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Simultaneous Determination of the Total Number of Aquatic Bacteria and the Number Thereof Involved in Respiration

REINHARD ZIMMERMANN,[1] * RODOLFO ITURRIAGA,[2] AND JENNY BECKER-BIRCK[3]

Institut für Meereskunde[1] and Hygiene Institut,[3] Universität Kiel, Kiel, West Germany, and Marine Laboratory, Duke University, Beaufort, North Carolina 28516[2]

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The electron transport system of respiring organisms reduces 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to INT-formazan. Respiring bacteria deposit accumulated INT-formazan intracellularly as dark red spots. Corresponding to electron transport system activity, these deposits attain a size and a degree of optical density which allows them to be examined by light microscopy. If polycarbonate filters and epifluorescence microscopy are applied to analyze an INT-treated water sample, it is possible to differentiate between respiring and apparently nonrespiring bacteria. This differentiation, which permits determinations of the total number of bacteria and the proportion thereof involved in respiration, is realized directly within one and the same microscopic image. Initial applications of the present method for hydrobiological purposes showed that the proportion of respiring aquatic bacteria ranged between 6 to 12% (samples taken from coastal areas of the Baltic Sea) and 5 to 36% (samples taken from freshwater lakes and ponds). Cells of 1.6 to 2.4 μm (freshwater) and 0.4 μm (Baltic Sea) account for the highest proportion of respiring bacteria.

A significant role for the flux of energy in aquatic environments has been attributed to the activity of heterotrophic and autotrophic bacteria. It is therefore a major aim of ecological microbiology to develop and improve methods which reflect metabolic activity of aquatic bacteria under conditions close to those in situ. When assessing those methods which have been used to date to measure activity, the approach towards determination of actual bacterial activity still appears limited. Biochemical or biophysical methods, such as the determination of electron transport system activity after extraction procedures, the measurement of oxygen consumption, and microcalorimetry, are used to determine activity parameters for faunal and floral aquatic communities. Only in those habitats where bacterial biomass presumably dominates (e.g., in certain sediments) do these methods possibly reflect bacterial activity exclusively. Techniques which are based on the uptake of certain traced organic compounds (1, 3, 4, 10, 15) are assumed to be specific for bacteria. However, as far as the determination of total heterotrophic activity is concerned, the incubation of the sample with certain labeled nutrients which are known to represent a minute selection of the diverse natural nutrients present in the investigated aquatic environment, is subject to selectivity. By assuming, in addition, the presence of a significant number of dormant bacteria in the water, incubation for 2 to 3 h under more or less modified ambient conditions may induce subsequent activity.

Certain artificial products of biochemical processes related to activity can be detected by light microscopy. If these processes are independent on the addition of nutrients to the water sample, and if both artificial products and organisms are simultaneously visible, it is possible to count specifically active bacteria. Because respiration is closely bound to active metabolism, the cytochemical detection of dehydrogenase activity was chosen to combine it with a technique for direct counting of aquatic bacteria.

The active electron transport system is an almost universal component of respiring organisms (9) which reduces 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to INT-formazan. In contrast to a mainly diffuse incorporation of red INT-formazan by respiring eucaryotic cells, respiring bacteria and many blue-green algae (cyanobacteria) deposit accumulated INT-formazan as optically dense, dark red intracellular spots. Corresponding to the intensity of respiration, these spots attain a size and a degree of optical density which allows light microscopic observation. To avoid the limits of those methods, which are based on the uptake of traced nutrients, Iturriaga and Rheinheimer (5) analyzed polycarbonate filters after prior filtration of an INT-treated water sample.

By counting INT-formazan spots with phase-contrast microscopy, numbers of respiring bacteria were determined. Because formazan deposits do not represent the definite outline of bacterial cells, it was difficult to define the nature of counted spots. According to the origin of the water sample, various amounts of nonspecific particles of similar size and optical density interferred with the identification of formazan deposits. To study morphology or arrangement of bacteria and to determine their total number, a separate preparation of the sample for epifluorescence microscopy was necessary.

Besides the advantage of specificity, the simultaneous representation both of products derived from activity and of organisms also yields information about structural components of bacterial populations and their habitats. By considering, for instance, a comparison of free and attached bacteria with regard to their activity or of the size spectrum of respiring bacteria, it is evident that a direct differentiation between active and inactive bacteria is required. By combining formazan detection and epifluorescence microscopy, a method was developed which allows differentiation of bacteria on one and the same microscopic image.

MATERIALS AND METHODS

Sampling. Water samples were taken from different near-shore areas (water depth, 1 m) of Kiel Bight (Baltic Sea) and of various freshwater lakes near Kiel (Federal Republic of Germany). Sampling was carried out with sterile bottles (volume, 1 liter) with a modified Sorokin sampler. The samples were processed immediately after sampling.

Processing of samples. A 10-ml quantity of each water sample was poured into sterilized, small glass bottles. A 1-ml amount of 0.2% (aqueous solution) INT dye (Baker Chemicals) was added to each subsample. After mixing carefully, the subsamples were kept for 20 min in the dark at in situ temperature. The reaction was stopped with 0.1 ml of 37% formaldehyde, which also served to preserve the subsample. Preserved subsamples were stored up to 1 month at 4°C in the dark. All aqueous solutions (double-distilled water), including the alcoholic solution needed for the following microscopic preparations, were purified by filtrations through cellulose nitrate filters (pore size, 0.2 μm) before use.

Microscopical preparation. A stock of polycarbonate filters (Nuclepore Corp.; 25 mm in diameter; pore size, 0.1 μm), which had been checked before use for uniform wettability, was immersed for at least 1 day in a solution of Sudan black B (Merck) in 50% ethanol. Final concentration of Sudan black B was 1:15,000 (Sudan black B has to be dissolved in absolute ethanol, which then is diluted to 50% with double-distilled water). Before use the filters were thoroughly rinsed in double-distilled water.

A 0.5- to 3-ml amount (eutrophic freshwater lakes, coastal marine waters) of the fixed sample was filtered (0.15-bar [ca. 1.5×10^4 Pa] pressure difference). Uniform distribution of particles was obtained by supporting the filter with a silver membrane (Selas Flotronics; pore size, 0.8 μm) instead of a normal filter

TABLE 1. *Effect of fixatives and dry heat (applied before incubation of the sample with INT) on the respiratory activity of a natural bacterial population*[a]

Fixation technique	Final concn (%) of fixative	Respiring bacteria (%)[b]
Formaldehyde	0.1	34.6
	0.25	3.6
	0.8	0
Lugol solution	0.6	0
Hg_2Cl	0.06	0
Dry heat (40 min at 70°C)		0
No fixation before incubation (control)		100

[a] See text.
[b] Total number of respiring bacteria (control) = 100%.

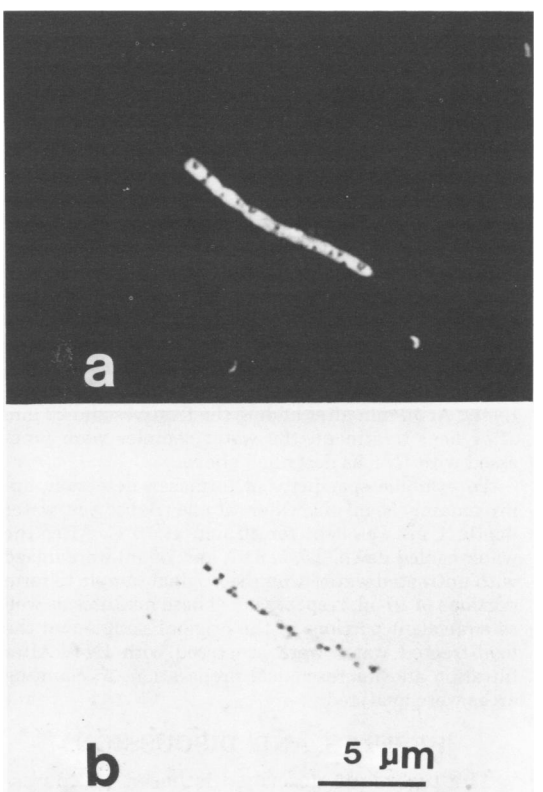

FIG. 1. *Fluorescent (a) and transmitted bright-field (b) image of a filamentous bacterium. Recognizability of formazan deposits is enhanced by bright-field illumination; however, cell contours are absent (b).*

support (sintered glass disk). When putting the filter on top of the silver membrane, care was taken to avoid inclusion of gas bubbles. After filtration, 1 ml of an acridine orange solution (1:10,000 in 6.6 mM phosphate buffer, pH 6.7) was pipetted onto the filter. Contact time was 2 min. After removing the fluorochrome solution by filtration, the filter was taken off the filtration device, air dried, and cut into wedges with a razor blade. One wedge was mounted with a drop of immersion oil (Cargille, type A) on a microscope slide and covered with a cover slip. Embedded filter wedges were not stored for longer than 2 h.

Microscopic examination. The mounted filter was examined by epifluorescence microscopy (Zeiss Universal microscope, HBO 200 W, KP 490, KP 500, dichroic beam-splitting filter 510, LP 520). By using an oil immersion objective (Planapochromat, 100×) a magnification of 1,560× was attained. Formazan spots were examined either by simultaneous (mixed light) or by alternating epifluorescence and transmitted bright-field illumination. In the latter case the diaphragms of the bright field and incident light paths were masked alternately by simple manipulation. The adsorption filter (VG 9, Zeiss) reduced the intensity of the transmitted light and transformed the background color of the bright field image into light green, which promotes convenient counting. The total number of bacteria and the proportion of respiring bacteria were determined within the area of a 10 × 10 graticulated eyepiece. Cell lengths were measured by an eyepiece micrometer. Bacterial biomass was calculated by the procedure given by Zimmermann (16). The time required for sample processing and microscopical preparation was less than 0.5 h. Microscopical examination of each filter preparation (50 areas counted, 10 areas of size classification) was performed within 20 to 40 min.

Supplementary investigations. Suppression of bacterial INT reduction by prior fixation of six subsamples of a water sample taken from the Baltic Sea (water depth, 1 m) was performed with formaldehyde (0.8, 0.25, and 0.1%), Lugol solution (0.6%), $HgCl_2$ (0.06%), and heat treatment (40 min at 70°C), respectively. At 10 min after adding the fixatives and 20 min after heat treatment, the water samples were processed with INT as described above.

To examine specificity of formazan detection, approximately 50 ml of a water sample (Baltic Sea; water depth, 1 m) was kept for 40 min at 70°C. After the water cooled down, 2.5, 5, 6.67, and 7.5 ml were mixed with untreated water from the original sample to form portions of 10 ml, respectively. These mixtures as well as equivalent portions of the original sample and the heat-treated water were processed with INT. After filtration and microscopical preparation, 15 counting areas were analyzed.

RESULTS AND DISCUSSION

The technique described is based on the assumption that the intracellular INT reduction and the formation of optically dense formazan spots occur in living cells exclusively. It is further assumed that the presence or absence of visible formazan spots in cells of bacteria and many blue-green algae is in general a function of respiratory intensity. By treating water samples with different fixation techniques (Table 1) before addition of INT, it was possible to demonstrate that this assumption is likely to be true.

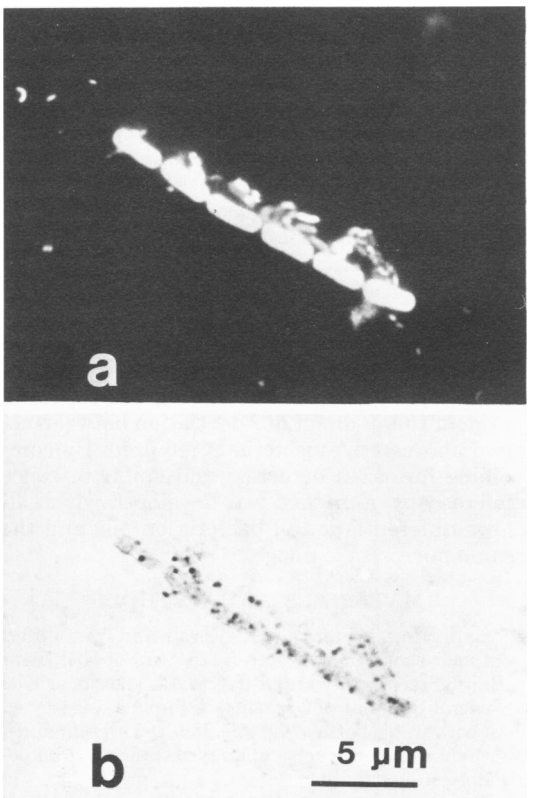

FIG. 2. *Fluorescent (a) and transmitted bright-field (b) image of a voluminous bacteria chain with adhering smaller bacteria. Due to strong fluorescence, formazan deposits can be detected only in the bright-field image (b).*

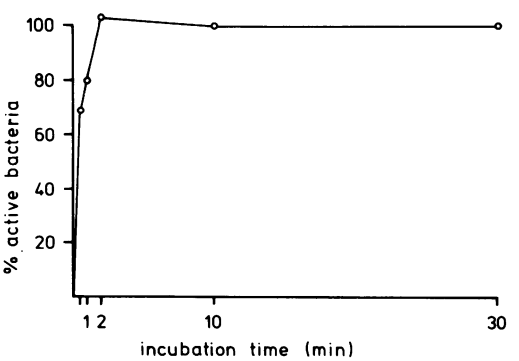

FIG. 3. *Effect of the incubation time on INT-treated natural water subsamples. Number of respiring bacteria after 30 min of incubation = 100%.*

Dependence of formazan production on the intensity of respiration is shown by the effect of graduated concentrations of formaldehyde. A 0.25% concentration of formaldehyde, which is assumed to be the minimal concentration needed to stop bacterial respiration (18), signifies also the limit of visible formazan formation.

When examining the microscopic preparation by epifluorescence illumination, previously respiring bacteria could be recognized by the presence of one or several round, dark red formazan spots inside the fluorescent cell (Fig. 1a). If deposits are small, they may show weak contrast due to irradiation by strong fluorescence. A sharp contrast between formazan deposits and the image background was obtained when transmitted bright-field illumination was applied. In the bright-field image, formazan spots generally appeared somewhat bigger and optically more dense. On the other hand, exclusive bright-field illumination did not allow detection of fluorescent bacteria (Fig. 1b). Formazan was often deposited near the cell wall. Therefore, sharp outlines of cells and formazan spots were seldom both on one focal plane when an objective of high magnification was used (Fig. 2a and b). Thus, microphotographs do not reflect the optical quality and information content of direct microscopic observation.

The intracellular accumulation of INT-formazan by the respiring bacterium is indispensable for the detection of deposits. Preliminary investigations with numerous bacterial pure cultures, which had been isolated from human blood as well as from water samples of the Kiel Firth and the Persian Gulf (cultured on ZoBell medium 2216 E) showed accumulation of INT-formazan in all strains observed. This supports the assumption that the deposition of formazan in this manner is a characteristic property of bacteria. For tetra-Nitro Blue Tetrazolium, which had been used to localize dehydrogenase in bacteria, a more diffuse and peripheral deposition of reduced compounds was observed (11). The optical density of these deposits may then be likely to be insufficient for light microscope analysis. The sites of dehydrogenase and INT-formazan deposits may be not necessarily identical. Like triphenyltetrazolium-formazan (13, 14), INT also seems to coalesce after its reduction to form the typical larger (secondary) formazan deposits located in the cytoplasm.

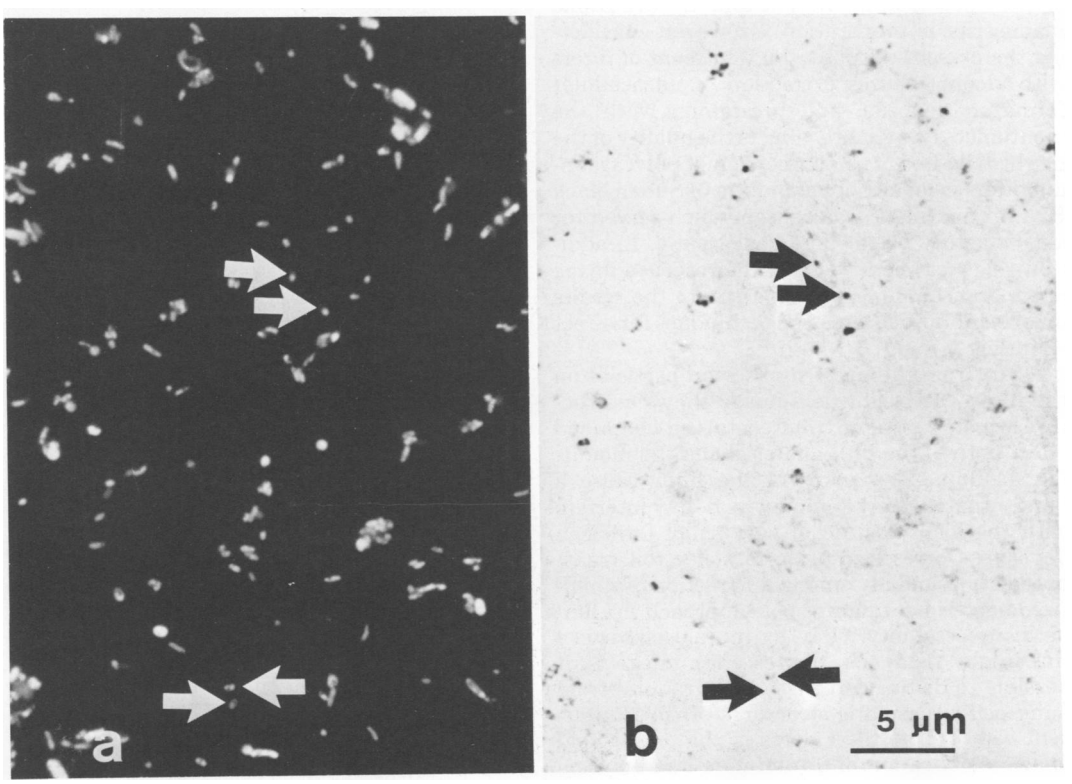

FIG. 4. *Fluorescent (a) and transmitted bright-field (b) image of a bacterial population (sample taken from the Baltic Sea), which was incubated for 20 h at 20°C (arrows: see text).*

To attain intracellular formazan deposits of maximal size and optical density, incubation of the water sample with INT has to last until there is no further increase in formazan deposition. As shown by different incubation times, maximal numbers of respiring bacteria (water sample taken from a freshwater pond; water temperature, 11°C) were obtained already after about 2 min (Fig. 3). Prolonged incubation (2 h) did not change considerably the initial number of respiring cells. Because there is still little information about factors which control the uptake of INT and its reduction, an incubation time of 20 min was chosen to be more certain of providing for a maximal deposition of formazan in the cell.

The adsorption of fluorochromes by the filter material (polycarbonate) signifies an important problem of direct counting. To enhance contrast properties, emphasis was given to treatments which reduce interfering background fluorescence. In former methods the fluorochromed polycarbonate filter was rinsed with isopropyl alcohol (17) or was prestained with Dylon (6) or irgalan black (2) before fluorochromation. Immersion in colorless compounds also can serve to some extent to saturate active adsorption sites of polycarbonate (Iturriaga and Becker-Birck, manuscript in preparation). However, considering the present purpose, the treatment of filters with alcohol causes extraction of intracellular formazan deposits, and prestaining with the mentioned dyes slightly affects the quality of the bright-field image. By immersion of polycarbonate filters in an alcoholic solution of Sudan black B, a pretreatment was chosen which served for a satisfactory bright-field background. In addition this treatment yields a fluorescence image of superior quality in comparison to the results of former fluorochromation techniques for direct counting.

Apart from the pattern of seston particles on the filter, the bright-field image shows a structure which can be attributed to the combined effect both of the filter material and the mounting medium. The pores of the filter cause a faintly spotted background which may interfere with the identification of very small formazan deposits. Corresponding to low electron transport activity mainly among the fraction of small-sized bacteria, formazan spots approach the limits of detectability. Only by mutual control of the bright field and fluorescence image is it possible to distinguish these tiny granules from nonspecific plasmatic structures of the fluorescent cell or from filter pores in the bright-field image. Application of 0.1-μm-pore size polycarbonate filters, which are only 5 μm thick, diminishes visible filter structure in comparison to thicker (10 μm) 0.2-μm-pore size polycarbonate filters. If emphasis is placed mainly on absolute numbers of bacteria, the thinner filter results additionally in a better retention of very thin bacteria which can pass pores of 0.2 μm in size, as shown by scanning electron microscopy (16). In terms of total biomass, this loss of cells can be ignored unless extremely oligotrophic waters are examined. In comparison to 0.2-μm polycarbonate filters, which usually are applied for direct counting, 0.1-μm filters reduce the flow rate by a factor of 3. However, because the microscopical analysis consumes by far most of the time needed for both preparation and counting, temporal differences of a few minutes are negligible.

Among various tested mounting media, Cargille immersion oil (type A) was chosen to achieve a fluorescent image with optimal contrast (2). Care was taken to avoid contamination by other mounting media. The optical refraction properties of polycarbonate (birefringent n = 1.616 and 1.584) and Cargille immersion oil (n = 1.515) are not identical. By applying immer-

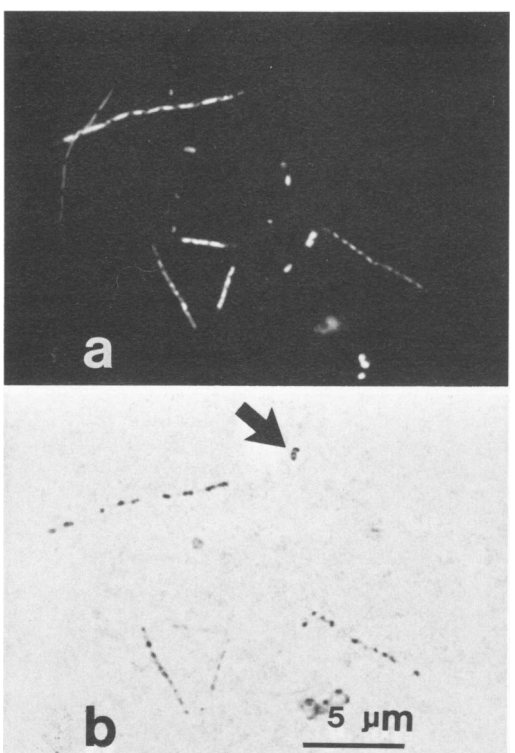

FIG. 5. *Fluorescent (a) and transmitted bright-field (b) image of filamentous bacteria from pond water (arrow: see text).*

sion oils with refraction indexes of about $n = 1.6$ (immersion oils mixed with halogenated compounds or cinnamic aldehyde), it was possible to attain a structureless bright-field background. However, when using these media, background fluorescence was quenched badly, and in some cases gradual extraction of intracellular formazan was noticed. Extraction of intracellular formazan also occurred after storage of filters embedded in Cargille immersion oil for longer than 2 h.

Whereas there were few problems when detecting formazan spots in voluminous fluorescent bacteria and blue-green algae, the additional application of the bright-field control image was indispensable for the differentiation of small single bacteria. In those cells formazan was often deposited at the polar ends of the rods (Fig. 4a and b, arrows) where it contrasted poorly to the dark background of the fluorescent image. The minimal cell size which allowed detection of formazan deposits was represented by short rods of only 0.4 μm in length (Fig. 4a). Sometimes formazan deposits filled out the entire volume of the cell (Fig. 5b, arrow). Because fluorescence was thus drastically reduced, these specimens may only be recognized in the bright-field image.

As determined also by microautoradiography (8), filamentous bacteria often showed high activity (Fig. 5a and b, and 6a and b). Despite the subdivision of filaments by formazan spots, it is not likely that fluorescent sections of the filaments would be mixed up with single bacteria. The background of the fluorescent image, which was slightly lighter than is shown by the microphotographs, still allowed cellular outlines of nonfluorescent sections (formazan deposition) to be recognized. Therefore, a completely black background, as can be obtained by applying other long-pass light filters (e.g., LP 540), need not be an advantage.

Aggregates and "Aufwuchs" often contain high proportions of respiring bacteria (Fig. 6a and b, and 7a and b). Bacterial aggregates are well suited for examining the metachromatic effect of acridine orange and its assumed relation to activity (2, 12). In the greater part of samples

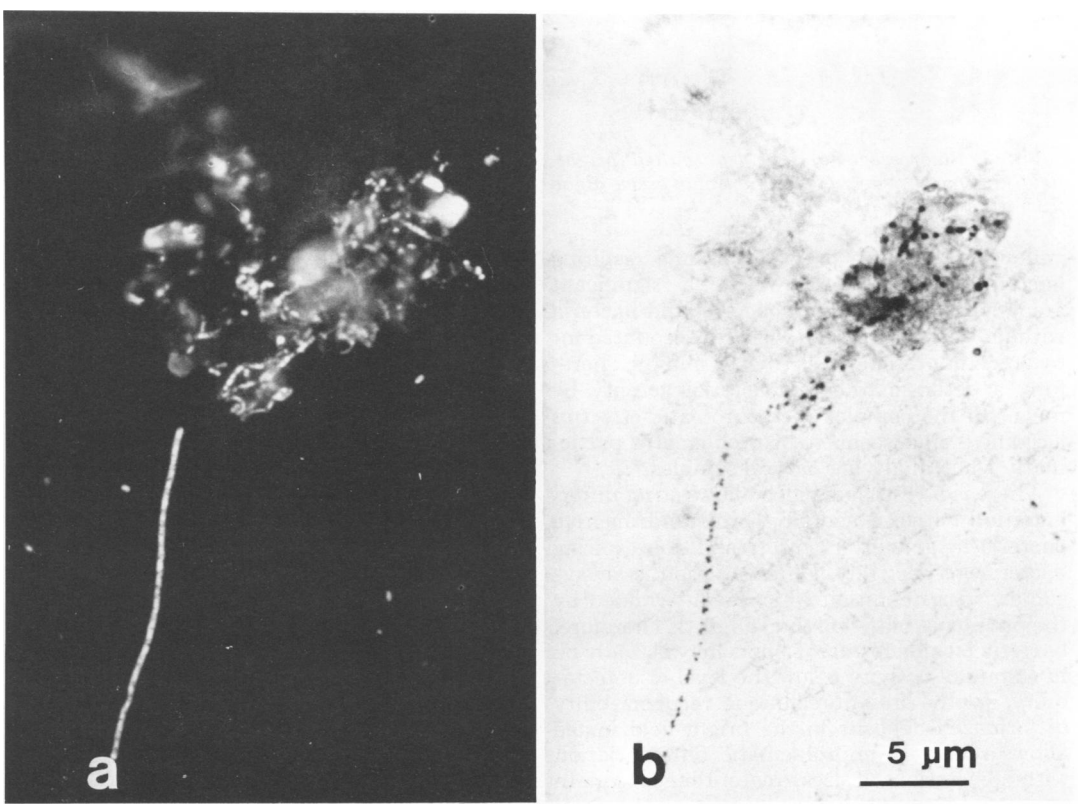

FIG. 6. *Fluorescent (a) and transmitted bright-field (b) image of Aufwuchs and a filamentous bacterium from pond water. Formazan spots contrast well to detrital matter (b).*

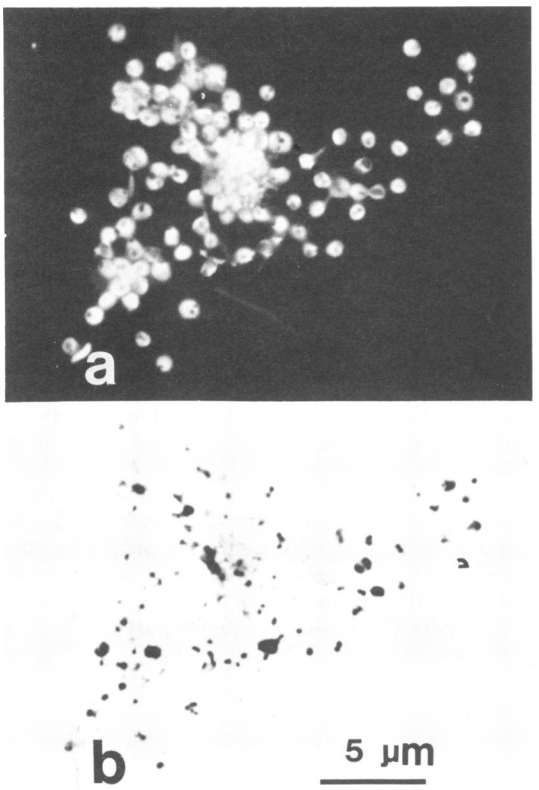

FIG. 7. *Fluorescent (a) and transmitted bright-field (b) image of an aggregate of blue-green algae from a freshwater lake.*

yellow fluorescence prevailed among respiring bacteria (Table 2). However, a significant amount of both respiring bacteria and bacteria without visible formazan was also accounted for by green fluorescing cells (Fig. 8a and b). Therefore, a determination of bacterial activity by means of the complex metachromatic effect of acridine orange seems to be problematic, particularly when analyzing natural samples.

There is no method which allows strict differentiation between actually active and inactive bacteria or actually respiring and nonrespiring bacteria, respectively. Differentiation is always subject to a definition which is determined by the properties of the method applied. Therefore, bacteria lacking formazan spots may actually be in a state of activity below the level of detectability. For the present technique, recognizability of formazan deposits in the bright-field image allows relatively unproblematic differentiation within bacterial cell sizes greater than 0.4 μm. In comparison to the determination of the total number of bacteria on polycarbonate filters by epifluorescence microscopy (16, 17), which has already been applied as a routine technique for some years in Kiel (Federal Republic of Germany), the identification of respiring bacteria does not appear to be more difficult. When counting respiring bacteria, errors due to subjectivity do not seem to be higher than those obtained by exclusive determination of the total number. This may also be confirmed by the specificity test shown in Fig. 9, which was performed with a sample taken from a freshwater pond rich in detritus.

Because there is still no mounting medium which is suited to accomplish the requirements for both microscopical images, emphasis was also given to treatments which intensify INT reduction. The presence of low concentrations of NaCN during incubation has been found to enhance electron transport system activity of phytoplankton homogenates (7). By adding NaCN (0.2 mM) to the water sample, it was possible to attain in some cases higher numbers of respiring bacteria, especially among the fraction of cells below 0.8 μm in length. The effect of NaCN on INT reduction has not yet been explained in detail. Further information is also required to evaluate whether addition of the activator promotes selectivity. The determination of all data presented here was performed without addition of the activator.

Some results of initial investigations by means of the present technique are shown in Table 3. The proportions of respiring bacteria were small. This may be a function of predominantly low temperatures of the water samples. When respiring biomass was measured, its proportion to the total biomass exceeded the proportion of the number of respiring bacteria. This was mainly due to the presence of voluminous filamentous bacteria which were counted as individual cells. The variable discrepancies between the percentages of the number of bacteria and the biomass indicate that interpretation of cell numbers exclusively may be misleading when evaluating bacterial activity.

TABLE 2. *Differentiation of natural bacterial populations by the metachromatic fluorescence of acridine orange*

Sample	Fluorescence of respiring bacteria		Fluorescence of nonrespiring bacteria	
	Green[a]	Yellow[a]	Green[a]	Yellow[a]
South River (S. Carolina)	7	93	15	85
Pond (Botanical Garden)	3	97	33	67
Kiel Bight (Baltic Sea)	7	93	24	76

[a] Number is percentage of the total number of respiring or nonrespiring bacteria.

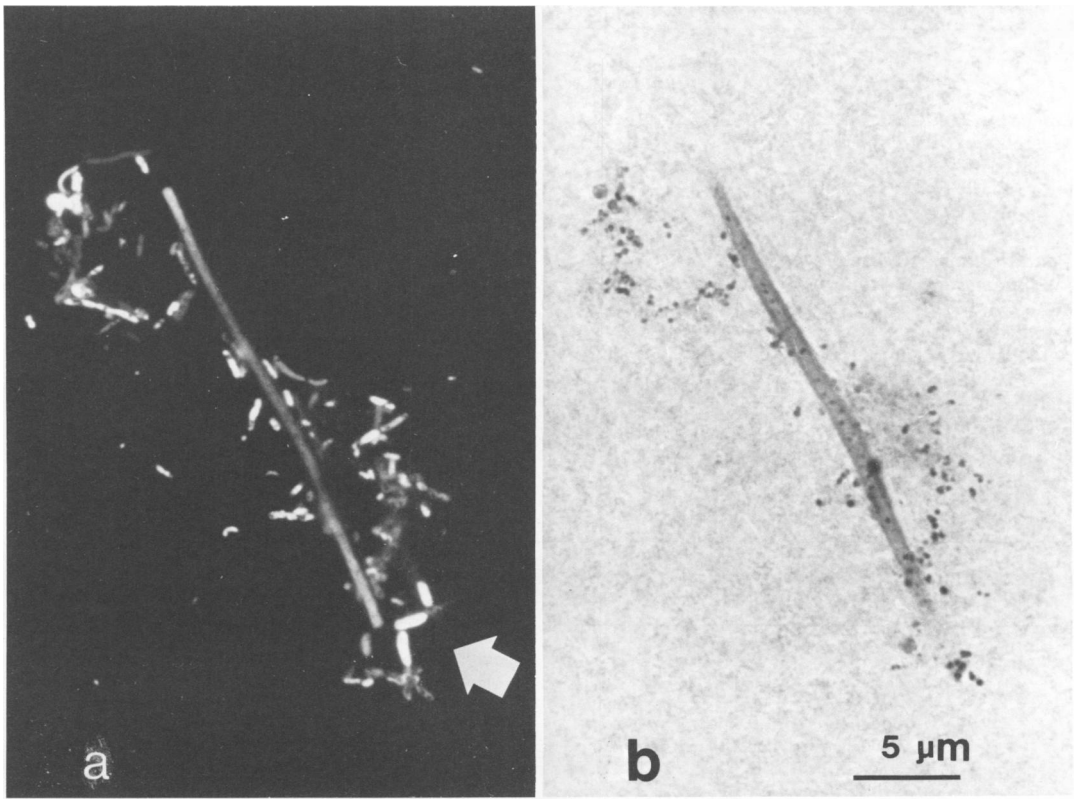

FIG. 8. *Fluorescent (a) and transmitted bright-field (b) image of a bacterial aggregate from a freshwater lake. In black and white microphotographs, green and yellow fluorescence of acridine orange is represented by a grey and a white (arrow) tone, respectively. Note the majority of green fluorescent respiring bacteria.*

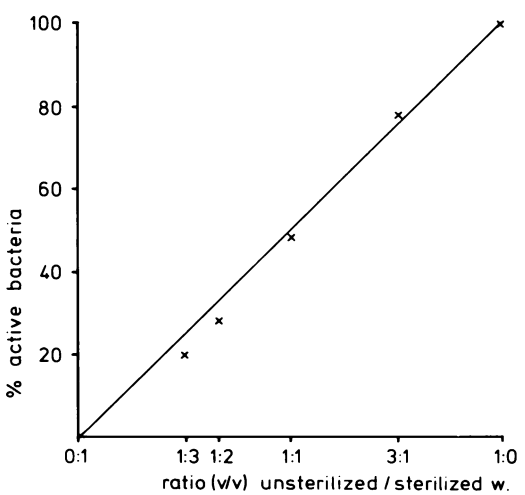

FIG. 9. *Specificity test (see text). Number of respiring bacteria in the unsterilized water sample = 100%.*

The initial investigations also proved that storage of processed water samples for up to 1 month did not cause loss of intracellular INT-formazan, which is a water-insoluble compound. This was confirmed by repeated counting of samples at different storage times.

Biomass calculation requires a size classification in the fluorescent image. However, size distribution of bacteria (including the registration of exceptional morphology) may also be regarded as an independent parameter which characterizes the structural composition of a population. When comparing different populations of respiring bacteria, differences in the size composition can be rather significant. As shown by the example in Table 4, the predominant size-class of one water sample coincided with the minimal proportion of classified bacteria of the other sample and vice versa.

The measurement of cell lengths by an eyepiece micrometer appears to be somewhat strenuous and time consuming. The application of a semiautomatic electronic particle analyzer (MOP/Kontron GmbH), which was tested recently, facilitated the microscopic procedure considerably and rendered differentiation more accurate.

It is intended to provide a further approach to

TABLE 3. *Number and biomass of optically differentiated bacterial populations from aquatic environments*

Sample	Water temp (°C)	Total no. ($\times 10^6$/ml)	Total biomass (mgC/m^3)	No. of respiring bacteria ($\times 10^6$/ml)	Biomass of respiring bacteria (mgC/m^3)	Share (%) of respiring bacteria	Share (%) of biomass of respiring bacteria
Westensee (lake nearby Kiel)							
14 March 1977	5.0	3.1		0.7		23	
18 July 1977	17.6	4.2		1.5		36	
Pond (Botanical Garden)							
4 January 1978	2.0	1.1	5.8	0.1	0.8	9	14
Pond (Kiel City)							
14 January 1978	1.9	3.7	16.7	0.2	3.4	5	20
Baltic Sea (Kiel Firth)							
28 March 1977	4.0	2.4		0.3		12	
Baltic Sea (Kiel Bight)							
2 March 1977	0.5	1.4		0.1		7	
10 January 1978	1.8	1.5	6.1	0.1	0.8	6	13

TABLE 4. *Share of respiring bacteria of different size classes*

Sample	Share (%)[a]					
	Rods or cocci 0.4 μm	Rods 0.4–0.8 μm	Rods 0.8–1.6 μm	Rods 1.6–2.4 μm	Rods >2.4 μm	Filaments
Westensee (lake nearby Kiel)	9	12	21	27	16	15
Baltic Sea (Kiel Firth)	25	17	19	9	13	17

[a] Total number of respiring bacteria = 100%.

the determination of actually active bacteria by using the technique described. Because selectivity due to incubation with certain labeled compounds is avoided, experimental conditions are assumed to be closer to in situ than with comparable methods. Due to the combined optical detection of respiratory activity and bacteria, it is likely that further studies with this technique will yield important information about the relation between bacterial activity, biomass, and the structure of their habitat.

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