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Denitrification and Removal of Heavy Metals from Waste Water by Immobilized Microorganisms

R. P. TENGERDY,[1]* J. E. JOHNSON,[2] J. HOLLÓ,[3] AND J. TÓTH[3]

[1]*Department of Microbiology, and* [2]*Department of Radiation Biology, Colorado State University, Fort Collins, Colorado 80523;* [3]*Institute of Agricultural Chemical Technology, Technical University of Budapest, Budapest, Hungary*

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Abstract

Pseudomonas fluorescens, immobilized on soft polyvinyl chloride granules containing up to 35% softeners as carbon source, was used for simultaneous removal of nitrate and heavy metals. In typical continuous column operation, a 100 mg/L nitrate input solution was reduced to a 20 mg/L output at a feeding rate of 1500 mL/h, with a capacity of 14 kg/day/m^3, and with an efficiency of 79%. In the same column, $Pb(NO_3)_2$ concentration was reduced from 1.0 to 0.05–0.1 mg/L and $ZnSO_4$ concentration was reduced from 10 to 5 mg/L. *Pseudomonas aeruginosa* immobilized on an O_2 plasma-treated melt blown polypropylene web was used for removing 95% of a 1.7 nCi $PuCl_4$ activity from a nuclear plant waste water in a batch operation.

Index Entries: *Pseudomonas fluorescens*, in waste water denitrification; *Pseudomonas fluorescens*, in heavy metal removal from waste water; immobilized bacteria, in waste water treatment; bacteria, immobilized, in waste water treatment; denitrification, of waste water by immobilized microbes; metals, removal from waste water by microbes; microbes, immobilized, in waste water denitrification; microbes, immobilized, in heavy metal removal from waste water; plutonium, removal from waste water by immobilized microbes.

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Introduction

Fixed-bed biological reactors with immobilized microorganisms are used increasingly for waste water treatment (*1–4*). The main advantage of such reactors is the possibility of continuous operation and the relative absence of microbes in the effluent.

This latter point depends on the method of immobilization of microorganisms in the fixed-bed reactor. Those methods that assure firm immobilization, microencapsulation (*5*) or entrapment in gel (*6*), are usually prohibitively expensive for waste water treatment; therefore, simpler and cheaper methods, such as simple physical adherence, were preferred in earlier applications (*1, 2*).

Inorganic carriers, such as limestone chips and ceramics, have been used, but they are too limited in their range of physical properties to allow flexible designs for maximal flow rate (*7–10*).

Plastics can be made in practically any geometric shape to satisfy design requirements, hence their increasing popularity in fixed-bed reactors (*2, 3, 11, 12*). A further advantage of using plastics as microbe carriers is the relative ease of modifying the surface properties of the plastic, or using it as a carbon source for the continued survival and metabolic activity of the microorganism.

In this paper we describe two methods that assure better immobilization of bacteria on plastic carriers, and better metabolic activity of the immobilized bacteria on the plastic. One method is plasma treatment, the other is enriching the plastic with special softeners that may serve as a sole carbon source. We describe the application of such immobilized microorganisms for biological denitrification and the simultaneous removal of heavy metals, zinc, and lead. We describe a separate application for plutonium removal. Microorganisms have been widely used for such purposes in different waste water treatment technologies.

Denitrification, as a tertiary treatment of waste water, is carried out by certain bacteria in fixed bed bioreactors more efficiently than in suspended culture reactors (*11, 12*).

Heavy metal uptake is primarily based on the ability of microbial surfaces to complex with metal cations (*13, 15*). The complexing units are exocellular polyphosphate groups chelating metals and/or negatively charged acidic polysaccharide fibrils extending from the cell walls of microorganisms (*16, 17*).

Microorganisms, including algae, have been used for the removal of heavy metals from industrial and domestic sewage (*13, 16*), for plutonium removal from holding ponds (*18*), and also for ore leaching (*19*).

Materials and Methods

Selection of Plastics as Microbial Carriers

The selection criteria were: availability, price, physical and mechanical properties for column design, surface properties and ease of surface alteration,

and finally the possibility of using the plastic as a limited carbon source. The intended final use influenced selection. For denitrification a long half life of the bioreactor was desirable for continuous operation, for plutonium removal a maximum surface area and maximum cell loading capacity was required for efficient metal complexing.

After screening a wide range of plastics, the two most suitable ones were selected: polyvinyl chloride (PVC) for denitrification and polypropylene (PP) for plutonium removal. The PVC was specially prepared in a soft granule form, containing up to 35% special softeners to be used as C-source for the bacteria (Hungarian Patent C02 B1/14). The softeners assure firm embedment since the bacteria burrow into the plastic and maintain the high metabolic activity necessary for efficient denitrification (*20, 21*).

Polypropylene is available commercially as a melt blown web that consists of very fine, less than 1.0 μm diameter filaments with a large surface area, which allows high cell loading and consequently high Pu or other heavy metal accumulation.

Plasma Treatment of PP

Oxygen plasma treatment, the exposure of the plastic surface to O_2 plasma in a glow discharge apparatus (*22*) is now a commercial and very economic procedure, used for instance for facilitating printing on plastic surfaces.

In our laboratory-scale experiments, we treated small, 1.5 × 12 cm strips of PP web in a sealed glass tube glow discharge apparatus at 1.5 mm HgO_2 pressure. A radiofrequency generator provided the energy: 13.56 MHz frequency, 8W output for a 2–3 s treatment was adequate.

In the treatment, ·OH radicals are grafted to the surface, and other oxidative changes also contribute to an increased wettability, which is a prerequisite for firm bacterial attachment. The treatment affects only the outermost 1–10 μm layer of the plastic, thus bulk properties remain unchanged. A longer treatment, however, leads to a rapid deterioration (scorching) of the plastic.

Immobilization of Bacteria on Plastic Surfaces

For denitrification and the simultaneous uptake of heavy metals, a *Pseudomonas fluorescens* biotype C culture was used. A glass column of 4 × 32 cm was loosely packed with soft PVC granules passing through a 5.25 mm sieve containing 35% dibutyl phtalate softener. The bulk packing density of the column was 588 g/L. The column was sterilized with dry steam for 20 min. The void volume (250 mL) was filled with a sterile mineral solution of: (NH_4NO_3, 0.1%; KH_2PO_4, 0.1%; NaCl, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.1%; $CaCl_2 \cdot 2H_2O$, 0.01%; $FeCl_3 \cdot 6H_2O$, 0.01%). The column was inoculated with 0.5-mL 24-h beef-broth culture of *P. fluorescens* containing about 1.0×10^9 viable cells/mL. Temperature was kept constant at 32°C. In Fig. 1 it can be seen that the change in viable cell numbers was rather slow, developing in the column reactor on the plastic surface in an order of magnitude of 10^7 cells/cm^2 in 14

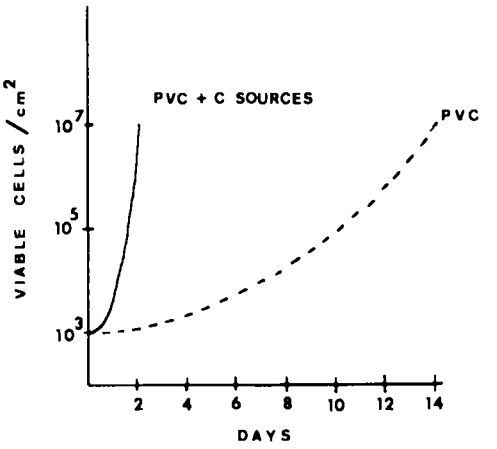

FIG. 1. Growth of *Pseudomonas fluorescens* on PVC with or without added carbon source.

days. If an additional C-source, glucose or methanol, was added, growth accelerated. It follows from the figure that the plastic may serve as sole carbon source required for the multiplication of bacteria, but an additional carbon source promotes the initial phase of attachment, owing to better adhesion of a large number of quickly multiplying bacteria, which become attached as a new layer to the layer of bacteria fixed on the plastic surface. The same holds true for the addition of various trace elements as nutrient supplements, e.g., calcium, magnesium, iron, manganese, and phosphate salts, which not only stimulate the multiplication of cells, but by surface charge alterations also affect physical attachment between negatively charged bacteria and positively charged surface groups of the plastic (*23*). Scanning electron micrographs of the immobilized bacteria clearly revealed a mass of rod-like viable bacteria in the process of cell division on the surface of the plastic (Fig. 2). The bacteria remained fully attached even in streaming water or during vigorous shaking. No bacteria could be detected, on the other hand, on the surface of less suitable plastic beds after such treatments.

The total number of bacteria attached to the plastic surface was determined by the Lowry test (*24*); the number of viable bacteria was determined colorimetrically by the triphenyl-tetrazolium-chloride (TTC) reduction test (*25*). Cell loading was 0.1–0.4 g/g plastic, dependent on the PVC used and on the growth of the bacteria.

For Pu removal *Pseudomonas aeruginosa* (ATCC 13388) was used because of our previous experience with this organism, although other *Pseudomonas* cultures, as well as other gram negative bacteria, may be used equally efficiently (*18*).

The plasma-treated PP strips were cut into 1.5 cm squares and immersed in a trypticase soy broth shaker culture of *P. aeruginosa* for 72 h. The squares were washed with running tap water. Detachment of bacteria from the surface under these conditions was minimal.

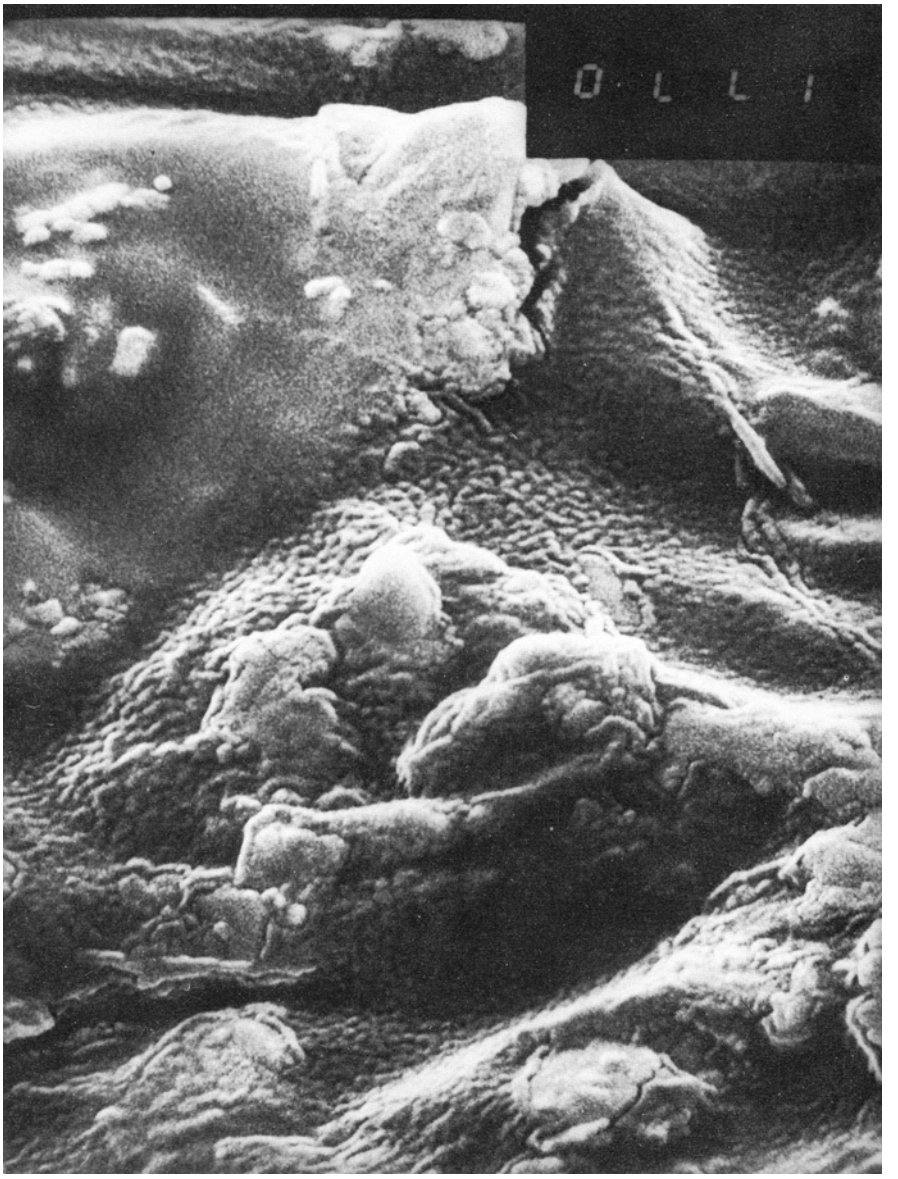

FIG. 2. Electronmicrograph of *Pseudomonas fluorescens* embedded in PVC granules.

Results

Denitrification

The PVC–bacteria colums prepared as described above have been used for studying the effect of flow rate and input nitrogen concentration on denitrification, and 60 mg/L methanol was added to the nitrate (KNO_3)

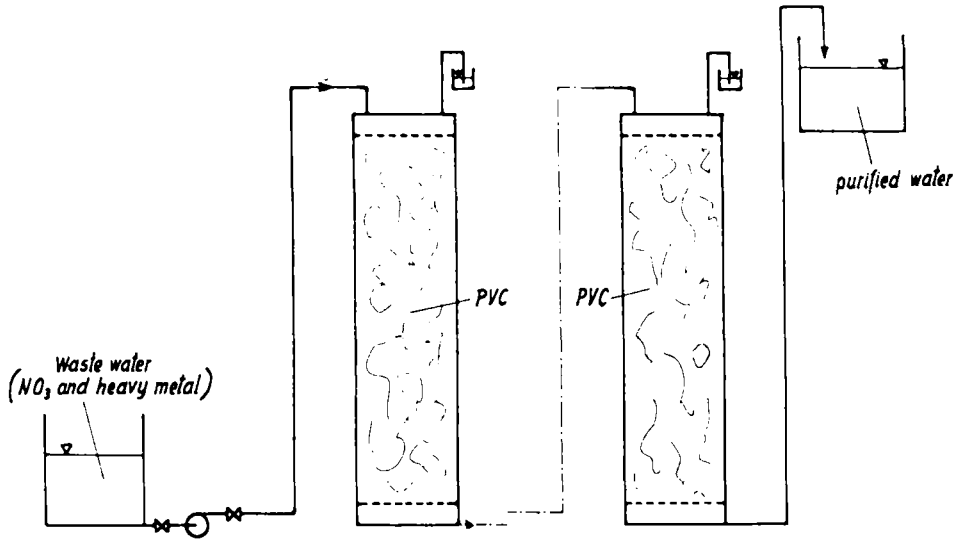

FIG. 3. Flow diagram of the PVC bioreactor.

solution as an additional carbon source. Input was from the top of the column and output was regulated through an overflow device (Fig. 3). Temperature was kept at 28°C pH at 7.0–7.2. In this system the granular PVC acts as a filter retaining the floating particles (cells, cell debris, others), while the immobilized bacteria convert the nitrate and adsorb the heavy metals. The gas outlet was at the top of the column through a liquid trap designed to ensure anaerobic conditions. Input and output nitrate concentrations were determined by standard water quality control procedures: after evaporation with salicylic acid and dissolution with sulfuric acid, the yellow salicylic acid nitrate complex obtained upon alkalization was determined photometrically. In spite of the effective immobilization technique and optimization of the flow rate, bacteria (10^2–10^3 cells/mL) could always be detected in the effluent water. To trap these bacteria, a freshly filled column was attached to the end of the system. After saturation of this new column with bacteria (about 2 weeks), the old column was disconnected, the new column became the denitrification column, and a fresh filter column was attached. The operation was maintained in this configuration continuously for over 2 years.

Table 1 shows average values for measurements of denitrification capacity and efficiency on various columns in experiments carried out during a period of approximately 2 years. As can be seen from the data, the increase in the denitrification capacity of each column (3.6–24.3 kg nitrate/m^3 reactor void volume per day) was nearly proportional to the increase in feeding rate and in daily load. At the same time, the efficiency of denitrification has dropped from 84 to 71% as the feeding rate was increased. With the increase in this rate, a simultaneous increase could be observed in the number of cells in the column, as well as in the effluent. The data measured on the individual columns prompted us to apply serially linked columns by which nitrate removal could be achieved with 99% efficiency.

TABLE 1
Dentrification Capacity and Efficiency of Column Reactors[a] Using Immobilized *P. fluorescens*

Nitrate concentration, mg/L		Feeding rate,	Load/day,	Capacity for decomposed NO_3,	Efficiency,
Input	Output	mL/h	g NO_3	kg/day/m^3	%
106	17	427	1.08	3.6	83.9
114	18	488	1.32	4.5	84.2
116	19.5	727	2.03	6.7	83.1
115	22	1134	3.7	11.9	80.8
119	30	2115	6.04	18.0	74.8
119	34	3093	8.83	25.2	71.4

[a]Reactor void volume: 250 mL.

Figure 4 shows the correlation between capacity, efficiency, and nitrate loads. Although there are some deviant points among the data plotted, when linear regression analysis was carried out on individual data, the correlation

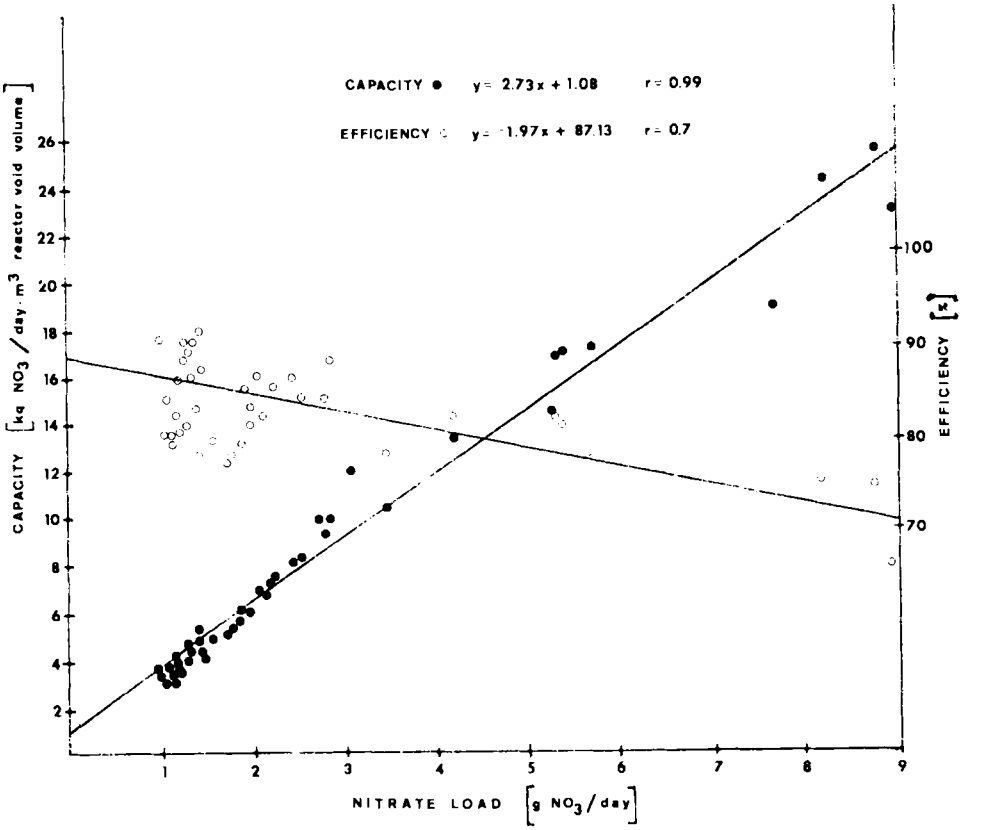

FIG. 4. Correlation between nitrate removal capacity and efficiency.

coefficient was found to be very good for capacity (0.99) and satisfactory for efficiency (0.7). This verifies the correctness of the relationships and the straight lines drawn. The intersection of the two lines is regarded as an operational parameter of the individual columns; thus with further experimental design modifications, we have calculated denitrification efficiencies of 75–80% and column capacities of 12–13 kg nitrate/m^3/day.

Simultaneous Removal of Heavy Metals and Nitrate

In addition to denitrification, the above series of columns were also used for the removal of heavy metals. A decrease in the denitrification capacity of the column was used as an index of the critical heavy metal concentrations causing toxicity to the *P. fluorescens*. The toxic effect of metals (lead, chromium, copper, cadmium, and zinc) was examined in a separate study (unpublished data). Of these metals, copper proved to have the highest toxicity since it fully prevented denitrification in a very short time at a concentration of 10 ppm. Regarding their toxic effects the next most toxic were, in order, lead, followed by cadmium, zinc, and chromium.

The removal of heavy metals on fixed-bed columns was carried out primarily with lead and zinc contaminated effluents. The metal content of both input and output waste waters was determined by atomic absorption spectrophotometry. In one example, a solution containing 10 mg/L $ZnSO_4$ and 100 mg/L KNO_3 was reduced to an output solution of 10 mg/L KNO_3 and 5 mg/L $ZnSO_4$ with a capacity of 30 g $ZnSO_4$/m_3/day. In another example, a solution containing 1.0 mg/L $Pb(NO_3)_2$ and 100 mg/L KNO_3 was fed to the column, and an output of 0.05–0.1 mg/L $Pb(NO_3)_2$ was measured. In this series of experiments, appropriately treated columns were kept in operation only for a certain period of time (300–400 h), when denitrification efficiency suddenly dropped in a few hours from 90–95 to 0%, indicating that the microbes had accumulated a lethal dose of the toxic heavy metal (pH 7.5, at 25°C, with continuous feedings at the 5 ppm level). Cell death was also verified by the lack of TTC reduction.

The equipment used for the recycling of waste water in a Hungarian chemical plant has been designed on the basis of the above experimental results. Contamination in the waste water of this plant exceeded permissible limits for zinc, chromium, barium, aluminum, nickel, lead, and nitrate. Based on above experiments, the metal ions present in toxic concentrations were first precipitated by the addition of lime hydrate and sedimented as hydroxide in the pH range: 8.5–9.0 (*11*). After readjustment to pH 7.5, the very diluted metal ions (about 1 ppm) in the supernatant were transferred into a biological reactor and then removed with or without nitrate by means of *Pseudomonas* cells immobilized on a PVC bed as described above. The direction of the flow in the biological reactor was changed weekly in order to improve the efficiency of filtering. Filtration of possible eluted microbes was carried out on a sand-filter, which assured microbe-free effluent water.

TABLE 2
$PuCl_4$ Uptake by *Pseudomonas aeruginosa* Immobilized on a Plasma-Treated Polypropylene Web[a]

Sample	Mean Pu uptake, %
Control	6.7 ± 2
Immobilized cells	96 ± 15

[a]$PuCl_4$ activity, 1.7 nCi; sample size, 1.5 cm square (~ 0.1 g), cell loading, 0.3 g/g polypropylene.

Biological Plutonium Removal

P. aeruginosa calls attached to PP were used efficiently for the removal of plutonium from aqueous systems. Pu removal experiments were conducted in small scale batch or continuous column operations.

In the batch experiment 1.5 cm squares of the bioadsorbent web were immersed in $PuCl_4$ solutions or PuO_2 suspensions for 6 h. A 1.5-cm square sample of the PP bioadsorbent was able to remove 96% of 1.7 nCi $PuCl_4$ activity (Table 2). The removal of PuO_2 depended on particle size. From 1-μm diameter monodisperse particles, 34% was removed, from 0.59 and 0.13 μm particles 13 and 12%, respectively, were removed under the same conditions as for $PuCl_4$ (Table 3). This finding suggests that the insoluble PuO_2 particles are merely entrapped in the filaments, rather than adsorbed to the bioadsorbent; smaller particles escaped entrapment.

In continuous Pu-removal experiments, small 10 × 100 mm glass columns were packed with PP bioadsorbent (cell loading: 0.3 g cells/g plastic; column load: 1.5 g bioadsorbent). This column removed 75–80% of 1.7 nCi/100 mL $PuCl_4$ activity at a 15 mL/h flow rate in a 12-h operation. When a second column was linked serially with the first one, efficiency increased to 98–99%. The useful life of the columns was about 2 weeks. In a continuous two-column

TABLE 3
PuO_2 Uptake by *Pseudomonas aeruginosa* Immobilized on a Polypropylene Web[a]

	Mean Pu uptake, %	
PuO_2 particle size, μm	Immobilized cells	Control
1.0	34 ± 9	22 ± 5
0.59	13 ± 8	11 ± 5
0.13	12 ± 1.5	4 ± 1

[a]PuO_2 activity, 1.5 nCi; sample size, 1.5 cm square (0.1 g), cell loading, 0.3 g/g polypropylene.

operation, a new column should replace the exhausted one after a 2-week period.

The entire operation is designed with disposability in mind. The trapped Pu can be buried with the bioadsorbent or incinerated first to reduce bulk. The inexpensive plastic carrier and the simple immobilization process permits disposal, making the process highly competitive with other Pu removal technologies.

Conclusions

Fixed-bed bioreactors using immobilized *Pseudomonas* cells are suitable for the simultaneous or separate removal of nitrates and heavy metals from very diluted solutions. Plastics are excellent microbe carriers: they have a flexibility in geometric design for column operations, their surfaces can be modified by plasma treatment, and they can serve as a carbon source when treated with appropriate softeners. The choice of plastic depends on the intended application: for denitrification, PVC is adequate, providing maximum viability of cells but less stability; for Pu removal, PP provides maximal microbial attachment. The plastic-based bioreactors are highly competitive with others both in performance and in economy.

Acknowledgments

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