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Journal of Microbiological Methods 52 (2003) 19–28

Journal of Microbiological Methods

www.elsevier.com/locate/jmicmeth

Direct estimate of active bacteria: CTC use and limitations

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Received 1 April 2002; received in revised form 24 May 2002; accepted 3 June 2002

Abstract

During the last 10 years, the dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) has been used to determine the in situ number of "active" bacteria in different ecosystems. A part of this success is due to a simple protocol, which does not require sophisticated equipment. However, it has not been established whether the method determines viable cells, e.g. those capable of growth and cell division, as opposed to cells that are active in the sense of having some detectable metabolic activity. In this study, the number of CTC-positive cells through the growth stages of *Escherichia coli* was estimated and compared to counts of the total number of bacteria, the culturability (CFU counts) and respiratory activity (CO_2 evolution). There was a good correlation between the number of CTC-positive cells and the CFU count, regardless of the growth phase. However, CTC could still be reduced by a large part of the population during the first hours of stationary phase even if the bacteria were no longer releasing CO_2. Thus, the reduction of CTC is a good estimator for cell viability, rather than cell activity. Additionally, a review of the literature showed that there is presently no standardized protocol for using CTC, which makes difficult at present the comparison of active bacterial numbers in different samples from different sites.

Keywords: Active bacteria; 5-Cyano-2,3-ditolyl tetrazolium chloride (CTC); Method

1. Introduction

In ecological studies, the determination of metabolically active bacterial numbers is an effective means of estimating system productivity, biomass turnover or substrate utilisation potentials (Rodriguez et al., 1992; del Giorgio and Scarborough, 1995). Dyes such as acridine orange (AO) or 4,6 diamino-2-phenylindole (DAPI) have traditionally been used to estimate bacterial numbers in diverse environmental samples: these dyes function by staining any DNA-containing cells. They eliminate unspecific staining of dead cells that do not contain a significant amount of DNA, but include inactive bacteria in the count. In contrast, plate counts estimate the numbers of culturable, active bacteria that are able to initiate cell division at a sufficient rate to form colonies (Boulos et al., 1999). However, a large proportion of bacteria encountered in environmental samples do not grow on conventional substrates. Other methods such as universal probe hybridisation have been used to assay all cells containing a threshold amount of rRNA (Karner and Furhman, 1997; Kallmbach et al., 1997). While the presence of rRNA is not direct

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0167-7012/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
PII: S0167-7012(02)00128-8

proof of metabolic activity, it indicates, at least, the potential viability of the cell. Alternatively, autoradiography, after incorporation of radio-labelled amino acids, also indicates cells with an active metabolism since there has been transport of the label through the cell membrane.

During the last decade, new fluorescent dyes and more sensitive detection equipment have been developed to improve the methods of separating living from dead cells. A useful method for estimating viability is to use a cytochemical procedure leading to the accumulation of the product of a metabolic reaction. The membrane of viable cells is impermeable to the charged molecules, which therefore accumulate intracellularly and can be detected by fluorescence. The use of the redox dye, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), for evaluating the metabolic activity of aerobic and anaerobic bacteria has gained wide application in recent years in environmental studies. The CTC method indicates respiratory activity and is therefore a direct indicator of oxidative metabolism as well as viability (Smith and McFeters, 1997). CTC is a colourless, membrane-permeable compound that produces a red-fluorescing precipitate in the cell when it is reduced by the electron transport system (ETS) of bacterial cells. This approach for estimating active bacterial cells has been used in a wide range of studies and is comparable in simplicity and reliability to any epifluorescence technique commonly used for the enumeration of total bacterial density (Dufour and Colon, 1992; del Giorgio and Scarborough, 1995). However, it has not been determined if there is a direct relationship between the amount of cells that are actively respiring, growing and dividing and the amount of cells that reduce sufficient dye to be detected as CTC positive.

The aim of this study was to determine whether the CTC method is a suitable approach for the determination of the number of active bacteria before using the method for routine ecological purposes. We compared the CTC response with other assays such as bacterial respiration (CO_2 production), culturability (colony-forming unit: CFU) and total cell number (Sybr-Green® staining) in lag, exponential and stationary phases of *Escherichia coli* cultures. Additionally, the results of methodological studies to determine optimal conditions for CTC reduction are summarized, as are studies in which CTC was compared with other techniques to obtain a better estimate of the pool of active bacteria.

2. Materials and methods

2.1. Bacterial strain and growth conditions

E. coli K-12 was provided by the clinical laboratory of Rennes Hospital (Laboratoire de Bactériologie, Rennes, France). The culture was grown in M_{63} mineral medium with 0.2% D-glucose at 37 °C. A subsample taken after 48 h was used as a standard inoculum for the experiments. All media were prepared with reagent-grade water (Milli-Q, Millipore), sterilised by autoclaving at 120 °C for 20 min. The glucose solution was filtered (0.2 μm). One milliliter of culture was inoculated into M_{63} medium containing 0.01% D-glucose and incubated at 37 °C with shaking at 120 rpm. For the study, two sets of triplicate *E. coli* cultures (55 ml) were followed: one for the bacterial and plate counts, and one for the [^{14}C]glucose experiment. We sampled at seven incubation times: two during the lag phase, three from the beginning to the end of the exponential phase, and two during the stationary phase.

2.2. Bacterial number

The total number of bacteria was determined from subsamples (1 ml), fixed with a 10% final volume of 37% formalin. Cells were stained with 1 mM final concentration of Sybr-Green® (Molecular Probes, Interchim, Montluçon, France) for 10 min. In addition, the culture was also followed by optical density (OD) measurement at 600 nm. A strong correlation was observed between OD and the bacterial number [$r = 0.99$, $p < 0.001$, and $n = 20$].

In parallel, 1 ml of CTC (Polyscience, Coger, Paris, France) was added to a 1-ml aliquot of culture, giving a final concentration of 5 mM. CTC kills bacteria by interrupting the respiratory chain, but this only happens after bacteria take up of the fluorogenic CTC and the fluorescent compound is produced (Ullrich et al., 1996). The assay was incubated in the dark for 60 min at 37 °C and the reaction was stopped with 10% formalin and stored overnight at 4 °C. Since CTC can

be reduced by exogenous chemical reductants in the absence of biological electron transport, the addition of CTC to a formaldehyde-treated sample provided a convenient negative control to account for any abiotic CTC reduction in experimental preparations (Rodriguez et al., 1992). The samples (CTC and Sybr-Green®) were filtered onto 25 mm (∅), 0.2-μm black-stained membrane filters (Poretics, Serlabo, Bonneuil sur Marne, France). The filters were rinsed with sterile, distilled water, mounted onto slides with a drop of FluoroGuard Antifade Reagent (Bio-Rad Lab., Ivry-sur-Seine, France) and examined under 1250 magnification with a Leitz microscope outfitted for epifluorescence microscopy (470–490 nm excitation filter, and a 590-nm cutoff filter).

2.3. Colony-forming units

The number of viable bacteria was estimated using the heterotrophic plate count procedure. The heterotrophic plate count procedure was done with autoclaved LB medium (Bactotryptone, yeast powder, NaCl, agar 10 g/l), which is nonselective for *E. coli* cultures. For each sampling time, an aliquot of culture was 10^4 and 10^5 diluted and 200 μl were spread on the counting plate.

2.4. [^{14}C] glucose respiration

Net respiration was measured with [^{14}C]glucose (Amersham BioScience, Orsay, France) as a tracer (specific activity, 310 mCi/mmol). The experiment was performed in 250-ml flasks with rubber caps equipped with a filter saturated with 6 N KOH to trap the released $^{14}CO_2$. The filter was replaced by a new one after each incubation time. The experiment was done in triplicate, in the dark at 37 °C after the addition of 47 μl of [^{14}C] glucose as the organic substrate at the beginning of the experiment. At the end of the experiment, 6 ml of 12 N HCl was added to release $^{14}CO_2$ from any dissolved $^{14}HCO_3$ in the water. The filters were placed into vials filled with 3.5 ml of Hionic scintillation cocktail (Packard BioScience, Rungis, France) and measured by a Packard Tri-carb 2200 Ca liquid scintillation counter.

2.5. Statistical analyses

Relationships between the different bacterial parameters were tested with Pearson's correlation analysis and Kruskall and Wallis' ANOVA. Statistical analyses were performed with SYSTAT and differences were considered to be significant at $p < 0.05$.

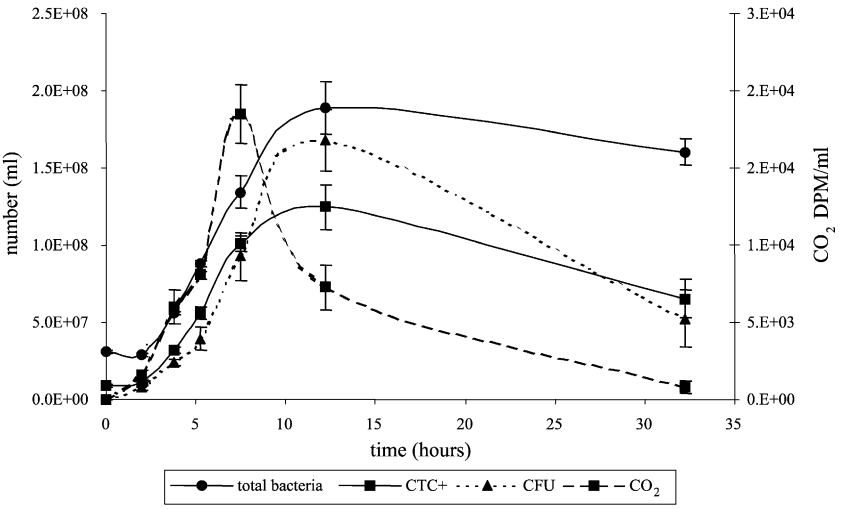

Fig. 1. Determination of total bacteria (ml^{-1}), CTC + cells (ml^{-1}), CFU (ml^{-1}) and released CO_2 during the growth of *E. coli* on M_{63} + 0.01% D-glucose. Data points are the mean and ± are the standard deviation for $n = 3$.

3. Results

Three different methods were used for bacterial counting. The total bacterial number (Sybr-Green®), the CFU count, and the number of CTC-positive cells all gave good agreement for the duration of the lag and exponential phases of growth, with values of 2 and 5 h, respectively (Fig. 1). All measurements reached a maximum at 12.3 h, indicating the beginning of the stationary phase, at which point the count values were 1.89×10^8 total cells ml^{-1}, 1.68×10^8 CFU counts ml^{-1} and 1.25×10^8 CTC-positive cells ml^{-1}. The maximum release of CO_2 was reached at 7.5 h, during the exponential phase (1.85×10^4 DPM ml^{-1}), before decreasing to 7.9×10^2 DPM ml^{-1} 24 h later. The same trend was observed with the CO_2 released per cell (Fig. 2). The highest percentage of CTC-positive cells compared to the total number of bacteria was 75% at 7.5 h (Fig. 2). This percentage decreased slowly to 66% at the beginning of the stationary phase, whereas the CFU count reached its maximum of total cells (89%) at this time. After 32.3 h, the two estimates of active cells were 41% (CTC-positive cells) and 33% (CFU counts).

A statistical analysis performed on pooled data from each of the three growth phases indicated a strong and significant correlation between CTC-positive cells and total bacteria (Pearson's $r = 0.93$; Table 1), as well as between CTC-positive cells and CFU counts ($r = 0.96$). In contrast, the quantity of CO_2 released was not related to CTC-positive cells or to the total bacteria. However, if the statistical analysis was performed without taking into account the data from the stationary phase, the correlation coefficients were improved to 0.99 between CTC-positive cells and total bacteria, and 0.98 between CTC-positive cells and CFU counts. In the same way, the correlation coefficient was improved from a nonsignificant value of 0.50 to a significant value of 0.99 between the amount of CO_2 released and the CFU counts.

Table 1
Pearson's correlation coefficient between bacterial parameters during growth of *E. coli* on M_{63} + 0.01% D-Glucose

	Total bacteria (ml^{-1})	CTC-positive bacteria (ml^{-1})	CO_2 DPM (ml^{-1})
Total bacteria (ml^{-1})	–		
CTC-positive cells (ml^{-1})	0.93[a]	–	
CO_2 DPM (ml^{-1})	0.38	0.64	–
CFU (ml^{-1})	0.88[a]	0.96[a]	0.50

[a] Values of r significant at $p < 0.05$ ($n = 28$).

The hourly growth rates calculated for the different measurements during the lag phase were similar for both the CTC-positive cells and the total bacteria, but they were lower than the growth rates derived from the CO_2 released and the CFU counts (Fig. 3). However, there was no significant between-method

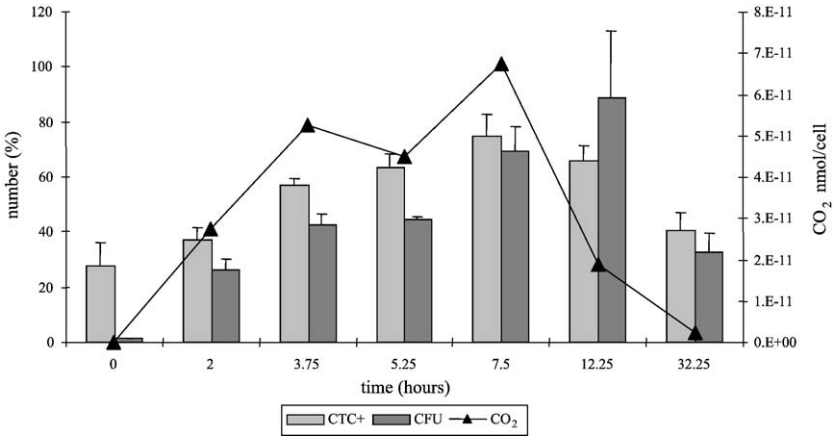

Fig. 2. Percentage of CTC + cells (ml^{-1}) and CFU (ml^{-1}) related to total bacteria (ml^{-1}) during growth of *E. coli* on M_{63} + 0.01% D-glucose. Data points are the mean and ± are the standard deviation for $n = 3$.

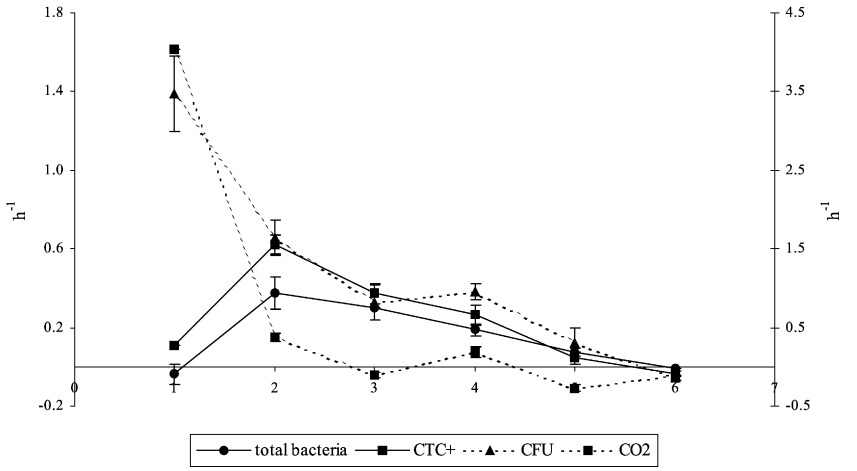

Fig. 3. Growth rate of the total bacteria, CTC + cells, CFU and CO_2 release during different phases of the *E. coli*'s growth (lag phase 1; exponential phases 2, 3, and 4; stationary phases 5, and 6) on M_{63} + 0.01% D-glucose. Data points are the mean and ± are the same standard deviation for $n = 3$.

differences in growth rates estimated during the exponential and stationary phases (Kruskall and Wallis' ANOVA, $n = 24$).

4. Discussion

General acceptance of this method depends on understanding how it works and on clear interpretation of the results (Sieracki et al., 1999). In the cellular electron transport system (ETS), tetrazolium salts function as artificial redox partners instead of the final electron acceptor, oxygen. Thus, CTC is thought to indicate that ETS activity or respiration is occurring. However, the CTC method remains controversial and it is not known if viable bacteria that are physiologically stressed (i.e. damaged or viable but nonculturable) reduce CTC (Schaule et al., 1993). In this study, we tested different physiological states related to the advancing growth stage of a bacterial culture. During the lag and exponential phases, there was a strong correlation between the number of CTC-positive cells and the total number of bacteria, as well as with the amount of CO_2 released. This showed that the increase of CTC-positive cells resulted from both the development of the population and the increased activation state of the cells. Thus, the maximum percentage of CTC-positive cells corresponded to the time of maximum CO_2 release per cell.

During the stationary phase, a sharp decrease of respiratory activity was observed, similar to that shown by Kaprelyants and Kell (1993). The number of total bacteria, the plate counts, and number of CTC-positive cells also decreased after stationary phase was reached, but not to the same extent as the CO_2 release. After 32.3 h, CTC-positive cells still represented 41% of the total population. Thus, bacteria that are physiologically in stationary phase can maintain enough energy to reduce CTC even when respiratory activity is decreased to a low level (Lopez-Amoros et al., 1995). However, in response to long-term stationary phase conditions, energy levels will decrease progressively in the cells (Caro et al., 1999), which will finally result in cells losing their ability to reduce CTC to fluorescent formazan (Lopez-Amoros et al., 1995).

A survey of the literature showed that the percentages of CTC-positive cells observed during the exponential and stationary phases were usually higher than those reported in this study. Values from 90% to 100% have been recorded for the lag phase (Yu and McFeters, 1994; Pyle et al., 1995b; Choi et al., 1996; Bhupathiraju et al., 1999) and values from 50% to 80% (Pyle et al., 1995b; Choi et al. 1996) for the stationary phase for cultures of *Klebsiella pneumoniae*, *Pseudomonas pseudoalcalegenes* and *E. coli*, as well as for environmental samples. However, it is difficult to compare the results between different studies because there is no standardized method for using CTC.

The highest variations in the protocol concern the final concentration and incubation time (Table 2). We found that a wide range of CTC concentrations have been used by different authors. The lowest and highest concentrations were 0.5 (Schaule et al., 1993; Epstein and Rossel, 1995; Kallmbach et al., 1997) and 10 mM (Choi et al., 1996). In the majority of studies, the final concentration varied between 2 and 6 mM with the commonest value being 5 mM (Rodriguez et al., 1992; Pyle et al., 1995a,b; del Giorgio et al., 1997; Bhupathiraju et al., 1999; Choi et al., 1999; Sherr et al., 1999a,b; Sieracki et al., 1999; Van Duyl et al., 1999; Proctor and Souza, 2001). However, at 5 mM, Ullrich et al. (1996) and Servais et al. (2001) demonstrated that the tetrazolium salt was toxic to bacteria and led to an underestimation of active cells. On the contrary, at low concentrations, CTC-stained bacteria grew at the same rates as unstained controls (Epstein and Rossel, 1995).

The incubation times reported by different authors were also variable (Table 2). del Giorgio and Scarobough (1995) showed for lake samples that labeled cells appeared as early as 1 h after the addition of CTC and that both the number and the proportion of CTC-positive cells continued to increase during 8–10 h of incubation. This gradual increase was due to the diversity of metabolic states within the bacterial community. Certain bacteria are present with a very low inherent activity; these need a longer incubation time before accumulating enough reduced dye product to be visible (Caro et al., 1999; Choi et al., 1999; Berman et al., 2001). Yu and McFeters (1994), Ullrich et al (1996) and Proctor and Souza (2001) selected a shorter time for their samples: only 2 h for a biofilm or 4 h in an oligotrophic lake and in a sediment sample (Table 2). The type of bacteria used in the study was also responsible for some of the variability in protocols, as some bacteria reduce the tetrazolium salt poorly (Winding et al., 1994; Cook and Garland, 1997; Smith and McFeters, 1997; Yamaguchi and Nasu, 1997). Species-specific differences in the ability for CTC reduction appear to be independent of aerobic or anaerobic conditions during the incubation (Bhupathiraju et al., 1999; Smith and McFeters, 1997).

All parameters that control the bacterial growth rate can also influence the rate of reduction of CTC such as temperature, pH, concentration of nutrients and substrates (Table 2). Additionally, certain proce-

Table 2
Summary of studies concerning optimal conditions for CTC reduction assay in different ecosystems

Parameters	References	Samples
Final concentration	Rodriguez et al. (1992)	culture
	Ullrich et al. (1996)	fresh/brackish water
	del Giorgio et al. (1997)	lake
	Choi et al. (1999)	seawater + culture
	Bhupathiraju et al. (1999)	aerobic culture
	Servais et al. (2001)	seawater
Incubation time	Rodriguez et al. (1992)	culture
	Schaule et al. (1993)	drinking water + biofilm
	Yu and McFeters (1994)	biofilm
	del Giorgio and Scarborough (1995)	lake
	Walsh et al. (1995)	starved aerobic/ anaerobic culture
	Yu et al. (1995)	soil
	Pyle et al. (1995b)	culture
	Ullrich et al. (1996)	fresh/brackish water
	del Giorgio et al. (1997)	lake
	Bhupathiraju et al. (1999)	anaerobic culture
	Proctor and Souza (2001)	sediment
	Servais et al. (2001)	seawater
Abiotic reductants	Walsh et al. (1995)	starved anaerobic culture
	Smith and McFeters (1997)	anaerobic culture
	Bhupathiraju et al. (1999)	anaerobic culture
Temperature	Pyle et al. (1995a)	culture
	Choi et al. (1999)	seawater
pH	Pyle et al. (1995a)	culture
Nutrients	Pyle et al. (1995a)	culture
	Choi et al. (1999)	seawater + culture
	Sieracki et al. (1999)	seawater + culture
Substrates	Schaule et al. (1993)	drinking water + biofilm
	Bartsch et al. (1999)	freshwater
	Bhupathiraju et al. (1999)	anaerobic culture
	Choi et al. (1999)	seawater
	Sieracki et al. (1999)	seawater culture
Procedure extraction	Yu et al. (1995)	soil
	Mayr et al. (1999)	soil
Storage	Yu et al. (1995)	soil + culture
	Choi et al. (1999)	seawater + culture
	Sieracki et al. (1999)	seawater culture

dures used to extract bacteria from soil using density gradient techniques can cause an underestimation of the amount of intracellular red-fluorescing formazan grains (Mayr et al., 1999). Other extractions using detergents (e.g. pyrophosphate) had no effect on the counting efficiency of CTC-positive cells (Yu et al., 1995). Finally, the conditions of sample storage are also important. Liquid samples retain the same percentage of CTC-positive cells as the original samples for several days in refrigerator (Sieracki et al., 1999) or for several months in liquid nitrogen (Choi et al., 1999).

All of the methods to determine the pool of active bacteria do not yield the same information. Certain methods measure only the actively growing bacteria; others count nongrowing cells as well. Few studies have compared the results of different methods (Table 3). The CTC method determines both growing and nongrowing bacteria, as demonstrated here. Iodonitrotetrazolium (INT) is a tetrazolium salt, similar to CTC, which is tightly coupled to ETS. In culture, it gave similar results as CTC for metabolically active cells during exponential phase (Walsh et al., 1995). However, INT use is more difficult because it is less easily reduced than CTC within bacteria, but is more likely to be reduced by agents other than the direct electron donors within the ETS itself (Smith and McFeters, 1997; Smith, 1998).

The respiration rate and different dyes related to the membrane potential and integrity have been also

Table 3
Studies with a comparison between CTC method and other techniques to determine the pool of active bacteria in different systems

Assay	Technique	References	Samples
Electron transfer system activity	INT	Rodriguez et al. (1992)	culture
		Walsh et al. (1995)	starved/non-starved culture
Respiration rate	CO_2 release and O_2 uptake measurements	Ullrich et al. (1996)[a]	fresh/brackish water
		Cook and Garland (1997)	biorector
		Smith (1998)	seawater
Membrane potential	$DIBAC_4$ (3)	Lopez-Amoroz et al. (1995)	culture
		Suller and Lloyd (1999)	culture
	Oxonol VI	Lopez-Amoroz et al. (1995)	culture
	Sytox green®	Suller and Lloyd (1999)	culture
	Rhodamine 123	Yu and McFeters (1994)	culture
Membrane integrity	Live/dead bacterial kit	Choi et al. (1996)	seawater
		Boulos et al. (1999)	culture + drinking water
		Caro et al. (1999)	culture
		Berman et al. (2001)	freshwater
Metabolic activity	Microautoradiography	Ullrich et al. (1996)[a]	fresh/brackish water
		Karner and Furhman (1997)	seawater
Production	Radioactive thymidine and leucine incorporation	Ullrich et al. (1996)[a]	fresh/brackish water
		del Giorgio et al. (1997)	freshwater
		Sherr et al. (1999b)	seawater
		Lovejoy et al. (2000)	seawater
		Servais et al. (2001)[a]	seawater
Cell enlargement	Direct viable count (DVC)	Yu and McFeters (1994)	culture
		Heijnen et al. (1995)	soil, rhizosphere culture
		Caro et al. (1999)	culture
Viability	Heterotrophic plate count (CFU)	Rodriguez et al. (1992)	ground/waste/seawater
		Schaule et al. (1993)	drinking water
		Yu and McFeters (1994)	culture
		Winding et al. (1994)	soil
		Pyle et al. (1995a)	culture
		Pyle et al. (1995b)	wastewater
		Ullrich et al. (1996)[a]	fresh/brackish water
		Kallmbach et al. (1997)	biofilm
		Caro et al. (1999)	culture

[a] Studies related to CTC toxicity.

compared with the CTC assay. Smith (1998) showed a positive correlation between the consumption of dissolved oxygen and CTC-positive cells, whereas Cook and Garland (1997) did not observe an increase of CTC-formazan (reduced CTC-formazan)-containing cells during a peak in CO_2 production in a bioreactor. In this study, we observed a good correlation between the two measurements during the exponential phase of *E. coli*. However, the rapid decrease of CO_2 production at the beginning of the stationary phase (Kaprelyants and Kell, 1993) showed that this activity was more closely related to substrate limitation. Anionic or cationic membrane-sensitive dyes (e.g. $DIBAC_4$ (3), Oxonol VI, Sytox® green, and Rhodamine 123) enter into inactive cells with depolarised plasma membranes and bind to lipid-containing intracellular components or nucleic acids. These assays give variable results because they are dependent on the strain and metabolic state of the bacterial population (Lopez-Amoros et al., 1995; Suller and Lloyd, 1999). This is not the case for the commercially available Live/Dead® Baclight™ bacterial viability kit, which has a combination of dyes for determining live bacteria (SYTO 9) and dead bacteria (propidium iodide). This kit always shows higher live bacteria counts than the CTC measurements, whatever the environmental conditions (Table 3). The addition of labeled substrates, which may activate cells during the protocol of microautoradiography, explains the higher number of active bacteria which are observed with this method compared to CTC (Ullrich et al., 1996; Karner and Furhman, 1997).

Other methods simply determine the number of growing bacteria by means of bacterial production measurements or cell enlargement (Direct Viable Count (DVC)). The comparison between these results and CTC results are often contradictory. Some researchers (Ullrich et al., 1996; del Giorgio et al., 1997; Sherr et al., 1999a,b; Lovejoy et al., 2000) found a positive correlation between the bacterial production and the number of CTC-positive cells, whereas others did not find a strong relationship (Choi et al., 1996; Servais et al., 2001). Yu and McFeters (1994) and Caro et al. (1999) determined the same or a higher percentage of CTC-positive cells compared to DVC (Kogure et al., 1979) in normal conditions of culture. These results probably depended on the physiological state of the bacterial community, and not on the nutrient quantity and organic substrate provided in the DVC method.

Finally, plate counts give information on the substrate responsiveness of cells and only show those cells that are capable of dividing and forming colonies (Button et al., 1993). The high correlation between the CFU counts and cells positive for CTC was already observed by Pyle et al. (1995a) and showed that CTC was a good estimator of cell viability (Sherr et al., 1999a). However, the number of CTC-labelled cells is generally higher than the CFU count in all studies, owing to the difficulty of growing all members of the bacterial community in artificial conditions (Table 3).

In summary, several authors have found that CTC staining represents a simple and effective method of counting active bacteria (Yu and McFeters, 1994; del Giorgio et al., 1997) in aerobic as well as anaerobic conditions (Bhupathiraju et al., 1999), and without background reduction (Rodriguez et al., 1992; Walsh et al., 1995; Smith and McFeters, 1997). The method detects not only respiring bacteria (Pyle et al., 1995b; Sieracki et al., 1999) but also the majority of metabolically active cells, which are able to initiate cell division (Rodriguez et al., 1992; Schaule et al., 1993; del Giogio et al., 1997; Sherr et al., 1999a). However, the inhibitory effect of high concentrations of CTC on bacterial metabolism may undermine the usefulness of the CTC assay for the evaluation of active bacterial cells, under these conditions it can be assumed that the count of active bacteria may be underestimated (Ullrich et al., 1996; Bartscht et al., 1999; Servais et al., 2001). Also, bacteria with a low level of metabolic activity are not detected as CTC-positive cells (del Giorgio et al., 1997; Choi et al., 1999; Sherr et al., 1999a; Sieracki et al., 1999). It is possible that some cells accumulate only very small amounts of formazan and are therefore below the limit of the detection of conventional epifluorescence microscopy (del Giorgio and Scarborough, 1995). The use of more sensitive equipment, such as flow cytometry, to detect these weakly-labelled cells will greatly increase the ability to detect small differences in the abundance of active bacteria, as well as giving additional indications about the size distribution and specific activity of individual respiring bacteria (del Giorgio et al., 1997; Sherr et al., 1999a). Finally, the diversity in experimental protocols presently in use for CTC staining make

impossible the drawing of meaningful comparisons between studies or between ecosystems.

Acknowledgements

This research was supported by European community DG XII (Contract ENV4-CT97-0582) and we thank R.M. Forster for his helpful review of the manuscript. This is Publication 3007 of NIOO-CEMO, Yerseke, The Netherlands.

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