PHYSIOLOGICAL RESPONSE OF SUBSURFACE MICROBIAL COMMUNITIES TO NUTRIENT ADDITIONS

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ABSTRACT: Studies were conducted to evaluate the physiological response of subsurface microbial communities to nutrient addition campaigns implemented as part of an landfill in situ bioremediation project. Different injection campaigns were performed throughout this study involving the addition of air, with triethylphosphate (TEP), nitrous oxide (N_2O), and methane. Samples were taken from saturated zone piezometer wells and analyzed for the following parameters: direct and viable counts, frequency of dividing cells, and percent of cells hybridizing to eubacterial rRNA targeting probes. Less than 2% of the microscopical counts from the saturated zone hybridized to an eubacterial probe before any of the injection campaigns. However, the number of hybridizing cells increased to nearly 42% after air injections, reaching a maximum of 63% a few weeks later. A steady decrease in the number of hybridizing cells was observed after maximum activity was reached, despite additional nutrient campaigns. In contrast, direct and viable counts, and the frequency of dividing cells remained relatively unchanged after the initial microbial biomass stimulation. Interestingly, the ratio of beta and gamma Proteobacteria changed during this period suggesting that nutrient campaigns had an effect in the in situ microbial community structure.

INTRODUCTION

It is now accepted that the subsurface is inhabited by physiologically diverse microorganisms capable of transforming compounds of environmental concern. The spectrum of transformations reported for subsurface microbial communities is broad, ranging from chlorinated aliphatics to polyaromatic hydrocarbons. However, despite the intrinsic degradation potential of subsurface environments, biotransformation proceeds at slow rates, mostly due to the lack of essential nutrients and electron acceptors. Thus, the addition of nutrients has become a common practice in the bioremediation of contaminated environments.

A treatability study using soil and groundwater samples from the Savannah River Site (SRS) Sanitary Landfill showed that oxygen, supplemental carbon sources (methane), and trace elements (phosphorous and nitrogen) were required to accelerate remediation of this site (WSRC, 1994). Thus, an in situ bioremediation optimization test was performed in 1995, during which air injection campaigns were implemented to deliver oxygen, methane (CH_4), triethylphosphate (TEP), and nitrous oxide (N_2O). Different physical, analytical and microbiological parameters were monitored to test the efficacy of this technology for the stimulation of the landfill microbial community.

Objective. The objective of this paper is to investigate the effect of the nutrient campaigns to the landfill aquifer microbiota.

MATERIALS AND METHODS

Nutrient campaigns. Saturated zone piezometer wells were set at the southwest corner of the SRS Sanitary Landfill at depths of 12.2 and 16.8 m, with 3 m screens at the bottom of each well. Several campaigns where implemented during the course of 1995 (WSRC, 1996). Nutrients were delivered as a mixture of air and

4% CH_4, 0.07% N_2O and 0.007% TEP; atmospheric air provided oxygen during all campaigns. Air injections started on 7/11/95 on an intermittent basis for a week, after which air was injected continuously until 8/8/95. Air campaign was resumed on 9/11/95, on a continuous basis until 11/1/95. N_2O was injected from 9/18/95 to 9/22/95 as three cycles of 8 hours N_2O + air and 40 hours of air alone. TEP and N_2O were injected from 10/6/95 to 10/19/95 as seven cycles of 8 hours of TEP, N_2O + air and 40 hours of air alone. A CH_4 injection was added from 10/20/95 to 11/1/95 as seven cycles of 8 hours CH_4, TEP, N_2O + air and 40 hours of air alone.

Microbiological analyses. Water samples used in this study were collected in sterile containers from 12.2 m piezometer wells using submersible in-line pumps and transported in ice coolers to the laboratory. For direct microscopical counts, frequency of dividing cells (FDC), and fluorescent in situ hybridizations (FISH), subsamples were immediately fixed with formaldehyde (3.7% final concentration) and stored at 4°C until processed. Microscopical counts were performed using the acridine orange direct counts (AODC) method (Hobbie et al., 1979). FDC were determine following the method of Newell and Christian (1981). FISH were performed in a hybridization oven using fluorescently labeled rDNA oligonucleotide probes (Amman et al., 1995). A general eubacterial probe was used to determine the total number of hybridizing bacteria while group-specific probes were used to monitor beta and gamma Proteobacteria (Manz et al., 1992). Probes were labeled with tetramethylrhodamine and HPLC purified by Genosys Biotechnologies, Inc. (The Woodlands, TX). Cells were counter stained with 4'6-diamidino-2-phenylindole (DAPI) to determine the number of cells per field (Hicks et al., 1992). Microscopical enumerations were performed using a Zeiss Axioskop epifluorescence microscope. Total heterotrophic plate counts were determined using 1% PTYG plates (Balkwill et al., 1989).

RESULTS

No significant increases in microscopical counts were observed after the initial air (oxygen) injection (7/11/95) campaign for most wells (data not shown). Densities remained at similar levels after additional air campaigns and nutrient amendments (Figure 1).

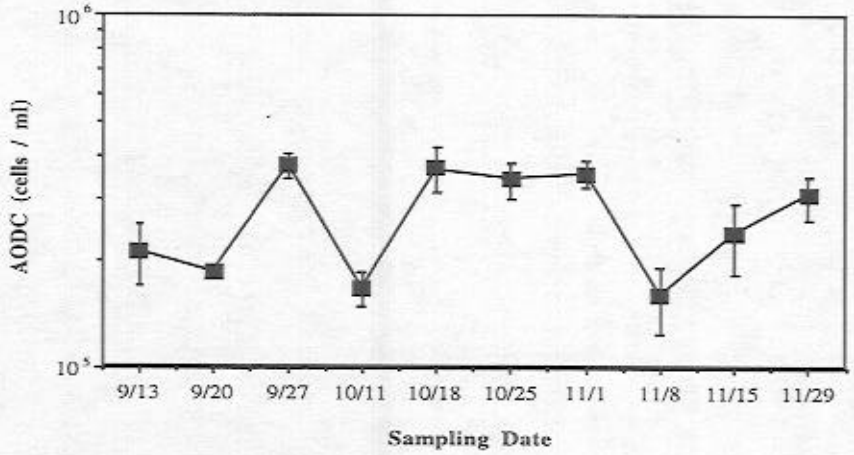

FIGURE 1. Direct microscopical counts of a landfill saturated zone piezometer well as determined by the AODC method.

However, a general increase in bacterial cell volume was observed during this period. Viable counts increased after the first air campaign (data not shown), although the addition of TEP or N_2O did not significantly increase total plate counts. The number of active cells as determined by FDC increased slightly after the second air campaign, although, no changes in activity levels were seen throughout the remainder of this study (Figure 2). In contrast, the number of hybridizing cells increased from less than 2% before air injections to approximately 42-63% in weeks subsequent the second air injection campaign (Figure 3). The highest number of detectable bacteria using the eubacterial probe (i.e., 63%) was recorded after N_2O + air injections. However, hybridizing bacteria decreased to less than 20% after additional nutrient campaigns. Samples taken from other piezometer wells showed similar decreases in detectable bacteria during the late stages of nutrient campaigns (data not shown).

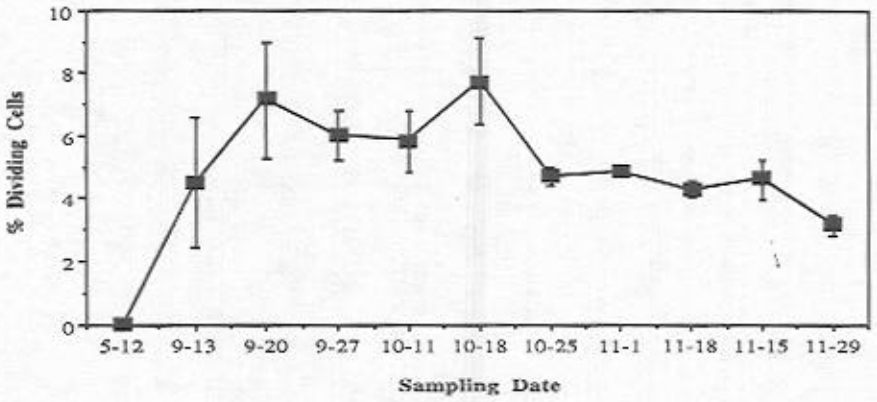

FIGURE 2. Frequency of dividing cells from samples collected from a piezometer well. Values represent means of triplicates and error bars represent standard deviations.

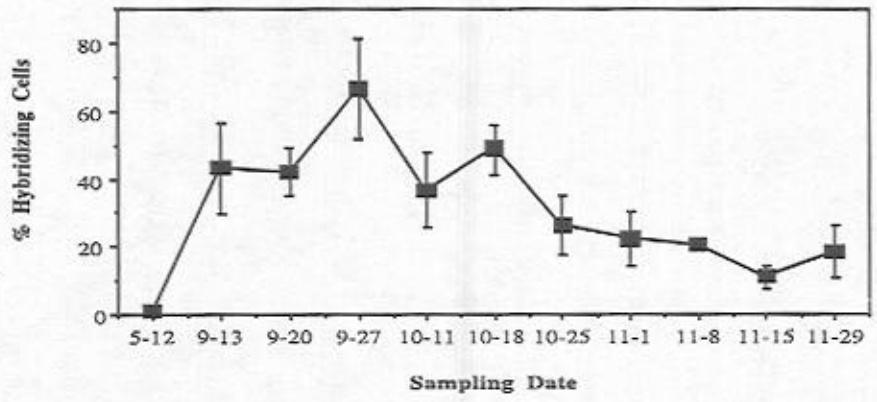

FIGURE 3. Per cent of total cells that hybridized to rRNA-targeting eubacterial probe. Values represent means of triplicates and error bars represent standard deviations.

Group specific phylogenetic probes showed that the number of detectable beta and gamma Proteobacteria increased after the second air injection campaign (Figure 4). This initial increase in detectable bacteria was more noticeable for beta-like populations. The ratio of beta to gamma changed throughout the study. Beta-like populations were more abundant than gamma-like bacteria after air and N_2O injections. However, gamma-like populations were more abundant than beta Proteobacteria during methane injection and after nutrient campaigns were completed.

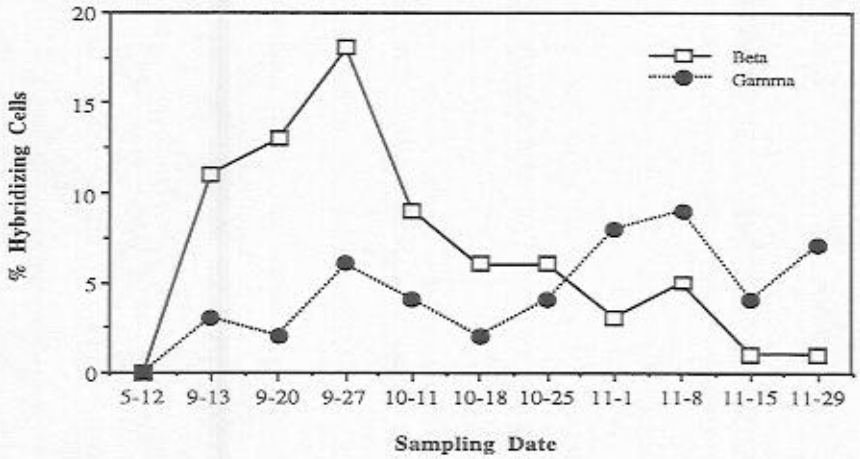

FIGURE 4. Per cent of beta and gamma Proteobacteria hybridizing cells from water samples used in figure 3. Values represent means of duplicate samples.

DISCUSSION

The results from the enumeration methods indicated that after initial stimulation subsequent injection campaigns had a small effect in total cell densities. Air injection increased the number of dividing cells short term. In contrast, a dramatic impact in activity was observed using the FISH method immediately after the second air campaign. The increase in total hybridizing cells was in agreement with the increase in dividing cells and the general increase in cell volume observed during this period. Since the FISH method depends on the cellular ribosomal content, the increase in cells detected using the eubacterial probe indicated that available carbon sources stimulated *di novo* ribosomal synthesis, and most likely, the metabolic activity of a significant fraction of the aquifer community.

Beta and gamma-like Proteobacteria also increased after the second air injection suggesting a significant response by these populations. The two phylogenetic groups made up approximately 20-35% of the total hybridizing bacteria during injection campaigns, and up to 40-70% after the nutrient campaigns were completed, indicating that these are numerically dominant populations and that might carry relative important functions in landfill aquifer community processes. The presence of organisms within the beta and gamma Proteobacteria (e.g., *Pseudomonas* spp., *Burkholderia* spp., *Alcaligenes* spp.) capable of transforming a broad range of chlorinated compounds suggests their potential role in in situ bioremediation in the subsurface. Nevertheless, their role has not been clearly stated yet, and further characterization must be implemented in future studies.

Moreover, other phylogenetic groups capable of relevant transformations have been shown to inhabit the subsurface, and thus it is reasonable to propose that these will also play key roles in the remediation of contaminated aquifers.

The number of hybridizing cells decreased after the nutrient amendments, suggesting that no further stimulation of microbial activity occurred. However, the fact that there was a shift in the ratio of beta and gamma Proteobacteria indicates that nutrient additions had an effect on the microbial community structure. These changes were not perceived by culturable methods, reflecting the limited and potentially biased information obtained with such conventional methods. These structural changes occurred concomitantly with the decrease in several chlorinated organics observed in this site (WSRC, 1996), suggesting that changes in the community might have played a role in the kinetics of biotransformation. The fact rDNA probes were capable of establishing changes in landfill aquifer bacterial populations indicates that the phylogenetic approach is suitable to characterize subsurface microbial communities.

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