Activity assessment of microorganisms eluted from sediments using 5-cyano-2,3-ditolyl tetrazolium chloride: a quantitative comparison of flow cytometry to epifluorescent microscopy

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Abstract

Enhanced natural recovery may be successfully implemented at contaminated sediment sites, which are often characterized by large volumes of sediments with low to moderate levels of contamination to cost-effectively reduce human and ecological risks. In order to evaluate the potential for microbial contribution to remediation strategies, physiological assessment of indigenous microorganisms is essential. We report here a method for rapid and accurate assessment of metabolically (5-cyano-2,3-ditolyl tetrazolium chloride [CTC]) active microorganisms eluted from sediment, based on flow cytometry (FCM). Microorganisms eluted from sediment and suspended in estuarine medium were stained with CTC and counterstained with the DNA stain Picogreen (PG). Optimal stain concentrations and incubation times were employed. FCM quantification of the dual-stained microorganisms was not statistically different (paired t test; \( t = 0.05; \) df = 10) from enumeration (total or active numbers) by an established method (fluorescent microscopy) over two orders of magnitude (approximately \( 10^4 – 10^6 \)/ml). This research suggests that FCM, which allows the collection and analysis of multiple parameters (light scatter and fluorescence emission), is a good candidate for microbial characterization in complex environmental matrices, such as sediments, across a broad range of activity levels (~ 2% to 84% of total). Potential applications for this FCM-based method include the rapid assessment of changes in sediment microbial activity in response to enhanced bioremediation strategies.

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Keywords: CTC; Sediments; Flow cytometry; Anaerobic

1. Introduction

Contaminated freshwater, estuarine, and marine sediments are a priority of national concern, due to potentially adverse impacts on aquatic ecosystems, human health, and economic development. For the vast majority of contaminated sediment sites, bioremediation technologies aimed at degrading and sequestering contaminants may represent an effective and affordable sediment management approach. For the development of potential bioremediation strategies, an accurate appraisal of the physiological state of indigenous subsurface bacteria is essential (Yu et al., 1995). Direct optical detection methods such as epifluorescent microscopy (EPM) and flow cytometry (FCM), which utilize stains specific to biological molecules (e.g., DNA stains), are increasingly being employed for microbial characterization due to the
awareness of problems associated with culture-based assays (Vives-Rego et al., 2000). FCM is advantageous because fluorescently stained microbial samples can be simultaneously analyzed for multiple parameters (e.g., red, yellow, green fluorescence emission and light scatter) (Kalmbach et al., 1997; Nebe-von-Caron et al., 2000). In addition, FCM detectors (photomultiplier tubes [PMTs]) provide superior sensitivity as compared to EPM. Despite the speed, sensitivity, and reproducibility of FCM, applications have been limited in subsurface microbial characterization.

Numerous stains have been developed for the assessment of cell function including reproductive ability, metabolic activity, membrane activity, and membrane potential. These methods have been employed to distinguish viable, but nonculturable, microorganisms that would be discounted in traditional, growth-based assays. Several of these probes are compatible with flow cytometry including: (i) lipophilic cationic dyes (e.g., Rhodamine 123-accumulated on inner side of energized membranes), (ii) DNA intercalators (e.g., propidium iodide-crossed only altered cell membranes), (iii) redox dyes (e.g., 5-cyano-2,3-ditolyl tetrazolium chloride [CTC]-reduced during respiration to form fluorescent intracellular precipitate) (Lopez-Amoros et al., 1997; Nebe-von-Caron et al., 2000). By simultaneously staining with selected multiple fluorochromes (e.g., non-specific stains and activity stains), FCM analysis can reveal the functional heterogeneity of the microbial sample (Kalmbach et al., 1997; Nebe-von-Caron et al., 2000). Rapid, high-throughput microbial techniques, such as FCM, that accurately quantify metabolic activity are of interest in the bioremediation field due to the need for spatially diverse analysis of ecological parameters in a wide range of applications.

For indicating metabolically active cells, the use of tetrazolium salts, such as CTC, is beneficial due to rapid reduction (minutes to hours) to formazan by intracellular reductase enzymes. The mechanisms for biotic CTC reduction under aerobic conditions have been researched extensively (Lopez-Amoros et al., 1995; Smith and McFeters, 1997). Although CTC application under anaerobic conditions has been documented in the literature (Smith and McFeters, 1997; Bhupathiraju et al., 1999a; Proctor and Souza, 2001; Gruden et al., 2003), a comprehensive understanding of the anaerobic processes involved in CTC reduction has yet to be disclosed (Bhupathiraju et al., 1999a; Gruden et al., 2003). To date, CTC has been used to assess microbiological activity in complex, anaerobic environmental matrices including wastewater, sludge, soil, and sediments (Rodriguez et al., 1992; Winding et al., 1994; Yu et al., 1995, Proctor and Souza, 2001; Gruden et al., 2003; Ziglio et al., 2002). For example, CTC was used to quantify fluctuations in microbial activity in response to varying environmental conditions (e.g., nutrients) in wastewater applications including activated sludge and anaerobic digester sludge (Gruden et al., 2003; Ziglio et al., 2002).

The fluorescent precipitate formed by CTC reduction has been quantified in whole cells using optical detection methods such as direct microscopy (Rodriguez et al., 1992, Pyle et al., 1995; Gruden et al., 2003). Subsurface microbial samples present challenges to optical detection methods due to an abundance of non-living bacterial sized particles and inherent background fluorescence (Yu et al., 1995; Proctor and Souza, 2001). As compared to other redox dyes, CTC works well in complex matrices because the fluorescent red intracellular precipitate is easily detected and retains its fluorescence upon storage for up to 2 days (Yu et al., 1995). Potential applications for this method include the rapid determination of microbial contribution to remediation of subsurface contaminants through the comparison of CTC-active bacterial counts from samples at contaminated sites to samples from geologically similar but uncontaminated background sites (Bhupathiraju et al., 1999b). This manuscript details a CTC-based flow cytometric method for assessing the metabolic activity of sediment microorganisms and provides a comparison to an established direct count protocol.

2. Materials and methods

2.1. Sediment sample preparation

Microorganisms were eluted, using an established protocol (Barkovskii and Adriaens, 1996, 1998) from Passaic River, NJ, Pearl Harbor, HI, and San Diego Bay, CA, sediments (VS = 10–20%). The eluted microorganisms were suspended in estuarine medium.
(40 mM phosphate buffer (KH₂PO₄/Na₂HPO₄; pH = 7.2); 2.0 g/l NaHCO₃; 0.9 g/l (NH₄)₂Cl; 0.07 g/l CaCl₂·2H₂O; 0.13 g/l MgSO₄·7H₂O; 0.02 g/l FeCl₂·4H₂O; cysteine–HCl; trace minerals; vitamin stock; resazurin). In order to evaluate the impact of organic acid (OA) amendment and incubation time on microbial activity, some samples were enriched with organic acid cocktail (45 mg/l acetate; 30 mg/l propionate; 15 mg/l butyrate; 10 mg/l benzoate) and incubated for up to 7 days while constantly stirring at room temperature prior to analysis. All transfers, enrichments, amendments, and incubations were carried out in an anaerobic chamber. The percentages of CTC-active microorganisms in OA amended samples and estuarine medium only samples were compared as a function of incubation time.

2.2. DNA stain

Picogreen (PG) (P-7589; Molecular Probes, Eugene, OR), a proprietary, high-affinity, green, nucleic acid dye (ex/em \( \lambda = 502/523 \) nm), was stored at \(-20^\circ \mathrm{C} \) for up to 30 days. A range of PG concentrations (\( 5 \times 10^{-4} \) to \( 1 \times 10^{-2} \) of stock) and incubation times (2 min to 4 h) were investigated for determining optimum conditions (\( 5 \times 10^{-3} \) of stock and 30 min) for enumeration of sediment microorganisms. After PG incubation, samples were split for independent FCM and fluorescent microscope (EPM) counts (Lange et al., 1997). In order to maximize fluorescence emission, samples were not fixed prior to analysis. In all instances, they were analyzed within 4 h of preparation.

2.3. Metabolic activity stain

The metabolic activity of the sediment microorganisms was determined using the tetrazolium redox dye CTC (Polyscience, Warrington, PA) (ex/em \( \lambda = 475/600 \) nm). The CTC assays were performed using filter-sterilized (0.2 \( \mu \)m) CTC stock solution (20 mM) prepared in ultrapure water and stored at \( 4^\circ \mathrm{C} \) for a maximum of 2 days (Rodriguez et al., 1992). The appropriate final CTC concentration and incubation time (5 mM and 1 h) for flow cytometric analysis were determined in optimization experiments (Smith et al., 1994; Bhupathiraju et al., 1999b; Proctor and Souza, 2001; Gruden et al., 2003).

2.4. Flow cytometry

The samples were analyzed using a FACSCalibur™ dual laser flow cytometer, with six parameters of detection: forward scatter (FSC), sidescatter (SSC), and four fluorescence intensity detectors (FL1, FL2, FL3, FL4) (Becton Dickinson, San Jose, CA). The specifications of the detector channels were as follows: FL1 (green light: \( \lambda = 515–545 \) nm); FL2 (yellow/orange: \( \lambda = 563–607 \) nm); FL3 (dark red: \( \lambda = 670 \) nm LP); and FL4 (red: \( \lambda = 653–669 \) nm). An air-cooled argon laser (488 nm) and red diode laser (635 nm) were used for excitation. All parameters were collected as logarithmic signals.

2.5. Calibration and instrument settings

Daily calibration was completed by analyzing unlabeled beads (to simulate unstained microorganisms) and fluorescein isothiocyanate (FITC: yellow-green), phycoerythrin (PE: red-orange), and peridinin chlorophyll protein (PerCP: red) beads (to simulate fluorochrome labeled microorganisms) (CaliBRITE™ beads [Cat. No. 349502], Becton Dickinson). The beads have expected values for FSC, SSC, and fluorescence, and these values are used to adjust instrument settings, to set fluorescence compensation, and to check instrument sensitivity (BD FACSComp™ software) before any samples were processed. The unlabeled beads were used to discriminate actual events from background debris using the FSC detector. The photomultiplier tube voltages were adjusted so that the mean channel values of the beads corresponded to the programmed target values. The threshold was adjusted to minimize background noise.

Each fluorochrome emits light over a range of wavelengths when excited by the laser. This spectral overlap was corrected using electronic compensation. Using a mixed suspension of equal amounts of unlabeled, green, yellow, and red beads, the software differentiated green-yellow signal from orange signal and orange from red signals by aligning labeled bead populations with the corresponding unlabeled bead population. After the instrument settings were established, FL1, FL2, and FL3 sensitivity (mean channel separation between the signal produced by labeled and unlabeled beads) and FSC and SSC sensitivity (mean channel difference between light scatter, beads signal,
Initial instrument settings for each experiment were: threshold-SSC; FSC-E01, logarithmic amplification; SSC-375, logarithmic amplification; FL1-600, logarithmic amplification; FL2-550, logarithmic amplification; FL3-800, logarithmic amplification. An unstained sample was processed prior to each run to optimize the instrument settings. Settings were deemed acceptable when the entire unstained population was visible on an FSC vs. SSC dot plot and the bell-shaped histogram showed a distinct gap from the noise signal on a histogram of both FSC and SSC events. In addition, the voltages of FL1, FL2, and FL3 were adjusted using fluorescence as a function of SSC to contain the target population within the first order of magnitude on the logarithmic y-axis (10⁰ to 10¹) to correct for background fluorescence in the unstained sample. The system threshold was set on SSC signals and all analyses were performed at the low setting (12 μl/min). Enumeration was carried out using standard procedures involving a lipophilized bead pellet that dissolved, releasing a known concentration of fluorescent beads (TruCount™ Tubes: 340567; Becton Dickinson) and in accordance with previously published protocols (Lange et al., 1997). Data analysis was performed manually using CellQuest™ software (Becton Dickinson).

2.6. Gating and control samples

A negative control (unstained sediment bacteria) was used to define the background or negative region on fluorescence contour plots. A positive control (PG- and CTC-stained pure culture of bacteria) was used to determine the location of the fluorescent signal or the region expected for a positive response. SSC, which is often considered a measure of the internal complexity of the labeled cell, and fluorescent (FL1/FL3) emission were used simultaneously to separate the target population (fluorescently labeled microorganisms) from background particles. Negative and positive controls were compared to stained samples. Events that generated a fluorescent signal above the negative region (background) were gated as the population of interest. In addition, each experiment included controls such as PG only, CTC only, and PG and CTC after microorganisms were exposed to sodium azide in an effort to determine background fluorescence associated with non-specific binding of the selected stains and to account for potential extracellular and/or abiotic reduction of CTC.

2.7. Microscopic analysis

The Zeiss Axioplan Universal Microscope (451888, Carl Zeiss MicroImaging, Thornwood, NY) was equipped with an epifluorescence attachment (448006, Carl Zeiss), a super high-pressure mercury lamp power supply (Carl Zeiss) and 50-W mercury lamp (HBO 50W L2, Toby’s Instrument Shop, Saline, MI) for epifluorescence illumination. Fluorescent turret and filter sets (Optavar Fl, 446421, Carl Zeiss) were standard with the Axioplan.

2.8. Filtration and enumeration

The fixation, filtration, and enumeration methods as well as the detection limit used in this research were adapted from widely accepted protocols and were previously reported (Gruden et al., 2003). After incubation on a shaker table at room temperature (± 25 °C) in the dark, samples were filtered through a 0.2-μm black polycarbonate filter (Poretics, Cat. No. K02BP02500) backed with a 25-mm glass-fiber pre-filter (11-394-106C, Fisher Scientific, Fair Lawn, NJ). Filters were dried and mounted on a slide with immersion oil and a cover slip and stored in the dark prior to microscopic analysis. Triplicates were counted. Laboratory controls were prepared including dead (sodium azide) samples and method blanks. A minimum of seven fields and 200 microorganisms were counted per slide. Only microorganisms that were both DNA stained (green) and contained red intracellular precipitate were considered metabolically active.

3. Results

The results from all three sediments studied in this work were similar. The data presented in this manuscript represent sediment samples from the Passaic River, NJ (Volatile Solids = 15%). Prior to each experiment, an unstained sample, which served as a negative control, was analyzed to determine back-
ground fluorescent response due to the sample matrix. Fig. 1 is a contour plot of the unstained sample, and it displays green fluorescence (FL1) as a function of SSC. Background fluorescence is shown below $10^{1}$ on the logarithmic FL1 axis. In addition, a dual-stained (PG and CTC) pure culture was analyzed, and both FL1 (green fluorescence) and FL3 (dark red fluorescence) were plotted as a function of SSC. This positive control sample was used to determine the region (R1) of expected response from PG-stained microorganisms (Fig. 2A) and region (R2) of expected response from CTC-active microorganisms as a subset of R1 (Fig. 2B).

Fig. 1. Control samples used to establish gating protocol. (A) Contour plot of green fluorescence emission (FL1) vs. SSC for unstained sediment microorganisms. R2 is defined as the gate or region of interest where green fluorescent emission is expected to occur. Contour plot of (B) green fluorescence emission (FL1) and (C) dark red fluorescence emission (FL3) as a function of SSC for a positive control sample (pure culture stained with both PG and CTC). R1 delineates DNA containing (stained green) microorganisms. R2 contains metabolically active microorganisms (dark red) that are a subset of the population defined by R1 only.

Fig. 2. Contour plot of green fluorescence emission (FL1) as a function of SSC for PG-stained sediment microorganisms. R1 is defined as the gate containing sediment microorganisms (82.5% of total events). Contour plot of dark red fluorescence emission (FL3) as a function of SSC for CTC- and PG-stained sediment microorganisms. R2 is defined as the gate containing metabolically active sediment microorganisms (83.9% of events in R1).
Green fluorescence (FL1) was plotted as a function of SSC to discriminate the PG-stained sediment microorganisms (Fig. 2A). In Fig. 3, the gated population (R1), which composed 82.5% of the total events, had a uniform fluorescent intensity (FL1) and SSC and was readily distinguished from background events (below $10^1$ on the logarithmic scale of FL1 and SSC axes). Metabolically active sediment microorganisms fluoresced both dark red and green when stained with CTC and PG. The dark red fluorescent (FL3) events, a subset of those in R1 (green fluorescent events from Fig. 2A), were plotted as a function of SSC (Fig. 2B). The subpopulation of microorganisms that were stained by both PG and CTC are circumscribed in R2 (83.9% of R1).

Optimization experiments were performed for FCM analysis of CTC-stained sediment bacteria (Fig. 3). No statistically significant change (paired t-test; $\alpha = 0.05$) in % CTC activity was observed at concentrations greater than 5 mM CTC (Fig. 3A) or after more than 1 h of incubation (Fig. 3B). Optimal incubation conditions for microscopic analysis of sediment bacteria were 5 mM CTC and 4 h of incubation (data not shown).

Sediment microorganisms stained with CTC and counterstained with PG were enumerated using FCM and compared to values estimated by an established direct count method (EPM). PG-stained (total) sediment microorganisms quantified using EPM and FCM.
ment microorganisms are bright green. Microorganisms that are considered metabolically active as judged by CTC contain a red fluorescent intracellular precipitate. In order to determine if the two methods (EPM and FCM) for enumerating both total and active sediment bacteria produced different results, the average of the differences was assessed (Fig. 4) (Berthoux and Brown, 1994). In Fig. 4, samples were plotted in ascending order (1–6) based on total concentration (EPM). At highest and lowest concentrations (samples 1 and 6), FCM appears to overestimate the number of bacteria, and the lowest concentration demonstrates the greatest difference between values (0.53). There was no statistically significant difference (paired t test; \( \alpha = 0.05; df = 10; |t| Stat = 0.33 \)) between the total number of microorganisms quantified using EPM and FCM. In addition, the number of CTC-active organisms determined using FCM was not statistically different than the number determined using EPM (paired t test; \( \alpha = 0.05; df = 10; |t| Stat = 0.46 \)). Flow cytometry and epifluorescent microscopy provided comparable quantification of CTC-active microorganisms across two orders of magnitude (10^4–10^6 cells/ml) (Fig. 5).

The percent of CTC-active microorganisms eluted from sediment with and without OA amendment was plotted as a function of incubation time (Fig. 6). OA addition resulted in a statistically significant increase in percent CTC active from approximately 16–72% after 12 h of incubation (Fig. 6). No statistically significant difference was noted in total numbers of sediment microorganisms for either treatment at any incubation time (data not shown). After several days of incubation (7 days), the percent of CTC-active microorganisms of the OA amended sample returned to a baseline value of approximately 4% CTC active.

4. Discussion

PG distinguished the microbial population from sediment background fluorescence, as compared to an unstained sample. Other stains tested (e.g., SYTO BC, Molecular Probes) resulted in non-specific staining of the background and an inability to distinguish the microorganisms (data not shown). As cited in previously reported sediment studies using direct count methods (Weinbauer et al., 1998; Griebler et al., 2001), high-affinity green nucleic acid dyes, such as PG, effectively achieved separation of the community. Optimum PG incubation conditions (3 × 10^-5 of stock and 30 min) for enumeration of microorganisms eluted from sediment (data not shown) were in the range of those previously reported (Weinbauer et al., 1998).

Several authors have successfully distinguished CTC-active microorganisms in aqueous environmental samples using FCM (Kaprelyants and Kell, 1993; del Giorgio et al., 1997; Sieracki et al., 1999). Proctor and Souza (2001) employed epifluorescent microscopy to enumerate CTC-active sediment microorganisms. However, FCM analysis of subsurface microorganisms has been considered difficult due to red autofluorescence (Davey and Kell, 1996). In order to eliminate interferences associated with red background fluorescence, the detection of sediment microorganisms was carried out using a green stain (PG) for microbial identification in this work. Gating protocols were adapted from Sieracki et al. (1999). CTC-active sediment microorganisms were readily separated from red autofluorescence (background) since only green events were further analyzed for CTC (dark red) activity. In addition, fluorescence intensity of labeled microorganisms was maximized because samples were not fixed prior to analysis, which occurred directly following (<4 h after) preparation. Preliminary tests indicated a 50% decrease in fluorescent signal following sample fixation (1% formaldehyde
for 30 min) (data not shown) as previously reported (Lebaron et al., 1998).

The CTC optimization values determined for this work (5 mM CTC and 4 h of incubation), used for both FCM and EPM analyses, are in agreement with previously published protocols for anaerobic microorganisms in soils and sediments (Proctor and Souza, 2001; Bhupathiraju et al., 1999b; Yu et al., 1995). As expected, the optimum incubation time for FCM analysis is significantly shorter (1 h) than for epifluorescent microscopy (4 h) because the FCM detectors (photomultiplier tubes), which are considerably more sensitive than EPM detectors (e.g., the human eye) (Sieracki et al., 1999), can identify lesser amounts of reduced formazan without difficulty.

FCM analysis (up to 10^3 cells/s) of dual-stained sediment microorganisms was 5–10 times faster than EPM analysis (approximately 1 cell/s) when the time required for sample preparation and instrument maintenance was incorporated. The amount of time saved per sample increased with increasing number of samples. FCM analysis eliminated the need to prepare slides over several dilutions for accurate counting, the laborious process of counting individual fields for each individual fluorescent stain, and the effort associated with manual data reduction.

Total and active microorganisms were accurately quantified using standardized counting beads using a previously published protocol (Lange et al., 1997). The greatest difference between the two methods occurred at the lowest concentration of active microorganisms (~1 × 10^4/ml) indicating the possibility for increased error. This concurs with preliminary laboratory data that indicated poor reproducibility and accuracy in instances where the concentration was outside of the acceptable signal to noise range as determined during instrument calibration with unstained samples (data not shown). The quantification of dual-stained sediment microorganisms by FCM corresponded to EPM numbers over two orders of magnitude (approximately 1 × 10^4 to 1 × 10^6/ml). In aqueous environmental samples, the concentration for accurate quantification of microorganisms, as compared to EPM, was in a similar range (5 × 10^5/ml to 5 × 10^6/ml) (Sieracki et al., 1999; del Giorgio et al., 1997).

A statistically significant increase in % CTC activity occurred after elution (with or without OA amendment), indicating metabolic stimulation (two- to fourfold) due to the elution protocol and subsequent suspension in estuarine medium only. In addition, OA amendment in the elution protocol (enrichment) significantly increased the number of CTC-active microorganisms only (total number of organisms did not increase) after several hours (12 h) of incubation. The amendment of OA is a common strategy to enhance metabolic activity in sediments prior to experimentation or to stimulate in situ activity for remediation purposes (Barkovskii and Adriaens, 1996). However, the implications of this protocol on microbial viability and respiration have not been quantified and are likely to be site specific. These results underline the requirement for a consistent elution protocol between samples and individual experiments in order to compare resulting data. These results also demonstrate that OA amendment is an ineffective method for increasing microbial activity in the short term (<12 h) for these samples and the specific OA cocktail. This data indicates that the FCM-based method can accurately measure percent CTC activity of sediment microorganisms across a broad range (2.1–83.8% CTC active).

5. Conclusions

Samples with varying sediments and biogeochemistry may require individualized protocols. Specific protocols should be developed including optimum incubation conditions for each of the selected fluorescent stains, microbial elution/enrichment methods, and appropriate FCM operating parameters. Several controls are required to distinguish the target cells and physiological stains from background fluorescence including positive and negative controls. If possible, sample fixation should be avoided to maximize the fluorescent intensity of stained microorganisms, facilitating the distinction between microorganisms and inert particles.

Although CTC use may be considered contentious (due to opposing views on the operational definition of active microorganisms) (Creach et al., 2003; Servais et al., 2001; Choi et al., 1999; Kaprelyants and Kell, 1993), it has been effectively applied as an indicator of variation in metabolic activity in complex matrices (e.g., biosolids and sediments) (Gruden et al.,
CTC has previously been employed to detect enhanced microbial activity corresponding to zones of organic contamination (Bhupathiraju et al., 1999b; Lopez-Amoros et al., 1998). This research demonstrates the utility of CTC activity assessment of sediment microorganisms using flow cytometry across a broad range of activity levels. This rapid and accurate method may be applied to evaluate the potential for microbial contribution to bioremediation in contaminated sediments.

**Acknowledgements**

This work was supported in part by the NOAA-Cooperative Institute for Coastal and Estuarine Environmental Technology (CICEET). Tom Komorowski in the School of Public Health at the University of Michigan and Jordan Peccia in the Department of Civil and Environmental Engineering at Arizona State University assisted in the preparation of the manuscript.

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