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A new direct microscopy based method for evaluating in-situ bioremediation

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Abstract

A new epifluorescent microscopy based method using 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and 5-(4,6-dichlorotriazinyl) aminofluoroscein (DTAF) was developed for quantifying total microbial biomass and evaluating levels of microbial activity. CTC is a tetrazolium dye that forms fluorescent intracellular formazan when biologically reduced by components of the electron transport system and/or dehydrogenases of metabolically active bacteria. DTAF is a fluorescein-based fluorochrome that selectively stains bacterial cell walls thereby enabling quantification of total bacterial biomass. CTC can be used in conjunction with DTAF to provide the optical resolution necessary to differentiate metabolically active cells from inactive cells in microbial populations associated with subsurface soils. The CTC/DTAF staining method has been shown to be effective for quantifying the metabolic activity of not only aerobic bacteria, but also diverse groups of anaerobic bacteria. This method allows for the rapid quantification of total and active bacterial numbers in complex soil samples without enrichment or cell elution. In this study, CTC/DTAF staining was applied to evaluate in-situ microbial activity in petroleum hydrocarbon contaminated subsurface soils from Sites 3 and 13 at Alameda Point, CA. At each site, subsurface microbial activity at two locations within contaminated plumes were examined and compared to activity at two geologically similar but uncontaminated background locations. Significant bacterial populations were detected in all soils examined, and the biomass estimates were several orders of magnitude higher than those obtained by conventional culture-based techniques. Both the total

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bacterial concentrations and the numbers of active bacteria in soils from contaminated areas were substantially higher than those observed in soils from background locations. Additionally, the percentages of metabolically active bacteria in the contaminated areas were consistently higher than those detected in background areas, suggesting that the enhanced microbial activity was due to microbial contaminant degradation. Although conventional heterotrophic plate counts failed to show significant microbial activity at either of the sites, soil gas carbon dioxide and methane measurements confirmed that hydrocarbon contaminant degradation was occurring in both areas. The CTC/DTAF staining protocol proved to be a rapid, reliable, and inexpensive method to evaluate the progress of in-situ bioremediation.

Keywords: Metabolic activity; Anaerobic; 5-Cyano-2,3-ditolyl tetrazolium chloride (CTC); In-situ bioremediation; 5-(4,6-Dichlorotriazinyl) aminofluoroscein (DTAF)

1. Introduction

To better understand the ecology of microorganisms in the subsurface environment and to quantify concentrations of microbial populations, methods are needed to differentiate between metabolically active and inactive microorganisms as they exist in situ, i.e. associated with subsurface soils and groundwater. Conventional culture-based detection methods are incapable of identifying non-viable or non-culturable microorganisms, and result in underestimations of microbial concentrations. Thus, the sole use of culture-based methods in bioremediation studies can lead to inaccurately measured responses of subsurface bacteria to pollution. For example, standard plate counts will not detect slow-growing, non-culturable, or inactive microorganisms and bacteria that require specific redox or nutrient conditions not provided by the chosen culture media. In addition, methods that require elution of cells from soil particles are plagued by low cell recovery and the potential for damaging cells during the elution process. A genetic amplification method, polymerase chain reaction (PCR), has been applied to quantify subsurface soil microorganisms [1]. While promising, PCR cannot yet assess subsurface microbial activity and is susceptible to biased amplification [2].

The technique of coupling fluorescent biological staining with membrane filtration has been successfully used to enumerate microorganisms in a variety of aquatic and terrestrial environments, and is an ideal method for use in bioremediation research. Fluorochrome-based enumeration has been used to measure total bacterial concentrations in subsurface soils using DNA intercalating agents such as acridine orange and 4′6-diamidino-2-phenylindole (DAPI) [3–9]. More recently, environmental studies on water samples and biofilms have reported the use of fluorochromes which have been coupled with various heterocyclic tetrazolium dyes or nalidixic acid to determine not only total microbial numbers, but also the fraction of metabolically active microorganisms [10–18]. Tetrazolium dyes are reduced from a colorless complex to a brightly colored, intracellular, formazan precipitate by components of the electron transport system and/or dehydrogenase enzymes of active bacterial cells. In several ecological studies, non-fluorescing tetrazolium dyes have been used concurrently with DNA intercalating agents or fluorescing antibodies to estimate fractions of active and inactive bacteria in environmental samples [14,19–26]. An intrinsic disadvantage to the use of non-fluorescing tetrazolium products is that they must be used with transmitted light

microscopy, which used in combination with fluorochromes, dictates the sequential use of epifluorescent and light microscopy. More importantly, in subsurface soil samples, the microscopic visualization of non-fluorescing formazan precipitates within bacteria is difficult because of visual interference from opaque and/or translucent soil particles, and the occlusion of transmitted light through slide or filter-mounted samples.

A new generation of tetrazolium-based redox dyes which reduce to fluorescing intracellular formazan precipitates are now commercially available in high purity. Fluorescing formazan offers analytical advantage over its non-fluorescent analogues because it provides superior resolution, decreased interference from opaque particulate matter, and can be used concurrently with other fluorochromes. A cyanted tetrazolium derivative, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), has been used in several aquatic studies to assess the activity of bacteria suspended in water and wastewater, and within biofilms [14,16,23,24,27–33]. CTC has recently been adapted for use in subsurface soils [25,34]. The combined use of DNA intercalating agents and CTC in soil samples, however, has presented some complications. DNA intercalating agents can irreversibly bind to soil mineral surfaces, causing interfering levels of background fluorescence. In addition, in both pure-culture and environmental samples, the intercalation of DAPI with bacterial nucleoids attenuates the fluorescent signal emitted from CTC-formazan precipitates [35]. Finally, many wide-band-pass commercial epifluorescent filter sets are not capable of concurrently producing optimized excitation of DAPI and CTC, resulting in underestimation of CTC-positive bacteria in environmental samples [16].

In response to the existing limitations of culture-based, staining, and genetic amplification methods, we developed a protocol that combined direct epifluorescent microscopy of stained bacterial cell walls with a fluorescent activity stain to characterize the metabolic state of subsurface microorganisms associated with soil particles. Our approach for measuring subsurface microorganism concentrations and activity was to modify a DNA staining method to selectively counterstain bacterial cell walls with a fluorescein derivative DTAF (5-(4,6-dichlorotriazinyl) aminofluoroscein) concurrent with CTC incubation. The developed method was tested in the laboratory using various pure cultures of aerobic and anaerobic bacteria [36]. Field investigations using subsurface samples containing a wide variety of contaminants ranging from 100 + -year old refinery wastes to recent jet fuel spills from Alameda Point, Alameda, CA (formerly the United States Naval Air Station at Alameda), were conducted to evaluate the applicability of the developed protocol as a tool for evaluating in-situ bioremediation. The strategy for assessing microbial activity with respect to remediation of subsurface contaminants was to compare microbial activity within samples of subsurface solids from outside contamination zones to those from geologically similar zones within contaminated areas.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Desulfovibrio desulfuricans subsp. *desulfuricans* (ATCC 27774), *Methanobacterium formicicum* (ATCC 33274), and *Pseudomonas pseudoalcalegenes* subsp. *pseudoalcale-*

genes (ATCC 17440) were obtained from American Type Culture Collection in Rockville, MD. *Geobacter sulfurreducens* (ATCC 51573) was obtained from Dr. D.R. Lovley. *Syntrophus aciditrophicus* (ATCC 700169) was obtained from Dr. M.J. McInerney. *Escherichia coli* K-12 strain W3110 was from Dr. L. Alvarez-Cohen's laboratory at the University of California in Berkeley, CA.

E. coli, *P. pseudoalcalegenes* subsp. *pseudoalcalegenes*, *D. desulfuricans* subsp. *desulfuricans*, and *M. formicicum* were grown in a basal medium that contained the following components (g/l): NaCl, 4.0; NH_4Cl, 1.0; KCl, 0.1; KH_2PO_4, 0.1; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.04; 50 mM Tris–HCl buffer (pH 7.4), 5 ml trace metal solution [37], and 10 ml vitamin solution [37]. *S. aciditrophicus* was grown in a basal medium described previously [38]. For growth of *D. desulfuricans* subsp. *desulfuricans*, *M. formicicum*, and *S. aciditrophicus* the basal medium was reduced using cysteine–HCl (3 mM). *G. sulfurreducens* was grown in a basal medium described previously [39]. Substrates and electron acceptors used for growth of cultures are listed in Table 1. Methods of Balch and Wolfe [40] were used for the preparation and use of anaerobic media. All cultures were incubated at 37°C.

2.2. Description of study area

Alameda Point is located on the north-western end of Alameda Island, in Alameda, CA. Alameda Point is approximately 2 miles long and 1 mile wide, and occupies 2634 acres. Site 3 consists of approximately 2 acres located in the southeastern portion of Alameda Point. This site was an abandoned fuel storage area containing four partially buried concrete tanks and one partially buried steel tank. Approximately 365,000 gal of

Table 1
CTC/DTAF staining of aerobic and anaerobic cultures[a]

Culture	Growth substrates (electron donor/ electron acceptor)	CTC-active cell count ($\times 10^6$ cells/ml)	Total DTAF cell count ($\times 10^6$ cells/ml)
P. pseudoalcalegenes subsp. *pseudoalcalegenes*	Acetate (30 mM)/O_2	330 ± 70	350 ± 70
E. coli	Glucose (30 mM)[b,c]	150 ± 10	180 ± 20
P. pseudoalcalegenes subsp. *pseudoalcalegenes*	Acetate (30 mM)/ NO_3 (30 mM)[c]	450 ± 40	510 ± 30
S. aciditrophicus	Crotonate (10 mM)[b,d]	150 ± 10	160 ± 20
G. sulfurreducens	Acetate (10 mM)/ fumarate (30 mM)[d]	215 ± 10	315 ± 40
D. desulfuricans subsp. *desulfuricans*	Lactate (40 mM)/ SO_4 (20 mM)[c]	1300 ± 100	1350 ± 150
M. formicicum	Formate (10 mM), H_2 (80%)/CO_2 (20%)	40 ± 1	70 ± 20

[a] CTC cell enumeration assays were done after cultures were incubated for 72 h. Data are averages of triplicates ± standard deviation.
[b] Cultures were grown fermentatively.
[c] Cultures were grown in basal medium with a head space of N_2 (100%).
[d] Cultures were grown in basal medium with a head space of N_2 (80%) and CO_2 (20%).

AVGAS is believed to have leaked from the tanks during the 1960s and early 1970s. The tanks were subsequently destroyed and buried in place. Site 13 consists of approximately 30 acres located in the southeast corner of Alameda Point. This site is the former location of the Pacific Coast Oil Works refinery which operated between 1879 and 1903. Refinery wastes and asphaltene residues were released at the site during the 24-year history of the refinery. A JP-5 release from a jet engine test cell was reported at the site in 1991. The hydrogeology, hydrocarbon-release history and distribution, and the soil and groundwater quality at the sites has been summarized previously [41].

2.3. Soil sampling, handling and processing

Subsurface soil samples were collected from Sites 3 and 13 of Alameda Point. Soil samples were collected from four locations at each site. Two of the sampling locations in the contaminated area, designated CA2 and CA3, were located within the area of petroleum hydrocarbon contamination. Two of the sampling locations in the background area, designated BG1 and BG2, were located outside of the area of contamination. Soil samples were obtained using a Geoprobe 5400 Direct Penetration Technology (DPT) rig equipped with a MacroCore sampler. The sampler was lined with sterilized clear 4 cm diameter by 120 cm long Polyethylene Terephthalate Glycol (PETG) sampling tubes and pushed into the ground until the desired depth was reached. Upon retrieval of the soil cores, sampling tubes were removed from the sampler and the ends were capped with sterilized polypropylene covers. Samples were then transported to the laboratory in a cooler and processed within 12 h. All sample collection and handling was done under aerobic conditions. For Site 3, soils obtained from a depth of 1.9 and 2.3 m were analyzed. 3CA2 was located in a grass covered area with predominantly aerobic groundwater (>2 mg/l oxygen), while 3CA3 was located in an asphalt covered area with predominantly anaerobic groundwater (<1 mg/l oxygen). Both Site 3 background locations had predominantly anaerobic groundwater (<1 mg/l oxygen). For Site 13, soils obtained from a depth of 1.6 and 2.3 m were analyzed. 13CA2 had predominantly anaerobic groundwater (>1 mg/l oxygen), while 13CA3 and both Site 13 background locations had predominantly aerobic groundwater (>4 mg/l oxygen). Approximately 25 g of the soil was diluted in 250 ml of phosphate buffer (pH 7.2) containing 0.1% sodium pyrophosphate in Erlenmeyer flasks and was subsequently mixed for 4 h with magnetic stirrers. Different dilutions of the soil suspensions were subjected to concurrent CTC/DTAF staining and direct epifluorescent microscopy (see below) for estimating the bacterial concentrations and bacterial activity in the soils.

2.4. Plate counts

Heterotrophic bacteria in soils were enumerated by spread-plating using the methods of Ghiorse and Balkwill [42]. Ten grams of soil were mixed with sterile 0.1% sodium pyrophosphate–10 H_2O diluted to 100 ml volume and placed on a shaker table at 160 rotations per minute (rpm) for 15 min. Aqueous suspensions of soil slurry were serially diluted with sterile 0.1% sodium pyrophosphate–10 H_2O (pH 7.0). Aliquots of the dilutions were plated in triplicate on PTYG agar plates. Samples were inverted and

incubated aerobically at room temperature for 28 days. Results were enumerated by visually counting individual colonies and are reported in colony forming units (CFU) per gram dry soil.

2.5. CTC / DTAF staining

The viability of pure cultures and bacterial activity in subsurface soils was determined using the tetrazolium redox dye CTC (Polyscience, Warrington, PA) with DTAF (Molecular Probes, Eugene, OR) as a counterstain. CTC is biologically reduced to fluorescent formazan precipitates by intracellular reductase enzymes and identifies metabolically active cells. DTAF is a nonspecific fluorescein derivative that covalently adheres to bacterial cell walls regardless of metabolic state.

CTC staining was performed using a procedure modified from Rodriguez et al. [16]. Staining was performed in 2.0 ml microcentrifuge tubes containing 0.5 ml of phosphate-buffered saline (10 mM Na-phosphate buffer [pH 7.4]; 138 mM NaCl; 2.7 mM KCl). CTC was added to achieve a final concentration of 5 mM from a stock solution (50 mM) prepared in deionized water. This concentration was chosen based on an optimization assay conducted with several pure cultures [36]. Samples were incubated aerobically for 4 h at either 37°C (for pure cultures) or 22°C (for subsurface soils) prior to counterstaining. No exogenous carbon substrates were added during CTC incubations. Because CTC is photoreactive, all samples were covered with opaque material to protect the contents from light. All staining solutions were prepared immediately prior to use and were filter sterilized. All staining assays were performed in triplicate. Autoclaved (121°C, 15 min) and/or formaldehyde-treated (3.7% final concentration, treated for 30 min) cultures were used as killed controls.

Following CTC incubation, a filter-sterilized DTAF concentrate (5 mg/l DTAF in 50 mM Na_2HPO_4; [pH 9.0]) was added to a final concentration of 0.5 mg DTAF/ml similar to the methods of Bloem et al. [43] and Sherr et al. [44]. Continuing to protect the samples from light, they were mixed with DTAF for 20 min at 22°C and then passed through a 0.22-μm, black, polycarbonate membrane filter (Poretics, Livermore, CA) supported by a silver filter (25-mm diameter; 5.0-μm pore size). The filter and support were thread sealed in a 50-ml capacity, autoclavable, polysulfone, filter funnel (Gelman Sciences, Ann Arbor, MI, effective filtration area 2.86 cm^2). Bacteria retained on the filter surface were then washed with a minimum of 100 ml of PBS (50 mM Na_2HPO_4; 145 mM NaCl; pH 9.0) to remove unbound DTAF. All filtration and washing was completed under a vacuum of no greater than 103.5 kPa. The DTAF stock solution was made fresh and stored for not more than 2 h.

Following the final wash, all filters were dried under vacuum and immediately transferred to clean microscope slides. Approximately 50 μl of Tris-buffered glycerol (1:1 v/v) containing 2% 1,4-diazobicyclo[2,2,2]octane (to retard quenching of the fluorescent signal) was applied to filter surfaces. The mounting solution was adjusted to pH 8.6 with glacial acetic acid to optimize the fluorescence of the cell-bound DTAF. Following mountant application, coverslips were immediately laid on the filters. Mounted filters were immediately examined by epifluorescent microscopy.

Epifluorescent microscopy was performed with an Olympus BH2-RFCA microscope equipped with epifluorescence illumination (100-W mercury burner). An Olympus filter cube unit with an excitation filter (BP490), a dichroic mirror (DM500), and a barrier filter (O515) were used to simultaneously observe CTC-formazan and DTAF fluorescence. An eyepiece with a graticule calibrated for 1100 × magnification was used for all bacterial counting. Formazan deposits in the CTC-stained cells were examined under the same epifluorescent conditions as the DTAF stained samples. Only cells that both retained the green fluorescence from DTAF and contained bright intracellular orange formazan deposits were considered active. All fluorescing particles consistent with common bacterial morphologies were counted. The counting protocol of Hobbie et al. [45] was modified to count 10 random fields per slide for each filtered sample (> 25 cells per field). The concentration of total and active bacteria were determined by the relation:

$$C_N = \left[(NA_f)/(Va) \right] \mathrm{DF} \quad (1)$$

where C_N = bacteria concentration (cells/ml or cells/g); N = number of bacteria per field; A_f = effective filtration area (286 mm^2); V = volume of dilution applied; a = area of microscopic field (0.008 mm^2); DF = dilution factor.

For soils, the cell count was normalized to the total suspended solids of the stained soil dilution.

3. Results

3.1. Variability of CTC reduction at different redox conditions

3.1.1. Pure culture assays

To help validate bacterially-mediated CTC reduction in different environments, pure cultures of batch-grown bacteria were exposed to CTC under carefully controlled redox conditions. Using different electron acceptors, seven independent, experiments were conducted to estimate the variability in CTC reduction by different pure cultures. Each culture, electron acceptor and substrate was chosen to represent a specific metabolic process (i.e. aerobic respiration, denitrification, sulfate reduction, fermentation, etc.). Collectively, all seven experiments spanned the reduction potential range between aerobic conditions (+600 mV) to methanogenic conditions (−400 mV).

The metabolic activity of aerobic bacteria, facultative anaerobic bacteria, and obligate anaerobic bacteria were effectively detected using CTC/DTAF staining with aerobic incubations (Table 1). On average, more than 80% of the total cells cultured were observed to reduce CTC regardless of redox condition.

3.2. Collection and recovery of bacteria from Alameda Point soils

3.2.1. Plate counts

Standard heterotrophic plate counts obtained from soils at the contaminated (CA) sample locations did not significantly differ from those obtained from soils at the background (BG) sample locations at Site 3 (Table 2). At Site 13, plate counts varied

Table 2
Bacterial biomass in Alameda Point soils[a]

Sample designator	Sampling depth (m)	Heterotrophic bacteria plate counts ($\times 10^2$ CFU/g soil)	Total-DTAF cell count ($\times 10^6$ cells/g soil)	CTC-active cell count ($\times 10^6$ cells/g soil)
3BG1	1.9	80 ± 5	100 ± 10	10 ± 3
	2.3	60 ± 3	100 ± 20	30 ± 5
3BG2	1.9	30 ± 4	100 ± 4	10 ± 2
	2.3	30 ± 4	300 ± 50	10 ± 2
3CA2	1.9	80 ± 3	2500 ± 400	1600 ± 300
	2.3	50 ± 2	1500 ± 100	1000 ± 100
3CA3	1.9	bd[b]	1000 ± 40	600 ± 50
	2.3	50 ± 5	800 ± 30	400 ± 30
13BG1	1.6	20 ± 4	200 ± 10	20 ± 10
	2.3	7600 ± 3000	300 ± 20	30 ± 2
13BG2	1.6	140 ± 20	100 ± 4	40 ± 10
	2.3	10 ± 2	100 ± 20	40 ± 10
13CA2	1.6	1800 ± 200	2100 ± 80	1000 ± 40
	2.3	70 ± 5	3400 ± 200	1400 ± 100
13CA3	1.6	760 ± 400	6200 ± 400	2800 ± 300
	2.3	10 ± 4	1800 ± 100	700 ± 60

[a] Data are averages of triplicates $\pm$ standard deviation.
[b] bd, Below detection limit.

significantly at different locations with the highest plate count occurring at one of the 2.3 m depth background location. No consistent pattern of microbial activity with respect to presence or absence of hydrocarbon contaminants was apparent at either site. Respectively, plate counts of culturable heterotrophic bacteria ranged from below detection to 8×10^3 CFU/g soil at the 3CA sample locations and from 3×10^3 to 8×10^3 CFU/g soil at the 3BG sample locations. At the 13CA sample locations, plate counts of culturable heterotrophic bacteria ranged from 1×10^3 to 1.8×10^5 CFU/g soil and at the 13BG sample locations from 1×10^3 to 7.6×10^5 CFU/g soil.

3.2.2. Total DTAF direct cell counts

Bacterial biomass was detected in all of the soils examined at Sites 3 and 13 (Figs. 1 and 2). The highest concentrations of bacterial cells were measured in samples taken from contaminated locations. Concentrations of total subsurface bacteria in the contaminated sample locations at Sites 3 and 13 ranged from 8×10^8 to 2.5×10^9 cells/g soil and from 1.8×10^9 to 6.2×10^9 cells/g soil, respectively. At both sites, the total bacterial cell concentrations from the background sample locations were nearly an order of magnitude lower than the average concentrations measured in soils from the CA sample locations.

3.2.3. CTC-active cell counts

CTC-active cells were detected in all of the soils examined at Sites 3 and 13 (Figs. 1 and 2). Subsurface soil concentrations of CTC-positive cells in the contaminated sample

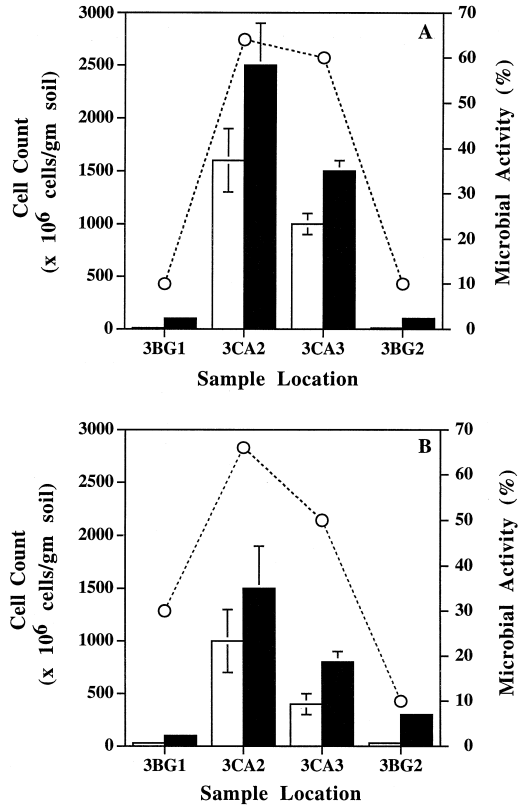

Fig. 1. CTC-positive cell counts (open bars), total DTAF direct counts (solid bars), and percent activity (open circles) in (A) soils from a depth of 1.9 m and (B) soils from a depth of 2.3 m at Alameda Point Site 3.

locations at Sites 3 and 13 ranged from 4×10^8 to 1.6×10^9 cells/g soil and from 7×10^8 to 2.8×10^9 cells/g soil, respectively. The active cell count in subsurface soils for the BG sample locations at both sites were substantially lower than those observed in the CA sample locations. For all soil samples analyzed, concentrations of CTC-positive cells were several orders of magnitude greater than plate counts (Table 2).

3.2.4. Variation of cell counts with depth and oxygen content

3.2.4.1. Plate counts. Subsurface soil samples were taken from two depths at each site. No consistent pattern of microbial activity with depth was observed with heterotrophic plate counts at either site (Table 2). At Site 3, no significant differences between the numbers of heterotrophic bacteria were observed in shallow and deep soils, regardless of contamination presence. At Site 13, the numbers of culturable heterotrophic bacteria in soils from shallow depth at both the contaminated locations and one uncontaminated background location (13BG2) were higher than those observed in the deeper soils. At

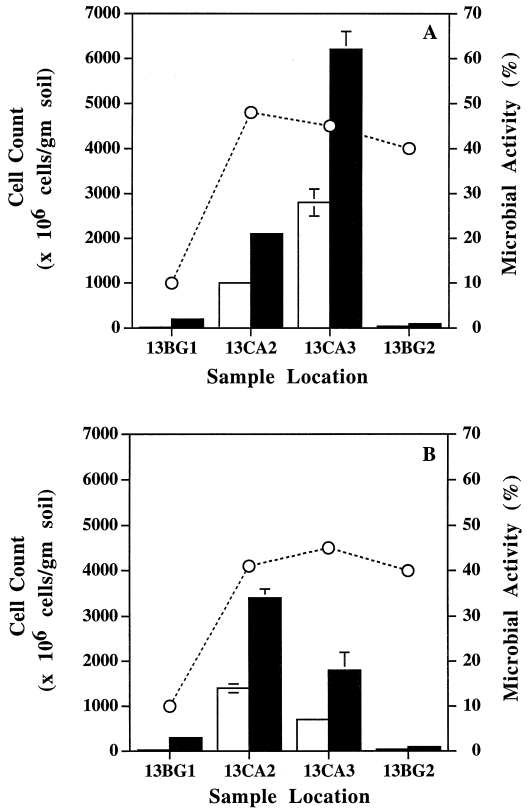

Fig. 2. CTC-positive cell counts (open bars), total DTAF direct counts (solid bars), and percent activity (open circles) in (A) soils from a depth of 1.6 m and (B) soils from a depth of 2.3 m at Alameda Point Site 13.

the other background location, 13BG1, the number of heterotrophic bacteria in the deep soil samples were substantially higher than those observed in the soil collected from the shallow depth.

No pattern with respect to plate counts and goundwater oxygen content was observed. That is, sample locations with aerobic groundwater (13BG1, 13BG2, 13CA3 and 3CA2) did not have a consistently higher or lower plate counts than sample locations with anaerobic groundwater.

3.2.4.2. Direct counts using CTC / DTAF method. The total and active bacterial cell concentrations in soils collected at shallow depth (1.6 m depth at Site 3, and 1.9 m depth at Site 13) were generally higher than those in soils collected at greater (2.3 m depth at both sites) depth (Table 2).

With one exception (2.3 m at 13CA3), contaminated sample locations with aerobic groundwater (13CA3 and 3CA2) generally had higher total and active bacterial numbers than contaminated locations with anaerobic groundwater (13CA2 and 3CA3).

4. Discussion

4.1. CTC / DTAF staining method

A new staining method was developed for quantifying microbial biomass and microbial activity in soil samples. The procedure involves incubation of soils with the tetrazolium redox dye CTC and subsequent counter-staining using a bacterial cell wall stain DTAF. The stained cultures are then enumerated by epifluorescent microscopy. Metabolically active bacterial cells are identified as those that are stained by both CTC and DTAF.

4.2. Comparison of CTC / DTAF with other fluorochromes used in soil systems

The CTC/DTAF staining method appears to be superior to the previously described CTC/DAPI staining method in several aspects. First, the CTC/DTAF staining method allows for reliable and concurrent identification of active and inactive cells using the same epifluorescent filter set. While the CTC/DAPI staining method allows for concurrent visualization of active and inactive cells using various epifluorescent filter settings, reduced detection of CTC-stained cells may cause underestimation of active cells [16,24]. Use of different epifluorescent filter sets for visualizing CTC-stained cells and DAPI-stained cells have also presented problems; bacteria accumulating multiple intracellular CTC-formazan crystals might be counted more than once and thereby introduce an overestimate of active cells [24]. Secondly, the intercalation of DAPI with bacterial nucleoids has been reported to dampen the fluorescent signal emitted from CTC-formazan precipitates [35]. DTAF binds to proteinatious material in bacterial cell walls, and does not appear to interfere with intracellular fluorescence emission from CTC-formazan. Lastly, because it can non-specifically bind to soil matrices and other organic particulate matter, DAPI has been reported to stain non-nucleoid-containing bacterium-like particles [46], and also to cause interfering levels of background fluorescence. While some background fluorescence was sometimes present, DTAF exhibited little nonspecific binding to the various soil constituents.

4.3. CTC reduction under different redox conditions

The use of CTC for evaluating aerobic microbial activity is well established [16,22–24,27–30,32,33,47–54]. Recent studies have suggested that CTC could also be used to detect the metabolic activity of bacteria under some anaerobic conditions [54,55]. Until recently however, the ability of facultative and obligate anaerobic bacteria to reduce CTC has not been reported. The results from this study along with a previous study [36] suggest that nitrate-reducing bacteria, iron-reducing bacteria, sulfate-reducing bacteria and methanogenic bacteria are able to reduce CTC. Further, these studies show that concurrent CTC/DTAF staining method could be used to quantify metabolic activity and cell concentrations of not only aerobic bacteria, but also a diverse group of anaerobic bacteria.

4.4. Field application of CTC / DTAF staining

In order to evaluate CTC/DTAF as a tool for evaluating in-situ bioremediation, subsurface soil samples collected from contaminated and uncontaminated background areas were subjected to CTC/DTAF staining. The strategy for assessing microbial activity with respect to remediation of subsurface contaminants was to compare total and active bacterial counts from soil samples taken from contaminated areas to those from geologically similar but uncontaminated background areas. Total DTAF and CTC-positive cell counts from contaminated areas were one to two orders of magnitude higher than those measured in uncontaminated background areas. More importantly, the percentage of total bacteria that were CTC-positive were higher in the contaminated areas (range 50% to 66% at Site 3, and 39% to 48% at Site 13) than those measured in the background samples (range 10 to 30% for Site 3, and 10% to 40% for Site 13). Direct epifluorescent microscopy of subsurface bacteria using CTC and DTAF suggest the following: (i) indigenous subsurface bacteria actively responded to contaminants present at Alameda Point Sites 3 and 13, and (ii) some of these bacteria used the contaminants as a growth substrate.

The enhanced bacterial activity measured with CTC/DTAF staining in CA sample locations at both sites appears to be due to contaminant degradation activity in soils. Carbon dioxide and methane, which are typical products of active bacteria, were consistently detected in the soil gas at these locations while they were not detected in the background locations [41]. Additionally, independent isotopic studies completed at this site demonstrated that most of the methane and carbon dioxide produced at the CA locations was from hydrocarbon degradation by microorganisms [41,56].

The higher biomass concentrations observed with CTC/DTAF staining in the shallow samples at both locations may be due to the influence of groundwater, since soil moisture analyses indicated that the water table at the time of sampling was situated at a depth between 1.0 and 1.9 m at Site 3 and at a depth between 0.9 and 1.6 m at Site 13 [41]. Elevated biomass numbers would be expected to occur in the vicinity of the water table since groundwater movement may replenish essential nutrients required for microbial metabolism while the presence of oxygen under unsaturated conditions enables aerobic respiration.

4.5. Comparison of CTC / DTAF counts and plate counts

Standard heterotrophic plate counts of soil bacteria were an average of 5 orders of magnitude lower than total bacterial counts determined using DTAF staining and direct microscopy. Plate counts were also approximately 4 orders of magnitude lower than active bacterial counts as determined by CTC staining. These findings are in accordance with previous studies with aquatic samples that reported direct-microscopy-based total bacterial counts to be orders of magnitude greater than CFU's or MPN's estimated from culture-based methods [16,22,23].

Since the heterotrophic plate counts cultured from contaminated soils did not significantly differ from those in uncontaminated background areas, the enhanced bacterial activity detected by the DTAF/CTC staining method was not enumerated by

conventional plate counts. The large differences between the CTC-positive counts and the plate counts may have been due in part to the significant amounts of anaerobic activity present at the two sites. However, there was no consistent pattern observed between the plate counts and the oxygen content of the groundwater. These results demonstrate some of the limitations of culture-based methods to gauge microbial activity associated with in-situ biodegradation.

In previous studies of aquatic and terrestrial environments, it has been demonstrated that tetrazolium reduction assays are rapid and convenient for quantifying metabolically active microorganisms. The data provided by this study, confirms that the CTC/DTAF staining of soils accurately detects enhanced microbial activity in zones of petroleum hydrocarbon contamination, and that direct microscopy had improved sensitivity compared to bacterial isolation using standard culture-based techniques. Thus, the CTC/DTAF staining method appears to be more reliable than conventional culture-based techniques for quantifying microbial activity associated with in-situ bioremediation. Further, since the soil sampling method required for CTC/DTAF is not different from that currently used for conventional microbial analyses, and the only required instrumentation is a fluorescent microscope, this methodology may be especially attractive for field-based bioremediation applications.

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