in habitat in individual fish, elemental fingerprints being used as natural tags. For fish that move between environments or water masses this information can be used to track movements and analyse stock mixing. Differences in the composition of otolith cores have also been used to identify spawning and nursery grounds as a first step toward evaluating the relative contributions of these components to the adult stock. Much of the work on identifying nursery grounds is preliminary, and has been restricted to demonstrating the application of the principle. Differences in otolith composition have been detected in juvenile *Cynoscion regalis*, (Thorrold *et al.*, 1998b), juvenile *Alosa sapidissima*, (Thorrold *et al.*, 1998a) and juvenile *Solea solea*, (Pontual *et al.*, 2000) from different estuaries. Oxygen and carbon isotope analysis has been used to distinguish between migratory and non-migratory individuals (Northcote *et al.*, 1992) and between salmon from different natal streams (Gao & Beamish, 1999). The differences in composition can be used to identify the source of individuals contributing to the adult population, provided that the characterisation of the spawning or nursery grounds and the analysis of the mixed group occur over a period of time during which the environment chemical characteristics remain stable. This point is discussed in two recent full-scale applications, the first based on analysis of the core of juvenile *Tenualosa toli* (Milton *et al.*, 1997) and the second on the analysis of whole otoliths of *Gadus morhua* (Campana *et al.*, 2000).

Otolith microchemistry data is usually analysed using multivariate statistical techniques for stock separation. These permit the inclusion of large numbers of elements (or isotopes) and present procedures for quantifying the importance of different elements to stock discrimination.

Principle component analysis (PCA) is a family of techniques that provides synthetic representations of vast data sets, usually by means of graphical visualisations. The relative proximity of variables (here otolith elemental/isotopic concentrations) is interpreted in terms of correlation, while the proximity of individuals is interpreted in terms of global similarity of observed values. Discriminant analysis is a family of techniques that are designed to classify (i.e. to assign to pre-existing classes; here, groups of fish) individuals that are characterised by a number of numerical variables. The most commonly used

method, linear discriminant analysis, is both descriptive and predictive. The discriminatory power of the discriminant functions should be assessed by means of bootstrapping or jack-knifing techniques which provide accurate estimates with confidence intervals. Maximum likelihood-based methods may be more suitable for classifying samples from mixed-stock origin in cases where discriminant analysis lacks sufficient discriminatory power.

5. Other applications

5.1. Habitat characterisation through elemental fingerprints

Differences in otolith composition of course provide valuable information about fish populations, but it may also be possible to make certain inferences about habitats and environments from otolith-derived information. Otolith microstructure has been used to indicate habitat quality, using otolith increment width as an indicator of fish growth rate (Szedlmayer & Conti, 1999). Our understanding of how various elements are incorporated into the otolith is still incomplete, and it is unlikely that the concentrations of individual elements can be used directly to indicate concentrations in the environment. However, variations in elemental concentrations across the otolith may indicate short-term fluctuations in the environment and may discriminate between stable and dynamic environments. There may exist key elements that can be used to trace specific water masses and identify the frequency of incursion into a habitat occupied by a fish population, especially when that population is known to be sedentary or migrating. Barium, for example, is associated with oceanic waters and its presence in the otolith could record exchanges between coastal and open ocean environments. Along with Sr, as mentioned above, other elements (or isotopic ratios) such as Pb and U may also prove to be valuable indicators of seawater or freshwater incursions.

5.2. Mass marking

Among the methods that have been developed to mark otoliths and more generally fish calcified structures (chap. IV.A.1.2), radiolabelling with the ^{85}Sr radioisotope and marking with particular elements depend specifically on OMC techniques. The former was employed by Lehtonen *et al.* (1992) to mark newly hatched anadromous *Coregonus lavaretus* before release into the field in order to study the timing of their downstream migration.

Immersion of *Oncorhynchus kisutch* fry in water enriched with lanthanides (La, Ce and Sm) resulted in detectable levels in bony tissues as late as 10 months after the treatment (Ennevor & Beames, 1993; Ennevor, 1994).

Immersion in a strontium chloride (SrCl_2) solution has also been proposed as an inexpensive and relatively environmentally safe method of tagging large numbers of small fish with good success. Otoliths of marked salmon fry (*Oncorhynchus keta* and *O. nerka*) presented clearly identifiable Sr mark after 21 months of rearing in freshwater (Schroder *et al.*, 1995). The potential of this method has recently been evaluated as a means of marking the dorsal spine of juvenile *Pagrus auratus* (Pollard *et al.*, 1999).

From a large-scale mark-recapture experiment, Clear *et al.* (2000), using injections of SrCl_2 , proved that increments are deposited annually in the otoliths of *Thunnus maccoyii* at least until the age of 13 years.

Depending on the experiments, various analytical methods of Sr measurement were used: backscattered electron microscopy, EDS, WDS and ICP-MS (chap. VII.G). Although useful for some fisheries research experiments, SrCl_2 is not approved for use on food fish in certain countries, limiting its widespread use.

5.3. Analysis of fossil biogenic carbonate for paleoclimatology and paleoecology

Fish bones, teeth and otoliths have been analysed for geological, paleontological and archaeological studies. Oxygen isotope ratios in fossil otoliths (Devereux, 1967; Patterson *et al.*, 1993) and fossil fish teeth (Kolodny *et al.*, 1983) can provide information about paleotemperatures. Smith & Patterson (1994) and Patterson *et al.* (1993) not only estimated temperatures from fossil otoliths, but also reconstructed the seasonal temperature variations experienced by fish populations. One aspect that is debated among paleontologists and geochemists is the accuracy of applying experimentally derived isotope-temperature relationships (tab. VII.2) to fossil material. During fossilisation, diagenesis can occur, which can transform aragonite into the more stable CaCO_3 polymorph calcite. Several studies have attempted to reproduce this process experimentally, and thus derive conversion factors (Yoshioka *et al.*, 1985; Nelson *et al.*, 1986). The effect of diagenesis on oxygen isotope ratios is inconclusive, but when aragonite transforms to calcite there is a release of Mg and Sr (Yoshioka *et al.*, 1985).

5.4. Pollution history and anthropogenic influences

Water quality monitoring programmes and studies of the impact of environmental contamination rely on a combination of measurements of the physical and chemical environment and the biota. The impact of environmental contamination on organisms can be measured at ecosystem, community, population, and individual levels. Unfortunately, monitoring and assessment require long-term studies, and detecting environmental contamination is difficult in areas where

there are no base-line data. Chemical analysis of fish otolith composition may offer significant improvements in our ability to detect current, recent and historical levels of contamination, and at the same time to assess the impact of contamination at the individual and population levels. Environmental contamination affects the physiology of individual fish, and this effect propagates to higher levels of organisation, altering fish populations and the whole aquatic community.

Otoliths can be analysed to reveal critical information about the environmental history of fish, comparative growth rates of fish from different environments, and the long-term effects of sub-lethal exposure to environmental contamination. Although fish movements and migrations may make it difficult to pinpoint just where an individual was exposed, the record of concentrations in the otolith may provide evidence about how long it was exposed. In species with local populations, especially in freshwater, the power of the technique in pinpointing specific pollution events and location is enhanced. Historical collections of otoliths may also reveal the record of environmental contamination over periods of several decades.

Heavy metal concentrations have been measured in the whole dissolved otoliths of a number of fish species sampled at coastal locations. In these studies, otolith concentrations generally reflect spatial differences in environmental concentrations of metals. For example, the content of zinc in the otoliths of fish from Greek coastal waters was higher near towns with industrial inputs of this metal (Papadopoulou *et al.*, 1978). Heavy metals have also been analysed in otoliths of Baltic *Gadus morhua* (Protasowicki & Kosior, 1988) and *Scomberomorus cavalla* (Grady *et al.*, 1989). More recent studies have used ICP-MS and LA-ICP-MS to study temporal changes in the exposure of fish to anthropogenic contamination (Dove & Kingsford, 1998; David Milton, CSIRO, pers. comm.).

In addition to this field evidence, the results of laboratory studies show that metal concentrations in fish otoliths respond to different exposure concentrations (Geffen *et al.*, 1998). Experimental work to date indicates that the incorporation of elements is not merely a reflection of their availability in the environment. The distribution coefficient of each element differs (Milton & Chenery, 1998; Campana, 1999; Bath *et al.*, 2000) both because of chemical characteristics and metabolic requirements and responses to toxic elements. Environmental conditions such as pH or temperature can change the bioavailability and uptake of metals and other toxic elements into the fish. Once taken up, the different elements, especially metals, are metabolised, sequestered, or detoxified via various pathways, and this affects how much is available for incorporation into the otolith. Toxins that are stored in fat tissue may become available only when fat

reserves are mobilised, and this may induce a time-lag between exposure and appearance in the otolith. The chemical characteristics of individual metals and conditions in the endolymph may cause differential precipitation or inclusion into the otolith. In addition, the interactions of metals and proteins are very complex and it is probable that many elements can be incorporated into the otolith via protein components. This aspect has received little attention to date, but may be inferred from studies of mineralised tissue in other taxa.

F. What about other calcified structures?

The need to assess “the extent to which organisms possess natural tags, chemo-prints, the duration during which these are retained and whether new prints are acquired” was early recognised (Calaptice, 1971) and subsequent investigations were undertaken on the chemical composition of skeletal tissues such as scales, *cleithrum*, and vertebral centres.

Although the otolith may be the most frequently used CS in ecological and fisheries studies, analyses involving scales and bones are often performed in order to address certain questions. Analyses of scales and fin rays may be the only viable method of sampling endangered or valuable fish (recreational fisheries, Salmonids, sturgeon, etc.), where individuals cannot be sacrificed or where otolith extraction would destroy the value of the specimen.

Both structural descriptions and the processes of mineralisation of these structures are beyond the scope of this chapter (but see chap. II.B and II.C and e.g. Francillon-Vieillot *et al.*, 1990). From a chemical point of view, all skeletal tissues consist of organic and mineral components. Briefly, the organic matrix is made of collagenous components and non-collagenous components (proteoglycans, phosphoproteins, phospholipids, etc.). The mineral phase consists of “impure” hydroxyapatites ($\text{Ca}_5(\text{PO}_4)_3\text{OH}$) containing carbonate, fluoride, which substitutes for the OH ions in fluoroapatite, and minor and trace constituents (Mg, Si, Sr, Mn, Ba, Na, etc.).

It is worth noting that, unlike otoliths except in very unusual situations, the skeletal tissues are involved to various degrees in the control of body homeostasis through the storage and release of calcium and phosphorus salts, and thus undergo resorption and remodelling processes. Such properties have to be taken into account when interpreting the chemical composition of these bony parts.

The range of applications approximately reflects that of otolith composition analysis (tab. VII.4).

Ecotoxicological research and pollution monitoring of aquatic systems have also made use of studies of fish scale and bone composition. The literature on this topic is abundant and undoubtedly represents a source of valuable information on mechanisms of elemental or radionuclide uptake from water or food and on differential body tissue distribution.

Table VII.4 - Examples of studies of CS chemical composition (the literature cited is far from being exhaustive).

Application	Species	Analysis	CS	References
Migratory pattern	<i>M. saxatilis</i>	Sr	Scale	(Coutant & Chen, 1993)
	<i>Acipenser transmontanus</i>	Sr	Pectoral fin ray	(Veinott <i>et al.</i> , 1999; Veinott & Evans, 1999)
Salinity	<i>Brevoortia patronus</i>	$^{87}\text{Sr}/^{86}\text{Sr}$	Vertebrae	(Chesney <i>et al.</i> , 1998)
Seasonal variation	<i>Thunnus thynnus</i>	Elemental composition	Vertebrae	(Calaprice, 1983)
	<i>Esox lucius</i>	Ca	Cleithra	(Casselman, 1974)
Differentiation between groups of fish	Salmonids	Elemental composition	Scale	(Lapi & Mulligan, 1981)
	<i>Scomberomorus cavalla</i>	$\delta^{15}\text{N}$	Vertebrae Bone collagen	(Yamada <i>et al.</i> , 1987) (Roelke & Cifuentes, 1997)
Diet sources and trophic position		$\delta^{13}\text{C}$ and $\delta^{15}\text{N}$	Scale	(Estep & Vigg, 1985; Wainright <i>et al.</i> , 1996)
Chemical marking	Salmonids	Sr	Opercular bone	(Guillou & de-la-Nouee, 1987)
			Scale	(Snyder <i>et al.</i> , 1992; Mulligan, 1997)
	<i>Pagrus auratus</i>		Dorsal spine	(Pollard <i>et al.</i> , 1999)
Paleosalinities	Shark	$^{87}\text{Sr}/^{86}\text{Sr}$	Tooth enamel	(Schmitz <i>et al.</i> , 1997)
Metal pollution	<i>Salvelinus alpinus</i>	Pb	Opercula	(Koeck <i>et al.</i> , 1996)
	Marine fish	Se, Cr, Co, Fe, Zn, Cs, Ag	Scale	(Papadopoulou & Moraitopoulou-Kassimati, 1977)
	Sharks	Cd, Mn, Zn	Vertebrae	(Vas <i>et al.</i> , 1990)
	<i>Fundulus heteroclitus</i>	Zn	Scale	(Sauer & Warabe, 1989)

G. Microchemistry: methodological requirements and issues

1. Analytical techniques

Numerous analytical techniques have been used in OMC studies. As a matter of fact no single method can fulfil all the requirements of the wide range of questions to be addressed. The decision as to which technique is most appropriate depends on the application, the aspect of composition to be measured, the sensitivity required and the level of spatial (temporal) resolution needed. These considerations are illustrated by a block diagram linking main applications, measurement, resolution and techniques (fig. VII.9). Concurrently, some characteristics of major analytical tools are provided in table VII.5. In the following sections, we summarise the basic principles and capabilities of the most important techniques without attempting to cover the complete capabilities or limitations of each one.

1.1. Elemental composition

Analytical techniques are broadly divided into “surface” analysis techniques which provide composition information at specific locations or spots on the otolith, and bulk techniques which provide global composition information, usually integrated over the whole otolith. Some techniques measure the concentrations of several elements simultaneously (multi-element techniques) while others are only capable of measuring the concentrations of one element at a time. The selection of the most appropriate technique for a particular otolith application depends on the hypothesis to be tested, as this determines the spatial resolution required and the elements to be assayed. Criteria such as limits of detection (LODs) in relation to expected concentrations, particular requirements regarding absolute concentrations (as opposed to elemental ratios or relative differences) and the accuracy of the determinations with respect to approved standard reference materials (SRMs) must be taken into account when selecting the appropriate methodology.

Table VII.5 - Analytical techniques used in OMC and their main characteristics.

Technique	Acronym	Type	Information	LODs Spatial resolution	Comments
Energy Dispersive Spectrometry (associated with SEM or TEM)	EDS (or ED-EM)	S	- Elemental analysis - X cartography	> 1000 ppm - x: 0.5 μm to 10 μm	Multi-element analysis Surface condition critical
Wavelength Dispersive Spectrometry	WDS (or WD-EM)	S	- Elemental analysis - X cartography	100-1000 ppm - x: 0.5 μm to 10 μm	Good for light elements (Na, K...) with Sr and Ca Mono or multi-elemental Surface condition critical
Secondary Ion Mass Spectrometry	SIMS	S	- Elemental analysis - Isotopic analysis - Depth profiling	< 1 ppm to 100 ppm - x: 0.1 μm to 0.5 mm - z: 1 nm to 10 nm	Surface condition critical
Particle-induced X-ray Emission	PIXE	S	- Elemental analysis	$\approx$ 1 ppm - x: 10 μm to 1 cm - z: 1-10 μm	Not appropriate for light elements
Laser Ablation Inductively Coupled Plasma Mass Spectrometry	LA-ICP-MS	S	- Elemental analysis - Isotopic analysis	$\approx$ 1 ppm - x: 5-20 μm - z: 5-20 μm	Best compromise between ranges of elements detected and sensitivity Multi-element analysis Surface condition not critical
Atomic Absorption Spectrometry	AAS (flame, furnace)	B	- Elemental analysis	0.1 ppb to 500 ppb (flame) 0.005 ppb to 50 ppb (furnace)	Mono or multi-elemental Best suited for light elements
Atomic Emission Spectrometry	AES (flame, arcs and sparks)	B	- Elemental analysis	> 5 ppb	Multi-element analysis Less vulnerable to matrix or signal interferences than AAS
Inductively Coupled Plasma-Atomic Emission Spectrometry	ICP-AES (or ICP-OES)	B	- Elemental analysis	< 1 ppb to 30 ppb	Multi-element analysis Less vulnerable to matrix or signal interferences than AAS
Inductively Coupled Plasma Mass Spectrometry	ICP-MS	B	- Elemental analysis - Isotopic analysis	ppt to ppb	Multi-element analysis, Some elements subject to interferences (e.g. Ca, Na...) Best compromise between ranges of elements detected and sensitivity
Thermal Ionisation Mass Spectrometry	TIMS		- Isotopic analysis		High precision and accuracy for isotope-ratio measurements Need for efficient chemical separation of the target analyte
Isotope Ratio Mass Spectrometry	IRMS	B	- Stable isotopes (H, C, N, O, S)		Automatic micro-sampling can give better spatial resolution (10 μm) Limited by sample size considerations

S: surface technique; B: bulk technique; SEM: Scanning Electron Microscopy; TEM: Transmission Electron Microscopy; x: spot sample area; z: sample penetration depth. For bulk techniques, LODs refer to solution. They must therefore be multiplied by the dilution factor to refer to solid material.

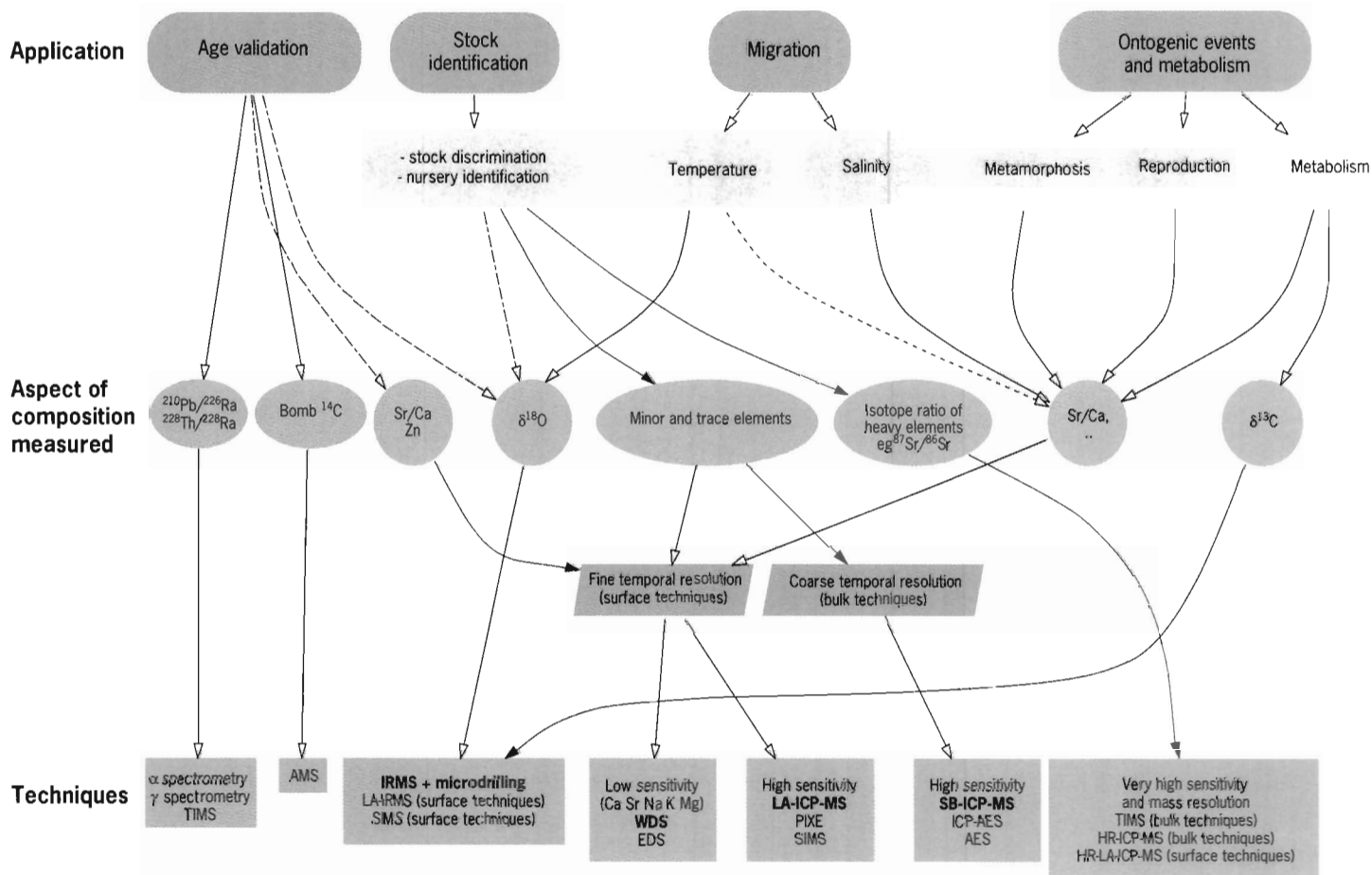


Figure VII.9 - Synthetic block diagram showing main applications of otolith microchemistry. For each application, the aspect of composition that is measured is shown, along with the relevant analytical techniques. Solid arrow: methods in general use; dash and dotted arrows: more rarely used methods; dotted arrow: method not recommended; bold text: most commonly used techniques.

The practise of expressing elemental compositions in terms of element ratios has become widespread throughout the microchemistry literature. For certain techniques this is a result, or limitation, imposed by the operation of the analytical machines. In specific applications it may also be reasonable to examine the relationships between elemental ratios, especially where the ratio is expected to be influenced by specific conditions. However, automatically expressing element concentrations as element ratios, especially as ratios to calcium, is inappropriate, and even biologically incorrect.

1.1.1. Surface analyses

A wide range of analytical techniques utilises the interaction of a primary energy particle beam (photons, electrons, ions) with the sample. The interaction results in the emission of a secondary beam (X-ray, electrons, or ions) whose characteristics depend on the nature, structure and composition of the sample. Of the instruments (see tab. VII.5 for expansion of acronyms) commonly used in OMC, EDS and WDS both use a primary electron beam whereas PIXE uses a proton beam. All these instruments analyse the emitted X-ray signal. They are multi-elemental (the number of elements that can be simultaneously analysed with WDS depends on the number of spectrometers the machine has), normally non-destructive and have very good spatial resolution. Their respective LODs, which are often element-specific, differ by several orders of magnitude (see tab. VII.5). They cannot measure isotopic composition.

Several other techniques are derived from mass spectrometry (MS). MS techniques separate ionised atoms or molecules from their differences in mass-to-charge ratio (m/z) and thus can be used to measure either isotopic or elemental compositions. The first step in an analysis consists of creating a gas phase ion from a given material. This is done in SIMS by using a primary ion beam accelerated and focused on the surface of a material. Note that SIMS allows depth profiling by continuous, regular sputtering of the surface material. In LA-ICP-MS, the excitation source (ICP for inductively coupled plasma) is argon plasma at very high temperature that efficiently excites and ionises atoms. ICP-MS was first designed to operate on liquid samples. The recent coupling of a laser ablation probe (LA) operating in the far UV now permits the sampling of discrete points on the surface of solid samples (rough surfaces, powder, polished sections or thin slides).

These techniques work with otoliths mounted on glass slides or in epoxy blocks. Some techniques are very sensitive to surface roughness (EDS, WDS, SIMS, PIXE). These therefore require a highly polished, mirror-flat surface with no defects. Other techniques such as LA-ICP-MS can be used with little preparation beyond sectioning and cleaning. More details on preparations are provided in chapter VIII.C.

1.1.2. Bulk analyses

Bulk analyses in OMC have used either atomic spectrometric methods or MS methods. The former are based on the interaction of electromagnetic radiations with matter. Atomic absorption spectrometry (AAS) exploits the absorption of light to measure the concentration of atoms in a gaseous phase. The light source is usually a hollow-cathode lamp made of the element to be measured so that this technique is generally mono-elemental. The sample is dried and vaporised (to obtain free atoms) in a flame or a graphite furnace. The former only accepts solutions whereas the latter can also accept solids and is a much more efficient atomiser. Atomic emission spectrometry (AES, also called OES: Optical emission spectroscopy) determines the elemental concentration of solution samples by measuring the optical emission of excited atoms. Various excitation sources may be used, including flame or inductively coupled plasma (ICP-AES) where atomisation is much more efficient. A major advantage of AES compared to AAS is that it is a multi-element technique.

Because of its advanced capabilities (sensitivity and less interference, except for a few elements; see below) ICP-MS is displacing the other techniques for the determination of trace and ultratrace elements. It can be operated in both standard and isotope-dilution (ID-ICP-MS) modes, the latter providing unparalleled measurement accuracy. Recently developed high-resolution HR-ICP-MS improves sensitivity even further and reduces interference in comparison with conventional quadrupole ICP-MS. Detection powers < 1 ppq are claimed by manufacturers for non-interfered isotopes.

Bulk analysis is generally performed on solution samples obtained by dissolving cleaned and dried otoliths in an acidic solution after weighing (see chap. VII.G.2 and VIII.C for more details).

1.2. Stable isotope measurement

Mass spectrometry techniques are used to measure stable isotopes but the exact analytical methods depend on the isotope being analysed. Heavier elements such as Sr can be measured using either surface or bulk techniques (LA- or SB- ICP-MS) but lighter elements such as C, N and O are measured using Isotope ratio mass spectrometry (IRMS). The vaporising source used in ICP-MS atomises the elements, but there is a risk that only some of the lighter and more volatile elements are channelled into the MS detector, leading to underestimates of their concentrations.

The procedure for the routine analysis of otolith C and O isotopes by IRMS requires the careful dissolution of the otolith in a sealed system, in order to capture evolved CO_2 . The gas molecules are ionized and stripped of an electron, causing each molecule to be positively charged. The charged molecules pass under a magnet that separates

them according to their mass. Faraday collectors then measure the intensity of each beam of ions of given mass after they have been separated by the magnet. Sample gas mass ratios are measured against a reference gas calibrated with respect to an international standard. Mass ratios should be corrected for ^{17}O effects in accordance with the procedures of Craig (1957) and temperature-dependent oxygen isotope fractionation between aragonite and CO_2 generated by reaction with phosphoric acid, using a fractionation factor (α) of 1.01034 (Friedman & O'Neill, 1977).

Prior to isotopic analysis, otoliths should be treated to remove organic components since the protein contains C and O with different isotopic ratios. The protein can be removed by roasting or bleaching. It is also possible to exploit plasma etching to remove organic material from otoliths, but this has not yet become a common pre-treatment technique.

Typical analytical precisions (σ_{n-1}) for replicate analysis of standard materials are 0.05‰ and 0.07‰ for carbon and oxygen isotope ratios, respectively. For oxygen, this precision corresponds to $\pm 0.2^\circ\text{C}$ for the estimate of temperature range.

IRMS may be equipped with automatic preparation devices that operate with ultra-small carbonate samples (5 μg) and are supported by an automated sampling device (computerised microdrill) capable of taking discrete samples across sectioned otoliths (Wurster *et al.*, 1999).

Recent developments in ion probes (SIMS) and laser probes (LA-IRMS) have permitted the measurement of C and O isotopes *in situ*, that is, from the surface of solid samples. SIMS offers a spatial resolution of 20 to 30 microns and has already proved to be a powerful tool in analysing oxygen isotopes in geological carbonates (Graham *et al.*, 1996). Analytical precision has been estimated to be $\pm 1\text{‰}$, giving a temperature range of $\pm 4^\circ\text{C}$. A laser probe (LA-IRMS) uses gentle heating to slowly extract CO_2 and as a consequence the spatial resolution is not as good as SIMS; however, analytical precision as good as 0.08‰ ($\pm 0.4^\circ\text{C}$) has been reported for oxygen isotopes measured in herbivore teeth (apatite) (Kohn *et al.*, 1996). These developments may soon offer a capacity for performing fine-scale surface analysis of otolith isotope ratios. For these surface analyses, it may be necessary to pre-treat the otoliths by bleaching or roasting before embedding to remove the protein components.

1.3. Radionuclides

A number of methods can be used to measure radioactive isotopes, depending on the mass, abundance, type of decay involved and accuracy desired. Methods that have been used in otolith radiometric ageing include alpha spectroscopy, gamma spectroscopy, thermal ionisation mass spectrometry (TIMS) and accelerator mass spectrometry (AMS).

Alpha spectroscopy and gamma spectroscopy are based on the type of decay of radionuclides by particle emission (either an α particle, which is a helium nucleus, or a β particle, which is an electron, or less often a positron β^+), as well as high-energy photons called gamma (γ) rays.

The α particles emitted from a given nuclide all have the same energy or are divided into a few monoenergetic groups between about 4 and 6 MeV. Alpha-emitting radionuclides are often quantified by alpha spectroscopy. This technique requires elements to be chemically separated before analysis in order to minimise interferences between different nuclides emitting α particles, whose energies can differ in a range near the resolution of the detectors used in alpha spectrometers. Like α particles, gamma rays are monoenergetic, and decaying gamma emitters provide gamma spectra with well-defined energy peaks that are often used to quantify specific nuclides.

With regard to otoliths, ^{210}Pb is generally assayed by α -spectroscopy through its α -emitting granddaughter ^{210}Po ($T_{1/2} = 138$ days), which is within 5% of secular equilibrium with ^{210}Pb for otoliths analysed one or more years after collection (Bennett *et al.*, 1982; Fenton *et al.*, 1990).

Otolith ^{226}Ra has been measured either directly by α -spectroscopy (Fenton *et al.*, 1990) or by the so-called ^{222}Rn emanation technique, ^{222}Rn ($T_{1/2} = 3.82$ days) being used as a proxy for its parent ^{226}Ra (Bennett *et al.*, 1982).

Alpha spectroscopy has also been used to measure ^{228}Th , while ^{228}Ra has been assayed by gamma spectroscopy using the gamma energies of its daughter product ^{228}Ac ($T_{1/2} = 6.13$ hours).

Note that prior to the analysis, samples must be submitted to thorough cleaning in order to remove any exogenous contamination, and subsequently to chemical separation processes. Detailed procedures may be found in Fenton *et al.* (1990) for $^{210}\text{Pb}/^{226}\text{Ra}$ analysis and Smith *et al.* (1991) for $^{228}\text{Th}/^{228}\text{Ra}$ analysis.

In order to improve the precision of radiochemical ageing of otoliths, an ion exchange separation technique of Ra and TIMS analysis has been recently used (Andrews *et al.*, 1999b). In comparison with conventional methods it has been proved to be more accurate and precise, less time-consuming and requiring less calcified material.

Due to the extremely low natural abundance of ^{14}C (there is one ^{14}C atom for 10^{12} ^{12}C atoms) and the inability of conventional MS to distinguish between ^{14}C and the very abundant nitrogen isotope ^{14}N , otolith ^{14}C measurements require the use of particle AMS.

2. Data quality

Several issues pertaining to data quality have been raised during the development and increase in applications of OMC. It is important to realise that there are many steps in the process of obtaining information about fish otolith composition and at each step there are factors that potentially affect the quality of the data. These issues directly affect the interpretation of microchemistry data, since they control the value (elemental concentration) assigned to each individual fish or area of the otolith. In addition it is important to evaluate the precision and accuracy of the various analytical tools employed in order to select the technique most suitable for an individual application. The quality of analytical data and the need to quantify uncertainty in analytical measurement is a topic of continuing interest to analysts. A practical guide to some of the procedures recommended for quantifying analytical uncertainty is available from the Eurachem/Citac Working Group (Ellison *et al.*, 2000). Although this guide is designed for more routine applications, it is a source of useful methods for evaluating data quality in OMC studies.

2.1. Potential sources of uncertainty

It is clear from the discussion and examples offered in this chapter that the study of OMC is of recent origin and is still developing rapidly. There is not yet sufficient information available to quantify precisely all the operations and processes that can alter the composition of fish otoliths. Some of the uncertainty is due to biological processes that are still poorly understood. Other factors act during the process of fish capture, sample preparation and analysis (fig. VII.10). The uncertainty, or range of error, has not yet been quantified for all of these factors, and only some can be manipulated to minimise the error. Not all of the uncertainty has been quantified for elements that are of interest in OMC, although there is now some evidence that elements bonded to the organic material or adsorbed onto the surface are less robust (i.e. more affected by the analytical procedure) than others. The following sections refer to the steps indicated in figure VII.10 and words in italics refer to the problems listed along the arrows emanating from each step.

2.1.1. Sampling

The *sampling strategy* employed for collecting fish samples can inadvertently result in problems in interpreting OMC results. Decisions need to be made to ensure either selective or representative samples, depending on the application. The method used to collect fish is also important

because *capture conditions* may exacerbate stress-induced or *post mortem* changes in the endolymph. When techniques such as trapping or gill netting are used, the interval between fish capture and retrieval may be sufficient to alter the concentrations of some elements.

2.1.2. Fish storage

Sometimes fish are stored after collection until their otoliths can be extracted and analysed. The *storage method* has been shown to alter otolith composition. Significant differences in the concentrations of Na and K were observed between otoliths of fish stored frozen or in ethanol (Milton & Chenery, 1998; Proctor & Thresher, 1998), and the purity of the storage medium may also be important. The effect of *storage duration* is unknown. Most studies have tested storage methods over weeks or months, but many applications require the analysis of samples that have been stored for much longer. There should be a limit to the amounts of individual elements that can leach out of, or be introduced into, the otoliths during storage, but this is likely to differ between elements. The effect of storage method and duration also varies with *fish size* and species, since the size of the otoliths and their position in the head will affect the rate of exchange between the otolith and external medium, or from the fish tissue during defrosting or storage in ethanol.

2.1.3. Otolith extraction

During otolith extraction there is a danger of *contamination* of the otolith, especially when using metal dissection tools and paper envelopes for storage. Likewise, *surface cleaning* after extraction needs to be consistent because of the need to remove any adhering tissue. The otolith may also be contaminated by elements (particularly Na) if they are cleaned using bare hands. The effects of contamination cannot be quantified, because they vary with each otolith and action, so plastic or ceramic tools should be used to extract otoliths and they should be stored in inert containers. Otolith composition is often measured after age reading, and sometime otolith readers use different media to enhance the rings. Otoliths are sometimes *immersed* in water, glycerine, or oil and these can contaminate the otoliths or mobilise various elements, changing their spatial distribution. Most otoliths are stored dry before OMC analysis, both for ease of storage and because of the potential of storage fluids to mobilise otolith elements. *Desiccation* also adds an unquantified error since the protein portion of the otolith can disintegrate together with any accompanying elements, and elements distributed in inter-lattice spaces may be displaced.

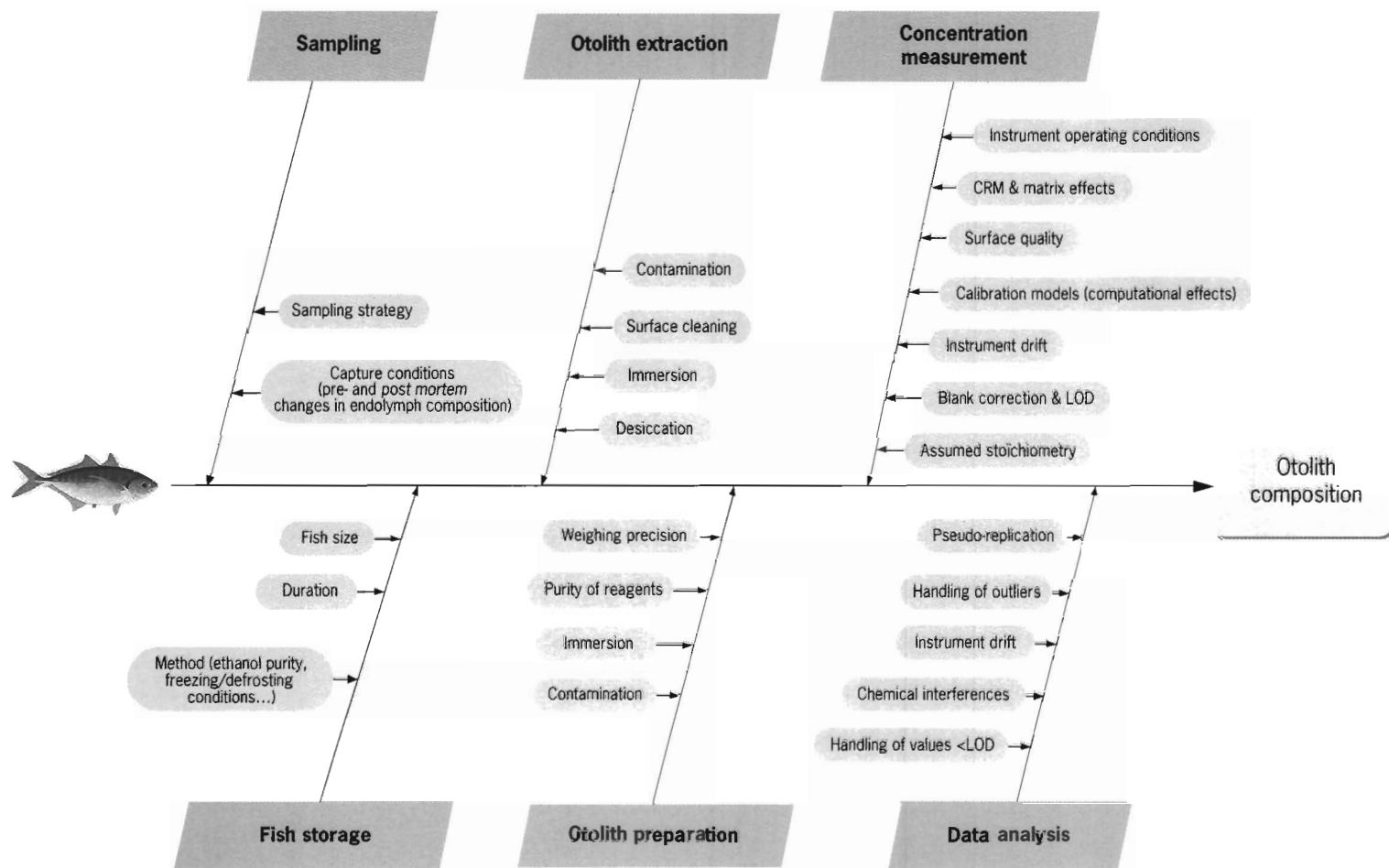


Figure VII.10 - Potential sources of uncertainty associated with the process of analysing the chemical composition of fish otoliths and other CS.

2.1.4. Otolith preparation

Otolith *contamination* is almost unavoidable during the preparation of samples for surface analysis. Otolith cores or edges that have been cut for bulk analyses are also vulnerable to contamination. The saws, lubricating fluids, grinding and polishing cloths and suspensions must be carefully selected to minimise contamination (see chap. VIII.C). Even if ultra-pure water is used for cleaning and lubricating, *immersion* itself may cause mobilisation of some elements (especially Na). Otoliths are porous, and fluid readily enters the crystal lattice by capillary action. It is important to ensure the *purity of reagents* used so as not to introduce contaminants. Some polishing compounds contain ethanol or oils, and grinding papers and compounds can include high levels of Al as well as other metals. Glass is a source of Si, as well as of S and Pb, and can contaminate the otolith surface. The resins used for embedding should have low levels of trace elements, and it is often helpful to analyse the resin alongside otolith samples in order to estimate background concentrations or potential levels of contamination. Gloves should be worn during sample preparation in order to avoid contamination from skin salts and oils.

Bulk analyses give results that must be normalised to the exact weight of the otolith. *Weighing precision* thus has a direct and quantifiable effect on the calculation of element concentration.

2.1.5. Composition measurement

The most difficult part of OMC is the measurement of the elements in the otolith samples. This is complicated by the fact that most applications require the biologist to understand some principles of physics and chemistry, and sometimes to learn to operate machines that are designed for use in other sciences. Biological applications also tend to push these machines to their limits of performance, because the concentrations of the elements of interest are low, as is the amount of available material, and the need for spatial resolution may be great. The sources of uncertainty associated with measuring composition are not always apparent to new users. Systematic analytical experiments can be used to quantify most of the measurement error, but only a few such studies have been published (chap. VII.G.2.2).

Instrument operating conditions have a profound effect on the measurement of element concentrations. For instance, for electron probes the quality of the data depends on the interaction between beam diameter, the intensity and the voltage of the excitation current and acquisition time. The sensitivity of bulk analyses is affected by sample dilution rates and the number of elements being measured, and also varies with the element or isotope measured. In all cases the instrument settings used represent a compromise between the competing needs for accuracy, precision, and speed of analysis.

One of the major sources of uncertainty in measurement results from problems in procuring suitable reference material (chap. VII.G.2.2). The magnitude of the error can be estimated by comparing analyses that include different CRMs, and the availability of new CRMs for otoliths should alleviate this problem. Even with suitable CRMs, *matrix effects* need to be controlled or taken into account. There are two types of effects to be considered in OMC. The otolith protein matrix takes uncertainty in the determination of otolith composition into account because the complete array of elements and their relative concentrations that are associated with the protein is not known. Some digestion protocols for bulk analysis will dissolve the protein so that both organic and inorganic components are measured. Other digestion protocols leave a visible organic “ghost” that represents different amounts of the original otolith protein. The effects of the protein matrix on the measurement of elements by surface methods are harder to quantify, but they include variations in the quality of the surface that alter the efficiency of the probes. Matrix effects also mean the analytical problems associated with measuring elements within compounds. In ICP-MS and WDS the detection of different elements in the otolith is complicated by the predominance of CaCO_3 . The error contributed by these *matrix effects* can be controlled by matrix-matching, where the analytical blanks and standards are also presented in a comparable CaCO_3 solution. This is easier to achieve for SB-ICP-MS than for LA-ICP-MS or WDS, where there are no suitable CRMs. The quality of the prepared otolith surface is an important consideration in surface-analysis techniques. Only LA-ICP-MS is insensitive to the *surface quality* (but the surface should still be free of contamination). Cracks in the surface of the otolith cause extra scattering of electrons and thus introduce errors in the detection of elements and the quantification of their concentrations.

The translation of the machine-measured signal into concentrations of elements depends on a *calibration model* based on the signal measured from the analytical standards. The fit of the calibration lines should be checked before proceeding to analyse otolith samples, otherwise *computational effects* will introduce measurement errors.

Instrument drift is a significant problem in these analytical techniques. The term refers to gradual, or sometimes abrupt, changes in the sensitivity of the machine over the course of one or more consecutive analysis sessions. There are several methods used to minimise the effects of drift on the determination of otolith composition. The usual approach is to try to analyse samples over a short time period. Sometimes a subsample of otoliths can also be re-measured at intervals to quantify instrument drift and correct for it during later calculation stages. Randomisation of samples helps to ensure that drift errors do not systematically affect any particular group.

One significant source of error in measurement is the analytical blank. The quality of the analytical and procedural blanks affects the *blank correction* used by instrument software to calculate concentrations. The blanks are also analysed for calculation of the LODs, and any contamination or matrix effects will result in lowered sensitivity. Blank correction software can produce negative values for element concentrations. These are equivalent to concentrations below the LOD.

In WDS the *limits of detection* (LOD) are estimated for each element from the standard deviation of the background signal when the standards are measured. For solution-based ICP-MS, the limits of detection are estimated from the standard deviation of replicated measurements on blanks corresponding to the solution that is used to dissolve samples. The estimations correspond to those of solution samples and have to be corrected by a dilution factor to refer to the otolith sample, i.e. to give the LOD in terms of $\mu\text{g}\cdot\text{g}^{-1}$ (= ppm) of otolith. To date, no agreement regarding methods of estimation of the limits of detection for LA-ICP-MS has been reached, and several possible methods have been suggested:

- estimation from measurements on the argon gas (i.e. without ablation);
- estimation from measurements on glass standards (e.g. NIST 612 & 610);
- estimation from measurements on powdered standards.

Each method leads to a different estimate of LODs but none of them is satisfactory because of the lack of matches with the otolith matrix. The first one is the least 'conservative' since it does not take into account the quantity of ablated material, which significantly influences LOD values.

Whenever the amount of otolith material actually analysed varies between spots (as in laser ablation) or is not measured, the element concentrations are usually reported relative to some constant measure. In stable isotope analysis the IRMS measures isotope ratios in the sample and compares this ratio to that in the standard (PDB, SMOW, etc). In LA-ICP-MS the usual method is to normalise the counts of the elements with respect to simultaneous counts of calcium. When calcium concentration is not known, calculation of elemental concentration depends on an *assumed stoichiometry* for calcium within CaCO_3 . If otoliths are regarded as pure CaCO_3 the proportion of calcium is 40%. The difference from the "true" value will affect the estimated concentrations of the otolith elements, but will not affect their relative abundances. However, any heterogeneity in the distribution of calcium within the otolith will result in under- or over-estimation of the true element concentrations.

2.1.6. Data analysis

In OMC applications there are many situations in which the concentrations measured are close to the LODs, and methods of *treatment of values below the LOD* must be decided before statistical analysis. The existing microchemistry literature unfortunately contains few details about either the calculation of LODs or the treatment of values that fall below the LOD. It is necessary to report how the LOD has been calculated, how the analytical results falling below the LOD are treated statistically, and what percentage of the results for each element fall below the LOD. This should be reported separately for each group of samples. Differences in these values between sessions can obscure the real comparisons between groups and produce misleading trends in the data.

There are several methods for dealing with analytical results below the LOD:

- include all data, using the values which are below the LOD in calculations of means to represent individual fish or otolith areas;
- set values below the LOD to 0, using 0 in calculations of means to represent individual or otolith areas;
- set values below the LOD to the LOD, using the LOD value in calculations of means to represent individual or otolith areas;
- enter values below the LOD as missing values;
- convert all values of elements which have measurements below or near the detection limit to presence/absence data and use appropriate statistical methods.

In some applications it is possible to ignore elements that have many values below the LOD. This is useful for improving data quality when the results are subjected to multivariate analyses, as in stock separation problems. In all cases, the occurrence of values below the LOD must be reported for each element, as:

- percentage of all points below LOD;
- percentage of individuals with points below the LOD.

Statistical methods that include the analysis of the proportion of points below the LOD should be developed to help characterise population and individual responses.

The values obtained for element concentrations are only reliable if the measurements are free of spectral or *chemical interferences*. Interference can artificially elevate the calculated concentrations or obscure their measurement. The presence of interference can usually be detected by examining the concentrations calculated for different isotopes of a single element. If the ratios between the isotope concentrations in the sample correspond to the *naturally occurring proportions*, then those data values can be regarded as being free of interference. It is helpful to be

familiar with likely sources of interference before beginning an OMC study. Spectral interferences in X-ray emission lines are published for WDS and EDS, while software in newer ICP-MS instruments offers the capability of checking likely interferences (with associated quantification) for each isotope of any given element with compounds of similar nominal masses. In ICP-MS, spectroscopic interference is most likely to occur with isobaric ions (e.g. ^{87}Sr and ^{87}Rb), refractory oxides (MO^+) and argon polyatomic ions (ArX^+ with $\text{X}=\text{H,C,O,N}$) formed from the argon plasma. With regard to the latter situation, the case that is often discussed is that of interference between $^{40}\text{Ar}^{16}\text{O}^+$ and $^{56}\text{Fe}^+$. Various ArX^+ ions may interfere with the various Fe isotopes, although to different extents, complicating the measurement of this element by this technique. Whether an interference will be separated from an isotope of interest depends on the instrument mass resolution which has been considerably improved in recent years.

As mentioned above, *instrument drift* can produce OMC data trends that are actually artefacts of the analysis. Some techniques use automatic software to correct for instrument drift before producing the final concentration values. It is useful to examine the concentration data before statistical analysis in order to detect the presence and influence of instrument drift. The data values for each element should be plotted sequentially against the order of measurement. If there are time trends in the data, it is sometimes possible to determine and correct for the drift using a regression model fit to measurements of the blanks or standards made at the start, middle, and end of each session.

Prior to statistical analysis, the data should be examined and selection criteria applied to exclude *outliers*, or aberrant analytical results. Certain data points should be rejected from further analysis, especially when they result from irregularities and fractures in the otolith surface, the inclusion of resin in a spot, etc. In WDS analysis, measurements are sometime inadvertently made at fractures or scratches if the visualisation attachment has low magnification or if measurements are made along pre-set radial tracks. Aberrant points in WDS can easily be identified by their Ca values, and the results for all elements measured at such spots should be rejected. In LA-ICP-MS fractures and resin at the edge of the otolith may inadvertently be included within ablation points because the exact ablation volume varies and cannot be controlled. These points should be identified by the values of abundant elements such as Sr or Mg. Continued improvements in laser technology may reduce the errors in ablation points. The identification and rejection of aberrant points should be based on criteria that depend on how far the results deviate from the average. Local areas of contamination may also produce aberrant points that should be rejected in the same way.

When suspect data points are the result of analytical problems they should be removed before statistical analysis. Occasionally, anomalous data values that cannot be attributed to analytical problems are observed. This can happen in multi-element determinations in which the concentrations of all other elements for this measurement are "normal" and one element is "abnormal". In some publications these outliers are removed from statistical analysis, but there is no real justification for this.

There is a danger of *pseudo-replication errors* in analysing otolith composition data. OMC is expensive and time-consuming and there is often pressure to reduce the number of samples analysed. In some studies spots measured along an otolith transect are pooled to represent a single value for an individual. Likewise, several spots measured in one area of the otolith are often analysed statistically as independent replicate samples of that individual. Values measured along a transect are usually not independent because of age- or size-related trends in composition. Independent replicate measurements for an individual fish should ideally be taken from different otoliths. A random subsample of transect measurements can be used for statistical analysis to avoid the problem of co-dependence. Thresher (1999) discusses other pseudo-replication problems associated with sampling strategy and fish handling.

2.2. Accuracy and precision of analytical tools

Very few studies have focused on methodological topics. Gunn *et al.* (1992) reviewed the principles of using Electron probe micro analysis (EPMA) for otolith composition analysis (Ca, Sr, Na, K and S). They provided the first quantitative comparison of the detection capabilities of EDS and WDS and concluded that EDS is not sensitive and precise enough to provide suitable data for otolith composition analysis. They discussed the effects of WDS operating conditions (beam area (A), accelerating voltage (E), current intensity (I) and acquisition time) on data quality and concluded that a beam power density ($BPD = E \cdot I/A$) of $3 \mu W \cdot \mu m^{-2}$ is an acceptable compromise. Toole & Neilsen (1992) also discussed the effect of measurement errors associated with Sr and Ca quantification by WDS on hypotheses drawn up from otolith Sr/Ca ratios.

More recently, an international otolith composition experiment (Campana *et al.*, 1997) compared the potential of the most popular surface techniques (EDS, WDS, LA-ICP-MS and PIXE) and examined differences among laboratories using similar instrumentation. The results showed that:

- there is no single instrument to be preferred for elemental analysis. Na and K were measured by only EPMA (EDS and WDS) whereas trace

elements were measured with LA-ICP-MS or PIXE. As expected, EDS showed the highest LOD and the poorest precision;

- accuracy, precision and LODs differed among laboratories using the same instrument. Such differences may be partly due to laboratory-specific operating conditions. This makes it difficult to compare published data and indicates that inter-calibration is needed for projects involving several laboratories;
- the development of certified reference materials (CRMs) for otoliths is critical to the further development of elemental analysis and is also a prerequisite for comparisons of published data. Actually, geological CRMs that are often used in OMC do not match the otolith matrix (aragonite and proteins) nor do substitutes that have been proposed for probe analysis such as otolith powder fused into glass beads (Campana *et al.*, 1997) or pressed carbonate pellets (Perkins *et al.*, 1991; Pearce *et al.*, 1992). A CRM for solution-based analyses, prepared from *Lutjanus sebae* otoliths, has been announced, with certified values for Na, Mg, K, Ca, Sr, and Ba and reference values for Cu, Zn, Cd and Pb (Yoshinaga *et al.*, 2000).

Chapter VIII

Preparation and observation techniques

As has been seen in the previous chapters, scales, otoliths and skeleton bones are used to estimate the age of fish in years, seasons or days. Preparation of CS implies a series of processing steps that may be complex and time-consuming. Before beginning such studies it is necessary to know the constraints on available time and costs, and especially the final objectives and expected results (chap. III). The choice of an age estimation method for a given species involves deciding on an appropriate CS (e.g. scales, otoliths, vertebrae, spines, etc.) and preparation method (e.g. whole CS, impressions, sections, thin sections, etc.) for that CS. This chapter will help the reader to choose from among the multitude of techniques available.

In any given species, the choice of preparation method for CS may also depend on the size of the fish, e.g. direct embedding of larval otoliths, or on the type of final analysis required, e.g. grinding and polishing otoliths for microstructure studies and “clean” extraction and preparation techniques for microchemistry studies. In the latter type of study the preparation techniques are primarily governed by the need to reduce sample contamination.

The aim of this chapter is to guide the reader's choice in the direction of one or more preparation methods, first by using flow charts to help his decision, secondly by choosing an extraction method for a given CS, thirdly by selecting a particular preparation technique and finally, by selecting an observation method. The selection of both the method of preparation and the observation technique to be employed should also reflect the growing demand for demonstrable quality assurance and quality control measures (McCurdy *et al.*, 2000) (see also Web site <http://www.mar.dfo-mpo.gc.ca/science/mfd/otolith/english/quality.htm>).

A. Aid to decision trees

H. Troadec, H. de Pontual

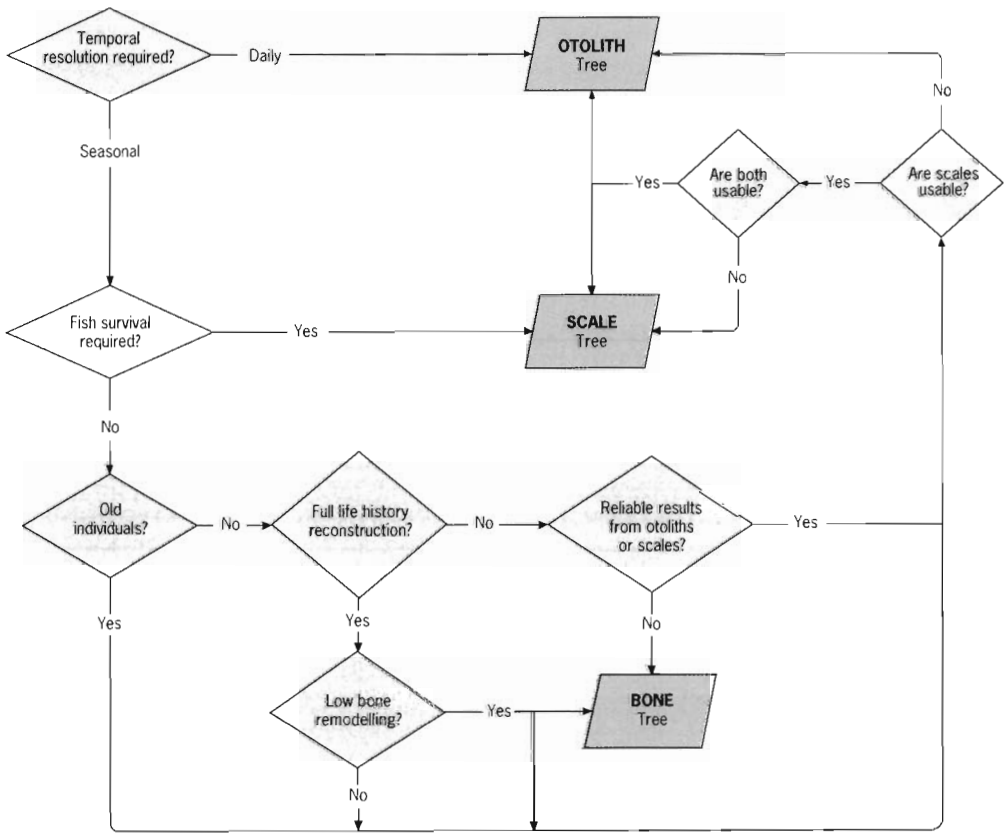
The primary aim of this sub-chapter is to provide the reader with an overview of the subsequent descriptions of extraction, preparation and observation procedures. It is also intended to give the readers direct access to the relevant procedures according to their individual goals and constraints. The task of outlining the decision trees was complex and exhaustive representation of the different ways to process each CS, in so far as this is possible, would have resulted in a huge and unusable flow chart. For this reason, we have chosen to reduce the scope of the decision tree to the principal CS processing procedures and to primarily target a public of novice users. These decision trees were based on the book material and constitute the unique link between theory and practice. However, CS experts may consider them incomplete, over simplified or even wrong as counter-examples can readily be found in the literature, while novices may even have difficulty in answering some of the questions. We hope that at least this sub-chapter will guide novice readers in their choice of a CS, specially when literature is poor, and accelerate their CS process adjustment.

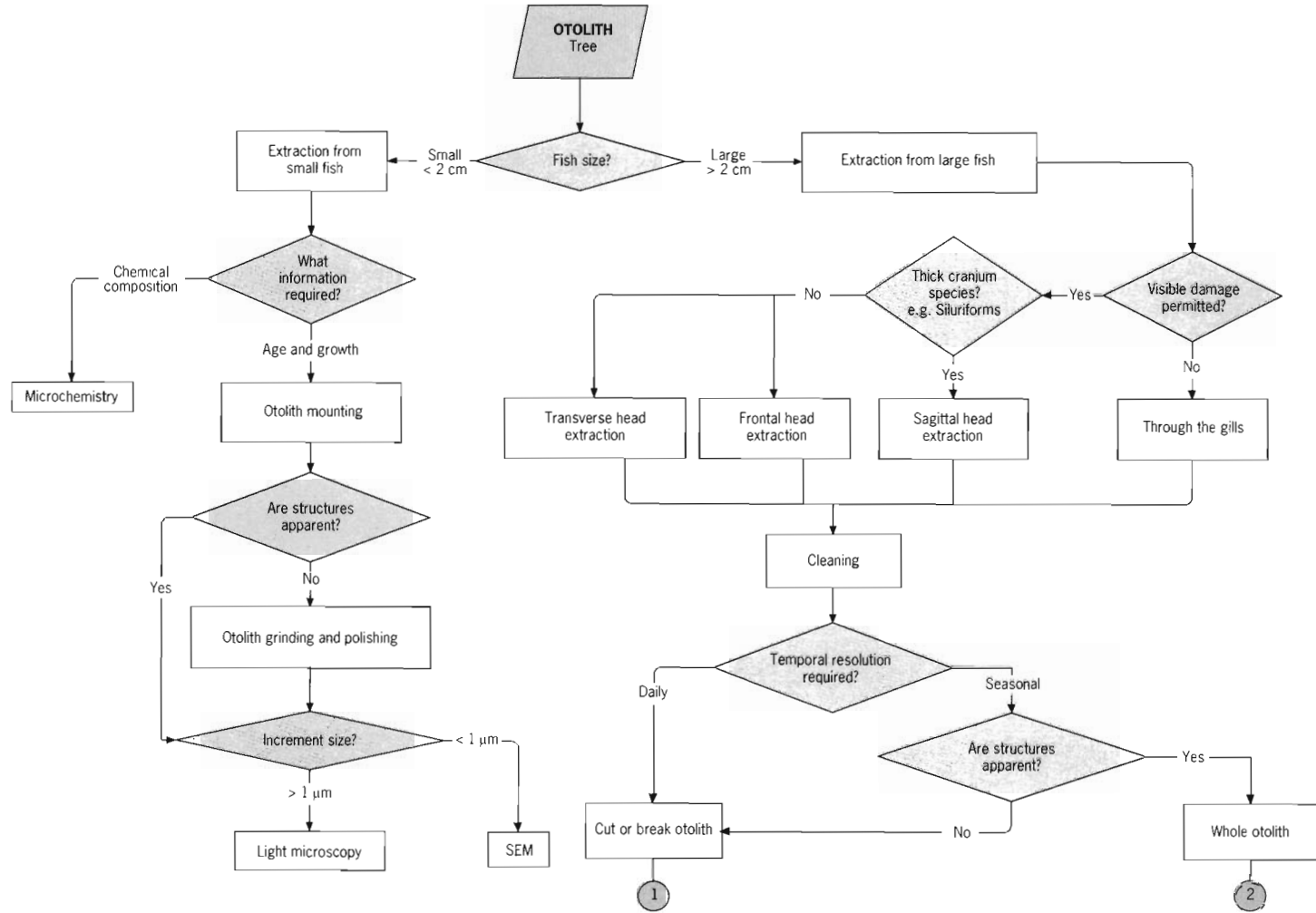
Trees are composed of:

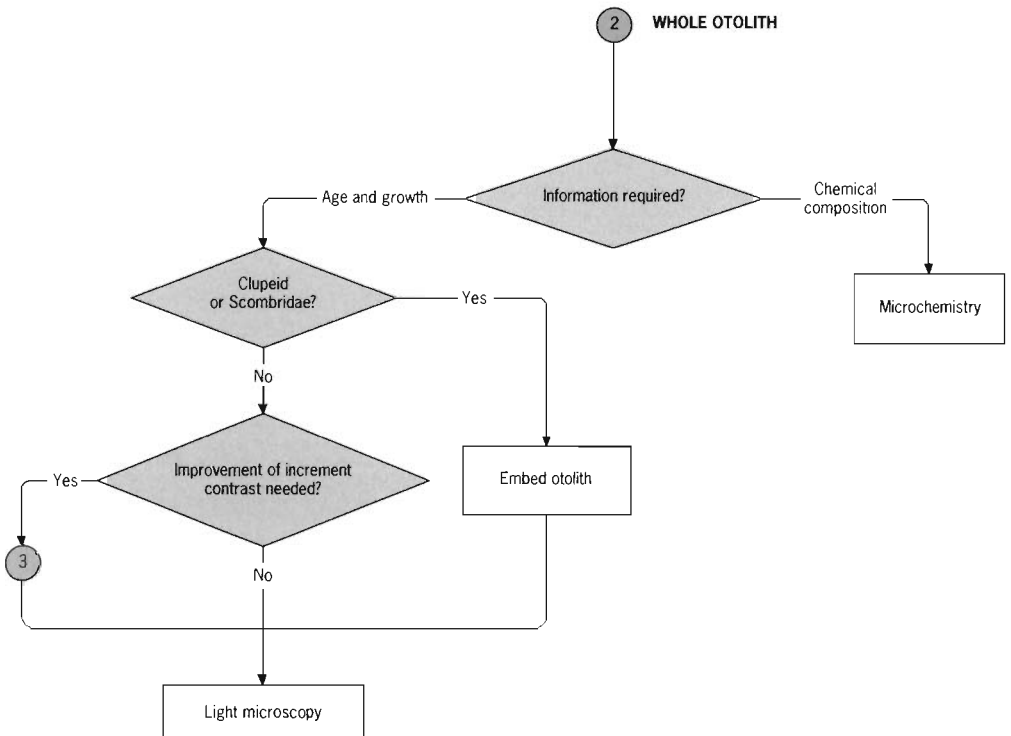
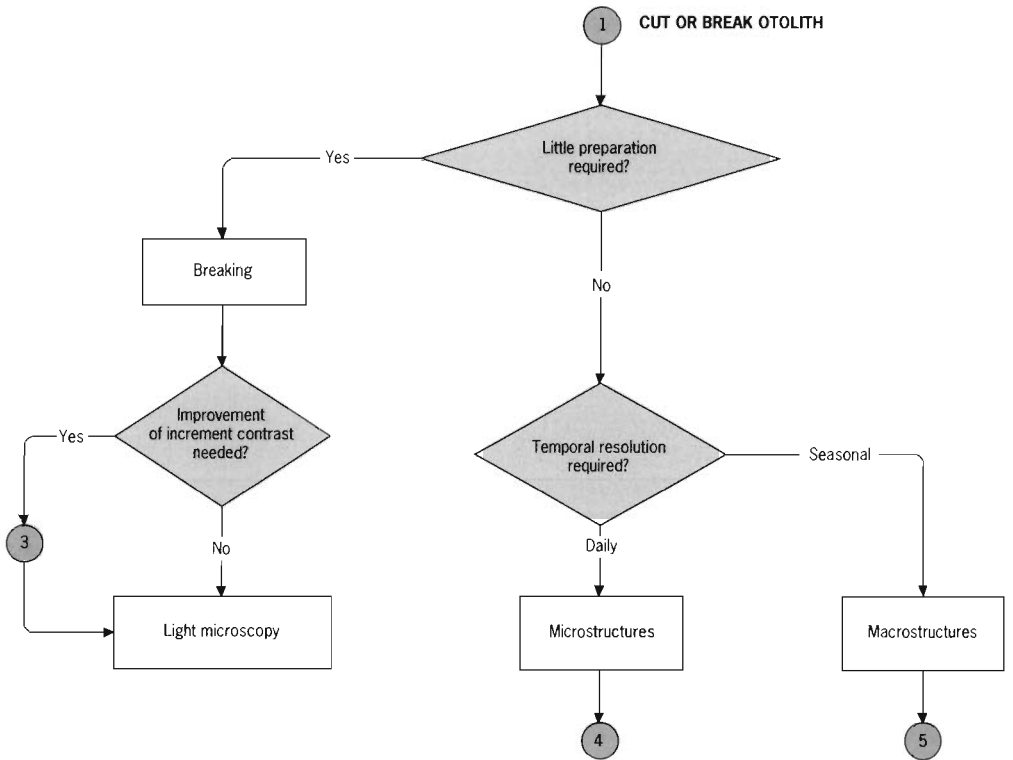
- leaves that are represented by rectangular boxes and correspond to procedures described in the following sub-chapter. The multimedia version has hypertext links between each leaf and the relevant sub-chapter. *Italic text indicates optional procedures*;
- control branches that are represented by diamond shaped boxes and contain conditions permitting orientation of the tree run when a node is reached. Most of the conditions relate to study aims, but some will require a CS preparation to be made before the question can be answered, (e.g., “Ring contrast enhancement needed?”).

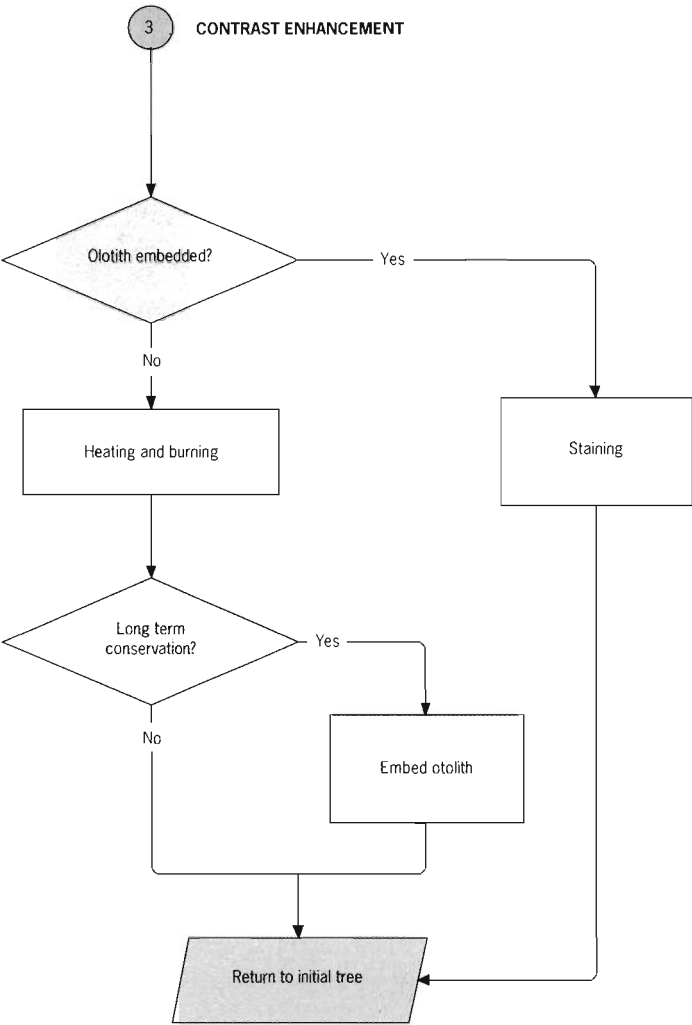
A tree run finishes when an end leaf is reached, the protocol being the collection of the procedures identified during the run. When encountering a multiple choice branch, or a question node that appears to be ambiguous, refer to the literature and/or your personal experience in order to compare the different solutions. If you intend to introduce a technical innovation (i.e. varying a preparation procedure or using a different CS), you should assess the relative benefit provided by this technical improvement versus your capability to compare your results with previous studies. Finally, if you intend to use several observation techniques, remember to check that all the preparation procedures are compatible and that they can be adequately combined at the different stages, before beginning the work.

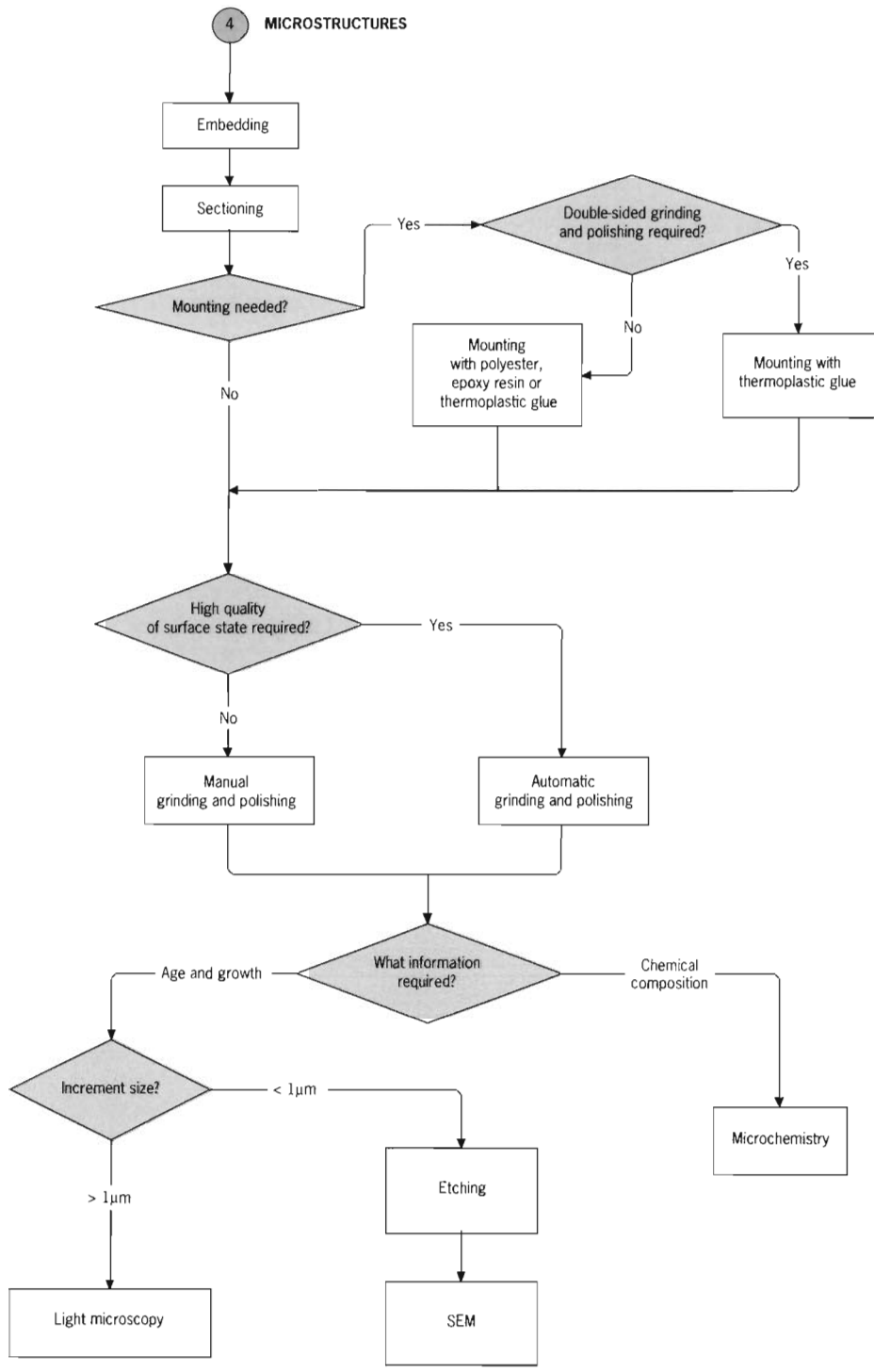
The first decision-tree uses very general criteria to select an appropriate CS, i.e. otolith, scale or bony tissue. The remaining three trees focus on these CS and on their different processing methods.

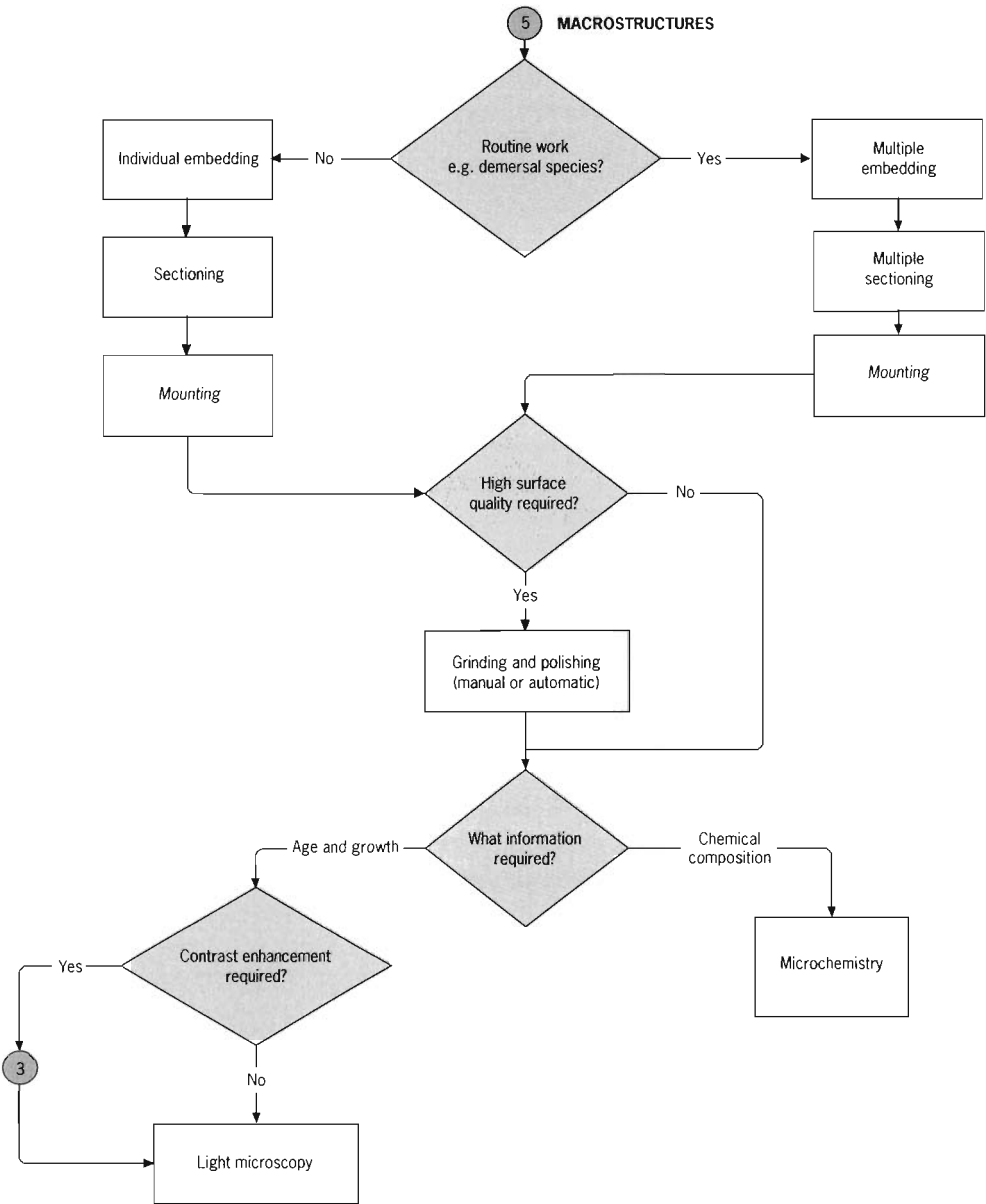


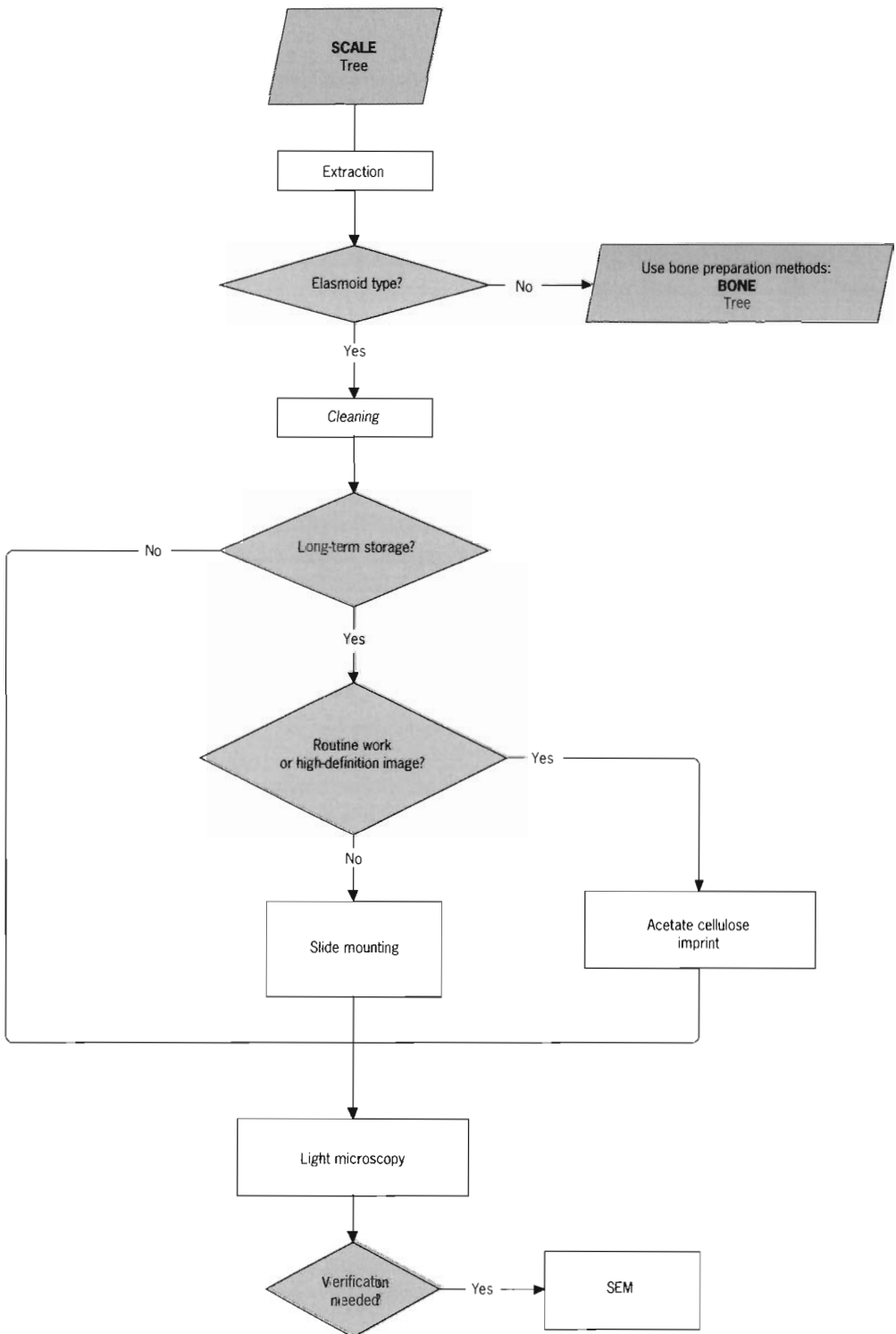


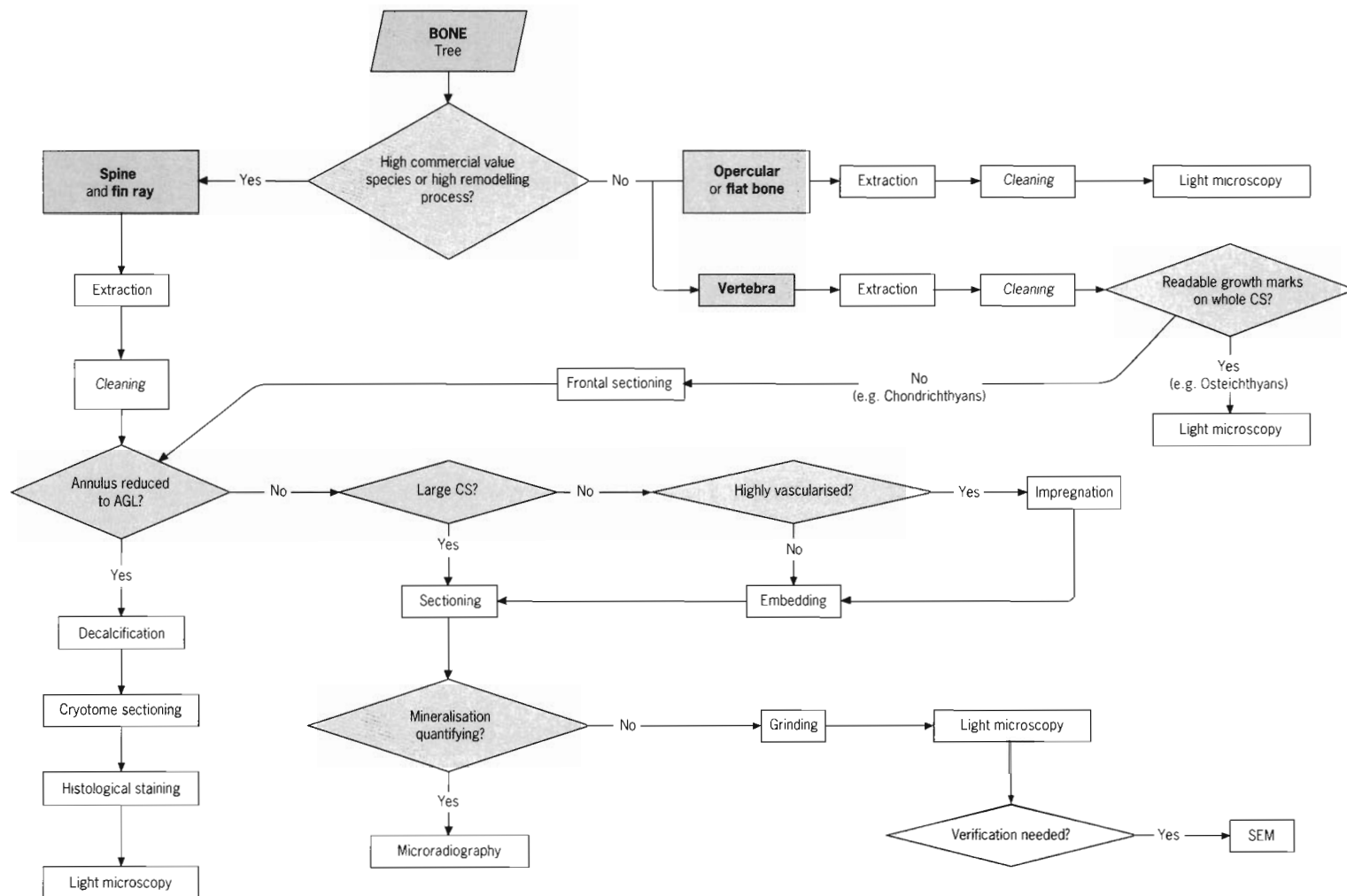












B. Extraction and conservation of calcified structures

J. Panfili

The stages of extraction, handling and conservation of CS are of prime importance for the success of the subsequent stages of the analysis to be undertaken: age estimation, microchemical analysis, etc. Inappropriate conservation techniques can cause irreversible degradation of the samples. All the planes of extraction, preparation and observation described in this chapter refer to the classical orientations of living symmetric organisms: sagittal, frontal and transverse planes.

When CS are being collected and conserved for later studies of their composition (microchemistry) it is important to reduce as far as possible any sources of sample contamination. Gloves should be worn to prevent contamination by salts and oils from the skin. Metal dissection tools are also a source of contamination and extraction should therefore be carried out using plastic or ceramic tools. Storage affects the composition of otoliths and potentially other CS. The samples should be stored in inert containers (not paper envelopes) and should not be stored in conserving fluid. As far as possible, the extraction and manipulation of samples for microchemistry studies should be carried out under clean conditions and if possible in a laminar flow cabinet.

1. Storage and preservation of whole fish

The method used to conserve the whole fish influences the preservation of CS. For example otolith structure can be altered by inappropriate conservation techniques, as *post mortem* degradation or dissolution inside the labyrinth of the internal ear is a possibility (Brothers, 1987). This is generally the case for any internal CS.

It is better to extract calcified material from freshly killed fish at any stage of their life history. Extreme care should be taken with larval and juvenile stages as their otoliths are smaller and more liable to degradation (Brothers, 1987). If conservation is necessary, this may be done (1) by freezing or (2) in alcohol. Several alcoholic solutions have been mentioned in the literature but we recommend the use of 95% ethanol (at lower concentrations some risk of deterioration remains). One should also check the volume of alcohol relative to the volume of the fish in order to avoid dilution of the effective concentration. It is of course strongly recommended to avoid acid solutions such as formalin for conservation because of the calcareous nature of the otoliths or other structures and the possibility of secondary destruction

(Williams & Bedford, 1974). Even buffered formalin should be avoided (Brothers, 1987).

2. Scales

The scales are the easiest structures to be extracted. They can be removed directly but carefully, even on living fish, with forceps (fig. VIII.B.1). The survival of individuals after scale removal is not a problem (scale loss is a natural phenomenon). The area from which the scales are sampled must be chosen carefully: the shape of the scale differs from one area to another within individual fish, so the sampling site must be standardized. This site may also differ from one species to another (Werder & Soares, 1984; Baglinière & Le Louarn, 1987). The first experiment on any new species should compare various sampling locations before choosing those that are least rich in regenerated scales or in variations in shape (Paul, 1968; Werder & Soares, 1984; Al-Absy & Carlander, 1988). Sampling locations are usually in the latero-dorsal area (e.g. under the dorsal fin) or an area where the scales are protected from damage (e.g. under the pectoral fins for some species). Some locations at which the scales are modified, such as the lateral line, should be avoided. Several scales should be extracted at the same location for the sake of future comparisons. The main advantage of scale extraction is that the fish can be kept alive during sampling.

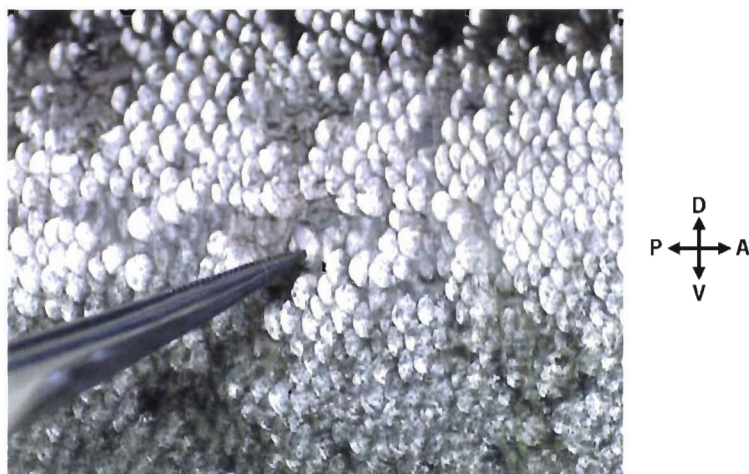
After extraction, scales should be cleaned. The cleaning operation is fairly simple, ranging from direct conservation with mild cleaning (e.g. wiping in absorbent paper) to ultrasonic bath cleaning. The bath may contain distilled water, normal water, potash, sodium peroxide or trypsin. The duration of immersion in the active solution must be controlled to avoid partial destruction of the scales.

Scales are usually conserved dry in labelled vials or envelopes. Scales are normally hydrophilic tissues and dry conservation distorts their original shape. When a scale that has been stored for a long time is to be studied it is sometimes rehydrated (e.g. for large or thick scales for some species), normally in water and without the need for any particular precautions to be taken. Scales are also often conserved after mounting between microscope slides (chap. VIII.C.1).

3. Otoliths

Otoliths should be extracted from newly killed or dead fish, or after conservation by freezing or in alcohol. If necessary, it may even be possible to use otoliths extracted from cooked fish, providing that suitable precautions have been taken to ensure that the otolith structure has not been altered by the cooking process. Extraction is relatively

Figure VIII.B.1
Scale extraction from
a Salmonid (*Salmo trutta*),
using forceps,
in the latero-medio-dorsal
area. A, anterior; D, dorsal;
P, posterior; V, ventral
(photo © Ifremer
O. Dugornay).



simple in large fish, depending on species and/or individual size, but is more difficult in larvae and juveniles. Obviously, prior knowledge of the cranial and vestibular anatomy of a given species will facilitate otolith removal. Usually only the larger otolith is extracted and stored for subsequent study (the *sagitta* for non-Ostariophysean and *asteriscus* for Ostariophysean species). The procedure is more complicated when we wish to extract all the otoliths, due to the small sizes of the 2nd and 3rd pairs: in such cases a binocular microscope is essential.

In a recent review of descriptions of the internal ears of cartilaginous fishes, Lychakov *et al.* (2000) point out that they do not have otoliths like bony fishes. The term “otolith” is therefore restricted to bony fish species. Age in Chondrichthyans is estimated using parts of the internal skeleton.

When removing the otoliths for the first time from a given species, one should be aware of the orientation planes of the material under study. This is very important for the subsequent stages of preparation and description. All otolith images should include an indication of the orientation.

3.1. Extraction from large fish

Except in very small species, otoliths can be extracted without the need for magnification (e.g. by binocular microscope). There are four main techniques of removal, the choice of which depends on the plane of the cranium section:

- frontal head section;
- transverse head section;
- sagittal head section;
- through the gills, ventral cranium section.

The frontal head section is universal and can be used for any kind of fish (species, size, cranium morphology). Nevertheless, one particular method is generally used for any given species after appropriate modifications. The cutting tools used vary according to the size of the head, but range from scalpel for small individuals to electric bone saws for larger fish (e.g. tunas, swordfish, marlin) and a kitchen knife is usually adequate. The section must be taken carefully in order to avoid cutting into the internal ear and the otoliths. After the appropriate cranium section, the otoliths are usually removed with forceps. Careful removal may permit extraction of the whole semi-circular canal system, containing the three otoliths, but normally only the largest otolith is extracted.

3.1.1. Frontal head section (fig. VIII.B.2)

A frontal section of the superior part of the cranium is made, normally passing through the dorsal part of the eye, parallel to the major axis of the fish, and ending at the outer edge of the opercular bone (fig. VIII.B.2a). The upper part of the cranium is removed (fig. VIII.B.2b,b'). The brain is carefully removed from the cut cranium (fig. VIII.B.2c, c', d, d'). The cavities of the internal ears containing the semi-circular canals are then visible from above: they are positioned at the bottom of the cranium cavity, in the posterior part of the brain cavity and lateral to the main axis of the fish. The otoliths are removed with forceps (fig. VIII.B.2e, e'). This method is used mostly in laterally compressed (most highly evolved) species and in flatfish.

3.1.2. Transverse head section (fig. VIII.B.3)

A transverse section of the head is made at the posterior part of the cranial bone, generally passing through the posterior part of the preopercular bone (fig. VIII.B.3a, a'). The whole head is cut off and the front part is separated. Looking from the back at the head section, the posterior part of the brain (rachidian bulb) is then visible in the upper part of the cranium and the two cavities of the internal ears are located laterally and below it. The brain does not normally have to be removed. The otoliths and the semi-circular canals are directly removed with forceps (fig. VIII.B.3b, b', c, c'). This method may require training, depending on the morphology of the head, in order to be sure of the antero-posterior level of the cut: there is often a risk of cutting the otoliths. It is used mainly for large species such as tuna and swordfishes, and also for Anguilliformes.

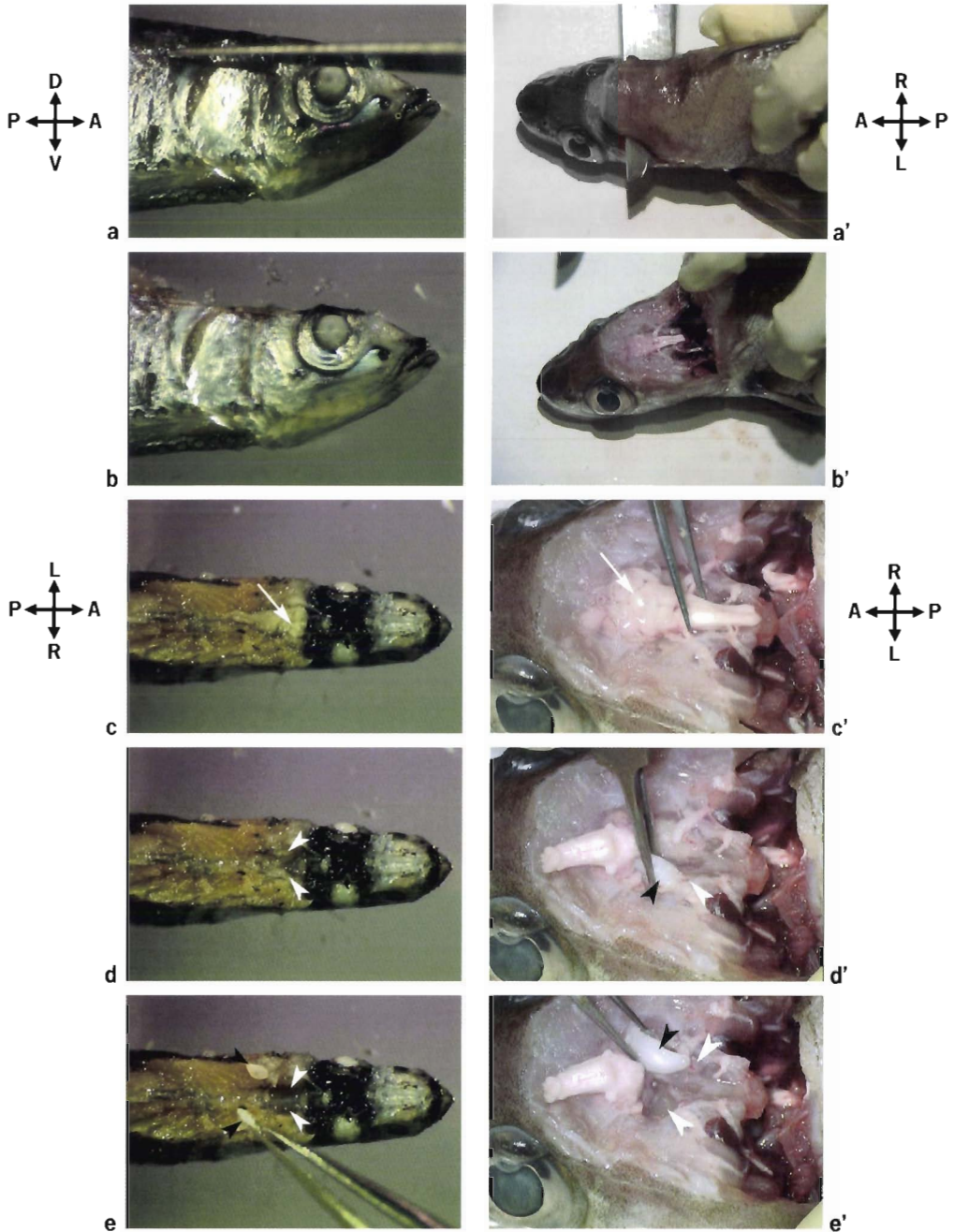


Figure VIII.B.2 - Otolith removal with a frontal head section. a, b, c, d, e: small species (*Vinciguerria nimbaria*, Photichthyidae, 4 cm SL). a', b', c', d', e': medium-sized species (*Trisopterus luscus*, Gadidae, 25 cm SL). a) Lateral view of frontal section with scalpel blade. a') Dorsal view of frontal section with knife. b, b') Views of frontal section after removal of the dorsal part of the cranium and muscles. c, c') Location of brain (white arrow) before removal. (d, d') Otolith location after brain removal; white arrows indicate location of internal ears; black arrows show sagittal otolith. e, e') Sagittal otolith extraction with forceps; white arrows indicate the location of the internal ear cavities; black arrows show sagittal otoliths. A, anterior; D, dorsal; L, left; P, posterior; R, right; V, ventral (photos© Ifremer O. Dugornay).

3.1.3. Sagittal head section (fig. VIII.B.4)

A sagittal section is made passing through the middle of the head (fig. VIII.B.4a, a'), from the mouth until at least the end of the opercular bone. Due to the latero-central position of the internal ears in the cranium the section must be done precisely. A transverse section of the head in the opercular area then permits separation of the two half heads. Each half head is treated separately. The brain is removed to locate the entire internal ear (fig. VIII.B.4b, b', c, c'). The otoliths and semi-circular canals are carefully extracted from the ear, at the

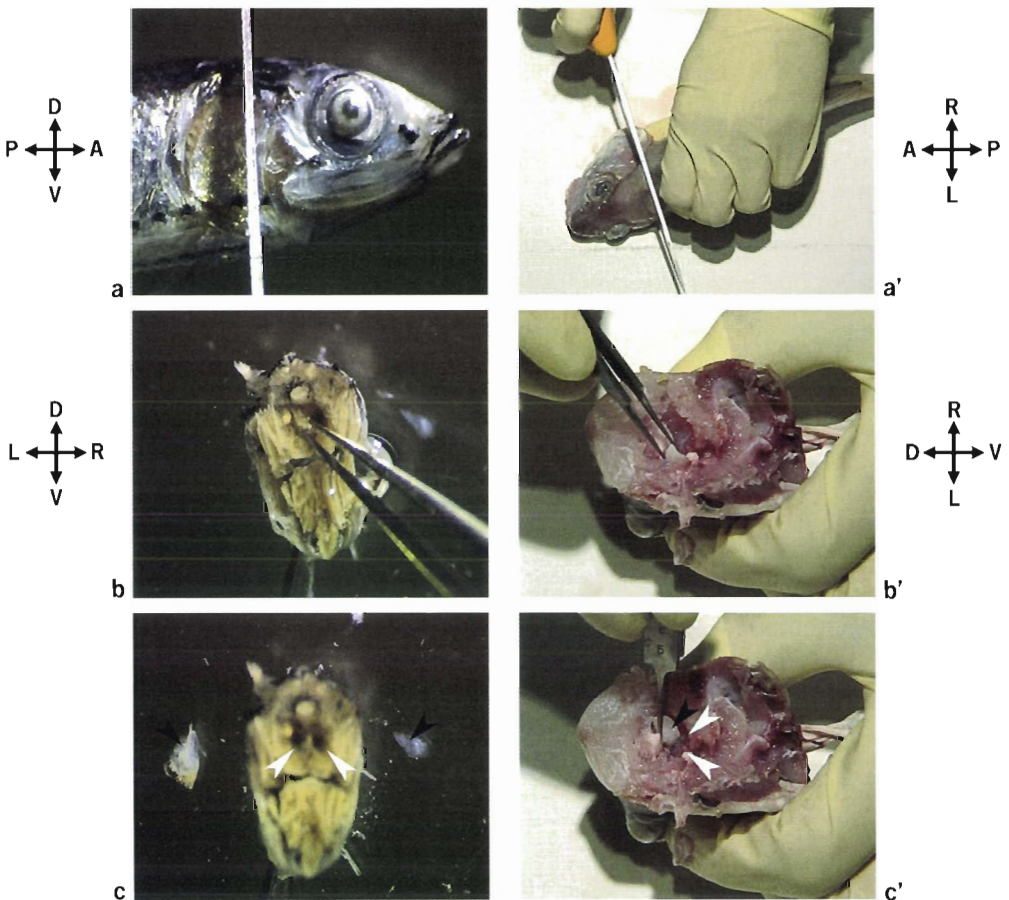


Figure VIII.B.3 - Otolith removal with transverse head section. a, b, c: small species (*Vinciguerria nimbaria*, Photichthyidae, 4 cm SL). a', b', c': medium-sized species (*Trisopterus luscus*, Gadidae, 25 cm SL). a) Lateral view of transverse section with scalpel blade. a') Dorsal view of transverse section with knife. b, b') Sagittal otolith extraction with forceps after removal of brain. c, c') Sagittal otolith extraction; white arrows indicate location of internal ear cavities; black arrows show sagittal otoliths. A, anterior; D, dorsal; L, left; P, posterior; R, right; V, ventral. (photos© Ifremer O. Dugornay)

back and beneath the brain (fig. VIII.B.4d, d'). This method is mainly used in species with large crania such as Siluriformes. It also makes it easier to extract the three otolith pairs as the anatomical position of the labyrinth is shown precisely, making this a useful method for unfamiliar species.

3.1.4. Through the gills, ventral cranium section (fig. VIII.B.5)

The *operculum* is spread out from the ventral face of the fish head (fig. VIII.B.5a). The gills are visible and the branchial arches are cut on their internal aspect. The ventral structure of the neurocranial bones appears (fig. VIII.B.5b) after all the surrounding tissues have been cleared away. The pro-otic bullae are located in the medio-lateral parts of the neurocranium. A small cut into the external part of the bulla opens the internal ear, from which the main otolith (usually the *sagitta*) can be removed (fig. VIII.B.5c). If the *sagitta* is pushed inside the brain it is very difficult to extract it without cutting all the cranial bones and removing the whole brain. This method is particularly suitable when there is a need to conserve the original aspect of the head after the otolith extraction, for example in commercial species destined for the market. It is therefore suitable for any kind of non-Ostariophysean species, though only for the extraction of the *sagitta*. Some other semi-automatic methods for sampling otoliths with drills in fish markets have been improved and are now suitable for important commercial species such as the Scombridae (Thorogood, 1986).

3.2. Extraction from small fish (fig. VIII.B.6)

This description concerns all fish species and stages with body lengths of less than 2 cm, i.e. usually juveniles and/or larvae. The extraction must be done in an immersing medium (we recommend 95% ethanol but some authors use water, glycerine or xylene). The use of 95% ethanol prevents secondary erosion of the otolith structure. The removal is normally done in the same conservation medium as is used for the whole individuals.

Otoliths can easily be extracted, even from the smallest larvae, using a dissecting microscope (generally binocular) with polarised light. They are known to be refringent to cross-polarised light, enabling them to be distinguished from the surrounding tissues (fig. VIII.B.6a). Otoliths are removed and separated using fine dissecting needles (e.g. 150 µm diameter) to tease apart the head, muscles, bones and other tissues (fig. VIII.B.6b). They are then cleared of surrounding tissue by scraping their surface with the dissecting needles. Manipulation during dissection requires steady hands, appropriate tools, practice and patience (Secor *et al.*, 1992). The most delicate step

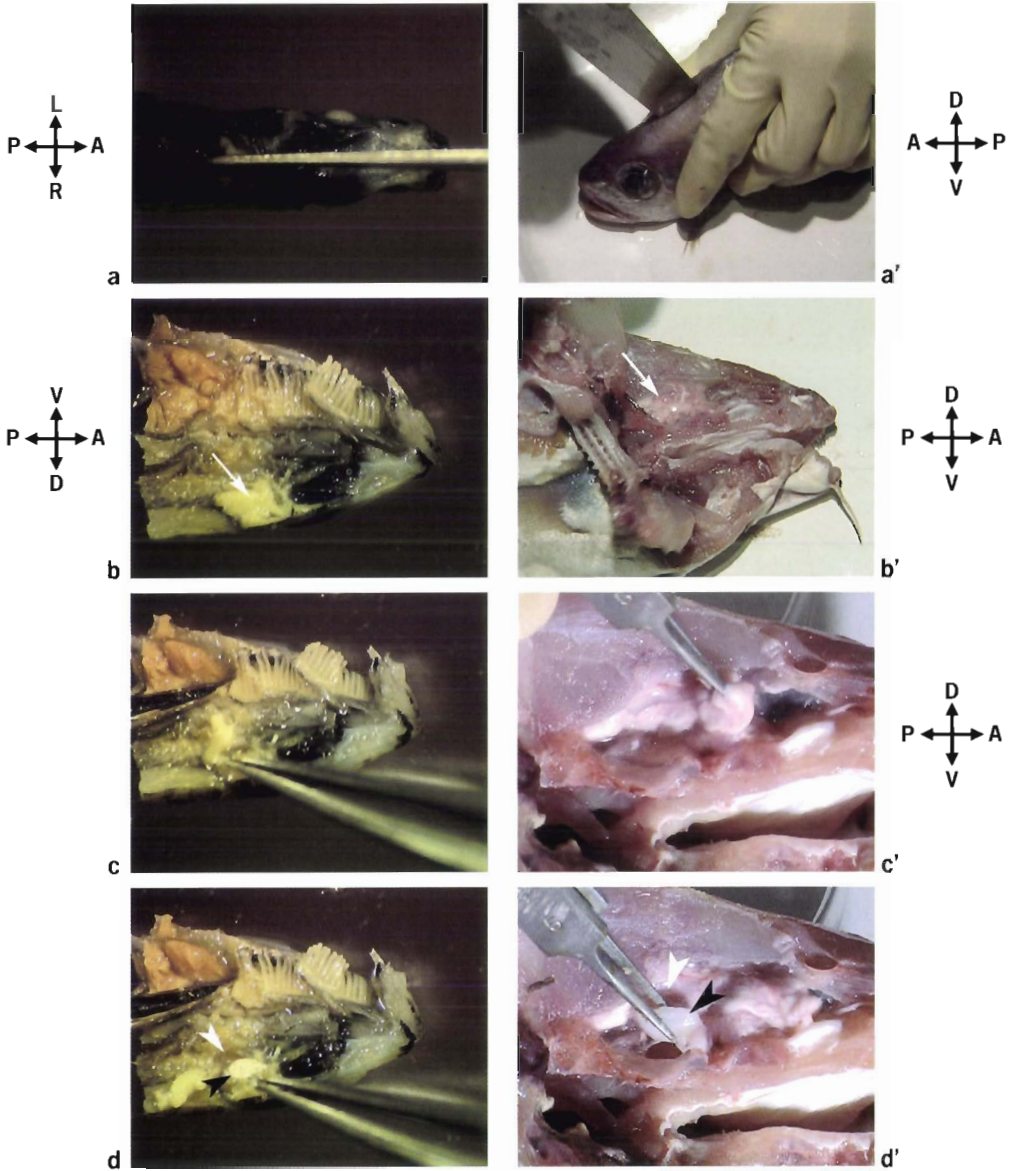


Figure VIII.B.4 - Otolith removal with sagittal head section. a, b, c, d: small species (*Vinciguerria nimbaria*, Photichthyidae, 4 cm SL). a', b', c', d': medium-sized species (*Trisopterus luscus*, Gadidae, 25 cm SL). a) Dorsal view of sagittal section with scalpel blade. a') Medio-dorsal view of sagittal section with knife. b, b') Views of brain location (white arrow) in half-head. c, c') Brain removal with forceps inside each half-head. d, d') Sagittal otolith extraction with forceps; white arrows indicate location of internal ear cavities; black arrows show sagittal otoliths. A, anterior; D, dorsal; L, left; P, posterior; R, right; V, ventral (photos® Ifremer O. Dugornay).

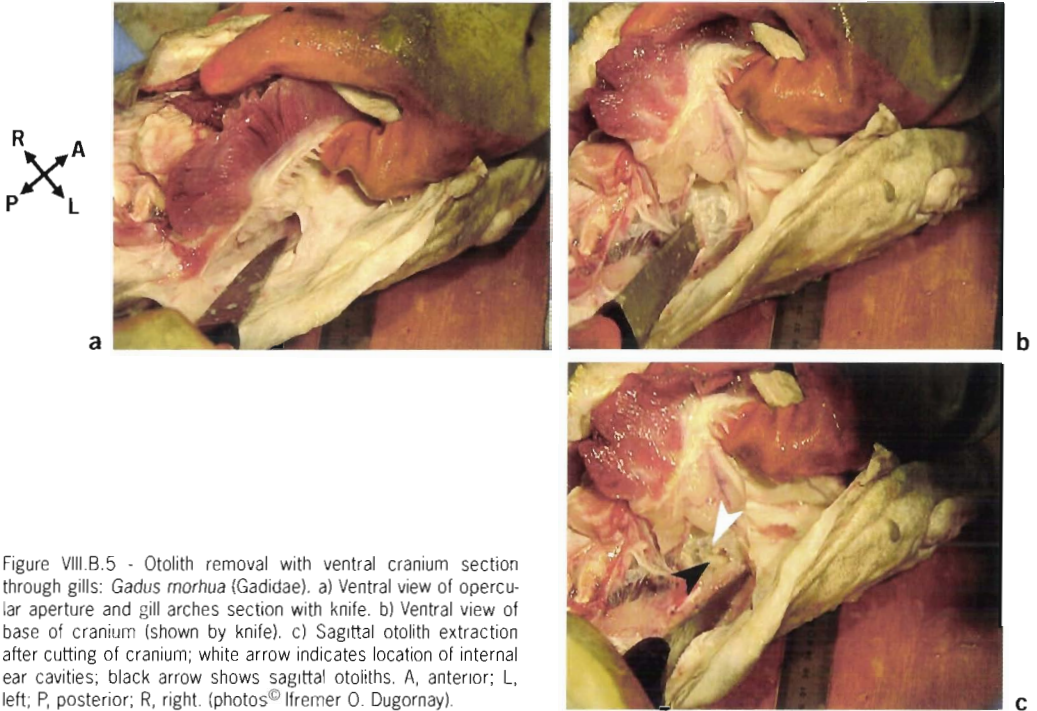


Figure VIII.B.5 - Otolith removal with ventral cranium section through gills: *Gadus morhua* (Gadidae). a) Ventral view of opercular aperture and gill arches section with knife. b) Ventral view of base of cranium (shown by knife). c) Sagittal otolith extraction after cutting of cranium; white arrow indicates location of internal ear cavities; black arrow shows sagittal otoliths. A, anterior; L, left; P, posterior; R, right. (photos© Ifremer O. Dugornay).

is handling. If the otoliths do not need any further preparation, the best method is to embed them directly in resin (chap. VIII.C.2.2.3). They are handled by sucking up them directly into a drop of liquid resin; otherwise otoliths must be handled dry for storage.

The same procedure can also be employed for removing otoliths from embryos in eggs. First, the embryo is extracted from the egg and then the otoliths are removed as described above for larvae.

Secor *et al.* (1992) described two other methods for extracting very small fish otoliths; bleaching and embedding, but these need much practice and time, and finally they are less successful: we do not recommend them. Bleaching consists of immersing the material in sodium hypochlorite and extracting the otoliths after tissue lysis. Other protein enzymes such as trypsin (Rojas-Beltran & Vincent, 1993) have also been used, but the success of the removal procedure seems to be variable and it is not possible to confirm that there has been no erosion of the protein content of the otolith. The embedding method requires the individual fish to be completely dehydrated and then embedded in synthetic resin (chap. VIII.C.2.2). The handling of the fish is facilitated and it can be cut (sectioning and/or polishing, chaps VIII.C.2.3 and VIII.C.2.5) in any plane and particularly at the otolith level. This method is nevertheless very complicated and needs careful

training at each step. Due to the thickness of the saws and the relative size of the otoliths, it is often very difficult to reach them in the brain area.

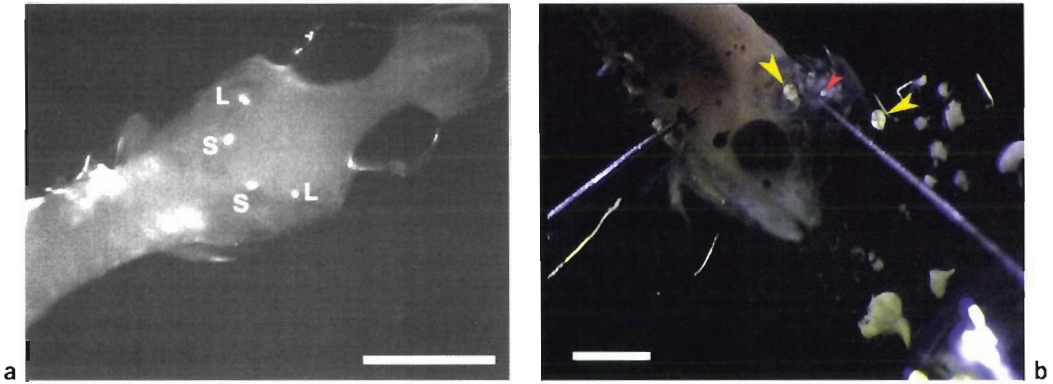


Figure VIII.B.6 - Otolith removal from larvae under binocular microscope and polarised light: *Vinciguerria nimbaria* (Photichthyidae). Otoliths are clearly located with their refringence against the polarised light. a) Dorsal view of anterior face of a larvae; S, sagitta; L, lapillus; Scale bar = 500 μ m (photo J. Tomás). b) Otolith extraction using dissecting needles; cranial bones and the brain are removed before isolation of the sagitta (yellow arrow) and lapillus (red arrow); Scale bar = 1 mm (photo⁶⁰ Ifremer O. Dugornay).

3.3. Otolith cleaning and conservation

3.3.1. Cleaning and handling

Otoliths must be cleaned before storage in order to remove any adhering remnants of the *macula* and vestibular tissues after dissection. After drying, any remains of such tissues would preclude good observation of whole otoliths or good quality embedding in synthetic media. The easiest way is to clean them during or immediately after extraction. Mechanical cleaning is done by teasing away the tissues with fine tools such as forceps and dissecting needles with the otolith in a liquid medium (e.g. water or ethanol) (Secor *et al.*, 1992). Otoliths can be simply cleaned by wiping them with absorbent paper. Reaction cleaning can be done by immersion in dilute bleach (10 to 100% sodium hypochlorite) for a given time (a few minutes to several hours), following which it is important to rinse them several times with water and/or ethanol, and then dry them.

Handling does not pose a major problem for large otoliths (> 500 μ m): they can be manipulated with forceps and/or by hand. Smaller otoliths (< 500 μ m) are much more difficult to handle, especially those from larvae, which have diameters of only a few microns. Small otoliths are also extremely fragile and can be crushed with very slight

pressure. We recommend considering the future use of these otoliths before adopting any given protocol. Secor *et al.* (1992) list four techniques for handling and transferring small otoliths:

- remove the otolith with a small amount of the dissecting medium, using a micropipette. Transfer the liquid with the otolith to a clean slide or storage container;
- shunt the otolith over to a clean area of the dissecting medium and let it dry. After drying press a finger down on the top of the otolith and transfer it to the storage container (perhaps using a binocular microscope);
- pick up dried otoliths with a wetted dissecting needle and place them in an aqueous medium;
- use small brushes, bacterial loops or invertebrate forceps to transfer otoliths.

The handling of very small otoliths nevertheless requires practice. If no further examination of the whole otolith is needed, we recommend embedding it directly in the media (chap. VIII.C.2.4.1). The otolith can be sucked into a drop of embedding medium (resin) at the end of the dissecting needle, or directly into the dissecting medium (e.g. water or ethanol): there is no risk of mixing the media as most resins are hydrophobic. The otolith inside the drop of resin is put directly into another drop of resin on a cover slip. Using a cover slip allows it to be observed on both sides and/or to be prepared by another technique (e.g. grinding, polishing).

3.3.2. Storage and conservation

The best method of storing and preserving otoliths is to store them completely dry. After cleaning they are dried by exposure to air or in a low-temperature oven. They can then be placed in referenced micro-tube vials (e.g. Eppendorf®, Treff®), which are preferable to “tissue culture plates” because they can be individually handled or stored in special supports. The microtubes are also rigid enough to protect the otoliths from fracture due to their concavo-convex shape, which often happens if they are stored in envelopes as earlier recommended (Williams & Bedford, 1974). Cool, dark storage areas are probably best and dry otoliths can be stored indefinitely (Brothers, 1987).

Some authors mention conservation in a medium such as alcohol, which can be useful for fragile and small otoliths. The concentration must be at least 95%. We have also mentioned direct mounting in media (see above) but care must be taken not to make the mount too thick because the working distance of the microscope may be very short at higher magnifications (Brothers, 1987).

4. Other skeletal parts

4.1. Extraction

All the other skeletal parts belong to the internal skeleton and must therefore be extracted by dissection. However, there is a difference between the “external” bones that belong to the fins (spine, fin ray) and are easily extracted, and the “internal” bones that frame the body (opercular, vertebrae, *cleithrum*, etc.). The cutting tools used vary according to the size of the fish but range from scalpels for small individuals, through various types of knives, to electric bone saws for large fish. Fin ray removal starts with the separation of the target ray (generally 1st and/or 2nd) from its neighbours with a knife (fig. VIII.B.7a) and continues when the surrounding muscles at the base of the ray are cut in order to extract the whole piece (fig. VIII.B.7b). The bone structure must be extracted carefully in order to conserve its integrity for further analyses. Removing internal bones (e.g. vertebrae) is also easy but the dissection is more complete: the muscles and the tissues surrounding the target bone are first carefully removed (fig. VIII.B.8a) and then the bones are cut at the level of their articulations and extracted in their entirety (fig. VIII.B.8b). If the dissecting planes are uncertain, it is better to take the bones with the muscles that are attached to them, even if this means cleaning everything afterwards.

4.2. Cleaning and conservation

Cleaning of skeletal parts is an essential precursor to good quality conservation. The integument and the muscles attached to the bones are usually removed by hand after a period in a bath of boiling water, diluted bleach, or potash or trypsin. We recommend the simplest method, that employs a bath of boiling water. The duration of the immersion must be controlled in order to prevent overheating of the skeletal elements, and may range from a few minutes for smaller pieces to several hours for the largest pieces. Progress should be checked at regular intervals.

Conservation is very simple and is generally performed dry. For all bones, storage in paper envelopes can be recommended because most bones are simultaneously flexible and resistant, while the paper of the envelopes allows some “ventilation” of the tissues, but they can also add some very severe contamination for later microchemistry analysis.

Storage in plastic packing often leads to the appearance of fungi which may subsequently destroy the bone. For dry conservation, care should be taken to ensure that the tissues are not attacked by specialized insects: insecticides or naphthalene may be added to the envelopes. For the most fragile samples alcohol can be used as a fixative, in order to avoid acid fixers (Meunier, 1988). Ethanol between 70 and 95% is recommended for this purpose.



Figure VIII.B.7 - Fin ray removal from large fish: white tuna *Thunnus alalunga* (Scombridae). a) Dorsal view of first fin ray separation with knife. b) A transverse section of the skin is made before and after the fin ray. The fin ray is extracted ensuring of the integrity of the bone (photos© Ifremer O. Dugornay).

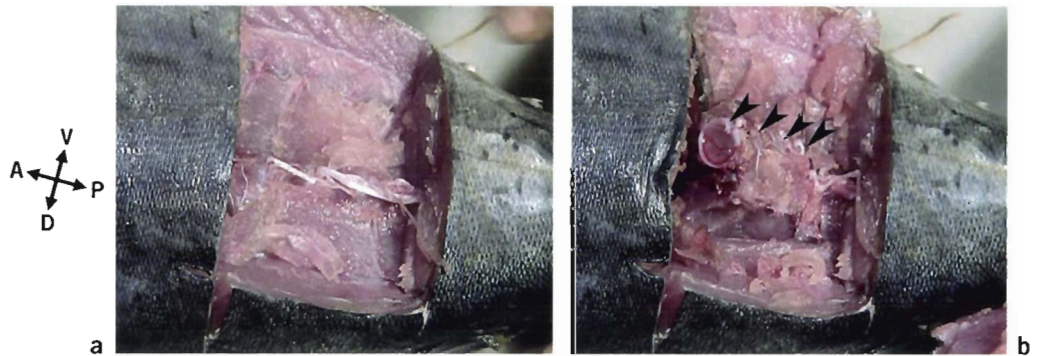


Figure VIII.B.8 - Vertebrae removal for large fishes: extraction of caudal vertebrae in the white tuna *Thunnus alalunga* (Scombridae). a) Sectioning with knife and removal of skin and muscles above the last vertebrae. b) A section is made between two vertebrae and the vertebral bodies (arrows) are extracted after sectioning the vertebral rays (photos© Ifremer O. Dugornay).

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C. Preparation of calcified structures

W.J. McCurdy, J. Panfili, F.J. Meunier, A.J. Geffen, H. de Pontual

1. Scales

Scales do not normally need to be prepared for observation of their growth marks. However, as they are hydrophilic structures, dry conservation will eventually result in tissue dehydration and deformation. It is therefore preferable to prepare them on a support in order to preserve their original shape.

1.1. Direct observation

Unmounted scales can be observed directly under a low-power binocular microscope or compound microscope: they are observed either dry or after immersion in a rehydration and clean-up bath (e.g. water, alcohol 70% to 95%, glycerol with water) (chap. VIII.D.1).

1.2. Slide mounting

The simplest mounting method consists of locking the scales between two microscope slides (fig. VIII.C.1). The scales are first rehydrated (in water or diluted alcohol) and then placed directly on a referenced microscope slide (fig. VIII.C.1a). A second slide is placed on top of the scales and fixed to the first slide with adhesive tape (fig. VIII.C.1b). It is essential to thoroughly check the structural integrity of the slide unit and reinforce it with additional tape if necessary. The preparation is therefore both immediately usable and storable. If the scales are large, it is possible that subsequent dehydration will deform them and

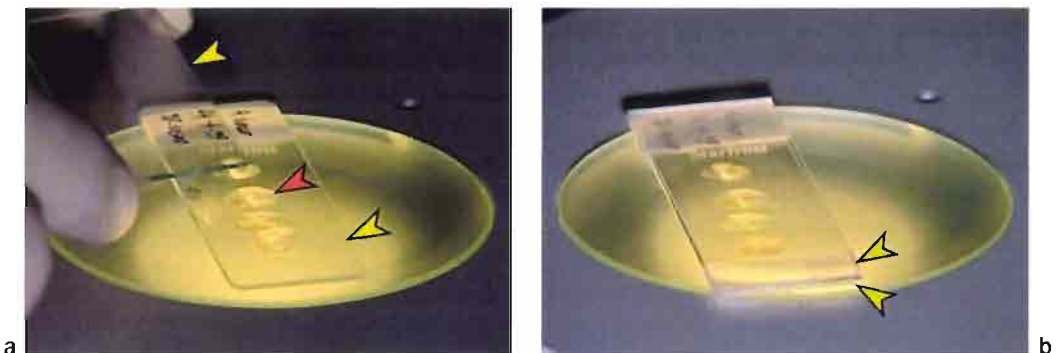


Figure VIII.C.1 - Scale mounting between two slides. a) The scales (red arrow) are positioned on the referred slide (yellow arrow). It is preferable that the scales are wet in order to be able to deform (to compress) them more easily. b) A second slide (yellow arrow) is deposited on the preceding one and comes to compress the scales. The two slides (yellow arrows) are fixed together with adhesive paper (photos[©] Ifremer O. Dugornay).

exert pressure on the slides, causing them to move apart slightly. With time the scales will tend to slip from their support. It is therefore occasionally necessary to ensure that the untaped edges of the slides are well sealed by using either adhesive tape or flexible glue (e.g. silicon mastic).

1.3. Cellulose acetate impression

An alternative method of preparing scales is to make cellulose acetate impression of the external face (fig. VIII.C.2). Cellulose acetate is a relatively flexible plastic that allows hard elements to be imprinted by applying mild pressure. The scales are placed between two slides of cellulose and the unit is passed through a jewellery press (fig. VIII.C.2a). The unit is then withdrawn and the scales removed (fig. VIII.C.2b). The acetate slide that was directly in contact with the external face of the scale presenting the *circuli*, retains the imprint of these *circuli* (fig. VIII.C.2b). The acetate slide is then directly observable under a low-power binocular or compound microscope and can be preserved in that state for many years.

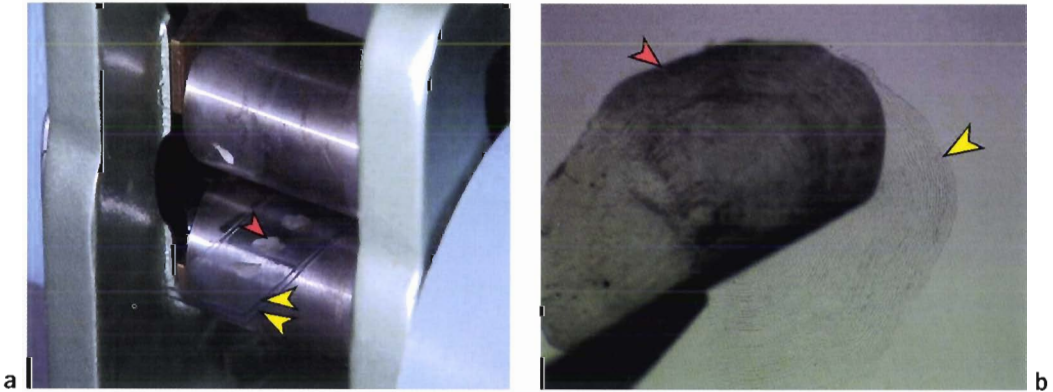


Figure VIII.C.2 - Scale impression on acetate peel. a) The scales (red arrow) are positioned between 2 acetate peels (yellow arrows). The whole is passed between the two rollers of a press (e.g. jeweler press). b) The external face of the scale (red arrow) is separated of acetate (yellow arrow) and its print remains visible (yellow arrow) (photos © Fremer O. Dugornay).

2. Other structures

2.1. Simple preparations

Some CS, e.g. opercular bone, can be observed directly in their original conserved state and others following a minimum of preparation, e.g. vertebrae, *cleithrum* and otoliths. None of these preparations require the CS to be embedded prior to observation. These calcified structures are typically observed either directly or immersed in a liquid observation medium (chap. VIII.D.1), using a simple optical magnification device with additional illumination or a binocular microscope.

2.1.1. Whole calcified structures

For some thin otoliths, notably *sagittae* from flatfish and certain pelagic species, it is possible to count the *annuli* without extensive preparation. Individual otoliths are immersed in water, alcohol or oil and viewed directly using either transmitted or reflected light (chap. VIII.D.1.1.2 and VIII.D.1.1.4). A range of oils are used to “clarify” or reduce the reflected glare from the observed surface of CS. Historically, these have included clove oil and immersion oils. Some oils such as Creosote are no longer used as they are considered to be carcinogenic. Oils with known safety hazards must not be used for this purpose. “Baby oil” is often used as a clearing oil by otolith readers. A cold light source should be used to reduce the evaporation of the oil during observation.

2.1.2. Breaking otoliths

Otoliths may be broken in two using the fingers, forceps, a scalpel or piano-wire cutters. The broken otoliths may be mounted in Plasticine™ (modelling clay) and painted with a clearing oil (e.g. baby oil) for observation under incident light. They may also be manipulated within a clear liquid-filled (water, alcohol, water & alcohol) observation cell.

2.1.3. Simple preparations for other structures

Some bones are observed without previous preparation, e.g. vertebrae, opercular, *cleithrum*, etc. They can be viewed directly, or after immersion in a clearing media, using either transmitted or reflected light (Chap. VIII.D.1.1.2 and VIII.D.1.1.4). Some other bones are simply sectioned (Chap. VIII.C.2.3) before observation, without embedding, e.g. spines from large fish or vertebrae. The section or the slice is then directly observed, dry or wet, under a binocular microscope.

2.2. Embedding and impregnation

2.2.1. Embedding media

All embedding media should be stored in a cool dark place and used in accordance with the manufacturers' instructions. High-transparency polyester resins have a short shelf life and should be purchased in small quantities as they can become opaque as they deteriorate. The principal embedding media and their applications are listed in table VIII.C.1, reprinted from Mosegaard *et al.* (1998). The most used embedding media for the next stages of preparation are the hard and irreversible synthetic resins (polyester, epoxy, etc.). These utilise a catalyst to polymerise them (fig. VIII.C.3): depending on the medium selected, mixing is by weight or by volume. For example, catalyst is used at 1-2% by weight of polyester resin (e.g. 25 g of clear casting polyester resin and 0.2 g of catalyst).

Table VIII.C.1 - Standard mounting materials (from Mosegaard *et al.*, 1998).

Material	Uses	Advantages	Disadvantages
Polyester resin (PR)	- high-quality permanent fixing - embedding and surface grinding	- multi-purpose - moderate optical clarity - good grinding/polishing properties - light stable	- non-reversible - variable setting quality - slow setting (12-24 h) - toxic - low shrinkage - catalyst required
Epoxy resin	- high-quality permanent fixing - embedding and surface grinding	- harder than PR - high optical clarity - light stable - low chemical contamination - minimal shrinkage - wide range of applications	- non-reversible - moderate/slow setting - toxic - expensive - catalyst required
Thermoplastics	- high-quality reversible fixing - embedding and surface grinding	- fast setting (minutes) - easily removed - high optical clarity - non-toxic - good grinding/polishing properties	- heat needed (70-160°C) - develops gas bubbles - moderate shrinkage - high Si content
Super Glue® (cyanoacetate)	- rapid setting, high strength fixing - surface grinding	- easy to use - sets without heat or chemical hardeners	- high S, Pb content - can detach from slide
UV dental glues	- rapid setting, high strength fixing - surface grinding	- sets without heat or chemical hardeners - non-toxic	- very expensive - optical clarity not known
Wax	- quick temporary fixing - embedding only	- fast, - cheap, - easily removed	- soft - poor optical properties - non-permanent
Nail varnish	- quick temporary fixing - surface grinding	- cheap, easily removed	- soft - dissolves in oils
Eukitt®	- good quality soft fixing medium - embedding only	- easy to use - no hardener - good optical clarity - easily removed	- slow setting - remains soft - unstable over time - can detach from slide

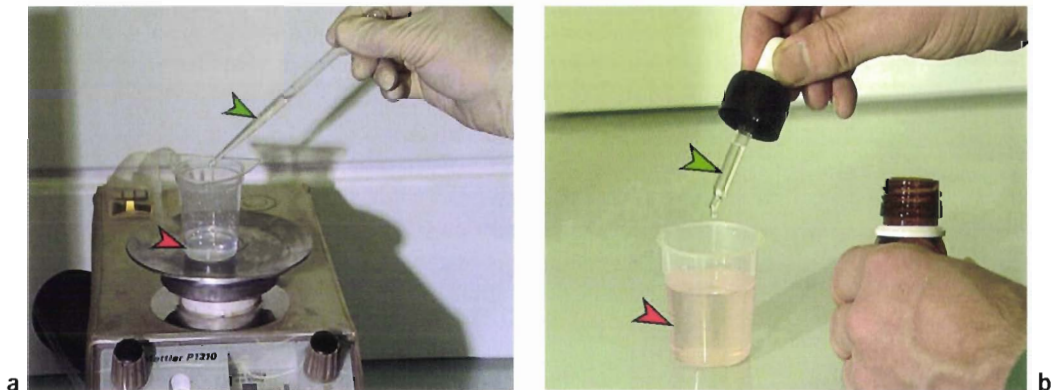


Figure VIII.C.3 - Preparation of the embedding resin under a fume extraction hood. a) Example of mixture according to the weight (e.g. epoxy resin). The catalyst (green arrow) is added to the resin (red arrow) in a polypropylene becher directly posed on a balance. The mixture have to be stirred thereafter. b) Example of direct mixture in a number of drops (e.g. polyester resin). The drops of catalyst (green arrow) are added to the resin (red arrow) in a polypropylene becher according to the resin volume. The mixture have to be stirred thereafter (photos© Ifremer O. Dugornay).

Table VIII.C.2 provides a simple calculation of the quantities of resin and catalyst to be mixed, depending on their ratio. Polyester resins are dissolved in styrene and requite the addition of both an accelerator and a catalyst to cure. As there is a risk of explosion if the undiluted accelerator and catalyst are mixed together, polyester resins are supplied pre-accelerated for safety reasons. Even so, an excessive amount of catalyst added to the pre-accelerated polyester resin still leaves a risk of explosion.

Table VIII.C.2 - Simple dosage of polyester resin (in volume and/or weight) and catalyst (in drops) depending on their ratio. The greater the quantity of catalyst the higher the speed of setting. Users should not exceed a maximum of 2% catalyst to avoid the risk of explosion.

Resin volume (ml)	Resin weight (g)	Number of drops of catalyst	
		1% mixture	2% mixture
91.0	~ 100	20	40
45.5	~ 50	10	20
23.3	~ 25	5	10
11.5	~ 12.5	2.5	5
9.1	~ 10	2	4

The media and its catalyst are mixed carefully in order to prevent bubbles forming in the resin mix, as these would make it difficult to prepare and/or observe the CS. The mixture is then left to rest for few minutes to allow the biggest bubbles to escape. The hardening time of the media varies from a few seconds (Super Glue®, UV dental glue)

to a few hours (epoxy, polyester). We recommend the use of a dry oven to complete the polymerisation process after the resin has set to the gel stage. For example, the polymerisation of polyester is better after a minimum of 24 h in a dry oven (around 30°C).

The resin can be coloured by the addition of a pigment, generally black, or it can be purchased pre-coloured. All the resins and catalysts, often known as “hardeners”, are available from specialist suppliers. Mounting kits containing small quantities of pre-coloured resin and catalyst are also available from some suppliers.

2.2.2. Embedding individual structures for sectioning

Individual CS are embedded before sectioning, grinding and/or polishing. They are then prepared for further processing by embedding in transparent media. The CS are cleaned and dried at room temperature or in an oven before embedding.

The use of individual moulds is necessary (fig. VIII.C.4). Moulds are usually made of silicon elastomer, and are dimensioned according to the size of the CS. They are often individually labelled (with a reference number or symbol impressed into the base of each cell in the mould). Silicon elastomer is a flexible material that facilitates the removal of the casts when the resin has set.

First, a base layer of the medium (e.g. polyester resin) is pipetted into the cells of the mould and allowed to set. After sufficient time for hardening (e.g. 4 to 24 h for polyester), a CS is placed in each cell (fig. VIII.C.4a) and a covering layer of embedding medium is poured over it (fig. VIII.C.4b and 4c), making sure that it completely covers the CS. At this step, it is important to turn over the CS in its mould to eliminate air bubbles that are often trapped, and also to re-position it in case it has floated out of position. Re-positioning should take into account the next steps of preparation (e.g. the sectioning plane). After approximately 20 minutes (the time will vary with the resin medium used and the age of the resin), the catalysed resin will begin to set, after which it will be impossible to remove the CS. The total time of setting is from a few minutes to a few hours, e.g. a minimum of 24 h for polyester (chap. VIII.C.2.2.1). The embedded structures are then ready for sectioning and/or polishing in preparation for further analysis (fig. VIII.C.4d).

2.2.3. Embedding for incident-light observations

Calcified structures, mainly otoliths, are embedded in a clear medium, usually high-transparency polyester resin, in cylindrical cavities on black plastic slides. The black background of the slide provides better contrast for observations of *annuli*. This technique, evolved from

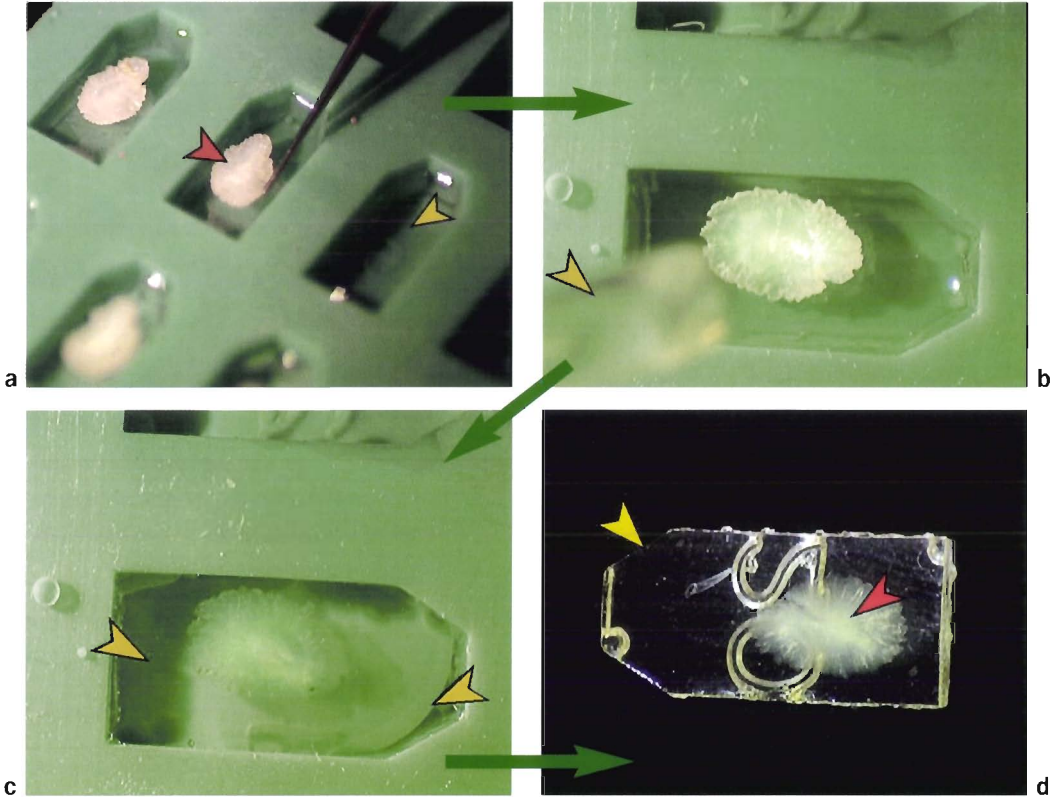


Figure VIII.C.4 - Example of embedding: otolith embedding in an elastomer mould. a) A layer of resin (yellow arrow) was run on the bottom of each location: after polymerisation, it is the base of the support of the otolith (red arrow). Each otolith is deposited on the resin bottom. b) The otolith is well oriented and it is embedded in one second layer of resin which has been just prepared (yellow arrow). c) It should be made sure that the liquid resin (yellow arrows) comes to fill the mould completely. Here the resin did not reach yet the right edge. The otolith must be then turned over to drive out the captive air bubbles. d) Otolith (red arrow) definitively embedded in the polymerized resin (yellow arrow). The block of resin carries an identification number (20) molded on the lower face (photos © Ifremer O. Dugornay).

methods first described by Parrish & Sharman (1959), Raitt (1961) and Watson (1965), and is used for incident light observations of the external faces of large numbers of *sagittae* from pelagic species, usually Clupeid and Scombridae. It is also suitable for incident light observations on the surfaces of small pieces of CS and provides an efficient long-term method of conservation for repeated observations at a later date.

Grids of twenty-five flat-bottomed cavities, each 7 mm in diameter and approximately 1.2 mm deep, are machined into 65 mm x 60 mm slides cut from 3 mm-thick Perspex™ sheet. The grid of cavities is offset to leave a 10 mm wide un-drilled strip at the top of each slide.

This allows each slide to be engraved with a reference number. Slide reference numbers can be engraved with a hardened steel stylus or an electric engraving tool fitted with a tungsten carbide tip. Reference numbers may be made more legible by rubbing yellow or white wax crayon into the engraved text.

Each pair of otoliths must be correctly positioned within its cavity on the plastic slide, i.e. with the *sulcus acusticus* facing downwards and thus not visible (fig. VIII.C.5a). Occasionally small otoliths e.g. Sprat (*Sprattus sprattus*) (fig. VIII.C.5a), acquire a static electrical charge that makes the positioning of the otoliths more difficult. Static electricity problems can sometimes be resolved by increasing the humidity of the atmosphere or changes in the type of clothing and shoes used by individual workers. A disposable plastic pipette is used to drop catalysed clear resin onto each pair of otoliths in the cavity, ensuring that the otoliths are completely covered (fig. VIII.C.5b). At this stage, it is vital to re-position any otoliths that have floated out of position. Catalysed clear casting resin is then dropped along the spaces between the pairs of otoliths to form a single layer of resin that completely covers the all otoliths on the slide. A custom-sized (54 mm x 54 mm x 0.5 mm thick) glass microscope cover slip is then floated over the resin (fig. VIII.C.5c) and left in the fume cabinet until completely set (fig. VIII.C.5d). Thin cover slips will deform above the cavities and may crack as the thicker layer of resin contracts more on hardening. The resin will set firm within a few hours, but observations must not begin until the resin has completely set and there is no risk of styrene being absorbed through the skin. Perspex™ is expensive and a number of alternative plastics that bond equally well with casting resin are now in use. Injection-moulded slides with embossed numbers are cheaper and are now available commercially. Where machined plastic slides are used, the plastic must be sufficiently hard and inert and the special drill bit must be sufficiently sharp to ensure that the base of each pit in the slide is smooth and level. If the base of the pits is rough and uneven, or if the resin does not completely adhere to the plastic, thin patches of partial vacuum may form between the base and the hardened resin when the resin sets and cools. It will then be very difficult to observe the CS zones as the interfaces of these patches will have the silvered effect of a mirror.

2.2.4. Multiple embedding of otoliths for sectioning

Otoliths must be embedded in a support medium before cutting or thin sectioning and polyester resins are frequently used as embedding media (often black polyester). Special moulds machined from solid aluminium blocks, constructed from flexible compounds, or assembled from machined aluminium parts, are used for this purpose

cut into the sides of the moulds to line up the otolith centres for cutting or sectioning at predetermined locations (fig. VIII.C.6a). Monofilament nylon located in the grooves of the mould (Bedford, 1983) or X/Y embedding tables fitted with a modified video camera (fig. VIII.C.6b) and monitor (Van Beek *et al.*, 1997) are used to minimise the effects of parallax when otoliths are being positioned in the moulds. In the latter case an indicator line across the centre of the monitor gives the operator a more comfortable working position when placing the otoliths in the mould.

The basic principle is the same for all aluminium moulds. The mould is sprayed or painted with a mould release agent before the first layer of catalysed black polyester resin is poured. The otoliths are placed on top of the first layer of resin when this has set to the gel stage (fig. VIII.C.6c). A covering layer of resin is pouted over the otoliths and allowed to harden; (the moulds can be placed on a level table to



Figure VIII.C.6 - Otoliths embedding in series in aluminium mould. a) View of aluminium moulds. b) System of alignment control in mould with a video (photos Bennett Ltd.). c) Lines of otoliths (red arrow) are laid out on a bottom of polyester resin beforehand colored in black and polymerized. The second layer of black polyester resin (yellow arrow) is run in the mould (photo[©] Ifremer O. Dugornay).

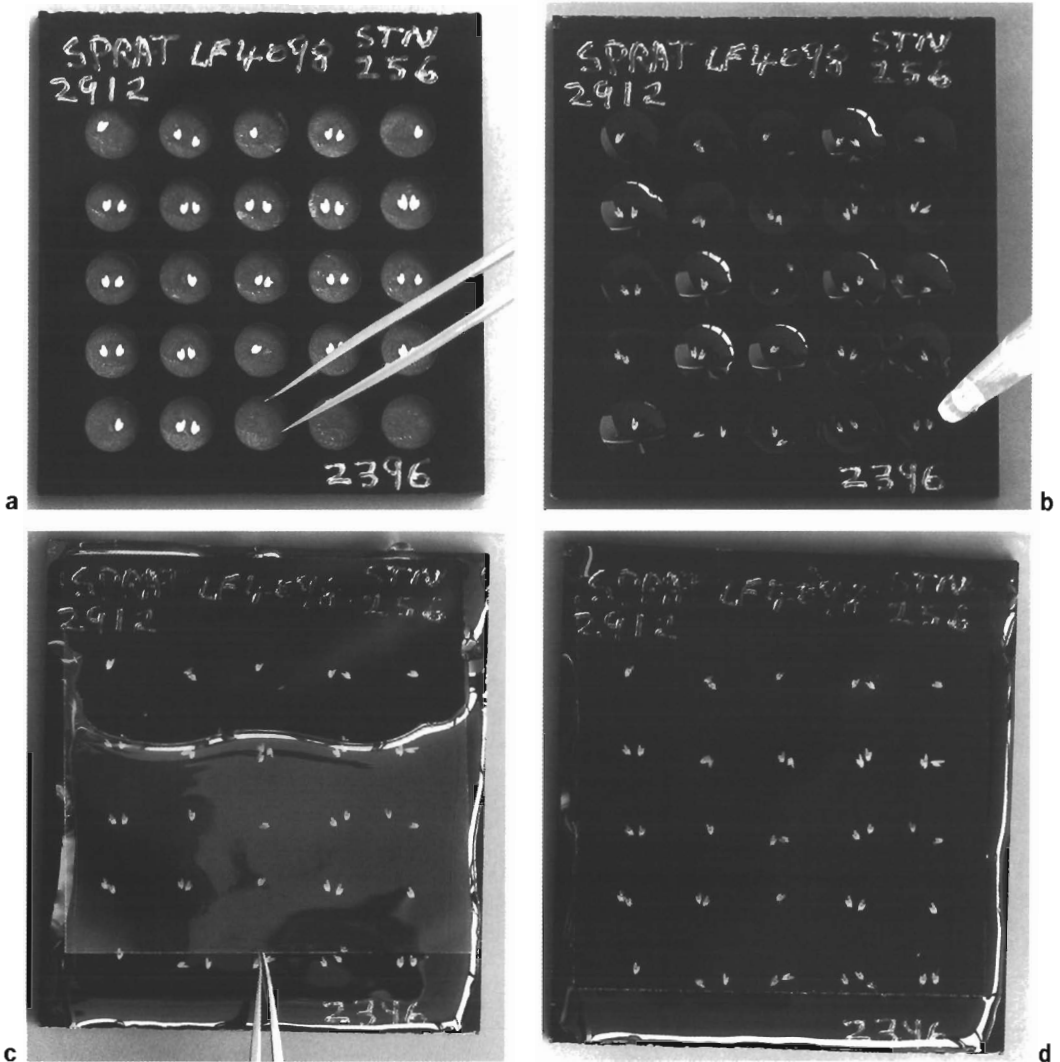


Figure VIII.C.5 - Embedding whole otoliths on a black plastic slide. a) Placing otoliths in the prepared slide. b) Filling the cavities with catalysed resin. c) Placing the cover slip on the slide. d) The completed slide (photos R. Rosell).

(fig. VIII.C.6a). The latter moulds are normally used where large numbers of otoliths have to be processed, for example from demersal species, where age information is required for stock assessments. These moulds can be partially disassembled in order to facilitate the easy removal of the resin blocks containing the embedded otoliths. One variant of this process now uses an ejection lever that permits the rapid removal of the resin blocks without the need to dismantle the moulds (Van Beek *et al.*, 1997). Aluminium moulds typically use guidelines

ensure that the layers of resin are of uniform thickness). The resin blocks containing the embedded otoliths are removed from the moulds when the covering layer of resin has completely set. It is very important to accurately record the sample number and the fish number in the sample for the otoliths placed in each part of the mould. This information will later be used to label the finished resin blocks containing the embedded otoliths.

Alternatively, the first resin layer can be allowed to set completely before the otoliths are fixed in position with a small amount of catalysed resin that is allowed to set before adding the covering layer of resin. The former method is quicker, but care must be taken to ensure that the correct amount of catalyst is added to the resin. If too much catalyst is used, the resin layer will not remain at the "gel" stage long enough to permit the otoliths to be fixed to the surface. This method is also slightly more subjective as concave otoliths must be positioned underneath the monofilament or camera monitor guideline and then turned upside down (*sulcus acusticus* facing upwards) before positioning on the resin surface, in order to prevent air bubbles from forming at the concave surface of the otolith.

Markers (e.g. fragments of dry spaghetti pasta) to indicate the start of each row of otoliths, are placed at the left hand side of each mould cavity on top of the first layer of resin when this has set to the "gel" stage. One type of mould (Van Beek *et al.*, 1997) has a bevelled edge at the left side of each mould cavity that performs this function.

Some procedures use only "lay-up" resin, the type of resin that is used to bond layers of glass fibre in boat-building (Bedford, 1983). In the three-stage embedding process, a mixture of "lay-up" resin and "flex" resin is used for both the first layer and the resin "glue", while a mixture of "gel-coat" resin and "flex" resin forms the base for the top resin layer. Using "gel-coat" resin, the type of resin that is used to form the hard glossy outer layer on GRP boat hulls, for the top layer ensures a completely smooth top surface on the resin blocks. Powdered talc or a similar product can be used to dry off the surface if "lay-up" resin is used for the top layer (McCurdy, 1985). The addition of small amounts of "flex" resin (tab. VIII.C.3) ensures that the finished resin blocks are slightly flexible (blocks can be bent slightly), and there is less risk of the otoliths being shattered by the action of the high-speed saw. If small otoliths are to be embedded, powdered chalk can also be added to the resin mixture as this prevents the diamond blades "gumming-up" with resin and improves the cutting speed.

Table VIII.C.3 - Polyester resin mixing tables used for the Otolin process.

	Weight of material (g)										
Bottom layer											
Total layer weight (g)	5	10	20	30	40	50	60	70	80	90	100
Lay-up resin	4.5	9	18	27	36	45	54	63	72	81	90
Flex resin (5%)	0.25	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
Black resin pigment (5%)	0.25	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
Hardener (MEKP) (1%)	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Top layer											
Total layer weight (g)	5	10	20	30	40	50	60	70	80	90	100
Casting resin	4.5	9	18	27	36	45	54	63	72	81	90
Flex resin (5%)	0.25	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
Black resin pigment (5%)	0.25	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
Hardener (MEKP) (0.6%)	0.03	0.06	0.12	0.18	0.24	0.30	0.36	0.42	0.48	0.54	0.60

2.2.5. Impregnation of bony tissue

Many bones consist of vascularised bony tissue, and when the osseous trabecules are relatively thin and surrounded by large cavities the whole organ is brittle. For this reason, it is necessary to strengthen the bony structures to avoid breakage during sectioning. Embedding should therefore follow careful impregnation with polyester, in order to ensure that the polyester enters all the bone cavities, where it hardens during polymerisation like the polyester that surrounds the CS. We now describe the polyester resin (stratyl) impregnation process.

The CS, i.e. a bone, is dehydrated in successive alcohol baths of 70%, 95% and 100%, using at least one bath of 24 h for each concentration, and possibly two baths of each for large pieces. It is then immersed in two successive baths of acetone for 24 to 48 h and finally in the monomer polyester (without catalyst) for 24 h. Depending on the polyester used, supplementary baths may be required between the monomer step and the embedding step. For the embedding stage, the CS is immersed in the prepolymerised resin (monomer + catalyst 1-2%) in a mould which already contains a hardened base layer. It is sometimes recommended to place the mould with the resin and the CS for a few minutes under vacuum in order to remove bubbles and improve impregnation. When the polymerised block has hardened (for example after 1-2 days at room temperature and then one week at 60°C, for stratyl) it can be sectioned with a low-speed sectioning machine (chap. VIII.C.2.3.1).

2.3. Sectioning

Sectioning structures is a primary step for a number of further preparation techniques such as etching, staining and preparation of thin slices, but it is also necessary for revealing the internal structures of CS. For example, where the *sagittae* are extremely opaque or are too thick for all the seasonal increments to be clearly observed on the upper surface, observations on the thin sections bisecting the core in the transverse plane provide more reliable observation. There is no single preferred method of sectioning CS, and the procedures and materials used in making simple and/or thin sections are very dependent on the nature of the application (Mosegaard *et al.*, 1998).

2.3.1. Sectioning individual structures (fig. VIII.C.7)

The major problem when sectioning CS is to ensure that the section includes the best plane (e.g. centre of CS, etc.) (Chap. III.C). Sectioning is done by a simple grinding (chap. VIII.C.2.5) or, more often, by low- or high-speed sawing. One of the most frequently used saws is the low-speed Isomet[®] (Buehler Ltd) equipped with a diamond disk. All saws of this type use liquid media as the cutting fluid: we recommend water for non-hydrophilic structures such as otoliths and diluted alcohol (70 to 95°) for hydrophilic tissues such as bones. Because of evaporation, the quantity of alcohol being used must be checked regularly. The preparation and sectioning of a CS generally take from one to five minutes.

The CS is usually embedded (chap. VIII.C.2.2) before being sectioned: it is easier to manipulate it and is essential in the case of small CS like certain otoliths. The section level, adjusted using the saw micrometer, must first be located (fig. VIII.C.7a), by marking guidelines on the resin with an indelible pen or a cutting tool. If two sectioning levels are required for further preparation, e.g. thin slice, they must be located first (fig. VIII.C.7a). When precise sectioning is necessary (e.g. with small otoliths) the thickness of the sectioning tool must be taken into account: a diamond disc is about 300 µm thick. With the Isomet[®] saw, the sample is then sectioned simply by exerting pressure on the cutting disk (fig. VIII.C.7b, c). Some large CS, such as spines can be cut whole without embedding them (fig. VIII.C.7d).

Double thin sections are sometimes made with a low-speed saw using a pair of blades separated by a spacer approximating to the desired thickness of the section. Spacer thickness may vary from 150 to 500 µm, depending on the viewing requirements for increments in the CS section.

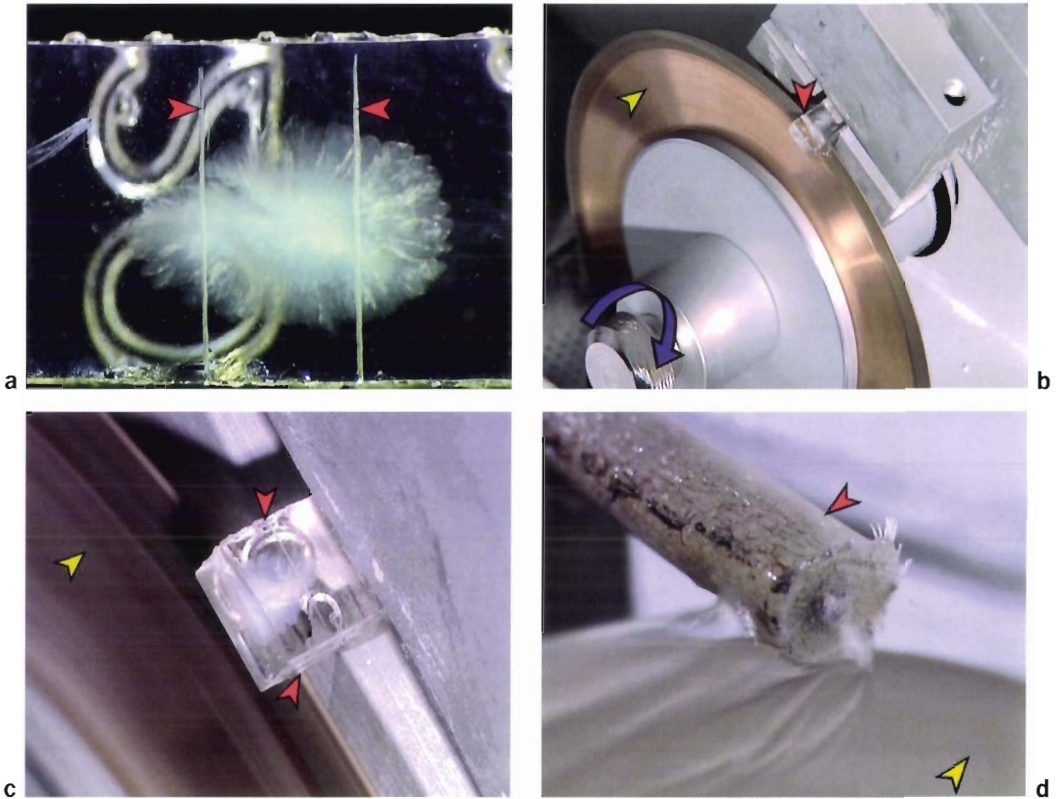


Figure VIII.C.7 - Section of the hard parts: example of section of an otolith embedded in resin and of a whole dorsal spine for obtaining a slice. a) Location of the levels of cut (red arrows) for obtaining the otolith slice including the *nucleus*. b) Cutting with a rotative saw with slow speed (Isomet[®], Buehler[®]). The resin block with the otolith (red arrow) is cut by a diamond disc (yellow arrow) in rotation (large blue arrow). The disk is humidified permanently. c) Detail of the preceding figure. The diamond disc (yellow arrow) turns while the resin block is supported above. The level of cut is indicated by the red arrows. d) Section of a slice in a whole dorsal spine. The water flow is visible at the interface spine (red arrow) - diamonds disk (yellow arrow) (photos[©] Ifremer O. Dugornay).

2.3.2. Multiple sectioning (fig. VIII.C.8)

Although low-speed saws have been used to cut multiple thin sections from otoliths embedded in polyester resin blocks (McCurdy, 1985), high-speed saws are more efficient when there is a need to process large numbers of otoliths. High-speed saws are usually adapted from precision milling machines (Bedford, 1983) or precision cutting/grinding machines (Van Beek *et al.*, 1997). Purpose-built high-speed otolith saws are also available. The choice of high-speed saw will depend both on the quantity of otoliths to be processed and the available budget for equipment purchase. The need for further processing after the otoliths have been cut or thin-sectioned may also have to be considered.

Multiple transverse thin sections are routinely prepared from the *sagittae* of demersal species, in laboratories where the results of fish age estimations are used for stock assessment purposes. European fish-age estimation laboratories tend to favour variations of the technique described by Williams & Bedford (1974), that produce thin strips of cured polyester resin containing transverse sections of otoliths (fig. VIII.C.8a-c). Elsewhere transverse thin sections are routinely prepared from individual *sagittae* for stock assessment purposes using variations of the method described by Nichy (1977). The Otolin system developed by Van Beek *et al.* (1997) was the first stage in the development of a prototype otolith production line for routine age reading of demersal species.

A number of variations of Bedford's method have evolved (tab. VIII.C.4). Some of these are not thought to affect the readability of the otolith growth zones; e.g. the number of cutting blades and the cutting speed (blade r.p.m.). However variations in otolith section

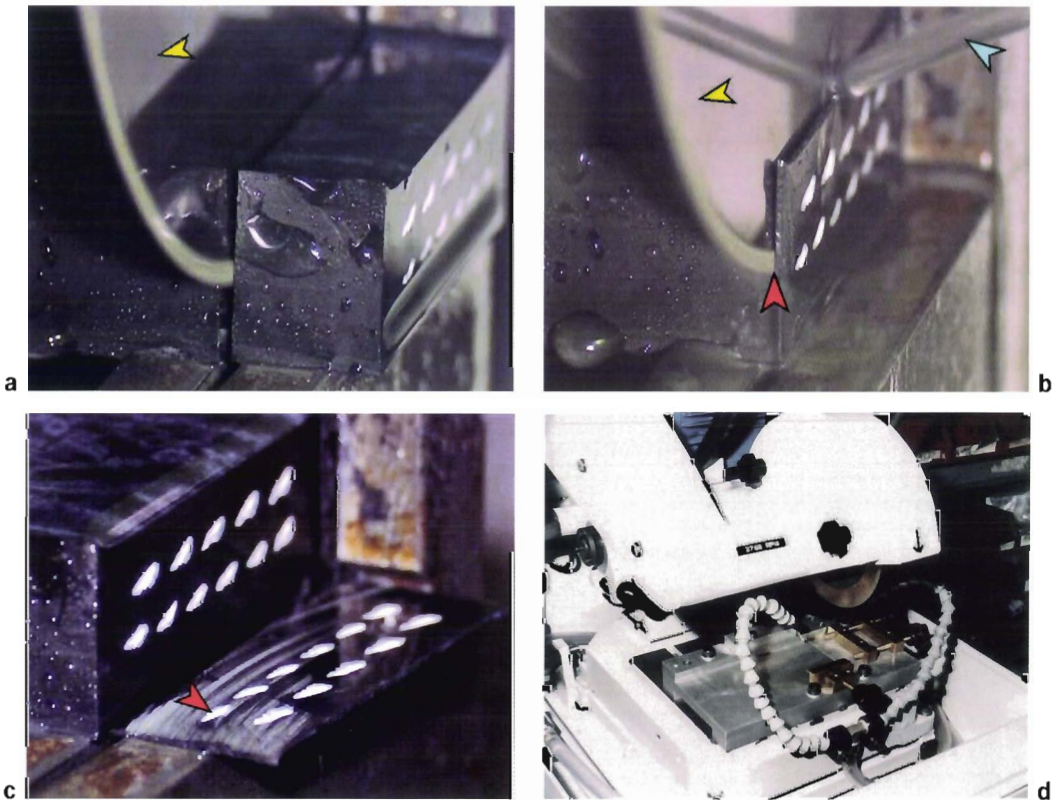


Figure VIII.C.8 - Section of otolith series with high speed diamond saw (yellow arrow). a) The first section crosses the otoliths. The section levels had been located as a preliminary on the black resin. b) The second section carries out the slice of the otoliths (red arrow). The water flow (blue arrow) arrives directly on the diamond disk (yellow arrow). c) Result of the slice of otoliths series (photos[®] Ifremer O. Dugornay). (d) View of the high speed diamond saw of the Otolin system (photo Bennett Ltd).

thickness will affect the readability of the otolith sections. All circular cutting blades have a preferred minimum section thickness and this is related to the amount of material lost on either side of the blade every time a cut is made, the kerf loss. Factors affecting kerf loss for a particular blade are the thickness of the blade itself and the grit size of the diamond compound. Attempts to produce thinner sections may result in the destruction of the otolith thin section. This is of particular importance for saws fitted with two blades, where thinner blades may be required to produce sections of the desired thickness. With all types of saw, the section must be thick enough for the embedding media to support the otolith section during the cutting process but thin enough to provide the required degree of transparency for observation of the growth rings.

Table VIII.C.4 - Thin-section preparations used for the age estimation of *Merlangius merlangius* otoliths at some European institutes (ICES, 1998).

Research institute	Section thickness	Colour of embedding resin	Unmounted sections	Mounted with cover slip	Sections mounted on glass microscope slides	
					Uncoated	Protective coating
RIVO-DLO Netherlands	0.8 mm	Black	No	No	Yes	No
FRC Ireland	0.4-0.5 mm	Black	No	No	No	Yes
CEFAS UK	0.6-0.7 mm	Black	No	Yes	No	No
AESD N. Ireland	0.3-0.4 mm	Black	No	Yes	No	No
Ifremer France	0.3 mm	Clear	Yes	No	No	No

2.3.3. Cryotome sectioning (fig. VIII.C.9)

The use of Ehrlich's hematoxylin to stain bone sections requires decalcified material (chap. VIII.C.2.6.2). Sectioning is managed on frozen samples with a cryotome, for example the Cryomat™ (a microtome with both the knife and the platine refrigerated) manufactured by Leica Ltd. The decalcified bones are frozen either in dry ice or directly on the sample-holder that is refrigerated with methanol in the Cryomat™. The frozen sample is then cut with the knife of the microtome (fig. VIII.C.9a) and the sections are caught in a petri-dish filled with distilled water (fig. VIII.C.9b). The sections are about 15-20 µm

thickness, depending on the material. The sections can be preserved in 70% alcohol before staining (chap. VIII.C.2.8.2).

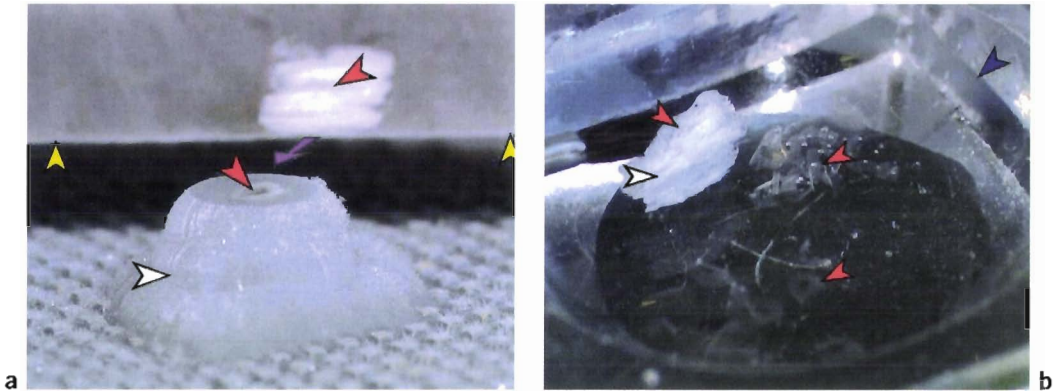


Figure VIII.C.9 - Spine section with a cryotome. a) A decaified spine (red arrow) is embedded inside ice (white arrow). The blade of the cryotome (yellow arrow) is moving from the back (pink arrow) and cut very thin slice of spine embedded in the ice (red arrow on the blade). b) The thin sections (red arrow), still embedded in the ice (white arrow), are deposited at the surface of a water bath (blue arrow). After defrosting, the sections float on the surface (red arrows) and can be recovered (photos© Ifremer O. Dugornay).

2.4. Mounting

2.4.1. Section mounting for subsequent stages of preparation (fig. VIII.C.10)

When transverse thin slices of otoliths (or other CS) are to be prepared, the resulting thick section including the core is attached to a glass slide with thermoplastic glue (Crystal Bond™) before being ground and polished (fig. VIII.C.10a, b). The block can subsequently be turned over, fixed again to a slide with the polished face down, and ground and polished on the other side (fig. VIII.C.10c, d).

2.4.2. Mounting otoliths from small fish (fig. VIII.C.11a)

Small otoliths are often directly mounted in embedding media (chap. VIII.C.2.2.1). After extraction the otolith is sucked into a drop of the same medium, and put directly in the medium on a cover slip. One can use polyester resin which permits the otolith to be manipulated for a while before it sets. The mounted otolith can be observed directly within a few hours of setting, and conserved as it is for a long period. The most important precaution is to check the thickness of the medium above the otolith, as the distance of the focal plane may be very short under high magnification. It is often necessary to remove a certain amount of embedding media before it sets: any excess resin can be trimmed from the cover slips with a scalpel; otherwise it will be necessary to grind and/or polish it before observation.

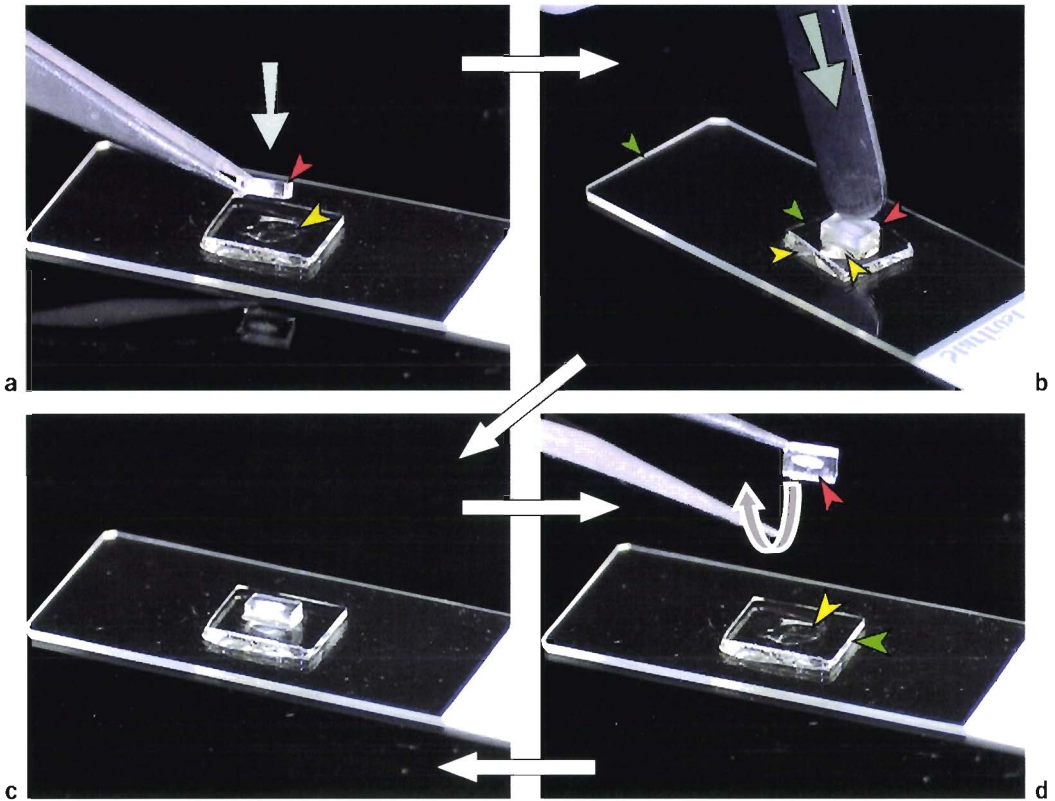


Figure VIII.C.10 - Preparation mounting: example of affixing otolith section with thermoplastic glue and turning over on hotplate. a) Affixing of the otolith slice on the thermoplastic glue (e.g. Crystal Bond™). The block of resin containing the slice (red arrow) is brought (grey arrow) on the thermoplastic glue at a viscous state (yellow arrow). b) the slice is supported (grey arrow) on the glue (yellow arrow). The pressure drives out the air bubbles of the glue captive under the preparation. In fact the slice (red arrow) is paste on the glue (yellow arrows) on a slide itself paste on a microscopic slide (green arrows). c) View of the finished preparation outside of the hotplate. d) Turn over of the resin block. When the thermoplastic glue is sufficiently liquid at high temperature (yellow arrow) the slice is taken off, turned over (grey arrow), and redeposited on the glue on the slide (green arrow). The finished preparation is withdrawn from the hotplate (photos⁶⁹ Ifremer O. Dugornay).

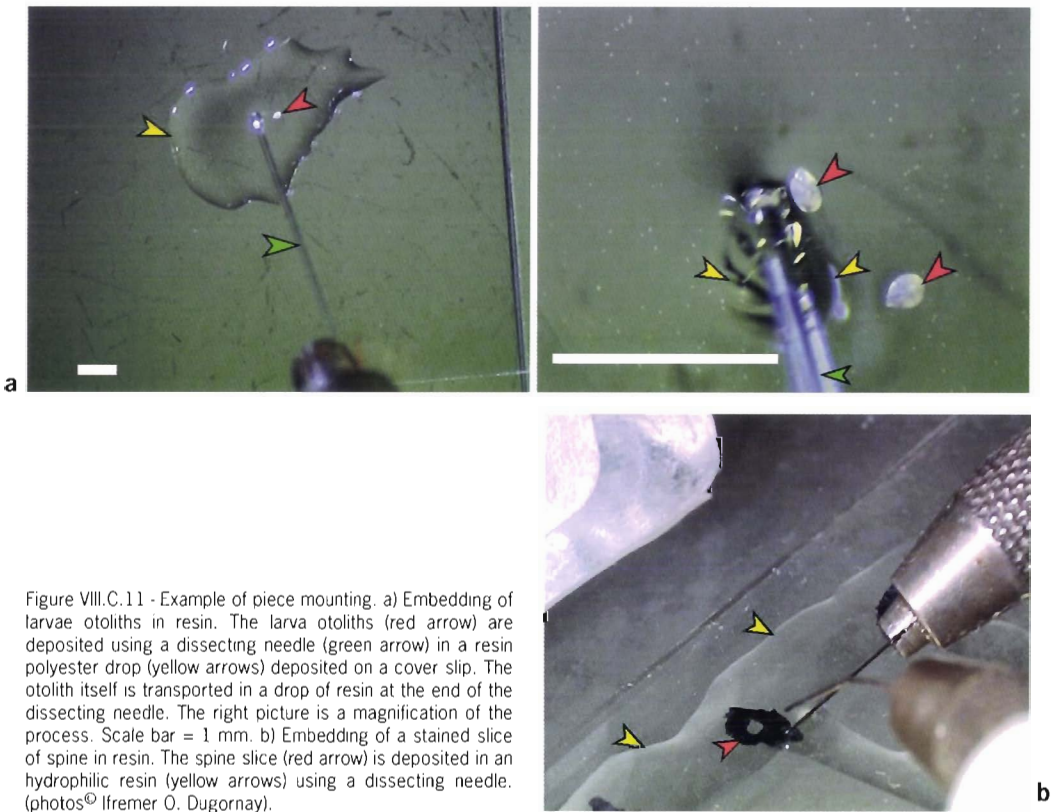
2.4.3. Slide mounting of multiple otolith sections

The black resin strips containing the transverse thin sections through the otolith growth centres (fig. VIII.C.8c) are fixed to custom-sized glass microscope slides (76 mm x 50 mm), covered with catalysed clear casting resin and protected by glass cover slips. Each microscope slide is engraved with the identification numbers of the otolith sections to be mounted on it.

A disposable plastic pipette is used to apply narrow strips of catalysed clear casting resin to the microscope slide and this is used to glue the slices of thin sections to the slide. The resin is then allowed to set. Catalysed clear casting resin is dropped onto the glass slide in the spaces between the black resin strips and on top of the strips, to form a complete layer of resin on top of the slide. Gentle stirring/mixing is

essential to prevent bubbles from forming in the resin as these would make it difficult to observe the *annuli* on the otoliths. After about 20 minutes the resin begins to set and a glass cover slip is gently placed over it. The slide is carefully placed inside a fume cupboard or under a fume extraction hood until the resin has completely set. Excess resin can be trimmed from the microscope slides with a scalpel once the resin has set to the gel stage, and any remaining resin smears can be removed by using a drop of acetone and a piece of tissue paper.

Very thin sections of stained bone (e.g. spinal sections) are mounted on microscope slides in a hydrophilic medium (e.g. Permount®) (fig. VIII.C.11b).



2.5. Grinding and polishing (fig. VIII.C.12)

There is not a great difference between the meanings of “grinding” and “polishing”. The latter term is probably best reserved for the final stages of the grinding/polishing procedure. Calcified structures can be

directly ground, or ground after embedding or sectioning. Like sectioning, CS grinding is employed in order: (1) to improve readability, (2) to distinguish marks that are not visible on the whole CS, (3) as a precursor to many other preparation techniques (e.g. burning, staining, SEM, impressed acetate, microradiography). As for sectioning, the grinding process requires a careful check of the CS plane (transverse, sagittal or frontal).

Grinding and polishing procedures are often carried out by hand using wet abrasive papers, with or without abrasive pastes. Grinding uses wet sandpapers with grit grades between 120 and 1 200. Polishing uses polishing cloths with different grades of alumina pastes (from 3 μm to 1/3 μm) or diamond powder. In manual preparation, grinding and polishing movements must be random, in order to avoid systematic distortion of the plane of preparation (fig. VIII.C.12).

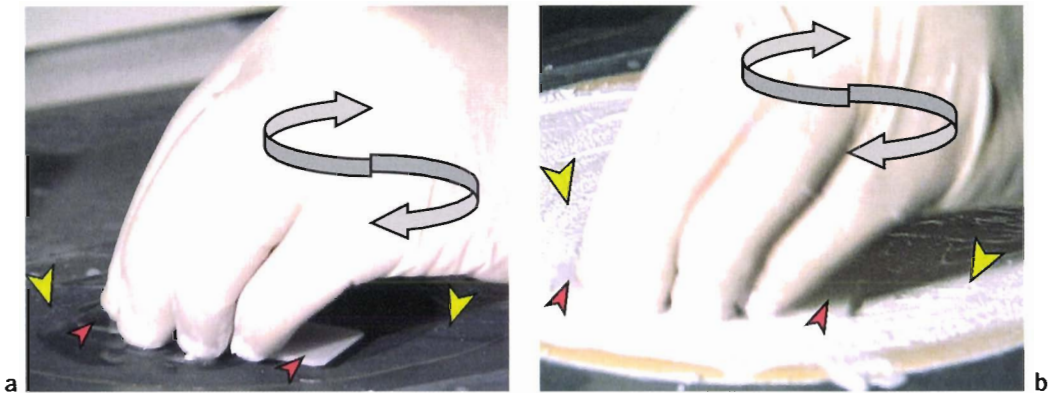


Figure VIII.C.12 - Grinding and polishing of preparations. a) Grinding. The preparation, an otolith slice mounted on microscopic slide (red arrows) is ground on a wet sand paper (yellow arrows). The hand movement is irregular (grey double-arrow) to avoid systematic scratches. b) Polishing. The preparation, an otolith slice mounted on microscopic slide (red arrows) is polished on a polishing cloth (yellow arrows) with and alumina slurry (white powder) and water. The hand movement is irregular (grey double-arrow) to avoid systematic scratches (photos® Ifremer O. Dugornay).

Grinding and polishing machines may be used both for otolith serial preparation and for specific applications in which a perfect surface state (perfect flatness and relief-free) must be obtained, as for thin sections prepared for microchemistry analysis, particularly with WDS, EDS or PIXE. Some grinding machines allow the amount of material to be removed to be specified (i.e. the final thickness of the sections). The grinding medium is silicon carbide (SiC) powder. It is used:

- to grind glass slides in order to standardise thickness and obtain a surface roughness that will enhance subsequent resin adherence, when the otoliths are mounted on the slides;
- to grind preparations in order to remove the maximal thickness of resin covering embedded otoliths.

After grinding, slides or preparations are carefully cleaned in ultrasonic baths (MilliQ water is required for microchemistry). Preparations are then processed by means of automatic polishing machines in several steps:

- fine grinding or pre-polishing, usually carried out in successively finer steps with SiC-paper (e.g. 800-1 200 grain size), using water lubrication (MilliQ water is required for subsequent microchemistry analysis) and involving regular optical inspections of the plane to be reached;
- polishing, which removes the scratches and damage introduced by previous grinding stages. It is carried out in successively finer steps with diamond suspensions or sprays in the aqueous phase (e.g. 9 μm - 3 μm - 1 μm - 0.25 μm grain sizes). The requirement for the finest steps depends on the final quality required (see above). Polishing is carried out using polishing cloths, whose characteristics depend on diamond grain size.

During polishing, preparations are regularly checked under the microscope in reflected light, as this is the only way to check surface quality (cracks, scratches, etc.). To facilitate this procedure, Plexiglass[®] specimen holders may be used, as these are also more advantageous in terms of contamination risk.

2.6. Acid etching, decalcification

2.6.1. Otolith acid etching

The action of the acid on the otolith or its section superficially destroys part of the calcium complexes. Several diluted acids have been suggested in the literature: nitric acid at a concentration of 0.2 N (Albrechtsen, 1968); hydrochloric acid at concentrations of between 0.1 and 1% (many authors); acetic acid at 1% (Richter & McDermott, 1990); ethylene diamine tetra-acetic acid (EDTA) at 5% (several authors). EDTA seems preferable because its attack is more gentle and degrades the surface of the otolith less rapidly (Campana & Neilson, 1985). Times of attack acid are very variable, and depend on species and otolith size: a few minutes are enough to obtain good preparations, but preliminary evaluation tests must be made for each particular case.

After etching the preparation must be rinsed in abundant clear water for several minutes. It must then be dried before further preparation (e.g. staining, SEM).

2.6.2. Decalcification of bony tissue

To prepare frozen sections for hematoxylin staining (chap. VIII.C.2.8.2), bones must be decalcified. For skeletochronological studies, the decalcifying agent is usually nitric acid at a concentration

of 5-10%, depending on the volume of the sample. The duration of the decalcification stage also varies, depending both on sample volume and on the degree of mineralisation of the bone. In any case, the decalcification process must be kept strictly under control because after the bone has been completely cleared of its mineral content the collagenous matrix may rapidly become damaged and thus no longer stainable.

Decalcification is stopped by washing in tap-water. Samples can then be preserved in 70% alcohol for several days before processing. The next step in sectioning is always preceded by careful washing in running tap-water for 12-24 h in order to remove all the nitric acid, which would otherwise disturb the staining process.

2.7. Heating and burning (fig. VIII.C.13)

In some cases (e.g. flatfish) the quality of the observations may be further improved by heating the otolith until its protein denatures and turns brown in colour, rendering the winter (translucent) growth zones more visible. Burning lasts for a few seconds to a few minutes,

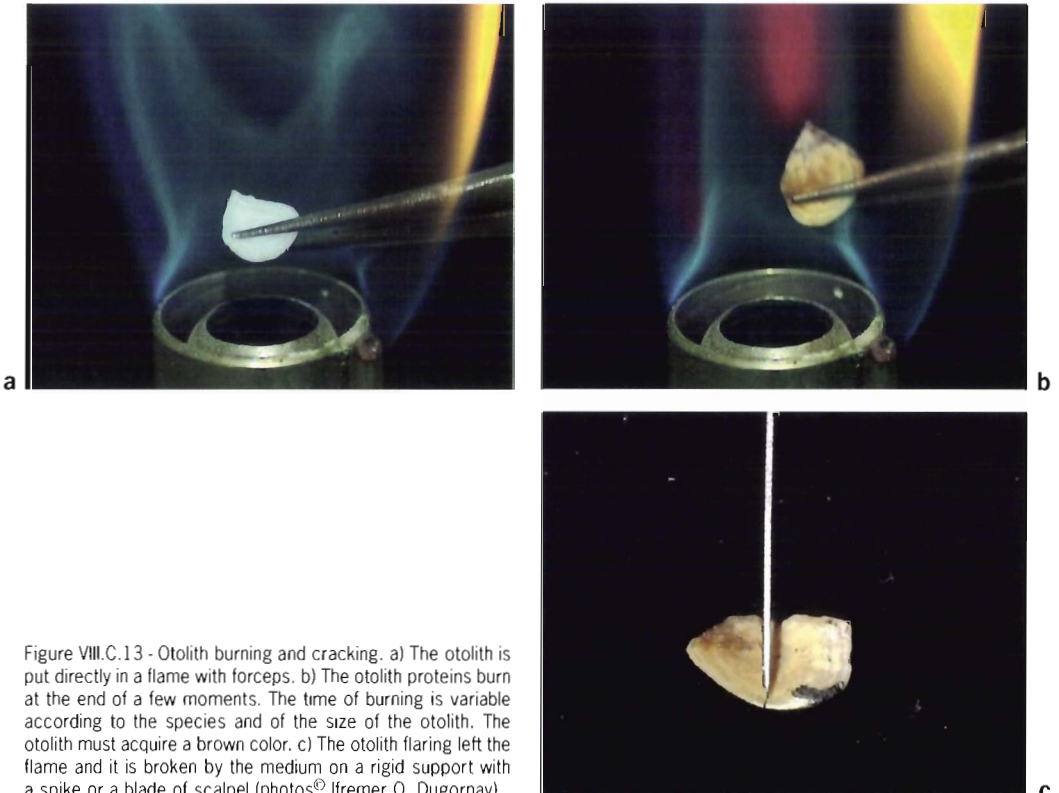


Figure VIII.C.13 - Otolith burning and cracking. a) The otolith is put directly in a flame with forceps. b) The otolith proteins burn at the end of a few moments. The time of burning is variable according to the species and of the size of the otolith. The otolith must acquire a brown color. c) The otolith flaring left the flame and it is broken by the medium on a rigid support with a spike or a blade of scalpel (photos[©] Ifremer O. Dugornay).

depending on the species and the otolith size. After burning the otolith is gently broken with a scalpel blade or other hard tool (fig. VIII.C.13c). A variety of heating methods have been tested at the NMFS Wood's Hole laboratory, but currently only *Gadus morhua* otoliths are heated by baking in a radiant heat oven at 275°C for three to six minutes (Almeida & Sheehan, 1997). In some cases, broken otoliths or otoliths that have been cut in half with a diamond bladed saw, are burned after breaking in order to improve *annulus* readability. The stability of the burned surfaces can be maintained by embedding the otolith pieces in polyethylene vials filled with clear casting resin.

2.8. Staining (fig. VIII.C.14)

2.8.1. Staining otoliths (fig. VIII.C.14a-b)

Staining is a method of preparation which reveals fine chromophilic increments, sometimes comparable to those obtained after burning. Developed for the first time by Albrechtsen (1968), the technique consists, after sectioning, polishing and etching the surface, of placing it in contact with a histological stain. The stain used by Albrechtsen was methyl violet (15%), a specific stain for cellular kernels, but a whole range of histological stains may be used. According to Richter & McDermott (1990), who tested several stains, 1% aniline blue (also called light blue) and toluidine blue (1%) give the best results, colouring respectively collagen and cartilage, and the cell kernel. It is not really known what is the specific target of these dyes in the otolith, which contains neither collagen nor cellular kernels. It may be that they are attracted by specific proteins adjacent to the organic components of the otolith. Gauldie (1990) suggested that the dyes act physically while settling in the discontinuities underlined by acid etching, rather than by reacting selectively with proteins. In summary, we recommend the use of toluidine blue for a few minutes, after acid etching, as this is a universal dye for otoliths.

Bouain & Siau (1988) proposed another method of staining after first breaking the otolith, immersion for 12-14 h in fuchsin acid, then passage for several minutes in starch black (5%): the growth increments appear dark blue on a pink background. It seems that the physical principle of coloration is not completely excluded. Another method of treatment consists of passing the otolith through a bath of acid and stain before sectioning (Richter & McDermott, 1990), but this is not often employed.

As in acid etching, the time of attack acid is very variable, depending on species and otolith size: a few minutes are sufficient to obtain good preparations, but preliminary trials should be carried out in each case. After staining the preparation must be gently rinsed with clear water for few second to preserve the staining.

2.8.2. Staining bony tissue (fig. VIII.C.14c)

The specific stain for bones is hematoxylin, which stains the cementing lines because of their richness in proteoglycans. This technique is thus very valuable for detecting the AGL.

Before staining, the decalcified bone sections are rehydrated if necessary, picked up with a paint brush, drained off and dropped in a bath of hematoxylin. After ten to twenty minutes (the time required will vary depending on the material and the quality of the hematoxylin), the sections are collected and washed in tap-water till the stain turns bluish. The sections are then delicately picked up using a paint brush and mounted in a hydrophilic resin (e.g. Aquamount®) (chap. VIII.C.2.4.3 and fig. VIII.C.11b).

Ehrlich's hematoxylin (Ganter & Jolles, 1969) is prepared using the following quantities:

- hematoxylin	4 g
- ethanol 95%	200 ml
- distilled water	200 ml
- glycerol	200 ml
- potassium alum	6 g
- acetic acid	20 ml

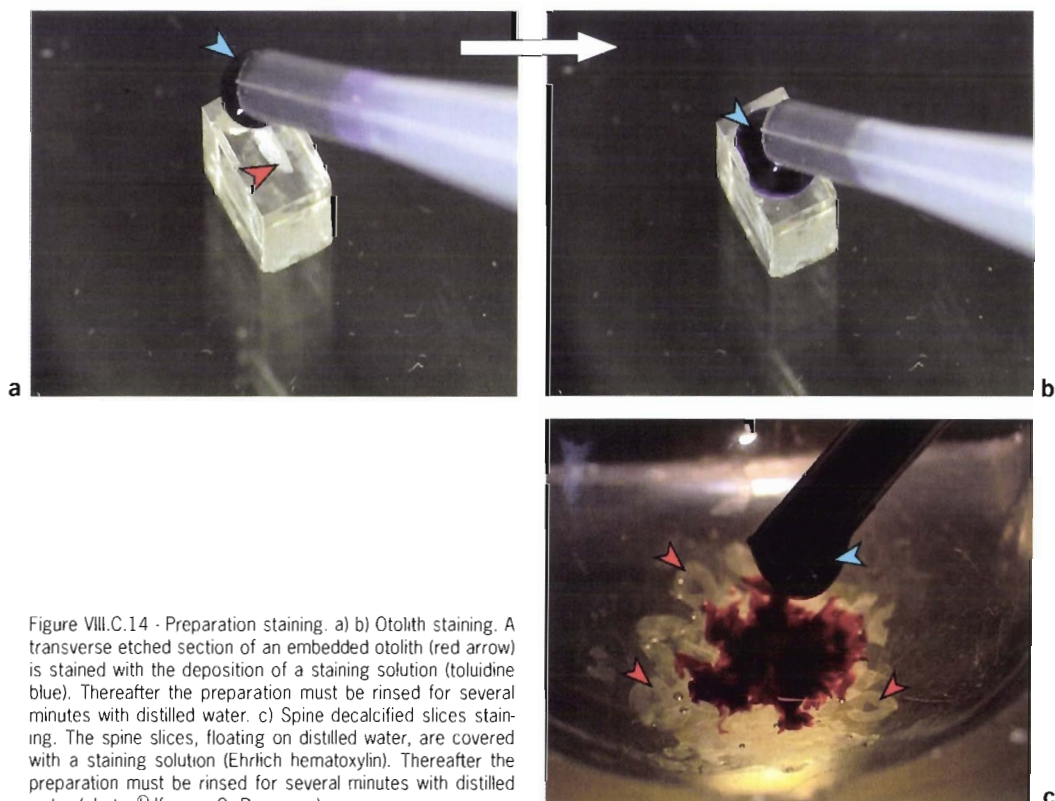


Figure VIII.C.14 - Preparation staining. a) b) Otolith staining. A transverse etched section of an embedded otolith (red arrow) is stained with the deposition of a staining solution (toluidine blue). Thereafter the preparation must be rinsed for several minutes with distilled water. c) Spine decalcified slices staining. The spine slices, floating on distilled water, are covered with a staining solution (Ehrlich hematoxylin). Thereafter the preparation must be rinsed for several minutes with distilled water (photos© Ifremer O. Dugornay).

First dissolve the hematoxylin in ethanol; then add the other materials; leave the mixture to mature slowly in the laboratory in light and air for at least two weeks. Ehrlich's hematoxylin keeps well for several years.

Furthermore, the older the staining solution, the better is the quality of Ehrlich's hematoxylin. For this reason, it is advisable to keep Ehrlich's hematoxylin permanently available in a bottle, though it should be regularly filtered in order to avoid precipitates, or at least just before use.

2.9. Some special considerations for microchemistry

Microchemistry is the analysis of the composition of calcified tissues. For the most part, the sample preparation techniques are the same as for observation of increments. However, there is always a risk of contaminating samples during handling and manipulation, and some of the analytical techniques have particular requirements for surface smoothness. In order to reduce analytical problems several special considerations in the choice of material and techniques for sample preparation should be borne in mind.

As a general guideline, all handling and storage equipment should be chemically inert, cleaned with dilute ultra-pure HNO_3 and rinsed in ultra-pure water (18-M Ω).

Surface techniques measure composition at discrete points on the surface of sectioned samples. The CS are embedded in resin and sectioned by sawing or grinding. The resin should be selected carefully, bearing two considerations in mind:

- Purity of the resin. The resin used should be basically inert so that it does not introduce any contamination during infiltration. Most epoxy resins do not contain detectable levels of the elements usually found in otoliths. However, cyanoacrylic glues and thermoplastic resins may contain high levels of S, Si, and sometimes Pb. Polyester resins should also be avoided because they can contain contaminants which can affect the measurement of otolith composition.
- Viscosity of the resin. When samples are analysed using laser probes (LA-ICP-MS), the energy of the laser can be very destructive. If the sample is held only loosely in the resin the energy can cause it to vibrate, shatter, and fly out of the resin. Low-viscosity resins such as Spurr or Araldite are most suitable for microchemistry preparations. It is particularly important to ensure the purity of the low-viscosity resins, precisely because they do infiltrate otoliths so readily.

The reagents and grinding and polishing media must be selected so as to reduce the risk of contamination. This may require the use of different compounds for microchemistry and for observation studies. AlO_2 grinding papers and powders are an obvious source of metal

contamination. The use of diamond sprays or suspensions in aqueous phase is therefore recommended. Less obvious sources of contamination include polishing suspension media and the lubricants used for saws (MilliQ water is recommended).

Some analytical techniques (EDS, WDS, PIXE) require a perfect final polished surface and flatness that are difficult to achieve by hand polishing. Automatic grinding and polishing (see chap. VIII.C.2.5) are recommended. Frequent checks on the progress of the preparation (with respect to the plane to be reached and the surface state) should be made using a reflected light microscope.

Final cleaning with MilliQ water in an ultrasonic bath has often been suggested in preparation protocols.

It is also important that preparation storage containers are free of contaminants. Certain analytical methods are very sensitive to moisture, so preparations should be stored in a desiccating cabinet until analysis. Various decontamination processes have been suggested prior to sample analysis:

- pre-ablation when preparations are analysed with LA-ICP-MS (Campana *et al.*, 1995);
- 15s rubbing with a tissue soaked in ultra-pure HNO_3 (Milton *et al.*, 1997). This procedure is not recommended when the analytical technique is sensitive to surface roughness.

Bulk analyses are microchemistry measurements that are made from dissolved samples of CS. The sample is usually dissolved in an acid, and the result is introduced into the analytical equipment in the form of a liquid.

Handling always carries an increased risk of contamination of samples, and extraction, cleaning and drying of bulk sample preparation should therefore ideally be done in clean conditions, in a class 100 laminar flow fume cupboard.

Care must be taken when weighing samples, because the results of bulk analyses are usually expressed relative to the amount of material analysed, and any uncertainty introduced into the procedure may affect the reliability of the results.

The purity of the acid used for digestion is extremely important for these measurements, because of sample contamination and also because impurities in the acid used for standard and procedural blanks will result in higher limits of detection (LODs) and thus poor sensitivity. Sub-boiling redistilled nitric acid diluted with ultra-pure water is recommended. A number of methods are used to dissolve CS samples for bulk analyses. Most use nitric acid because it readily attacks CaCO_3 . The sample can be dissolved at room temperature, but the digestion is not always complete. In the case of otoliths, room temperature digestion may leave a residual “ghost” composed of the

otolith protein that resists acid digestion. Hot digestion in nitric acid will completely decompose the protein matrix, but involves extra handling that may result in sample contamination. A compromise is to use a microwave oven to heat the sample in acid in order to achieve complete digestion. The protein matrix is an important consideration in microchemistry studies because there is very little information on what elements are associated with the protein (as compared to the mineralised components). While it might appear to be preferable to develop a digestion protocol that would leave the protein matrix untouched, in fact it is probably better to ensure complete digestion of the sample in order to avoid any variability introduced by excluding the protein.

Campana *et al.* (2000) reported the result of a decontamination experiment that compared the results of five preparation procedures. Mg was the only element of the four tested (Mg, Ba, Li and Sr) that differed significantly among treatments.

D. Observation

B. Morales-Nin, J. Panfili

There are about as many CS observation techniques as there are preparation techniques, ranging from simple to very complicated. The researcher should select from them according to their suitability for the objectives of his study. As is the case for preparation, the choice of observation tool depends on the time required and available but also on the experimental costs of the study. For example standardising illumination conditions is very important in image analysis (Mosegaard *et al.*, 1998): the accurate description of illumination conditions is of the utmost importance when the optical properties of CS are being compared. Increments can appear to vary depending on the light source used.

In the following sections, we summarise some of the most frequently used observation techniques available for sclerochronological studies.

1. Light microscopy

1.1. Low-power magnification (binocular)

Large skeletal elements such as *cleithra* of Esocids can be viewed and measured without magnification. Age can be assessed with the naked eye, even under field conditions and even up to 30 years (Casselman, 1974). Nevertheless incremental structures are rarely large enough to be observed accurately by the naked eye, and it is usual to view them under low-power magnification. At this scale only seasonal and annual increments are visualised. This observation method is the cheapest and most rapid, permitting large numbers of samples to be observed. The usual range of magnification is from 1x to about 100x.

1.1.1. Whole structures

Whole-CS observation is most suitable for relatively thin translucent structures such as scales, opercular bones, *cleithra* and certain otoliths. For fish with thick CS that are too dense for the innermost increments to be observed, sections are used (chap. VIII.C.2.3).

1.1.2. Clearing media

The use of clearing media is recommended in any type of observation with or without preparation. A clearing medium is a liquid or hardening compound which enhances the visualisation of the increments. It has often the same or nearly the same optical density as that of the CS, allowing the light to penetrate more easily in order to reveal

the internal structures. Several liquid clearing media exist: the most used is probably water, saline solutions (more isotonic), followed by alcohol, mixtures of water-alcohol (various percentages), glycerine, glycerine-alcohol (30:70), immersion oil. Essential oils (clove, rosemary, pine, cedar, camomile, etc.) also offer very good results. Other media such as creosote, xylene, methyl benzoate, are now less used because many of them are health hazards. Care should be taken to ensure that the increment structures are not damaged and/or rendered unreadable by secondary action of the clearing media. After observation in viscous and/or oily media, the CS must be cleaned using a tissue impregnated with a solution of ether-alcohol (50:50). For otoliths, for example, the choice of clearing medium depends on species: for small and/or thin otoliths, water or physiological serum can be recommended while for thicker refringent otoliths oily media are most suitable (e.g. rosemary oil). Most fixing media clear CS to some extent.

1.1.3. Mounting media

Mounting media refer to media that simply fix the CS, usually to a slide (Secor *et al.*, 1992). Some mounting media are also used to conserve CS preparations and enhance the visibility of the increments. These also act through their optical density properties. They are mostly synthetic resins: polyester, Protexx[®], Flo-texx[®], acrylic glue, thermoplastic glue, Permout[®], etc. There are difficulties associated with some specific mounting media due to their relative opacity and colour, their hardness and/or their poor quality after long storage; such media include Eukitt[®], cyanocrylate, Canada balsam and epoxy. Permanent media that cannot be removed limit the possibilities of manipulating the CS if the observation requires this: for instance mounting pelagic fish otoliths on black Perspex[®] sheeting with polyester resin. We recommend mounting non-hydrophilic CS preparations with thermoplastic glue that melts at low temperatures (40-60°C) or with polyester resin, and hydrophilic CS preparations (e.g. thin spine sections) with Permout[®].

1.1.4. Illumination

Two means may be employed to illuminate the surface being viewed. Light is either directed through the CS from below (transmitted light) or directly on to the surface from above (reflected light). Reflected light is usually used to observe seasonal increments, while for microstructural (e.g. daily) otolith examination we use transmitted light. Nevertheless, some trials comparing transmitted and reflected light should be carried out on seasonal increments (e.g. bones) before deciding on a routine method. The aspect of the seasonal increments will depend on the type of illumination employed (fig. VIII.D.1):

under transmitted light translucent increments are bright and opaque increments are dark, while under reflected light translucent increments are dark and opaque ones are bright. The fact that one and the same increment can be described as “bright” or “dark”, depending upon the form of illumination, can lead to considerable confusion and for this reason the terms “opaque” and “translucent” should always be used (Casselman, 1974; Williams & Bedford, 1974; Casselman, 1983).

1.1.4.1. Transmitted light (fig. VIII.D.1a and 1b)

When using transmitted light a transparent container should be used if the CS is to be observed immersed in a clearing medium. Two sources of light are also available and should be tested: direct (fig. VIII.D.1a) or diascope (fig. VIII.D.1b) transmission. When observing thick CS sections, the best view is obtained when the base of the CS is illuminated from the side by fibre-optic light sources, and the surface shaded. Surface shading causes light to strike the lower part of the section and scatter upward to the surface, thereby enhancing the contrast between the translucent and opaque increments. The brightness and contrast of the CS surface can be controlled by varying the position of the illuminating fibre-optic sources (Estep *et al.*, 1995).

1.1.4.2. Reflected light (fig. VIII.D.1c)

The quality of observations is improved by using a container with a black background. The light source must come from either the side or the top of the surface of the CS preparation under examination. Reflected light requires the use of a clearing medium to avoid surface light distortion. The orientation of the light source is very important and it may have to be moved in order to obtain the best contrast between growth increments. It is preferable to use a cold light source for incident light observations to minimise evaporation of the liquid observing medium: fibre-optic bundles are used to direct light from a high-intensity lamp.

1.1.4.3. Profile projector

The profile projector is mainly used for scale-reading. In the early 1920s the profile projector was developed to project an enlarged image of a scale on ground glass. This was copied in many laboratories and was available commercially (Carlander, 1987). This apparatus allows the manual measurement of distances on the ground glass. Nowadays computerised image analysis systems are employed in high-tech research laboratories.

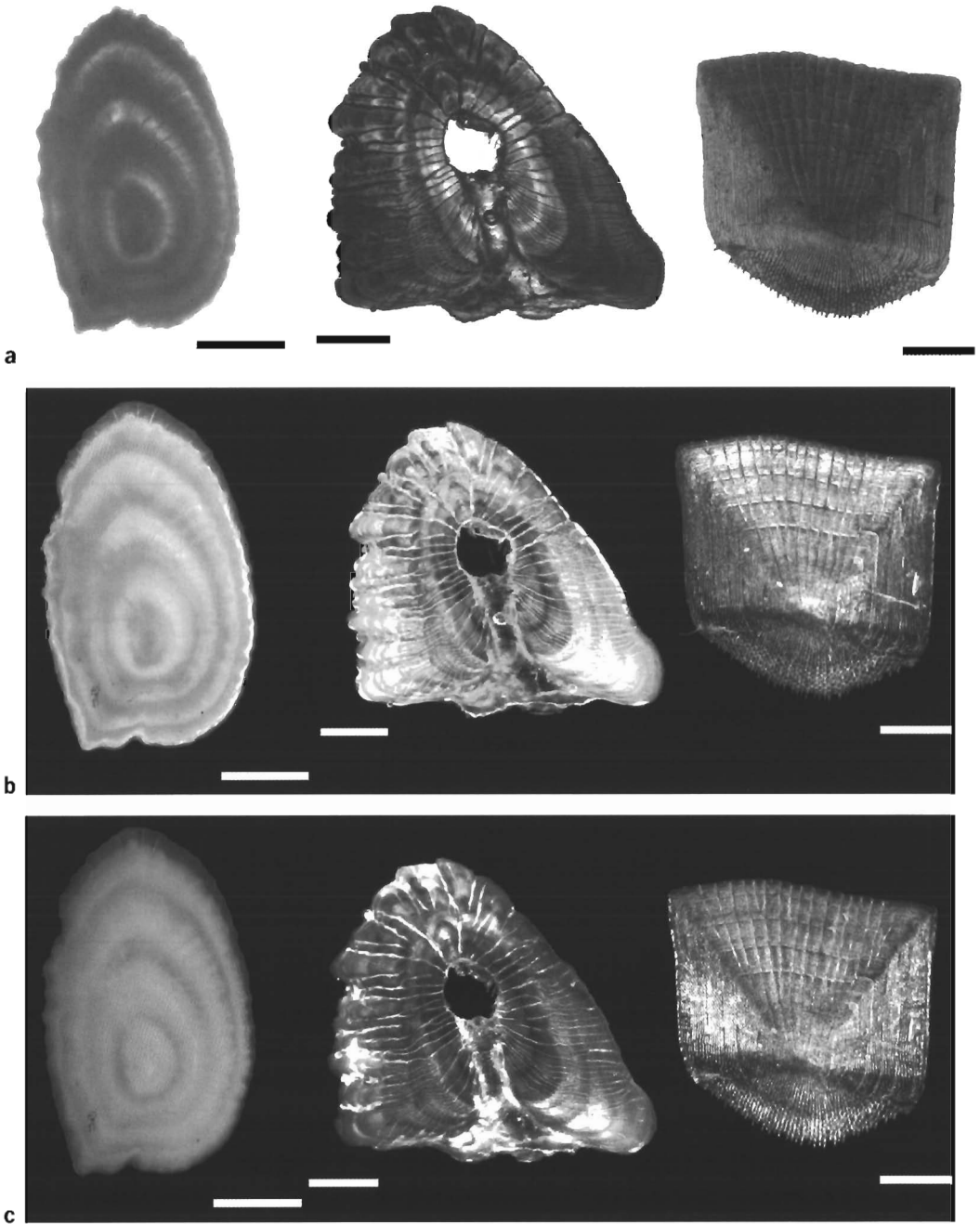


Figure VIII.D.1 - From left to right, whole otolith of *Pleuronectes platessa*, spine section of *Pangassius hypophthalmus*, and scale of *Dicentrarchus labrax*, observed in direct transmitted light (a), diascopic transmitted light (b) and reflected light against a dark bottom (c). Scale bar = 200 μm (photos J. Panfili).

1.2. High-power magnification (compound microscopy)

High-power magnification using compound microscopy is mainly used for microstructural examination of otoliths, for reading primary increments, and sometimes for observations of the internal structure of other CS (e.g. bone constituents). As in binocular microscopy, various sources of light are used: although transmitted light is most often used, reflected, polarised, phase contrast, and fluorescent illuminations also exist. At this level the observer needs to make a choice after comparison of different light sources. Microstructural examinations are best made with a compound microscope with the following minimum features (Campana, 1992): binocular eyepieces, at least one of which can be focused, objective lenses with nominal magnifications of 20x, 40x and 100x (plus others such as 10x and 60x), a movable specimen stage (or better a motorised stage moved by means of a joystick), a substage condenser lens, an aperture diaphragm, and a variable intensity illuminator with its own focusing/condenser lens. Poorer microscope characteristics induce bias when interpreting the microincrements. Image visualisation is facilitated by use of a video camera link to a videoscreen projector.

1.2.1. Resolution

The limit of resolution corresponds to the smallest distance that separates two adjacent visible structures. The resolution of light microscopy depends mainly on the hardware and particularly on the objective lens available. Lenses corrected for colour and spherical aberration, and with a low numerical aperture are essential. Campana (1992) provides an interesting table that illustrates the limiting characteristics of the main types of objective lens: the numerical aperture of the objective ultimately controls both the magnification and the resolution that can be obtained, and the latter increases as the numerical aperture falls. Nevertheless, the resolution limit of a perfectly set up compound microscope is lower than the theoretical limit (around 0.3 μm), and often limits observation of the smallest structures, i.e. increments < 1 μm (Campana, 1987, 1992; Morales-Nin, 1988). In such cases the use of a SEM is essential. On the other hand, the possibilities of magnification are limited only by the hardware available. The maximum objective used is normally 100x (either with or without oil) and, with the appropriate combination of eyepieces and body tubes, the maximum useful magnification is 1000x-1250x (Campana, 1992).

1.2.2. Immersion v. dry objectives

Low-power lenses are dry objectives (up to 60x) while immersion lenses are used for high magnifications. In biology most users believe

that the maximum magnification can be obtained by using oil immersion objectives of 100x magnification. However 100x dry objectives are used in geology, and these can be also utilised for sclerochronological purposes. One of their main advantages is the preservation of the original state of the CS preparation (e.g. thin otolith slices) as it is not necessary to clean it after the observation. When using immersion oil the preparation must be cleaned afterward using an ether-alcohol solution (50:50). We thus absolutely recommend the use of dry objective lenses at all magnifications.

1.2.3. Illumination

Apart from the objectives and eyepieces, an optimised source of illumination has the greatest influence on image quality (Campana, 1992). All steps when adjusting illumination in compound microscopy are much the same, including centring the light source, focusing the light on the plane of observation with the condenser, and adjusting the aperture and field diaphragms. A properly adjusted aperture diaphragm will balance contrast, depth of field and resolution. All these parameters should be checked at the beginning of a sample series of examinations but the same parameters should be used between series in order to standardise the process.

1.2.3.1. Filters

Achromatic lenses tend to improve image quality when the aperture diaphragm is closed down, and when light of a single colour is used. The change in image quality can be attributed to the fact that lenses are not fully corrected for all wavelengths of light or for the entire field of view. Green light is the most frequently used. A coloured filter over the light source can raise the limit of resolution by 15-20% (Campana, 1992). Polarising filters improve observation of increments, and are also recommended (chap. VIII.D.1.3).

1.2.3.2. Confocal microscopy

Confocal microscopy, also named Laser scanning microscopy (LSM), permits observations of confocal sections (single-focal plane, thickness around 0.5 μm) within internal planes of thick specimens. It is used to examine CS, mainly otoliths, morphology and microstructures of larvae and juveniles in order to evaluate growth and the choice of section plane (Lagardère *et al.*, 1995). Compared to conventional light microscopy, the advantages of LSM include simultaneous measurements in 3D and improved contrast and resolution in certain applications. LSM microscopy can operate in epi-illumination (confocal) and transmission modes. Observations based on the epi-illumination mode are dependent on otolith autofluorescence. The technique can be

also used to detect fluorescent marks beneath the otolith surface. However the confocal technique cannot delineate the deposition of increments into otoliths, because it depends on the fluorescence of the specimens. The examination of otolith microincrements requires transmission-mode LSM.

1.2.3.3. Transmitted v. reflected light

In compound microscopy, there are two possible sources of light, the most frequently used being transmitted light which allows observations to be made throughout a shallow depth of field, and reflected light (also named epi-reflected) which allows the surface to be observed. The latter source of illumination was originally developed for geological studies and requires special equipment on a standard microscope. This allows the light beam to pass through the objective lens, reflect off the surface, and return through the lens to generate the image. It is particularly useful for observing the surface after special preparation such as grinding and/or polishing to check its quality. Another application is control of the acid etching of CS surfaces for SEM preparation. Etching control can also be done directly under the microscope (chap. VIII.C.2.6). It is possible to combine both sources of light, transmitted and reflected, when necessary.

1.3. Polarised light

Polarised light is obtained by means of two polarising filters and transmitted light. The first filter is placed between the source of light and the preparation, and the other between the preparation and the eyepieces. The relative position of the two filters can be adjusted and offers the possibility of revealing different states of polarisation of the light through the CS preparation. This may reveal some structures which are not normally visible. Otoliths or bones themselves have polarising characteristics. Otoliths are refringent to polarised light and this property allows them to be visualised even in very small individuals such larvae of a few mm in length (chap. VIII.B.3.2). Polarised light is necessary to view certain otolith increments, particularly the microincrements near the otolith edge (Mosegaard *et al.*, 1998). For ultrastructural bone examination, polarised light is used to reveal the orientation of the collagen fibres, for example isotropic fibres in woven-fibred bone matrix, or anisotropic fibres in parallel-fibred bone matrix (Francillon-Vieillot *et al.*, 1990) (chap. II.C.1.1.2).

1.4. Ultraviolet light

Ultraviolet light (UV) is only used to reveal fluorescent marks incorporated into CS (chap. IV.A.1.2.1). The equipment needed to produce UV light is usually specially adapted for compound microscopes, which unfortunately restricts its use to small preparations. The route of the light beam is similar to that of the reflected light (chap. VIII.D.1.1.4.2) arriving from above and passing through the objective. The name “epi-fluorescent light” is also given to this type of illumination. The user must check the compatibility of the UV source and the microscope objectives. Depending on the fluorescent substance which is to be revealed, specific filters may be needed. Table IV.A.1 summarises the characteristics of fluorescent dyes, their excitation wave-lengths, their fluorescence wave-lengths, their excitation rays and the filters required. In order to view both fluorescent marks and CS increments, it is possible to simultaneously use epi-fluorescence and transmitted light (fig. IV.A.2).

2. Electron microscopy

Electron microscopy uses electrons instead of photons and thus offers much higher magnification and resolution. Two principal types of microscope are available: the scanning electron microscope (SEM) and the transmission electron microscope (TEM). Most sclerochronological studies use only SEM. For this reason we offer only descriptions and recommendations for the use of SEM, and the reader should refer to the numerous specialised references available for the use of TEM. SEM falls into an intermediate position between light microscopy and TEM in term of resolution and image information. It is also similar to reflected compound microscopy (chap. VIII.D.1.2.3.3).

2.1. SEM

The SEM is a surface topographic examination tool which is widely used in the study of otoliths. This instrument offers a better than 300-fold improvement in the depth of field over the highest quality light microscope, as is reflected in the superb 3D images it provides. In addition, studies of beam-specimen interactions using the SEM can provide useful information about the chemical composition of the surface of the specimen as well as the crystallographic, magnetic, and electrical characteristics of the specimen. For microchemical analysis purposes the SEM is coupled to an EDS (Energy dispersive spectrometer) (chap. VII.G.1).

2.1.1. SEM characteristics

The electron beam used to “illuminate” the specimen is accelerated by a voltage of 1 to 30 kV. Interactions between the beam and the specimen used to generate the image have three possible outcomes:

- some primary electrons, depending upon the accelerating voltage employed, penetrate the solid to depths as much as 10 μm . Their trajectories vary and they lose most of their energy as they penetrate the specimen;
- some primary electrons interact with the uppermost atoms of the specimen so that there is a change in momentum (but no exchange of energy) with the result that the electron is backscattered through a large angle and is effectively reflected from the specimen. Such elastically reflected primary electrons are known as “backscattered electrons”;
- some primary electrons interact with the host atoms with the result that collisions cause a cascade of “secondary electrons” to form along the penetration path. Some of these electrons that are located in the outermost *stratum* diffuse towards the surface, losing energy as they do so. However, if they retain enough energy they escape, in a process known as “secondary electron emission”;
- specimen-beam interactions, as well as producing backscattered and secondary electrons, also produce photons, specimen currents, Auger electrons and X-rays, which are characteristic of the probed specimen. While any signal generated can be utilised in principle to produce an image, in practice it is the low-energy secondary electrons released from the sample that are most frequently used. Using surface-emitted electrons, rather than those that pass through the specimen as in TEM, surface images with some three-dimensional quality can be obtained. The impression of the image of the 3D surface is the result of the distribution of light and dark areas. This distribution is largely due to the fact that the incident beam generates more collectable secondary electrons per unit area when it strikes a sharply curved edge or sloping surface than when it hits a flat surface. Biological specimens, being heavily contoured, facilitate the induction of such a differential effect. Where the surface is smooth, tilting the sample at an angle to the probe will enhance the desired variation in collected secondary electrons. More subtle effects involving the manner in which structures lying above a primary surface either deflect or absorb the probe electrons also come into play in the process. In any case, the “shadows” seen are a true representation of the 3D character of the specimen surface under study.

Depending on the beam accelerating potential used, which is generally around 15 kV for otolith observation, the ratio between the low-energy secondary to high-energy backscattered electrons will be higher. Higher voltages, although they penetrate the sample surface further, result in electronic charges due to the low conductivity of aragonite.

2.1.2. Sample preparation

SEM preparation techniques depend on the objective of the study: (1) ultrastructure examination or (2) morphology.

To reveal the ultrastructure of an otolith it is necessary to cut it along one principal sectioning plane (chap. VIII.C.2.3). Due to the low penetrating power of the SEM, the section must be made through the required otolith area. Once the observation plane has been reached and checked the surface must be polished (chap. VIII.C.2.5). Reflected light showing the surface sample enables the quality of the preparation to be checked (chap. VIII.D.1.1.4.2). In order to eliminate any remaining attached material the otolith should then be cleaned in a deionised ultrasonic water bath for 15-30 seconds. After cleaning, the section must be etched to reveal the ultrastructure. The two most common etching reagents are dilute HCl (2%, pH 2.0-5.0) and EDTA (pH = 7.2-7.6) (chap. VIII.C.2.6). The etching time (1-10 min) depends on the room temperature and the species being studied. Trials should therefore be carried out to determine the time required to obtain good results (chap. VIII.C.2.6).

For morphological study, the whole CS is observed and cleaning alone is required before the next preparatory steps for SEM.

Samples should be dried at 30°C for 6-8 hours and kept free of dust. They are then attached to a SEM stub with carborundum paint, thermoplastic glue or double-sided tape. A thin line of colloidal silver should join the stub to the surface of the preparation in order to prevent charges from building up during observation. The section is then sputtered with gold (100 ångströms for topographic examination) or carbon (10-50 ångströms for microchemistry) before SEM observation.

3. Microradiography (F.J. Meunier)

In some cases the best technique for visualising growth marks on bones is microradiography of ground sections (fig. VIII.D.2) (Caillet *et al.*, 1983; Casselman, 1983; Gruber & Stout, 1983; Yudin & Caillet, 1990; Boujard & Meunier, 1991; Francis & Mulligan, 1998). The principle of the technique is the same as that of medical radiography, but using an apparatus adapted to thin calcified objects such as ground sections (Boivin & Baud, 1984, and others). Sections (chap. VIII.C.2.3.1) are carefully laid on high-resolution film (Kodak SO643) (fig. VIII.D.2a) and then exposed in an X-ray apparatus which delivers X-rays of 10-30 kV at 10-15 Marintek, depending on the parameters of the apparatus. The films are developed in HRP developer, washed and fixed. Then they are observed with a binocular microscope after mounting between glass slides (fig. VIII.D.2b).

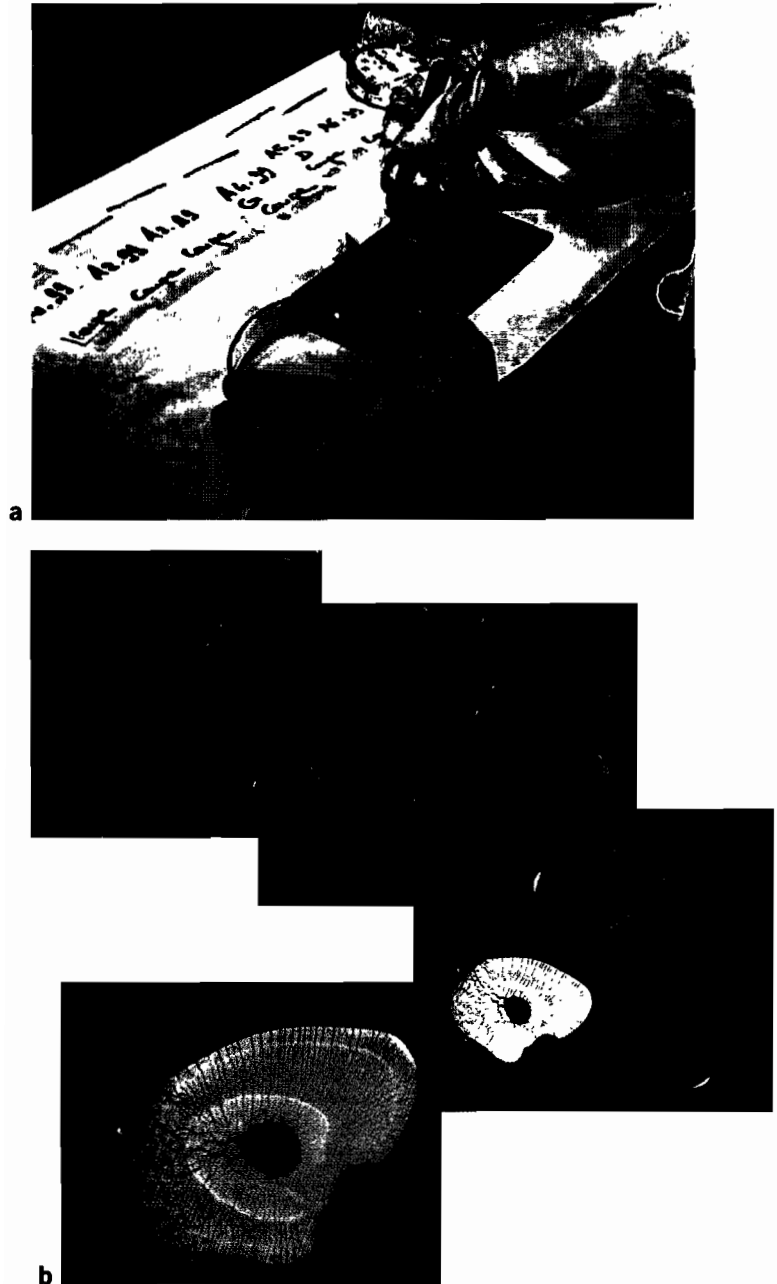


Figure VIII.D.2 - Microradiograph of fin ray slices (*Hoplosternum littorale*). a) In a dark room, the fin ray slices (red arrow) are deposited on a photographic film (yellow arrow) before being subjected to X-rays. b) Result of microradiography under different magnifications (photos© Ifremer O. Dugornay).

E. Conservation of preparations

W.J. McCurdy

1. Conservation of calcified structure preparations

Cool dark storage is the best conservation environment for many CS preparations. The chosen method of conservation should protect the CS from deterioration and also from loss or mechanical damage. All conserved CS should be properly referenced and a copy of the reference data should be kept in a secure place. Table VIII.E.1 lists the principal conservation methods for calcified structure preparations.

Table. VIII.E.1. - Conservation methods for calcified structure preparations.

Calcified structure preparation	Conservation	Storage containers
Unmounted scales	Conserve dry	Microtube vials, paper envelopes or plastic scale packets
Scales mounted on microscope slides	Conserve dry	Slide storage trays and boxes
Cellulose acetate scale impressions	Conserve dry	Storage boxes
Unmounted small otoliths	1. Conserve dry 2. $\geq 95\%$ alcohol	1. Microtube vials or plastic slides 2. Microtube vials
Unmounted large otoliths (whole and broken)	Conserve dry	Subdivided containers or paper envelopes
Directly embedded larval otoliths	Mounting media	Microscope slide trays and storage boxes
Plastic slides with many otoliths	Mounting media	Custom slide trays and storage boxes
Mounted otolith thin sections	Mounting media	Microscope slide trays and storage boxes
Many otolith thin sections mounted on custom microscope slides	Mounting media	Custom slide trays and storage boxes
Unmounted bones and other skeletal parts	Conserve dry protected from insects and fungi	Paper envelopes, individual containers or subdivided containers
Mounted thin sections of bones and other skeletal parts	Mounting media	Microscope slide trays and storage boxes
Bones and other skeletal parts prepared for sectioning	Embedding media	Individual containers or subdivided containers

2. Scales

2.1. Unmounted scales

Scales are usually conserved dry (chap. VIII.B.2). Preprinted paper envelopes and plastic scale packets are often used where large numbers of scale samples are processed, e.g. for North Atlantic Salmon, *Salmo salar* and other Salmonid species. Envelopes and packets containing

scales should be stored in labelled boxes to reduce the risk of loss or damage. Referenced microtube vials can be used to store both dry scales and otoliths. The vials should be stored in special support racks (chap. VIII.B.3.3.2).

2.2. Mounted scales and scale impressions

Preparation methods for scales mounted on microscope slides and cellulose acetate impressions are described in (chap. VIII.C.1.2) and (chap. VIII.C.1.3). Prepared microscope slides should be conserved by storage on slide trays and cellulose acetate impressions should be stored in suitable boxes in a cool place.

3. Otoliths

3.1. Otolith collections

Sometimes pairs of otoliths are collected, even when only one otolith is needed for the analysis. The reasons for doing so vary, but it is almost routine practice when sampling *sagittae* in adult fishes. Sometimes one particular otolith is preferred for analysis e.g. Plaice, *Pleuronectes platessa*, but often the otolith to be read is selected at random. In such cases consideration should be given to conserving the unused otoliths, as these are a valuable repository of sclerochronological information. The combination of a shortage of otoliths from older specimens in some habitats and advances in otolith microchemistry has highlighted the value of these otoliths. Existing collections of otoliths need to be evaluated, catalogued and conserved, in a way that will make both the otoliths and the corresponding fish life history information, more accessible to other researchers.

3.2. Whole and broken otoliths

Many otoliths are concavo-convex in shape and some smaller otoliths are thin and fragile. If they are stored in paper envelopes as previously recommended (Williams & Bedford, 1974), there is a significant risk of damage every time the collection is handled. Unmounted otoliths can be stored in labelled vials (chap. VIII.B.3.3.2) or in custom plastic slides and trays with covers. Some authors recommend conservation in alcohol >95%, which can be useful for fragile and small otoliths (chap. VIII.B.3.3.2). Large Gadoid otoliths are frequently stored in Repli dishes®, clear plastic trays with twenty-five 2 cm compartments and a lid that can be taped to the base.

When alcohol or water have been used as observation media, both whole and broken otoliths are best wiped dry with soft tissue paper and conserved in their original storage vials (chap. VIII.B.3.3.2). If clearing oils (chap. VIII.D.1.1.2) have been used to improve the

quality of the observation, the oil may be gently wiped off before returning the otoliths to their original storage containers. Small pelagic otoliths e.g. Sprat, *Sprattus sprattus* are often stored and observed on custom moulded plastic slides. Any water or alcohol used to observe these otoliths should be allowed to evaporate and the otoliths allowed to dry out thoroughly, before the glass or plastic protective cover is secured to the slide. The slides may then be stored in a suitable tray or container.

3.3. Embedded otoliths and otolith thin sections

Individual otoliths, e.g. from larvae, can also be conserved by direct mounting in media (chap. VIII.C.2.4.2). Otoliths or otolith thin sections mounted on glass microscope slides and plastic slides containing many pairs of embedded whole otoliths (chap. VIII.C.2.2.3), may be stored on plastic or metal trays. The trays containing these slides should be stored in suitable containers with lids to protect their contents. It is very important to maintain the highest possible standards of slide preparation, as the cover slips will break easily if they are not completely supported by the embedding medium. Excess embedding media may also cause slides to stick together during storage. We recommend the use of custom cover slips with a thickness of 0.5 mm, when using polyester resins to prepare plastic slides with many embedded whole otoliths and custom microscope slides with many otolith thin sections (chap. VIII.C.2.3.2 and chap. VIII.C.2.4.3). Thinner cover slips may crack or shatter during handling and observation.

4. Other skeletal parts

Conservation is very simple and is usually done dry. Generally speaking, bones are best conserved by returning them to their original paper storage envelopes. Precautions must be taken to prevent the growth of fungi and attack by specialised insects (chap. VIII.B.4.2). For very fragile samples, ethanol between 70% and 90% can be used as a fixative (Meunier, 1988), (chap. VIII.B.4.2). If alcohol is used the calcified materials should be stored in suitable referenced vials. Tissue sections mounted on microscope slides may be stored in standard slide storage racks or boxes. Larger samples and embedded material prepared for sectioning should be stored in suitable referenced individual or sub-divided containers.

Glossary

J. Panfili, F.J. Meunier, H. Mosegaard, H. Troadec,
P.J. Wright, A.J. Geffen

From Francillon-Vieillot *et al.*, 1990; Kalish *et al.*, 1995.

Legend: (O), terminology reserved for otoliths; (S), terminology reserved for scales; (Sk), terminology reserved for skeleton.

Accessory growth centre (O): A growth centre formed beyond the otolith core that leads to a new plane of growth and from which a new series of growth increments appears to emanate. Formation of these structures is often associated with life history transitions such as metamorphosis. Accessory growth centres are often referred to as accessory *primordia*; however, the term accessory growth centre is preferred because these features are different structurally from *primordia* (e.g., they do not contain primordial granules). The term “secondary growth centre” has also been used. See figure II.A.8.

Accuracy: The closeness of a quantity estimation (measured or computed value) to its true value.

Age estimation: This term is preferred when discussing the process of assigning ages to fish. The term aging (ageing) should not be used as it refers to time-related processes and the alteration of the composition, structure, and function of an organism over time. The term “age estimation” is preferable to “age determination” due to the uncertainty in assigning ages.

Age-group: The cohort of fish of a given age (e.g., the 5-year-old age-group). The term is not synonymous with year-class or day-class.

Annulus (pl. annuli): One of a series of concentric zones on a structure that may be interpreted in terms of age. In some cases, an *annulus* may not be continuous or obviously concentric. The optical appearance of these marks depends on the calcified structure and the species and should be defined in terms of specific characteristics of the structure. This term has traditionally been used to designate year marks even though the term is derived from the Latin “*anus*”, meaning ring, not from “*annus*”, which means year. For otoliths, the variations in microstructure that make an *annulus* a distinctive region of an otolith are not well understood. See figures II.A.9, II.A.10 and II.C.9.

Anterostrum (O): see *Antirostrum*.

Antirostrum (O): Anterior and dorsal projection of the *sagitta*. Generally shorter than the *rostrum*. See figure II.A.4.

Asteriscus (*pl. asterisci*) (*O*): One of the three otolith pairs found in the membranous labyrinth of Osteichthyan fishes. It lies within the *lagena* ("flask") of the *pars inferior*. In non-Ostariophysan fishes the *asteriscus* is small and shaped like a flattened hemisphere or quarter moon. In the Ostariophysi the *asteriscus* is roughly circular and laterally compressed and is considerably larger than the *sagitta*. See figure II.A.1.

Axis of measurement: A line along which growth increments are numbered and measured.

Band: See zone.

Bone remodelling (*Sk*): The process of reshaping of the bone tissue which occurs internally or at the edge of the bone. It can affect primary or secondary bone and every type of tissue. It is due to morphogenesis during the early life, or to physiological demand or mechanical constraints.

Bone resorption (*Sk*): The action of the erosion of the bone surface by osteoclasts or osteocytes at their own periphery.

Calcification: The process of deposition of calcium carbonate crystals in otoliths and calcium phosphate crystals in bones and scales.

Canaliculus (*pl. canaliculi*) (*Sk*): The thin space of the bone tissue including the cytoplasmic extension of the osteocysts and osteoblasts. See figure II.C.2.

Cementing line of resorption (*Sk*): There are two kinds of cementing lines: resorption lines (reversal lines) that occur on irregular resorbed surfaces and resting lines (rest line) that occur on unresorbed surfaces. Both are thin chromophilic lines that show a greater degree of mineralisation than the surrounding bone tissue. The resorption line separates the secondary bone from the primary bone. The resting line is a line of discontinuity within the bone tissue which corresponds to temporary but complete cessation of growth. See figure II.C.8.

Check (*O*): A discontinuity (e.g., a stress-induced mark) in an otolith zone, a pattern of opaque and translucent zones, or microincrements. Microstructural checks (e.g. hatching checks) often appear as high-contrast microincrements with a deeply etched D-zone or an abrupt change in the microstructural growth pattern. If the term is used, it requires precise definition. See figure II.A.11.

Circulus (*pl. circuli*) (*S*): A concentric crest on the external face of elasmoid scales caused by tissue elevation of the superficial layer of the scale.

Cohort: A group of fish of a similar age that were spawned during the same time interval. Used with both age-group, year-class, and day-class.

Compact bone (Sk): A kind of bone architecture in which the tissue volume is greater than that of the vascular cavities. Compact bone can be avascular.

Core (O): The area or areas surrounding one or more *primordia* and bounded by the first prominent D-zone. See figure II.A.5. Some fish (e.g., Salmonids) possess multiple *primordia* and multiple cores.

Corroboration: A measure of the consistency or repeatability of an age estimation method. For example, if two different readers agree on the number of zones present in a hard part, or if two different age estimation structures are interpreted as having the same number of zones, corroboration (but not validation) has been accomplished. The term "verification" has been used in a similar sense; however, the term "corroboration" is preferred as verification implies that the age estimates were confirmed as true.

Ctenius (pl. ctenii) (S): The outgrowth (spine) on the external face of the ctenoid scales, mainly deposited in the posterior field, particularly on the posterior edge. See figure II.B.2.

D-zone (O): That portion of an otolith microincrement that appears dark when viewed under transmitted light, and as a depressed region when acid-etched and viewed with a scanning electron microscope. This component of a microincrement contains a greater amount of organic matrix and a lesser amount of calcium carbonate than the L-zone. Referred to as the discontinuous or matrix-rich zone in earlier works on daily increments; "D-zone" is the preferred term. See L-zone and figure II.A.5.

Daily increment (O): An increment formed over a 24-hour period. In its general form, a daily increment consists of a D-zone and a L-zone. The term is synonymous with "daily growth increment" and "daily ring". The term "daily ring" is misleading and inaccurate and should not be used. The term "daily increment" is preferred. See increment and figure II.A.5.

Day-class: The cohort of fish spawned or hatched on a given date (e.g., the 22 September 1990 day-class). Whether this refers to the date of spawning or hatching must be specified.

Discontinuity (O): See check.

Double zone (or ring or mark): Two zones/rings that are close together relative to the size of the calcified structure and the distance between two *annuli*, which are considered as one *annulus*. As such a double zone includes both a secondary zone and an *annulus*. This structure has also been termed a "split ring".

Excisura major (O): The cleft separating *rostrum* and *antirostrum*.

Excisura minor (O): The cleft separating *postrostrum* and *pararostrum*.

Field: An area of a calcified structure, defined on a side or a section, and delimited at least by the centre and the edges (e.g., anterior, posterior, dorsal, ventral fields). It has also been termed as "region".

Focus (*pl. foci*) (S): The central part and the centre of origin of the scale.

Growth mark (*or ring or zone*): See mark or zone.

Growth pattern: The notion of the relative growth of increments during a period of the life of the calcified structure (e.g. *annuli* or daily increments).

Hatch date (O): The date on which a fish has hatched, typically ascertained by counting daily increments from a presumed hatching check (see check) to the otolith edge.

Haversian system (Sk): See secondary osteon.

Hyaline zone: A zone that allows the passage of greater quantities of light than an opaque zone. The term should be avoided; the preferred term is "translucent zone". See translucent zone.

Increment: A reference to the region between similar zones on a structure used for age estimation. The term refers to a structure, but it may be qualified to refer to portions of the hard part formed over a specified time interval (e.g. sub-daily, daily, annual). Depending on the portion of the hard part being considered, the dimensions, chemistry, and period of formation may vary widely. A primary increment consists of a D-zone and an L-zone, whereas an annual increment comprises an opaque zone and a translucent zone. There may also be secondary structures such as sub-daily increments and false and double zones within annual increments.

Initium (Sk): The centre of origin of growth of the fin ray.

L-zone (O): That portion of a microincrement that appears light when viewed under transmitted light, and as an elevated region when acid-etched and viewed with a scanning electron microscope. The component of a microincrement that contains a lesser amount of organic matrix and a greater amount of calcium carbonate than the D-zone. Referred to as "incremental zone" in earlier works on daily increments: "L-zone" is the preferred term. See D-zone and figure II.A.5.

Lapillus (*pl. lapilli*) (*O*): One of the three otolith pairs found in the membranous labyrinth of Osteichthyan fishes. The most dorsal of the otoliths, it lies within the *utricle* ("little pouch") of the *pars superior*. In most fishes, this otolith is shaped like an oblate sphere and it is smaller than the *sagitta*. See figure II.A.1.

Lobes (*O*): The minor rounded protrusions of the *sagitta* along the dorsal and the ventral edge.

Macrocentric scale (*or regenerated scale*) (*S*): See regenerated scale.

Macroincrement: Increments that are typically more than 50 μm in width; the prefix "macro" serves to indicate that the object denoted is of relatively large size and that it can be seen with a binocular microscope. Often used to describe seasonal increments. See increment.

Marginal increment: The region beyond the last identifiable mark at the margin of a structure used for age estimation. Quantitatively, this increment is usually expressed in relative terms; that is, as a fraction or proportion of the last complete annual or daily increment.

Mark: A general expression describing an histomorphological mark of similar structure or optical density laid down during the growth of hard parts. See zone.

Medular cavity (*Sk*): The first vascular cavity in the median position in the long bones, for example in spiny rays of catfishes. See figure II.C.6.

Microcentric scale (*S*): A scale present since the early life of the individual, which has not been resorbed.

Microincrement (*O*): An increments that is less than 50 μm in width, typically from one to 20 μm ; the prefix "micro" serves to indicate that the object denoted is of relatively small size and that it can only be observed with a compound or electron microscope. Often used to describe daily and sub-daily otolith increments. See increment.

Microstructural growth interruption (*O*): A discontinuity in crystallite growth marked by the deposition of an organic zone. It may be localized or a complete concentric feature. See check.

Mineralisation: The biological process of the deposition of crystalline or amorphous mineral material in or on an organic matrix.

Multiple zone (*or ring or mark*): A number of closed zones, compared to the size of the calcified structure and the distance of the *annuli*, which are regarded as one *annulus*. See also false zone.

Nucleus, Kernel (O): Collective terms originally used to indicate the *primordia* and core of the otolith. These collective terms are ambiguous and should not be used in descriptions of microstructure. The preferred terms are “*primordium*” and “core” (see definitions). When viewed macroscopically, the term “*nucleus*” has been used to refer to the region around the core, although the precise extent of this area is generally ill defined. If the term is used in describing macrostructure, it requires precise definition.

Opaque zone: A zone that restricts the passage of light in comparison with a translucent zone. The term is a relative one because a zone is determined to be opaque on the basis of the appearance of adjacent zones in the otolith (see translucent zone). In untreated otoliths under transmitted light, the opaque zone appears dark and the translucent zone appears bright. Under reflected light the opaque zone appears bright and the translucent zone appears dark. An absolute value for the optical density of such a zone is not implied. See translucent zone.

Optical focus plane (O): A plane at a certain depth in the 3D otolith structure where microincrements can be distinguished when viewed under a light microscope. The orientations of the radial growth directions at an optical focus plane are perpendicular to the direction of observation.

Ossification (Sk): All the processes involved in bone formation.

Osteoblast (Sk): A specific bone cell which synthesizes the bone matrix and is located on the inner (endost) or on the outer (periost) surfaces of the bone tissue.

Osteoclast (Sk): A specific bone cell which is involved in bone resorption, generally multinucleated in higher Vertebrates; they may also be mononucleated in fish.

Osteocyte (Sk): A specific type of bone cell embedded in the bone tissue and carrying out the trophic needs of the bone. It is an osteoblast incorporated in tissue.

Osteogenesis (Sk): The process of bone tissue formation by the specialized cells (osteoblasts).

Otolithometry (O): Age estimation from marks recorded in the otoliths of Teleost fish.

Pararostrum (O): The posterior and dorsal projection of the *sagitta*. Generally shorter than the *postrostrum* (used in connection with Clupeid otolith morphology).

Postrostrum (O): The posterior and ventral projection of the *sagitta*. Generally longer than the *parastrostrum* (used in connection with Clupeid otolith morphology).

Precision: The closeness of repeated measurements of the same quantity. For a measurement technique that is free of bias, precision implies accuracy but the two terms are not equivalent.

Primary bone (Sk): The bony tissue which is deposited where antecedent bone does not exist.

Primary osteon (Sk): A vascular canal surrounded by concentric bone *lamellae* which are deposited centripetally and which does not depend on previous resorption.

Primordial granule (O): The primary or initial components of the *primordium*. There may be one or more primordial granules in each *primordium*. In *sagittae* the granules may be composed of vaterite, whereas the rest of the *primordium* is typically aragonite.

Primordium (pl. *primordia*) (O): The initial complex structure of an otolith; it consists of granular or fibrillar material surrounding one or more optically dense *nuclei* from 0.5 μm to 1.0 μm in diameter. In the early stages of otolith growth, if several *primordia* are present, they generally fuse to form the otolith core.

Pseudo-lamellar bone tissue (or *parallel-fibred bone tissue*) (Sk): A bone tissue which is composed of a matrix with parallel collagenous fibres from one to another bone layer.

Radius (pl. *radii*) (S): A radially oriented groove, generally starting from the scale focus, and corresponding to the absence of an superficial (external) layer of the scale. See figure II.B.1.

Read, Reader, Reading: Special terms used in the jargon of sclerochronologists. The reading of a calcified structure consists of interpreting its growth patterns. A reader is a person who tries to interpret the marks recorded in a given calcified structure.

Regenerated scale (or *macrocentric scale*) (S): A scale which has been rapidly regenerated after the removal of the original (microcentric) scale.

Resorption: The loss of the original material of a calcified structure through a physiological process (e.g. removed from its original place).

Rest line (or *line of growth stop*) (Sk): See cementing line.

Ring: See increment and zone.

Rostrum (O): Anterior and ventral projection of the *sagitta*. Generally longer than the *antirostrum*.

Sagitta (pl. sagittae) (O): One of the three otolith pairs found in the membranous labyrinth of Osteichthyan fishes. It lies within the *sacculus* ("little sack") of the *pars inferior*. It is usually compressed laterally and is elliptical in shape; however, the shape of the *sagitta* varies considerably among species. In non-Ostariophysan fishes, the *sagitta* is much larger than the *asteriscus* and *lapillus*. The *sagitta* is the otolith used most frequently in otolith studies. See figure II.A.1.

Scalimetry (S): Age estimation using marks recorded in the scales of Teleost fish.

Sclerochronology: The method of estimating age and the duration of life history events (or temporally-based events), from marks recorded and conserved in calcified structures.

Secondary bone (Sk): Bony tissue which is deposited in an area where the primary bone has been resorbed (bone of substitution).

Secondary osteon (or Haversian system) (Sk): An erosional cavity initiated from a vascular canal and secondarily filled with concentric bone *lamellae* deposited centripetally.

Secondary structure: A term used for all macroscopic zonations that do not appear to conform to the opaque and translucent zones of an *annulus*. The main examples are false and split or double rings/zones.

Skeletochronology (Sk): Age estimation using marks recorded in the skeletal structures of Teleost fish.

Spongy bone (Sk): A type of bone architecture in which the tissue volume is very vascular. The volume of the vascular cavities is much larger than that of the tissue.

Stained ring (or line): A chromophilic/stainable ring or zone with a variable intensity.

Sub-daily increments (O): An increment formed over a period of less than 24 hours. See increment.

Sulcus acusticus (usually shortened to *sulcus*) (*O*): A groove along the medial surface of the *sagitta*. A thickened portion of the otolithic membrane lies within the *sulcus acusticus*. The *sulcus acusticus* is often referred to in otolith studies because of the clarity of the increments near the *sulcus* in transverse sections of *sagittae*. See figure II.A.4.

Supernumerary mark (or ring or zone): A mark which is not accepted for age estimation or retained as an *annulus*. This mark is generally aperiodic.

Transition zone (O): A region of change in otolith structure between two similar or dissimilar regions. In some cases, a transition zone is recognized due to its lack of structure or increments, or it may be recognized as a region of abrupt change in the form (e.g. width or contrast) of the increments. Transition zones are often formed in otoliths during metamorphosis from larval to juvenile stages or during significant habitat changes such as the movement from a pelagic to a demersal habitat or a marine to freshwater habitat. If the term is used, it requires precise definition.

Translucent zone: A zone that allows the passage of greater quantities of light than an opaque zone. The term is a relative one because a zone is determined to be translucent on the basis of the appearance of adjacent zones in the structure (see opaque zone). An absolute value for the optical density of such a zone is not implied. In untreated calcified structures under transmitted light, the translucent zone appears bright and the opaque zone appears dark. Under reflected light the translucent zone appears dark and the opaque zone appears bright. The term “hyaline” has been used, but “translucent” is preferable.

Ultrastructure: The structure of a tissue observed at high levels of magnification (particularly with electron microscopy).

Validation: The process of estimating the accuracy of an age estimation method. The concept of validation is one of degree and should not be considered in absolute terms. If the method involves counting zones, then part of the validation process involves confirming the temporal significance of the zones being counted. Validation of an age estimation procedure indicates that the method is sound and based on fact.

Verification: The process of establishing that something is true. Individual age estimates can be verified if a validated age estimation method has been employed. Verification implies the testing of something, such as a hypothesis, that can be determined in absolute terms to be either true or false. See corroboration.

Vertebral body (Sk): The circular and central part of a vertebra.

Year-class: The cohort of fish that were spawned or hatched in a given year (e.g., the 1990 year-class). Whether this term is used to refer to the date of spawning or hatching must be specified, as some high-latitude fish species undergo a long period of development before hatching.

Zone: A region of similar structure or optical density. Synonymous with “ring”, “band” and “mark”. Where possible the use of this term should be illustrated.

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Acronyms and chemical symbols

2D	2-dimensional view
3D	3-dimensional view
A	age
AAS	atomic absorption spectrometry
ADC	analog-to-digital converter
AES	atomic emission spectrometry
AGL	arrested growth line
AGR	absolute growth rate
Al	aluminium
ALK	age-length key
AMD	absolute marginal distance
AMS	accelerator mass spectrometry
ANOVA	analysis of variance
APE	average percent error
ASA	age-structured stock analysis (+ see SPA, VPA)
Ba	barium
BCF	back calculation formula
BPH	body proportional hypothesis (in back-calculation)
C	carbon
Ca	calcium
CAAGE	computer-assisted age and growth estimation
CaCO ₃	calcium carbonate
CCD	charge-coupled device
CCIR	Comité Consultatif International des Radio-communications
Cd	cadmium
Ce	cerium
Cl	chlorine
CO ₂	carbon dioxide
CRM	certified reference materials
CS	calcified structure
Cu	copper
CV	coefficient of variation
DAC	digital-to-analog converter
DCT	discrete cosine transform
DGR	daily growth rate
EDS	energy dispersive spectrometry
EFAN	European Fish Ageing Network
Fe	iron
FFT	fast Fourier transform
g	instantaneous growth rate
GAG	glycosaminoglycans

GRE	growth rate effect
H	hydrogen
HR-ICP-MS	high resolution-inductively coupled plasma-mass spectrometry
ICES	International Council for the Exploration of the Sea
ICP-AES	inductively coupled plasma-atomic emission spectrometry
ICPMS	inductively coupled plasma-mass spectrometry
ID-ICP-MS	isotope dilution-inductively coupled plasma-mass spectrometry
IRMS	isotope ratio mass spectrometry
JPEG	joint photographic experts group
K	potassium
LAC	“Ligne d’arrêt de croissance” (French)
LA-ICP-MS	Laser ablation-inductively coupled plasma-mass spectrometry
Li	lithium
LOD	limit of detection
MANOVA	multivariate analysis of variance
Mg	magnesium
Mn	manganese
MSVPA	multispecies virtual population analysis
N	nitrogen
Na	sodium
Ni	nickel
O	oxygen
OES	optical emission spectrometry
OMC	otolith microchemistry
P	phosphorus
PA	percentage agreement
PAS	periodic acid Schiff
Pb	lead
PCA	principle component analysis
PDB	Peedee Belemnite
PIXE	particle-induced X-ray emission
Ra	radium
RAM	random access memory
Rb	rubidium
RGR	relative growth rate
RMD	relative marginal distance
Rn	radon
S	sulphur
SEM	scanning electron microscope
SGR	specific growth rate
Si	silicon
SIMS	secondary ion mass spectrometry
SL	standard length
SLAP	standard light Antarctic precipitation

Sm	samarium
SMOW	standard mean ocean water
SPA	sequential population analysis
SPH	scale proportional hypothesis (in back-calculation)
Sr	strontium
Sr/Ca	strontium/calcium
SRM	standard reference materials
TAC	total allowable catch
TC	thin collagenous (fibres)
TEM	transmitted electron microscope
Th	thorium
TIFF	tagged image file format
TIMS	thermal ionisation mass spectrometry
Tl	thallium
U	uranium
VGBF	von Bertalanffy growth function
VPA	virtual population analysis
VPDB	vienna-Peedee belemnite
VSMOW	vienna standard mean ocean water
WDS	wavelength dispersive spectrometry
WIP	water-insoluble protein
WSP	water-soluble protein
Zn	zinc

Author addresses

Abdesslam Benzinou

ENIB, laboratoire RESO, Technopole Brest-Iroise, Site de la Pointe du Diable, BP 30815, 29608 Brest Cedex, France
Tel. : +33 2 98 05 66 92 - Fax : +33 2 98 05 66 89
E-mail : benzinou@enib.fr

Arild Folkvord

University of Bergen, Department of Fisheries and Marine Biology
Bergen High Technology Center, 5020 Bergen, Norway
Tel. : +47 55584456 - Fax : +47 55584450
E-mail : arild.folkvord@ifm.uib.no

Audrey J. Geffen

School of Biological Sciences, University of Liverpool, Port Erin
Marine Laboratory, Port Erin, Isle of Man IM9 6JA GB, UK
Tel. : +44 1624 83 10 16 - Fax : +44 1624 83 10 01
E-mail : geffen@liv.ac.uk

Willie McCurdy

Department of Agriculture and Rural Development for Northern
Ireland, Aquatic Systems Group,
18a Newforge Lane, Belfast BT9 5PX, Northern Ireland, UK
Tel. : +44 2890255513 - Fax : +44 2890382244
E-mail : willie.mccurdy@dardni.gov.uk

Benoît Mesnil

Ifremer, centre de Nantes, laboratoire MAERHA
BP 21105, 44311 Nantes Cedex 3, France
Tel. : +33 2 40 37 40 09 - Fax : +33 2 40 37 40 75
E-mail : Benoit.Mesnil@ifremer.fr

François J. Meunier

MNHN, FR CNRS 1451, laboratoire d'Ichtyologie générale
et appliquée, 43 rue Cuvier, 75231 Paris Cedex 05, France
Tel. : +33 1 40 79 37 61 - Fax : +33 1 40 79 37 71
or,
Université Denis Diderot-Paris 7, UMR CNRS 8570, 2 place Jussieu,
75251 Paris Cedex 05, France
E-mail : meunier@mnhn.fr

Beatriz Morales-Nin

GOI-IMEDEA (UIB/CSIC)

Miguel Marqués 21, 07190 Esporles, Spain

Tel : +34 971611721 - Fax : +34 971611761

E-mail : ieabmn@clust.uib.es

Henrik MosegaardDanish Institute for Fisheries Research, Charlottenlund Castle,
2920 Charlottenlund, Denmark

Tel : +45 33 96 34 61 - Fax : +45 33 96 33 33

E-mail : hm@dfu.min.dk

Jacques PanfiliIRD, centre de recherche halieutique méditerranéenne et tropicale,
BP 171, 34203 Sète Cedex, France

E-mail : panfili@ird.fr

Hélène de Pontual

Ifremer, centre de Brest, Drv/rh/LASAA,

BP 70, 29280 Plouzané, France

Tel : +33 2 98 22 46 92 - Fax : +33 2 98 22 46 53

E-mail : pontual@ifremer.fr

Peter J. Wright

Fisheries Research Services Marine Laboratory,

PO Box 101, Victoria Road, Aberdeen, AB11 9DB, Scotland, UK

Tel. : +44 1224 295436 - Fax. : +44 1224 295511

E-mail : wrightp@marlab.ac.uk

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Manual of Fish Sclerochronology

Sclerochronology, the study of calcified structures to reconstruct the past history of living organisms, is central to fish biology and fisheries management. This manual aims to provide an overview of the current theoretical and practical aspects of sclerochronological studies. By providing information on the nature of calcified structures (otoliths, scales, skeleton), their uses in fish research and methods for preparation and examination, the manual constitutes a comprehensive guide for researchers, technicians and students either new to the field or interested in expanding their range of expertise. The enclosed multimedia version (DVD) is supplemented by videos illustrating the main technical procedures with an alternative navigation mode based on decision trees.

Keywords : otolith, scale, skeleton, age, growth, image analysis, microchemistry.

Manuel de sclérochronologie des poissons

La sclérochronologie, discipline qui étudie les pièces calcifiées pour reconstruire l'histoire individuelle des organismes vivants, est essentielle pour la connaissance de la biologie des poissons et la gestion des pêches. Ce manuel présente une synthèse actualisée sur les aspects théoriques et pratiques des études de sclérochronologie. En donnant des informations sur la nature des pièces calcifiées (otolithe, écaille, squelette), leur utilisation dans les recherches en ichthyologie et les méthodes de préparation et d'observation, ce manuel constitue un guide détaillé pour les chercheurs, techniciens et étudiants novices ou désirant étendre leurs domaines d'expertise. La version multimédia (DVD) incluse est enrichie de séquences vidéos et bénéficie d'un mode de navigation alternatif basé sur des arbres d'aide à la décision.

Mots-clés : otolithe, écaille, squelette, âge, croissance, analyse d'image, microchimie.

IRD Éditions

213, rue La Fayette
75480 Paris Cedex 10, France
Tel. +33 (0)1 48 03 76 06
Fax +33 (0)1 48 03 76 12
editions@paris.ird.fr

Diffusion

IRD
32, avenue Henri Varagnat
93143 Bondy Cedex, France
diffusion@bondy.ird.fr
Tel. +33 (0)1 48 02 56 49
Fax +33 (0)1 48 02 79 09

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Éditions Ifremer

BP 70, 29280 Plouzané, France
Tel. +33 (0)2 98 22 40 13
Fax +33 (0)2 98 22 45 86
editions@ifremer.fr

Diffusion

ALT Brest
Service Logistique
3, rue Edouard Belin - BP 23
29801 Brest Cedex 9, France
Tel. +33 (0)2 98 02 42 34
Fax +33 (0)2 98 02 05 84
alt.belin@wanadoo.fr

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