

The tetrazolium reduction method for assessing the viability of individual bacterial cells in aquatic environments: improvements, performance and applications

Philippe Dufour & Michel Colon

Antenne ORSTOM – Station d'Hydrobiologie lacustre de l'INRA, 74203 Thonon les bains, France

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Abstract

The electron transport system of respiring organisms reduces 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to INT-formazan. Active bacterial cells may be recognized under the microscope by epifluorescence and by the simultaneous presence, seen under bright light field of optically dense intracellular deposits of INT-formazan. An improved procedure that leads to a sharp definition of cells and formazan deposits is presented here. Cells are concentrated on cellulose membrane filters of 0.1 μm porosity which are rendered further transparent prior to immersion of the cells in a layer of 4', 6-diaminidino-2-phenylindole (DAPI) s' fluorochrome. This process leads to two significant improvements: (1) the fluorochrome is not trapped inside the membrane, which decreases the background fluorescence and leads to a better detection of the small cells; (2) the cells are immersed in an aqueous solution, which prevents rapid dissolution of the formazan crystals which would be expected if they were in contact with oily clearing agents. Tests on formazan labelling and on storage of INT-processed samples suggest other precautions for reliable use. Improved in this way, the method is simple, rapid and has numerous applications in environmental studies, ecophysiology and ecotoxicology. Some examples are given, with 2 to 98% of INT reducing cells observed, depending on different environmental conditions.

Introduction

It is now well known that only a fraction of the bacterial community present in a natural aquatic environment is metabolically active at any given moment (Novitsky & Morita, 1978; Stevenson, 1978). It is therefore pertinent to ask what changes in a bacterium's environment are responsible for the activity, dormancy or death of the cells. This requires a method of distinguishing between active

and inactive cells. The widely used epifluorescence microscopy of fluorochrome-stained cells fails to make such a distinction (Jones, 1974). A method, first proposed by Von Bielig *et al.* (1949) allows the identification of active bacteria by the presence of active electron transport systems (ETS). This system reduces a water-soluble, membrane-permeable and colourless tetrazolium salt to an optically dense intracellular deposit of formazan which can be detected under bright field

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microscope. Zimmermann *et al.* (1978) improved the method by combining the identification of individual cells showing formazan deposits with direct counts made by fluorescence microscopy. In spite of the significant improvements proposed by Tabor & Neihof (1982), Bitton *et al.* (1983), Newell (1984) and King & Parker (1988), the method has received little attention. In our opinion, this is due to 3 drawbacks:

- (1) the complexity of the procedure making the method long and thus inadequate to routine uses;
- (2) the lack of sensitivity preventing the detection of the small but active cells, numerous in natural ecosystems;
- (3) the misunderstanding of the INT reduction kinetics and of the optimal storage conditions of the samples making the method non reliable.

We propose here a new protocol that leads to minimize or to suppress these imperfections. Moreover, the improved method is tested for its applicability in different aquatic environments.

Materials and methods

Sampling and preliminary processing

Tests were conducted on two bacterial populations derived from Lake Geneva: (i) small cells (mean vol. of $0.1 \mu\text{m}^3$) from natural assemblages and (ii) large cells (mean vol. of $0.5 \mu\text{m}^3$) resulting from *in situ* samples amended with yeast extract (0.005% wt/vol. final concentration) and allowed to grow for one to three days in the dark at 20 °C.

The procedure for INT respiring and total bacteria counts, as we have modified it, involves the five steps detailed below.

Addition of INT to samples

Filter-sterilized 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) is added to the freshly collected samples at a final concentration of 0.02% (wt/vol). Duplicates serving as controls are fixed with filter-sterilized formalde-

hyde to a final concentration of 2% before addition of INT.

Incubation with INT

Samples are incubated for periods between 10 min and 2 h (see discussion) in the dark, at *in situ* temperature before fixing with formaldehyde.

Storage

Processed samples are kept at 4 °C for at least 1 hour and no longer than 1 day before preparation for microscopy.

Microscopical preparations

Aliquots of the fixed samples are filtered through $0.1 \mu\text{m}$ pore size cellulose membranes (Millipore VC). Membranes are air dried for about half an hour in a desiccator with silica gel and then are cleared by floating, organisms-side up, on a drop of Cargille immersion oil (code 848, $n_D^{25} \text{ °C} = 1.506$) on a microscope slide. After a few seconds the filter becomes transparent and a small drop of filter-sterilized DAPI (Boehringer, 10^{-5} wt/vol) is added to stain the cells. The preparation is then covered with a coverslip.

Microscopic examination and counts

The mounted filter is immediately photographed or examined through a microscope. Fields are alternately illuminated with bright light transmitted through the transparent filter to detect formazan spots and UV light (350 nm) to observe DAPI fluorescence of the cells. Fluorescent cells with a formazan deposit are enumerated as INT respiring micro-organisms (INTRM). Usually, more than 400 cells on 20 fields are counted. Counts of formazan spots on controls are subtracted from counts of formazan spots on the corresponding samples.

Results and discussion

Addition of INT to samples

The method was first used by Von Bielig *et al.* (1949) with the reduction of TTC (triphenyl tetrazolium chloride). McDonald (1980) tried a

variety of other electron acceptors and concluded that INT was the best. Optimum staining was found to occur between 0.01 and 0.15% (wt/vol) of INT. We used a final concentration of 0.02% and noticed that increases over this concentration did not increase the size or the number of formazan deposits even in the most active and dense cell cultures examined. No differences in active cell counts were observed in samples incubated with INT of different origin (Koch Light, Sigma and Aldrich chemical suppliers).

Incubation with INT

Previous papers indicate that maximal numbers of cells with formazan spots were obtained after 2 min. (Zimmerman *et al.*, 1978), 10 min. (Harvey & Young, 1980; Maki & Remsen, 1981; Tabor & Neihof, 1982), 20 min. (Lovell & Konopka, 1985), 4 h or more (Mac Donald, 1980; Quinn, 1984). Consequently, a wide variety of incubation times, ranging from 10 min. (King & Parker, 1988) to 4 h (Quinn, 1984) and more (Mac Donald, 1980), have been used. The incubation time is a critical step in this procedure. Below a certain threshold, too little formazan will be deposited to be seen in the cells having low activity, especially in the smallest ones, while if the time of incubation is too long, there will be a possibility to activating dormant cells *in vitro*.

The time-course of INT labelling was noted in two 6 h experiments (Fig. 1). With large and active cultured cells, a maximal number of respiring bacteria was obtained after 2 min. With small and weakly active natural cells, as much as two hours was necessary to obtain the maximum number. These results explain the discrepancies in incubation times noticed above. From these observations, it would be unrealistic to advise an optimum incubation-time which has to be assessed rather from the results of preliminary time-course experiments of INT labelling for different conditions.

Storage of processed samples

Tabor & Neihof (1982) observed an increase in formazan deposits when samples were held for 12 hours at 4 °C before preparation for microscopy.

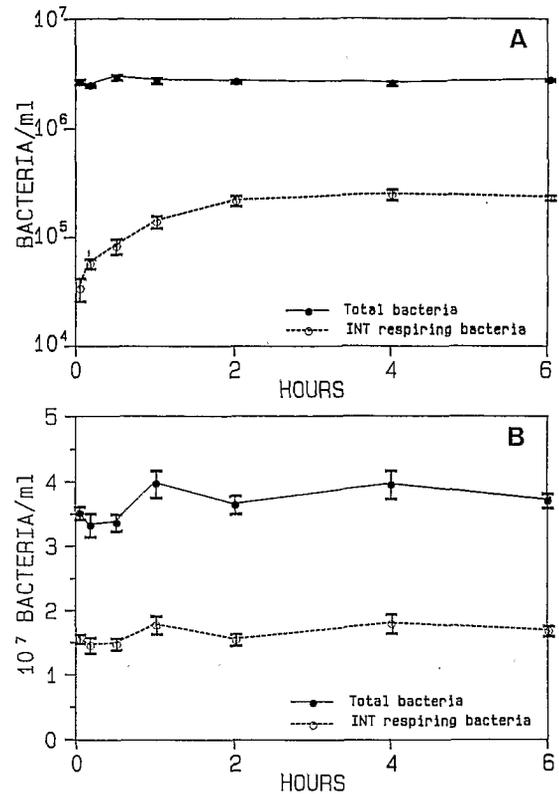


Fig. 1. Effect of prolonged exposure to INT on counts (+/- standard deviation) of both total and INT reducing bacteria. A = small ($0.1 \mu\text{m}^3$) cells of natural samples. B = large ($0.5 \mu\text{m}^3$) cultured cells.

This was attributed to a secondary reduction of INT or to a coalescence of formazan. In this study, the number of cells with formazan spots was maximal after one hour in the dark at 4 °C and then decreased constantly (Fig. 2). Such a decrease during storage of cells in water at 4 °C was observed for all the samples ($n = 7$) tested. These results contradict those of Zimmerman *et al.* (1978) who suggested that storage of processed water samples for up to one month did not cause loss of intracellular INT formazan. They conform with Ellar *et al.* (1978) who reported that during incubation and storage, some components of the ETS may be lost by diffusion. We attempted the storage of processed cells on dry filters at 4 or -20 °C in the dark without any success. Consequently, until a satisfactory process of storage is found, we recommend the counting (or micro-

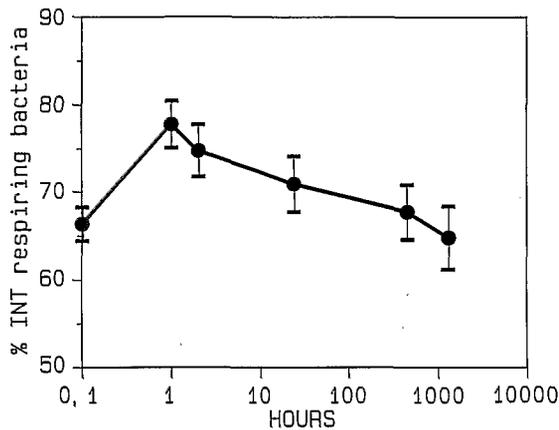


Fig. 2. Effect of prolonged storage of fixed water samples on counts (\pm standard deviation) of both total and INT reducing bacteria.

photography) of processed samples within a few days of fixing.

Microscopical preparations

Bacterial cells need to be concentrated on a transparent support to allow the detection of formazan granules. Vacuum evaporation is long and tedious; centrifugation is not convenient for small cells. Concentration on membrane filters is preferable.

Polycarbonate membranes (Nuclepore or Isopore) have a double refractive index and cannot be made fully transparent like cellulose membranes. Some small cells may pass through such membranes when a $0.2 \mu\text{m}$ pore size is used. We used the $0.1 \mu\text{m}$ Millipore model VC which becomes transparent when it is immersed in an oil with a refractive index of about 1.500.

The process consisting of the collection of the fluorescent and INT-reducing cells on a cellulose membrane before clearing it by immersion in an oily clearing agent has two drawbacks: (i) a bright background due to the fluorochrome that is trapped inside the membrane and that cannot be entirely eliminated by rinsing; (ii) a dissolution of formazan granules by the immersion oil (Tabor & Neihof, 1982; Zimmerman *et al.* 1978 and Fig. 3).

To overcome these problems, Tabor & Neihof (1982), Newell (1984) and King & Parker (1988)

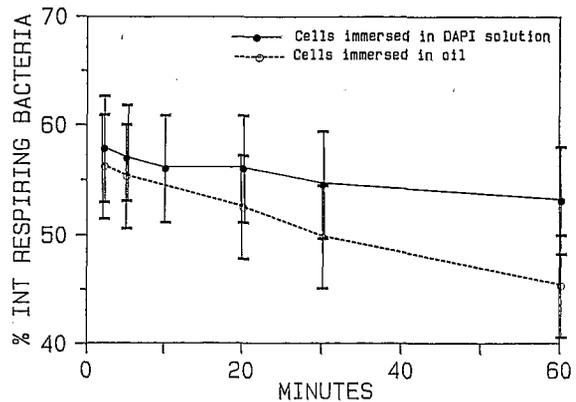


Fig. 3. Compared effect of prolonged immersion of bacteria in oil and in DAPI solution on the proportion (\pm standard deviation) of INT-respiring cells.

proposed the transfer of acridine orange-INT or DAPI-INT cells from the filter to a gelatin film. The process is laborious and not always reliable. To solve the first problem, Bitton *et al.* (1983) proposed filtering the INT treated sample through a cellulose membrane before drying and clearing it by floating on immersion oil, the organisms being on the top. The cells are then colored by the addition of a stain (malachite green in their paper) that is dripped on until the filtration surface is covered. After five minutes, the excess stain is removed with bibulous paper and is replaced by a drop of immersion oil. Staining the cells once the oil has already saturated the membrane prevents an excessive absorption of the stain. We applied this process with two significant modifications: (i) Non fluorescent stain was replaced by a fluorescent one which has been proved to be more effective for the recognition of small cells (Jones, 1974); (ii) the water solution of fluorescent stain was not replaced by immersion oil; in this way, cells are indeed immersed in an aqueous solution; this prevents the formazan crystals from rapid dissolution which would be expected if the cells were in contact with oil and solves the second problem (Fig. 3). This procedure improved greatly the sensitivity of the method.

DAPI (4'-diamidino-2-phenylindole) was preferred to acridine-orange as it resulted in a less background interference under epifluorescence

illumination. Acridine-orange also resulted in a slight residual orange color in the cells under transmitted illumination which resulted in a poor recognition of the smallest crystals of formazan.

Microscope examination and cell counts

Counts were conducted at a magnification of $1200\times$ with a Laborlux Leitz microscope fitted for transmitted light and epifluorescence.

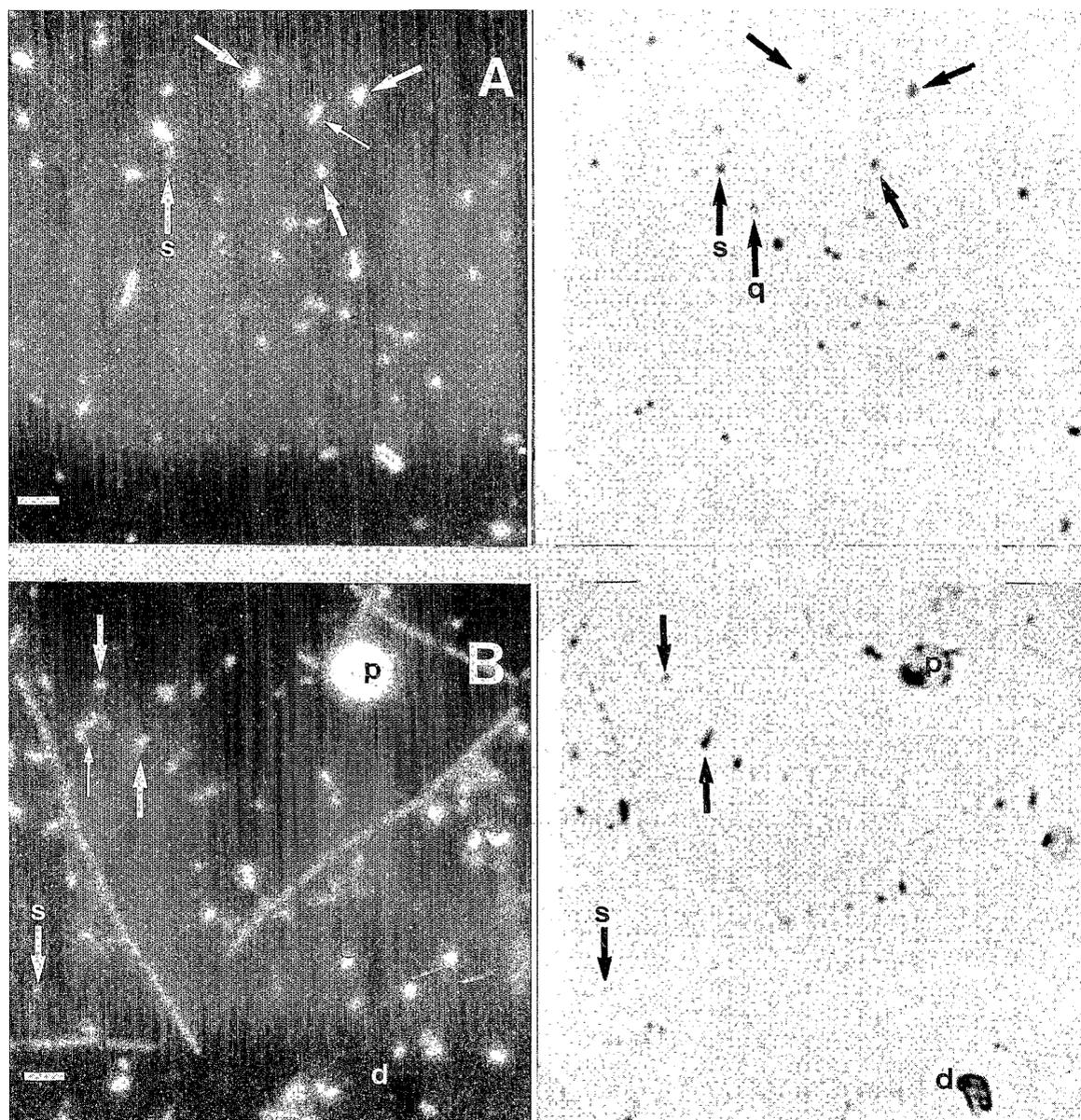


Fig. 4. Photomicrographs of bacterioplankton, (A) from Lake Geneva (may 1989, surface, coastal zone), (B) from a fish pond in the Ivory Coast (march 1988). On the left the UV excitation shows all the cells; on the right, on the same field under transmitted light, formazan crystals are accumulated in active cells. Fine arrow: an inactive cell; large arrow: an INT reducing cell; bars: $2\mu\text{m}$. Note that even the smallest cells may present detectable formazan (see for example cells 's' on the Fig.). In some cases formazan deposits quench the fluorescence (cell 'q' on Fig. A). In panel B a phytoplanktonic cell 'p' and non fluorescing detritus 'd' are identified.

In our procedure, we noticed a sharp contrast between the fluorescing cells and the dark background (Fig. 4). We did not find any significant difference ($P < 0.05$) between total cell counts of duplicate samples prepared according to our process and the standard process (Table I). This suggests that cells are immersed essentially in DAPI solution rather than in oil and explains why formazan dissolution on the membrane is slow (Fig. 3). This also suggests that 0.1 μm pore size Millipore filters trap cells as well as the 0.2 μm Nuclepore standard filters. Fluorescing cells are homogeneously distributed on the membrane and very few are detected away from the surface of filtration. This suggests that our membrane preparation does not perturb the cell distribution.

The intensity of the bright transmitted light was attenuated to 10% by a neutral density filter to avoid dazzle. When such a bright light is employed, there is a sharp contrast between the formazan spots and a white structureless background (Fig. 4). This allows the detection of formazan deposits even in the smallest cells ('s' on Fig. 4). In some cases formazan deposits fill out the entire volume of the cells ('q' on Fig. 4A). This results in non-fluorescing bacterial shapes that have to be included in counts of INT respiring cells as has been previously noted (Zimmer-

man *et al.*, 1978; Maki & Remsen, 1981). Some cells do not contain definite INT formazan granules but show varying amounts of a dispersed red color which indicates a weak INT reduction. Formazan coloration is absent in controls, which conforms with the method's basic assumption that the formation of optically dense formazan spots occurs exclusively in living cells.

Advantages and limitations of the improved method

Other microscopic methods have been used to make the distinction between active and dormant or dead bacteria. Differential coloration of cells stained by acridine orange (Jones, 1974; Hobbie *et al.*, 1977), microautoradiography (Meyer-Reil, 1978), the 'nalidixic acid method' (Kogure *et al.*, 1979) and the fluorescein diacetate method (Chrzanowski *et al.*, 1984). Each one has its own uses, advantages and drawbacks.

The INT method offers several advantages. Disturbance of the environment is minimal because it involves neither substrate addition nor long incubation times. Based on the ETS activity, the reduction of INT is linked to many metabolic pathways of virtually all bacteria in aerobic as well as in some anaerobic environments (Smith & Hoare, 1977; Packard *et al.*, 1983; Packard, 1985). The method may be applied to benthic and

Table 1. Comparison of direct counts of samples prepared according to Porter & Feig (1980) and the procedure proposed in this paper. Data are reported as mean \pm standard deviation.

Procedure membrane pore size (μm)	10^6 cells per ml			
	Porter and Feig nuclepore		This paper millipore	
	0.2	0.1	0.2	0.1
<i>Natural assemblage from</i>				
Fish pond (Ivory coast, 30/3/88)	7.10 \pm 0.31	7.17 \pm 0.40	6.10 \pm 0.34	6.85 \pm 0.37
Lake Geneva (9/2/89) 0 m	1.69 \pm 0.12	1.75 \pm 0.11	1.43 \pm 0.10	1.79 \pm 0.12
Lake Geneva (22/5/89) 5 m	4.70 \pm 0.27			4.28 \pm 0.21
7.5 m	3.90 \pm 0.24			4.32 \pm 0.20
10 m	3.97 \pm 0.28			3.76 \pm 0.22
<i>Cultured cells from</i>				
Lake Geneva (19/9/89) 0 m	330 \pm 16			309 \pm 17
Lake Geneva (20/9/89) 0 m	63.6 \pm 2.8			74.8 \pm 3.9

attached bacteria (Itturiaga, 1979; Harvey & Young, 1980; Bright & Fletcher, 1983; Quinn, 1984).

One problem inherent in this method relates to the bacteria size. Very small cells are difficult to see with epifluorescent microscopy and the resolution of formazan deposits within such bacteria is even more difficult. However, bacterial cells with such a low ETS activity probably do not have an important metabolic role relative to the whole community. A more serious difficulty of the method is the dissolution of intracellular formazan which occurs during the storage of fixed samples. The samples need to be counted, or photographed, within a few days. Another drawback of the method is that the maximum number of INT respiring microorganisms is reached after incubation times which vary from several minutes to several hours, depending on the sample. This necessitates preliminary time-course experiments of INT labelling for different environmental conditions.

The advantages of the procedure we propose here, as compared with the previous ones, lie in its simplicity and rapidity. Handling from the incubated sample to the microscope slide is as simple as the standard procedure used for total cell counts (Hobbie *et al.*, 1977; Porter & Feig, 1980). Data on inactive and active bacteria can be made available within a few hours of sampling. Since microscopic examination is easy this method is more sensitive and more reliable.

Applications

Thus improved, the INT method is applicable for routine purposes and may have numerous applications in environmental studies, ecophysiology, ecotoxicology, microbiological control of water quality and waste water treatment. It has been used on different bacterial assemblages (Table 2). Two to 71% of cells were shown with an intracellular crystal of formazan among natural communities, whereas this proportion could increase to nearly 100% during the exponential phase of populations cultivated on yeast extract. It may be possible to make assumptions from activity levels about the factors that control bacterial activity. Thus, bacteria, in winter in Lake Geneva and in the Mediterranean sea, may be controlled by the low temperature, nutrient starvation, or other adverse metabolic factors as shown by the low percent of active cells. On the contrary, the high proportion of active cells in summer in Lake Geneva and in a fish pond of the Ivory Coast suggests that grazing may be important. In all cases when it was tested, bacterial activity as determined by the uptake of ^3H thymidine or ^{14}C glucose, or frequency of dividing cells was better correlated to the counts of INT reducing cells determined by our improved method than with the total number of cells (Dufour *et al.*, 1990). This proves the metabolic significance of the method. Other examples of uses and performance of modified method are given in Dufour *et al.* (*ibid.*).

Table 2. Total counts of bacteria and percent of INT-reducing (active) bacteria in different environments determined with the improved method.

Location and date	Temperature °C	Total count 10^6 ml^{-1}	% INT active
Lake Geneva, epilimnion, march	5	0.60	23
Lake Geneva, hypolimnion, march	5	0.65	9
Lake Geneva, epilimnion, september	18	2.58	65
Lake Geneva, hypolimnion, september	6	0.44	34
Lake Geneva, culture, exponential growth	25	4.1	98
Fish pond, Ivory coast, march	27-35	2-15	28-71
Mediterranean sea, off marseille surface water, march	13	0.08	2

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