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Use of substrate responsive-direct viable counts to visualize naphthalene degrading bacteria in a coal tar-contaminated groundwater microbial community

C. Bakermans, E.L. Madsen*

Department of Microbiology, Cornell University, Ithaca, NY 14853-8101, USA

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Abstract

A microscopy-based method was developed to distinguish naphthalene-degrading bacteria within the microbial community of a coal tar-contaminated groundwater system. Pure cultures of *Pseudomonas putida* NCIB 9816-4 were used to develop the substrate responsive-direct viable count (SR-DVC) method. Cells were concentrated on membrane filters, placed on agar plates of Stanier's minimal basal salts media containing antibiotics (nalidixic acid, piromidic acid, pipemidic acid, and cephalixin), and exposed to vapors of naphthalene. Following brief incubation, samples were fixed in 2% formaldehyde and examined by epifluorescent microscopy. Pure cultures displayed the expected cell elongation response to the SR-DVC assay and required a minimum incubation time of 9 h for differentiation of elongated cells. When applied to groundwater samples from the study site, naphthalene responsive cells in the groundwater community were easily distinguished from unresponsive cells and debris (350 ± 180 substrate responsive cells/ml, relative to negative controls with no added growth substrate). In an attempt to reduce background counts of elongated bacteria and fungi, the SR-DVC procedure was modified by adding a wash step prior to incubation and a fungal inhibitor, cyclohexamide, to the plates. When groundwater samples were subjected to the modified procedure, only cells in washed samples showed a significant response to naphthalene (150 ± 25 cells/ml), indicating the presence of inhibitory substances in the groundwater. Variations in response of the groundwater microbial community to the two SR-DVC procedures suggest that subsurface conditions (microbial and chemical composition) vary temporally. SR-DVC allows the phenotypes of individual naturally occurring cells to be assessed. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Direct viable count; Groundwater; Naphthalene

1. Introduction

Pure cultures of microorganisms have been used to obtain the majority of physiological, biochemical, and molecular biological information describing the

metabolism of environmental contaminants. However, the relevance of pure culture-based information to biodegradation processes that occur in soils, sediments, and groundwaters is not always clear (Madsen, 1998; Madsen et al., 1991). Techniques in 16S rRNA and catabolic gene analysis are bridging the gap in knowledge between naturally occurring microbial communities and pure cultures. These techniques have contributed significantly to new

*Corresponding author. Tel.: +1-607-255-3086; fax: +1-607-255-3904.

E-mail address: elm3@cornell.edu (E.L. Madsen).

information about taxonomic and catabolic diversity of in situ microbial communities (e.g., Amann et al., 1995; Pace, 1997; Stapleton et al., 1998; van Elsas et al., 1998; Wilson et al., 1999).

Methods for examining the activities of microorganisms in their natural environments remain limited. Of the possible approaches (e.g., Amann and Kühn, 1998; Chen et al., 1997; Hodson et al., 1995; Jansson and Prosser, 1997; Lee et al., 1999; Lisle et al., 1999; Nybroe, 1995; Ouverney and Fuhrman, 1999; Ward, 1984), the direct viable count (DVC) method is promising. DVC measures substrate uptake and potential division yet does not completely rely on culturability, and is easily extendable to the examination of specific substrates. Since its introduction in 1979, DVC has been used extensively as an indicator of the general metabolic activity of bacteria in the marine environment (Desmonts et al., 1992; Holmquist and Kjelleberg, 1993; Joux and LeBaron, 1997; Kogure et al., 1979). DVC has been used to study physiological states of cells (Joux et al., 1997; Lisle et al., 1999), biofilms (Kalmbach et al., 1997a,b; Yu and McFeters, 1994a; Yu et al., 1993), the response of biofilms to disinfection (Yu and McFeters, 1994a), and the survival of bacteria introduced into the rhizosphere (Heijnen et al., 1995). The original assay has been gradually improved by examining samples on membranes (Braux et al., 1997; Desmonts et al., 1992) and by using suites of antibiotics (Guyard et al., 1999; Joux and LeBaron, 1997). The suitability of DVC as an indicator of general heterotrophic metabolic activity has been well documented. To our knowledge, only one other study (Someya et al., 1998) has used DVC to examine the response of natural microbial communities to individual substrates (glucose, starch, and carboxymethylcellulose).

In our subsurface study site, coal tar waste is a major resource for heterotrophic activity. Coal tar waste consists primarily of polycyclic aromatic hydrocarbons; naphthalene being the most abundant (approximately 30% of the initial contamination, Taylor et al., 1996). Dissolution and transport processes have produced a plume in which naphthalene groundwater concentrations range from 4.3 mg/l near the point of deposition to 0.5 mg/l 300 m downgradient (Taylor et al., 1996). Therefore, naphthalene-based metabolic assays are appropriate for

studying microorganisms at this site. In this study, a substrate responsive-direct viable counts (SR-DVC) procedure was developed to assess the ability of microorganisms native to coal tar-contaminated groundwater to utilize naphthalene. The procedure was first refined with *Pseudomonas putida* NCIB 9816-4 to determine the required incubation time. SR-DVC was then used on groundwater samples from our contaminated site to characterize the native microorganisms. Subsequently, the procedure was modified to address cell elongation seen in the negative (no added substrate) controls.

2. Materials and methods

2.1. Preparation of model bacteria

Pseudomonas putida NCIB 9816-4 was grown in 10 ml 5% PTYG (0.25 g/l Bacto-Peptone, 0.25 g/l tryptone, 0.5 g/l yeast extract, 0.5 g/l glucose, 0.6 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.07 g/l CaCl_2 , pH 7.0) at 30°C, 250 rpm, for 2 days. Cells were pelleted in a refrigerated Sorvall (SS-34 rotor) centrifuge at $8600 \times g$ for 10 min. Pellets were washed 3 times in 10 ml phosphate-buffered saline (PBS) containing 120 mM NaCl, 2.7 mM KCl, 10 mM potassium phosphate buffer at pH 7.6, with a final resuspension in 10 ml of PBS. Following incubation for 3 h at 30°C, 250 rpm, cells were diluted to an appropriate concentration in PBS for use in the SR-DVC assay.

2.2. Field site and groundwater sampling

The site studied is located in South Glens Falls, NY (e.g., Madsen et al., 1991; Murarka et al., 1992; Stuart-Keil et al., 1998; Taylor et al., 1996; Wilson et al., 1999; Wilson and Madsen, 1996). Groundwater was pumped from monitoring wells using a portable GeoPump (Geotech Environmental, Denver CO) fitted with a size 15 pump head and Pt-treated silicone tubing in the peristaltic rollers. New polyethylene tubing was inserted into each well to the screening depth and groundwater samples were collected after purging for four well volumes at a flow rate of 300 ml/min.

2.3. Substrate responsive-direct viable counts

In the laboratory, pure cultures were prepared as above and 10 ml of sample was used in the following procedure with overnight incubation at 30°C. In the field, quadruplicate 25- or 50-ml groundwater samples from each well were prepared (within 10 min of collection) according to the following procedure and incubated for 48 h, at a combination of room and outdoor temperatures.

All samples were concentrated on Isopore polycarbonate membrane filters (0.2 µm pore size, 13 mm diameter, black; Millipore, Bedford, MA). Filters were placed (cell side up) on agar plates of minimal basal salts (Stanier et al., 1966) medium containing antibiotics as follows: 20 mg/l nalidixic acid, 10 mg/l piromidic acid, 10 mg/l pipemidic acid, and 10 mg/l cephalexin (Joux and LeBaron, 1997). Plates were exposed to vapors of naphthalene in an airtight glass chamber and negative controls were incubated in the absence of naphthalene. Plates were incubated in the dark. Cells were fixed prior to analysis by immersing filters in 1 ml of 2% formaldehyde in PBS in 1.5-ml Eppendorf tubes. Samples were stained with acridine orange and visualized by epifluorescence microscopy as described below. Following the statistical procedures of Trolldenier (Anguish and Ghiorse, 1997; Trolldenier, 1973), at least 15 fields per sample were counted for total cells and at least 50 fields per sample were counted for elongated cells. Cells were counted as 'elongated' if their length exceeded 4 µm.

Following initial trials, the method was modified to include: washing the filters with 20 ml PBS plus antibiotics (nalidixic acid, piromidic acid, pipemidic acid, and cephalexin) prior to incubation on plates and incorporating 0.1 mg/ml cyclohexamide into the plates.

2.4. Acridine Orange direct counts of groundwater bacteria

Ten-ml groundwater samples were concentrated on Isopore polycarbonate membrane filters (0.2 µm pore size, 13 mm diameter, black) at the site and filters were immediately fixed in 2% formaldehyde in PBS. All solutions that came into contact with any counted sample were filter-sterilized with 0.2-µm

pore size disposable cellulose acetate filters prior to use. Upon return to the laboratory, samples (filters) were stained with 100 µl 0.01% Acridine Orange for 2 min, washed with 2 ml 1 M NaCl, and washed with 2 ml distilled, deionized H₂O. Samples were blotted with bibulous paper to dry and mounted under a 22-mm² cover slip with 15 µl 32.7 mg/ml DABCO. Coverslips were sealed with vaspar (vaseline: paraffin 1:1) and samples were viewed immediately by epifluorescence microscopy. Samples were counted for total and elongated cells as above following the statistical procedures of Trolldenier (Anguish and Ghiorse, 1997; Trolldenier, 1973).

2.5. Epifluorescence microscopy

All samples were examined with a Zeiss Standard 14 microscope equipped for bright field and epifluorescence with a mercury arc lamp. Fields were viewed under oil immersion with a bright field ×100 Plan-apochromat objective lens (numerical aperture, 1.25). Conventional epifluorescence imaging was done at an excitation wavelength of 440–490 nm with a 510-nm reflector and a 520-nm barrier filter.

2.6. Naphthalene determinations

Groundwater samples (≈ 20 ml) were collected from the field site in sterile 40-ml vials (I-Chem 100 series, Hayward, CA) prefilled with 6 ml of hexanes: butanol (9:1) and 1 ml of 1 N HCl. Vials were weighed before and after sample addition in order to determine the amount of sample added. After sample addition, vials were shaken vigorously to facilitate extraction of naphthalene into the organic phase. Triplicate samples were taken from each well and kept on ice or at 4°C until analysis. One-microliter samples were drawn from the organic phase for analysis on a Hewlett-Packard Model 5890 Series II gas chromatograph equipped with a 30 m × 0.25 mm × 0.25 µm film thickness, HP-5 (5% phenyl methyl silicone; Hewlett-Packard) fused-silica capillary column connected to a Hewlett-Packard Model 5971A quadrupole mass selective detector operated at an electron energy of 70 eV and a detector voltage of 2000 (Wilson and Madsen, 1996). A splitless injection was used, with a 1-min delay before septum

purge. The carrier gas was helium traveling at a linear velocity of 30 cm/s. Injector and detector temperatures were 250 and 300°C, respectively. The ion source pressure was maintained at 1.0×10^{-5} Torr. The GC temperature profile was 40°C for 1 min, followed by increasing the temperature at a rate of 10°C/min until the final temperature of 250°C was reached. Naphthalene retention time was 9.80 min. The detection limit was 10 ppb naphthalene.

2.7. Statistical analysis

The average elongated cell count data of triplicate or quadruplicate samples was normalized by subtracting the average elongated cell count of the negative control. The normalized average elongated cell count of the sample is referred to as the substrate responsive cell count. The Student's *t*-test was performed on the normalized count data in order to determine statistical significance of the results.

3. Results and discussion

3.1. Development of SR-DVC using *Pseudomonas putida* NCIB 9816-4

SR-DVC is based on the direct viable count (DVC) procedure. DVC is traditionally performed on cells in suspension in the presence of nalidixic acid and a complex growth substrate such as yeast extract. SR-DVC elucidates the response of organisms to a single, specific growth substrate. In this study, the environmentally relevant substrate is naphthalene, a bicyclic aromatic hydrocarbon that is volatile and only mildly soluble in water (32 ppm, Taylor et al., 1996). This low water solubility limits aqueous phase substrate delivery to bacteria. Instead, delivery of naphthalene in the vapor phase is commonly used to grow colonies on minimal media agar plates with naphthalene as the sole carbon and energy source (Herrick et al., 1997; Kleinheinz and Bagley, 1997; Stuart-Keil et al., 1998). Therefore, minimal media agar plates were used, so that naphthalene could be introduced by vapor phase delivery. The low concentration of cells in groundwater samples also limits the feasibility of using a suspension-based assay. For this reason, cells were concen-

trated onto polycarbonate membranes and these membranes were placed onto the agar plates for analysis by SR-DVC (Braux et al., 1997; Desmonts et al., 1992). In an attempt to ensure antibiotic susceptibility of the native microbial community, a mixture of cell division-inhibiting antibiotics was used and included nalidixic acid, piromidic acid, pipemidic acid, and cephalexin (Guyard et al., 1999; Joux and LeBaron, 1997). The SR-DVC procedure that was developed based on these criteria was subsequently tested on pure cultures and natural microbial communities in groundwater.

Pure cultures of *P. putida* NCIB 9816-4 showed the expected elongation response in the SR-DVC assay (Fig. 1). Substrate responsive cells were quantified as the number of elongated cells in the sample minus the number of elongated cells in the negative control (the same sample incubated with no added growth substrate during the SR-DVC assay). Significantly more cells of *Pseudomonas putida* NCIB 9816-4 elongated in the presence of naphthalene than in its absence during the SR-DVC assay. The percent of substrate responsive *P. putida* NCIB 9816-4 cells in the SR-DVC assay varied from experiment to experiment within a range of 28–43% of the total cell count. This level of response of pure cultures to the SR-DVC assay is not surprising. Cultures that have been grown to stationary phase, centrifuged, washed, and starved should not remain 100% viable. Many dead and damaged cells are expected to be present. In addition, the AODC procedure can overestimate the number of cells due to the presence of non-living particles that are not easily distinguished from cells (Kogure et al., 1979). It is also possible that the antibiotics (necessary for the cell elongation assay) may inhibit the growth of some cells.

A concern in DVC is that the incubation time must be long enough to discern responsive cells, while also short enough to prevent growth of the cells (by division). For *P. putida* NCIB 9816-4, a minimum incubation time of 9 h was required in order to easily distinguish substrate responsive (SR) cells from nonresponsive cells (Fig. 2). Total cell numbers did not change significantly over the course of the assay. Substrate responsive cells did not exceed 43% of the total cell counts (Fig. 2). An incubation time of 9 h is within the range (6–24 h) employed by other DVC

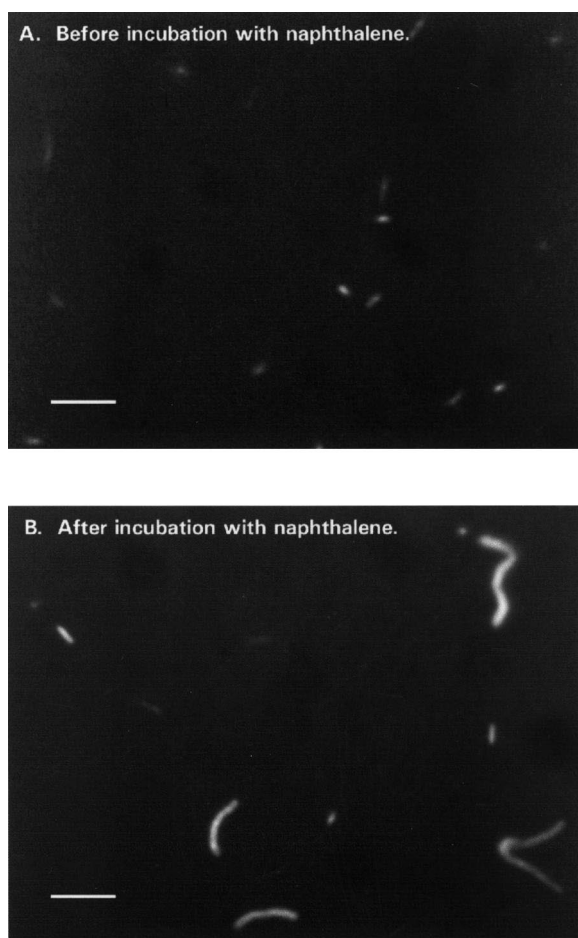


Fig. 1. Pure cultures of *P. putida* NCIB 9816-4 before (A) or after (B) SR-DVC incubation with naphthalene were stained with acridine orange and viewed with epifluorescence microscopy. Magnification, $\times 100$. Scale bar = 5 μm .

methods (Desmonts et al., 1992; Guyard et al., 1999; Heijnen et al., 1995; Kalmbach et al., 1997b; Kawai et al., 1999). Incubation time is expected to vary depending on the conditions (antibiotics used, temperature, etc.) of the assay. For example, Kogure et al. (1979) found that 5–6 h at 20°C was optimal for their system using nalidixic acid; while Joux and LeBaron (1997) found that 18–24 h of incubation at 20°C was required when using a suite of antibiotics.

Starvation of the cells prior to the SR-DVC assay by incubation in PBS for several hours was designed to mimic nutrient-limited growth conditions expected to be present in the groundwater environment.

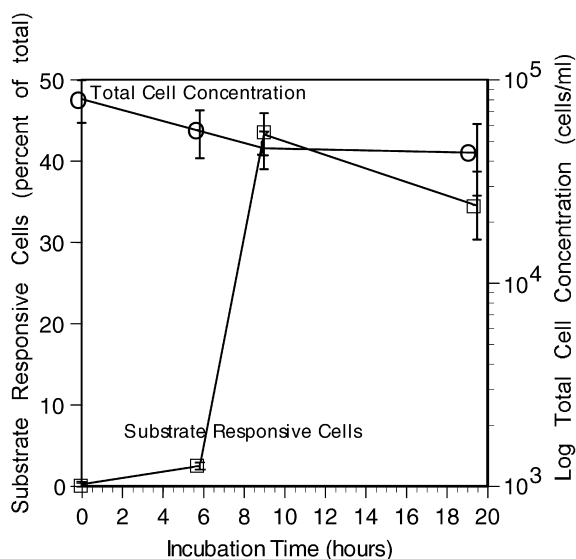


Fig. 2. Change in substrate responsive cells (squares) and total cell numbers (circles) during the SR-DVC assay. Two-day cultures of *P. putida* NCIB 9816-4 were washed three times in PBS with a final resuspension in PBS. Cultures were returned to a shaking water bath at 30°C for 3 h to be starved. Following starvation, samples were analyzed by the SR-DVC assay. Quadruplicate samples were removed from the incubation chamber at varying times during the SR-DVC assay for fixation and analysis.

Starvation of pure cultures is an accepted technique for approximating natural conditions (Guerin and Boyd, 1995; Nelson et al., 1996). The starvation step was crucial for understanding the response of *P. putida* NCIB 9816-4 to the SR-DVC assay because the physiological state of bacteria influences their ability to take up and utilize growth substrates. The effect of starvation for 0, 0.125, 1, 2, and 3 days on response in the SR-DVC assay was examined for *P. putida* NCIB 9816-4 cells. Without starvation, only 11% of the total cells were substrate responsive (data not shown). Three hours of incubation under starvation conditions provided an optimal response (in this experiment) of 34%. With prolonged starvation (3 days), the percent of responsive cells diminished to 7%. The gradual decrease in response, particularly notable after 2 days of starvation (data not shown), was most likely due to diminished viability of the cells.

The concentration of elongated cells in negative controls receiving no added growth substrate was often higher than expected (up to 14% of total cell

counts). Background levels may have been high for pure cultures because cells were grown on 5% PTYG prior to SR-DVC incubations which may have caused a delayed response to naphthalene or allowed the intracellular storage of carbon reserves for growth. In addition, stresses such as temperature, light, nutrients, and salinity can affect the response of laboratory organisms to the DVC assay (Joux and LeBaron, 1997; Nelson et al., 1996; Nybroe, 1995). Nitrogen starvation has been shown to contribute to the presence of elongated cells of *Escherichia coli* (Nelson et al., 1996); however, carbon starvation-mediated responses of *Vibrio* sp. strain S14 (which did not include cell elongation) have been shown to dominate over other nutrient starvation responses (Holmquist and Kjelleberg, 1993).

3.2. Field site studies

On two occasions, the SR-DVC assay was used to quantify a subset of the groundwater community that was capable of degrading naphthalene.

The first implementation of the SR-DVC assay was performed on groundwater samples from monitoring wells in the contaminated field site in July 1998. The concentration of naphthalene in the contaminated groundwater from monitoring well (MW) 36 was 3.0 mg/l and below detection in the uncontaminated groundwater (MW10). Total cell counts (by AODC) were 8700 ± 2400 cells/ml in MW36 and 4500 ± 1100 cells/ml in MW10. Cells in the groundwater community from MW36 displayed the expected cell elongation response in the SR-DVC assay (Fig. 3). Initial counts of long cells present in the microbial community at time zero were 340 ± 140 and 180 ± 120 cells/ml for MW36 and MW10, respectively (Fig. 4). A response to naphthalene (relative to the no substrate treatments) of 350 ± 180 cells/ml ($P < 0.05$) was detected in the contaminated well samples (Fig. 4). In contrast, there was no significant difference between the naphthalene and no substrate treatments from the uncontaminated well samples. The response of cells in the contaminated well was detected over a fairly high background of elongated cells which appeared during the assay (690 ± 160 cells/ml). Both initial counts of elongated cells and cell elongation in negative controls of groundwater samples are difficult to

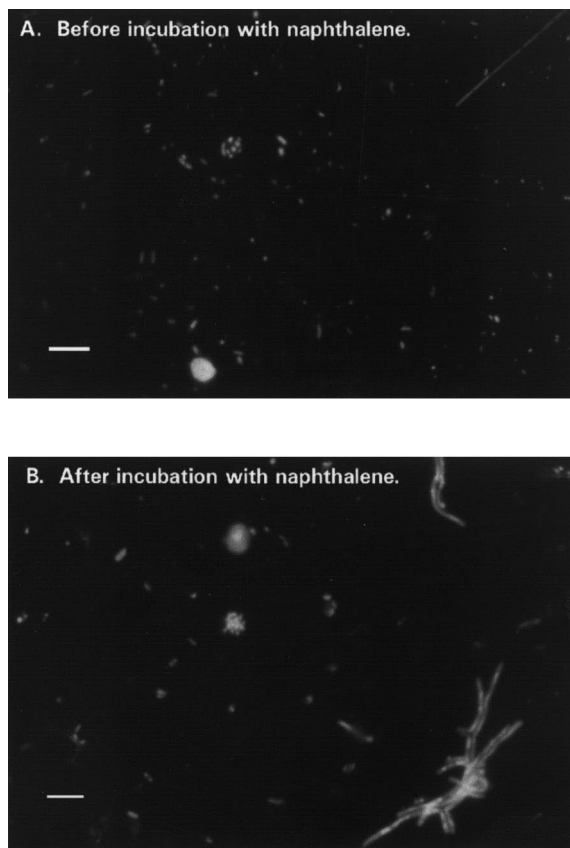


Fig. 3. Response of groundwater community to SR-DVC incubation. (A) The microbial community present in MW36 at time zero. Many small rods and cocci are present. Any cell exceeding $4 \mu\text{m}$ in length was included in initial counts of long cells. (B) The microbial community from MW36 after incubation with naphthalene in the SR-DVC assay. Note the presence of elongated cells (right side, top and bottom) and, as in (A), the presence of many small rods and cocci. Representative photographs of the microbial community are shown. Samples were stained with acridine orange and viewed with epifluorescence microscopy at a magnification of $\times 100$. Scale bars = $5 \mu\text{m}$.

interpret because little is known about the nutritional and physiological status of cells in nature.

Yeast extract, commonly used as the growth substrate in DVC, was also included as a treatment at 0.05 g/l in the plates (Fig. 4). For unknown reasons, no cell elongation occurred in the presence of yeast extract (370 ± 140 and 60 ± 17 cells/ml for MW36 and MW10, respectively). In addition, fungal growth was evident on the filters (and plates) from MW36 samples following SR-DVC incubation.

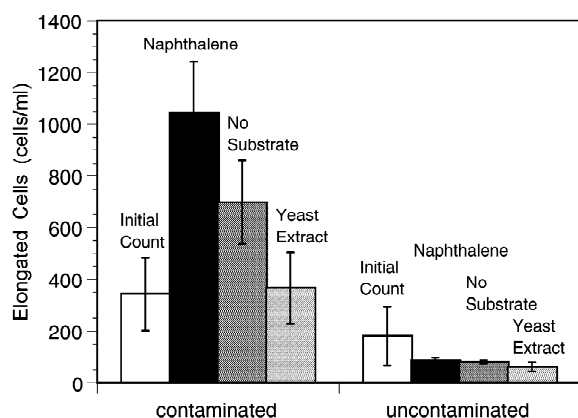


Fig. 4. Enumeration of groundwater bacteria using the SR-DVC assay, July, 1998. Quadruplicate groundwater samples from contaminated and uncontaminated wells were analyzed.

In an attempt to reduce the background counts of elongated cells, a filter washing step (20 ml PBS plus antibiotics) was added to the SR-DVC procedure. To curtail fungal growth, 0.1 mg/ml cyclohexamide was incorporated into the plates. A second analysis of the groundwater community was performed in October 1999 with the modified SR-DVC procedure. At this sampling date, naphthalene concentrations of the contaminated (MW36) and uncontaminated (MW4) groundwater were 2.4 mg/l and below detection, respectively. The total cell concentrations at MW36 and MW4 were $57\,000 \pm 5800$ and 4700 ± 560 cells/ml, respectively, as determined by AODC.

Fig. 5 displays the results of the modified SR-DVC procedure (October 1999 samples). Long cells present in the microbial community at time zero were 230 ± 28 and 37 ± 9 cells/ml for MW36 and MW4, respectively. The contaminated well (MW36) showed a response to naphthalene of 150 ± 25 cells/ml ($P < 0.05$) in washed samples relative to washed, no substrate added controls. Surprisingly, the wash step did not alter the number of background elongated counts in the samples from MW36 (180 ± 37 cells/ml in unwashed, no substrate control; 230 ± 10 cells/ml in washed, no substrate control; 230 ± 28 cells/ml present in direct counts). However, washing did affect the response to naphthalene: 180 ± 38 cells/ml in unwashed, naphthalene-exposed samples versus 380 ± 34 cells/ml in washed, naphthalene exposed samples. The lack of response of unwashed

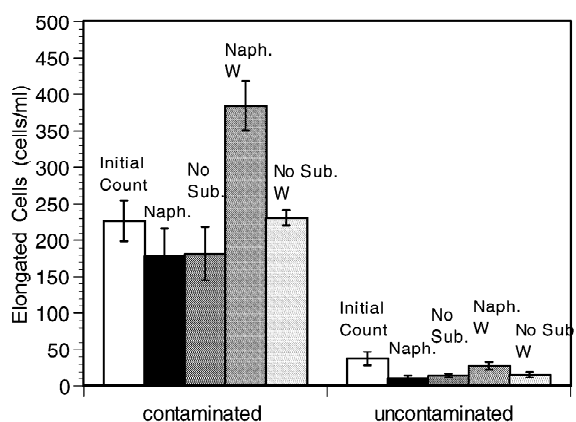


Fig. 5. Enumeration of groundwater bacteria using the SR-DVC assay, October, 1999. SR-DVC (Fig. 4) was modified for this sampling date by adding a wash step prior to incubation and incorporating cyclohexamide into the plates. Abbreviations: Naph., naphthalene; No Sub., no substrate; W, washed.

samples suggests the presence of growth inhibitors in the groundwater that co-concentrated on the filters with the cells. Toxic effects of polycyclic aromatic hydrocarbons and uncontaminated groundwater have been previously documented (Haeseler et al., 1999; Helma et al., 1998). Thus including a wash step prior to SR-DVC analysis did not aid in reducing the background counts of elongated cells, but it did reveal the presence of growth inhibitors in the groundwater. Furthermore, the incorporation of cyclohexamide into the agar successfully prevented fungal growth, as fungi were not seen on plates or filters. Numbers of elongated cells in the uncontaminated samples (Fig. 5) were too low to distinguish effects of the treatments.

4. Summary

We have developed the SR-DVC assay using pure cultures and applied the assay twice to field samples. In the initial (July 1998) assay, 4.0% (350 substrate responsive cells/ml of 8700 total cells/ml) of the community was involved in the response to naphthalene in the SR-DVC assay. When the modified SR-DVC procedure was applied to field samples 15 months later (October 1999), 0.27% (150 substrate responsive cells/ml of 57 000 total cells/ml) of the bacterial community was involved. Given the low

solubility of naphthalene, only a small proportion of the microbial communities examined was expected to be active in naphthalene metabolism. A low proportion of active cells is also consistent with plate count data obtained from the seep area of the site where bacteria that use naphthalene as their sole carbon and energy source comprise only 2.9% of the total culturable bacteria (Herrick, 1995). Whether the temporal changes in SR-DVC data were due to changes in the microbial and chemical composition of the samples over time or due to the differences between the original and modified SR-DVC assays are uncertain; an awareness of such variations is important for designing and implementing SR-DVC investigations.

Like related assays (microcolony formation, DVC, CTC reduction, microautoradiography), SR-DVC still has the disadvantage of requiring an incubation time for the discernment of responsive cells. If the objective of these assays is to distinguish the active members of a microbial community at the time of sampling, then the shortest possible incubation time is desired so that induction of gene expression by the assay can be minimized. Because induction and expression of naphthalene degradation genes requires only 15 min in pure cultures (King et al., 1990), it is unlikely that any of these assays prevent induction of naphthalene degradation. Hence these assays are all measures of 'metabolic potential' and not 'in situ activity'. In addition, the SR-DVC assay depends on adding nutrients to the samples; thus cells must be able to respond to this nutrient. Organisms that require additional nutrients (beyond minimal salts) in order to grow will not be detected by this assay. Many groups of bacteria and fungi can degrade naphthalene and may play a role in naphthalene degradation at the site examined here; however, bacteria that are resistant or highly sensitive to the antibiotics used will not be detected by this assay and neither will fungi. Furthermore, the physiological state of cells will affect their response to the SR-DVC assay resulting in underestimation of activity by the SR-DVC assay. Due to these limitations, SR-DVC should be considered a conservative indicator of metabolic potential.

SR-DVC is a useful method for identifying phenotypes of cells; in particular, those cells within a population that degrade specific substrates. If used in

combination with fluorescent in situ hybridization (as has been done for other techniques including CTC reduction (Kalmbach et al., 1997a) and microautoradiography (Lee et al., 1999; Ouverney and Fuhman, 1999)), SR-DVC may become a useful technique for the examination of bacterial phenotypes and genotypes in microbial communities without the need for extensive culturing.

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