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Advantages of distinguishing the active fraction in bacterioplankton assemblages: some examples

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Abstract

The difficulty of distinguishing between active and dormant or dead bacterial cells is an important problem for the aquatic microbiologist.

Active cells can be detected under the microscope by the presence of an intact electron transport system able to reduce the colourless INT [2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride] to an optically dense intracellular deposit.

An improvement of this method has been applied to Lake Geneva and to a fish pond in the Ivory Coast. The portion of INT-reducing bacterial cells ranged from 1 to 71%, depending on place, depth, season and time of the day. In all cases bacterial activity, determined by uptake of ^{3}H Thymidine or ^{14}C glucose, and frequency of dividing cells were better correlated with the number of INT-reducing cells than with the total number of cells. This means that counts of cells able to reduce INT have a better metabolic significance than total cell counts. Some examples are developed which show the advantages of applying this method in cases where it is useful to distinguish active cells in a bacterial assemblage.

Introduction

It has been recognized that only part of the bacterial aquatic flora is metabolically active at a given moment (Stevenson, 1978). Because of resting or dead cells, total abundance of bacteria does not reflect activity. Therefore, it may be pertinent to distinguish between active and inactive cells in a bacterial assemblage. This may further lead us to inquire what changes in a bacterium's environment could determine its activity, dormancy or death.

A method first proposed by Von Bielig *et al.* (1949) and further developed by Iturriaga & Rheinheimer (1975) allows the enumeration of respiring bacterial cells. Respiring cells are defined as being able to reduce colorless INT [2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride] to INT formazan recognized as an opaque intracellular deposit. Zimmermann *et al.* (1978) improved this method by combining the recognition of individual cells with respiring activity and direct counts by fluorescence microscopy. We further improved this method and applied it to samples from three different trophic environments. The aim was to evaluate its interest and metabolic significance in conjunction with growth indicators such as thymidine incorporation or ^{14}C glucose uptake.

Material and methods

Water samples were collected from a central station in Lake Geneva at 12 levels from surface to bottom (300 m) in March and May 1989, and in a fish pond in Ivory Coast in March 1988 at a unique level (0.10 m) every two hours over 34 h.

Every sample was assayed for ^{3}H Thymidine (TdR) incorporation (Riemann *et al.*, 1982) and ^{14}C glucose assimilation (Wright & Hobbie, 1966) at *in situ* temperature immediately after collection. Total counts of bacterial cells were obtained after staining with Dapi (Porter & Feig, 1980) and frequency of dividing cells was also noted (Hagström *et al.*, 1979). Grazing on bacteria was estimated by comparing the change of bacterial abundance in undisturbed water and in water filtered by gravity onto 35 μm mesh size plankton net and 10 μm, 3 μm and 1 μm pore size nuclepore membranes. The percentage of respiring bacterial cells was determined by counting those showing an electron transport system (ETS) activity (Zimmermann *et al.*, 1978) by means of an improved procedure briefly described below.

Freshly collected samples were incubated with INT (Aldrich, 0.02% final concentration) and NaCN (0.2 mM) for one hour in the dark at *in situ* temperature. Formalin-fixed controls (2% final concentration) were included for each sample. Incubation was stopped by formalin and samples were then stored in the dark at 4 °C. Samples were further filtered onto 0.1 μm pore size cellulose membranes (Millipore VC). Air-dried filters were then cut into 6 wedges. One wedge was cleared by floating it on a drop of Cargille immersion oil (code 848, $n_D^{25\,°C} = 1.506$) on a microscopic slide, organisms up. Bacteria were then immediately stained by a drop of Dapi (Boehringer, 10^{-5} wt/vol) and a cover slip was added. The preparation was then examined under microscope. Each field was photographed, first under bright light transmitted through the membrane in order to detect intracellular granules of formazan (Fig. 1A), and secondly with a standard epifluorescence procedure for total cell detection (Fig. 1B). The procedure was repeated on other wedges, until at least 400 cells were photographed for each sample.

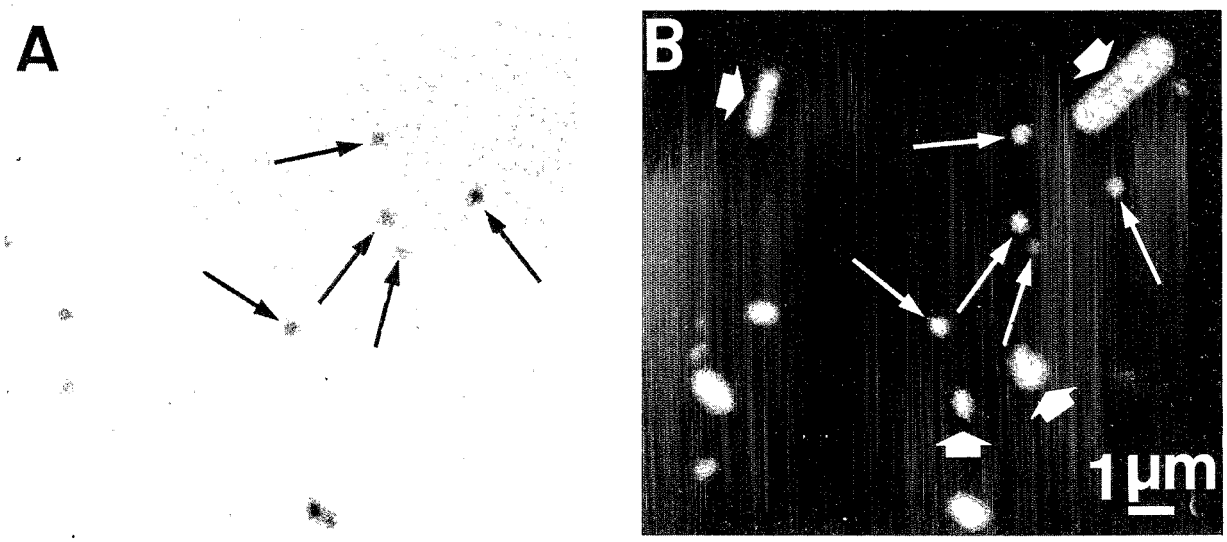

Fig. 1. Photomicrographs of bacterioplankton from Lake Geneva (9/6/89, surface, coastal zone). A: transmitted light; formazan cristals are accumulated in active cells. B: the same field under epifluorescent excitation shows all the cells. Large arrow: an inactive cell; fine arrow: an INT reducing cell. Note that even the smallest cells may present detectable formazan.

Results and discussion

Total bacterial abundance ranged from 0.1 to 9.4×10^6 cells ml^{-1}, in agreement with the trophic states of the different water bodies sampled. In Lake Geneva the average abundance in the whole water column (0–300 m) was 6×10^5 cells ml^{-1} in March and 9×10^5 cells ml^{-1} in May. Seasonal differences were more pronounced in the epilimnion (0–10 m) where 6×10^5 and 4.1×10^6 cells ml^{-1} were enumerated in March and May respectively. In the fish pond, abundance ranged from 5.6×10^6 at night to 9.4×10^6 cells ml^{-1} at sunset (average 7.1×10^6).

The percent of cells able to reduce INT ranged from 0.6 to 47% in Lake Geneva (average 10%) and from 28 to 71% in the tropical aquaculture pond (average 47%). These two values are included in the range reported in literature (Table 1). It may be interesting to check the significance of the large variations in the proportion of 'respiring' bacteria all through the water column for lake Geneva and during a diurnal cycle in the tropical aquaculture pond.

Spearman's rank correlation matrix (Table 2) shows that every index of activity (thymidine incorporation, glucose assimilation, abundance of dividing cells) was more closely related to INT-reducing cells than to total cell counts. As a matter of fact, in Lake Geneva in May, the profile of thymidine incorporation was more closely related to INT-reducing bacteria than to total bacteria (Fig. 2). In the epilimnion, the minimum of thymidine incorporation at depth 7.5 m was asso-

Table 1. Range of values for percent of INT-reducing bacteria in different water bodies.

Location	% active	References	Method
Kiel Fjord (FRG)	19–23	Iturriaga & Rheinheimer (1975)	Iturriaga & Rheinheimer (1975)
Westensee (lake, FRG)	23–36	Zimmermann *et al.* (1978)	Zimmermann *et al.* (1978)
Pond (FRG)	5– 9	Zimmermann *et al.* (1978)	
Baltic Sea (Kiel Firth, FRG)	12	Zimmermann *et al.* (1978)	
Baltic Sea (Kiel Bight, FRG)	7	Zimmermann *et al.* (1978)	
Eckernförde Bight, Baltic Sea setlting particles	8–97	Iturriaga (1979)	Iturriaga & Rheinheimer (1975)
Salt marsh creek (Calif., USA)			
surface layer	16	Harvey & Young (1980)	Zimmermann *et al.* (1978)
subsurface	5	Harvey & Young (1980)	cells $>0.4\ \mu m$
Rice field, Camargue (Fr.)	5	Baldensperger (1981)	Zimmerman *et al.* (1978)
Salt marsh (Georgia, USA)			
Duplin river	31–36	Newell (1984)	Newell (1984)
Marsh creek	29–30	Newell (1984)	
Chesapeake Bay (Md, USA)			
1 m	25–94	Tabor & Neihof (1984)	Tabor & Neihof (1982)
8.5 m (near bottom)	29–62	Tabor & Neihof (1984)	
Lough Neagh (Ireland)	21–28	Quinn (1984)	Zimmermann *et al.* (1978)
Little Crooked lake (Ind., USA)	28–30	Lowell & Konopka (1985)	Zimmermann *et al.* (1978) cells $>0.4\ \mu m$
Mc Loud lake (Florida, USA)	9	Dutton *et al.* (1985)	Bitton & Koopman (1982)
Wauberg lake (Florida, USA)	25	Dutton *et al.* (1985)	
Lake Geneva (Fr.)	1–47	This study	This study
Fish Pond (Ivory Coast)	28–71	This study	

Table 2. Spearman's rank correlation matrix between 3 measurements of bacterial activity and 2 abundance determinations (total and INT-reducing bacteria) in the three situations investigated. ns = not significant; * = significant $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

	Total abundance	INT reducing bacteria	Sampling site
TdR incorporation	0.02 ns	0.64*	Lake Geneva (winter 89)
Glucose assimilation	o.30 ns	0.69*	Vertical profile (12 levels)
Abundance of dividing cells	0.71**	0.72**	Mesotrophic
TdR incorporation	0.67*	0.99***	Lake Geneva (spring 89)
Glucose assimilation	0.64*	0.79**	Vertical profile (12 levels)
Abundance of dividing cells	0.62*	0.71**	Mesotrophic
TdR incorporation	0.17 ns	0.58*	Fish pond (Ivory Coast)
Abundance of dividing cells	0.22 ns	0.61**	March 1988, 16 samples in 32 h
			Eutrophic

ciated with a minimum in INT-reducing bacteria (less than 3% of total counts), while total abundance was nearly constant from 0 to 10 m. This suggests that this minimum of thymidine incorporation rate per cell, an index of growth rate for the whole population, may be due to inadequate growth conditions for 97% of the total population. In March, again in Lake Geneva, total abundance was nearly constant in the whole water column (average 0.60×10^6 cell ml^{-1} in epilimnion, 0.62×10^6 cell ml^{-1} in hypolimnion), while thymidine incorporation rate decreased 12 times from epilimnion to hypolimnion. The enumeration of INT-reducing bacteria shows that the decrease in thymidine incorporation rate may be partly due to decreasing proportion of 'active' bacteria, from a 23% average for the epilimnion to a 9% average for the hypolimnion.

In the tropical fish pond, thymidine incorporation rate and abundance of dividing cells, both indices of bacterial cell production, increased by 1.4 and 1.2 respectively between sunrise and sunset, while the total bacterial abundance decreased 1.2 times during the same period (Table 3). The cells produced during the day may have disapeared by grazing, lysis and/or sedimentation. However, the active INT-reducing fraction

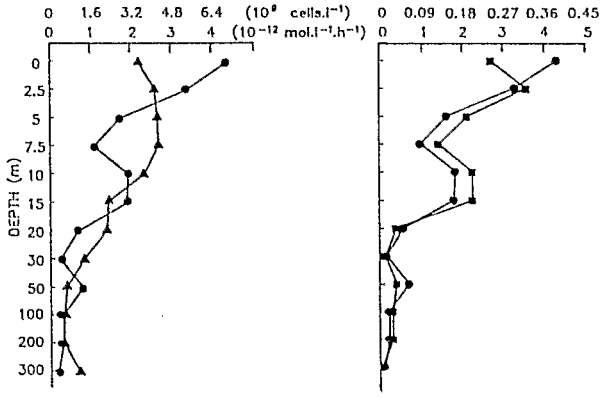

Fig. 2. Vertical profiles of bacterial abundance and activity in Lake Geneva (26/5/89). Circles: TdR-incorporation rates; triangles: total cell abundance; squares: INT-reducing bacteria abundance.

Table 3. Examples of bacteria-related parameters during a diurnal period in a fish pond in Ivory Coast (20/3/88).

	6 a.m.	8 p.m.
Total abundance 10^9 cells l^{-1}	7.6	6.4
INT reducing bacteria 10^9 cells l^{-1}	2.2	4.1
Dividing bacteria 10^9 cells l^{-1}	2.4	2.9
TdR incorporation rate 10^{-9} mol l^{-1} h^{-1}	1.7	2.5

rose from 29 to 64% of the total (Table 3). A constant or slightly increasing lysis of the cells (abundance is decreasing) is not compatible with the increase of the active fraction. Sedimentation is likely to be independent of the active proportion of the bacterial cells, but related only to the presence of sedimenting particles colonized by bacteria. Thus an increase of grazing on bacteria, acting probably equally on the growing and the inactive fraction, might be the major reason for bacterial losses. This is in agreement with the observed increase of thymidine incorporation per cell. An independent estimate of grazing by differential filtration confirmed that grazing was effective during the day and inexistent during the preceeding night.

The enumeration of INT-reducing bacteria allows also the more realistic estimation of generation time for bacteria. For example, in Lake Geneva, assuming exponential growth *in situ*, the experimentally determined conversion factor (according to Kirchman *et al.*, 1982) of 3.9×10^{18} cells per mol of incorporated thymidine allowed the estimation of a generation time of 146 h for the whole bacterial population of the surface sample in May (Fig. 2). Interpretation of INT reducing bacterial abundance suggests that there were, in that sample, only 7% of active cells with an average of 10 hours generation time.

An example of application in ecophysiology is given for Lake Geneva, where we sought to discover whether the bacterioplankton population in winter was mainly composed of strictly psychrophylic bacteria. A sample of surface water collected in February (6 °C *in situ* temperature), with only 9% of the total bacteria reducing INT, was incubated at 25 °C with 0.025% yeast extract (wt/vol, final concentration). Five hours later, the total number remained unchanged, but cell volumes were larger and 98% of the cells were found to be able to reduce INT. Cell-multiplication occurred later, after 9 h incubation. Direct examination of INT-reducing bacteria thus revealed that these bacteria are not strictly psychrophilic, but are able to grow at a higher temperature and are limited *in situ* by low temperature, and/or nutrient concentration.

This method could also be applied to check whether the lag phase before exponential growth in a water culture (Ammerman *et al.*, 1984) is due to 'awakening' of dormant bacteria and/or to the development of only a small part of the bacterial population. This question is important for the evaluation of the representativity of growing bacteria, since these cultures are often used to determine a conversion factor between thymidine or leucine incorporation and cell-production (Kirchman *et al.*, 1982).

These examples show the importance of distinguishing between active and dormant cells. Other methods have been used to make this distinction. Microautoradiography (Meyer-Reil, 1978) has been widely used, with an addition of significant amounts of substrate that may shift some bacteria from dormancy to activity, thus leading to an overestimation of *in situ* active bacteria. Moreover, it usually takes days before data can be provided. The colour emitted by cells stained with acridine orange is not directly related to cellular activity. (Jones, 1974; Quinn, 1984). The method proposed by Kogure *et al.* (1979), involving several hours incubations of bacteria with yeast extract and nalidixic acid, may also lead to overestimation, due to the substrate addition (Peele & Colwell, 1981), or to underestimation due to nalidixic acid resistant cells (Kogure *et al.*, 1979; Maki & Remsen, 1981). The fluorescein diacetate method proposed by Rotman & Papermaster (1966) has also been shown to be a method which underestimates active bacteria (Chrzanowski *et al.*, 1984; Quinn, 1984).

The INT-reducing bacterial count method implies neither substrate addition, nor long incubation times. The procedure of filter preparation used here is as simple as that described by Hobbie *et al.* (1977) for total cell count. It allows the enumeration of respiring and total cells on the same microscope field. When counting INT-respiring bacteria, errors due to subjectivity do not seem to be higher than those obtained by exclusive determination of the total number (Zimmermann *et al.*, 1978). Tetrazolium salt has shown no visible toxic effects during short incu-

bations (Guha, 1957). Based on ETS activity, the reduction of INT is a component of virtually all bacterial types, including lithotrophs and methylotrophs (Smith & Hoare, 1977), facultative or obligate anaerobes (Packard *et al.*, 1983) and aerobic heterotrophs (Packard, 1985). The limit of the method is due to the size of formazan deposit into the cell. Cells with low activity and low-size deposits under detection may be considered as dormant or dead, leading to an underestimation of the active fraction. However, cells presenting such a low ETS activity may not be important for total community metabolism.

Conclusions

The above examples show that, with the use of this method of determination of active cells in conjonction with other specific methods, it may be possible to differenciate factors controlling bacterial production. Thus, in mesotrophic Lake Geneva at the time of experiment, bacteria seemed to be limited by starvation and/or low temperature, as attested by the low percentage of active cells, while in the eutrophic tropical fish pond, a high percentage of active cells suggests an important contribution to grazing. Therefore, we think that the improved INT method used here should be applied whenever the distinction between active and dormant or dead cells is necessary. It could help to improve knowledge of life strategies of bacteria, particularly in aquatic environments. This method is appropriate for a number of applications in ecophysiology and ecotoxicology.

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