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Mineralization of pentachlorophenol in a contaminated soil by *Pseudomonas* sp UG30 cells encapsulated in κ-carrageenan

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A contaminated soil from Ontario, Canada containing 350–370 ppm pentachlorophenol (PCP) and 21 000 ppm total petroleum hydrocarbons (TPH) was inoculated with PCP-degrading *Pseudomonas* sp UG30 cells either encapsulated in κ-carrageenan or as free cells. Uninoculated control soil produced 18.8 ± 3.9% of $^{14}CO_2$ from 100 000 dpm [U-^{14}C]-PCP after 30 weeks incubation at 22°C. Addition of phosphate increased PCP mineralization, whereas addition of nitrogen inhibited mineralization almost completely in soil not inoculated with UG30 cells. No enhancement of mineralization was observed in soil with the addition of 10^8 CFU g^{-1} dry soil free UG30 cells. κ-Carrageenan-encapsulated UG30 cells at the same inoculum density mineralized 64.7 ± 0.3% PCP in soil after 26 weeks. Repeated inoculations with encapsulated UG30 cells up to six times over 6 weeks resulted in 64.8 ± 1.9% mineralization of radiolabelled PCP within 9 weeks, although by 12 weeks all treatments with encapsulated cells had mineralized to this level. Addition of sterile beads (controls) led to less than 16.6 ± 9.2% mineralization within 16 weeks. Varying initial inoculum densities of encapsulated cells were compared to determine effects on PCP mineralization. Cells grown inside the beads, and higher initial cell densities exhibited greater mineralization activity in the first weeks, but by 20 weeks PCP mineralization was approximately 70.0 ± 8.0% and was not significantly different between all soil treatments. Our results show encapsulation can enhance pollutant mineralization in a chemically contaminated soil.

Keywords: bioremediation; contaminated; encapsulation; mineralization; pentachlorophenol; *Pseudomonas*; soil

Introduction

Bioremediation of contaminated soil is a potentially non-disruptive and economical alternative to current non-biological remediation technologies. The most effective or appropriate method of remediation often needs to be determined on a case-by-case basis due to heterogeneity of soil, the pollutants present and numerous parameters which may influence the soil site. In many contaminated soils, the levels of nutrients such as C, N, P and K may be low. Biostimulation of indigenous microorganisms by introducing fertilizer or organic amendments [15] can be an effective and inexpensive solution in some soils if certain additional criteria are met. The indigenous microorganisms must be capable of degrading the pollutant, able to utilize the additional nutrients, and at the same time, compete with pollutant-tolerant microorganisms present in the soil. However, the addition of fertilizers or degradable carbon [2] does not necessarily enhance or improve degradation of pollutants such as PCP.

If there are no indigenous pollutant-degrading microorganisms, or they exist at very low numbers and activity, bioaugmentation, the addition of active pollutant-degrading microorganisms, may be a suitable alternative. Additions of suspensions of microbial cells to remediate contaminated soils have been explored and relatively equal numbers of successes and failures have been reported [1,8,13]. The formulation of bacterial cells may be a crucial factor in the survival and activity of the microorganisms in soil, affecting the success or failure of the bioremediation effort [10,18]. Although immobilization technology, and encapsulation in particular, have been successfully used for degradation of recalcitrant compounds in controlled bioreactor conditions [4,5], there are few investigations on their use as carriers for soil inocula.

Studies of pollutant biodegradation in contaminated soils have used soil slurries or agricultural soils 'spiked' with a certain concentration of pollutant [2,7,9,20]. It has been suggested that a more realistic evaluation of the potential of a biological treatment should be done with historically contaminated soils [3]. For example, Lestan and Lamar [10] observed 82–90% mineralization of PCP in a 'spiked' agricultural soil using a pelleted solid substrate (agricultural and wood industry by-products) coated with an alginate suspension of fungi, whereas no mineralization occurred in an actual PCP-contaminated soil using the same inoculum.

In this study, we investigated PCP mineralization in an aged industrial soil from Ontario, Canada contaminated with high levels of PCP and total petroleum hydrocarbons (TPH). The effect of nutrient amendments and addition of free or encapsulated PCP-mineralizing *Pseudomonas* sp UG30 on PCP mineralization in this soil was examined. Mineralization effectiveness of encapsulated cells as a function of inoculum density was also investigated. Inoculum density was compared in two ways: cells grown before encapsulation, or growth within the beads for 24 h after encapsulation.

Materials and methods

Soil

Soil was used from a wood-treatment site in Ontario, Canada. It was kindly provided by Dr AG Seech of Grace Bio-

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remediation Technologies, Mississauga, Ontario, Canada. Analyses by Grace Bioremediation Technologies showed the soil to be contaminated with 350–370 ppm PCP, 20 900 ppm TPH, including 1224 ppm of polyaromatic hydrocarbons (PAHs), and to have very low original nutrient levels (Table 1). Soil was sieved through a 2-mm mesh and kept in a sealed container in the dark at 4°C until use. Soil was adjusted so that the final water holding capacity for each experiment was 60%.

Pentachlorophenol

Sodium pentachlorophenate (NaPCP) (Aldrich, Milwaukee, MI, USA) was dissolved in sterile distilled water and filter-sterilized before use. Radiolabelled [UL-^{14}C]PCP (Sigma Chemicals, St Louis, MO, USA) (specific activity 6.5 mCi mmol^{-1}; purity >98%) was used in experiments where a radiolabelled tracer was required.

Fertilizers/nutrients

Nutrients were added to final concentrations of 1000 ppm each. Carbon (C) was added as dextrose; Nitrogen (N), as ammonium nitrate (NH_4NO_3); phosphorous (P), as super triple phosphate [$Ca(H_2PO_4)2H_2O$]; and potassium (K), as potassium sulfate (K_2SO_4). Each nutrient was added either alone or in combination with other nutrients. Resulting treatments were C, N, P, K, CN, CP, CK, NP, NK, PK, CNP, CNK, CPK, NPK and CNPK.

Microorganism

Pseudomonas sp UG30 was initially isolated in our laboratory [11,16]. UG30 exhibited a growth rate of 0.2 h^{-1} in minimal salt medium supplemented with 0.4% (w/v) glutamate (MMG) at 30°C. A cell density of 8×10^7 CFU ml^{-1} mineralized NaPCP up to 250 μg ml^{-1} in a minimal salt solution. Cells were grown in MMG to an OD_{600} of 0.5 at which time NaPCP was added at a concentration of 50 ppm to induce PCP-mineralizing ability. Cells continued to grow to an OD_{600} of approximately 1.0 (equalling 1.0×10^9 CFU ml^{-1}) before use. Cell numbers were estimated using a standard curve generated from cell suspension turbidities and cell counts from plating.

Table 1 Characteristics and components of historically contaminated soil from wood processing site in Ontario, Canada[a]

NH_4-N	1.17 ppm
NO_3-N	3.17 ppm
% Total N	<0.01 ppm
Phosphorous (P)	4.0 ppm
Potassium (K)	25.8 ppm
Magnesium (Mg)	47.0 ppm
% Total carbon (C)	8.44 ppm
% Organic carbon(C)	2.45 ppm
pH	8.1
Initial soil moisture	13.4%
Sand	76.2%
Silt	21.3%
Clay	2.5%
Soil texture	Loamy sand
Pentachlorphenol (PCP)	350–370 ppm
(TPHs)[b]	20 900 ppm

[a]Soil analysis was performed by Grace Bioremediation Technologies, Mississauga, Ontario, Canada.
[b]Total Petroleum Hydrocarbons—includes 1224 ppm polycyclic aromatic hydrocarbons (PAHs).

Encapsulation procedure

The κ-carrageenan formulation and encapsulation procedure were described previously in Cassidy *et al* [6]. Briefly, 5×10^{10} CFU were encapsulated in 25 ml of a 1% (w/v) κ-carrageenan formulation amended with 5% (w/v) clay and 1% (w/v) skim milk powder at 30°C. The mixture was extruded through a sterile syringe with a 21-gauge needle into a cold (4°C) 0.3 M KCl solution to produce gel-beads of about 3 mm in diameter. Gel-beads were allowed to harden in 0.3 M sterile KCl solution for 30–45 min, and washed twice with sterile cold (4°C) distilled water.

For the experiment on repeated applications of encapsulated cells, approximately 2×10^{11} CFU of UG30 cells were encapsulated in 200 ml of a 1% (w/v) κ-carrageenan formulation amended with 5% (w/v) clay and 1% (w/v) skim milk powder at 30°C. The resulting encapsulated cells were dried for 24 h at 22°C inside a laminar flow cabinet. This resulted in approximately 13 g of dried beads. Sterile beads were similarly produced in the same quantity, although without inoculum addition. Dried beads were stored in sterile glass bottles at 4°C in the dark until used. At the beginning ($t = 0$), 0.6 g dry beads (inoculated beads contained 10^8 CFU g^{-1} dry soil) were applied to 18 of the 21 microcosms, distributed as evenly as possible over the soil surface. Three microcosms remained untreated (control) for the duration of the experiment. At day 7, an additional 0.6 g dried beads were applied to 15 of the 18 previously treated microcosms. At day 14, an additional 0.6 g dried beads were applied to 12 of the 15 microcosms receiving two applications. At day 21, an additional 0.6 g dried beads were applied to nine of the 12 microcosms receiving three applications. At day 28, an additional 0.6 g dried beads were applied to six of the nine microcosms receiving four applications. At day 35, an additional 0.6 g beads were applied to three of the six microcosms receiving five applications.

For the inoculum density experiment, 5×10^{10} CFU UG30 cells were serially diluted six times in water, and each dilution was encapsulated in 50 ml of a 1% (w/v) κ-carrageenan formulation amended with 5% (w/v) clay and 1% (w/v) skim milk powder at 30°C. One half of the resulting encapsulated cells were dried for 24 h at 22°C inside a laminar flow cabinet. These were designated as regular cell-encapsulated treatments. The other half were incubated for an additional 24 h in an MMG solution (containing twice the levels of potassium phosphate as found in the standard MMG solution) at 30°C, and dried for 24 h at 22°C inside a laminar flow cabinet. These were designated as enhanced cell growth treatments. The original cell dilutions were surface plated to confirm accuracy of initial counts, and 20 beads were disintegrated and plated prior to the experiment to determine the cell counts in each set of beads. To effect disintegration, beads were dissolved in 1 ml water in a 1.5-ml sterile Eppendorf tube and kept at 4°C for 1 h. Beads were then shaken on a Mini Beadbeater (Biospec Products, Bartlesville, OK, USA) for 5 min at high speed setting or until beads dissolved as determined by visual examination. Serial dilutions were made and 100 μl

plated on MMG plates. Cell counts were an average of three replications.

Microcosms

Soil microcosms used were 250-ml Erlenmeyer modified biometer flasks [13]. Soil, 25 g in dry weight, was added to each flask. Amendments were added as necessary by pipetting as evenly as possible over the entire soil surface. Approximately 100 000 dpm of [UL-^{14}C]-PCP was added by pipetting over the entire soil surface as the final amendment before cells or encapsulated cells were added. Flasks were aerated for 15 min bi-weekly. Evolved $^{14}CO_2$ was trapped by 5 ml of 1.0 N NaOH held in a 10-ml plastic scintillation vial which was placed in the centre glass well. The trap solution was replaced periodically and the radioactivity in the trap solution determined by liquid scintillation counting [13]. The flask was sealed with a rubber stopper and kept static in the dark at 22°C. All experiments included three replications of each treatment.

Statistical analyses

Three independent microcosms were set up for each treatment. Statistical analyses were performed using Sigma Stat (SPSS Inc, Chicago, IL, USA) on an IBM-compatible PC. Significant differences reported are at the 95% confidence level.

Results

Biostimulation of indigenous microorganisms

In the unamended control microcosms, 18.8 ± 3.9% of the added radiolabelled PCP was mineralized over 30 weeks at 22°C in the dark. Addition of 1000 ppm of C, P and K either alone, or in combination resulted in some increase in PCP mineralization (Figure 1). Addition of phosphorous alone and carbon with phosphorous resulted in almost 50% mineralization of PCP. Any of the amendments containing nitrogen inhibited mineralization, except for the nitrogen and phosphorous combination, and all of these treatments led to significantly lower PCP mineralization than the control after 30 weeks (usually resulting in no mineralization).

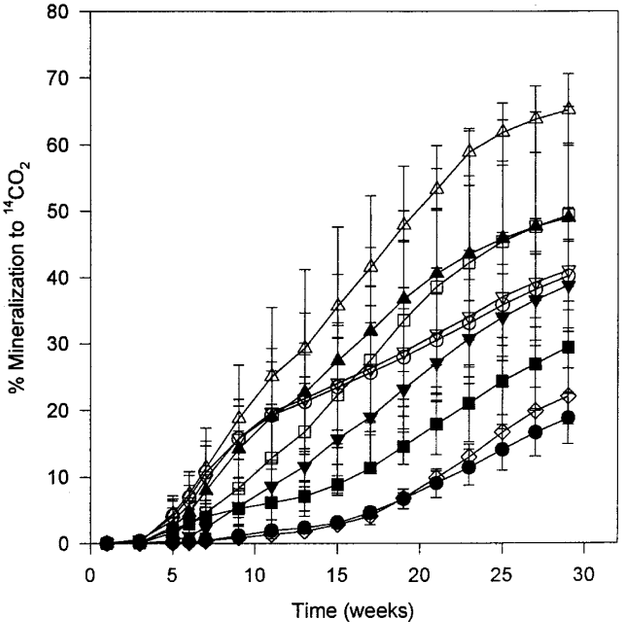

Figure 1 Mineralization of PCP in Ontario soil by indigenous microorganisms with fertilizer amendments (◇, C; □, P; ▽, K; ▲, CP; ○, CK; ■, PK; ▼, CPK; ●, control; △, NP; all others—N, CN, NK, CNK, CNPK, CNP were almost zero.) Each point is the average of three values from three independent microcosms. Each error bar represents ± 1 s.d.

Bioaugmentation with free and encapsulated cells with or without fertilizer

The addition of a liquid inoculum of PCP-degrading UG30 cells at 10^8 CFU g^{-1} dry soil did not result in significantly higher levels of PCP mineralization in any treatments. Overall, the addition of phosphorous alone or combined with C or K resulted in higher mineralization levels than the control (Figure 2). Treatments containing N inhibited mineralization, to the extent that PCP mineralization was significantly lower than the control when nitrogen was used either alone or in combination.

The addition of encapsulated UG30 cells at 10^8 CFU g^{-1} dry soil resulted in significantly higher levels of PCP mineralized (64.7 ± 0.3%) and faster rates of degradation than either the uninoculated treatments or the free-cell inoculated treatments over 30 weeks (Figure 3). Amendment with N inhibited mineralization completely. Without fertilizer, soil treatments receiving encapsulated cells mineralized PCP at similar rates and extents as compared to any of the treatments with fertilizer amendments.

To determine if the skim milk powder in the beads had a stimulatory effect, mineralization was compared between amendments with sterile beads, an equal amount of skim milk powder as found in the beads, and an equal amount of beads containing encapsulated cells. Treatments receiving

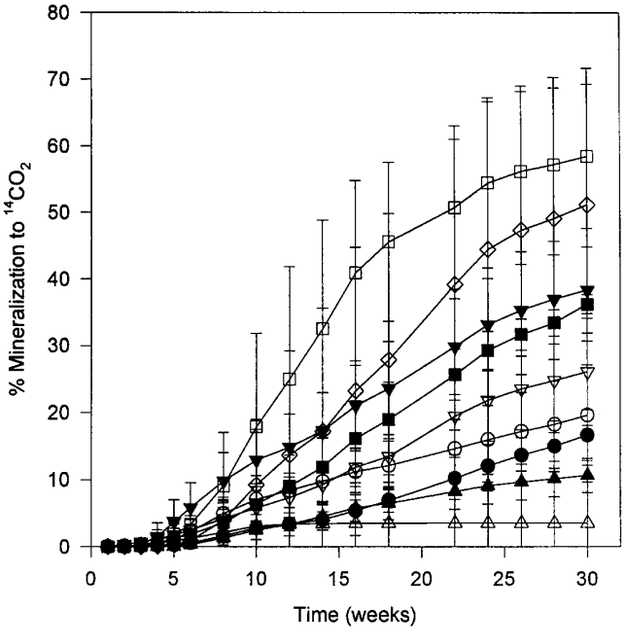

Figure 2 Mineralization of PCP in soil by free *Pseudomonas* sp UG30 cells (at 10^8 CFU g^{-1} dry soil) with fertilizer amendments (◇, C; □, P; ▽, K; ▲, CP; ○, CK; ■, PK; ▼, CPK; ●, control; △, NPK; all others—N, CN, NP, NK, CNK, CNPK, CNP were almost zero). Each point is the average of three values from three independent microcosms. Each error bar represents ± 1 s.d.

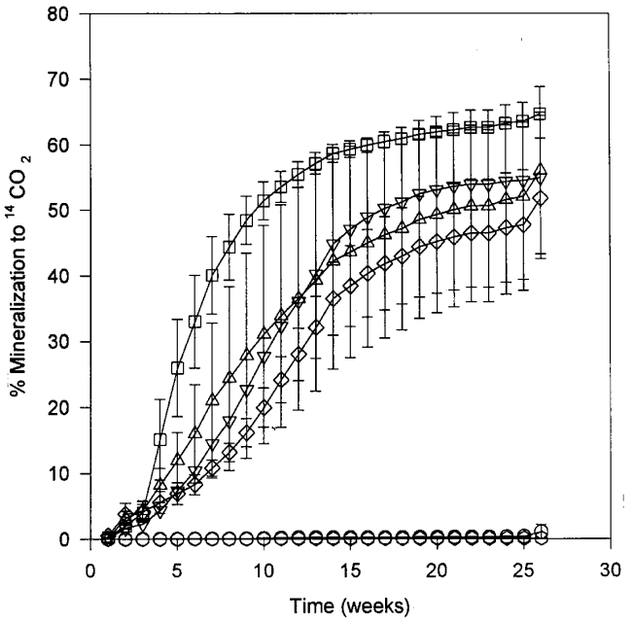

Figure 3 Mineralization of PCP in contaminated soil by κ-carrageenan-encapsulated *Pseudomonas* sp UG30 cells (at 10^8 CFU g^{-1} dry soil) with fertilizer amendments (○, control; □, beads with no fertilizer; △, beads + P; ▽, beads + K; ◇, beads + K + P; □, beads + N). Each point is the average of three values from three independent microcosms. Each error bar represents ± 1 s.d.

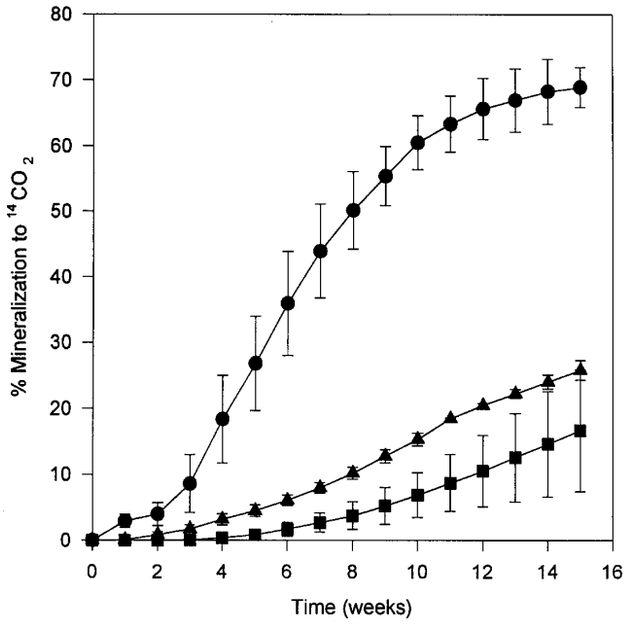

Figure 4 Mineralization of PCP in contaminated soil by single applications of sterile carrageenan beads, encapsulated cells, or skim milk powder (●, 0.6 g encapsulated cells; ■, 0.6 g sterile beads; ▲, skim milk powder). Each point is the average of three values from three independent microcosms. Each error bar represents ± 1 s.d.

encapsulated cells exhibited significantly higher levels of mineralization than the other treatments after 15 weeks (Figure 4).

Bioaugmentation with repeated applications of encapsulated cells

There was no significant difference in PCP mineralization using repeated applications of encapsulated UG30 cells in contaminated soil, from one through six applications (Figure 5) over 15 weeks. Each treatment mineralized 64.7 ± 1.9% of the radiolabelled PCP within 9 weeks. Faster onset of mineralization was observed using three or more applications of dried encapsulated cells. One application of 0.6 g sterile beads resulted in PCP mineralization of 16.6 ± 9.2%. However, no mineralization resulted from four or more applications of sterile beads. All sterile bead treatments exhibited significantly lower PCP mineralization than encapsulated cell treatments.

Bioaugmentation with varying density of encapsulated cells

After 20 weeks, regardless of inoculum density, there was no significant difference in PCP mineralization by encapsulated cells (Figure 6). When cell growth was allowed inside the bead prior to drying (enhanced cell growth treatment), cells mineralized PCP at a faster rate than treatments where cells were initially grown to a high level, added to beads and dried (regular cell encapsulation). In the enhanced treatments, there appeared to be a slight lag period of about a week as the inoculum density decreased. The regular

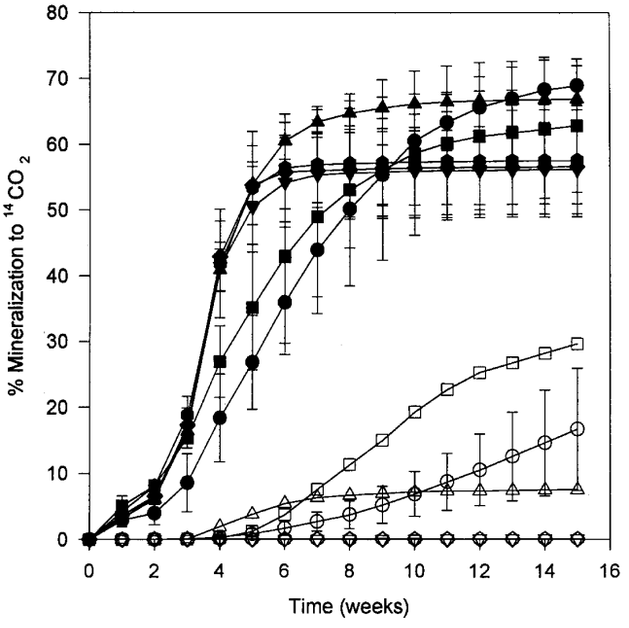

Figure 5 Mineralization of [^{14}UL]-PCP in soil by repeated applications of sterile κ-carrageenan beads or encapsulated *Pseudomonas* sp UG30 cells. Each application consisted of 0.6 g (dry wt) sterile beads or beads containing 10^{10} CFU UG30 cells. Repetitions were done as a series of weekly applications over 6 weeks. One application ($t = 0$) of sterile beads (○) or encapsulated cells (●); two applications ($t = 0$, wk 1) of sterile beads (□) or encapsulated cells (■); three applications ($t = 0$, wk 1, wk 2) of sterile beads (△) or encapsulated cells (▲); four applications ($t = 0$, wk 1, wk 2, wk 3) of sterile beads (▽) or encapsulated cells (▼); five applications ($t = 0$, wk 1, wk 2, wk 3, wk 4) of sterile beads (◇) or encapsulated cells (◆); six applications ($t = 0$, wk 1, wk 2, wk 3, wk 4, wk 5) of sterile beads (⬡) or encapsulated cells (⬢). Each point is the average of three values from three independent microcosms. Each error bar represents ± 1 s.d.

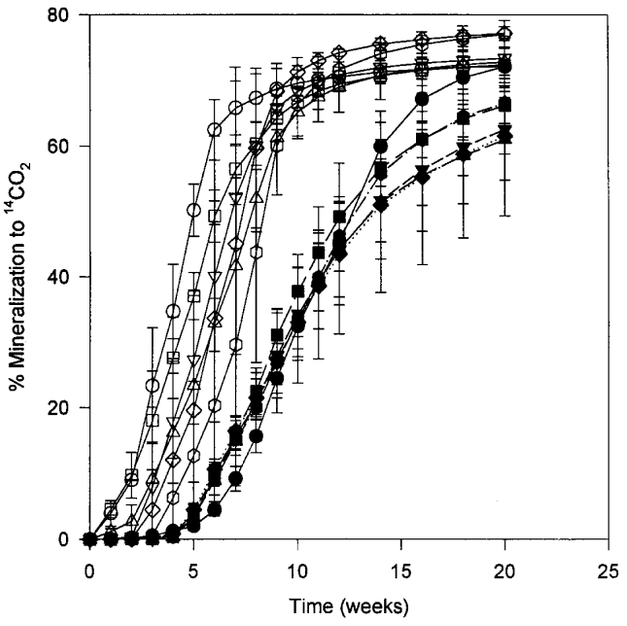

Figure 6 Mineralization of PCP in soil by encapsulated *Pseudomonas* sp UG30 cells at various cell densities, with cells either grown prior to encapsulation (regular encapsulated cells) or grown inside the bead after encapsulation (enhanced cell growth). (Units are CFU g^{-1} dry soil.) Enhanced: ○, 4.8×10^8; □, 2.4×10^6; △, 2.4×10^4; ▽, 2.4×10^2; ◇, 4.0×10; ⬡, 1.0. Regular: ●, 2.4×10^4; ■, 4.0×10^2; ▲, 4.0; ▼, 4.0×10^{-2}; ◆, 4.0×10^{-4}; ⬢, 4.0×10^{-6}). Each point is the average of three values from three independent microcosms. Each error bar represents ± 1 s.d.

treatments appeared to lag by about 4 weeks as compared to the enhanced treatments. However, all treatments eventually mineralized the [UL-^{14}C]-PCP with $^{14}CO_2$ recovery ranging from 62 to 77% after 20 weeks. Addition of enhanced encapsulated cells led to a faster rate of PCP mineralization than the regular encapsulated cells at the same inoculum density.

Discussion

There have been few studies on the use of encapsulated cells for bioremediation of historically contaminated soil. Our study is the first to our knowledge to demonstrate mineralization of PCP from a historically contaminated soil using bacterial cells encapsulated in κ-carrageenan.

Depending on soil characteristics and conditions, PCP may remain in the soil for months to years (Figure 1). Soil analysis indicated a deficiency of C, N, P and K (Table 1). Although PCP-degrading microorganisms have been isolated from this soil [11], their growth and degradative activity may be limited by this nutrient deficiency. Results from the addition of 1000 ppm P demonstrated that it was useful in stimulating PCP mineralization.

Addition of 1000 ppm nitrogen inhibited PCP mineralization in almost every experiment. The reason(s) for this effect is not known. The contaminated soil also contained PCP-tolerant cells unable to degrade PCP, some of which formed colonies on MMG plates in 2 days, *versus* 10 days for *Pseudomonas* sp UG30 colonies [11]. It is possible that these PCP-tolerant cells outcompeted any PCP degraders for nitrogen. Alternatively, it is possible that N at 1000 ppm was toxic to the cells.

Bioaugmentation may increase mineralization, but its effectiveness depends on the survival and degradative activity of the inoculated microorganisms in the soil [10]. In our experiment, the addition of 10^8 CFU g^{-1} dry soil UG30 free cells did not enhance mineralization, and often resulted in less mineralization than indigenous microorganisms with fertilizer amendments. This lack of activity is not uncommon for free cell inoculations into soil [1,8,13]. It has been suggested that there may be advantages in using encapsulated cells for *in situ* biodegradation [5], including a significant increase in bacterial survival [12,17,19,20] and degradative activity [2] in soil.

PCP-mineralizing UG30 cells isolated from this historically contaminated soil were returned encapsulated in κ-carrageenan. Significantly greater PCP mineralization rates and extents were observed with encapsulated cells compared with 10^8 CFU g^{-1} soil free cells. Encapsulated cells, not the beads themselves nor skim milk powder, were responsible for increased PCP mineralization activity (Figure 4). Cells encapsulated in this κ-carrageenan formulation with clay and skim milk amendments effectively mineralized PCP without fertilizer (Figure 3), although the addition of 1000 ppm P in each experiment allowed comparison of results from all treatments.

Although repeated applications of encapsulated cells and increased cell densities inside the beads increased PCP mineralization rates, the differences were not significant and over the course of the experiments the amounts of $^{14}CO_2$ recovered were similar. Ramadan *et al* [14] observed improved degradation of PNP in water with increased free cell inoculum density, but suggested it may not be feasible to inoculate large volumes with sufficient bacterial cells in natural environments. Our results suggest that the use of specific encapsulated cells may only require infusion of κ-carrageenan with a small number of bacterial cells and only one soil application, achieving mineralization effectiveness with lower costs.

For some contaminated soils, microbial cells encapsulated in a biodegradable carrier may have the potential to reduce remediation time and costs, while minimizing physical disruption of the site. The ability to effectively mineralize large amounts of soil pollutant(s) in a significantly shorter time period can be beneficial in temperate and cold climates where conditions for optimal microbial growth and activity may be limiting.

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