Absorption of Pentachlorophenol (PCP) by Bark Chips and Its Role in Microbial PCP Degradation

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Abstract. A pentachlorophenol (PCP)-degrading mixed bacterial population was enriched in a biofilter filled with soft wood bark chips. We found that bark chips were essential for the degradation to proceed at PCP concentrations higher than 10 μM. PCP-degrading bacteria were found to be extremely sensitive to PCP. Bark chips absorbed PCP reversibly, thus detoxifying the medium and allowing degradation to proceed at higher concentrations of PCP (beyond 200 μM).

Introduction

The worldwide industrial production of pentachlorophenol (PCP) is over 45,000 tons a year [5]. Roughly 80% of PCP is used in wood preservation. Other uses are as a herbicide, insecticide, algicide, and bactericide [9]. Intensive worldwide application and spills from industries using or manufacturing PCP have resulted in extensive pollution. Lake water, for example, has been found to contain micrograms of PCP per liter [12, 15], and sediments of freshwater lakes and of the Baltic Sea to contain several hundred micrograms to milligrams per kilogram of dry matter [1]. Up to tens of micrograms of PCP per kilogram of dry matter has been detected as a soil contaminant [2].

PCP is toxic to all forms of life. In a water ecosystem it has been found to bioaccumulate in the food chain [11]. Two hundred micrograms of PCP per liter is lethal to fish [13], and 10 μg/liter inhibits chlorophyll synthesis in algae [7]. The inhibitory concentration of PCP to bacteria varies, depending on the species, from a few milligrams to several grams per liter [9].

In nature, PCP is quite recalcitrant to microbial attack. In the laboratory, however, bacterial cultures capable of degrading PCP have been enriched [4, 6, 19, 20]. We studied a PCP-degrading bacterial culture enriched in a fixed film biocontactor [14]. Electron microscopic examination and results from experiments with antibacterial agents indicated that the organisms responsible for the degradation were bacteria (R. Valo, J. Apajalahti, and M. Salkinoja-Salonen, submitted for publication). We also found that the presence of uninoculated bark chips shortens the half-life of PCP from 10 months (liquid phase) to 3 months (bark chips added) and that the addition of a PCP-degrading inoculum further shortens the half-life to less than a week [16]. During further

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characterization studies of a PCP-degrading mixed population, it was found in liquid culture experiments that degradation of PCP was inhibited at low PCP concentrations unless bark chips were added [14]. In this paper we describe the mechanism by which bark chips enhance biodegradation of PCP.

Materials and Methods

Inoculation

As an inoculum we used bark chips from a PCP-degrading biofilter [14], a previous flask culture (a single culture was used to inoculate 6–10 new cultures), or a suspension prepared from the bark chip inoculum by violent shaking in a mineral salt medium. After settling of the coarse particles, the supernate was withdrawn and used as an inoculum.

Media

A mineral salts medium [18] supplemented with vitamin [18] and trace element [3] solutions (1.5 ml of each) was used.

Unlabeled PCP (Merck technical quality) was found to contain 0.07% of different isomers of tetrachlorophenol but no other chlorinated impurities [14]. Uniformly 14C-labeled PCP (25.6 mCi/g) was synthesized by M. Haag.

Experimental

The experiments were carried out in duplicate in 100 ml shake flasks containing 10 ml of mineral salts medium, an inoculum, substrate, and bark chips. Each flask was provided with a rubber stopper onto which was anchored a 2 ml cup containing 500 µl of 1 M NaOH to absorb CO2. The NaOH solution was collected daily, apportioned into 2 scintillation vials for the measurement of radioactivity, and replaced by a fresh NaOH solution. The flasks were incubated in a gyratory shaker (150 rpm) at 28°C in the dark.

Measurement of Radioactivity

Radioactivity was measured on a scintillation counter (LKB Rac Beta) for 10 min at 10°C using 10 volumes of Picofluor 30 scintillation fluid (Packard).

Results

Noninvolvement of Solid Phase Surface Effects

Fig. 1 shows evolution of 14CO2 from 14C-PCP in shake flasks containing solid inoculum (0.5 g, wet wt) and 0, 3, or 6 g of autoclaved bark chips. Control flasks contained 6 g of autoclaved bark chips but no inoculum. PCP was added in each flask to a final concentration of 200 µM. The figure shows that PCP was degraded in the flasks containing 3 or 6 g of bark chips, but not in the
Fig. 1. (left) Degradation of PCP in the presence and absence of bark chips. Each of 8 flasks contained 10 ml of mineral salts medium and PCP to a final concentration of 200 µM. Six (■, ■), 3 (●), or 0 (○) g of wet uninoculated bark chips were added into duplicate flasks. Inoculated bark chips (0.5 g) were added in all flasks but the 2 control flasks (▲). The degradation of PCP was monitored as evolution of 14CO2.

Fig. 2. (right) Degradation of PCP in the presence and absence of contact between the microbial inoculum and the solid phase. Six flasks contained 10 ml of mineral salts medium and PCP to a final concentration of 200 µM. Inoculated bark chips (0.5 g) were placed in flasks 1–4 inside a dialysis tubing (●) and in flasks 5 and 6 without tubing (■). Wet uninoculated bark chips (5 g) were added in flasks 1 and 2 (●) and 5 and 6 (■) at 0 hours and in flasks 3 and 4 (▲, indicated by an arrow) at 118 hours. PCP degradation was monitored as 14CO2 evolution.

control flasks nor in the flasks containing 0.5 g of bark chips (inoculum). The onset of PCP degradation was slower in the flasks containing 3 g of bark chips than in the flasks with 6 g of bark chips, but there was no difference in the rates of degradation.

Fig. 2 shows how degradation of 14C-PCP proceeded in flasks containing 0.5 g of bark chip inoculum and 5 g of bark chips. In flasks 1–4, the inoculum was inside a dialysis tubing to prevent the degraders from spreading from the inoculum onto the added bark chips. The figure shows that in the presence of bark chips degradation started immediately whether or not the inoculum was sealed inside the dialysis tubing. In the flasks with no solids but the inoculum (0.5 g, wet wt), no PCP was degraded until more bark chips were added (indicated with an arrow in Fig. 2). This experiment was repeated, with similar results, using suspended instead of solid inoculum in the dialysis tubing. These results show that although the degradation was promoted by addition of bark chips, no direct contact with the inoculum was required.

**Role of Bark Chip Solids as a Possible Cosubstrate for the PCP-degrading Bacteria**

To find out whether the bacteria degrading PCP utilize compounds diffusing from the bark chips, we extracted the chips in Soxhlet’s apparatus, first with water and then with diethyl ether, to remove soluble components. An experiment similar to that shown in Fig. 1 was then carried out with 2 flasks con-
taining bark chip inocula and 5 g of extracted bark chips and 2 similar flasks containing extracted bark chips and water extract prepared from them. Control flasks contained no solids but the inoculum. Fig. 3 shows that in the flasks with extracted bark chips, PCP (200 μM) was degraded similarly to the results shown in Fig. 1 and that addition of the extract did not significantly enhance degradation. In the control flasks (without added bark chips), PCP again was not degraded. Therefore the soluble components of bark chips were not responsible for the promotion of PCP degradation by bark chips.

The capability of the PCP-degrading bacteria to reproduce on bark chips in the absence of PCP was investigated by first incubating inocula in a mineral salt medium with bark chips and then measuring the capacity of the cultures to degrade PCP. Control flasks were not preincubated. If the PCP-degrading bacteria had reproduced on the bark chips, the capacity of the test cultures to degrade PCP would increase during preincubation. However, no difference in the rate of PCP degradation was observed between the test flasks and the control flasks (Fig. 4), indicating that the PCP-degrading organisms had not multiplied on the bark chips.

Effect of Bark Chips on the Concentration of Dissolved PCP

Since the solids did not seem to interact with the microorganisms, they might interact with PCP. To study absorption of 14C-PCP onto the bark chips, an
Fig. 5. Effect of bark chips on PCP concentration in a liquid phase. Nine flasks contained a mixture of $^{12}$C- and $^{14}$C-PCP (final concentration, 200 $\mu$M). At 18 hours, duplicate flasks were supplemented with 5 (○), 3 (■), or 1 (□) g of uninoculated bark chips in a dialysis tubing. An empty dialysis tubing was added in 3 control flasks (○) containing no solid phase. At a moment indicated by the arrows, liquid inoculum in a dialysis tubing was added to all but 2 control flasks (○). Samples were taken from the liquid phase of each flask and centrifuged. Radioactivity of the supernatant was measured and PCP concentration calculated.

Table 1. Degradation of different concentrations of PCP in the presence of 5, 0.5, or 0 g of bark chips

<table>
<thead>
<tr>
<th>Amount of bark chips (g)</th>
<th>Conc of PCP ($\mu$M)</th>
<th>500</th>
<th>200</th>
<th>20</th>
<th>10</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>n.t.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n.t.</td>
</tr>
<tr>
<td>0.5</td>
<td>n.t.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>n.t.</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

- = less than 5% of PCP degraded in 500 hours, + = more than 60% of PCP degraded in 500 hours, n.t. = not tested

experiment with flasks containing 5, 3, 1, or 0 g of autoclaved bark chips and 200 $\mu$M PCP was carried out. Samples were taken from the liquid phase, centrifuged to remove suspended solids, and measured for radioactivity. Fig. 5 shows that after incubation of 71 hours, radioactivity had become adsorbed from the liquid phase onto the solids, so the remaining label corresponded to concentrations of 17, 29, and 48 $\mu$M of PCP in the flasks containing, respectively, 5, 3, or 1 g of bark chips per 10 ml of medium. In the control flasks (no solids), the radioactivity recovered corresponded to a PCP concentration of 183 $\mu$M. Suspended inoculum, enclosed in a dialysis tubing, was then placed in all test flasks and 1 control flask. The cultures with 3 and 5 g of bark chips were observed to start degrading PCP (not included in the figure). This experiment shows that the more bark chips were added the more PCP was adsorbed from the supernate, leaving a lower concentration of PCP in the liquid phase (Fig. 5).

The next experiment was designed to find out whether degradation of a lower concentration of PCP would be possible in the absence of bark chips. Five g, 0.5 g, or no bark chips were added in a series of flasks. PCP was added in the
flasks to different (3–500 μM) final concentrations. Suspended inoculum was added, and degradation of PCP by each culture was assessed. The results (Table 1) show that in the flasks with 5 g of bark chips, PCP was degraded at concentrations of up to 200 μM, whereas with 0.5 g of bark chips degradation proceeded at concentrations of up to 20 μM. Without bark solids PCP was degraded at concentrations of up to 10 μM. The critical concentration of PCP that allows degradation in the absence of bark chip solids appeared to be <20 μM.

**Kinetics of PCP Degradation**

The kinetics of PCP degradation in the cultures was studied in the absence of bark chips. A series of flasks was supplied with an increasing concentration of PCP (0.12–23 μM). Liquid inocula from the preceding experiment (flasks with no solids) were added. The data obtained are shown in Table 2. The degradation of PCP followed conventional enzyme kinetics; the degradation rate increased with increasing substrate concentration up to 12 μM. At higher concentrations of PCP, degradation was inhibited. The PCP concentration at which half the maximum rate of degradation was achieved (apparent Kₘ constant) was 5.1 μM (calculated from the data shown in Table 2). The lag phase preceding the onset of degradation lasted longer at higher concentrations of PCP: 72 hours at 0.12 μM, 530 hours at 12 μM. The yield of ¹⁴CO₂ from ¹⁴C PCP was 60–70% in most cases but decreased at the lowest concentrations tested.

**Discussion**

We developed a mixed bacterial culture which utilizes PCP in the presence of soft wood bark chips [14]. In this study we showed that the presence of bark chip solids promotes PCP degradation at concentrations higher than 10 μM, but these solids or associated solutes are not utilized as substrates. We also found that at sufficiently low concentration of PCP, degradation proceeds in the absence of bark chips or other solids. The promotive role of bark chips was shown to result from absorption of PCP by the bark chips, which detoxified

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**Table 2.** Relative rates of degradation of PCP at different substrate concentrations in liquid phase cultures

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Conc of PCP (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>23</td>
</tr>
<tr>
<td>Vₘₐₓ (nmol/h)</td>
<td>0.0034</td>
</tr>
<tr>
<td>Lag phase (h)</td>
<td>72</td>
</tr>
<tr>
<td>Degradation-%</td>
<td>0.65</td>
</tr>
</tbody>
</table>

* a Nmols/h of PCP degraded per culture; the rate of spontaneous degradation (without inoculum) was subtracted; mean values for duplicate flasks given
  
* b Time to attain maximum rate of degradation
  
* c Percentage degraded in 760 hours
the surroundings of the PCP-degrading bacteria. The microorganisms responsible for the degradation propagated on PCP. This was indicated by the fact that a serial 6- to 10-fold dilution of biomass from each flask, when used as inoculum in a subsequent experiment, was able to support the same rate of PCP degradation.

The PCP-degrading culture described here is in fact unusually sensitive towards PCP. Degradation proceeded only at concentrations below 20 $\mu$M (5.3 mg/liter) of PCP in liquid phase. Izaki et al. [8] observed that most gram-positive bacterial species are sensitive to PCP, tolerating only 3–10 mg of PCP per liter, but the gram-negative bacteria were more resistant, usually tolerating 100–200 mg of PCP per liter. They found no correlation between bacterial resistance to and bacterial degradation of PCP. Lipopolysaccharide (LPS) probably shielded the bacteria towards PCP, since LPS-deficient mutants were more sensitive [8]. In our PCP-degrading mixed population, gram-negative bacteria seemed to dominate. PCP-degrading bacteria described earlier by other authors were gram-negative or gram-variable [4, 6, 10, 19, 20] and were reported to degrade PCP at the concentrations of 40–300 mg/liter. These bacteria were therefore both resistant to PCP and sufficiently permeable to transport PCP through the membrane into the cell.

In our cultures the presence of bark chips led to a shortened lag phase prior to the onset of degradation of PCP (Fig. 1). This may be due to the absorption of PCP by the bark chips, which lowered the concentration of PCP in the liquid phase. Once the degradation started, it proceeded at a rate proportional to substrate concentration (Table 2). The lag phase was probably not involved with de novo synthesis of enzymes for the PCP metabolism or other mechanisms of metabolic adaptation, since the inocula were grown with PCP as a sole added source of carbon. Toxicity of PCP may somehow be involved. Perhaps at the sublethal concentrations, PCP was first metabolized only very slowly. As the concentration of PCP gradually decreased, metabolism became faster. A similar phenomenon has been observed in the microbial degradation of phenol in soils [17] where the lag phase was observed to become longer when phenol concentration was increased from 10 $\mu$M to 10 mM.

Our PCP-degrading bacterial community was enriched in a biofilter containing 500 ml of liquid and 200 g of bark chips [14]. We repeatedly observed that freshly added PCP soon disappeared from the medium. We now know that most of the PCP was absorbed by bark chips (1 g, wet wt, of bark chips absorbed 1.5 $\mu$mol of PCP; Fig. 5), and hence PCP concentration in the liquid phase remained low. However, the percentage of PCP degraded was about the same whether or not bark chips were added. This shows that the absorption of PCP by the bark was reversible. Such conditions made it possible for the PCP-sensitive but efficiently PCP-degrading bacteria to evolve. Bacteria degrading a certain toxic compound are usually found among bacteria resistant to that compound. For example, the PCP-degrading bacteria so far described in the literature were enriched in solutions containing 40–200 mg of PCP per liter, which is much above the concentration tolerated by the culture described here. The sensitive degraders described in this paper would not survive under the conventional chemostat conditions used for studies of laboratory evolution.

We have described above a mechanism that protects organisms against toxic
effects of substances without preventing the organisms to degrade these compounds. Such mechanisms may play an important role in environmental self-purification. Materials similar to those used here as a solid and protecting phase are amply available in the environment, and many other materials besides bark may exert similar effects.

Acknowledgments. We thank Professor Dr. M. Fischer (Institut für Wasser-, Boden- und Lufthygiene des Bundesgesundheitsamtes, Berlin, FRG) for his gift of 14C-labeled pentachlorophenol and the Maj and Tor Nessling Foundation for financial support. M.S.-S. was supported by the Academy of Finland.

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