RECOVERY OF MICROBIALLY MEDIANED PROCESSES IN SOIL AUGMENTED WITH A PENTACHLOROPHENOL-MINERALIZING BACTERIUM

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Abstract—Specific physiological groups can be used to evaluate the recovery of soil microbial communities following disturbance. In this study, soil was contaminated with pentachlorophenol (PCP) to assess the resiliency of microorganisms responsible for carbon and nitrogen cycling. Methane fluxes were monitored in soil microcosms to evaluate the effects of contamination and augmentation on microbial populations involved in carbon cycling. The addition of a PCP-mineralizing bacterium, Sphingomonas chlorophenolica strain RA2, enhanced the recovery of methane-oxidizing capacity relative to uninoculated treatments when soil was contaminated with low concentrations of PCP (10 and 50 μg/g soil, ppm). At the highest level of PCP contamination tested (300 ppm), there was no recovery of methane-oxidizing capacity whether or not the PCP-mineralizing bacterium was added to soil. The nitrogen-cycling capacity of contaminated and augmented soil was tested by measuring nitrification potentials. The addition of PCP resulted in a concentration-dependent reduction in nitrification rates. After three months of incubation, all augmented soils had nitrification rates equivalent to the uncontaminated control, and the uninoculated soils contaminated with 100 and 300 ppm PCP still were impaired in their nitrification potentials. These results indicate that biological removal of a contaminant has the potential to restore microbially mediated processes to levels observed prior to contamination.

Keywords—Pentachlorophenol Nitriﬁcation Methane oxidation Augmentation Sphingomonas chlorophenolica

INTRODUCTION

The importance of nutrient cycling by soil microorganisms for the maintenance of soil health is well established [1–4]. Two of the most closely scrutinized microbially mediated processes are carbon and nitrogen cycling. However, these processes also have been shown to vary in their respective responses to anthropogenic disturbance. Carbon flux, as measured by soil microbial respiration, has been shown to be suppressed [5–8], stimulated [9,10], or unaffected [8,11] upon exposure to various contaminants. Although the importance of respiration should not be underestimated, it is a process that is ubiquitous among soil microorganisms. Such functional overlap makes it less likely to detect the effects of contamination on individual populations, because the susceptibility of some species might be masked by the increased response of several others. The redundancy of soil microbial respiration diminishes its potential as an accurate indicator of disturbance and has been reported to be less sensitive than other processes [4,11,12]. Unlike respiration, the microbial production and consumption of methane is carried out by specific populations, and these processes are important in controlling atmospheric methane concentrations [13,14]. For these reasons, monitoring methane fluxes through soil has potential as a sensitive process-level indicator of carbon cycling [15–17].

Similar to carbon cycling, different components of the nitrogen cycle appear to be better indicators of disturbance [4]. As with microbial respiration, ammonification is one process that is carried out by many microorganisms and has been found to be suppressed [18] or unaffected [18–20] following contaminant exposure. Nitrification, however, appears to be consistently sensitive to disturbance [8,11,18–23]. Nitrification is an essential microbially mediated process that is carried out by few groups of microorganisms. Consequently, nitrifiers have the potential to serve as sensitive indicators of soil health, because any adverse effects should not be masked by a few resistant organisms that may not respond to disturbance. Although a vast number of studies have investigated the effects of contamination on soil microorganisms, relatively few have followed the recovery of microorganisms after the biological removal of the contaminant [16,24,25]. We, and others, have previously shown that Sphingomonas chlorophenolica strain RA2 rapidly mineralizes high concentrations of pentachlorophenol (PCP) when inoculated into contaminated soil [26–30]. In this study, we investigate whether the addition of S. chlorophenolica strain RA2 to soil contaminated with PCP restores the soil’s carbon- and nitrogen-cycling capacity.

MATERIALS AND METHODS

Soil incubations

Boulder Creek (Colorado, USA) soil was collected from the upper 15 cm in July 1995. This soil is a sandy loam and has been described in detail [26,27,29]. Immediately following collection, the soil was sieved (2 mm) and stored at 4°C and used within six months. Prior to the incubation, 30 g of soil (dry wt equivalent) was added to biomter flasks or 50-ml polyethylene specimen cups. All treatments described herein were carried out in triplicate unless indicated otherwise. On day 0 of each experiment, PCP (Fluka Chemical, Ronkonkoma, NY, USA) was added to the contaminated treatments at the appropriate concentration (0, 10, 50, 100, or 300 ppm). S. chlorophenolica strain RA2 was added to inoculated soils, a killed inoculum was added to uninoculated soils, and a sterile

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mineral salts medium [31] was added to the uncontaminated controls. Enough sterile water was added to each replicate sample to obtain a water content equivalent to 70% of water-holding capacity. The inoculum consisted of a stock culture of *S. chlorophenolica* strain RA2 that was maintained in a mineral salts medium [26] containing 300 ppm PCP as the sole carbon and energy source. One milliliter of stationary phase cells containing approximately \(2.7 \times 10^8\) cells/ml was added to the augmented treatments. This resulted in a final inoculum density of \(9.0 \times 10^7\) cells/g soil. The inoculum and PCP were mixed thoroughly into the soil using a Teflon®-coated spatula. Parafilm (American National Can, Chicago, IL, USA) was used to cover specimen cups to minimize water loss, and the microcosms were sampled for methane fluxes or potential nitrification rates at appropriate intervals. Water lost to evaporation was compensated for by the addition of sterile water at weekly intervals throughout the incubations. All incubations were carried out at 22.5 ± 1°C.

**PCP mineralization**

The biological removal of PCP was monitored in parallel flasks as described previously [26,27,29]. As described above, soil samples (30 g) were placed in 250-ml biometer flasks but were amended with uniformly \(^{14}\)C-labeled PCP (11.9 mCi/mmole, Sigma Chemical, St. Louis, MO, USA), unlabeled PCP, and deionized water. *Sphingomonas chlorophenolica* strain RA2 was added to inoculated soils and contaminated controls received sterile mineral salts medium. Evolved \(^{14}\)CO\(_2\) was capped to inoculated soils and contaminated controls. Enough sterile water was added to each replicate sample to obtain a water content equivalent to 70% of water-holding capacity. The inoculum consisted of a stock culture of *S. chlorophenolica* strain RA2 that was maintained in a mineral salts medium [26] containing 300 ppm PCP as the sole carbon and energy source. One milliliter of stationary phase cells containing approximately \(2.7 \times 10^8\) cells/ml was added to the augmented treatments. This resulted in a final inoculum density of \(9.0 \times 10^7\) cells/g soil. The inoculum and PCP were mixed thoroughly into the soil using a Teflon®-coated spatula. Parafilm (American National Can, Chicago, IL, USA) was used to cover specimen cups to minimize water loss, and the microcosms were sampled for methane fluxes or potential nitrification rates at appropriate intervals. Water lost to evaporation was compensated for by the addition of sterile water at weekly intervals throughout the incubations. All incubations were carried out at 22.5 ± 1°C.

**Aerobic methane oxidation**

To measure methane oxidation, specimen cups with soil were uncovered and placed into individual mason jars, each fitted with a Teflon-coated silicone septum. After closing the jars, two 10-ml volumes of air were removed to determine initial methane concentrations in the headspace of each jar. Duplicate 10-ml headspace samples were removed within 24 h, and linear regression analysis was used to determine the rates of net methane consumption over the incubation period. Significant differences in rates of methane oxidation among treatments were determined using a Student’s t test. Methane concentrations were measured with a Hewlett-Packard 5890A gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) fitted with a flame ionization detector at 150°C [32].

**Anoerobic methane fluxes**

An additional set of specimen cups with soil was used to assess methanogenesis potentials. The treatments were set up as described above and included 300 ppm of PCP augmented with *S. chlorophenolica* strain RA2, 300 ppm of PCP with no inoculum, and uncontaminated soil. replicate soil containers (3 of each treatment) were placed into mason jar assemblies as described above. Sodium borohydride (0.5 g) and sodium bicarbonate (3 g) were placed into glass vials and inserted into each mason jar along with a palladium catalyst (Becton Dickinson, Franklin Lakes, NJ, USA). A 10-ml water addition to each vial resulted in the removal of \(O_2\) and subsequent production of \(CO_2\) and \(H_2\). After at least 24 h, three 10-ml volumes of air were removed from each mason jar. A second measurement was made 24 h later and analyzed with a gas chromatograph to determine the methanogenesis potential.

**Nitrification rates**

The nitrification potentials of the treatments were measured with a soil-slurry method [33]. For each treatment, one soil container was sacrificed at every sampling interval. The samples were divided into two 15-g replicates and placed into a flask containing 100 ml of a dilute ammonium phosphate solution [33]. The flasks were covered with Parafilm and shaken for 24 h. For every treatment, a total of four 10-ml slurry samples (2 early and 2 late) were taken during the 24-h incubation. Each sample was filtered through a Whatman 1 filter (Florham Park, NJ, USA) and analyzed colorimetrically for nitrite accumulation using a Lachat flow injection analyzer (Milwaukee, WI, USA). Nitrate was converted to nitrite by passing the filtrate through an inline Cd-reduction column. Subtracting the initial nitrite concentration from the total nitrite after reduction yielded the amount of nitrate in the sample. Rates of nitrification over the 24-h period were then determined using linear regression analysis. A Student’s t test was used to determine significant differences in nitrification potentials between the contaminated and uncontaminated treatments.

**Ammonium levels in soil**

At the end of the incubations used to measure nitrification potential, the different soil treatments were sampled for ammonium by extracting 10-g subsamples with 50 ml of the nitrification potential solution minus the ammonium. The samples were filtered through a Whatman 1 filter and analyzed colorimetrically after reacting ammonium with hypochlorite and phenol using the technique outlined by Bundy and Meisinger [34]. Replicate samples were dispensed into 96-well assay plates (Rainin Instrument, Emeryville, CA, USA) and the absorbance at 650 nm read with a plate reader (Molecular Devices, Menlo Park, CA, USA).

**RESULTS**

**PCP removal**

At least 70% of added PCP was recovered as \(^{14}\)CO\(_2\) in soils augmented with *S. chlorophenolica* strain RA2, and these data have been published elsewhere [26,27,29]. After accounting for the amount of PCP incorporated into biomass [31], this corresponds to the removal of at least 85% of added PCP within the first week of incubation. As for soils not receiving *S. chlorophenolica* strain RA2, only the soil contaminated with 10 ppm PCP yielded any \(^{14}\)CO\(_2\). In this incubation, roughly 10% of the added PCP was captured as \(^{14}\)CO\(_2\) in the first week, with 30% recovery by three weeks. No \(^{14}\)CO\(_2\) was captured in controls of all other treatments.

**Effects of PCP contamination and augmentation on methane oxidation**

Contaminating soil with different levels of PCP resulted in varied responses of the methane-oxidizing populations. Prior to PCP amendments, Boulder Creek soil consumed atmospheric methane (0.00634 ± 0.00086 ppm per hour). When soil was contaminated with 10 ppm of PCP, net methane oxidation rates were not significantly different from the initial rate three weeks after contamination (Fig. 1). At this level of PCP contamination, augmenting soil with *S. chlorophenolica*
strain RA2 did not influence significantly the rate of methane oxidation.

When a higher concentration of PCP (50 ppm) was applied to soil, the differences between the inoculated and uninoculated samples were more pronounced (Fig. 1). Three weeks following PCP additions, the rate of net methane oxidation was significantly higher in treatments where *S. chlorophenolica* strain RA2 was added relative to the uninoculated, contaminated soil ($p < 0.05$). In addition, the rate of net methane oxidation in the augmented treatment was not significantly different from uncontaminated soil following the addition of PCP.

The effects of PCP contamination on methane oxidation at the highest level tested (300 ppm) was by far the most extreme. The net rates of methane oxidation were significantly suppressed relative to the uncontaminated control ($p < 0.05$). Throughout the three-week incubation there were no significant differences in net methane oxidation rates between PCP-contaminated treatments that were or were not augmented with *S. chlorophenolica* strain RA2.

**Methanogenesis potential of soil**

The highest level of PCP contamination tested (300 ppm) was used to assess whether or not PCP influenced methanogenesis potentials in Boulder Creek soil. Within 24 h of placing aerobically incubated soil containers into anaerobic containers enriched in CO$_2$ and H$_2$, all treatments exhibited net methane production. Overall, the methanogenesis potentials following PCP contamination were never significantly different from the uncontaminated controls ($p < 0.05$) throughout the three-week incubation (data not shown). These results were consistent whether or not soil was augmented with *S. chlorophenolica* strain RA2.

**Nitrification potential in contaminated and remediated soil**

Soil contaminated with different levels of PCP (0–300 ppm) yielded varied responses in nitrification potential. The production of nitrite and nitrate was measured in all of the samples; however, no substantial accumulation of nitrite was ever seen in any samples (data not shown). Therefore, all of the nitrification potentials reported are in terms of nitrate production.

The initial response to PCP contamination was a concentration-dependent reduction in nitrification potential (Fig. 2). The nitrification potentials were measured immediately following PCP and inoculum additions. Treatments contaminated with higher concentrations of PCP were suppressed to a greater degree than those receiving lower concentrations. No differences were found in nitrification potentials between the augmented and uninoculated treatments, regardless of the amount of PCP added.

Two weeks of incubation appeared to be sufficient time for the recovery of the nitrifying population exposed to the lowest concentration of PCP tested. The uninoculated and augmented treatments that were contaminated with 10 ppm PCP had equivalent nitrification potentials after 14 d of incubation (Fig. 3). Significant differences were found in nitrification potentials between the uninoculated and augmented treatments contaminated with 50 and 100 ppm PCP ($p < 0.05$). Soil that was contaminated with the highest amount of PCP (300 ppm) resulted in suppressed nitrification potentials relative to the uncontaminated control. At this level of contamination there was no significant difference between the augmented and uninoculated treatments.

After an extended, three-month incubation, all of the treatments augmented with *S. chlorophenolica* strain RA2 had nitrification potentials that were equivalent to the uncontaminated control (Fig. 4). The effect of PCP on the uninoculated treatments still was dependent upon the initial PCP concentration. Uninoculated controls that were contaminated with 100 and 300 ppm PCP had significantly lower nitrification potentials than the augmented treatments ($p < 0.05$).
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Fig. 3. Nitrification potentials 14 d after pentachlorophenol and *Sphingomonas chlorophenolica* strain RA2 amendments. Duplicate samples were used and error bars are standard errors of the mean.

**Fig. 5.** Extractable ammonium in the different soil treatments after the three-month incubation. Duplicate samples were used and error bars are standard errors of the mean. PCP = pentachlorophenol; RA2 = *Sphingomonas chlorophenolica* strain RA2.

**Extractable ammonium**

At the end of the three-month incubation, all soil treatments had ammonium present. Extractable ammonium concentrations ranged from 60 to 152 μmoles ammonium per g soil. The uninoculated treatment that was contaminated with 300 ppm of PCP had the highest ammonium concentration (Fig. 5).

**DISCUSSION**

Both carbon and nitrogen cycling were affected by PCP contamination in this soil. However, augmenting soil with the PCP-mineralizing bacterium *S. chlorophenolica* strain RA2 helped to reverse some of these deleterious effects in soil contaminated with moderate to high PCP concentrations.

Overall, net methane oxidation was impacted negatively by PCP contamination (Fig. 1). Soil contaminated with low levels of PCP (10 ppm) did not appear to benefit from the addition of *S. chlorophenolica* strain RA2, as there were no significant differences in net methane oxidation between augmented and uninoculated treatments. Given that there is an indigenous population capable of mineralizing up to 10 ppm PCP in this soil [29], this result is not surprising. Higher PCP concentrations had a more drastic impact on net methane oxidation. The only treatment in which the addition of *S. chlorophenolica* strain RA2 resulted in greater rates of net methane oxidation than the uninoculated treatment was when soil was contaminated with 50 ppm of PCP. At this PCP concentration, the rate of net methane oxidation in the augmented soil did not differ significantly from the measurement taken prior to PCP contamination. Alternatively, the net methane oxidation rate in uninoculated soil was lower than the precontamination rate. Similarly, methane consumption was suppressed throughout the three-week incubation in soil that received the highest application of PCP (300 ppm). However, the addition of *S. chlorophenolica* strain RA2 did not help to facilitate the recovery of methane oxidizers. These findings indicate that methane-oxidizing populations may be irreversibly impacted by high concentrations of PCP. Because *S. chlorophenolica* strain RA2 rapidly removed 300 ppm PCP in this soil, these results were somewhat unexpected. It is possible that the initial addition of PCP was so acutely toxic that any surviving meth-anotrophs were unable to regrow in the remediated soil within the three-week incubation period.

Although there was an obvious deleterious effect of PCP on methane-oxidizing populations, the impact of contamination and augmentation on the organisms involved in carbon cycling could have been underestimated had respiration been
used as an indicator. This especially may be the case for PCP, because its toxicity is largely due to its ability to uncouple oxidative phosphorylation, resulting in higher respiration rates after exposure [35]. Bauer and Capone [12] found that PCP additions to marine sediments significantly increased respiration while decreasing radio-labeled glucose incorporation. This potential effect, coupled with the increased respiration due to PCP-degrading bacteria, could result in concluding little to no impact on respiration in contaminated soil. Hence, methanotrophy appears to be a sensitive indicator of the potential effects of PCP contamination on microbial processes.

Net methane fluxes depend on the balance between methanogenesis and methanotrophy in soil. For example, West and Schmidt [32] showed that soils that normally oxidize methane will emit methane if methanotrophy is inhibited, even when soils are quite dry. In preliminary experiments, we occasionally observed methane production in highly contaminated treatments [29]. Therefore, controlled anaerobic incubations were carried out to determine if this soil has the capacity for methane production, and whether methanogenesis is impacted by PCP contamination. In agreement with the preliminary results, methane production in this soil was evident after 24 h of incubation under a H$_2$-CO$_2$ atmosphere. A three-week incubation of soil contaminated with 300 ppm of PCP resulted in no significant differences in methanogenesis potentials among uncontaminated, augmented, and uninoculated soil. In contrast, PCP has been shown to be highly toxic to pure cultures of methanogens, resulting in decreases in both methane evolution and growth of methanogens [36]. Similarly, when PCP was added to glucose-fed chemostats, acetate was produced continually, yet methane production was suppressed significantly, indicating that methanogens are more sensitive to PCP than are acetogens [37]. This leads us to believe that the soil environment may offer more protection to methanogens than to methanotrophs. Because methanogenesis requires anaerobic conditions, methanogens live deep within soil aggregates, where they are less likely to be exposed to oxygen [38] and, fortuitously, PCP. Conversely, methanotrophs typically require aerobic conditions and are more likely to encounter the contaminant.

As with carbon cycling, different components of the nitrogen cycle appear to be better suited as indicators of disturbance [4]. For example, PCP has been shown to strongly inhibit nitrification, but not ammonification, in glycine-amended soil [18]. Similarly, our uninoculated treatment contaminated with 300 ppm PCP had the highest concentration of ammonium (presumably due to ammonification) at the end of the incubation (Fig. 5). Selective toxicity to nitrification has been observed with other contaminants as well. Fuller and Scow [19] reported that toluene contamination had no significant effect on ammonification but had a large effect on nitrification. Unlike our findings, they did not observe a recovery of the nitrifying population after a 30-d incubation that followed venting-off the highly volatile contaminant. Remde and Hund [11] also showed a concentration-dependent decrease in nitrification potential in soil contaminated with dicyandiamide or anthracene oil. The nitrification potential of the soil was restored in the anthracene oil–contaminated samples when applied at low concentrations, but no recovery was observed in highly contaminated soil.

**CONCLUSION**

In conclusion, specific microbially mediated processes are appropriate indicators of soil disturbance. Augmenting PCP-contaminated soil with the PCP-mineralizing bacterium *S. chlorophenolica* strain RA2 helped accelerate the recovery and/or restore the nutrient-cycling capacity of the soil in most cases. Methane-oxidizing populations in soil contaminated with a high concentration of PCP (300 ppm) were impacted irreversibly, yet methanogens appeared to be unaffected by the application of the contaminant. The irreversible impact on methanotrophs may be due to the lack of colonizing organisms from an uncontaminated soil or even competitive exclusion of survivors by *S. chlorophenolica* strain RA2. Conversely, nitrogen-cycling populations in this soil responded favorably to the addition of *S. chlorophenolica* strain RA2. Overall, it appears that augmentation of PCP-contaminated soil with a PCP-mineralizing bacterium has the potential to restore the nutrient-cycling capacity of the soil.

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