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Use of conventional methods and whole cell hybridization to monitor the microbial response to triethylphosphate

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Abstract

The effect of triethylphosphate (TEP) on the activity of a landfill aquifer microbial community was evaluated using standard techniques and in situ hybridizations with phylogenetic probes. Benzene was used as an external carbon source to monitor degradation of an aromatic compound in TEP amended microcosms. Microscopic and viable counts were higher in TEP containing microcosms when compared to unamended controls. A significant increase in ribosomal activity was also observed for TEP amended samples as determined by the number of cells hybridizing to an eubacterial probe. In addition, the number of beta and gamma Proteobacteria increased from undetectable levels prior to the study to 15-29% of the total bacteria in microcosms containing TEP and benzene. In these microcosms, nearly 40% of the benzene was degraded during the incubation period compared to less than 5% in unamended microcosms. While TEP has previously been used as an alternate phosphate source in the bioremediation of chlorinated aliphatics, this study shows that it can also stimulate the microbial degradation of aromatics in phosphate limited aquifers. © 1997 Published by Elsevier Science B.V.; copyright held by the U.S. government

Keywords: Triethylphosphate; Benzene; rDNA probes; Degradation; Hydrocarbon

1. Introduction

One factor that limits bioremediation success in many environments is lack of essential nutrients needed for promoting microbial growth. Although nutrient addition generally accelerates bioremediation of contaminated sites, no universal formula has been found suitable for most bioremediation projects. In general, the type and concentration of nutrients needed vary depending on the compounds to be degraded, the level of nutrient deficiency, the form the nutrients must be delivered, and the ecosystem to be remediated [1]. In biosparging practices, for example, air is added to increase oxygen concentration and stimulate contaminant degradation by aerobic microorganisms. While some biosparging projects might not require carbon supplements since the contaminants can serve as a carbon source (e.g., petroleum hydrocarbons), bioavailability of nitrogen and phosphorus could significantly limit biostimulation. Thus, injecting gases like nitrous oxide (N_2O) and triethylphosphate (TEP) has been used to circumvent nitrogen and phosphorus deficiency in biosparging projects [2,3]. Despite the documented success of N_2O and TEP in the stimulation of in situ bioremediation of chlorinated aliphatics [3], few studies have been conducted to test the efficacy of

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these nutrients in the degradation of other contaminants [4]. Since all field studies to date have involved the addition of either N_2O or TEP in conjunction with other nutrients (e.g., oxygen and/or methane), virtually nothing is known regarding their specific effect on natural communities. Moreover, little evidence is found in the literature on the effect of N_2O or TEP on the microbial metabolic activity of nitrogen or phosphate limited ecosystems.

In this study we examined the response of landfill aquifer microbial communities to TEP additions. The microbial response was monitored in benzene containing microcosms using conventional methods and phylogenetic probes.

2. Materials and methods

2.1. Site characteristics, sampling and microcosm

Aquifer samples were collected from a saturated zone piezometer well located in the southwest of the Savannah River Site (SRS) sanitary landfill. This site was selected due to the presence of aromatics (e.g., chlorobenzenes) and previous exposure to TEP as part of a in situ bioremediation optimization test performed in the fall and winter of 1995/1996 [5]. Chemical characterization of this site before the optimization test revealed that phosphate was limiting [5]. The lithology of this site was mostly sand (>48%). The dissolved oxygen concentration in the saturated zone was of 20% of saturation and the hydraulic conductivity of >2.30E-03 m/s. Additional hydrogeological information regarding this site can be found elsewhere [5].

Three well volumes were purged before water collection to prevent sampling of stagnant well water [6]. Water samples were collected in sterile 500 ml polypropylene flasks, immediately placed in coolers and transported to the laboratory within 1 h of collection. Subsamples were fixed with formaldehyde (3.7%, final concentration) upon arrival to the laboratory. Microcosms were constructed by aseptically adding 10 ml of landfill water to 22 ml sterile glass vials. TEP (99% pure; Eastman Kodak Co., Rochester, NY) was added to the vials at two different concentrations (0.07% and 0.007%, final concentration). Benzene (approximately 5500 ppb, final concentration) was added to TEP microcosms using a glass syringe. After benzene was added, vials were immediately sealed using crimp tops and Teflon coated caps. Controls consisted of microcosms with only benzene (no TEP) or unamended water samples. Microcosms were incubated for 3 weeks at room temperature (ca. 21±2°C) without agitation. Benzene concentrations were determined by head space analysis on a Hewlett-Packard (HP) 5890 series II plus gas chromatograph equipped with a 30 m HP-5MS fused silica capillary column, and a HP 5972A mass selective detector.

2.2. Microbial enumeration

Aliquots from microcosms were fixed with formaldehyde for microscopic counts and in situ hybridization analyses. Direct counts were determined using the Acridine Orange Direct Count (AODC) method [7]. Total heterotrophic bacteria were determined as culturable counts by spreading different dilutions onto Peptone-Tryptone-Yeast extract-Glucose (PTYG) agar and 1% PTYG agar plates [8]. PTYG and 1% PTYG plates were incubated at 28°C for 7 days. Benzene degraders were determined using basal salts medium (BSM) plates [9] and benzene vapors as the sole carbon source. Minimal media plates were incubated in a glass desiccator for 3 weeks at room temperature.

2.3. In situ hybridizations

Oligonucleotide probes were labeled with 5(6)-carboxytetramethyl rhodamine and purified using high performance liquid chromatography (Genosys Biotechnologies Inc., The Woodlands, TX). Two types of probes were used in the in situ hybridization experiments. EUB338, capable of targeting all eubacteria [10] was used to determine the percent of hybridizing cells. In addition, group specific probes (BET and GAM) targeting beta and gamma Proteobacteria [11] were used to monitor changes in community structure. Fixed samples were transferred to centrifuge tubes and spun at 10,000 rpm at 4°C for 10 min to remove formalin. The supernatant was discarded and the cells were resuspended in autoclaved water filtered through Nuclepore filtering units (0.2 mm pore size). Aliquots were immediately

fixed onto gelatin coated toxoplasmosis slides as described by DeLong et al. [12]. Hybridization solutions consisted of 20 mM Tris(pH 7.2), 0.9 mM sodium chloride, 0.1% sodium dodecylsulfate (SDS), and appropriate concentration of formamide as suggested by Amann et al [13]. In situ hybridization conditions were performed by modifying the method of Amann et al. [13]. Prewarmed hybridization solution was used to resuspend probes to a final concentration of 0.3 ng/ml. The hybridization solution (10 μl) was applied to each slide well and the slides were then transferred to conical tubes containing prewetted paper towels. Hybridizations were carried at 37°C for the eubacterial probe, and 45°C for the group specific probes for 16 h in an hybridization oven (Bellco Glass, Inc., Vineland, NJ). Excess probe was removed by submerging the slides in hybridization buffer three times for 10 min. Excess salt was removed by washing the slides with generous amounts of sterile deionized water. Slides were then stained with 4, 6-diamidino-2-phenylindole (DAPI) [14] and observed under a Zeiss Axioskop epifluorescence microscope using filter sets 2 and 15.

3. Results

Microscopic counts in all microcosms increased more than two orders of magnitude over initial levels (3.3×10^5 AODC/ml, data not shown). The density increase in the control microcosms suggested that available carbon supported cryptic growth despite the phosphate limiting conditions that prevail in this aquifer [5]. Microcosms with TEP contained up to two to four times as many bacteria as those with benzene or no amendments (Fig. 1). However, TEP amendment increased culturable counts much more dramatically than direct counts (i.e., >2-3 orders of magnitude, Fig. 2). No significant differences in viable counts were observed between 0.07% or 0.007% TEP containing microcosms. Although similar trends were noted in all media used, viable counts were always higher in 1% PTYG plates. Nonetheless, viable counts never exceeded 4% of the direct counts. Benzene degrader counts were 2-3 orders of magnitude higher in TEP containing microcosms than in control microcosms (Fig. 3). Viable counts of

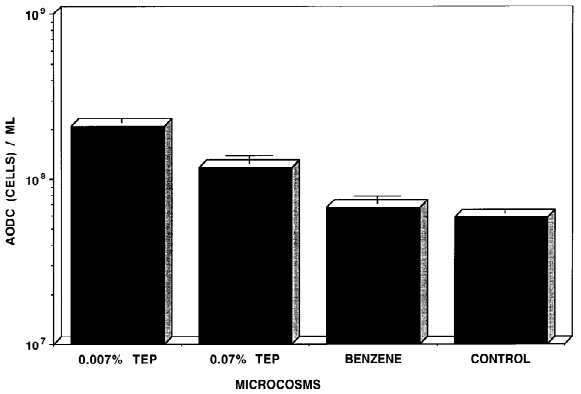

Fig. 1. Effect of TEP on microcosms microscopic counts. TEP was added at 0.007% and 0.07% final concentration (v/v) and microcosms incubated for 3 weeks at room temperature (ca. 21°±2°C) without agitation. Final concentration of benzene was approximately 5ppm. Benzene microcosms contained only benzene, while controls were unamended microcosms. Values represent means of triplicates and bars represent standard deviations.

benzene degraders represented on average 14, 5, 3, and 2.5% of total heterotrophic counts on 0.07% TEP, 0.007% TEP, benzene only, and unamended microcosms, respectively.

Gas chromatography analysis showed that microcosms containing 0.07% TEP degraded approximately 41% of the benzene added while unamended samples degraded only 5% during the incubation period (Fig. 4). Although, the effect of lower concentrations of TEP on benzene degradation was not

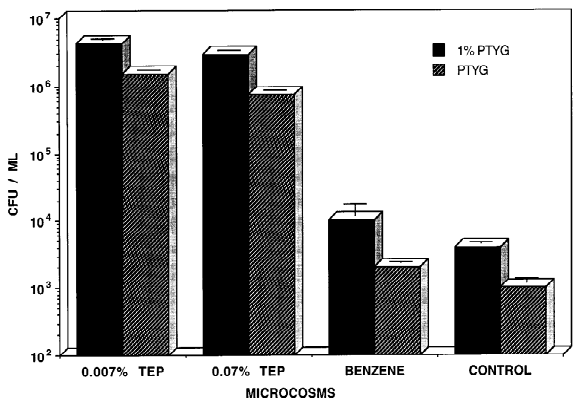

Fig. 2. Effect of TEP on total heterotrophic counts. Samples were taken from microscosms described in Figure 1 and appropiate dilutions spread onto PTYG or 1% PTYG plates. Values represent means of triplicates and bars represent standard deviations.

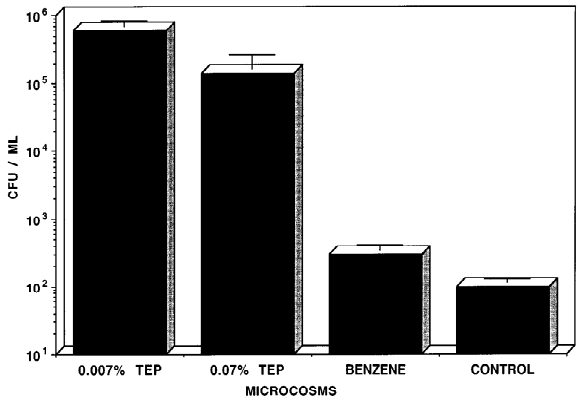

Fig. 3. Effect of TEP on benzene degrader densities. Samples were taken from microscosms described in Figure 1. Appropiate dilutions were spread onto minimal media (BSM) plates and benzene vapors used as sole carbon source. Values represent means of triplicates and bars represent standard deviations.

studied, the lower number of benzene degraders in 0.007% TEP microcosms (Fig. 3) suggests that benzene degradation was not significantly higher.

The percent of cells that hybridized to the eubacterial probe was higher on TEP amended samples than in control microcosms (Fig. 5). TEP microcosms showed an increase in hybridizing cells from the initial levels (7%, data not shown), while in benzene only microcosms the percent of detectable cells was similar to the levels prior to incubation. In contrast, hybridizing cells decreased to less than 1% in unamended controls. Microcosms with 0.07% TEP showed a higher number of hybridizing cells than

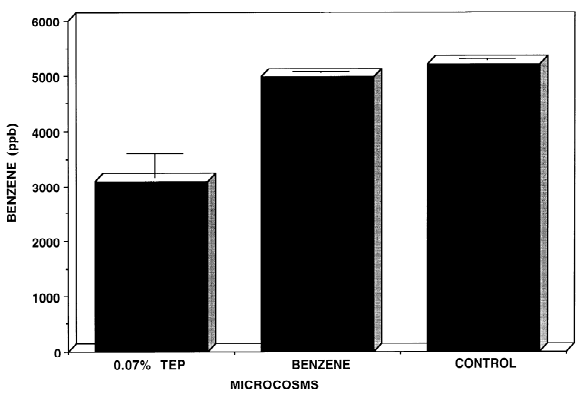

Fig. 4. Effect of TEP on benzene degradation. Values represent means of triplicates and bars represent standard deviations.

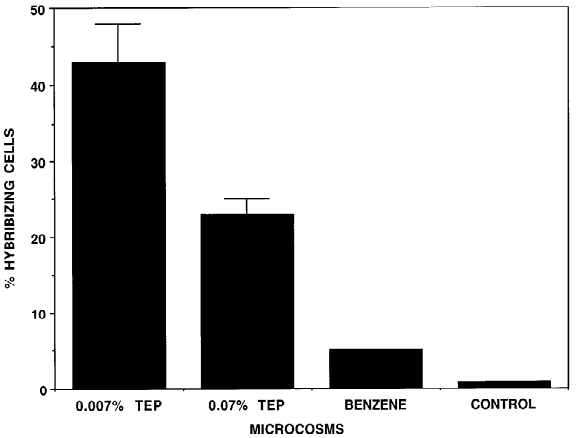

Fig. 5. Per cent of cells hybridizing to an rRNA-targeting eubacterial probe. Values represent means of triplicates and bars represent standard deviations.

0.007% TEP microcosms (43 to 23%, respectively). This was in agreement with the higher heterotrophic counts and benzene degraders in 0.07% TEP microcosms than in 0.007% TEP microcosms, although it was in disagreement with the direct counts.

Beta and gamma Proteobacteria were detected in TEP and benzene only microcosms (Fig. 6). Combined, beta- and gamma-like populations made up to 29, 15, and 3% of the total bacteria in 0.07% TEP, 0.007% TEP, and benzene only microcosms, respectively. Interestingly, these bacterial groups were not detected at the beginning of the study, although

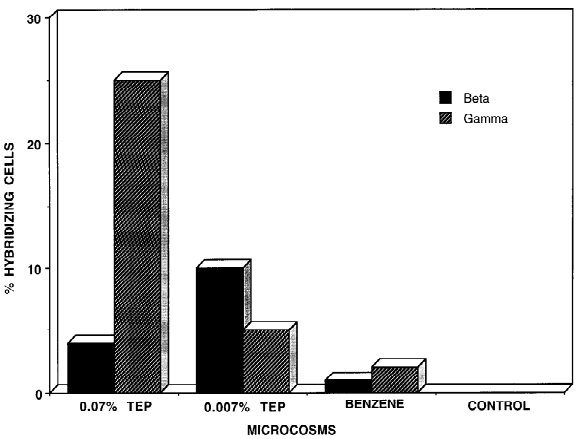

Fig. 6. Per cent of cells hybridizing to an rRNA-targeting group probes specific for beta and gamma Proteobacteria. Values represent means of duplicates.

approximately 7% of the bacterial community hybridized to the eubacterial probe. This represented an increase of more than an order of magnitude of detectable beta and gamma populations. In addition, bacteria identified by group probes represented between 60-67% of the total hybridizing cells after the incubation period. However, the proportion of beta and gamma Proteobacteria was different in the TEP microcosms. Beta-like bacteria were more abundant in 0.007% TEP microcosms (2:1) while gamma Proteobacteria were numerically dominant in 0.07% TEP microcosms (>6:1).

4. Discussion

This study showed that TEP can promote microbial growth of landfill aquifer communities. TEP had the biggest effect on microbial activity as determined by the significantly higher number of detectable cells using fluorescent ribosomal probes. This study also demonstrated that TEP can stimulate degradation of benzene by subsurface microbes. Palumbo et al. [15] showed that TEP can stimulate trichloroethylene (TCE) mineralization in the presence of methane. In addition, Brockman et al. [16] demonstrated that TCE degradation potential on subsurface samples increased approximately three orders of magnitude after N_2O and TEP additions. However, very little research has been conducted to examine the use of TEP in the remediation of aromatic hydrocarbon contamination under phosphate limiting conditions. Thus, our data provide laboratory evidence for use of TEP as an supplemental phosphorus source in the degradation of aromatic compounds. These conclusions are based on both the analytical results and the increase in total microscopic counts, heterotrophic counts, benzene degraders, and the number of bacteria that hybridized to eubacteria and group specific phylogenetic probes.

Heterotrophic counts were approximately two orders of magnitude lower than direct counts, suggesting that a significant percent of the cells were not viable or could not be cultured. Similar results have been documented for many oligotrophic and extreme environments [17]. In contrast, results from the in situ hybridization studies indicated that more than 20% of the bacteria in the TEP amended microcosms were sufficiently active to be detected with fluorescently labeled rRNA targeting probes. Thus, the culturing media used in this study severely underestimated viable microbial biomass. It should be noted that while the phylogenetic approach used here provided a reasonable estimation on the effect of TEP on total microbial activity, successful detection of bacteria in whole cell hybridization with rDNA probes depends on relatively high concentrations of ribosomes per cell, and therefore, the number of detectable hybridizing cells might have underestimated microbial activity in these microcosms. However, the results obtained with both conventional methods and in situ hybridizations strongly suggest that TEP had a beneficial effect on the aquifer microbiota. Moreover, these results underline the importance of using complementary methods to assess the microbial response to nutrient additions in environmental remediation projects.

Detecting beta and gamma Proteobacteria in the benzene containing microcosms could indicate intrinsic degradation potential in this aquifer community since several species (e.g., *Burkholderia* spp., *Alcaligenes* spp., and *Pseudomonas* spp.) belonging to these phylogenetic groups are known to metabolize aromatic hydrocarbons [9,18,19]. Although no studies have been previously performed to detect these bacterial genera in this landfill aquifer, their presence in aquifer ecosystems is well documented [20,21]. In fact, subsurface isolates from other South Carolina aquifers have been identified as *Pseudomonas* spp. using 16S rDNA sequencing analysis [22] or substrate utilization profiles [23]. Moreover, the increase of detectable beta and gamma populations coincided with benzene transformation, suggesting that these bacterial groups might have been involved in benzene degradation, especially in TEP containing microcosms. While it is likely that other potential carbon sources (landfill leachates) present in this aquifer partially supported microbial metabolism in the microcosms, adding TEP does not normally increase the number of aquifer hybridizing bacteria unless external carbon sources are also added (J. Santo Domingo, unpubl. data), further supporting the potential role of these bacterial groups in benzene degradation. The effect of TEP concentration on benzene degradation was not evaluated, thus it is presently unknown if there is a relationship

between community structure and degradation potential. Our results suggest that in situ hybridization studies with group-specific rDNA probes is a viable method to examine the assemblage of landfill aquifer microbial communities. Therefore, future studies using this phylogenetic approach might shed some light on the relationship between structure and function of aquifer degradative communities.

This study showed that beta and gamma Proteobacteria groups comprised no more than 29% of the microcosm communities. Thus, a significant fraction of the aquifer community was unidentified. Since other phylogenetic groups are expected to be part of this community, their relative abundance and population dynamics should also be studied. However, while other phylogenetic groups might play key roles in aromatic degradation, the fact that the identified groups responded to TEP amendments is relevant to the bioremediation of this contaminated site, for example, in designing strategies to specifically increase the biomass of these phylogenetic groups. Interestingly, the number of beta and gamma populations increased after air injection campaigns that were part of an in situ bioremediation optimization test in this aquifer [24]. Furthermore, changes in the proportion of beta and gamma Proteobacteria were observed after TEP was injected, suggesting that TEP also had an effect on the in situ population dynamics of these phylogenetic groups. Since other nutrients were injected simultaneously (namely, nitrous oxide and oxygen) it is difficult to compare the present study with data obtained from these field studies. Thus, future field studies should address the specific effect of TEP on the abundance and activity of these environmentally relevant bacterial groups.

The addition of TEP as gas (PHOSterTM) [2] to promote remediation of contaminants was recently tested in field studies with BTEX contaminated soils [4]. Evidence on hydrocarbon degradation was obtained using indirect methods, namely, in situ respiration data. Our results represent laboratory evidence for the stimulation of benzene degradation by TEP under phosphate limited conditions. These results are relevant to the bioremediation of hydrocarbon contaminated soils since delivery of TEP in the gas form has the advantage of influencing larger areas, while preventing microbial overstimulation that results in the formation of clogging. It also allows injection of supplemental phosphate into lower permeability soils and into unsaturated areas to promote bioventing.

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