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Journal of Hazardous Materials 41 (1995) 287–298

JOURNAL OF HAZARDOUS MATERIALS

Effect of gaseous nitrogen and phosphorus injection on in situ bioremediation of a trichloroethylene-contaminated site

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Received 11 August 1994; accepted in revised form 6 December 1994

Abstract

Methane and air were injected through a horizontal well into a trichloroethylene-contaminated site at a depth of 160 ft below ground surface to stimulate methanotrophic biodegradation of trichloroethylene (TCE). Sediment samples were analyzed after 35 weeks of methane and air injection, and after 13 weeks of methane and air injection supplemented with injection of the gases nitrous oxide and triethyl phosphate. Methanotroph most-probable-number (MPN) values were very low in most of the samples prior to the addition of nitrogen and phosphorus to the site, and increased several orders of magnitude following the addition. Similarly, the frequency of TCE biodegradative potential in methanotrophic enrichments increased approximately three orders of magnitude after the addition of nitrogen and phosphorus to the site. The MPN and biodegradative potential data indicated that the zone of influence after the addition of nitrogen and phosphorus extended to at least 60 ft from the injection well in both the vertical and horizontal directions.

1. Introduction

Trichloroethylene (TCE) and other chlorinated solvents are major contaminants on industrial and government sites. Cost-efficient strategies are needed for bioremediating these contaminants in deep (>50 ft) subsurface environments. The use of horizontal wells to efficiently deliver multiple gaseous nutrients to stimulate the growth and activity of indigenous microflora able to degrade TCE has been the focus of an Integrated Demonstration funded by the US Department of Energy at the Savannah

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SSDI 0304-3894(94)00119-7

River Site near Aiken, South Carolina [1,2]. Horizontal injection and extraction wells, as compared to vertical wells, maximize the volume of the treatment zone with a minimum number of wells. In addition, the injection of carbon, nitrogen, and phosphorus as gases promotes nutrient transport over greater distances. The use of methane as the carbon source targeted the stimulation of the indigenous methanotrophic microorganisms at the site [3]. Under aerobic conditions, these microorganisms oxidize both methane and TCE using the methane monooxygenase enzyme, but do not derive energy from TCE oxidation [4,5].

TCE contamination of the site occurred between 1952 and 1982 from a leaking process sewer line [1]. At the beginning of the bioremediation demonstration, ground water TCE concentrations were <1–14 ppm and sediment concentrations were <1 ppm with most samples below detection (2 ppb) [6]. The highest TCE concentrations were in the layers with high clay content. While non-aqueous phase TCE exists at the end of the sewer line, several lines of evidence indicate that non-aqueous TCE probably did not exist at the site of the bioremediation demonstration [6–8]. The majority of the TCE was located at 100–140 ft below ground surface (bgs) in a stratum termed the tan clay zone, which is composed of discontinuous, interlayered sand and clay beds of varying thickness [9]. The water table at the site was 130–140 ft bgs. The lower horizontal well was located in the aquifer at 160 ft bgs and the upper horizontal well was located in the unsaturated zone at 70 ft bgs. Injection of gaseous nutrients through the lower well and a vacuum exerted on the upper well moved nutrients through the contaminated region to promote the growth and activity of methanotrophic microorganisms. A 21-week air stripping demonstration (i.e., air injection only) was performed prior to the bioremediation demonstration (air, methane, and later, nitrogen and phosphorus injection) as a control experiment to monitor TCE removal in the absence of injected microbial nutrients [2]. The geology, hydrology, geochemistry, and microbiology of the site and the distribution of contaminants have been summarized [9]. Complete descriptions of sampling, permits, the oversight panel, and the components and operating conditions of the field system have been published [1, 10].

The objective of the bioremediation demonstration was to demonstrate and document that injection of microbial nutrients would result in enhanced TCE removal compared to air-stripping alone. The bioremediation demonstration consisted of a 35 week injection of 1–4% methane (by volume) in air, followed by a 13 week injection of 4% methane supplemented with nitrous oxide (0.07% by volume) and triethyl phosphate (0.007% by volume). Analyses included contaminant inventories in ground water, sediment, soil gas, and the extraction well; methanotroph numbers, biodegradative potential, and activity; and site-specific numerical simulations of the bioremediation demonstration versus air-stripping [1, 6].

In this paper we report on the effect of the nitrogen and phosphorus addition, by comparing the density of culturable methanotrophs and TCE biodegradative potential under methanotrophic enrichment conditions, in sediment samples from the 100 to 140 ft depths immediately before, and after, nitrogen and phosphorus addition to the site.

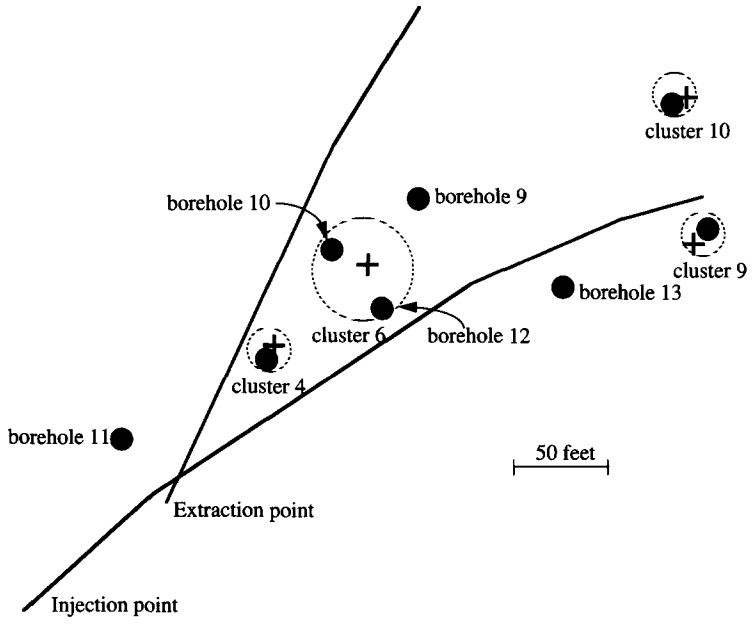

Fig. 1. Plan view of borehole locations, borehole clusters, and horizontal injection and extraction wells at the Savannah River Area M bioremediation site: (crosses) boreholes sampled before nitrogen and phosphorus addition; (solid circles) boreholes sampled after nitrogen and phosphorus addition.

2. Procedures

2.1. Sampling

Fig. 1 shows the location of boreholes from which sediment samples were analyzed. Aseptic sampling techniques were used, including sterile lexan liners, steam cleaning of core barrels, and use of sediment from only the inner portion of the cores [1]. The four locations selected for resampling at the end of the N/P/pulsed 4% methane injection are designated as clusters. At 10 ft intervals, 2 ft of core was homogenized and approximately 200 g was bagged, and samples were shipped by overnight Federal Express in insulated boxes containing ice to maintain a temperature of approximately 4 °C.

2.2. MPN enumerations

The number of culturable methanotrophs was estimated in enrichments set up in a 3-vial/dilution MPN format [11]. Upon receipt of sediment samples, a 10-g aliquot of homogenized sediment was added to 95 ml 0.1% pyrophosphate (pH 7.0) and shaken at 180 rpm for 30 min on a reciprocating shaker before carrying out serial dilutions (1 ml inoculum) to 20 ml headspace vials containing 10 ml of media. The medium was Shelton's mineral salts [12] amended with 2 μM cupric sulphate and 1 ml

of a vitamin mixture [13] per liter. After inoculation, the vials were closed with silicon septa, methane was added to 25% of the headspace, and the vials were sealed with aluminum crimp closures. Inoculated vials were incubated at room temperature. The presence of turbidity, a biofilm, or suspended or floating pellicles after 4 months incubation was scored as a positive result. MPNs below the detection limit (<3/g) and exceeding the upper detection limit (>2400/g) were assigned values of 50% and 200% of the calculated MPN, respectively, to allow an approximate mean value to be calculated for specific boreholes [14].

2.3. Biodegradative potential

Biodegradative potential under methanotrophic enrichment conditions was assessed using the same format, medium, and inoculum as described above. An additional medium was employed by omitting copper from the Shelton's mineral salts medium. A very low (<1 μM) to zero copper concentration results in expression of the soluble form of the methane monooxygenase (sMMO) enzyme, whereas higher copper concentrations result in expression of the membrane-associated or particulate form of the methane monooxygenase (pMMO) enzyme [5, 15]. Glassware was not acid-washed to remove all traces of copper from the latter medium because low levels of copper are present in most sediments and ground water. Immediately before vials were sealed, a gas-tight syringe was slipped alongside the 20-mm-thick Teflon-lined rubber septa and 10 µl of methanol containing 10.9 mM TCE was delivered to the headspace to give an actual concentration (calculated using Henry's constant) of 7.8 µM (1.0 µg/ml) in the medium. Enrichments from samples taken after the addition of nitrogen and phosphorus were set up in replicate sets of vials and an equal amount of TCE was delivered in water instead of methanol. Vials were immediately sealed with the septa and an aluminium crimp closure. No-sediment controls with TCE added were included to account for abiotic losses. Vials were incubated inverted in the dark at room temperature. Headspace in the vials was analyzed after 14–22 weeks using a Hewlett-Packard 5880A series gas chromatograph equipped with a Supelco Vocal capillary column (105 m, 53 mm i.d., 3 µm film thickness), an electron capture detector, and an automatic headspace sampler. The column was operated at 50 °C for 1 min, 7 °C increase/min to 150 °C, and a 25 °C increase/min to 200 °C with a helium carrier gas flow of 58 ml/min and a flow of 24 ml/min from the headspace sampler. TCE had a retention time of 12.8 min under these conditions. Due in part to the extended incubation time, 5–15% loss of TCE was common in the control vials. Much greater losses were infrequently (<5% of the time) encountered, probably due to a poor seal. Therefore, a positive result was conservatively defined as removal of $\geqslant 75$% of the TCE in at least two of the triplicate vials.

3. Results

3.1. Methanotroph MPN index in response to addition of nitrogen and phosphorus

The methanotroph MPN index was <3/g in 79% of the sediment samples prior to the addition of nitrogen and phosphorus to the site (Fig. 2). The addition of nitrogen

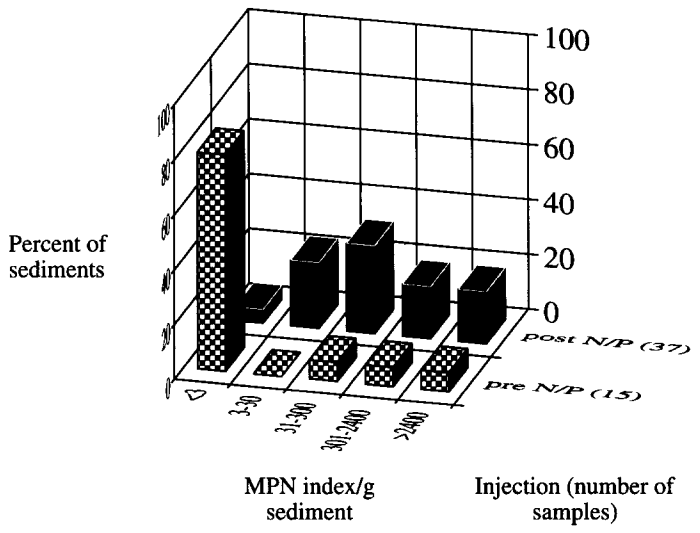

Fig. 2. Percent of 100–140 ft sediment samples containing levels of methanotrophs before and after nitrogen and phosphorus addition.

and phosphorus resulted in an approximately one to three order of magnitude increase in the methanotroph MPN index, with only 3% of the samples containing <3/g and 19% of the samples containing >2400/g.

3.2. *Zone of influence as defined by methanotroph MPN index*

Mean values for methanotroph MPN indexes had high standard deviations (Fig. 3). Due to the assumptions made for the purpose of determining approximate means, actual means and standard deviations were likely greater than shown in Fig. 3. Prior to the addition of nitrogen and phosphorus, 12 of the 15 samples were below detection and methanotrophs were not detected in clusters 4 and 6, the two locations closest to both the injection and extraction wells. The mean for cluster 10 resulted from a single sample (165 l/g) above detection. Following 13 weeks of nitrogen and phosphorus addition, methanotroph MPN means increased approximately two orders of magnitude in clusters 4 and 6, and one order of magnitude in cluster 9. Only one of the 37 samples was below detection after nitrogen and phosphorus addition. MPN indexes >2400/g were present in borehole 12 (three samples), borehole 9 (one sample), cluster 10 (one sample), and cluster 9 (two samples). The mean MPN index at 140 ft (3192/g) was 4 times greater than the means at the 100, 110, 120, and 130 ft depths (data not shown).

3.3. *TCE biodegradative potential in response to addition of nitrogen and phosphorus*

Prior to the addition of nitrogen and phosphorus to the site, TCE degradation under methanotrophic enrichment conditions (copper omitted, TCE delivered in

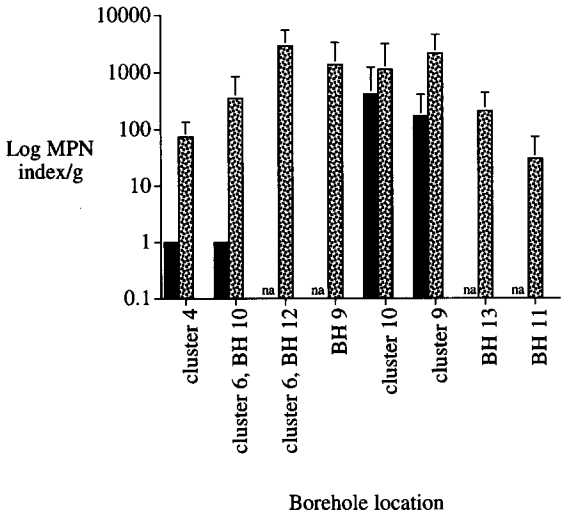

Fig. 3. Mean methanotroph MPN index in 100–140 ft sediment samples by borehole location. The positive standard deviation is shown by the vertical lines: (solid bar) before nitrogen and phosphorus addition; (stippled bar) after nitrogen and phosphorus addition. na, borehole location was not analyzed for methanotroph MPN before nitrogen and phosphorus addition.

methanol) was not observed with an inoculum equivalent to 100 mg sediment/vial. After the addition of nitrogen and phosphorus to the site, TCE degradation (copper omitted, TCE delivered in methanol) occurred in 81% of the samples with an equivalent of 100 mg sediment/vial, and in 68% of the sediment samples with a equivalent of 1 mg sediment/vial (data not shown). Thus, the frequency of TCE biodegradative potential increased by approximately three orders of magnitude in response to the addition of nitrogen and phosphorus.

The presence of methanol can inhibit methane and TCE oxidation in some methanotrophs [16, 17]. To ascertain if the use of methanol as the carrier for TCE affected the measurement of TCE biodegradative potential, separate enrichments were performed with and without methanol as the carrier for samples taken after the addition of nitrogen and phosphorus. The results for enrichments in the absence and presence of methanol were very similiar (data not shown). This was true for enrichments lacking exogenous copper (permissive of soluble methane monooxygenase [sMMO] expression) and containing exogenous copper (permissive of particulate methane monooxygenase [pMMO] expression). Thus, over the length of the incubation and with the criteria used for a positive result, the presence of methanol did not significantly effect TCE biodegradative potential.

TCE biodegradative potential was examined under methanotrophic conditions selective for both forms of the methane monoxygenase enzyme. Three of 7 locations (boreholes 10, 9, and 13) showed a much higher frequency of TCE biodegradative potential under conditions permissive of one form of the methane monooxygenase versus the other form (Fig. 4). Because all methanotrophs are thought to contain the

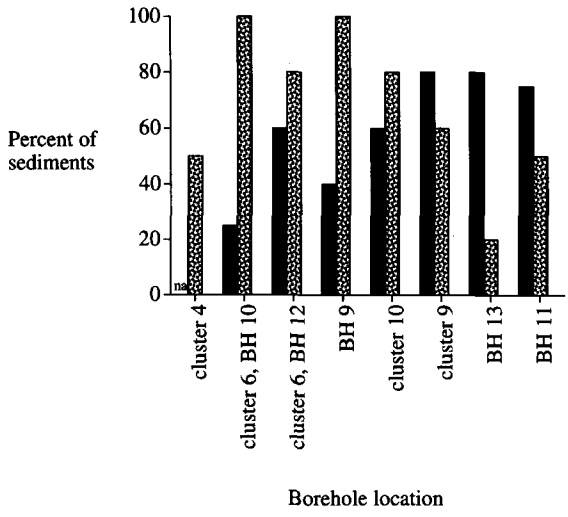

Fig. 4. Percent of 100–140 ft sediment samples from each borehole showing TCE biodegradative potential under methanotrophic conditions (TCE delivered in water), after the addition of nitrogen and phosphorus to the site: (solid bar) conditions permissive of sMMO expression; (stippled bar) conditions permissive of pMMO expression). na, not analyzed for TCE biodegradative potential.

pMMO gene while only some contain both the sMMO and pMMO genes [18], the common occurrence across the site of TCE degradation under enrichment conditions selective for both forms of the methane monooxygenase enzyme suggest that methanotrophs containing the sMMO gene were dominant at the site after the addition of nitrogen and phosphorus. However, the form of the enzyme that was actually expressed in situ cannot be determined from these results.

3.4. Zone of influence as defined by TCE biodegradative potential

The zone of influence after the 13-week injection of nitrogen and phosphorus included all borehole locations (Fig. 4). TCE biodegradative potential showed a trend of increased frequency with greater depth over the 100–140 ft interval (data not shown).

4. Discussion

The Savannah River bioremediation demonstration represents the first use of horizontal well technology to deliver nutrients for bioremediation and the first time that carbon, nitrogen, and phosphorus nutrient sources have all been injected as gases. The well configuration and gaseous nutrient injection strategy were used to maximize the volume of the treatment zone with a minimum number of wells and to promote more efficient delivery of nutrients to subsurface microorganisms. Culturable

methanotroph populations and methanotrophic TCE biodegradative potential in sediment samples were analyzed before and after the injection of gaseous sources of nitrogen and phosphate to assess the magnitude and spatial extent of the nitrogen and phosphorus addition.

Methane injection was initially at 1% by volume in air (15 weeks), followed by 4% methane (11 weeks), and pulsed 4% methane (9 weeks) [19]. Prior to methane injection, air was injected for 21 weeks as a control experiment to monitor TCE removal in the absence of injected microbial nutrients. Samples analyzed after 15 weeks of 1% methane injection, as compared to after 21 weeks of air injection, showed a rapid and large increase in the density of methanotrophic microorganisms and in methanotrophic TCE biodegradative potential as measured by $^{14}CO_2$ evolution from ^{14}C-TCE [19–21]. This increase was followed by a decline in methanotroph populations and TCE biodegradative potential over the next 20 weeks of methane injection. Total microbial biomass in sediments, as measured by acridine orange direct microscopic counts, increased only 30-fold during the 35 weeks of methane and air injection. These results suggested that the increase in biomass was limited by bioavailable nitrogen and/or phosphorus. This situation may have led to a transient, less stable microbial community that was subject to successional processes (i.e., one or more groups of organisms replace other groups of organisms). In an effort to increase the methanotrophic population and improve biodegradative performance, the oversight panel decided to add the gases nitrous oxide and triethyl phosphate to the site. Injection of the nutrients as gases served to maximize the travel distance of the nutrients and minimize the potential for plugging of the injection well by excessive microbial growth in two ways. First, injection of air and methane caused water saturation in the 130–140 ft bgs (initially saturated) sediments to drop 40–50% over the entire site, resulting in much higher diffusivities for any gaseous nutrient (diffusivities in a pure air phase are approximately 10 000 times greater than in a pure liquid phase) [6]. Secondly, nitrous oxide and triethyl phosphate are not readily assimilated by most microorganisms and must be transformed before they can be taken into the cell.

Delivery of nutrients as gases resulted in a zone of bioremediation influence that extended at least 60 ft above and to each side of the horizontal injection well. Prior to the injection of nitrogen and phosphorus, methanotroph MPN indexes were high in clusters 9 and 10, the locations with the least communication with the injection well (based on methanotroph populations and biodegradative potential, and TCE and methane present in ground water and soil gas [19–21]), and below detection in clusters 4 and 6, the locations in good communication with the injection well. The pattern of lowest methanotroph populations near the injection well is consistent with nitrogen and phosphorus limitation caused by injection of electron donor and electron acceptor, and replacement of the methanotrophs by other microorganisms. Indirect evidence that nitrous oxide and triethyl phosphate were delivered 60 ft above and to each side of the horizontal injection well was shown by the two order of magnitude increase in methanotrophic MPN indexes in clusters 4 and 6 and the one order of magnitude increase in cluster 9, and the much higher levels of TCE biodegradative potential at all sampled locations of the site. The addition of nitrogen and phosphorus in previous field bioremediation efforts had been unsuccessful [22, 23],

probably because the nutrients (ammonia, nitrate, and trimetaphosphate) were delivered to the vadose zone by surface irrigation and did not reach the volume of sediment being remediated due to sorption to the solid phase and utilization by microorganisms near the surface.

After the addition of nitrogen and phosphorus to the site, increases in methanotrophic TCE biodegradative potential were greater than increases in methanotroph MPN indexes. This result may be, in part, because the addition of nitrogen and phosphorus to the site improved the physiological status of the methanotrophic population and/or caused changes in other portions of the community structure in situ, resulting in improved ability to degrade TCE in the subsequent enrichments.

Methanotroph MPN indexes are generally assumed to be an estimate of the numbers of culturable methanotrophs in sediments. Given that the methanotrophs were the population targeted for stimulation by addition of methane and air, the relatively low methanotroph MPN indexes (generally <2400/g) were surprising. Methanotroph MPN indexes in sediment samples were several orders of magnitude lower than in ground water samples [19–21]. A contributing factor may be that sampling of ground water favors recovery of water from high conductivity regions and preferential flow paths. Methane and oxygen availability to microorganisms is likely to be greater at these locations as compared to the average sediment sample. A second factor may be that sediment-associated methanotrophic microcolonies are not easily disrupted into individual cells or small groups of cells despite the rigorous treatment prior to carrying out dilutions in the MPN method. In support of this possibility, a nucleic acid probe specific for the soluble methane monooxygenase gene suggested that the MPN method underestimated methanotrophic biomass in sediment samples [24].

The absence of TCE biodegradative potential in 12 of the 15 samples prior to the addition of nitrogen and phosphorus to the site was not unexpected due to the low MPN indexes. However, three of the 15 samples had MPN indexes >30/g (with one sample at >2400/g), yet TCE degradation was not observed with an inoculum equivalent to 100 mg of sediment. This result could be an artifact of sampling (i.e., due to spatial heterogeneity). It is also possible that methanotrophs unable to oxidize TCE were selected for at the site, because intermediates of TCE degradation inhibit cellular metabolism [25]. In sediment samples taken after the addition of nitrogen and phosphorus, the opposite situation was observed: 32 of the 37 samples showed TCE biodegradative potential in one or both of the media with an inoculum equivalent to 1 mg sediment, yet 23 of the samples had MPN indexes <250/g. This may result from the underestimation of methanotrophic biomass by the MPN method due to inability to disrupt sediment-associated microcolonies, or due to a growth habit in the enrichment that prevents the attainment of cell densities which are high enough to detect with the naked eye.

Bioremediation performance was also assessed by measuring TCE concentrations in soil gas, ground water, and sediment, and by numerical simulations that modeled the bioremediation process. Simulations were critical because contaminated water and air were constantly moving into the treatment zone due to vertical recharge (i.e., heavy precipitation events), horizontal recharge, induced water flow created by the injection process, and influx of air from the very large areal influence of the (vadose

zone) extraction well. The simulations showed that the addition of nitrogen and phosphorus to the site resulted in a 5-fold lower residual level of TCE and a doubling of the TCE removal rate [6]. Thus, the higher culturable methanotroph populations and greater methanotrophic TCE biodegradative potential after, as compared to before, the methane–air–nitrogen–phosphorus injection were consistent with TCE inventories and the numerical simulations.

Considering the entire bioremediation demonstration (35 weeks of methane and air injection plus 13 week of methane–air–nitrogen–phosphorus injection), simulations showed that TCE removal was 41% higher than for air-stripping alone [6]. In addition, in situ bioremediation acheived a final TCE concentration 3–6 times lower than that acheived by in situ air-stripping alone. Bioreactor studies using Savannah River sediment, ground water, and groundwater flow rates estimated that an average of 1.5 mg TCE was biodegraded/m^3/d throughout the demonstration, a rate similar to that estimated by the simulations [26]. Sediment concentrations of TCE declined to below detection (<2 ppb) over most of the site [19]. Soil gas TCE declined by more than 99%, with samples from near the injection well consistently being below detection by the end of the methane–air–nitrogen–phosphorus injection. Ground water concentrations of TCE decreased by as much as 95%, reaching concentrations below detection in some ground water monitoring wells. Moreover, direct chemical evidence that losses were due to bioremediation was indicated by the inverse correlation between ground water chloride concentrations and TCE concentrations in most samples.

5. Conclusions

1. Methanotroph MPN indexes in sediment samples taken after the addition of nitrogen and phosphorus, as compared to before the addition, increased approximately one to three orders of magnitude.
2. The frequency of TCE biodegradative potential increased by approximately three orders of magnitude in response to the addition of nitrogen and phosphorus to the site.
3. Spatial analysis of the methanotroph MPN and TCE biodegradative potential results indicate that the delivery of methane, oxygen, nitrogen, and phosphorus as gases resulted in a zone of bioremediation influence that extended at least 60 ft above and to each side of the horizontal injection well.
4. The higher culturable methanotroph populations and greater methanotrophic TCE biodegradative potential after, as compared to before, the methane–air–nitrogen–phosphorus injection were consistent with TCE inventories and the results of numerical simulations.

Acknowledgements

This research was funded by the Office of Technology Development, within the Department of Energy's Office of Environmental Management, under the Non-arid

Soils Volatile Organic Compounds Integrated Demonstration. Pacific Northwest Laboratory is operated by Battelle Memorial Institute for the US Department of Energy under Contract DE-AC06-76RLO 1830.

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