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Toxic Effects on Bacterial Metabolism of the Redox Dye 5-Cyano-2,3-Ditolyl Tetrazolium Chloride

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The monotetrazolium redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) has been used as a vital stain of actively respiring bacteria for several years. In this study, inhibitory effects on bacterial metabolism of this redox dye have been examined in a brackish water environment (Kiel Fjord, Germany) and a freshwater environment (Elbe River, Germany). As the results from time series experiments (1 to 10 h) show, bacterial growth and respiration of the investigated natural communities were clearly reduced by CTC supply. Compared with untreated controls (100%), CTC-treated samples showed distinctly lower heterotrophic bacterial plate counts (0 to 24 and 11.8 to 23.7%, respectively), bacterial production (0.9 to 14.1 and 1.1 to 9.6%, respectively), bacterial respiration (4.1 to 9.4 and 6.8 to 43.8% for several concentrations of ^{14}C-labeled glucose), and [^{14}C]glucose incorporation (0.2 to 4.2%). Additionally, toxicity of CTC was demonstrated by luminescence in a Microtox bioassay. CTC concentrations of 0.1 and 5.0 μM required only 15 min for decreases of approximately 50 and 100%, respectively. The suppression of CTC on several bacterial metabolic processes suggests that determination by the CTC technique underestimates the actual number of active cells distinctly. This conclusion is confirmed by the comparison of generation times calculated on the basis of thymidine uptake data and active bacterial counts determined by the CTC assay and microautoradiography. While unrealistic short generation times (0.5 to 5 h) resulted from the CTC assay, the generation times calculated according to microautoradiography ranged within values (7 to 21 h) reported elsewhere for comparable aquatic environments. The inhibitory effect of CTC demonstrated in our experiments is an aspect with regard to the application of this tetrazolium dye for the estimation of active bacteria in natural aquatic environments which hitherto has not been considered.

Determination of the number of actively metabolizing bacteria is an important objective of aquatic microbiologists. Several methods have been applied in this context, but none of them have been proved to be fully adequate. Previous investigations have shown that the number of metabolically active bacteria is underestimated by the plate count method. A more realistic approach is the use of nalidixic acid, a specific DNA gyrase inhibitor, which suppresses cell division in many gram-negative bacteria. Growing cells become elongated and can be detected microscopically (19). Viable counts detected by this method were up to 3 orders of magnitude higher than plate counts (20). The problem with this technique is that elongation may occur too slowly in the absence of an exogenously supplied carbon source, or it may be insufficient for microscopic detection (30).

Microautoradiography—a tracer technique based on the assimilation of radiolabeled organic solutes—combined with epifluorescence microscopy has also been applied as an alternative approach for enumeration of active heterotrophic bacteria (15, 23, 40). There are potential problems with substrate selectivity and standardization of the procedure, however (26).

Counts comparable to those reported for the nalidixic acid and microautoradiographic techniques resulted from measurements of electron transport system (ETS) activity (22, 23, 40). The universality of ETS in living cells allows this indirect measurement of respiratory activity (25) in a wide range of organisms, including procaryotes (an exception are, e.g., the group N streptococci) and eucaryotes (reviewed by Savenkoff et al. [32]). Tetrazolium salts are used as artificial electron acceptors which are reduced within the respiratory chain. This results in the intracellular formation of colored formazans, equivalent to the respiratory activity of cells. The tetrazolium dye 2-(*p*-iodophenyl)-3-*p*-(nitrophenyl)-5 phenyltetrazolium chloride (INT) was mainly used for this purpose in aquatic environments (1, 7, 19, 22, 32, 40, 47). Zimmermann et al. (47) first combined the INT assay with the acridine orange direct count (AODC) method for the simultaneous determination of total bacterial numbers and the number of INT-reducing (actively respiring) bacterial cells by counterstaining INT-treated samples with acridine orange. This method was later modified to improve visual detectability (7, 40).

In recent publications, the tetrazolium dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) was introduced to determine the number of metabolically active bacteria. This tetrazolium salt had been described as an indicator of respiratory activity in tumor cells (34, 37, 38) and was first applied in ecological studies by Rodriguez et al. (30) in natural and nutrient-amended water samples. The advantage of CTC compared with other dyes (e.g., INT) is the simple detection due to the red fluorescence in the reduced form (30). The CTC method has been used extensively in studies of several aquatic environments, including municipal wastewater and groundwater (30), seawater (12, 30), drinking water (5, 33), biofilms (33), as well as with soil samples (43, 45). The CTC assay has been applied to determine the viability of coliform bacteria exposed to antarctic conditions (35) and was also used in combination with a fluorescent antibody technique to detect enterohemorrhagic *Escherichia coli* in water (28). Furthermore, CTC reduction combined with rhodamine 123 and [^{3}H]uridine uptake has been applied in the assessment of disinfections on metabolic activity of bacteria associated with biofilms (44).

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Although the numbers of active bacteria determined by the CTC assay were found to be higher than the numbers determined by other methods (5, 30, 33, 35, 43), a relatively small percentage of the total bacterial numbers (determined by the AODC or 4′,6-diamidino-2-phenylindole method) was found to be respiratorically active in unsupplemented natural samples, especially from oligotrophic environments. Generally, ratios of CTC-reducing bacteria were reported as being below 10% of the total bacterial numbers under natural environmental conditions (12, 30, 33, 43). There are several explanations for these low ratios. (i) A common hypothesis is that up to 90% or more of the bacterial community inhabiting oligotrophic environments is dormant (22, 24, 39). Zimmermann et al. (47) deduced from results obtained with the INT method that bacteria lacking formazan spots may be "in a stage of activity below the level of detectability." The tendency towards increasing portions of inactive bacteria in more oligotrophic waters (reviewed by Gasol et al. [12]) has been related to nutrient limitation. (ii) Not all bacteria are able to reduce tetrazolium salts (42). (iii) The assay is not sensitive enough to detect low respiration rates of microorganisms, which might especially be a problem for the detection of small bacteria (7, 12). This methodological problem has been discussed previously for the INT approach (22, 47). (iv) Bacterial metabolic activity is directly suppressed by the chemical CTC.

To our knowledge, there are only a few hints about suppression of bacterial metabolic activity by tetrazolium salts and their reduced components (5, 17, 29). We therefore decided to study possible inhibitory effects of CTC on bacterial metabolic activity in natural freshwater and brackish water samples in greater detail to test the validity of the commonly applied CTC method.

MATERIALS AND METHODS

Sampling. Experiments were performed during the summer of 1995. Water samples were obtained from a brackish water environment and a freshwater environment (the inner part of the Kiel Fjord [Germany] and the Elbe River close to the city of Magdeburg [Germany], respectively). Samples were collected with sterilized glass bottles.

CTC assay. The monotetrazolium redox dye CTC (Polyscience, Inc., Warrington, Pa.) was used for fluorescence microscopic detection of intracellular insoluble CTC-formazan (30). CTC working solutions were prepared with sterilized distilled water by diluting a 50 mM stock solution (according to Polyscience data sheet 486 [27]) immediately before use. Stock solution and chemical were stored at 4°C. Triplicate 5-ml water samples treated with different concentrations of CTC were incubated in the dark under agitation at room temperature. Incubation was terminated by addition of 1% formalin. After incubation, bacteria were collected on 0.2-μm-pore-diameter black polycarbonate filters (Poretics Co., Livermore, Calif.) and stored in the dark at 4°C until counted. CTC-formazan fluorescence was detected with an Axioplan fluorescence microscope (Zeiss) equipped with a 450-nm excitation filter, a 510-nm beam splitter, and a 520-nm emission filter combination. This filter set was found to be most effective for the detection of the red-orange formazan fluorescence compared with other filter combinations. Formazan-containing cells were counted with a New Porton grid, an oil immersion objective (Zeiss), and a total magnification of ×1,000. After the testing of several concentrations of CTC (1, 2, 5, and 10 mM) and incubation times (1, 2, 4, 6, 8, and 10 h), the highest numbers of CTC-reducing bacteria were obtained for 5 mM CTC and 4 h of incubation in both environments. A CTC concentration of 5 mM was therefore used in all time series experiments.

Total bacterial number. Total bacterial counts were estimated by the AODC method (14, 46). By this method, a simultaneous differentiation of red-orange formazan-containing cells and acridine orange-stained cells was not possible. Therefore, each of the corresponding acridine orange- and CTC-treated subsamples was filtered on black 0.2-μm-pore-diameter polycarbonate filters (Poretics Co.) and counted. Total bacterial counts were determined with the same fluorescence microscopic equipment as described above, but with a filter combination with a 365-nm excitation filter, a 395-nm beam splitter, and a 397-nm emission filter (Zeiss).

Microautoradiography. Microautoradiography was used to determine the metabolic activity of individual bacterial cells. We employed [^{3}H]leucine (10 μCi ml^{-1}) as a substrate for bacterial uptake. In principle, preparation was performed according to the protocol described by Carman (4). After 1 to 10 h of incubation at room temperature, samples were fixed with formalin (1% [vol/vol]) and filtered onto 0.2-μm-pore-diameter polycarbonate filters (Poretics Co.). The filters were mounted top down on slides coated with semisolid autoradiographic emulsion (EM-1; Amersham, Little Chalfont, Buckinghamshire, United Kingdom). After solidification of the emulsion by cooling, slides were stored in the dark at 4°C and exposed for 9 days. The preparations were then developed (Kodak D19) and dried. The filters were subsequently gently recovered from slides. By this procedure, cells remained embedded in the emulsion. The uptake of [^{3}H]leucine by individual bacterial cells was identified microscopically by the accumulation of silver grains (spots) in the emulsion. The active part of the investigated natural bacterial community was derived from the relationship between the number of cells marked with silver grains and the total bacterial numbers, determined by the AODC method as described above.

Heterotrophic bacterial plate counts. Numbers of CFU were determined on ZoBell agar 2116e (48). The medium was prepared by adding 8 g of NaCl per liter of distilled water for the samples taken from the inner Kiel Fjord, while tap water was used for the samples from the Elbe River. Samples were incubated for 1 to 10 h at room temperature with 5 mM CTC or without additions (controls). Triplicate 0.2-ml treated and untreated samples were spread on agar plates. CFU were counted after 14 days. Furthermore, the effect of several CTC concentrations (1, 2, 5, and 10 mM) on viable counts after an incubation of 4 h was determined in the same manner.

[^{3}H]thymidine uptake. The incorporation of tritiated thymidine was used as an indirect measure of bacterial production (10). Triplicate samples of 5 ml each were treated with 20 nM [^{3}H]thymidine (Amersham; specific activity, 43 Ci mmol^{-1}) and, together with one formalin (1% [vol/vol])-treated control, were incubated for 1 to 10 h in plastic vials in the dark at room temperature. Incubations were terminated by addition of 1% (vol/vol) formalin, and the samples were then collected on 0.2-μm-pore-diameter polycarbonate filters (Poretics, Co.) by vacuum filtration (<200 mm Hg [<26,664, Pa]). These filters were subsequently rinsed several times with ice-cold 5% trichloroacetic acid. [^{3}H]thymidine incorporation was measured by the liquid scintillation technique. Filters were placed into picovials (Beckmann) filled with 5 ml of scintillation cocktail (Lumagel) and measured in a Beckmann scintillation counter 12 h after filtration.

[^{14}C]glucose incorporation and respiration. Net incorporation and respiration of glucose were measured with [^{14}C]glucose as a tracer (Amersham; specific activity, 295 mCi/mmol). This procedure was applied to only the Kiel Fjord samples. Forty-milliliter water samples were treated with two different concentrations (0.4 and 17.5 nmol liter^{-1}) of D-[U-^{14}C]glucose according to the method of Gocke (13) for determining glucose turnover and maximal glucose uptake. Duplicates and one formalin (1%)-fixed control of CTC-treated (5 mM, final concentration) and untreated samples of each glucose concentration were incubated over 1, 2, 4, 6, and 10 h in glass bottles at room temperature. Incubation was terminated by 1% (vol/vol) formalin. After incubation, 20-ml portions of the fixed samples were filtered on 0.2-μm-pore-diameter polycarbonate filters to determine the incorporation of [^{14}C]glucose according to the procedure described for [^{3}H]thymidine uptake. The remaining 20 ml of sample was transferred to respiration bottles to determine [^{14}C]glucose respiration. Five hundred microliters of 5 N HCl was then injected to drive out the $^{14}CO_2$. A strip of filter paper soaked with 200 μl of ethanolamine and located above the sample in the respiration bottle was used to absorb the emitted $^{14}CO_2$ over a period of 12 h. The efficiency of ^{14}C recovery was derived from a similar volume of water treated with [^{14}C]bicarbonate. The amount of $^{14}CO_2$ was measured by the liquid scintillation technique described above. One hundred microliters of Methyl Cellosolve was added to the scintillation cocktail to intensify the solution of ethanolamine.

Toxicity bioassay. The toxicity of CTC on bacteria was determined with a standard luminescence test, the Microtox bioassay (2, 3). In this highly reproducible biotest, the acute toxic effect of chemical compounds is related to the decrease of light emission by a bacterial test organism under short-time exposure in reference to an untreated control. We used a commercial test set (LUMIStox; Dr. Lange GmbH, Düsseldorf, Germany), including a test strain (*Photobacterium phosphoreum*), a luminometer, and chemical solutions. After rehydration of the test strain in the reactivation solution provided by the manufacturer, 0.5 ml of bacterial suspension was amended with 0.5 ml of 2% NaCl solution containing several CTC concentrations (0.1, 0.5, 1.0, 5.0 μM [final concentration]). Samples were incubated at 15°C in cuvettes. The relative luminescence decrease in CTC-treated samples was measured in reference to a control (untreated 2% NaCl solution) after incubation times of 15 and 30 min (duplicates per concentration and control). Toxicity is expressed in terms of the EC_{50}, which is the CTC concentration causing 50% inhibition. Because of a slight drift in the light emission of the controls over time, all values were rectified by a correction factor. The quality of the test strain was examined by addition of 0.5 ml of LUMIStox standard solution to 0.5 ml of bacterial suspension. A quality standard was considered acceptable if the resulting light inhibition ranged between 40 and 60%.

RESULTS

Relationship between CTC-reducing bacteria and other bacterial counts. The number of formazan-containing bacteria (CTC) as a measure of actively respiring bacteria was compared with total bacterial numbers (AODC) and the number of

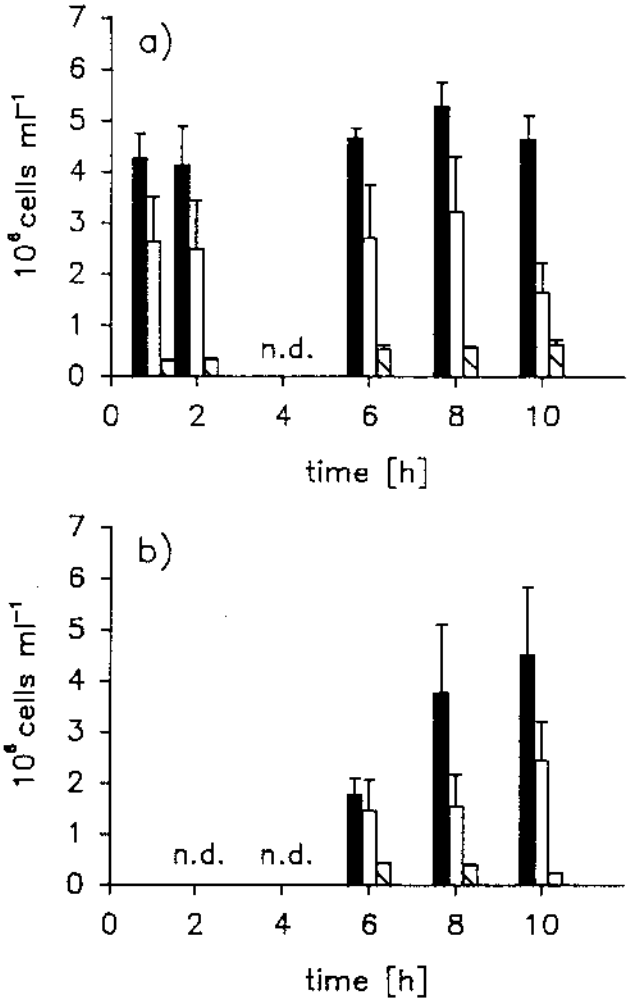

FIG. 1. Comparison of AODC (solid bars), numbers determined by microautoradiography (open bars), and numbers of formazan-producing cells (CTC [hatched bars]) throughout a time series experiment (0 to 10 h of incubation at room temperature). (a) Elbe River. (b) Kiel Fjord. n.d., not determined. Error bars indicate standard deviation ($n = 3$).

metabolically active bacterial cells determined by microautoradiography over an incubation period of 1 to 10 h. Totals of 6.4 to 13.6 and 5.4 to 19.4% of CTC-reducing bacteria were obtained in the Elbe River and Kiel Fjord, respectively, in relation to the corresponding total bacterial numbers (AODC, 100%) throughout this experiment (Fig. 1). Numbers determined by microautoradiography clearly ranged above the number of formazan-producing cells (CTC) by factors of 2.6 to 9.6 and 3.3 to 10.1 in the Elbe River and Kiel Fjord, respectively (Fig. 1).

Effect of CTC on viable counts. The number of heterotrophic bacteria growing on ZoBell agar (CFU) was drastically reduced by CTC (5 mM) during the time series experiment (Fig. 2). CFU in the CTC-treated samples ranged between 11.9 and 23.7 and 0 and 2.4% for the Elbe River and Kiel Fjord, respectively, in reference to the untreated controls (100%). When the 1, 2, 5, and 10 mM CTC-treated samples were compared, CFU continuously decreased with increasing CTC concentrations (Fig. 3). Even a 1 mM CTC solution reduced viable counts from 26,000 (control) to 7,000 and from 52,700 (control) to 1,200 in the Elbe River and Kiel Fjord, respectively.

Inhibition of thymidine uptake. Bacterial growth as measured by [^{3}H]thymidine incorporation was distinctly affected in the 5 mM CTC-treated samples compared with the controls. While thymidine accumulated continuously in the untreated samples from 71.8 to 1,034.4 pmol liter^{-1} (Elbe River) and from 39.4 to 580.5 pmol liter^{-1} (Kiel Fjord) throughout the 10-h incubation period, incorporation of the substrate ceased after 2 h in the CTC-treated samples (Fig. 4). Thymidine uptake was only 8.2% (Elbe River) and 14.1% (Kiel Fjord) of the values measured for the controls (100%) after 1 h. The various other CTC concentrations (1, 2, 5, and 10 mM) reduced thymidine uptake clearly to <6% (Elbe River) and <4% (Kiel Fjord) (data not shown).

Suppression of glucose incorporation and respiration. Similar results were found for the incorporation and respiration of the two concentrations of [^{14}C]glucose applied in samples from the Kiel Fjord. In the untreated controls, glucose incorporation increased from 4.8 to 105.0 pmol liter^{-1} for the lower glucose concentration (0.4 nmol) and from 29.4 to 1,034.1 pmol liter^{-1} for the higher glucose concentration (17.5 nmol) (Fig. 5a and b). In the CTC-treated samples (5 mM), uptake ranged distinctly below these values (0.1 to 0.9 pmol liter^{-1} and 0.1 to 2.9 pmol liter^{-1}, respectively) and stagnated over the 10-h incubation period (Fig. 5a and b).

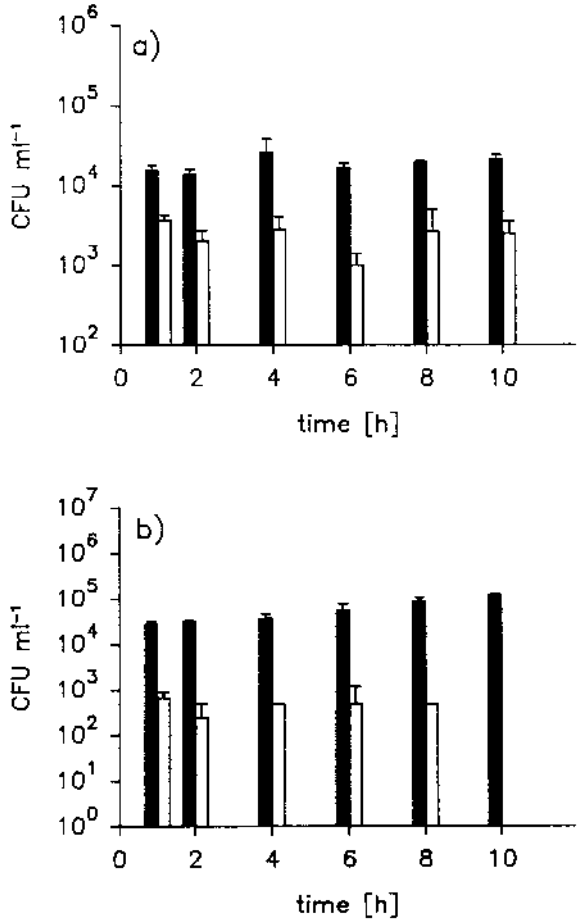

FIG. 2. Effect of CTC supply on heterotrophic bacterial plate counts (CFU) determined on ZoBell agar throughout 10 h of incubation at room temperature. Results represent a comparison of CTC-treated (5 mM [open bars]) and untreated (solid bars) samples. (a) Elbe River. (b) Kiel Fjord. Error bars indicate standard deviation ($n = 3$).

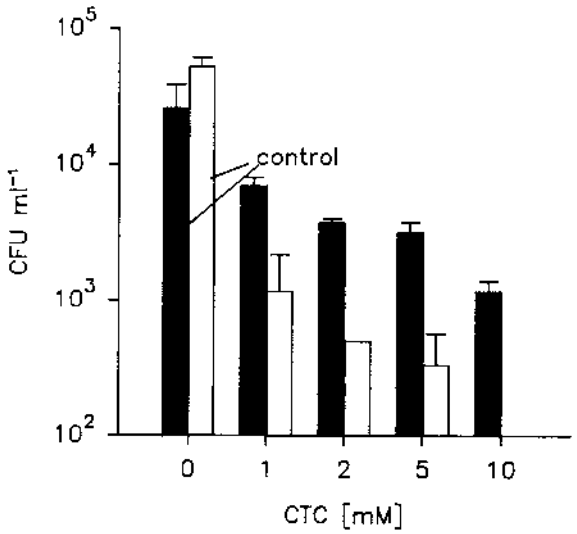

FIG. 3. Effect of increasing CTC concentrations on heterotrophic bacterial plate counts (CFU) determined on ZoBell agar (4 h of incubation at room temperature). Error bars indicate standard deviation ($n = 3$). Solid bars, Elbe River; open bars, Kiel Fjord.

Respiration was inhibited in a similar way (Fig. 5c and d). While $^{14}CO_2$ accumulated in the controls for both [^{14}C]glucose concentrations over time (2.8 to 16.7 and 11.6 to 197.5 pmol liter^{-1}, respectively), the values after CTC treatment clearly ranged below control values (1.2 to 1.5 and 0.6 to 18.5 pmol liter^{-1}). $^{14}CO_2$ respiration was more or less stagnant for the lower glucose concentration after 1 h of incubation, while a slight increase was observed for the higher glucose concentration after 10 h (Fig. 5c and d). The $^{14}CO_2$ respiration of the CTC-treated samples amounted to less than 10% of that of the controls for both concentrations.

Calculated ratios of incorporation versus respiration averaged 84% (± 2%) incorporation to 16% respiration in the untreated controls, while a distinct shift towards a higher level of respiration (60 to >90%) appeared for the corresponding CTC-treated samples (derived from data of Fig. 5).

Luminescence test. After the quality of the test kit (46.5 and 44.6% after 15 and 30 min of incubation [see Materials and Methods]) had been tested, the decrease in light emission was measured for samples treated with 0.1, 0.5, 1.0, and 5.0 mM CTC in reference to untreated controls under short-time exposure (15 and 30 min). Even for a concentration of 0.1 mM, an inhibition of 57 and 62%, respectively, was measured, which increased dramatically up to 100% in the 5 mM CTC-treated samples (data not shown). The EC_{50} was extrapolated from the data to be 0.85 mM CTC.

DISCUSSION

By the CTC assay, counts of formazan-producing bacteria detectable by epifluorescence microscopy amounted to 5.4 to 19.4 and 6.4 to 13.6% of the total bacterial counts in a natural brackish water (Kiel Fjord) and a freshwater (Elbe River) environment, respectively. These portions are in agreement with values reported elsewhere for this technique and comparable environments (12, 30, 33), and they are in accordance with estimates derived from the INT approach (8, 22, 47). On the other hand, microautoradiographic determination of metabolically active bacteria resulted in distinctly higher numbers in this study compared with those determined by the CTC assay (Fig. 1), confirming results reported by Hoppe (15, 16) and Meyer-Reil (23).

It can be concluded from our results that this discrepancy is due to an inhibitory effect of the CTC dye on several important metabolic processes. The most dramatic inhibitory effect occurred with respect to bacterial growth. Cell production measured by thymidine uptake after CTC treatment was strongly reduced to values of <4% (Kiel Fjord) and <6% (Elbe River) of those of the untreated controls (100%), even for a 1 mM CTC solution. The increase in CTC concentrations was clearly reflected by a continuous decrease in heterotrophic bacterial plate counts, (Elbe River) finally resulting in a total inhibition of colony growth for a 10 mM solution in samples taken from the brackish water environment.

The results of the Microtox assay used in our study showed that light emission of the test organism was suppressed up to 100% at CTC concentrations of ≥5 mM. The EC_{50} of CTC, calculated to be 0.85 nM, is distinctly below the CTC concentrations normally used as working solutions.

It may be argued that longer incubation times increase the number of CTC-containing cells, as already reported (12), especially if samples were taken from oligotrophic environments and incubated under low temperatures. However, the 4-h incubation used in our experiments corresponds well to the findings of several other authors (5, 30, 33), who recommended 4 to 6 h of incubation as the optimal incubation time for samples from aquatic environments in general. These studies showed that longer incubation times did not increase the number of CTC-reducing bacteria.

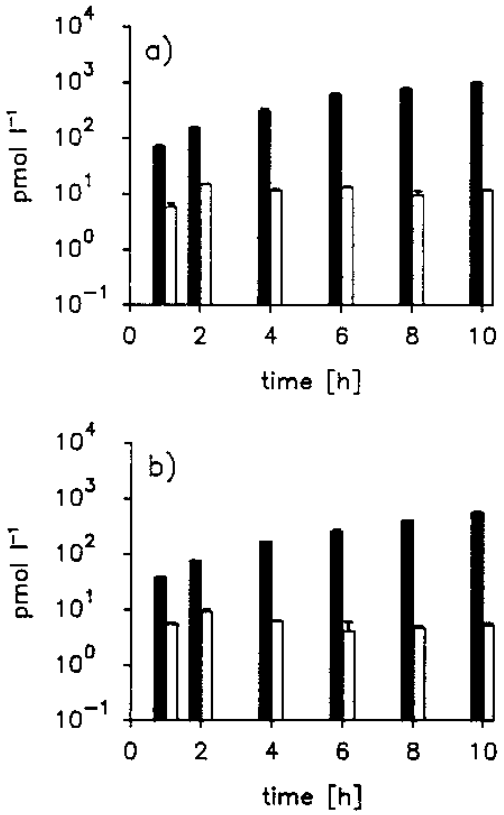

FIG. 4. Effect of CTC supply on [^{3}H]thymidine uptake. Results represent a comparison of CTC-treated (5 mM [open bars]) and untreated (solid bars) samples throughout 10 h of incubation at room temperature. (a) Elbe River. (b) Kiel Fjord. Error bars indicate standard deviation ($n = 3$).

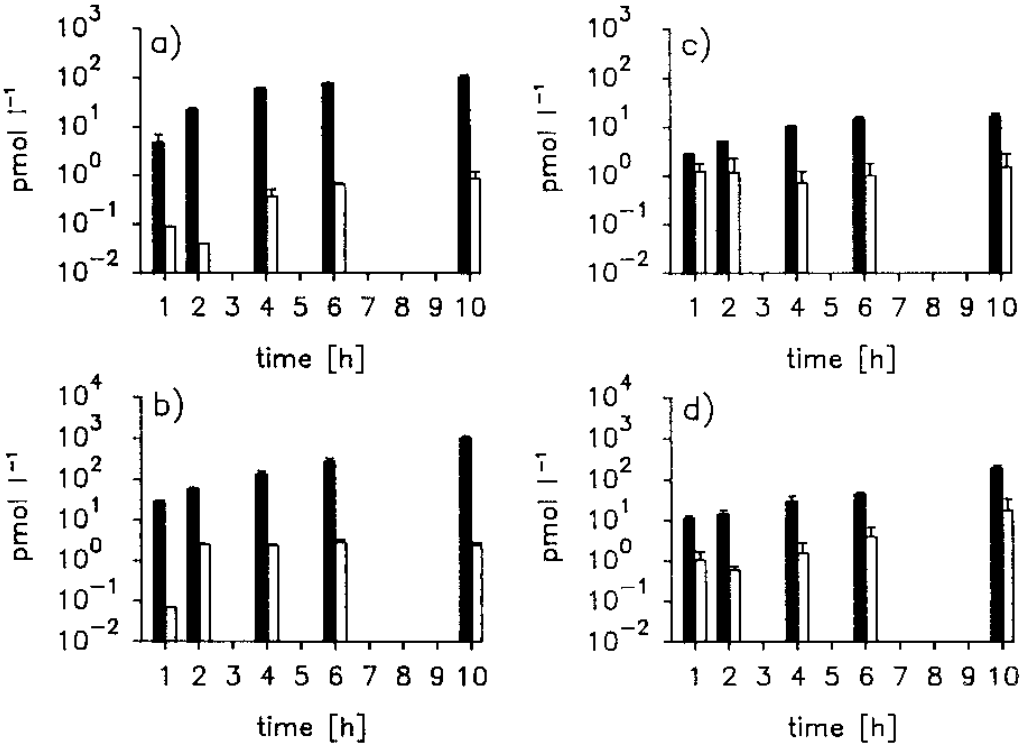

FIG. 5. Inhibition of [^{14}C]glucose incorporation and respiration by CTC throughout 10 h of incubation at room temperature. Results represent a comparison of CTC-treated (5 mM [open bars]) samples and untreated (solid bars) controls from the Kiel Fjord. (a) Incorporation of [^{14}C]glucose at the lower concentration (0.4 nmol). (b) Incorporation of [^{14}C]glucose at the higher concentration (17.5 nmol). (c) Respiration of [^{14}C]glucose at the lower concentration (0.04 nmol). (d) Respiration of [^{14}C]glucose at the higher concentration (17.5 nmol). Error bars indicate standard deviation ($n = 3$).

In our time series experiments, inhibition of CTC-treated samples occurred after a short exposure time. Heterotrophic bacterial plate counts, which were clearly reduced (98 to 100 and 76 to 88% inhibition in the Kiel Fjord and Elbe River samples, respectively) compared with the untreated controls, stagnated throughout 10 h of incubation (there was one exception in the Kiel Fjord after 10 h, in which no CFU could be detected, but this might be due to the inadequate dilution used in this case [Fig. 2b]).

Thymidine uptake and glucose uptake over time as measures for bacterial growth were affected in a similar way. While a continuous incorporation of substrates occurred in the untreated controls (100%), uptake stagnated after 1 to 4 h at distinctly lower levels (<14% of thymidine uptake and <4% of the glucose uptake, respectively) in the CTC-treated samples. A similar time-dependent effect could be shown for glucose respiration. Inhibition of bacterial growth was generally superior to suppression of respiration activity. The slight accumulation of $^{14}CO_2$ observed for the high glucose concentration over time (Fig. 5d) is probably due to a general activation of previously dormant cells by the high substrate concentration supplied.

A remarkable result was the observation that glucose incorporation was superior in the untreated controls, while glucose utilization shifted dramatically towards respiration in the CTC-treated samples. It can be hypothesized that the competitive collection of reducing equivalents by CTC in the respiratory chain finally leads to a loss of energy. Cells might try to compensate for this lack via an increase in respiratory activity to ensure energy supply instead of biomass production. Nevertheless, as shown by the respiration experiment, the absolute respiratory activity comes to a standstill after 1 to 2 h. This can be explained by biochemical reactions of tetrazolium salts (and most probably also of CTC) within the respiratory chain. The ETS "consists of a complex chain from catabolized foodstuffs to oxygen" (25). In this redox chain, tetrazolium salts function as artificial redox partners instead of the final electron acceptor, oxygen. It was shown by several authors using different electron transport inhibitors that INT reduction to INT-formazan is probably situated at or near the coenzyme Q (ubiquinone)-cytochrome *b* complex (11, 18, 21). Thus, the ETS is terminated at this redox step. This means that the linked oxidative phosphorylation of ADP to ATP for one H_2 molecule may be reduced to the generation of only one ATP molecule instead of three ATP molecules, the full energy yield if O_2 is the final electron acceptor. This inevitably leads to a severe energy depletion. Additionally, a severe mechanical effect of the big and water-insoluble formazan crystals on intracellular structures cannot be excluded.

There are already some indications for a toxic effect of CTC on bacterial cell metabolism in recent literature, although CTC has generally been assumed to be nontoxic (9, 34, 37). Rodriguez et al. (30) observed a suppression of CTC reduction activity in *Pseudomonas putida* at elevated reagent concentrations (>6 mM) and hypothesized that this may reflect a toxic effect of CTC at higher concentrations or the presence of impurities associated with the commercial product. A similar assumption was made by Coallier et al. (5). Roslev and King (31) discussed the effect of large intracellular formazan deposits on cell activity mentioned above. Pyle et al. (29) reported from their experiments that CTC reduction in cultured bacteria may be inhibited with respect to pH and nutrient conditions. However, some results (the addition of nutrients in particular) contradict results reported elsewhere in the literature

(30, 35), demonstrating that there might be differences concerning the suppression of CTC reduction between pure cultures and natural mixed populations.

The addition of carbon sources may increase the number of CTC-reducing cells (30). Similar observations have been made by using such additives for the INT method (42). As demonstrated in our glucose experiment, however, an artificially high concentration of substrates may induce cell activity of bacteria which have been in a dormant state before. An estimation of metabolically active cells under natural environmental conditions is not possible in this way.

Calculation of generation times provides additional evidence that the CTC assay distinctly underestimates the number of active bacterial cells. Generation times were calculated on the basis of the thymidine uptake data (production conversion factor of 2×10^{18} cells M^{-1} according to the method of Smits and Riemann [36] and Ducklow and Carlson [6]), which were attributed either to the number of active cells determined by microautoradiography or to the number of active cells determined by the CTC assay. While the generation times calculated from microautoradiography ranged within values (7 to 13 and 17 to 21 h in the Elbe River and Kiel Fjord, respectively) reported elsewhere for comparable aquatic environments and temperature conditions (reviewed by Thingstad [41]), unrealistic short times were derived from the CTC assay (0.5 to 1.3 and 2 to 5 h, respectively).

It can be concluded from our experiments that the inhibitory effect of CTC on bacterial metabolism contradicts the usefulness of the CTC assay for the evaluation of active bacterial cells in natural aquatic environments, and it can be assumed that the portion of active bacteria is underestimated by this method.

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