Microbiology, Vol. 70, No. 5, 2001, pp. 594–599. Translated from Mikrobiologiya, Vol. 70, No. 5, 2001, pp. 687–693.

EXPERIMENTAL ARTICLES

Enumeration of Active Cells in the Bacterioplankton of the Rybinsk Reservoir Using 5-Cyano-2,3-Ditolyl Tetrazolium Chloride: A Comparison with Other Methods

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Received September 27, 2000; in final form, March 19, 2001

Abstract—The enumeration of actively respiring bacterial cells in different biotopes of the littoral zone of the Rybinsk Reservoir during the spring period of ice thaw using the fluorescent dye 5-cyano-2,3-ditolyl tetrazolium chloride showed that bacterial communities growing on the bottom surface of the ice cover and in water overgrown by higher aquatic plants were most active. The number of active cells among individual bacterial cells averaged 20% and reached about 40% among aggregated and filamentous bacterial cells. The results of the count of active bacteria by this method were compared with those obtained by other methods.

Key words: the number of active bacteria, the total amount of bacterioplankton, tetrazolium salts, epifluorescence microscopy.

The enumeration of bacteria in bodies of water by direct microscopic count takes into account not only active but also inactive (resting, damaged, and even dead) cells. The inactive cells can comprise an essential portion of aquatic microbial communities [1, 2]. Total bacterial count is by no means an important microbiological characteristic of bodies of water. However, when studying many ecological problems, such as the cycling of biogenic elements and the distribution of energy fluxes in ecosystems, the concentration of just metabolically active bacteria is of interest.

Among many methods proposed for this purpose [3], those using tetrazolium salts (artificial electron acceptors) seem to be most adequate. In metabolically active and, hence, actively respiring cells, tetrazolium salts are reduced to formazanes by cellular dehydrogenases. The number of the tetrazolium salts used in microbiological, histochemical, cytochemical, and enzymatic studies for the analysis of electron-transport (or respiratory) chains is about 50. One of the most frequently used fluorescent dyes in microbial ecology is 2-(*p*-iodophenyl)-3-*p*-nitrophenyl-5-phenyltetrazolium chloride (INT) [1, 4–7].

Beginning in the early 1990s, active cells in natural microbial populations were detected using another tetrazolium salt, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), which is similar to INT in its molecular structure and function [8–11]. The electron-transport chain reduces colorless CTC to water-insoluble formazane, which, when exposed to shortwave light at 420 nm, will emit red light at about 620 nm. Due to formazane accumulation, actively respiring cells can easily be detected by epifluorescence microscopy. In this case, both the total number and the number of actively respiring bacterial cells can be determined in a water sample using only one filter for its filtration.

The aim of the present work was to evaluate the fraction of active bacterioplankton in different biotopes of the littoral zone of the Rybinsk Reservoir during the spring period of ice thaw. To do this, we optimized experimental conditions for the enumeration of actively respiring bacterial cells with CTC, evaluated the number of CTC-reducing cells in different morphological groups of bacterioplankton, and compared these results with those obtained by other methods.

MATERIALS AND METHODS

Water and ice samples were collected on April 19–25, 1999, in the littoral zone of the Rybinsk Reservoir on the Volga. Water with temperatures from 0.2 to 2.9°C was sampled at depths of 0.2 to 1.5 m. The thickness of ice floes during sampling was 32–37 cm. The water and ice samples were placed in sterile glass flasks, promptly transported to the laboratory, and analyzed within 2 h after their sampling.

The total number of bacteria was determined by epifluorescence microscopy using the fluorescent dye 4',6-diamidino-2-phenylindole (DAPI) purchased from Sigma [12]. Viable heterotrophic bacteria were counted by plating appropriate water dilutions onto one-tenth–strength agar that contained fish meal and peptone (FPA/10). Actively respiring cells were detected by epifluorescence and light microscopy using CTC (Poly-

0026-2617/01/7005-0594$25.00

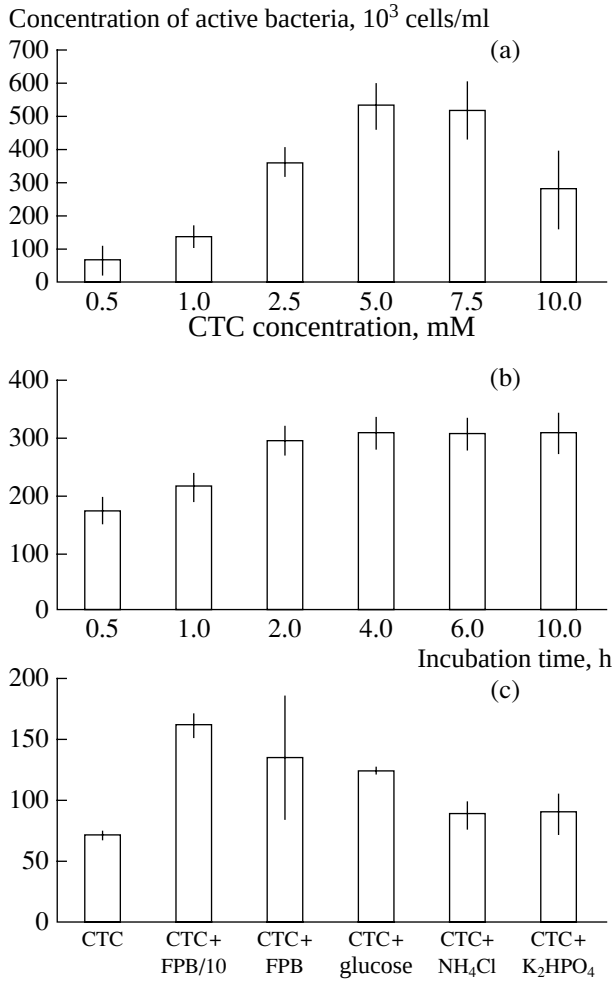

Fig. 1. Effect of (a) CTC concentration, (b) incubation time, and (c) incubation conditions on the count of actively respiring cells in the littoral water of the Rybinsk Reservoir.

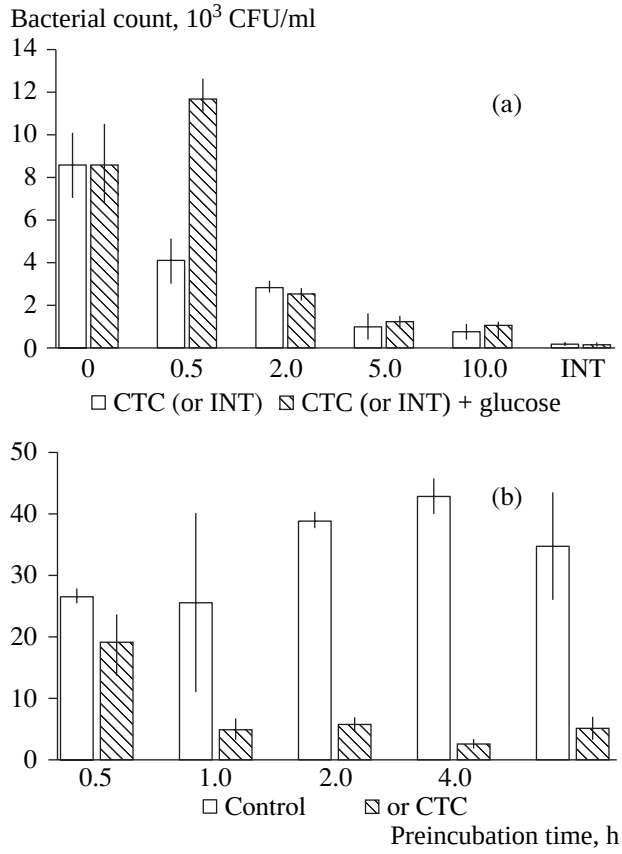

Fig. 2. Effect of (a) tetrazolium salts and (b) preincubation time on the count of bacterial cells producing colonies on FPA/10.

science Inc., Germany) and INT (Sigma), respectively [4, 8]. The number of bacterial cells with intact nucleoids was determined by the method of epifluorescence microscopy with DAPI, modified as described by Zweifel and Hagstrom [13]. Metabolically active bacteria were identified using nalidixic acid, an inhibitor of cell division [14]. No less than 400 cells were examined on each filter, using either a Lumam-I1 luminescence microscope or an Ergaval light microscope operated at a magnification of 1000×. All counts were performed in triplicate.

RESULTS

In preliminary experiments, we optimized conditions for the identification of actively respiring bacterial cells to avoid interfering factors, such as the extracellular accumulation of formazane and its formation through the chemical reduction of tetrazolium salts. According to some data in the literature, the optimum concentration of CTC for the detection of bacteria from different habitats varies from 0.5 to 7 mM [10, 11]. In our experiments, the maximum number of actively respiring cells in the bacterioplankton of the Rybinsk Reservoir was detected at a CTC concentration of 5 mM (Fig. 1a).

Another important parameter is the incubation time. If this time is too short, small and slow-respiring cells produce low amounts of formazane, so that such cells can hardly be detected under a microscope. Conversely, if the incubation time is too long, this may lead to the activation of resting cells (the so-called flask-induced effect) and to the extracellular formation of formazane granules, which can be confused under a microscope with microbial cells. In the case of CTC, the incubation time is varied from 10 min to 1 day [8, 10, 15]. In our experiments, no noticeable increase in the number of CTC-reducing cells was observed when the incubation time was longer than 2 h (Fig. 1b). In view of this, all further experiments were performed by incubating the bacterioplankton for 2 h. At this incubation time, the flask-induced effect could not be very strong, as the experiments were performed at low temperatures close to those in situ.

It is known that the enrichment of samples with organic substrates enhances the formation of forma-

Table 1. The abundance of different groups of bacterioplankton in the littoral zone of the Rybinsk Reservoir and the relative content of CTC-reducing cells as of April 24–25, 1999

Sample no.	Sampling location	Individual		Microcolonies		Attached to detritus		Filamentous	
		10^3 cells/ml	CTC-reducing cells, %	10^3 cells/ml	CTC-reducing cells, %	10^3 cells/ml	CTC-reducing cells, %	10^3 cells/ml	CTC-reducing cells, %
Nearshore littoral zone									
1	Open water	3008.4	21.0	223.4	23.5	188.5	42.3	69.8	32.0
2	The bottom surface of thawing ice	7182.1	23.9	577.7	62.7	5343.9	61.4	26.3	71.4
3	Water overgrown by macrophytes	5335.9	31.6	2967.7	58.8	6081.5	44.8	233.9	69.1
4	Water beneath the ice	2690.2	17.8	194.6	24.3	508.7	34.0	20.4	21.3
Offshore littoral zone									
5	The bottom surface of thawing ice	6419.8	26.0	47.8	50.6	5478.3	40.9	11.9	55.6
6	Water overgrown by macrophytes	4857.4	10.1	98.5	50.0	191.8	37.6	36.3	50.0
7	Open water	2237.8	9.3	40.2	66.7	579.5	35.8	11.5	22.2

Table 2. The abundance of active bacteria in the littoral zone of the Rybinsk Reservoir determined by different methods

Sample no.	DAPI-stained cells, 10^3/ml	CTC-reducing cells		INT-stained cells		Cells with nucleoids		Cells responding to nalidixic acid		Cells producing colonies on FPA/10	
		10^3 cells/ml	as a percentage of DAPI-stained cells	10^3 cells/ml	as a percentage of DAPI-stained cells	10^3 cells/ml	as a percentage of DAPI-stained cells	10^3 cells/ml	as a percentage of DAPI-stained cells	10^3 cells/ml	as a percentage of DAPI-stained cells
1	3490 ± 265	971 ± 155	27.8	901 ± 165	25.8	1768 ± 53	50.7	1197 ± 186	34.3	63 ± 33	1.8
2	13130 ± 286	5241 ± 229	39.9	4548 ± 580	34.6	4672 ± 147	35.6	4056 ± 1414	30.9	949 ± 476	7.2
3	14619 ± 532	6153 ± 176	42.1	3071 ± 446	21.0	5708 ± 461	39.1	2871 ± 419	19.6	329 ± 154	2.3
4	3414 ± 369	820 ± 96	24.0	1179 ± 116	34.5	1268 ± 287	37.2	1204 ± 186	35.3	44 ± 4	1.3
5	11955 ± 668	3994 ± 391	33.4	2229 ± 756	18.6	2532 ± 523	21.2	698 ± 180	5.8	16 ± 8	0.1
6	5184 ± 317	614 ± 90	11.8	567 ± 113	10.9	1298 ± 197	25.0	985 ± 90	19.0	84 ± 17	1.6
7	2869 ± 130	448 ± 51	15.6	392 ± 43	13.7	886 ± 138	30.8	543 ± 239	18.9	56 ± 5	2.0

zane by microbial cells [8, 11]. Among the substrates tested (glucose, fish meal–peptone broth (FPB), FPB/10, and some nitrogen- and phosphorus-containing compounds), the most beneficial effect on the estimation of CTC-reducing cells was exerted by FPB/10, which was added to water samples at a ratio of 1 : 1 (Fig. 1c). The addition of FPB intensified the extracellular formation of formazane crystals, thereby interfering with bacterial count. It should be noted that Rodriguez *et al.*, who proposed to use CTC for the enumeration of viable bacteria, incubated their samples of marine, ground, and waste waters with a nutrient R2A broth added at a ratio of 1 : 2 [8]. Plating the Rybinsk Reservoir water samples onto FPA, FPA/10, and R2A plates revealed viable bacteria in amounts of 868 ± 341, 12272 ± 1844, and 14694 ± 1617 CFU/ml, respectively, the difference between two last counts being statistically insignificant. In view of this, further bacterial counts were performed using FPA/10 as the nutrient medium. The incubation of heterotrophic bacterioplankton from the Rybinsk Reservoir with glucose stimulated the reduction of CTC to a greater degree than its incubation with NH_4Cl or K_2HPO_4, suggesting that, at the time of sampling, the bacterioplankton was limited in carbon sources, rather than in nitrogen or phosphorus sources.

Because of the toxic effect of CTC on microbial cells [16], the number of active bacteria determined using CTC may be underestimated. Our experiments also showed that the preliminary 2-h incubation of the Rybinsk Reservoir water with CTC decreased the number of bacterial colonies grown on FPA/10 (Fig. 2a).

The relative amounts of bacterial colonies grown on FPA/10 at CTC concentrations of 0, 0.5, and 10 mM were 100, 47.7, and 8.6%, respectively. The addition of glucose diminished the toxic effect of CTC. At the CTC concentration of 0.5 mM, the number of bacterial colonies grown in the presence of glucose turned out to be even greater (135.8%) than in the control. The estimated number of bacteria decreased as soon as after 1 h of incubation with CTC (Fig. 2b). In these experiments, we evaluated the effect of CTC on the ability of bacteria to produce colonies on solid media; therefore, the results of these experiments refer only to a culturable portion of the Rybinsk Reservoir bacterioplankton. The results presented in Fig. 2a show that INT, which is used for the enumeration of active bacterial cells for many years [1, 4–7], is a more toxic dye than CTC. Indeed, in the presence of 1 mM INT, the percentage of grown colonies was as low as 1.8% of the control.

Analysis showed that individual cells are the most abundant and the least active group of the bacterioplankton. The fraction of actively respiring cells in this group varied from 9.3% in the littoral water to 31.6% in the water that was overgrown by macrophytic plants, averaging 20% (Table 1). Among filamentous and aggregated bacterial cells (i.e., those associated with detritus and those aggregated into microcolonies), the fraction of CTC-reducing cells was about two times larger than among individual cells. Among the filamentous cells grown on the bottom surface of the thawing ice (sample 2), the fraction of CTC-reducing cells reached 70%. The total concentration of CTC-reducing cells varied from 0.47×10^6 to 6.4×10^6 cells/ml, making up 12–43% (on the average, 27%) of the total bacterioplankton count (Table 2). The fraction of active cells in the bacterioplankton than grew on the bottom surface of the ice, where it could use the organic substances of attached detritus and algae (samples 2, 5), and in the water overgrown by higher aquatic plants (sample 3) was greatest.

The average percentage of actively respiring cells estimated with the use of CTC was higher than that estimated with the use of INT (26.8 and 21%, respectively) (Table 2). The difference between these two estimates increased with the concentration of detritus. The method of viable bacterial count with the use of nalidixic acid yielded a lower estimate for actively dividing cells, which made up about 18% of the total bacterial count. The fraction of dividing cells in the offshore littoral zone (samples 1–4) was lower than in the nearshore littoral zone. The number of bacterial cells with intact nucleoids estimated using DAPI was higher (31.3%) than the number of such bacteria estimated by other methods. The enumeration of viable bacteria by plating the water and ice samples on FPA/10 showed that such bacteria amounted to 0.1–7.2% of the total bacterial count.

DISCUSSION

The enumeration of active bacteria using tetrazolium salts is a rapid, simple, low-cost, and high-sensitivity procedure based on the determination of the activity of the electron-transport chain, i.e., the metabolic activity of cells. The most significant shortcomings of this method are the toxic effect of tetrazolium salts on bacteria, because of which it is applicable to the bacteria that grow only under aerobic or microaerobic conditions. Furthermore, formazane granules are poorly visible against the background of opaque substances (such as filters) and are soluble in oil, which is used in immersion microscopy. Small bacterial cells and those with a low activity of the electron-transport chain accumulate formazane in small amounts and therefore are poorly visible under light microscopes. Upon the complex analysis of samples by epifluorescence microscopy, the metabolically active bacteria accumulating formazane are poorly visible against the background of acridine orange and reduce the fluorescence of DAPI, another dye used in such studies [7].

CTC, which emits red fluorescent light, lacks some of these shortcomings and has already found extensive use in the detection of active bacteria in the sea, fresh, ground, and waste waters, soils, and biofilms [8–11]. The data obtained with the use of this fluorescent dye are much easy to interpret than those obtained with the use of other tetrazolium salts or nalidixic acid. The formazane-containing bacterial cells exposed to blue light are easily visible on polycarbonate filters under epifluorescence microscopes. This allows a simultaneous count of the total and actively respiring bacterial cells on the same filter. Unlike the INT-based method, the method of CTC-based bacterial count can easily be automated. CTC can be used for the detection of not only aerobic but also some facultatively and even obligately anaerobic metabolically active bacteria [17]. Of great promise is the use of CTC in studying biofilms formed on various opaque materials.

On the other hand, to be reduced by metabolically active cells, CTC requires the addition of high concentrations of substrates, which may activate some dormant cells and thus interfere with the count of active bacterial cells. Actually, the CTC-based method detects not really but potentially active bacterial cells. Another disadvantage of CTC is that it can be reduced abiotically by many chemical compounds that may be present in analyzed samples. In view of this, the application of CTC requires the optimization of experimental conditions for an adequate reduction of this dye by metabolically active microbial cells. The optimization includes the choice of nutrient media, CTC concentration, the time and the temperature of incubation, the proper choice of light filters for epifluorescence microscopy, etc. In combination with other modern research methods employing molecular and immunofluorescent probes, the CTC-based method can provide useful

information on relationships in natural microbial communities and the role of bacteria in various ecosystems.

CTC can reveal the factors that control the development of microbial communities in various natural ecosystems. For instance, a low content of actively respiring bacterial cells in a community may suggest that the development of this community is limited by low ambient temperatures or nutritional deficiency. Conversely, a high content of active bacterial cells may suggest that bacteria are consumed by protozoans, since it is known that aquatic microbial communities evolved various mechanisms (such as the activation of metabolism and cell division) protecting them from annihilation by predators [7].

As was shown earlier, the bacterioplankton of the Rybinsk Reservoir in winter contains 24–59% of actively respiring cells, which are stained by INT, and 3–18% of dead cells, which are stained by primulin. The concentration of bacteria reached a maximum at the ice–water interface, where detritus and phytoplankton accumulate, by the end of the period of ice growth [18]. The relative content of actively respiring cells in the bacterioplankton in winter was greater than during the spring period of ice thaw, although the concentrations of actively respiring cells in winter and spring were about the same. This suggests that drastic changes in environmental conditions in spring are accompanied by a concurrent increase in the number of metabolically active, resting, and dead cells.

The population density of active bacteria determined by different methods in the same water and ice samples appreciably differed (Table 2), presumably because of the ability of various dyes to interact with different metabolic processes in microbial cells. As a result, tetrazolium salts detect cells with the active electron-transport chain and intact cell membranes. It remains unclear whether or not inactive or physiologically stressed bacterial cells can reduce tetrazolium salts, particularly CTC. Although all viable cells, including damaged and resting ones, must have nucleoids, the number of CTC-reducing cells is sometimes greater than the number of nucleoid-containing cells (samples 2, 3, 5). It is possible that some bacterial cells fail to be stained with DAPI because of a poor diffusion of this dye through cellular membranes. Furthermore, the intensity of the fluorescence of bacterial cells treated with DAPI depends on the tertiary structure of DNA and environmental conditions. For instance, UV radiation alters the structure of cellular polymers in bacteria and thus diminishes their fluorescence, which leads to an underestimation of their number [2, 13].

Plating on solid nutrient media makes it possible to detect the bacterial cells that can produce colonies. In nature, however, most bacteria occur in the viable but nonculturable state, in which they are unable to form colonies. Moreover, some bacteria are basically nonculturable [19].

The data presented in this paper show that, typically, the number of active cells in the bacterioplankton of the Rybinsk Reservoir during the period of ice thaw does not exceed 50% (Table 2). Therefore, more than half the cells are resting, damaged, or dead. When considering these data, it should be taken into account that, unfortunately, neither of the bacterial count methods employed yields adequate results [20].

To conclude, the CTC-based method of bacterial count can efficiently be used in ecophysiological and ecotoxicological studies, as well as for the microbiological monitoring of natural waters and for controlling the process of wastewater treatment, provided that this method is optimized with respect to the influence of oxygen, pH, redox potential, temperature, and some other physicochemical factors on the process of CTC reduction and accumulation.

ACKNOWLEDGMENT

This work was supported by grants nos. 98-04-48417 and 99-05-64378 from the Russian Foundation for Basic Research.

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