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Abstract

The 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) staining method is commonly and increasingly used to detect and to enumerate actively respiring cells (CTC+ cells) in aquatic systems. However, this method remains controversial since some authors promote this technique while others pointed out several drawbacks of the method. Using flow cytometry (FCM), we showed that CTC staining kinetics vary greatly from one sample to another. Therefore, there is no universal staining protocol that can be applied to aquatic bacterial communities. Furthermore, using ^{3}H-leucine incorporation, it was shown that the CTC dye has a rapid toxic effect on bacterial cells by inhibiting protein synthesis, a key physiological function. The coupling of radioactive labelling with cell sorting by FCM suggested that CTC+ cells contribute to less than 60% of the whole bacterial activity determined at the community level. From these results, it is clearly demonstrated that the CTC method is not valid to detect active bacteria, i.e. cells responsible for bacterial production.

Introduction

In aquatic ecosystems, bacterial communities play a fundamental role in both the organic matter decomposition and the conversion of dissolved organic carbon into bacterial biomass generally transferred to higher trophic levels through the microbial food web [1,2]. Over the past 20 years, methods have been developed by microbial ecologists to quantify the biomass and activity of total bacterial communities [3]. However, as bacterial communities are made of complex assemblages of numerous species having different metabolic characteristics and because nutrients are not always distributed homogeneously at the microscale, only a fraction of bacterial cells may contribute to the overall activity at a given moment and location. Therefore, a major question to be answered now is which fraction of the bacterial community of an aquatic system is really active and contribute to the production at the time of sampling?

In order to estimate whether a single bacterial cell is active or not, different methodologies have been proposed in the literature. Microautoradiography after incubation in the presence of a labeled substrate (amino acids, glucose, thymidine, etc.) [4], direct viable counts based on cell enlargement in the presence of cell division inhibitors and substrates [5,6] and activity of the electron transport system (ETS) as estimated by the reduction of the tetrazolium salt *p*-iodonitrotetrazolium violet (INT) [7,8] are the most common methods. More recently, different fluorescent dyes were used for bacterial activity or viability assessment at the cellular level [9]. The most commonly used dyes are fluorescein diacetate and its derivatives to assess esterase activity [10,11], dyes indicating the membrane potential of cells such as rhodamine 123 [12] or DiBAC4 [13] and tetrazolium salts such as 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), which represents a fluorochrome analogue to INT [14].

The CTC staining method has been used increasingly in recent years to detect the single-cell activity of bacteria in aquatic systems. CTC is reduced within individual respiring cells by the ETS into a fluorescent CTC-formazan product. Fluorescent respiring cells (CTC+) are usually detected by epifluorescence microscopy sometimes coupled to an image analysis system [15]. More recently, CTC+ cells have been detected by flow cytometry (FCM) [16,17]. The fraction of CTC+ cells found in natural aquatic ecosystems is generally low and varies in the range 0-10% [15,18-25]. In contrast, Smith [26] reported some high percentages of respiring cells (47%) in the Cheasapeake bay. However, comparison between different studies is often impossible because of the lack of standardization of the method. In

the literature, important differences in the CTC staining procedures are found which can influence CTC+ counts: CTC concentrations vary from 0.5 [27] to 10 mM [20] and incubation times from 1 [17] to 12 h [15].

Today, the CTC method remains controversial since an increasing number of authors promote this technique while others recently pointed out several drawbacks of the method. Comparative analyses have shown that the proportion of CTC+ cells in lakes tends to increase with increasing system productivity [28]. Some authors [16,19] reported positive correlations between the abundance of CTC+ cells and bacterial production. Smith [26] showed a good correlation between CTC+ cell abundances and respiration of the <3-µm planktonic community. Gasol et al. [15] showed that actively respiring bacteria tended to be larger than non-active bacteria leading to a strong size dependence for the probability of a bacterium to be active. In contrast, Karner and Fuhrman [22] found, in a study on marine bacterial assemblages, that the abundance of metabolically active cells determined by microautoradiography (using a mixture of tritiated amino acids) was higher than the number of respiring cells detected by the CTC method. In other reports, growth rates calculated from the bacterial production and the number of CTC+ cells were very high and, therefore, generation times were very low and unrealistic [21,29].

These data suggest that active cell counts are underestimated by the CTC method probably due to several limitations. The detection limit of fluorescence signals may contribute to this underestimation since the most weakly active cells generally do not provide a sufficient fluorescence signal to be detectable by microscopy and visual examination. Sieracki et al. [17] have found that FCM counts of CTC+ cells are often higher than microscopic counts, probably due to the fact that rapidly fading cells are not detectable by microscopic examination. Choi et al. [25] showed that most of the CTC+ bacteria found in Oregon coastal waters can become CTC- after incubation in the presence of substrates, probably by increasing the fluorescence signal of the cells. Toxicity of the CTC dye may constitute a second limitation. Ulrich et al. [21] demonstrated by several techniques (thymidine incorporation, glucose incorporation and respiration, Microtox bioassay) that CTC at millimolar concentrations severely reduces bacterial activity. Finally, some authors showed that some bacterial strains cannot be efficiently stained with CTC [30] and some active bacteria cannot reduce CTC under certain conditions [31]. In contrast, Sherr et al. [32] reported that all the bacteria isolated from this environment were able to reduce CTC.

Obviously, the CTC method requires more investigation to further understand the ecological meaning of CTC+ counts and the relationships which exist between such counts

and bacterial production commonly determined in aquatic ecosystems. In this study, several points of the CTC staining protocol (CTC concentration, incubation time) were investigated using flow cytometric detection. The toxicity of CTC on bacterial activity was tested by ^{3}H-leucine incorporation, a classical method used to estimate bacterial production [33]. Finally, the relationships between CTC+ bacteria and bacterial production were investigated by coupling tritiated leucine incorporation with cell sorting of CTC+ cells by FCM. Experiments were performed on samples collected in various aquatic environments including marine, brackish and fresh waters.

Materials and methods

Water samples

Samples were collected between March and June 2000 along the Mediterranean coast (France) at sites with different physico-chemical characteristics. Seawater samples were taken in the coastal area near Banyuls-sur-Mer: in the bay at a station called SOLA (42°29'N, 3°08'E) and in the harbour (42°28'N, 3°08'E). Brackish water samples were taken in a coastal lagoon (salinity 30x): the Leucate lagoon (42°49'N, 2°9'E). Samples of freshwater were collected in the Tech river at 5 km above the river mouth (42°35'N, 2°58'E). Five liters were taken just below the surface in the different studied environments except for the SOLA station where the water was collected at 24 m depth. Samples were processed in the laboratory within 2 h after collection.

Incubation of bacteria with CTC

CTC (Polysciences Europe) reduction was used to identify active respiring cells as proposed by Rodriguez et al. [14]. Three CTC final concentrations (1, 2.5 and 5 mM) were tested on different samples. When the CTC concentration is not explicitly mentioned, a final concentration of 2.5 mM was used. A stock solution of CTC (50 mM concentration) was freshly prepared on the day of experiment and stored at 4°C. Different incubation periods ranging from 10 min to 6 h were tested. The incubation period in the presence of CTC is reported for each experiment. Incubations were performed in the dark at in situ temperature, ended by adding formaldehyde (2% final concentration) and samples were then stored in liquid nitrogen until examination.

Enumeration of CTC+ cells by FCM

Enumeration of CTC+ cells was performed with a Facs-Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with an air-cooled argon laser (488 nm, 15 mW). CTC-stained cells were excited at 488 nm, discriminated and enumerated on the basis of their orange and red fluorescence emissions collected using a 585/42 nm band pass filter and a 650 nm long pass filter, respectively. The accurate volume analyzed and subsequent cell concentration estimation were calculated by weighing the sample before and after a 5-min run of the cytometer. Fluorescent beads (1.002 Wm; Polysciences Europe) were systematically added to each analyzed sample to normalize cell fluorescence emission and light scatter values. A blank made of sterile water (seawater or freshwater depending on the salinity of the

sample) filtered through 0.2-µm pore-size membrane and incubated with CTC (under the same conditions as the sample) was enumerated by FCM before each estimate of CTC+ cells in order to control the absence of non-specific signals (i.e. CTC precipitate) in the tested experimental conditions. A blank was also analyzed in the absence of CTC dye to check the absence of auto-fluorescent orange-red cells or particles in the CTC+ window. The proportion of CTC+ bacteria was calculated for each sample with regards to total cell numbers estimated by FCM (see below).

Total bacterial enumeration by FCM

For FCM analyses, 1 ml of fixed sample with formaldehyde (2% final concentration) was incubated with 0.5 µl of SYBR-II (nucleic acid stain; Molecular Probes) for 15 min at room temperature in the dark [34]. Counts were performed with the FacsCalibur flow cytometer. Stained bacteria, excited at 488 nm, were discriminated and enumerated according to their right angle-light scatter (SSC, related to cell size) and green fluorescence measured at 530/30 nm.

Impact of CTC on bacterial activity

The impact of CTC on bacterial activity was investigated using the 3H-leucine incorporation method commonly used to estimate bacterial production in aquatic ecosystems [33,35]. Incorporation of ^{3}H-leucine (151 Ci mmol^{-1}, Amersham) was measured at 80 nM (5 nM of tritiated leucine and 75 nM non-radioactive leucine) to saturate leucine incorporation in the different aquatic systems tested (data not shown). Two types of experiments were performed to test the impact of CTC on leucine incorporation: in the first set of experiments, leucine incorporation was compared in the presence and absence of CTC during a 2-h incubation while, in the second set of experiments, CTC was added during the course of incubation of bacteria with tritiated leucine. In the first type of experiments, two subsamples (20 ml each) were incubated in the presence of leucine for 2 h in the dark at in situ temperature. One subsample was supplemented with CTC (final concentration 2.5 or 5 mM) immediately after leucine addition. After 0.5, 1, 1.5 and 2 h of incubation, 5 ml were removed from each subsample and filtered through 0.2-µm pore-size cellulose acetate membrane. After filtration, 10 ml of ice cold 5% trichloroacetic acid (TCA) was added on the filter and filtered after 10 min. Then, filters were rinsed four times with 5 ml of cold TCA. Radioactivity associated with the filters was estimated by liquid scintillation. In the second type of experiments, two subsamples (10 ml each) were incubated in the presence of leucine for 2 h in the dark at in

situ temperature. In one subsample, CTC (final concentration 2.5 mM) was added after 1.5 h in order to test if the CTC addition has a direct effect on leucine incorporation. After incubation, subsamples were treated as reported for the first type of experiments.

Leucine incorporation followed by cell sorting using FCM

In order to estimate the contribution of CTC+ cells to total bacterial activity, we used a procedure similar to that developed by Servais et al. [36] and applied by Bernard et al. [37] to estimate the contribution of different cell-size classes to bacterial activity in various aquatic systems. This procedure consisted of labeling bacteria with ^{3}H-leucine and then sorting different bacterial subpopulations by FCM in order to estimate their contribution to the activity of the whole bacterial community. In this study, CTC+ cells were sorted after ^{3}H-leucine labeling in order to determine the specific leucine incorporation rate of CTC+ cells. In parallel, total bacteria were sorted after similar ^{3}H-leucine labeling to estimate the average specific leucine incorporation rate of the total bacterial community. From a practical point of view, leucine incorporation was performed at 80 nM final concentration. Only radioactive leucine (151 Ci mmol^{-1}, Amersham) was added to maximize the detection limit of labeled bacteria. Two subsamples of 3 ml were incubated at in situ temperature during 2 h in the dark and in the presence of ^{3}H-leucine. In subsample 1, CTC (2.5 mM final concentration) was added 1.5 h after ^{3}H-leucine addition. The incubation of both subsamples was stopped by addition of formaldehyde (2% final concentration) so that the incubation period of subsample 1 in the presence of CTC was 30 min. Labeled CTC+ bacterial cells from subsample 1 were then sorted with the FacsCalibur flow cytometer. For all sorting experiments, the salinity of the sterile sheath fluid was adjusted to that of the sample to avoid cell lysis or protein release due to osmotic shock. The sorting window was defined on each cytogram where FL2 (585/42 nm band pass filter) was plotted against FL3 (650 nm long pass filter) (Fig. 1a). We selected on the flow cytometer the ‘single sort mode’ where a sort occurs only if a single target cell is identified. The result gives high purity with less emphasis on recovery and the best accurate counts of sorted cells. Sorted cells were thus enumerated and collected at the outlet of the flow cytometer directly onto a 0.2-µm pore-size membrane (acetate cellulose filter). Sorting was complete when the number of CTC+ sorted cells ranged between 300 000 and 500 000 cells to obtain a measurable radioactivity on the filter. Ten milliliters of cold 5% TCA were added to the filter in order to precipitate macromolecules and to rinse the membrane. After 10 min, TCA was eliminated by filtration and the radioactivity associated with the sorted bacteria was estimated by liquid scintillation. Immediately after the end of the incubation, bacteria of

subsample 2 were stained with SYBR-II following the procedure used to determine total counts. Labeled and stained bacterial cells were then sorted using a window defined around the cells corresponding to the total bacterial community on a cytogram where green fluorescence was plotted against SSC (Fig. 1b). In a previous paper, this sorting procedure of the total bacterial population was shown to give a similar result to a direct measurement of the bacterial activity without cell sorting [36]. The specific activity (SA) of respiring cells (mol leucine incorporated $cell^{-1}$ h^{-1}) was determined from the radioactivity incorporated in the sorted CTC+ cells. Similarly, the average SA of the total bacterial cells was calculated on the basis of the sort of total bacteria after SYBR-II staining. The total bacterial activity (mol incorporated l^{-1} h^{-1}) and the activity of CTC+ cells were calculated by multiplying the total cell number by the average SA of the total bacterial community and the CTC+ abundance by the SA of the respiring cells, respectively. Duplicate labeling experiments were not made because of both the high cost and long time required for these experiments. However, on several samples, a coefficient of variation of 9% was estimated for the SA of the total bacterial population as determined by radioactive labeling followed by cell sorting after SYBR-II staining.

Results

Effect of CTC concentration and incubation period on the detection of respiring bacteria

The impact of different CTC concentrations on both the abundance and the fluorescence of respiring cells determined by FCM was first tested. Concentrations of 1, 2.5 and 5 mM of CTC were used (Fig. 2). Data are reported as the ratio between the number or the fluorescence of CTC+ cells measured at 2.5 and 1 mM versus the number and fluorescence values determined at 5 mM and at different incubation times. A concentration of 5 mM was used as a reference in Fig. 2 as it represents the most often used concentration reported in the literature. Our data obtained from several marine and freshwater environments suggest that CTC+ abundances were not significantly different (comparison of average values; t-test, K= 0.01) at concentrations of 2.5 M and 5 mM of CTC for all incubation times but average ratios were closer to 1 for incubation times higher than 20 min. The fluorescence of CTC+ cells was not significantly different at 2.5 and 5 mM CTC concentrations. In contrast, when 1 mM was used, the number of CTC+ cells was significantly lower at all incubation times while the average fluorescence of CTC+ cells was not significantly different to that measured at the other CTC concentrations. From these data, a concentration of 2.5 mM was applied for all subsequent CTC+ cell enumeration. In order to test the influence of the staining period on the number and fluorescence of CTC+ bacteria, kinetics of staining was performed on several samples including marine, brackish and freshwater environments (Fig. 3). The kinetics showed a first phase of increase of CTC+ counts up to a maximum followed by a second phase of decrease or stability of CTC counts. For all samples, the peak of CTC+ counts was reached after an incubation ranging from 0.5 to 2 h. The decrease after the peak was more or less pronounced from one sample to another. For instance, CTC+ cells enumerated after 4 h represented 63% of the maximum in the case of the Tech river sample (Fig. 3e) and 90% in the case of the Leucate lagoon sample (Fig. 3f). This indicates that CTC+ cells detected after a short incubation period (30 min to 2 h) can become CTC- by increasing the incubation. The fluctuations of the average fluorescence of CTC+ cells versus incubation were also very variable from one sample to another. Even within a similar environment (the marine SOLA station), the patterns in CTC+ abundances and average fluorescence observed at different sampling dates were not similar (Fig. 3a-c).

CTC toxicity

The toxicity of CTC on bacterial activity was investigated using 3H-leucine incorporation on water samples from various origins. The leucine incorporation method measures the protein synthesis rate, which is an essential and universal physiological activity in a bacterial cell, and is traditionally used to estimate bacterial production [33]. Fig. 4 shows the incorporation of radioactivity in the macromolecular fraction of bacteria when incubated in the presence of tritiated leucine with and without CTC. As expected in the absence of CTC, radioactivity plotted against time followed a linear pattern with a slope depending on the activity of bacterial communities. In the presence of CTC, leucine was slightly incorporated into protein during the first 30 min of incubation and then, protein synthesis was completely inhibited in all the tested samples. Data on the impact of CTC addition during the incubation with ^{3}H-leucine are reported in Table 1. In these experiments, one subsample was incubated for 2 h with ^{3}H-leucine and another was incubated for 2 h with ^{3}H-leucine but CTC was added during the last 30 min of incubation. The ratios of radioactivity incorporated into both subsamples were similar (close to 0.75) (Table 1) indicating that the incorporation of leucine and, thus, protein synthesis was immediately inhibited after CTC addition.

Contribution of CTC+ cells to bacterial production

A major consideration concerning the CTC technique is to gain a better understanding of the differences in metabolism between CTC+ and CTC- bacteria and their relative contributions to the whole community activity as commonly estimated by bacterial production. To investigate these contributions, the following procedure was used [36] : bacteria were labeled with ^{3}H-leucine and different bacterial subpopulations were then sorted by FCM in order to estimate their contribution to bacterial activity. After labeling bacterial cells with tritiated leucine, total bacterial cells stained with SYBR-II were sorted from one subsample and CTC+ bacteria were sorted from another subsample stained with CTC for 30 min. This allowed us to compare the SA (leucine incorporation rate per cell) of the total community with the SA of CTC+ bacteria in a marine, a brackish and a freshwater sample (Table 2). In these samples, CTC+ cells represented from 5 to 47% of total bacterial counts. For calculating the SA of CTC+ cells, an incubation time of 1.5 h (incubation period in the presence of ^{3}H-leucine and in the absence of CTC) was considered and not 2 h as for the total community because our data reported in Table 1 showed that leucine incorporation immediately stopped after CTC addition. As expected, the average SA of CTC+ bacteria was always higher than the average SA of the total community. Data reported in Table 2 allowed

us to calculate the average SA of CTC- cells which were, respectively, $219x10^{-21}$, $47x10^{-21}$ and $41x10^{-21}$ mol leucine incorporated $cell^{-1}$ h^{-1} for the Banyuls harbour, the Leucate lagoon and the Tech river. In the same samples, the ratios between SA of CTC+ and SA of CTC- bacteria were 1.37, 7.47 and 1.51, respectively. From the SA data and the abundance of total and CTC+ bacteria, we found that the contribution of CTC+ cells to total bacterial activity ranged from 27 to 57%. These results showed that, on the tested samples, even if the SA of CTC+ cells was always higher than the SA of CTC- cells, the difference was sometimes not important (sample from Banyuls harbour, for example). In addition, the contribution of CTC+ cells to total bacterial production was not predominant.

Discussion

A review of literature on the protocols used to estimate the fraction of actively respiring cells in natural aquatic systems by the CTC technique revealed large differences in both the concentration of CTC and the incubation time. These different protocols have been established from staining kinetics performed at various CTC concentrations [14,17-19,25,27]. The differences in the proposed protocols may be explained by the fact that the method was applied to different types of environments (pure culture of bacteria, lake water, drinking water, marine systems) which induced differences in the bacterial SA and thus in the CTC staining. Discrepancies between protocols may also be due to the use of different instruments for the detection of fluorescent cells since microscopy and FCM have different detection limits [16,17]. The enumeration by FCM minimises the effect of fading which constitutes one of the most important limitations when using microscopy. In addition, FCM offers the advantage of quantifying the fluorescence of an important number of cells in a few minutes. Del Giorgio et al. [16] estimated that flow cytometric counts of CTC+ cells were five- to 10-fold more accurate than microscopic counts.

In this paper, we showed using FCM that for both natural marine and freshwater systems, CTC+ counts and cell fluorescence intensities obtained using a CTC concentration of 2.5 mM were similar to those obtained at 5 mM, the most often used concentration reported in the literature [25,26,32]. In contrast, CTC+ counts were underestimated with 1 mM of CTC. Concentrations higher than 5 mM were not tested since several papers clearly showed a decrease of CTC+ counts at these concentrations [14,17]. For the incubation time, kinetic experiments performed on various aquatic systems (including fresh, brackish and marine waters) and at different periods for a given environment revealed different patterns from which no general rule could be defined. From our data, it appears clearly that it is impossible to define a standard staining protocol to enumerate CTC+ cells, probably due to the toxic effect of CTC on viable cells (see below). Therefore, it seems necessary to perform a kinetics study of CTC staining before each analysis and to consider the maximum of CTC+ counts as the correct number of actively respiring cells but this procedure is extremely expensive and time-consuming.

The decrease of CTC+ counts observed by increasing the incubation time suggests that CTC may be released or lost by respiring cells after a short period of incubation (30 min to 2 h). This loss of fluorescence may be the result of a passive efflux of formazan due to the toxicity of the dye leading to cell death and to the permeabilisation of membranes which may

occur more or less quickly depending on species. This observation has important consequences since interpretations of results from other studies that used long and variable incubation periods are questionable [15,17,26].

The inhibitory effect of CTC on bacterial activity measured by ^{3}H-thymidine incorporation, the uptake and respiration of ^{14}C-glucose, growth on plate count and Microtox bioassay was demonstrated by Ulrich et al. [21,38]. The two types of experiments presented here demonstrated the drastic toxic effect of CTC on protein synthesis at CTC concentrations (2.5 and 5 mM) which are commonly used to estimate the fraction of respiring bacteria. In addition, the toxic effect of CTC was very rapid as protein synthesis was immediately inhibited after CTC addition (Table 1). This toxic effect of the CTC dye on major physiological activities (substrate uptake and respiration, DNA and protein synthesis) probably leads to an underestimation of active cell counts estimated by this technique. This underestimation may explain why Karner and Fuhrman [22] found higher numbers of active cells by microautoradiography than by the CTC technique. It could also explain why unrealistically high production values per cell and short generation times were found in natural environments when determined from production measurements and CTC+ counts [21,29,38].

Several authors have suggested that CTC+ bacteria are responsible for the bulk of total bacterial activity [16,19,26]. These assumptions were based on positive correlations found between the total bacterial production [16,19,32] or respiration of the 63-µm planktonic community [26] and the absolute number of CTC+ bacteria but never on a quantitative estimation of the contribution of CTC+ cells to total activity. Sherr et al. [32] suggested that CTC+ cells represent bacteria with a high level of metabolic activity whereas some CTC- cells have sufficient RNA content and the ability to assimilate substrates to be identifiable as active cells by indices of cell-specific RNA content and microautoradiography. These authors also pointed out the fact that quantitative differences in metabolism between `highly active' CTC+ cells and `less active' CTC- cells have still to be determined. To our knowledge, this is the first report in which the absolute activity of CTC+ and CTC- cells has been quantitatively estimated and compared to total bacterial activity. The data obtained by cell sorting after radioactive labeling showed that, even if the SA of CTC+ bacteria is higher than that of CTC- cells, some CTC- cells significantly contribute to the total bacterial activity. In our samples, the contribution of CTC- cells to total activity ranged between 43 and 73%. This means that we cannot consider CTC- cells as inactive cells nor as weakly active cells. Thus, some active bacteria are probably not stained by CTC because of its toxicity and/or because some cells are

not able to reduce the CTC in the conditions met in natural aquatic environments as suggested by Smith and McFeters [31] while they are able to do so after an incubation in the presence of substrate as shown by Choi et al. [25]. Although only a few samples from different environments were analysed by the time-consuming cell sorting procedure, these data alone suggest that CTC+ cells are not responsible for the bulk of the bacterial production in natural aquatic systems.

As the CTC dye is not suitable for the detection and enumeration of active bacteria (i.e. cells contributing to bacterial production), other fluorescent dyes should be used for this purpose. For instance, when nucleic acids dyes such as TOTO, SYTO 13, SYBR green dyes are applied to natural bacterial communities, they allow us to discriminate two cellular sub-populations characterized by a high DNA content (HDNA) and a low DNA content, respectively [39,40]. Interestingly, it was recently hypothesized that cells with a high DNA content are responsible for the total bacterial activity [39,40]. The approach based on radioactive labeling followed by cell sorting applied in this paper has been recently used to validate this hypothesis [41].

Conclusions

The results reported in this study clearly indicate that the CTC procedure is not valid for estimation of the fraction of active cells in natural aquatic environments. Firstly, CTC+ bacteria are not responsible for a predominant part of the total bacterial activity because CTC- bacteria significantly contribute to this total activity. Secondly, CTC has a strong toxic effect on key metabolic functions. Finally, because CTC staining varies greatly from one sample to another, it requires expensive and time-consuming staining kinetics to optimize the staining protocol.

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Table 1. Impact of CTC addition during incubation of a sample with ^{3}H-leucine

Sample	*n*	Ratio (radioactivity incorporated in subsample 2/radioactivity incorporated in subsample 1)
Marine samples	4	0.76±0.04
Freshwater samples	3	0.75±0.04

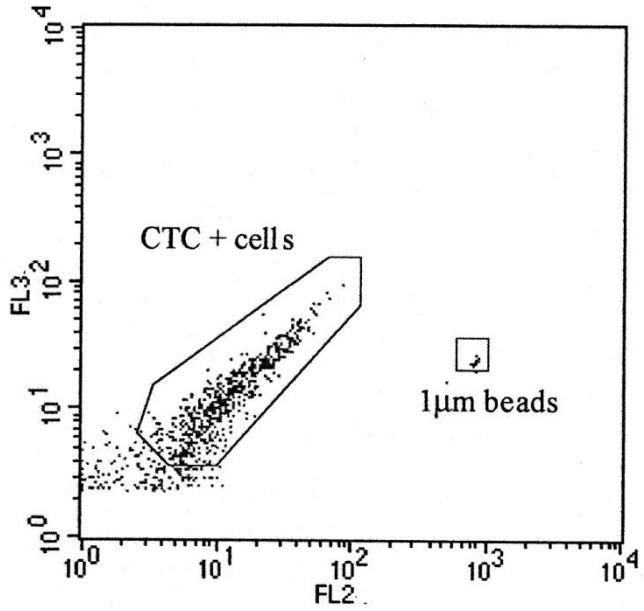
Subsample 1 was incubated for 2 h with ^{3}H-leucine. Subsample 2 was incubated for 2 h with ^{3}H-leucine and CTC was added 1.5h after the beginning of the incubation. Data are presented as ratios of radioactivity incorporated in subsample 2 and 1 for different (*n*) marine and freshwater samples.

Table 2. Contribution of the CTC+ cells to total bacteria activity determined by ^{3}H-leucine labeling followed by cell sorting in 3 water samples

	Sample (date)		
	Banyuls harbour (29/6/00)	Leucate lagoon (30/5/00)	Tech river (15/5/00)
Total cell abundance (10^{9} l^{-1})	2.4	6.02	1.61
CTC+ abundance (10^{9} l^{-1})	0.72	0.29	0.75
% of CTC+ cells	30	5	47
Average SA of total cells (10^{-21} mol leucine incorporated $cell^{-1}$ h^{-1})	244	62	51
Total bacterial activity (pmol l^{-1} h^{-1})	585	373	82
Average SA of CTC+ cells (10^{-21} mol leucine incorporated $cell^{-1}$ h^{-1})	300	351	62
Activity of the CTC+ bacteria (pmol l^{-1} h^{-1})	216	102	47
% of the bacterial activity due to CTC+ cells	37	27	57

SA =specific activity.

Figure 1. (a) Cytogram of red (FL3) versus orange (FL2) fluorescence used for cytometric detection of CTC+ cell. The window used for cell sorting is also reported. (b) Cytogram of green fluorescence (FL1) versus right angle-light scatter (SSC) used for flow cytometric detection of total cell number after stainng with SYBR-II. The window used for cell sorting is also reported. Sample collected in the Banyuls harbor (29/06/2000).



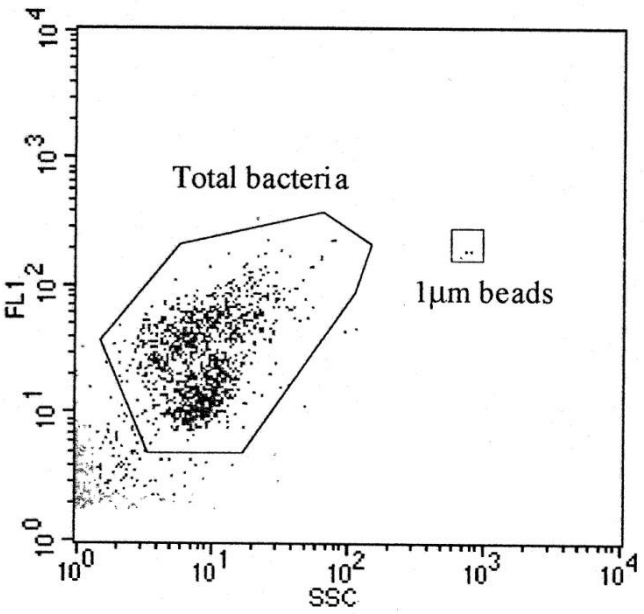


Figure 2. Effect of CTC concentration on the detection of respiring bacteria. (a). Ratio at different times of incubation between the number of CTC+ cells enumerated at 2.5 mM (black symbols) and 1 mM (grey symbols) versus CTC+ enumerated at 5 mM. (b) Ratio at different times of incubation between the fluorescence of CTC+ cells measured at 2.5 mM (black symbols) and 1 MM (grey symbols) versus fluorescence of CTC+ cells measured at 5 mM.

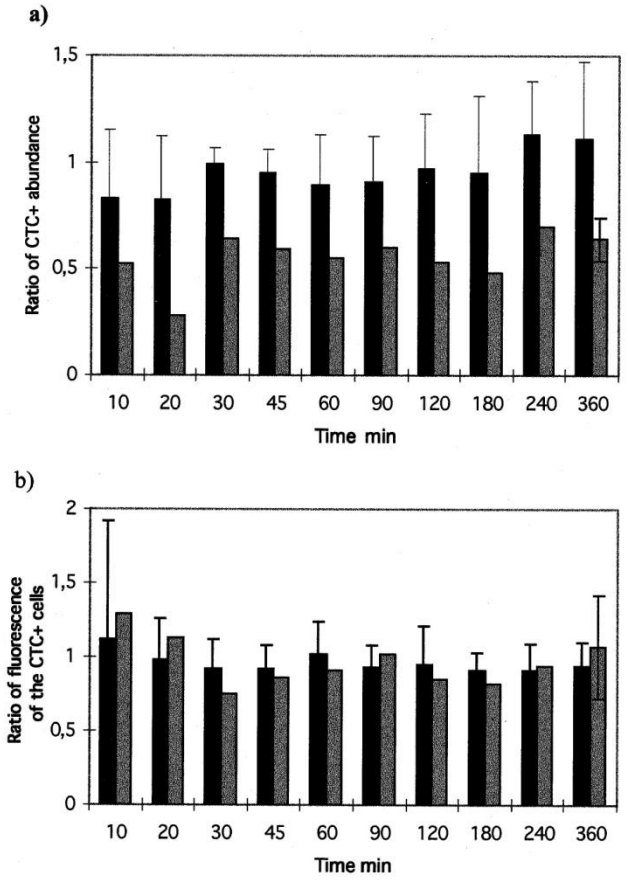


Figure 3. Effect of time of incubation on the number (●) and the average fluorescence (○) of CTC+ bacteria corresponding to (a, b and c) samples from the marine SOLA station (collected, on 8 of March, 11 of April and 9 of May, respectively) ; (d) sample from Banyuls harbor (29 of June) ; (e) sample from Leucate lagoon (30 of May) ; (f) sample from Tech river (15 of May).

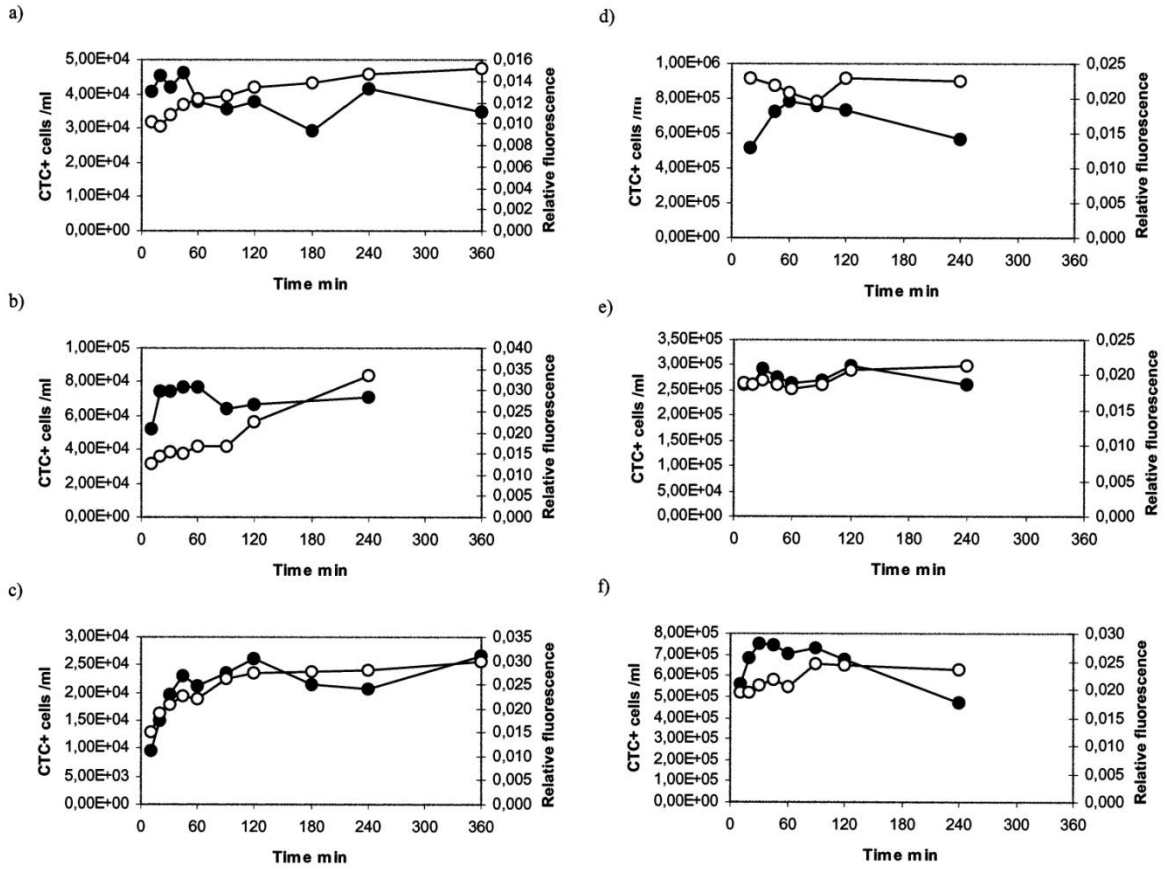


Figure 4. Leucine incorporation as a function of incubation time without CTC (●) and with a CTC concentration of 2.5 mM (○) and 5 mM (Δ). Samples from (a) seawater (marine SOLA station), (b) brackish water (Leucate lagoon) and (c) freshwater (Tech river).