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Biodegradation of *p*-Nitrophenol in an Aqueous Waste Stream by Immobilized Bacteria

MICHAEL A. HEITKAMP,[1]* VALÉRIE CAMEL,[2] THOMAS J. REUTER,[1] AND WILLIAM J. ADAMS[1]

Environmental Sciences Center, Monsanto Company, St. Louis, Missouri 63167,[1] and Ecole Supérieure de Physique et Chimie Industrielle de Paris, Paris, France[2]

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Microbiological analyses of activated sludge reactors after repeated exposure to 100 mg of *p*-nitrophenol (PNP) per liter resulted in the isolation of three *Pseudomonas* species able to utilize PNP as a sole source of carbon and energy. Cell suspensions of the three *Pseudomonas* sp., designated PNP1, PNP2, and PNP3, mineralized 70, 60, and 45% of a 70-mg/liter dose of PNP in 24, 48, and 96 h, respectively. Mass-balance analyses of PNP residues for all three cultures showed that undegraded PNP was <1% (<50 μg); volatile metabolites, <1%; cell residues, 8.4 to 14.9%; and water-soluble metabolites, 1.2 to 6.7%. A mixed culture of all three PNP-degrading *Pseudomonas* sp. was immobilized by adsorption onto diatomaceous earth biocarrier in a 1.75-liter Plexiglas column. The column was aerated and exposed to a synthetic waste stream containing 629 to 2,513 mg of PNP per liter at flow rates of 2 to 15 ml/min. Chemical loading studies showed that the threshold concentration for acute toxicity of PNP to the immobilized bacteria was 2,100 to 2,500 mg/liter. Further studies at PNP concentrations of 1,200 to 1,800 mg/liter showed that >99 and 91 to 99% removal of PNP was achieved by immobilized bacteria at flow rates of 10 and 12 ml/min, respectively. These values represent hydraulic retention times of 48 to 58 min and PNP removal rates of 0.99 to 1.1 mg/h per g of biocarrier at 25°C under optimal conditions. This study shows the successful use of immobilized bacteria technology to remove high concentrations of PNP from aqueous waste streams.

p-Nitrophenol (PNP) is a commodity chemical commonly used in the manufacture of pesticides and pharmaceuticals. In addition, PNP is the major metabolite resulting from the microbial degradation of parathion (11) and is a U.S. Environmental Protection Agency priority pollutant (8). Microbial degradation is primarily responsible for the removal of PNP from the environment. Several studies have reported that natural bacteria readily degrade PNP in soil (10, 23, 24), sediment (14, 18–20, 25), activated sludge (2, 3), water (7, 13, 21, 25), and groundwater (1). In most of these studies, lag phases ranging from 2 to 42 days were followed by periods of faster PNP degradation. Since a lag phase was usually not observed on subsequent exposures (19, 20, 24), it is believed to represent a period of selection or adaptation of PNP-degrading bacteria (1, 20, 24, 26). Several studies have also examined the degradation of PNP at low concentrations (17, 26), in the presence of inorganic nutrients (16, 24, 26), and by bacteria in granular activated-carbon columns (22). However, there are a lack of reports on the use of bacteria for the degradation of PNP at high concentrations in liquid wastes.

There is growing interest in the use of immobilized bacteria technology (IBT) for the cost-effective biological treatment of chemical wastes. IBT utilizes highly selected, chemical-degrading bacteria in bioreactors designed to provide optimal conditions for microbial activity. The immobilization of high cellular densities of chemical-degrading bacteria in an optimal environment offers the potential for chemical degradation rates which are much higher than those occurring in conventional waste treatment systems. However, the successful use of IBT depends on the availability of active and stable chemical-degrading bacteria. In addition, knowledge of the physical, chemical, and nutritional requirements of the bacteria is essential for maintaining high microbial activities. In this study, we report the isolation and identification of three *Pseudomonas* sp. strains able to degrade PNP as a sole source of carbon and energy. In addition, we compared the performances of these three *Pseudomonas* sp. strains by determining the rate of PNP mineralization for each strain and the total mass-balance of PNP residues in the cultures. Finally, we used the bacteria in laboratory-scale immobilized bacteria columns to test the feasibility of IBT for the removal of high concentrations of PNP from a synthetic aqueous waste stream.

MATERIALS AND METHODS

Chemicals. Radiolabeled 4-nitrophenol (uniformly ringed labeled) was purchased from Sigma Chemical Co., St. Louis, Mo. The specific activity was 6.9 mCi/mmol, and radiochemical purity exceeded 99% as indicated by high-pressure liquid chromatography analysis. Nonradiolabeled PNP was obtained from the Monsanto Co. PNP production facility at Aniston, Ala., and chemical purity exceeded 99% as determined by high-pressure liquid chromatography analyses. The synthetic waste stream for the immobilized bacteria columns consisted of 25% inorganic mineral salts (L-salts [9]) containing PNP concentrations ranging from 100 to 2,200 mg/liter. The pH of the synthetic waste stream was adjusted to 7.5 to 7.8. Bacterial growth media were purchased from Difco Laboratories, Detroit, Mich. Inorganic reagents and organic solvents were purchased from Burdick and Jackson, Muskegon, Mich., and were of the highest available purity.

Isolation of PNP-degrading bacteria. Municipal sludge was collected from the American Bottoms sewage treatment plant located in Sauget, Ill. Enrichment cultures for PNP degradation were established in the laboratory by adding a 1-liter aliquot of the municipal sludge to a continuously aerated, glass, 1.5-liter, semicontinuous activated sludge vessel (12). The municipal sludge was maintained at pH 7.5 to 7.8 and incubated with 100 mg of PNP per liter. The

* Corresponding author.

concentration of PNP in the unit was determined twice weekly by measuring optical A_{414} after adjustment of samples to pH >8.0. Authentic PNP standards showed linear detection of PNP at concentrations ranging from 0.2 to 50 mg/liter with this method. Once the removal of three successive doses of 100 mg of PNP per liter was achieved, 1-ml mixed-liquor samples were removed, serially diluted, and plated onto agar plates containing L-salts and 100 mg of PNP per liter. The agar plates were incubated at 30°C in the dark and examined daily. Several small bacterial colonies with similar morphology were present after 3 days, and complete loss of yellow color was observed on the plates. A total of eight colonies from the agar plates were streaked as pure cultures onto fresh L-salts agar plates containing 100 mg of PNP per liter.

Microbial isolates were examined with an Axioskop light microscope (Zeiss, Federal Republic of Germany) for cellular morphology and Gram stain reactions. The isolates were further characterized and identified with a VITEK AMS microbial identification system (McDonnell Douglas Co., St. Louis, Mo.). Strains which reproducibly showed identical cellular morphology, Gram staining reactions, and reaction patterns on the VITEK system were assumed to be identical and were counted as a single isolate. The identity of the *Pseudomonas* strains was confirmed by separate analyses, using the Biolog Identification System (Biolog Inc., Hayward, Calif.). These analyses showed that the PNP-degrading bacteria isolated in this study consisted of three distinct *Pseudomonas* strains. Permanent cultures of each isolate were prepared by both lyophilization and ultra-low-temperature storage.

Preparation of bacterial cultures. Cultures were streaked onto L-salts agar plates containing 200 mg of PNP per liter and incubated for 48 h at 30°C to provide a sufficient number of chemical-induced bacteria for initiating mineralization studies or inoculation onto immobilized bacteria columns. The PNP-degrading *Pseudomonas* strains were then inoculated into L-salts broth containing 100 mg of PNP, 100 mg of yeast extract, 1,000 mg of pyruvate, and 1,000 mg of dextrose per liter and grown to high cellular densities overnight at 30°C. The purity of the cultures was checked by light microscopy after Gram staining and by replating onto L-salts agar containing 100 mg of PNP per liter. The cells were harvested by centrifugation at 6,000 × *g* for 10 min. The supernatants were discarded and the cells were suspended in sterile L-salts broth containing 100 mg of PNP per liter. The cells were incubated at 30°C until the yellow color from the PNP was cleared from the flasks.

Bacterial cell densities throughout this study were monitored by measuring optical density at 540 nm. Plating studies showed a relationship of 5.2×10^8 cells for an optical density at 540 nm of 0.1 for these *Pseudomonas* strains. This relationship was linear for optical densities at 540 nm ranging from 0.05 to 0.30. The cellular density was determined for each culture by measuring optical density at 540 nm, and the cells were harvested as described above. The cells were suspended at a known concentration in an amount of L-salts broth which was 10% of their original culture volume. Aliquots of these cell suspensions were then used in mineralization studies or were inoculated onto the biocarrier in the immobilized bacteria column.

Mineralization studies. The rate and extent of PNP degradation were examined in pure cultures for each of the three *Pseudomonas* strains. Triplicate 125-ml flasks containing 50 ml of L-salts containing 3.5 mg (70 ppm) of PNP and 0.92 μCi of [^{14}C]PNP were inoculated with about 1.4×10^8 cells of bacteria per ml. The flasks were mixed with stir bars, purged continuously with air, and incubated at 25°C in the dark. Controls lacking bacteria were included to detect abiotic degradation or volatilization of PNP. The concentration of PNP in the flasks was measured as described above.

The gaseous effluents from each flask were passed through 200 mg of Tenax-GC resin (Alltech Associates Inc., Deerfield, Ill.) to remove any volatilized PNP or metabolites and then passed through 5 ml of an ethanolamine-based CO_2-trapping solution. The selectivity and efficiency of Tenax-GC and ethanolamine trapping solutions for volatile organics and CO_2 have been reported previously (6). The CO_2-trapping solutions were exchanged at sample times during incubations ranging from 3 to 7 days. The total radioactivity in the $^{14}CO_2$-trapping solutions was counted in a scintillation vial containing 15 ml of Instagel (Packard Instrument Co. Inc., Downers Grove, Ill.) scintillation cocktail. All radioactive values were counted on a model LS 6800 liquid scintillation counter (Beckman Instrument Co., Fullerton, Calif.) and were corrected for counting efficiency, quench, and background. Radioactive residues in the TENAX-GC were measured at the end of each experiment by flushing each TENAX-GC column with 5 ml of acetone and counting as described above. The total cumulative $^{14}CO_2$ evolved from each flask was compared with ^{14}C-labeled residues evolved from sterile controls.

Radioactive cell-associated residues and water-soluble residues were determined by passing 1-ml aliquots of the cell suspensions through a 0.45-μm Acrodisc 25 filter (Gelman Sciences, Ann Arbor, Mich.). Radioactivity passing through the filter was reported as water-soluble radioactive residue, and radioactivity retained on the filter was reported as cell-associated radioactive residue.

Construction and inoculation of the immobilized bacteria column. The column consisted of a 24-in. (61-cm)-long piece of 3.25-in. (8.25-cm) inside diameter by 0.25-in. (0.635-cm) wall Plexiglas tubing sealed with a rubber gasket and screw clamps onto a Plexiglas collar which was slipped around a Corning 350-ml Büchner funnel containing a glass frit of medium porosity. A schematic of an immobilized bacteria column is shown in Fig. 1. The glass frit served as the lower support for the packing and enabled continuous aeration of the column. Compressed air was passed through the Büchner funnel into the bottom of the column at a flow rate of 750 ml/min. This aeration rate maintained an oxygen-saturated headspace on the column throughout the entire study.

A single, 0.25-in.-inside diameter, stainless-steel waste inflow port discharged the synthetic waste stream into the center of the column about 1 in. (2.54 cm) above the glass frit. A second hole located 14 in. (35.5 cm) above the inflow port contained a 0.375-in. stainless-steel waste outflow port. The column was packed with 12 in. (30.48 cm) (829 g [dry weight], 1.6-liter volume) of R-635 diatomaceous earth biocarrier (Manville Co., Denver, Colo.) as support for the immobilized bacteria. The synthetic waste stream was pumped onto the column with an FMI model RP-G150 pump (Fluid Metering, Inc., Oyster Bay, N.Y.) at flow rates ranging from 1 to 15 ml/min. The interstitial fluid volume in the immobilized bacteria column was 580 ml.

The immobilized bacteria column was inoculated with a mixture of all three PNP-degrading *Pseudomonas* strains. Induced cultures were grown as described above, suspended in 1 liter of sterile L-salts medium containing 100 mg of PNP per liter, and recycled through the immobilized bacteria column for 48 h at a flow rate of 10 ml/min, which resulted in a 58-min hydraulic retention time (HRT). The synthetic

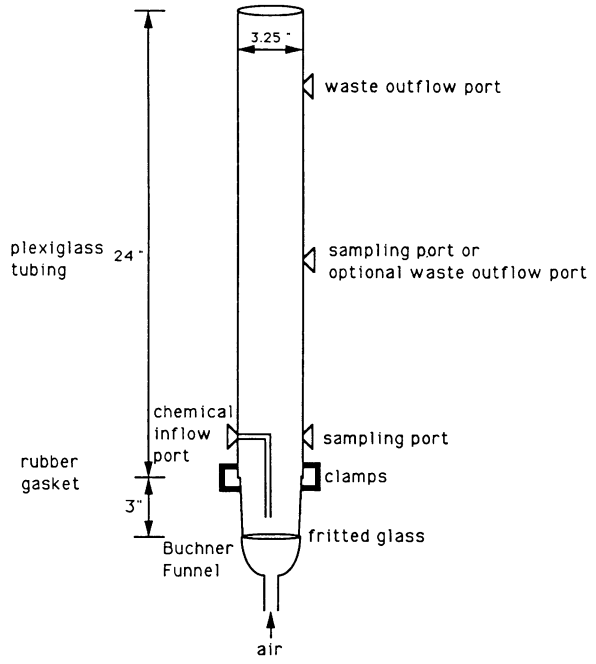

FIG. 1. Schematic of the immobilized bacteria column used in this study.

waste stream containing PNP was pumped continuously through the IBT column throughout the remainder of the study.

Chemical loading studies. The degradation of PNP by the immobilized bacteria was monitored while the chemical loading of PNP onto the column was increased. The total chemical loading of PNP for the immobilized bacteria, expressed as milligrams per hour, was calculated according to the following equation: chemical loading (mg/h) = {PNP (mg/liter) × [flow (ml/min) × 60 min/h]}/1,000 ml/liter.

In the first phase, the flow rate for the waste stream through the column was held constant at 5 ml/min and the concentration of PNP was increased until the immobilized bacteria failed to degrade PNP. It was assumed that this failure resulted from acute toxicity of PNP to the immobilized bacteria. In the second phase, the concentration of PNP was held relatively constant at a concentration which was about 25% below that which produced acute toxicity. At this point, the total chemical loading of PNP to the immobilized bacteria column was boosted by increasing the flow rate of the waste stream through the column.

Chemical analyses. All aqueous samples and standards were filtered through 0.45-μm Acrodisc 25 filters (Gelman Sciences) prior to analysis. A 9095 Autosampler (Varian Instrument Division, Walnut Creek, Calif.), using a Rheodyne 7126S sample injection valve (Rheodyne Inc., Cotati, Calif.) equipped with a 100-μl sample loop, was used to inject samples onto a Varian 9010 liquid chromatograph. The 100-μl sample loop was routinely overfilled with 400 μl of sample. A Chromegabond C22 column (4.6 mm by 25 cm; ES Industries, Marlton, N.J.) was eluted with a solvent gradient program of 1-min isocratic at 38:62 (vol/vol) acetonitrile-water followed by a 10-min linear gradient to 100% acetonitrile with a constant 0.1% (by volume) acetic acid throughout the gradient program. Standards were injected before, during, and after each sample set, and duplicate injections were made for all samples. Detection was accomplished by a Varian 9050 UV detector operating at 310 nm.

RESULTS

Identification of bacteria. Microbiological examination of the eight isolates showed that all were small gram-negative rods having a thickness of 0.5 to 1 μm and length of 2 to 4 μm. All isolates were catalase positive. The results from the analysis of fresh cultures of the isolates on a VITEK AMS showed a >98% probability for *Pseudomonas fluorescens* or *P. putida* or *P. mendocina* for all eight isolates. The strains which showed identical reaction patterns on the VITEK and Biolog systems were assumed to be identical and were counted as a single isolate. These analyses showed three distinct PNP-degrading *Pseudomonas* strains which were designated PNP1, PNP2, and PNP3. *Pseudomonas* sp. strain PNP1 differed from the other strains in that it was able to produce acid from the aerobic oxidation of maltose. *Pseudomonas* sp. strain PNP3 differed from the other strains in that it was able to ferment glucose in the presence of the inhibitor 2,4,4′-trichloro-2′-hydroxy-diphenylether. Repeated analyses of the strains at different times showed that the differences between the strains were reproducible.

Degradation of PNP by three *Pseudomonas* strains. The rate and extent of PNP degradation were determined for *Pseudomonas* strains PNP1, PNP2, and PNP3. Figure 2 shows the disappearance of PNP and evolution of CO_2 by *Pseudomonas* strain PNP1. Although this culture was grown in the constant presence of PNP prior to this experiment, a lag phase for PNP degradation of about 8 h was observed after PNP exposure. However, PNP was rapidly degraded after 8 h to the limits of detection (0.2 mg/liter) by hour 20. The evolution of $^{14}CO_2$ totaled about 4.6% at 12 h and rapidly increased to >66% by hour 24. The total PNP mineralized by *Pseudomonas* strain PNP1 did not significantly increase after the first 24 h of incubation. The cell density of *Pseudomonas* strain PNP1 increased from 1.51×10^8 cells/ml at the beginning of the experiment to 2.34×10^8/ml after 72 h of incubation. The total mass balance of ^{14}C-labeled residues after 72 h showed that undegraded PNP was <50 μg/liter, mineralization totaled 70%, volatile metabolites were <1%, radioactive cell residues totaled 8.4 to 11.8%, and water-soluble metabolites totaled 3.7 to 6.7%. Chemical analysis of the water-soluble metabolites showed the presence of unidentified, highly polar residues which eluted in the void volume of the reversed-phase high-pressure liquid chromatography system. No abiotic degradation of PNP or evolution of $^{14}CO_2$ was detected in sterile controls.

Similar to *Pseudomonas* strain PNP1, an initial lag phase in PNP degradation by *Pseudomonas* strain PNP2 was followed by a period of rapid PNP degradation (Fig. 3). However, the lag phase for *Pseudomonas* strain PNP2 lasted about 12 h and PNP concentrations did not drop to below 0.2 mg/liter until after 48 h of incubation. The evolution of $^{14}CO_2$ totaled about 60% after 48 h and did not increase significantly during the final 24 h of incubation. The cell density of *Pseudomonas* strain PNP2 increased from 1.40×10^8 cells/ml at the beginning of the experiment to 2.03×10^8/ml after 72 h of incubation. The total mass balance of ^{14}C-labeled residues after 72 h showed that undegraded PNP was <50 μg/liter, mineralization totaled 60%, volatile metabolites were <1%, radioactive cell residues totaled 10.7 to 12.3%, and water-soluble metabolites totaled 3.3 to 3.9%. No abiotic degradation of PNP or evolution of $^{14}CO_2$ was detected in sterile controls.

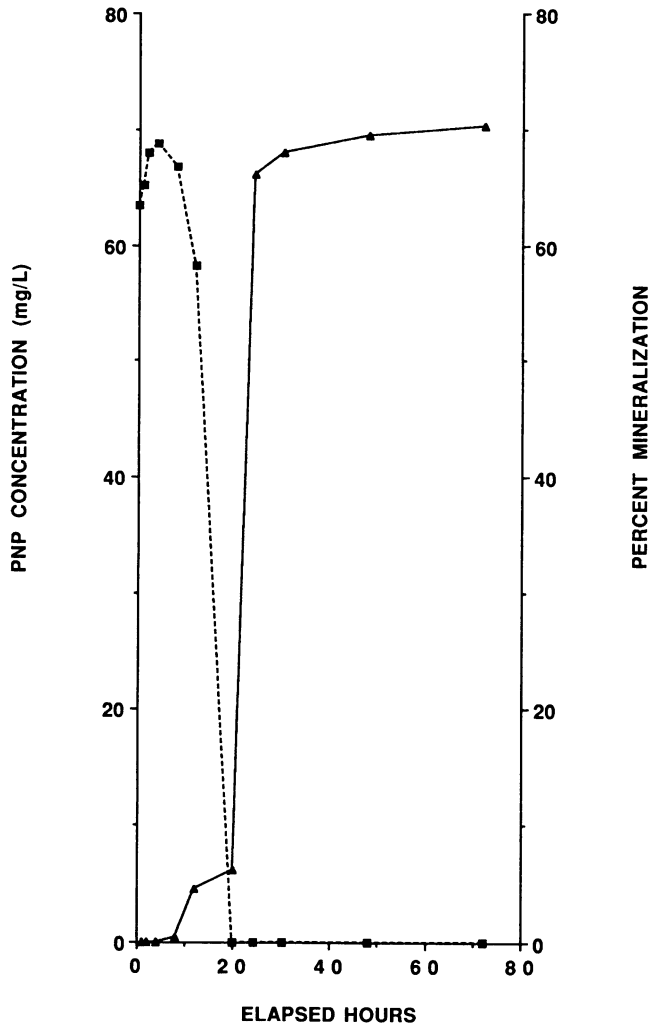

FIG. 2. Disappearance of PNP (■) and evolution of carbon dioxide (▲) resulting from the mineralization of PNP by *Pseudomonas* sp. strain PNP1.

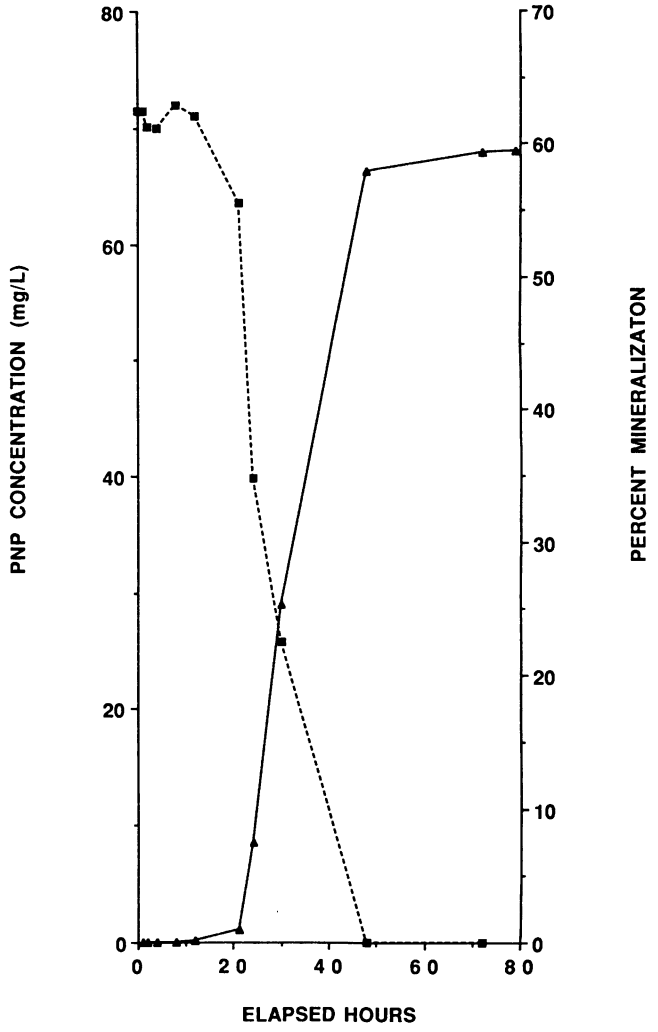

FIG. 3. Disappearance of PNP (■) and evolution of carbon dioxide (▲) resulting from the mineralization of PNP by *Pseudomonas* sp. strain PNP2.

A lag phase in PNP degradation by *Pseudomonas* strain PNP3 of about 4 h was observed in these cultures (Fig. 4). In addition, the rate of PNP degradation was much slower than for the other two PNP-degrading *Pseudomonas* strains since 98 h were required for the removal of PNP to the limits of detection. The total mineralization of PNP only totaled 41.3% after 96 h and only increased to 42.4% mineralized by hour 166. The cell density of *Pseudomonas* strain PNP3 increased from 1.09×10^8 cells/ml at the beginning of the experiment to 1.72×10^8/ml after 166 h incubation. The total mass balance of ^{14}C-labeled residues after 72 h showed that undegraded PNP was <50 μg/liter, mineralization totaled 42.4%, volatile metabolites were <1%, radioactive cell residues totaled 14.3 to 14.9%, and water-soluble metabolites totaled 1.2 to 1.3%. No abiotic degradation of PNP or evolution of $^{14}CO_2$ was detected in sterile controls.

Degradation of PNP by immobilized bacteria. Figure 5 shows the removal of PNP from a synthetic waste stream by immobilized bacteria. The chemical loading of PNP (milligrams per hour) to the IBT reactor was increased first by adjusting chemical concentration and then by increasing liquid flow rate. Initially, a constant flow of 5 ml/min (HRT, 116 min) was maintained through the IBT reactor and the concentration of PNP was increased from 629 up to >2,500 mg/liter. The removal of PNP dropped briefly to 3.6% early in the study when the concentration of PNP in the synthetic waste stream was increased from 628 to 850 mg/liter on day 5. The removal of PNP by immobilized bacteria remained ≥96% on days 6 to 21 as the concentration of PNP was increased from 850 (255 mg/h) up to 2,095 (628 mg/h) mg/liter. However, PNP removal dropped to 89 to 94% as the PNP concentration was increased to 2,121 to 2,202 mg/liter (636 to 660 mg/h) on days 22 to 23 and dropped suddenly to 6% removal on day 27 when the concentration of PNP was increased to 2,513 mg/liter (753 mg/h). Visual examination of the IBT column showed no detectable changes in the amount or appearance of microbial biomass. The sudden failure of the immobilized bacteria to degrade PNP at this concentration was attributed to acute toxicity of the PNP to the immobilized bacteria. The flow rate was decreased to 2 ml/min (HRT, 290 min), and the concentration was lowered to 1,761 to 1,930 mg/liter (PNP loading, 211 to 340 mg/h) on days 28 to 33 to allow the IBT column to recover PNP-degrading activity. The removal of PNP re-

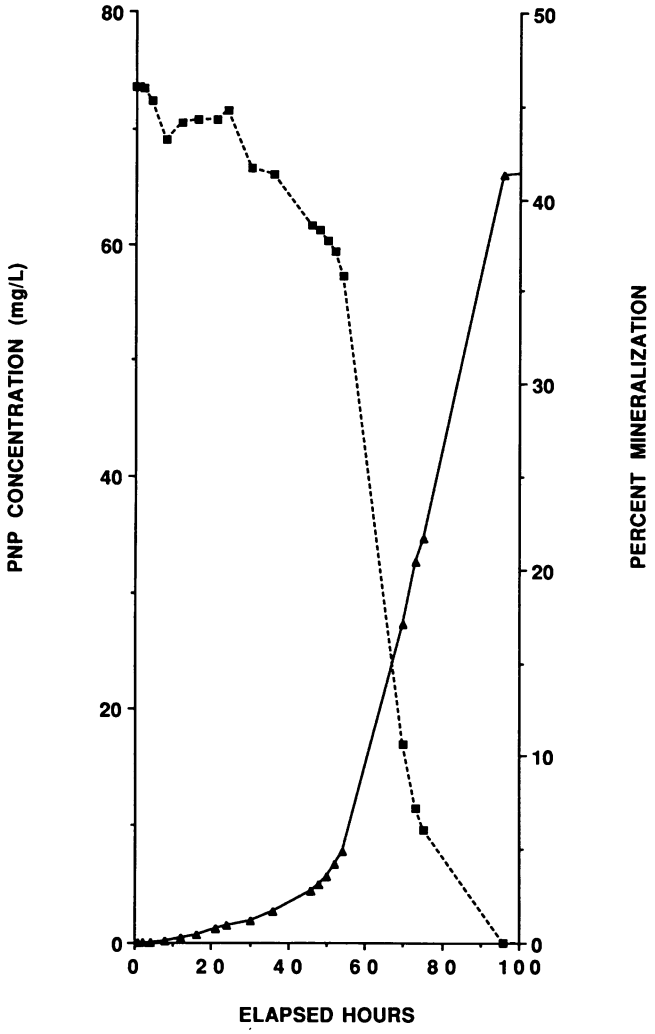

FIG. 4. Disappearance of PNP (■) and evolution of carbon dioxide (▲) resulting from the mineralization of PNP by *Pseudomonas* sp. strain PNP3.

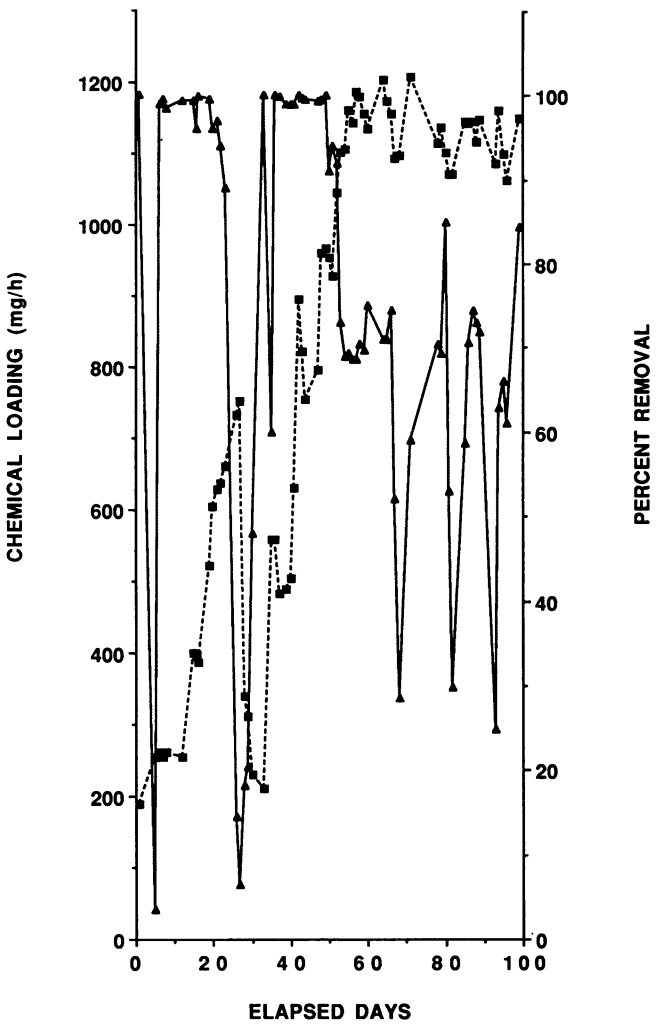

FIG. 5. Degradation of PNP in a synthetic waste stream by immobilized bacteria. The data are presented as percent removal of PNP (▲) at various levels of chemical loading (■).

turned to >99% on day 31. Since these data suggest that the threshold for acute toxicity of PNP to immobilized bacteria occurs at 2,100 to 2,500 mg/liter, the concentration of PNP in the synthetic waste stream was maintained at 1,200 to 1,800 mg/liter throughout the duration of the study and chemical loading of PNP (milligrams per hour) was increased by increasing flow rate of the synthetic waste stream through the IBT column.

The flow rate for the synthetic waste stream was returned to 5 ml/min (HRT, 116 min; PNP loading, 483 to 661 mg/h) on day 34 and PNP removal dropped to 60% for 1 day, but quickly recovered to >99% removal for days 35 to 40. The flow rate through the IBT column was increased to 10 ml/min on day 41 (HRT, 58 min; PNP loading, 754 to 896 mg/h) and PNP removal remained >99% for days 40 to 44. This 5-day interval represents 124 HRTs on the IBT column in which >99% removal of PNP was maintained by the immobilized bacteria. The flow rate for the synthetic waste stream was increased to 12 ml/min (HRT, 48.3 min; PNP loading, 928 to 960 mg/h) on day 47, and 91 to 99% removal of PNP was maintained on the IBT column for 4 days (119 HRTs). This PNP loading of 928 to 960 mg/h exceeded the previous PNP loading level of 753 mg/h which was acutely toxic to the immobilized bacteria due to the high concentration of PNP (2,100 to 2,500 mg/liter).

Although some low levels of PNP were passing through the IBT column at this point, the total chemical loading of PNP was further increased by raising the flow to 15 ml/min (HRT, 38.7 min; PNP loading, 1,045 to 1,209 mg/h) on day 52 to achieve consistent breakthrough of PNP. The continued presence of significant levels of PNP passing through the IBT column indicates that the full capacity of the immobilized bacteria for PNP degradation had been achieved. The removal of PNP by the immobilized bacteria during high chemical loading ranged from 68 to 75% over a 14-day period from days 52 to 65. This period resulted in 521 HRTs through the IBT column. However, a pattern of periodic failure in PNP degradation by the immobilized bacteria was observed during days 66 to 99 as the high rate of PNP loading was maintained. The removal of PNP dropped to 28.5% on day 67, but recovered on its own to 70.4% removal within 2 days. Similar patterns of losses in PNP degradation to about 30% removal followed by recovery within 48 h to 60 to 70% removal were observed on days 81 and 89. Visual examina-

tion of the IBT column during these periodic upsets showed no detectable changes in the amount or appearance of microbial biomass. In addition, no differences were observed in fluid movement, pumping rate, or aeration patterns on the IBT column during these periodic times of failure. The experiment was stopped on day 99, and plating studies showed that the microbial population on the IBT column consisted primarily of PNP-degrading *Pseudomonas* species.

DISCUSSION

This study shows that PNP was readily degraded by bacteria in activated sludge collected from a municipal sewage treatment plant after an acclimation period of 2 to 3 days. The relatively rapid adaptation of PNP-degrading activity by the bacteria in this study was not unexpected since the microbial degradation of PNP has been reported previously to occur after lag phases of 7 days in soil (24), 6 to 12 days in sewage (26), 40 h to 2 weeks in sediment-water ecocores (18, 19), and 6 days in a pond (21). Although the microbial degradation of PNP in the environment has been well documented, there is a lack of reports for the microbial degradation of PNP at high concentrations (>100 mg/liter).

Microbiological analyses of activated sludge in this study detected the presence of three *Pseudomonas* strains able to utilize PNP as a sole source of carbon and energy. The apparent stability of PNP-degrading activity shown by the *Pseudomonas* stains in this study is a desirable trait for the use of these bacteria for the long-term biotreatment of PNP. Furthermore, these results suggest that these *Pseudomonas* strains may be cultured to high cellular densities in rich medium to obtain sufficient cell mass for the inoculation of a large-scale biotreatment system and still retain their ability to degrade PNP.

The mineralization studies comparing the rate and extent of PNP degradation among pure cultures of the three PNP-degrading *Pseudomonas* sp. strains clearly indicated that *Pseudomonas* strain PNP1 performed better than the other two strains. In addition, the degradation of PNP by *Pseudomonas* strain PNP3 was significantly slower than that observed for *Pseudomonas* strain PNP2. It is noteworthy that the levels of radioactive cell residues for the three *Pseudomonas* strains were inversely related to the rate of PNP mineralization. For example, *Pseudomonas* strain PNP3 showed the slowest rate of PNP degradation and the highest levels of radioactive cell residues. The slower growth and lower PNP degradation observed for *Pseudomonas* strain PNP3 may have been a result of growth medium which was not optimal for strain PNP3. Alternatively, this relationship may indicate that *Pseudomonas* strain PNP3 only partially degraded PNP or had a higher rate of incorporation of PNP into cellular biomass. Although the reasons for these differences are not known, they could be related to differences in either the expression or the activity of PNP-degrading catabolic enzymes or to differences in the occurrence and accumulation of degradation intermediates. Catechol is believed to be an oxidation product occurring in the chemical pathway for the microbial degradation of PNP (15). Catechols are known to be common substrates for ring-opening enzymes and subsequent degradation to carbon dioxide by microbial catabolic enzymes (5). However, the occurrence of catechol, identity and expression of catabolic enzymes, and the chemical pathway for the degradation of PNP by the *Pseudomonas* species in this study are unknown and warrant further investigation. In all cases, the complete degradation of PNP to carbon dioxide with low levels of residues remaining in the aqueous phase is a desirable characteristic since it alleviates concerns over the possible occurrence of significant levels of toxic chemical intermediates produced by the bacteria.

Since a need exists for the development and evaluation of new biotreatment technologies for the removal of high concentrations of chemicals from aqueous waste streams, the three *Pseudomonas* sp. strains isolated in this study were used to evaluate IBT for the removal of high concentrations of PNP. The use of large populations of chemical-degrading bacteria in IBT reactors offers the potential for chemical biodegradation rates which are much faster than those occurring in conventional waste treatment systems. However, there is a concern that concentrated chemical wastes may be acutely toxic to the immobilized bacteria. Since the total loading of chemicals onto an IBT reactor is a function of both chemical concentration and flow rate, early in this study we held the flow rate constant and increased the concentration of PNP to determine the concentration threshold for acute toxicity to the immobilized bacteria. The results from this study indicate that the threshold for acute toxicity of PNP to the bacteria occurs between 2,100 and 2,500 mg/liter. These results show that the immobilized bacteria in this study were very tolerant of high PNP concentrations since the toxicity threshold in the cell multiplication inhibition test for PNP has been reported as 4 mg/liter for *Pseudomonas putida* and 100 mg/liter (lethal dose) for *Escherichia coli* (4). Whether the tolerance for PNP observed in this study is a characteristic of these particular *Pseudomonas* strains or occurs as a result of protective effects from immobilization onto the biocarrier is unknown, but does warrant further investigation. Maintaining PNP concentrations of ≤1,800 mg/liter should be an acceptable operating strategy to avoid acute toxicity to these PNP-degrading *Pseudomonas* spp. in an IBT reactor used for the degradation of PNP in aqueous wastes.

Throughout the remainder of the IBT reactor studies the PNP concentrations were maintained at ≤1,800 mg/liter, and chemical loading was elevated by increasing the flow of the synthetic waste stream through the IBT reactor. The purpose of this approach was to monitor the performance of immobilized bacteria at increasing levels of chemical loading to determine the full capacity of IBT for the removal of PNP from aqueous waste streams. Calculation of the PNP removal rate per unit weight of the biocarrier provides useful information for the sizing of full-scale IBT units. The average PNP loading (milligrams per hour) for each of the final three phases of the chemical loading study at flow rates of 10, 12, and 15 ml/min were 817, 953, and 1,155 mg/h, respectively. The average removal of PNP by the immobilized bacteria for each of these final three levels of chemical loading was 99.5, 95.3, and 70.9%, respectively. The dry weight of the biocarrier in the IBT reactor was 829 g. The PNP removal rate (milligrams per hour per gram of biocarrier) may be calculated for each of these three phases by using the following equation: PNP removal (mg/h/g) = [fraction removed × PNP loading (mg/h)]/829 g of biocarrier. These calculations show that the rate of PNP removal for PNP loading of 817 mg/h was 0.98 mg/h per g of biocarrier. Similar calculations for PNP loading rates of 953 and 1,155 mg/h showed PNP removal rates of 1.1 and 0.99 mg/h per g of biocarrier, respectively.

The PNP removal rates of 0.98 to 1.1 mg/h per g of biocarrier observed for the bacteria in this study represent the maximum PNP removal rates under optimal conditions.

For example, the synthetic waste stream used in this study was maintained at 25°C and at neutral pH, consisted solely of PNP, contained low salt, and was supplemented with inorganic nutrients. The presence of salt, other degradable organic chemicals, temperature extremes, or insufficient inorganic nutrient levels could significantly inhibit the rate of PNP degradation by immobilized bacteria. In addition, the rate of PNP degradation by immobilized bacteria may be different for high-volume waste streams containing low levels of PNP and warrants further investigation.

The results of this study indicate that IBT reactors are a useful alternative for the biotreatment of high concentrations of PNP in aqueous waste streams. Although this study shows the magnitude of the capacity for PNP removal from aqueous waste streams by IBT, feasibility testing would be required to evaluate IBT for specific waste streams and to determine the most efficient operating strategy for a full-scale IBT reactor.

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