Biodegradation **13:** 285–295, 2002.
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Microbial activity in soils following steam treatment

Ruth E. Richardson[1], C. Andrew James[2], Vishvesh K. Bhupathiraju[3] & Lisa Alvarez-Cohen*
*Department of Civil and Environmental Engineering, University of California, Berkeley 94720-1710, USA (*author for correspondence)*

Accepted 28 September 2002

Key words: bioremediation, bacterial survival, CTC, heat tolerance, steam enhanced extraction

Abstract

Steam enhanced extraction (SEE) is an aquifer remediation technique that can be effective at removing the bulk of non-aqueous phase liquid (NAPL) contamination from the subsurface, particularly highly volatile contaminants. However, low volatility compounds such as polynuclear aromatic hydrocarbons (PAHs) are less efficiently removed by this process. This research evaluated the effects of steam injection on soil microbial activity, community structure, and the potential for biodegradation of contaminants following steam treatment. Three different soils were evaluated: a laboratory-prepared microbially-enriched soil, soil from a creosote contaminated field site, and soil from a chlorinated solvent and waste oil contaminated field site. Results from field-scale steaming are also presented. Microbial activity before and after steam treatment was evaluated using direct epifluorescent microscopy (DEM) using the respiratory activity dye 5-cyano-2,3, ditolyl tetrazolium chloride (CTC) in conjunction with the fluorochrome 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF) to yield a quantitative assessment of active and total microbial numbers. DEM results indicate that steamed soils that were analyzed while still hot exhibited microbial activity levels that were below detection. However, soil samples that were slowly cooled, more closely reflecting the conditions of applied SEE, exhibited microbial activity levels that were comparable to presteamed soils. Samples from a field-site where steam was applied continuously for 6 weeks also showed high levels of microbial activity following cooling. The metabolic capabilities of the steamed communities were investigated by measuring cell growth in enrichment cultures on various substrates. These studies provided evidence that organisms capable of biodegradation were among the mesophilic populations that survived steam treatment. Fluorescent in situ hybridization (FISH) analysis of the soils with domain-level rRNA probes suggest that both Archaea and Bacteria survived steam exposure.

Introduction

Steam enhanced extraction (SEE) is a remediation technique in which steam is injected into an area of subsurface soil contamination while water, contaminant, and steam are removed using extraction wells. The fundamental concept of SEE is that increased temperature will enhance non-aqueous phase liquid (NAPL) displacement by decreasing NAPL viscosity, and will promote increased mass transfer of residual contaminants into the extraction fluid. Considerable removal efficiencies of volatile and semi-volatile organics have been documented using this technique in both laboratory and field studies (Hunt et al. 1988; Itamura et al. 1993; Udell & Stewart 1989; Udell 1997; Betts 1998). In a notable field operation at the Southern California Edison Visalia Pole Yard Superfund Site (where subsurface NAPL contained a mixture of pentachlorophenol, polynuclear aromatic hydrocarbons (PAHs), and diesel fuel) 635,000 pounds of NAPL were removed in the first 6 months of thermal treatment. Prior to this treatment, conventional pump-

[1] Current address: School of Civil and Environmental Engineering, Cornell University, 220 Hollister Hall, Ithaca, NY 14853, USA, E-mail: rer26@cornell.edu
[2] Current address: Department of State, 2330 Maputo Place, Washington D.C. 20521-2330 USA
[3] Current address: Bayer Corporation, Berkeley, CA

and-treat operations were removing only 10 pounds per week (Betts 1998). During SEE, two removal mechanisms exist: steam displacement of subsurface NAPL pools followed by steam distillation of remaining contaminants. During steam distillation, preferential removal of lighter compounds is expected, such that the residual contamination is enriched in heavier, low-volatility compounds such as PAHs.

The literature describing the microbial degradation of PAHs is extensive (Rockne & Strand 2001; Pothuluri & Cerniglia 1994; Wischmann & Steinhart 1997; Kastner & Mahro 1996). Indeed, the biodegradation rate of PAHs in some contaminated soils may not be limited by the microbial kinetics, but rather, by the slow rate of contaminant dissolution into soil pore water (Yeom & Ghosh 1993; Manilal & Alexander 1991). When PAHs exist in NAPLs they can act as continuous, slowly dissolving sources of contamination with lifetimes that may range from hundreds to thousands of years. It would be desirable, then, to utilize SEE to remove the bulk of the NAPL while allowing biodegradation to act as a polishing step to remediate the less labile contaminants. For SEE to be effective at creosote contaminated sites in particular, where contamination is often >50% PAHs by weight, it would be of particular importance for biodegradation potential to exist in post-steam subsurface microbial communities.

The effects of SEE on the survival and PAH-degradation potential of soil microbial communities, however, have not been previously documented. The microbial communities within soil environments are complex. Even deep subsurface soils have been characterized as highly diverse (Chandler et al. 1998). Within the soil matrix, microniches of various combinations of pH, water potential, carbon source, and redox potential harbor unique consortia that may differ dramatically from a neighboring niche a mere millimeter away (Madigan et al. 2000). Steam exposure is commonly used as a technique for disinfecting or sterilizing solid surfaces. Although it may be expected that steam exposure is insufficient for sterilization of soils due to the occurrence of spores, cysts and other microbial protection mechanisms, the effects of SEE on subsurface soil communities and their biodegradation capabilities has not been well characterized. In fact, it is understood that germination of some spores is enhanced by, or even dependent on, heat treatment (Maeda et al. 1978; Weaver et al. 1994). In addition, many vegetative cells have some level of resistance to heat sterilization (Pflug & Holcomb 1991; Ueki et al. 1997). Transformation into a viable but non-culturable (VBNC) state may also impart heat resistance to some species (Oliver et al. 1995). Preliminary studies on diesel contaminated field soils subjected to either SEE alone or in conjunction with electrical heating suggested that microbial populations can recover rapidly following a thermal remediation process (Dablow et al. 1995).

In this study, the effects of steam duration and post-steam cooling on the microbial communities in a number of soils were investigated. Direct epifluorescent microscopy (DEM) was used to quantify the number and activity of microbes in soil communities, while enrichment cultures were used to assess PAH-degrading capabilities. Flourescent in situ hybridization with domain-level probes was used to investigate whether Bacteria or Archaea dominated in the soil following steam exposure and cooling.

Materials and methods

Soils

One artificially enriched soil and two field-sampled soils were evaluated in this study. Salicylic acid was used to enrich a soil community designated SAES (for salicylic-acid enriched soil) by mixing 200 g clean #60 graded sand (RMC Lonestar, Pleasanton, CA), 20 g of clean beach sand and 2 g of contaminated soil from investigation site 13 at Naval Air Station (NAS), Alameda, CA that was contaminated with PAHs and heavy petroleum hydrocarbons (Bhupathiraju et al. 1999a). The mixture of soils was placed in 200 mL of single source carbon (SSC) media (Stringfellow & Alvarez-Cohen 1999) in a sterile 500 mL Erlenmeyer flask capped loosely with a 100 mL beaker and shaken at 160 rpm and 25 °C (± 3 °C). Twenty mL of 1 g/l salicylic acid (Fisher Scientific, Pittsburgh, PA) solution in SSC media were added to each enrichment culture as the carbon source. Additional salicylic acid was added at an average rate of 4 mg/day for a minimum of two weeks to establish active microbial communities.

One of the field-sampled soils was obtained from aseptic borings of silty sand aquifer material collected at NAS, Alameda, CA. This soil (designated Alameda soil) was taken from a sump area where a mixture of chlorinated solvents and waste oil was dumped over the course of decades. The boring was taken from a depth of 2–4 meters and was stored at room temperature for 2 months prior to the steam experiments. The

average TCE concentration was 2.5 mg/kg and TPH was near 4000 mg/kg (Bhupathiraju et al. 2001).

The second field-sampled soil was obtained from the Wyckoff/Eagle Harbor Superfund site in Washington State. This site was the location of a wood treatment facility where creosote and, to a lesser extent, pentachlorophenol were used for imbibing wood products. The groundwater at this site is rather saline due to the intrusion of salt water from Puget Sound. The soil (designated Wyckoff soil) is large-grained sand and gravel that was collected from sampling depth of 12 meters. The soil contained an average of 650 mg/kg PAHs (Eva Davis, personal communication – analysis performed by the Region IX EPA lab). Additionally, an uncontaminated background soil was collected from a depth of 6 meters. After sampling, the soils were stored at 4 °C for 3 months, but were equilibrated to room temperature for 3 weeks before steaming experiments began.

Field steamed soils

Small-scale field steaming was performed at the site from which the Alameda soil was taken. Multiple borings were taken from throughout the steam zone before exposure and after six weeks of steam treatment followed by six weeks of *in situ* cooling. Ethanol-sterilized steel sleeves were used to collect samples. Pre-steam soils were at ambient temperature and were assayed within 48 hours of sampling. Post-steam soils were at a temperature of 50–55 °C when borings were taken. While some soil was analyzed immediately (while hot), additional soil from each boring was allowed to cool at room temperature for four days prior to analysis.

Column preparation

Soil steaming was performed in 8 in. × 3/4 in. internal diameter sterilized, stainless steel columns. Each column was packed with 100 grams of test soil. The test soil for each event was homogenized in a 500 ml beaker before packing. The inlet and outlet were packed with approximately 13 g of sterile, #14 gravel and covered with a stainless steel mesh screen to aid in steam dispersion. Columns were sealed with stainless steel endcaps and connected to Teflon™ influent and effluent lines. All columns, endcaps, glassware and associated hardware were cleaned and autoclaved for 1 hour before use and packing was performed in a sterile laminar-flow hood.

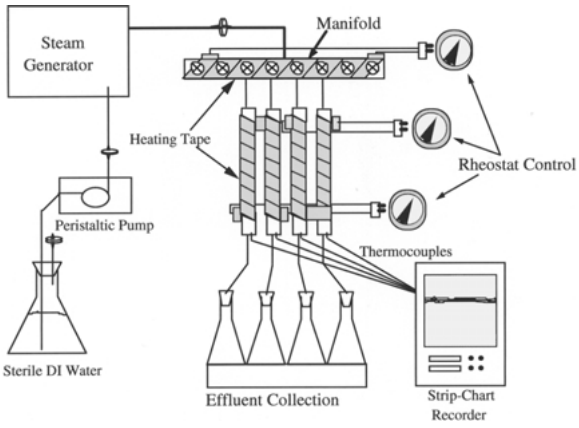

Figure 1. Illustration of the steam generator and parallel column steaming apparatus. Filters (0.2 μm pore diameter) were placed in-line before and after the steam generator to prevent introduction of microorganisms. Individual bonnet needle valves controlled flow to each column and heat loss was countered using heating tape attached to Rheostat controllers.

Steaming apparatus

Each steaming event involved up to five columns that were connected in parallel to a steam generator using a stainless steel manifold (Figure 1). The flow to each column was monitored and controlled through stainless steel integral bonnet needle valves (Whitey Co., Highland Heights, OH) to maintain equal steam flux to each column.

Steam was produced at a rate of 30 ml/hr/column (mass flux = 9.5 g $H_2O/cm^2/hr$) at a temperature of 102 °C to 115 °C (± 2 °C) (depending upon the soil grain structure – finer soils required higher temperature steam to break through the soil column) and controlled by a REX-C10 direct digital controller (Syscon International, Inc., South Bend, IN). The steam generator was fed sterile, distilled water by peristaltic pumps. In-line 0.2 μm filters were placed between the pump and steam generator to prevent microorganisms from being introduced into the steam generator. In most experiments, a second filter was added in-line after the steam generator.

Columns were wrapped with silicone resistance heater tape (Barnstead/Thermolyne, Dubuque, IA) to maintain adiabatic conditions during steam events. Temperature was controlled by adjusting power to the heater tapes using Ohmite Powerline Rheostats and monitored by Cole-Parmer thermocouple recorders (Vernon Hills, IL). The heat tape was turned on only after temperature breakthrough was achieved by steam flow in order to avoid artificial, external heating.

Steaming experiments

Steaming experiments were performed either with the salicylic-acid enriched soil (SAES) or the field-sampled soils as described above. Some steamed soils were analyzed while still hot immediately following steaming. Other samples were cooled in-place after steaming; column temperatures were decreased from steam temperature to 25 °C over a period of 36 hours by slowly lowering the temperature of influent water as well as power to the heater tape. Columns were then incubated by flushing with sterile water at 5 g/hour at room temperature after cooling. In this study, "incubation" time includes the 36 hours of cooling as well as time spent at 25 °C. Water-flushed control columns were fed autoclaved, deionized water at a rate of 30 g/hr. After the desired duration of steaming, incubation, or water flush, a single column was removed from the manifold system and immediately opened in a sterile, laminar-flow hood. The gravel and one centimeter of soil were discarded from either end of the column and the remaining soil was homogenized prior to analysis by direct microscopy or inoculation of enrichment growth cultures. For FISH samples soil was fixed in 4% paraformaldehyde overnight, pelleted, then resuspended in 95% ethanol/5% sterile water and stored at −20 °C until analysis. Soil moisture analysis was also performed on each sample. In each experiment, presteamed soil was analyzed prior to the steaming event.

Soil moisture content

Soil moisture content was measured gravitametrically after soil samples were homogenized and dried at 103 °C overnight. The soil water content for all sample soils was between 0.05 and 0.20 g H_2O/g dry soil.

Microbial enumeration

Enumeration of total and metabolically active microorganisms in the soils was performed by direct epifluorescent microscopy (DEM). Ten grams of homogenized soil were placed in a 250 mL Erlenmeyer flask with 100 mL of Phosphate Buffered Saline I buffer (PBS I) pH = 7.2 + 0.1% $Na_4P_2O_7$ (Bhupathiraju et al. 1999a). The soil slurry was mixed with a magnetic stirrer for 1 hour at room temperature. Magnetic stirring broke up soil clumps to yield microbial counts that were consistent and comparable to those obtained using a Waring blender (data not shown). Soil samples that did not have a specified cool-down or incubation period were abruptly cooled by placement into 25 °C PBS I while still hot.

The fluorescent cell wall stain 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF) was used along with the tetrazolium redox dye cyano-2,3-ditolyl tetrazolium chloride (CTC), in order to quantify both total and active microbial populations, respectively. DTAF quantifies total microbial numbers while CTC quantifies the activity of both aerobic and anaerobic microbial populations (Bhupathiraju et al. 1999a; Bhupathiraju et al. 1999b; Rodriguez et al. 1992; Smith & McFeters 1997). The protocol developed by Bhupathiraju et al. (1999a) was used. Briefly, a CTC concentration of 5 mM was used and no exogenous carbon was provided during the 8-hour CTC incubation. Modifications to the published procedure were as follows. The black polycarbonate filters were washed three times: initially with Phosphate Buffered Saline II buffer (PBS II; 50 mM Na_2HPO_4, 145 mM NaCl; pH = 9.0) followed by PBS I + 0.1% $Na_4P_2O_7$ and then again with PBS II. Between fifteen and twenty randomly selected fields were chosen for counting under 1100x magnification. Both total (DTAF-positive) and active (DTAF and CTC positive) cells were counted in each field and the average normalized per gram of dry soil.

FISH analyses

Forty μL aliquots of fixed soil suspensions were spotted onto the bottom of a 2 mL microfuge tube. FISH was carried out in suspension within a microfuge tube as described previously (Richardson et al. 2002) with the following exception: hybridizations were run overnight at 44 °C. The probes used were Cy3 -labeled Arch344 (Stahl et al. 1995) and Eub338 (Amann et al. 1990). Stock probe concentrations were 50 ng/μL. After a brief, low setting sonication (setting "5" on a Fisher Brand sonic dismembrator), samples were collected onto polycarbonate membrane filters (0.22 μm pore size) as in the CTC/DTAF assays. Here, however, warmed wash buffer was used to rinse membranes before mounting with Citifluor anti-quenching agent. Hybridizing percentages were calculated by dividing individual probe counts by DAPI counts. Counts were corrected for nonspecific binding by subtracting out the percent of cells that probed positive with a negative control probe (NON338 – the complement of Eub338). At least 500 cells were counted for each soil/probe combination.

Enrichment cultures

Enrichment cultures were generated from field-sampled soils by first dispersing one gram of soil in 100 mL SSC media and shaking at 160 rpm for 30 min. Aliquots of the slurry were then added to 100 mL SSC in 250 mL Boston Round bottles that were sealed with Teflon^{TM}-lined caps and incubated at 25 °C and 160 rpm. The size of the aliquot used for different soils depended on the amount of fine colloids present in each soil and was varied to yield an initial turbidity below 0.1 absorbance unit (at 420 nm). Four mL were used for Alameda soil, and 10 mL were used for Wyckoff soil. For cultures made from the SAES, one gram of soil was used directly as the inoculum. Each aerobic culture was supplied with a single carbon source: 200 or 400 mg/L tryptic soy broth (TSB; Sigma Chemicals, St. Louis, MO), 200 mg/L salicylic acid or 130 mg/L crystalline phenanthrene (Aldrich Chemical Company, Milwaukee, WI) made from hexane dissolved stock as described previously (Stringfellow & Aitken 1994). Growth was tracked by comparing the change of liquid absorbance in inoculated samples with an inoculum-free control measured using a light spectrometer (Perkin Elmer model "Coleman 55") at a wavelength of 420 nm (Koch 1994). The existence of microbes in enrichment cultures was checked by inspection of culture samples using light microscopy at 1100× magnification.

Results

Benchtop Steaming of SAES

The DEM data showing total and metabolically active microbial counts for presteamed soil, water flushed control soil, and soils after various durations of steaming for SAES are presented in Figure 2. Steamed samples that were allowed to incubate at room temperature after the steam period are also included. After two hours of steaming, the average total number of microbes dropped approximately 50% from $3.5 \pm 0.3 \times 10^8$ per gram of soil for the presteamed soil to $1.8 \pm 0.2 \times 10^8$ bacteria per gram of soil. The total numbers fluctuated only slightly with increased steam duration up to 6 days. In contrast, the numbers of active microorganisms measured in steamed soil samples that were not allowed to slowly cool prior to analysis were below the detection limit of 10^5 per gram of soil regardless of steaming duration. Samples from columns that were allowed to slowly cool after steaming, however, had active microbial numbers of $1.8 \pm 0.3 \times 10^7$ and percent activity similar to presteamed and water-flushed control soils. Similar trends with respect to slow-cooling were observed with each of the field-sampled soils (data not shown).

The water-flush control column showed no statistically significant change from the presteamed soil indicating that wash-out or indigenous cell decay did not significantly affect cell numbers for the duration of the experiment. Similar results were obtained for 8 separate experimental runs performed with SAES.

A negative control was run to test the possibility that the steaming apparatus could introduce microorganisms into the steamed soils. The sterile sand used to pack the negative control columns as well as the gravel used in column ends were sterilized by placing in a 550 °C furnace for at least 24 hours. The sterilized sand was then steamed for 1 day using the same apparatus used for experimental samples. This control had total cell numbers that were below 10^7 per gram and active numbers that were below the detection limit of 10^5 per gram even after 3 days of slow cooling and incubation (data not shown).

Benchtop steaming of Alameda and Wyckoff soils

Figure 3 shows total and active microbial counts for the two field-sampled soils before and after benchtop steaming. Microbial counts for the chlorinated solvent and waste oil contaminated soil (Alameda soil) are presented in Figure 3a. The total and active microbial numbers in the presteamed soil were $5.3 \pm 1.1 \times 10^8$ and $0.46 \pm 0.07 \times 10^8$ per gram, respectively. Counts in the water flushed control soil were similar to the presteamed soil suggesting that significant microbial washout was not occurring. All steamed columns were slowly cooled and incubated at room temperature after steaming and active populations rebounded above 10^7 per gram in all steamed/cooled soils.

Results from steaming experiments with the creosote-contaminated soil (Wyckoff soil) are shown in Figure 3b. Also included are data from an uncontaminated background soil taken from a geologically similar area at this site. For this soil, there was a significantly lower number of active microbes in the water-flushed column than in the presteamed soil ($0.3 \pm 0.1 \times 10^8$ versus $1.3 \pm 0.02 \times 10^8$). Recalling that the Wyckoff groundwater is influenced by salt water intrusions, it is possible that flushing with fresh water may have disturbed the established microbial

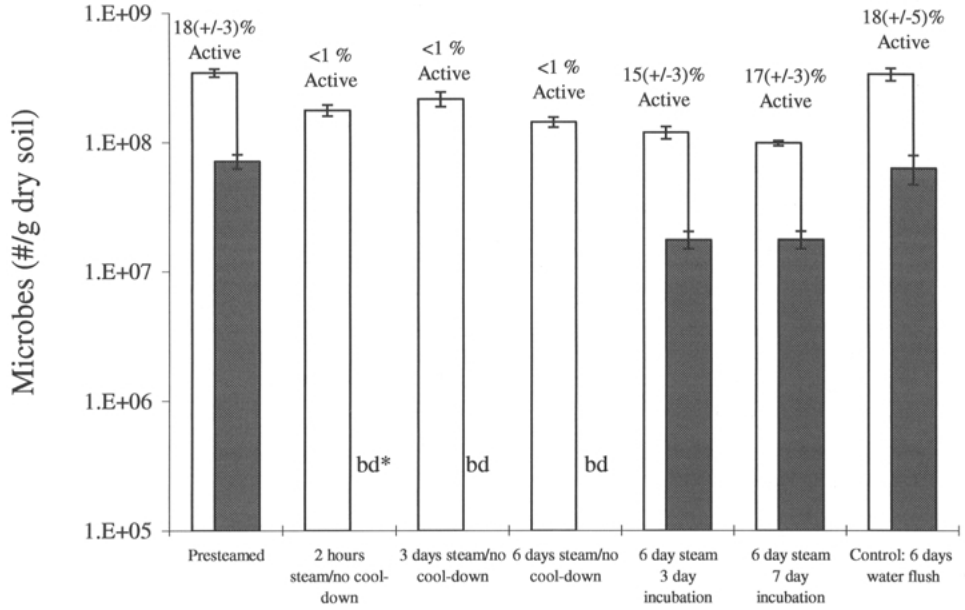

Figure 2. Total microbes (white bars) and active microbes (patterned bars) per gram of soil and corresponding percent activity in SAES exposed to different durations of steam exposure. Presteamed and water flushed control soils are also shown. The limit of detection for the CTC/DTAF assay is 10^5 microbes per gram of soil. Error bars represent one standard deviation. *bd = below detection limit.

communities resulting in decreased microbial activity. While the numbers of both total and active microorganisms in the soil that was steamed for 11 days and slowly cooled and incubated for 12 days were significantly lower than in the presteamed and water-flushed soils, they were within an order of magnitude of those values. Interestingly, the percentage of bacteria active in the column that was steamed for 11 days and incubated for 30 days was 29 ± 3%, close to that of the presteamed soil (33 ± 5% 5%). Uncontaminated background soil from the Wyckoff site had only 50% of the total microbial numbers of the presteamed contaminated soil and an order of magnitude lower active numbers.

Enrichment cultures from benchtop-steamed soils

In all SAES experiments, no enrichment culture growth was observed for samples taken from steamed soil that was cooled abruptly. In addition, all water flushed control soils generated growth trends and substrate utilization patterns that were very similar to presteamed soils and sterile soils steamed for 1 day and incubated for 3 days did not generate growth on any of the substrates used in this study (data not shown).

Figures 4a–c show the growth of enrichment cultures over time for the chlorinated solvent and hydrocarbon-contaminated Alameda soil. With the unsteamed soil, enrichment culture growth occurred on TSB and salicylic acid within 3 days while phenanthrene had an 8 day lag period (Figure 4a). For soil that had been steamed for 7 days and incubated for 3 days, no growth was observed on phenanthrene during the 18 day enrichment, however salicylic acid and TSB both supported growth within 3 days (Figure 4b). In contrast, soil that had been steamed for 7 days and incubated at room temperature for 17 days before use as an inoculum yielded growth on phenanthrene within 14 days of incubation (Figure 4c).

For the creosote-contaminated Wyckoff soil, enrichment culture growth trends differed in the phenanthrene data only. While trends for TSB and salicylic acid were similar to Alameda soil, no growth occurred on phenanthrene with steamed/incubated soil. However, presteamed soil generated growth on phenanthrene only after a 12 day lag period (data not shown).

FISH analysis of wyckoff soil

Samples of benchtop-steamed Wyckoff soil were fixed and analyzed with fluorescently labeled domain-level 16S rRNA probes to determine whether there were broad shifts in the microbial ecology of the soil following steaming. Table 1 presents total (DAPI-

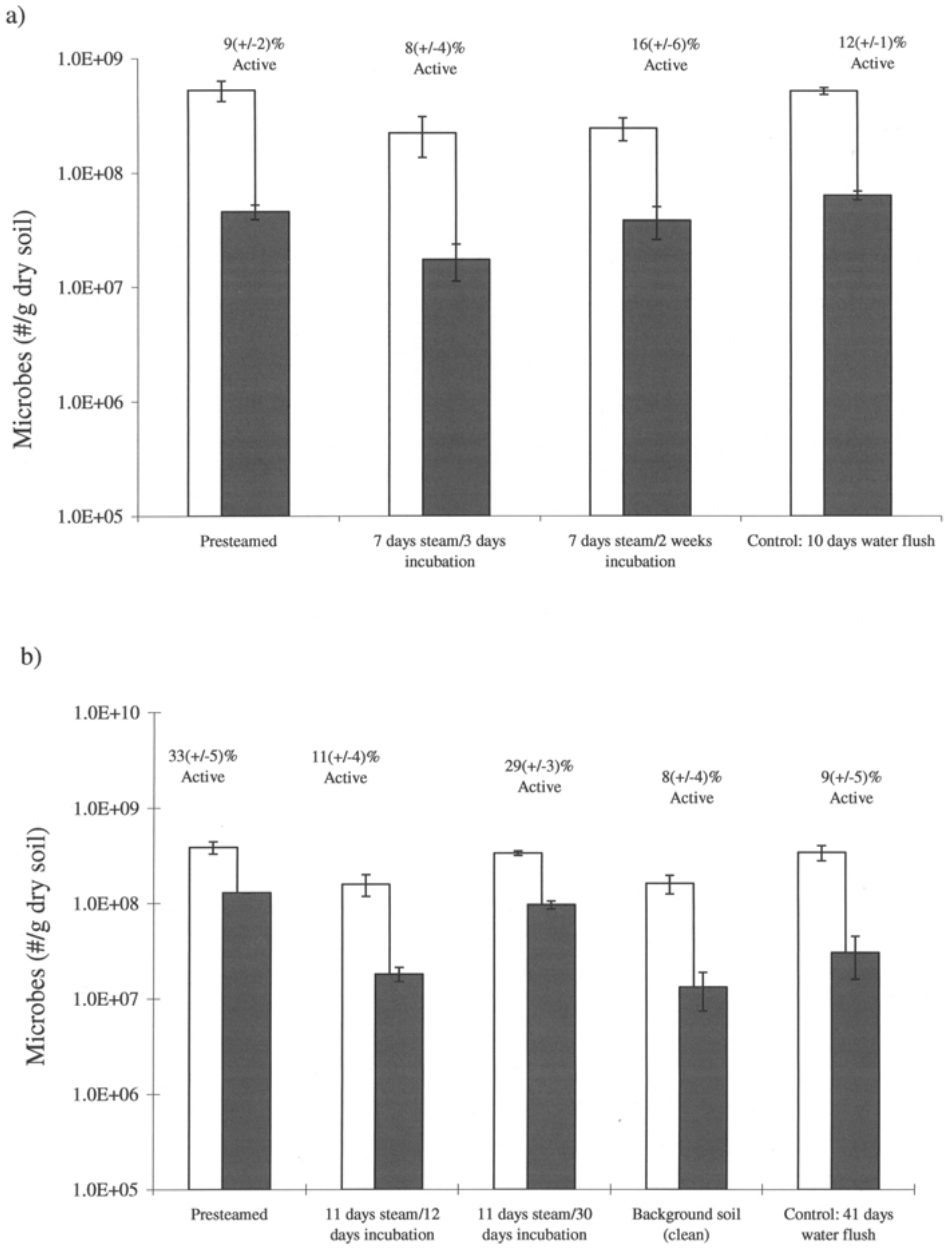

Figure 3. Total microbes (white bars) and active microbes (patterned bars) per gram dry soil and corresponding percent active for soil steamed and then slowly cooled and incubated at room temperature: (a) Alameda soil, (b) WYCK soil. Presteamed and water flushed control soils are also shown. The limit of detection for the CTC/DTAF assay is 10^5 microbes per gram of soil. Error bars represent one standard deviation. *bd = below detection limit.

determined) microbial counts and the percentage of organisms probing positive with Bacterial and Archaeal probes. In all soils the majority of cells did not probe positive with either domain-level probe. Absence of detectable probe signal from a cell could be caused by a number of reasons including a low ribosome content, the impermeability of cell walls to probe molecules, and the existence of sequence mismatches between probe and target rRNA. Of those cells that did hybridize with the probes, Bacteria were dominant. In presteamed soil, $30 \pm 7\%$ of organisms hybridized with the Bacterial probe whereas only $2.7 \pm 0.7\%$ hybridized with the Archaeal probe. In the water-flushed control soil, a slightly lower over-

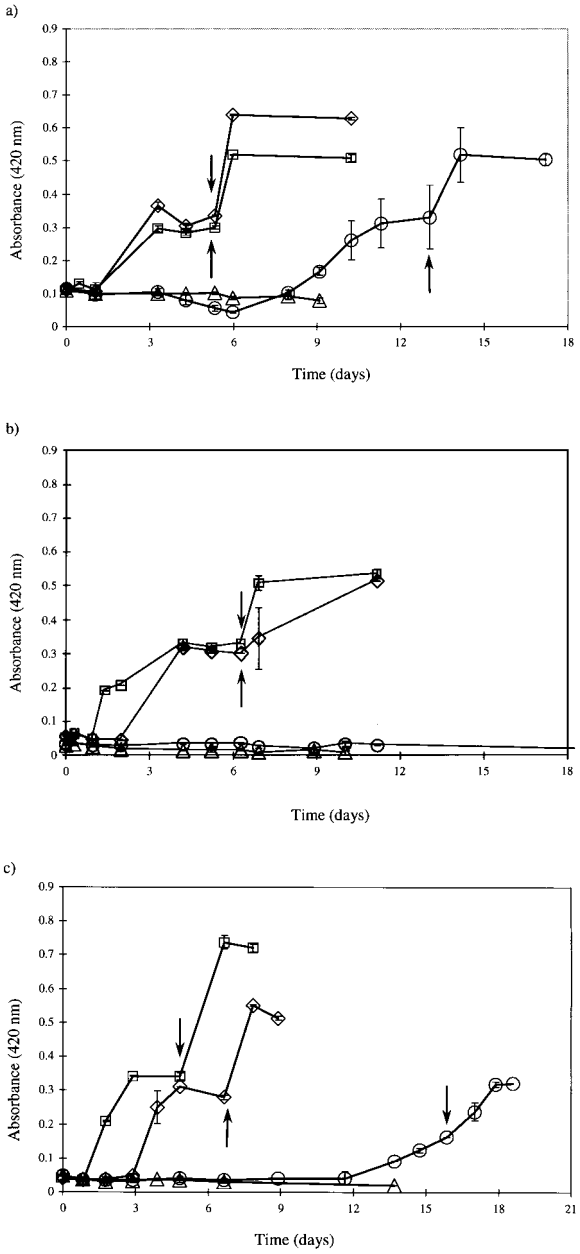

Figure 4. Microbial growth as measured by absorbance versus time for enrichment cultures from soil Alameda Soil with various compounds as sole carbon and energy sources: (a) presteamed soil, (b) soil steamed 7 days and incubated 3 days prior to inoculation, and (c) steamed 7 days, incubated 17 days prior to inoculation. Additional carbon was spiked into cultures at times indicated by arrows. Initial substrate concentrations were 400 mg/L for tryptic soy broth (···□···), 200 mg/L for salicylic acid (···◇···), and 130 mg/L for phenanthrene (···○···). The control enrichment, with no carbon addition, is represented by —△— .

all fraction of microbes hybridized with both probes. This is consistent with – although not as significant as - the trend shown in Figure 3b where metabolic activity was diminished by water-flushing. In steamed Wyckoff soils, Archaea made up a greater percentage of the hybridizable microbes, with the Archaeal probe hybridizing with 5–7% of the total community and the Bacterial probe hybridizing with 24–27% of the total community.

Field steaming of site 5 soil

Table 2 presents total and active microbial counts for Alameda soil borings taken before and after field-scale SEE was performed for six weeks. For each location and depth, three conditions are presented: pre-SEE, post-SEE while soil temperatures were still at 55 °C, and post-SEE for soil that was cooled and incubated at room temperature for 4 days. In general, activity was detected in all pre-SEE and post-SEE/cooled soils, but not in soils that were assayed while still warm. Exceptions occurred in core TMW-4 where microbial activity levels were still undetectable even after cooling and in core TMW-7 where detectable active populations were found at two depths (6′ and 10′) even without cooling and incubation.

Enrichment cultures from field-steamed soil

Post-SEE soil from Alameda (core TMW-20) was used to inoculate enrichment cultures as was done with benchtop-steamed soils. The data are shown in Figure 5. Soil that was used for enrichments while still warm generated growth only on TSB whereas soil that was cooled and incubated at room temperature for 2 weeks prior to enrichment displayed growth on TSB and salicylic acid after a two-day lag period. While growth on phenanthrene did not occur within the first month of monitoring, later observation (after 6 months) showed growth on that substrate in duplicate cultures (data not shown).

Discussion

The results of this study suggest that exposure to steam for up to 6 weeks followed by a gradual cooling period, does not destroy microbial activity in soils. In fact, direct microscopy assays suggest that although soils analyzed immediately after steaming failed to exhibit detectable microbial activity, all soils subjected

Table 1. FISH results for Wyckoff soil before and after treatment

Soil treatment	Total microbes ($\times 10^6$/gram dry soil)	Percent hybridizing with Eub338 probe	Percent hybridizing with Arch344 probe	Percent not hybridizing
Presteamed	230 ± 4	30 ± 7	2.7 ± 0.7	67
Control (water flushed 41 days)	240 ± 15	27 ± 1	2.0 ± 0.1	71
Steamed 11 days/incubated 12 days	90 ± 20	27 ± 1	6.5 ± 0.5	66
Steamed 11 days/incubated 30 days	80 ± 10	24 ± 1	5.5 ± 0.9	70

Table 2. Total and active microbes before and after field-scale SEE at the Alameda site

Core location	Sample depth (ft)	Pre-SEE		Post-SEE (soil at 55 °C)		Post-SEE with 4 days cooling	
		Total microbes ($\times 10^6$ cells/g)	Active microbes ($\times 10^6$ cells/g)	Total microbes ($\times 10^6$ cells/g)	Active microbes ($\times 10^6$ cells/g)	Total microbes ($\times 10^6$ cells/g)	Active microbes ($\times 10^6$ cells/g)
TMW-1	6	1110	30	160	bd*	1770	1470
	8	1850	280	150	bd	700	540
	10	1250	820	100	bd	210	90
TMW-4	6	1750	830	270	bd	190	bd
	8	800	320	90	bd	40	bd
	10	1650	680	70	bd	60	bd
TMW-5	6	1520	1110	70	bd	670	540
	8	890	270	70	bd	60	bd
	10	1640	830	300	bd	1750	330
TMW-7	6	1900	900	690	80	730	70
	8	1560	580	100	bd	1800	330
	10	620	150	180	20	550	160
TMW-20	8	2330	480	1570	bd	1420	550

* bd: below detection.

to a gradual cool-down and incubation yielded significant numbers of metabolically active cells. Within 2 weeks of reaching room temperature, activity levels were within an order of magnitude of presteam levels, regardless of soil or contaminant type. It is possible that the low levels of microbial activity observed for post-steam and post-SEE soils that were not allowed to cool gradually prior to analysis was due to the shock experienced by the heated cells upon abrupt immersion in room-temperature solution. In this case, the inherent slow cool-down associated with SEE applied in the field should result in significant microbial rebound such as that observed for most of the post-steam and post-SEE cooled and incubated soils. The findings reported here suggest that even without nutrient amendments, the activity of native microbial communities can rebound following steam exposure.

It is possible that some of the increase in microbial activity observed with increasing incubation time is attributable to germination of spores or viable but not culturable (VBNC) organisms (Oliver et al. 1995). Physiologically and phylogenetically, the various members of the rebounding microbial communities may require different time frames to germinate from dormant or VBNC states. It is also possible that many species are indeed decimated by the steaming process, leaving a less diverse population to regrow upon cooling. FISH results with Wyckoff soil do indicate that both Bacteria and Archaea can survive the steaming process but that Bacteria still dominate in post-steam communities.

The metabolic potential of the post-steam microbial communities varied slightly with soil. Phenanthrene-degrading organisms were culturable members of the rebounding communities in all but one of the tested soils - the creosote-contaminated Wyckoff soil. Phenanthrene degraders seemed to take longer to establish themselves in the post-steam communities than the generic TSB-utilizing populations. In Alameda soil, detectable microbial growth on phenanthrene was not

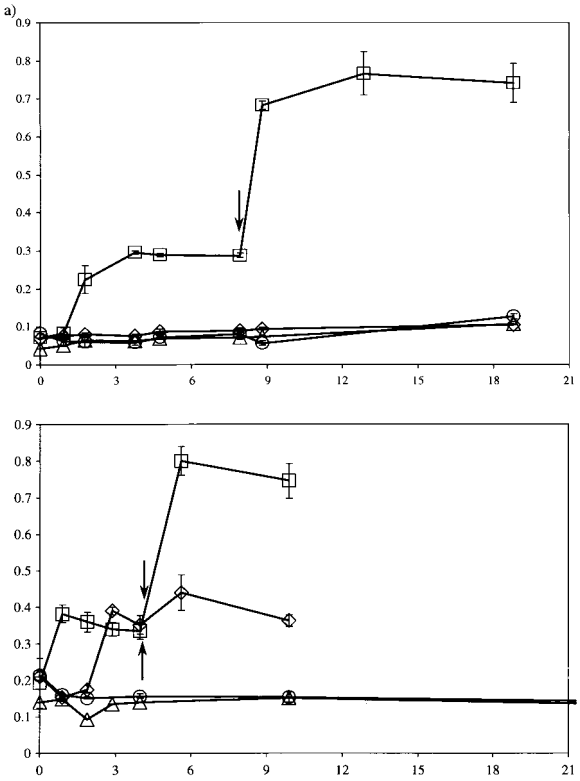

Figure 5. Microbial growth as measured by absorbance versus time for enrichment cultures inoculated with post-SEE soil from the Alameda field site with various compounds as sole carbon and energy sources: (a) post-SEE soil that was used as inoculum while still warm (∼55 °C), (b) post-SEE soil that was cooled to room temperature and incubated for 2 weeks before inoculation. Additional carbon was spiked into cultures at times indicated by arrows. Initial substrate concentrations were 400 mg/L for tryptic soy broth (· · · □ · · ·), 200 mg/L for salicylic acid (· · · ◊ · · ·), and 130 mg/L for phenanthrene (· · · ○ · · ·). The control enrichment, with no carbon addition, is represented by —△—.

apparent in soil incubated for 3 days prior to enrichment but emerged in soils incubated for 17 days (Figure 4b–c). In Wyckoff soil, which exhibited microbial growth on phenanthrene only in presteamed soils, it is possible that the PAH-degraders were killed by the steaming process or decreased in numbers so drastically, that even with a 30 day incubation period following steam, the soil inoculum failed to produce detectable growth in enrichment cultures (monitored daily for three weeks). It is also possible, however, that the 11 days of continuous steam (equivalent to 400 pore volumes of condensed steam) may have removed so much of the relatively bioavailable low molecular weight (two and three ring) PAHs that the residual high molecular weight PAHs could not support active PAH-degrading populations. In separate studies with soil from the Wyckoff site, 12 pore volumes of condensed steam removed >80% of the total PAHs (by mass) – with 95% removal of the two and three-ringed PAHs (data not shown).

SEE may actually stimulate the germination of spores and other dormant forms of microorganisms in soils. Evans & Curran (1943) first noted that the germination of spores of mesophilic aerobes might be accelerated by exposure to heat. They also reported that certain strains of *Bacillus* would not germinate in the absence of heat treatment. They later found that the number of thermotolerant spores that germinate increased after heat activation (1945). Further studies found this to be a more complicated process whereby complete germination of spores may require both heat and the presence of germination-inducing reagents (Maeda et al. 1978; Stringer et al. 1999).

Although data from the benchtop experiments suggest that bioremediation should resume after SEE, it is difficult to extrapolate directly from bench-scale tests to field sites. However, data from a field-scale SEE application generally agree with the benchtop results despite the temporal and spatial scale-up. In conclusion, this report presents bench-scale and field-scale evidence that mesophilic microorganisms can survive during SEE and that the surviving community has the potential for post-steam bioremediation.

Acknowledgements

We thankfully acknowledge the significant contributions of advice and equipment of Professor Kent Udell. Thanks to Justin Remais for help in experiments and apparatus design, the US Navy and US EPA (in particular Eva Davis and Hanh Gold) for soil samples. NIEHS grant P42-ES04705, NSF Young Investigator Award BES-9457246, the NSF Graduate Fellowship Program, and the UC Toxic Substances Research and Teaching Program provided funding for this work.

References

Amann RI, Krumholtz L & Stahl DA (1990) Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. 172: 762–770

Amann RI, Ludwig W & Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microb. Rev. 59: 143–169

Betts KS (1998) Technology combination cuts DNAPL cleanup time at Superfund site. Environ. Sci. Technol. 32: 359A

Bhupathiraju VK, Hernandez M, Krauter P & Alvarez-Cohen, L (1999a) A new direct microscopy based method for evaluating in situ bioremediation. J. Haz. Mater. 67: 299–312

Bhupathiraju VK, Hernandez M, Landfear D, & Alvarez-Cohen L (1999b) Application of a tetrazolium dye as an indicator of viabilityin anaerobic bacteria. J Microb. Meth. 37: 231–243

Bhupathiraju VK, Holman HN, Krauter P, Conrad ME, Daley PF, Templeton AS, Hunt JR, Hernandez M, & Alvarez-Cohen, L (2002) Assessment of in-situ bioremediation at a refinery waste-contaminated site and an aviation gasoline-contaminated site. (Biodegradation, in press)

Chandler DP, Brockman FJ, Bailey TJ & Fredrickson JK (1998) Phylogenetic diversity of Archaea and Bacteria in a deep subsurface paleosol. Microb. Ecol. 36: 3–50

Curran HR & Evans FR (1945) Heat activation inducing germination in the spores of thermotolerant and thermophilic aerobic bacteria. J. Bacteriol. 49: 335–346

Dablow J, Hicks R & Cacciatore D (1995) Steam injection and enhanced bioremediation of heavy fuel oil contamination. In: Hinchee, RE, Kittel, JA and Reisinger, JH (Eds) Third International In-Situ and On-Site Bioremediation Symposium. Battelle Press, Columbus, OH, 3(6): 115–121

Evans FR & Curran HR (1943) The accelerating effect of sublethal heat on spore germination in mesophilic aerobic bacteria. J. Bacteriol. 46: 513–523

Hunt JR, Sitar N & Udell KS (1988) Non-aqueous phase liquid transport and cleanup – Part II: Experimental studies. Water Resour. Res. 24: 1259–1267

Itamura MT & Udell KS (1993) Multiphase transport in porous media, FED-Vol. 173/HTD-265: 57–62

Kästner M & Mahro B (1996) Microbial degradation of polycyclic aromatic hydrocarbons in soils affected by the organic matrix of compost. Appl. Microbiol Biotech. 44: 668–675

Koch AL (1994) In: Gerhardt, Murray, Wood & Krieg (Eds) Methods for general and molecular bacteriology. American Society for Microbiology, Washington, DC

Madigan MT, Martinko JM & Parker J (2000) Brock Biology of Microorganisms, 9th Edition, Prentice Hall, Upper Saddle River, NJ

Maeda Y, Kagami I & Koga S (1978) Thermal analysis of the spores of *Bacilius cereus* with special reference to heat activation. Can. J. Microbiol. 24: 1331–1334

Manilal VB & Alexander M. (1991) Factors affecting the microbial degradation of phenanthrene in soil. Appl. Microbiol. Biotechnol. 35: 401–405

Oliver JD, Hite F, McDougald D, Andon N & Simpson LM (1995) Entry into, and resuscitation from, the viable but nonculturable state by *Vibrio vulnificus* in an estuarine environment. Appl. Environ. Microb. 61: 2624–2630

Pflug IJ & Holcomb RG (1991) In: Block, SS (Ed) Disenfection, Sterilization and Preservation. Lea and Felager, Malvern, PA

Pothuluri JV, Cerniglia CE (1994) Microbial metabolism of polycyclic aromatic hydrocarbons. In: Chaudhry (Ed) Biological Degradation and Bioremediation of Toxic Chemicals. Dioscorides Press, Portland, OR

Richardson RE, Bhupathiraju VK, Song DL, Goulet TA & Alvarez-Cohen L (2002) Phylogenetic Characterization of Microbial Communities that Reductively Dechlorinate TCE Based upon a Combination of Molecular Techniques. Envir. Sci. & Technol. 36: 2562–2662

Rockne KJ & Strand SE (2001) Anaerobic biodegradation of naphthalene, phenanthrene, and bipheynyl by a denitrifying enrichment culture. Water Res. 35: 291–299

Rodriguez GG, Phipps D, Ishiguro K & Ridgway HF (1992) Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. Appl. Envir. Microb. 58: 1801–1808

Smith JJ & McFeters GA (1997) Mechanisms of INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride), and CTC (5-cyano-2,3-ditolyl tetrazolium chloride reduction in Escherichia coli K-12. J. Microb. Meth. 29: 161–175

Stahl DA, Amann RI, Poulsen LK, Raskin L & Capman WC (1995) Use of fluorescent probes for determinative microscopy of methanogenic Archaea. In: Archaea: Methanogens: A laboratory manual. In: Sowers KR & Schreier HJ (Eds) Cold Spring Harbor Laboratory Press, Plainview, New York, p. 111–121

Stringer SC, Haque N & Peck MW (1999) Growth from spores of nonproteolytic *Clostridium botulinum* in heat-treated vegetable juice. Appl Env Microb. 65: 2136–2142

Stringfellow W & Aitken M (1994) Comparative physiology of phenanthrene degradation by two dissimilar pseudomonads isolated from a creosote-contaminated soil. Can. J. Microbiol. 40: 432–438

Stringfellow W & Alvarez-Cohen L (1999) Evaluating the relationship between the sorption of PAHs to bacterial biomass and biodegradation. Water Res. 33: 2535–2544

Udell KS & Stewart LD (1989) Mechanisms of in situ remediation of soil and groundwater contamination by combined steam injection and vacuum extraction. AIChE Meeting, San Francisco, CA

Udell KS (1997) Thermally enhanced removal of liquid hydrocarbon contaminants from soils and groundwater. In: Ward CH (Ed) Subsurface Restoration. Ann Arbor Press, Chelsea, MI

Ueki A, Ono K, Tsuchiya A & Ueki K (1997) Survival of methanogens in air-dried paddy field soil and their heat tolerance. Wat. Sci. Tech. 36: 517–522

Weaver RW, Angle JS & Bottomley PS (Eds) (1994) Methods of Soil Analysis Part 2. Soil Science Society of America, Madison, WI

Wischmann H & Steinhart H (1997) The formation of PAH oxidation products in soils and soil/compost mixtures. Chemosphere 35: 1681–1698

Yeom I-T & Ghosh MM (1998) Mass transfer limitation in PAH-contaminated soil remediation. Wat. Sci. Tech. 37: 111–118