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Evaluation of Aquatic Sediment Microcosms and Their Use in Assessing Possible Effects of Introduced Microorganisms on Ecosystem Parameters

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In this paper we describe a sediment microcosm system consisting of 20 undisturbed, layered sediment cores with overlying site water which are incubated under identical conditions of temperature, light, stirring rate of overlying water, and water exchange rate. Ecosystem parameters (nutrient level, photosynthetic potential, community structure of heterotrophic bacteria, thymidine incorporation rate, and oxygen microgradients) of the laboratory microcosms and the source ecosystem were compared and shown to be indistinguishable for the first 2 weeks. In weeks 3 and 4, small differences were detectable in the nutrient level, community structure of heterotrophic bacteria, and thymidine incorporation rate. However, the photosynthetic potential, depth profiles of heterotrophic bacterial community structure, and oxygen microgradients were maintained throughout the incubation period and did not differ between laboratory microcosms and the source ecosystem. The microcosm system described here would thus appear to be a valid model of aquatic sediments for up to 4 weeks; the actual period would depend on the sediment source and incubation temperature. The validated systems were used with Rhine river sediment to assess possible effects on ecosystem parameters of *Pseudomonas* sp. strain B13 FR1(pFRC20P), a genetically engineered microorganism (GEM) that had been constructed to degrade mixtures of halo- and alkylbenzoates and -phenols. The GEM survived in the surface sediment at densities of 5×10^4 to 5×10^5/g (dry weight) for 4 weeks and degraded added chloro- and methylaromatics. The GEM did not measurably influence ecosystem parameters such as photosynthesis, densities of selected heterotrophic bacteria, thymidine incorporation rate, and oxygen microgradients. Thus, the microcosm system described here would seem to be useful for the study of the ecology of biodegradation and the fate and effect of microorganisms introduced into the environment.

The potential for microbial degradation of xenobiotics, in particular for bioremediation of soils, sediments, and groundwater contaminated with toxic chemicals, is widely recognized (1). However, basic ecological mechanisms must be understood in more detail before this potential can be fully realized. Some of the technologies that are being developed require the introduction of large numbers of specialized bacterial strains into the environment (45). Microbial strains selected in the laboratory, however, sometimes fail to produce the desired effect in the natural habitats because their survival and activity in the environment are not adequate (13, 25, 26, 37). To predict the consequences of bioremediation treatments, microcosms can be used to model essential characteristics of the natural environment. Moreover, some bioremediation applications may involve the use of genetically engineered microorganisms (GEMs) either in situ (introduction of bacteria into the contaminated site) or on site (application of bacteria in a semicontrolled facility) (7, 44). Here, not only must the functioning of the bacteria in the environment be assessed prior to application, but also their possible influence on the indigenous microflora and the ecosystem as a whole must be considered. Microcosms constitute an important means of assessing such effects. Some of the important ecological questions that must be addressed include the fate of the GEM and its recombinant DNA and its possible effects on ecosystem structure and processes (e.g., the flow of carbon and energy and the cycles of major nutrients such as nitrate, sulfate, and phosphate [15, 23, 35, 42–44]). The development of useful microcosms requires that they be validated to study basic ecological mechanisms related to biodegradation (30), the efficacy of bioremediation treatments, and the ecological consequences of the introduction of large numbers of bacteria into the environment.

A microcosm is defined as ". . . a laboratory system which attempts to actually simulate as far as possible the conditions prevailing in the environment or part of the environment under study." (47) Microcosms have been used successfully to study ecotoxicologal questions (see, e.g., reference 31), biodegradation (see, e.g., references 19 and 41), ecological mechanisms (17), and the fate and effect of GEMs (2, 6, 21, 25, 26, 29, 36). Since they are contained laboratory systems, the introduction of toxic chemicals, radioactive chemicals, and/or GEMs is feasible. Well-designed microcosms should overcome the technical problems inherent in field experiments but should retain most of the complexity of the real-world situation. Moreover, they allow control of environmental parameters (e.g., temperature, oxygen, nutrient level) and manipulation of ecosystem features (e.g., inhibition of protozoans) when this is desired.

Aquatic sediments are an important target ecosystem for bioremediation because they are one of the ultimate sinks of persistent industrial chemicals. They house complex bacterial communities capable of biodegradation (33) and offer completely different survival conditions from those of surface waters (14, 38). We describe here an aquatic sediment

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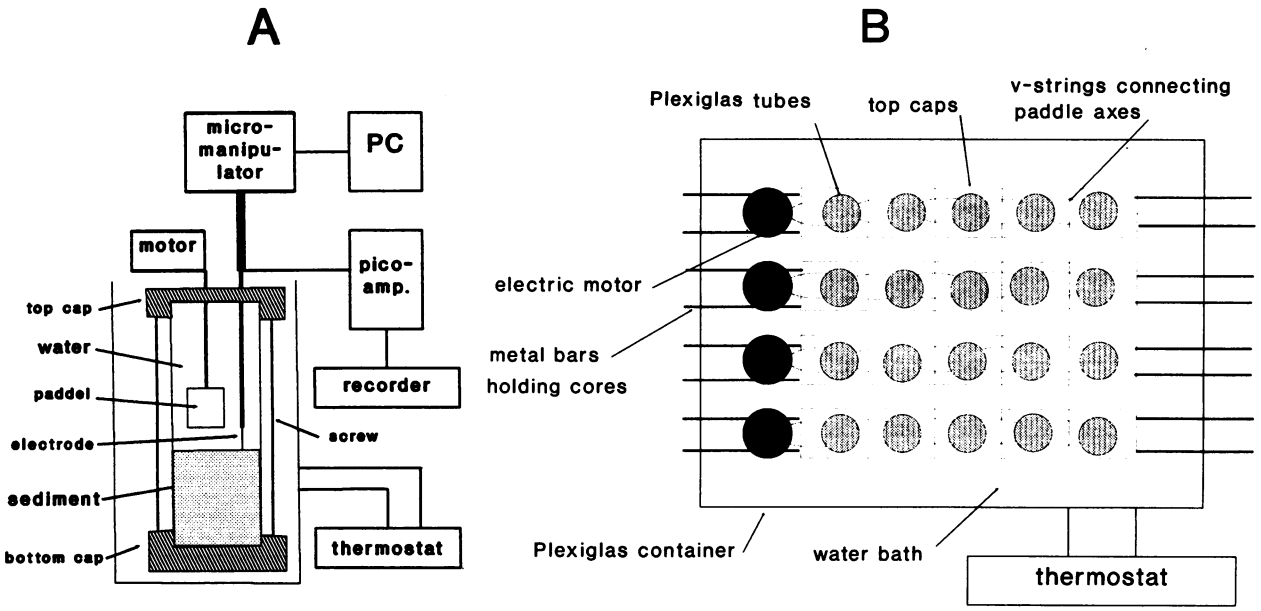

FIG. 1. Sediment microcosm system. (A) View of a single sediment core at the electrode workplace, with a micromanipulator (controlled by a personal computer [PC]), electrode, amplifier, and recorder attached. (B) View of the complete microcosm system from above, showing the water bath with incubated cores, electric motors driving paddles, v-strings connecting paddle axes, and metal bars holding the cores. The final dimensions of the system holding 20 cores are 660 by 730 by 370 mm, and the distance between core caps is ca. 5 mm.

microcosm system which allows maintenance of 20 intact layered sediment cores with overlying site water. Whole cores retain the sophisticated vertical structure of sediments (4, 9, 40) and are therefore a much more realistic model of nature than are simpler systems, e.g., slurries.

The usefulness of a microcosm depends strongly on the extent to which results can be transferred to the source ecosystem. As a basis for interpretation of microcosm data, therefore, the microcosm must be validated; i.e., important ecosystem parameters must be compared with the natural environment (3). This was the first aim of the present investigation. For each type of microcosm there is an optimal size and time span for experiments. These depend on the basic features of the source ecosystem and their temporal and spatial variation (patchiness) and also on the "bottle effect" in the microcosm, which is caused by containment (32, 47). As a result, the microcosm structure gradually changes over time. The source ecosystem is subject to diurnal, seasonal, and other fluctuations of physical, chemical, and biological parameters, some of which are difficult or impossible to reproduce in the laboratory but which nonetheless cause changes in its structure. As a consequence of these factors, the structures of the source ecosystem and the microcosm diverge as a function of time. The validation experiment described in the first part of this investigation was designed to analyze this divergence and determine the optimum time span for experiments. This experiment was performed with sediment from an unpolluted small lake (Grumbacher Teich) in the Harz mountains, Germany.

The second aim of the investigation was to study the response of ecosystem parameters to the introduction of a model, pollutant-degrading GEM. We used *Pseudomonas* sp. strain B13 FR1(pFRC20P), which has been designed through the patchwork assembly of appropriate genes (34) to degrade mixtures of chloro- and methylaromatics. This experiment was carried out with sediment microcosms from the Rhine river, which has a long history of pollution with man-made chemicals.

In both experiments, the same ecosystem parameters were analysed, but from different points of view. We chose to monitor parameters which are easy to measure and at the same time are important structural characteristics of the ecosystem, namely nutrient level (phosphate and nitrate concentrations), photosynthetic potential (concentration of chlorophyll *a*), community structure of heterotrophic bacteria (densities of cellulose and starch degraders, pseudomonads, fluorescent bacteria, enterobacteria, and denitrifying bacteria), incorporation of [^{3}H]thymidine, and respiratory activity of the sediment community (oxygen flux across the sediment/water interface).

MATERIALS AND METHODS

Microcosm system. The microcosm setup (Fig. 1) is a modification of the system used at the Netherlands Institute for Oceanic Sciences (formerly Netherlands Institute for Sea Research), Texel, The Netherlands (42a). A Plexiglas tube (length, 31 cm; diameter, 7 cm; volume, 1.2 liters) serves as both coring and incubation device. The bottom end is sharpened to facilitate insertion into the sediment, while the top end has a Plexiglas ring glued to it for attaching a cap and a stirring device. There is a bottom cap with a rubber washer and a top cap with an O-ring fitting the Plexiglas tube tightly for sampling. After the sediment core has been obtained, the caps are fastened firmly with screws. After transport to the laboratory, the top cap was replaced by one which carries a stirring paddle. Twenty tubes were placed in a water bath at the in situ temperature, in four rows of five tubes. The five paddles in each row are driven by one electric motor (Bürklin 9904120/52707) by means of v-strings at 0 to 24 V (0 to 24 rpm). The paddle is adjusted at 3 cm above the sediment surface and stirs the water slowly to facilitate the diffusion of oxygen. The top cap of the Plexiglas tube has a

hole which allows insertion of tubes (inflow and outflow of water), electrodes, and sampling pipettes. Filtered site water was introduced into the microcosms at a flow rate of about 0.1 ml/h by using peristaltic pumps and removed at the same rate either by using peristaltic pumps or by overflow out of a small hole.

Sampling procedure. Sediment cores were obtained by scientific divers from the Technical University, Clausthal-Zellerfeld, Germany. The divers carefully lowered themselves to the lake bottom and inserted the Plexiglas tube vertically into the undisturbed sediment in front of them until half of it had disappeared into the ground. The top cap was then fitted to the tube, closing it tightly. The tube was pulled out of the ground, filled half with sediment and half with overlying site water, and closed at the bottom end with the second cap. While holding the tube vertically, the diver transported it to the boat or shore, where both caps were immediately secured with screws. Tubes were stored on ice and transported to the laboratory on the same day.

Site characteristics and experimental design. (i) Validation of microcosm system. Samples were taken from Grumbacher Teich, a small, unpolluted lake in the Harz mountains in northern Germany in an area which serves as a drinking water supply. The lake has a maximum depth of 9 m. Twenty cores were taken on 12 June 1990 and incubated in the laboratory for up to 4 weeks at the in situ temperature (10°C). Samples for comparison (three cores each) were taken on 13, 18, and 25 June and 2 and 10 July. The sampling depth was 8 m, the temperature at the sediment surface increased from 10°C on 12 June to 12°C on 10 July, the conductivity was 11 to 14 μS, and the pH was 7.4 to 7.5. The sediment was fine grained, had a low lime content, and was light grey. On the top there was a loose layer of filamentous green algae. The experiment started on the day following the first sampling (13 June, day 0). On days 1, 6, 13, 20, and 28 three cores incubated in the laboratory for the above-mentioned number of days (called microcosms in the rest of the paper) were analyzed, together with three freshly sampled cores from Grumbacher Teich (referred to as lake sediment).

(ii) Effect of GEMs on ecosystem parameters. Twenty cores were taken from the Rhine river on 15 February 1990 behind a dam at Rhine kilometer 334 close to the village of Iffelzheim in Baden-Württemberg, Germany. The water depth was 10 m, the conductivity was 157 μS, the temperature was 7°C, and the pH was 7.6. The sediment was fine grained with a high content of lime and a clearly defined surface. Microcosms were incubated at the in situ temperature (7°C). They were subdivided into four sets of five cores, each set being treated differently. Treatment of sets 1 to 4 on day 0 involved addition of GEM to the water phase (set 1), addition of GEM and substituted aromatics (set 2), addition of substituted aromatics alone (set 3), and control with no addition of GEMs or aromatics (set 4). After the exact volume of the water column of each core had been determined, the GEM was added to the water phase to a density of 5×10^6/ml. The substituted aromatics 3-chlorobenzoate (3CB) and 4-methylbenzoate (4MB) were added to the water phase at 25 μM each. One microcosm from each set was sacrificed and analyzed on days 1, 5, 12, 19, and 26.

Microorganisms and media. *Pseudomonas* sp. strain B13 FR1(pFRC20P) (34) was routinely grown on standard M9 minimal medium (24) supplemented with mineral salts, 4MB, and 3CB (2.5 mM each) as sources of carbon and energy. The mineral salt solutions consisted of (i) 10.75 g of MgO, 2.0 g of $CaCO_3$, 4.5 g of $FeSO_4 \cdot 7H_2O$, 1.44 g of $ZnSO_4 \cdot 7H_2O$, 1.12 g of $MnSO_4 \cdot 4H_2O$, 0.25 g of $CuSO_4 \cdot 5H_2O$, 0.28 g of $CaSO_4 \cdot 7H_2O$, and 0.06 g of $H_3BO_4 \cdot 4H_2O$ dissolved in 51.3 ml of concentrated HCl; (ii) 1 M $MgSO_4$; and (iii) 36 mM $FeSO_4 \cdot 7H_2O$. Solutions i and ii were autoclaved separately, and solution iii was filter sterilized. Then 50 ml of solution i and 25 ml each of solutions ii and iii were combined. The medium was supplemented with 0.25 ml of the resulting mineral salts solution per 100 ml. The bacterial inoculum for the microcosms (200 ml) was grown to stationary phase, harvested by centrifugation (10,000 × *g* for 10 min at 4°C), washed twice with 200 ml of 10 mM phosphate buffer (pH 6.5), and finally resuspended in 20 ml of phosphate buffer. Bacteria were enumerated by the agar plate count method with appropriate media: (i) M9 minimal medium supplemented with 4MB (5 mM) and kanamycin (100 μg/ml) for *Pseudomonas* sp. strain B13 FR1(pFRC20P); (ii) 10-fold-diluted Luria broth (LB) (12) for total CFU of heterotrophic bacteria; (iii) King agar B (Merck no. 10991) for fluorescent bacteria; (iv) MacConkey agar (Merck no. 5465) for enterobacteria; (v) starch mineral salts agar (medium 252) for starch-degrading bacteria (5); (vi) cellulose medium for cellulose degraders (M9 minimal medium supplemented with 1% [wt/vol] cellulose MN300 [Fluka]); (vii) medium for nitrate-reducing bacteria (LB supplemented with 0.5% [wt/vol] KNO_3; nitrogen produced was collected in a Durham tube and bacterial density was determined by using the most-probable-number method after 4 weeks); and (viii) Cetrimid (Merck no. 5284) for pseudomonads. MacConkey agar plates were incubated at 37°C for 48 h, whereas all other plates were incubated at room temperature for 3 days [*Pseudomonas* sp. strain B13 FR1(pFRC20P)] to 4 weeks (cellulose degraders).

Experimental procedure. Each sediment core was analyzed in the following way. First, the core was transported to an electrode workplace, and the vertical gradient of oxygen concentration was measured in steps of 100 μm at the incubation temperature with an oxygen microelectrode (Diamond Inc., Ann Arbor, Calif.), mounted on a micromanipulator (Märzhäuser, Wetzlar, Germany). The oxygen uptake rate (J) was calculated as $J = D \times (O_2/Z)$, where J is the oxygen flux (micromoles per square meter per hour), D is the molecular diffusion coefficient (1.5×10^{-5} cm^2 s^{-1} for oxygen at 10°C), O_2 is the oxygen gradient across the diffusive boundary layer (micromoles per liter), and Z is the depth of the diffusive boundary layer (millimeters) (18). Two water samples (5 ml) were then taken for determination of nitrate (Spectroquant 14773; Merck) and phosphate (22). The water above the sediment was removed from the cores with a silicon tube and passed through a 50-μm plankton sieve. The contents of the sieve were transferred to a glass bottle and fixed with formalin (final concentration, 4%), and zooplankton organisms were enumerated with the aid of a dissecting microscope. The sediment was carefully pushed up through the Plexiglas tube and sliced with a stainless steel blade to obtain three slices, each about 5 mm thick, which were collected in petri dishes, weighed, and stirred well. Subsamples (0.5 ml) were taken from each slice sample with an Eppendorf pipette fitted with a wide-hole plastic tip (i.e., with the end cut off) and processed immediately for extraction of bacteria. A subsample (1 g) was transferred into a preweighed glass tube and dried overnight at 100°C to determine the sediment dry weight. Another subsample (1 g) was extracted for chlorophyll *a*. For determination of [^{3}H]thymidine uptake rate, triplicate subsamples (100 μl) were taken from the top sediment slice.

Extraction of bacteria from the sediment. A subsample of

wet sediment (0.5 g) from a well-mixed sediment slice was transferred to an Eppendorf tube (2 ml) containing 1.5 ml of 0.1 M sterile phosphate buffer (pH 5.5). The samples were mixed vigorously by vortexing (1 min), and then the sediment was separated by centrifugation at a speed which does not pellet bacteria (10 min at 2,500 rpm in an Eppendorf centrifuge 5415). The supernatant fluid (1 ml) with the extracted bacteria was collected in a sterile glass tube, and 1 ml of fresh phosphate buffer was added to the sediment sample. The extraction procedure was performed four times, and the supernatant fluids were combined to yield 4 ml of bacterial extract. This was then serially diluted with filtered site water, and 50 μl was plated for determination of viable cells. Control experiments revealed that the yield of this extraction method was 31%, comparable to that of a method involving homogenization with a Waring blender but less time-consuming and involving the use of less sediment (data not shown). The bacterial density is given by the following equation: Bacterial density per gram (dry weight) of sediment = counts per plate × (1 ml/sample volume) × 5 × dilution × dry weight^{-1}.

Determination of chlorophyll *a*. A 1-g subsample of the sediment slice was taken, and 4 ml of methanol was added. The sample was mixed by vortexing, boiled in a water bath for 1 min, and separated by centrifugation (10 min at 5,000 rpm; SS34 rotor, Sorvall RC-5C centrifuge). The A_{665} of the supernatant fluid was measured. The concentration of chlorophyll *a* was calculated (11) by using the following formula: chlorophyll *a* concentration (micrograms/liter) = $10^3 V_e E / V_s A l$, where V_e is the volume of solvent (milliliters), V_s is the volume of sample (liters), E is the absorbance in the solvent, A is the absorption coefficient (74.9 g^{-1} cm^{-2}), and l is the light path of the cuvette (1 cm).

[^{3}H]thymidine uptake rate. The protocol of van Duyl (45a), modified as described in reference 27, was used to measure [^{3}H]thymidine uptake rates. [*methyl*-^{3}H]thymidine (specific activity, 1.48 TBq/mmol; concentration, 20 nmol/ml) was purchased from Amersham. Triplicate samples of wet sediment (100 μl) were taken with an Eppendorf pipette with a wide-hole disposable tip and transferred to Falcon tubes (15 ml) containing [^{3}H]thymidine (0.5 nmol of labeled thymidine, i.e., 25 μCi; 9 nmol of unlabeled thymidine). After a 10-min incubation at room temperature, growth was stopped by addition of 10 ml of 80% ethanol and the samples were stored (10°C). Three blanks were prepared by mixing ethanol with thymidine and adding sediment afterwards. For further processing, ethanol was removed by centrifugation (10 min at 2,000 rpm and 4°C; Sorvall T600B centrifuge, H1000B rotor). The pH was lowered to below 4 by removing Ca_2CO_3 from the sediment by addition of 10 ml of 15% cold acetic acid and cooling the mixture for 4 to 6 h (10°C) until effervescence stopped. The sediment was pelleted by centrifugation (10 min at 2,000 rpm and 4°C; Sorvall T600B centrifuge, H1000B rotor), and the acetic acid was removed. The pellet was suspended in 2 to 5 ml of 80% ethanol, and the resulting liquid was filtered through a 0.45-μm-pore-size polycarbonate filter; the tube was rinsed with more ethanol, which was also filtered. The filter was washed with 2 ml of 5% ice-cold trichloroacetic acid four times. Trichloroacetic acid was sucked directly through the filter in less than 2 min. The filter was folded with tweezers and transferred to a centrifuge tube containing 2 ml of 5% trichloroacetic acid. The tube was capped, heated at 90°C for 30 min, and then cooled, and the sediment was separated by centrifugation (1 min at 14,000 rpm and room temperature; Eppendorf centrifuge 5415). A subsample of the supernatant fluid (0.5 ml) was transferred to a scintillation vial, and 10 ml of scintillation fluid (Scintillator 299; Packard, Groningen, The Netherlands) was added. For calculation of the uptake rate, counts per minute were corrected by a quench curve to give disintegrations per minute and then multiplied by 4 to account for the proper sample volume. Average background values of blanks were subtracted. The thymidine pool in the sediment was determined by the isotope dilution assay to be 15 μmol. Then the rate was determined as follows: thymidine incorporation rate (nanomoles of thymidine per liter per hour) = dpm × (unlabeled thymidine added + thymidine pool in sediment)/radioactivity added per sample × (1/2,220,000) × (60 min/incubation time) × 10,000. $Thym_{inc}$ = dpm × 0.0162 (nanomoles of thymidine per liter per hour).

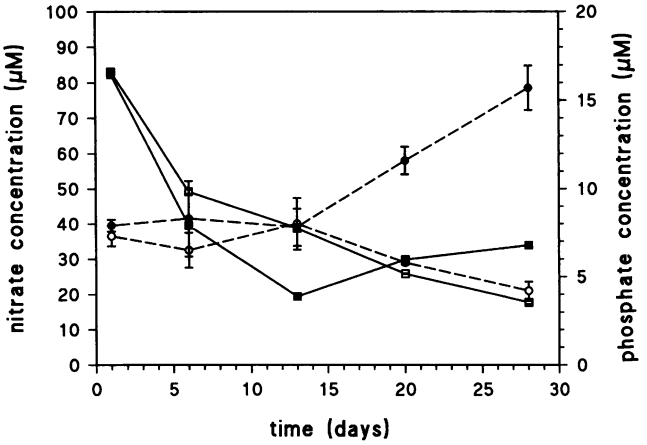

FIG. 2. Nitrate and phosphate concentrations in the overlying water of laboratory microcosms and lake sediment samples from Grumbacher Teich. Error bars represent the standard deviation between triplicate cores. Symbols: ■, nitrate concentration in microcosm; □, nitrate concentration in lake; ●, phosphate concentration in microcosm; ○, phosphate concentration in lake.

Statistics. If the standard deviations of two means did not overlap, the difference between the mean values was said to be significant. If the standard deviations of two means overlapped, the difference between the two groups of data was analyzed by using Fisher's randomization test for independent matched samples with the distribution-free statistics package Disfree (Biosoft Inc., Cambridge, United Kingdom) (20).

RESULTS

Validation of sediment microcosms. To validate the microcosm system, we measured ecosystem parameters in triplicate Grumbacher Teich sediment cores kept in the laboratory for up to 4 weeks (microcosms) and fresh triplicate cores from the lake (lake sediment), to analyze the variability between cores, the stability of ecosystem parameters, and differences between laboratory microcosms and fresh lake sediment. Comparison yielded the following observations.

Visual inspection of the sediment microcosms showed an intact surface layer and a vertical zonation of color which did not change during the 4 weeks of incubation. Numerous organisms from higher trophic levels were present. The water overlying the sediment contained zooplankton (daphnids, copepods, water mites, and protozoa), while benthic invertebrates (tubificids, small clams and snails, and insect

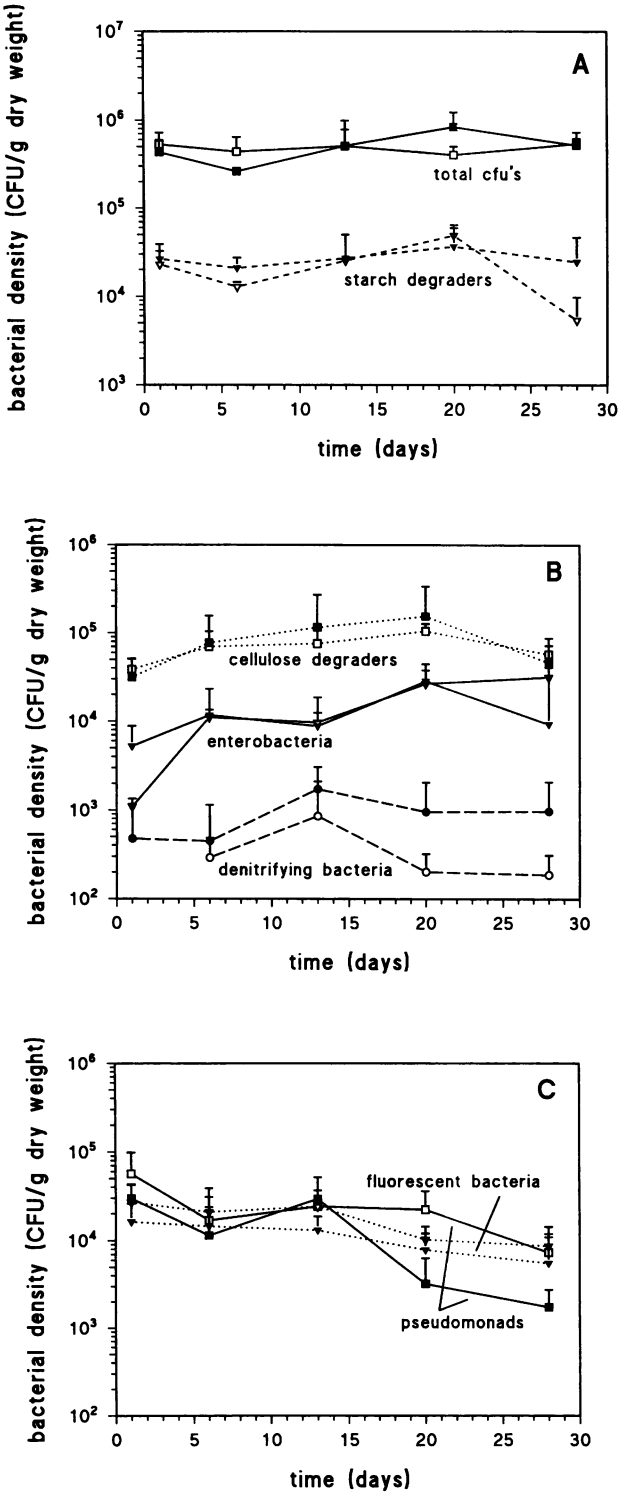

FIG. 3. Densities of bacterial subpopulations in the top layer of microcosms and lake sediment from Grumbacher Teich. Solid symbols, microcosms; open symbols, lake. Error bars represent the standard deviation between triplicate cores. (A) Total CFU and starch-degrading bacteria. (B) Cellulose degraders, enterobacteria, and denitrifying bacteria. The data for the denitrifying bacteria are averaged over the first 15 mm of the sediment. (C) Pseudomonads and fluorescent bacteria.

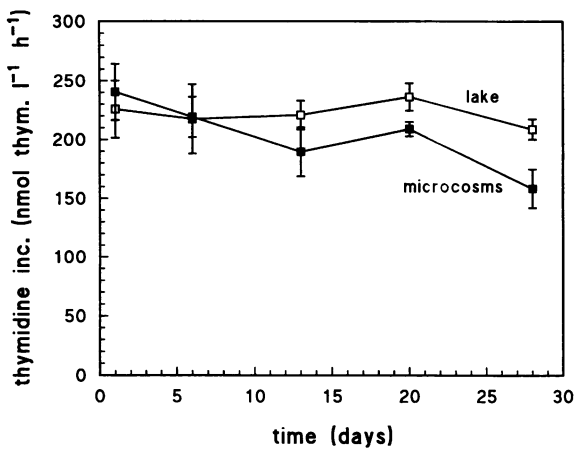

FIG. 4. Incorporation of [^{3}H]thymidine in the top sediment layer of microcosms and lake sediment from Grumbacher Teich. Error bars represent the standard deviation between triplicate cores.

larvae) were observed in the sediment. The presence of zooplankton and benthic invertebrates, especially tubificids, whose behavior could be monitored by eye, indicated that the microcosms maintained an intact sediment ecosystem with most of the complexity of the natural food web. Visual observation indicated that benthic organisms actively disturbed the sediment.

Nitrate concentrations decreased slightly over time in both the microcosms and the lake sediment (Fig. 2). Phosphate concentrations, however, increased in the microcosms from days 20 to 28, while in the lake samples there was a slight decrease during the same period (Fig. 2).

Concentrations of chlorophyll *a* in the sediment of the microcosms were not significantly different from those in the lake sediment (data not shown).

Total bacterial CFU and numbers of starch-degrading bacteria, cellulose degraders, enterobacteria, and fluorescent bacteria were stable over the incubation period and did not differ between microcosms and lake sediment (Fig. 3). The numbers of denitrifying bacteria were generally smaller in the lake sediment than in the laboratory microcosms. This difference increased with time and was statistically significant after day 20 (Fig. 3B). Numbers of pseudomonads were significantly reduced in the microcosms from day 20 onward (Fig. 3C).

On days 1 and 6, there was no difference in thymidine incorporation rates between lake sediment and microcosms. From days 13 to 28, thymidine incorporation rates decreased in the microcosms compared with fresh lake sediment (Fig. 4). Microcosms which had been incubated for 28 days exhibited 70% of the activity of fresh lake sediment.

Population densities of heterotrophic bacteria showed a pronounced vertical zonation within the first 15 mm of the sediment (data not shown). There was no significant difference between microcosms and lake sediment with respect to vertical zonation.

The oxygen flux was higher in the lake samples than in the microcosms during the first 2 weeks. However, in weeks 3 and 4 of the experiment, the oxygen flux was the same in the lake and in the microcosms (Fig. 5A). Therefore the microcosms did not experience a shortage of oxygen. The penetration depth of oxygen was greater in the microcosms and also showed a larger variability (Fig. 5B).

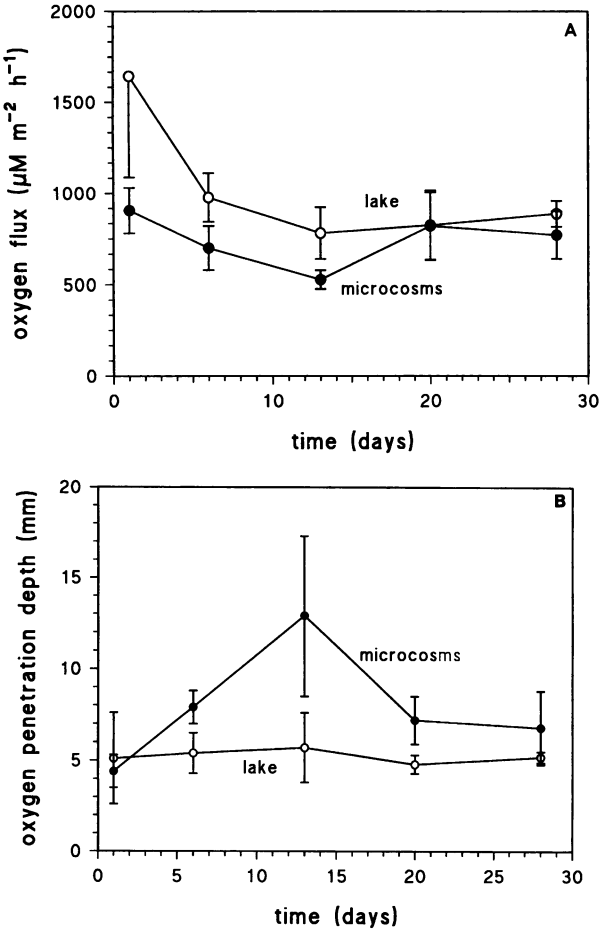

FIG. 5. Oxygen flux across the sediment/water interface (A) and oxygen penetration depth (B) in microcosms and lake sediment from Grumbacher Teich. Error bars represent the standard deviation between triplicate cores.

TABLE 1. Average percent variability of ecosystem parameters in microcosms and lake sediment from Grumbacher Teich[a]

Environmental parameter	% Variability of parameter in:	
	Microcosm	Lake
Nitrate	11	15
Phosphate	11	11
Chlorophyll *a*	20	22
Total CFU	38	39
Starch degraders	68	44
Cellulose degraders	87	38
Enterobacteria	83	39
Denitrifying bacteria	112	111
Pseudomonads	69	36
Fluorescent bacteria	61	58
[^{3}H]thymidine incorporation rate	8	8
Oxygen uptake rate	16	21
Oxygen penetration depth	24	17

[a] Variability between cores was calculated by averaging the standard deviation between triplicate cores of the five sampling dates (n = 15) and expressing it as a percentage of the mean value of the variable in question.

Variability between triplicate cores (Table 1) depended on the ecosystem parameter measured and reflects the patchiness of the environment for a specific parameter. It was smallest for thymidine uptake rate and larger for phosphate, nitrate, and chlorophyll *a* concentrations; oxygen uptake rate; and oxygen penetration depth. For bacterial population densities, the smallest variability between triplicate cores was observed for total CFU, whereas denitrifying bacteria had the largest variability. This was probably the result of both the small numbers occurring in Grumbacher Teich and the large error inherent in the most-probable-number technique. Variability between cores was smaller in fresh lake sediment than in the microcosms kept in the laboratory for population densities of starch degraders, cellulose degraders, enterobacteria, and pseudomonads.

Lack of effect of a GEM on some ecosystem parameters. The addition of the model pollutant-degrading GEM *Pseudomonas* sp. strain B13 FR1(pFRC20P) to a Rhine river sediment microcosm had no effect on the ecosystem parameters studied here. The GEM accounted for 1 to 5% of the total CFU. Cores inoculated with the GEM did not differ from untreated controls during the incubation period (tested by using Fisher's randomization test for independent matched samples) with respect to concentration of chlorophyll *a* (data not shown); total CFU; population densities of starch degraders, cellulose degraders, enterobacteria, fluorescent bacteria (Fig. 6), and nitrate reducers (data not shown); incorporation of thymidine (Fig. 7); oxygen flux; and penetration depth of oxygen (data not shown). Depth profiles of the ecosystem parameters measured showed no difference between cores inoculated with the GEM and untreated controls (Fisher's randomization test [data not shown]).

Independently of the addition of the GEM, i.e., in all four treatments, the densities of starch-degrading bacteria in the microcosms decreased from days 17 to 26 (Fig. 6B). Moreover, bacterial production, measured as the incorporation rate of [^{3}H]thymidine, decreased with time in all three treated cores but not in the untreated controls (Fig. 7). After 26 days it had dropped to 69% compared with day 1. Cores treated with chemicals showed a lower thymidine incorporation rate than did untreated controls or cores treated with the GEM.

DISCUSSION

Validation of sediment microcosms. A variety of important parameters which describe microbial ecosystems did not differ between microcosms derived from the Grumbacher Teich sediment and the source ecosystem after 2 weeks of laboratory containment. Thereafter, small but significant differences occurred in a few parameters, but overall the microcosms reflected the microbial community structure and microbial activities present in the target ecosystem.

Ecosystem parameters were chosen for their ability to reflect the basic structural characteristics of the sediment ecosystem at various trophic levels and for ease of measurement with appropriate sensitivity. Some parameters, such as phosphate, nitrate, and oxygen concentrations, are chemical parameters that are easy to measure but are, however, strongly affected by biological processes. Phosphate and nitrate concentrations are the limiting nutrients for photosynthesis and are also involved in numerous microbial metabolic pathways; oxygen flux across the sediment/water interface is a result of respiration of the benthic community as a whole, and the amount of dissolved oxygen also controls the activity of aerobic microorganisms. Photosynthesis was not measured directly, but the concentration of chlorophyll *a*

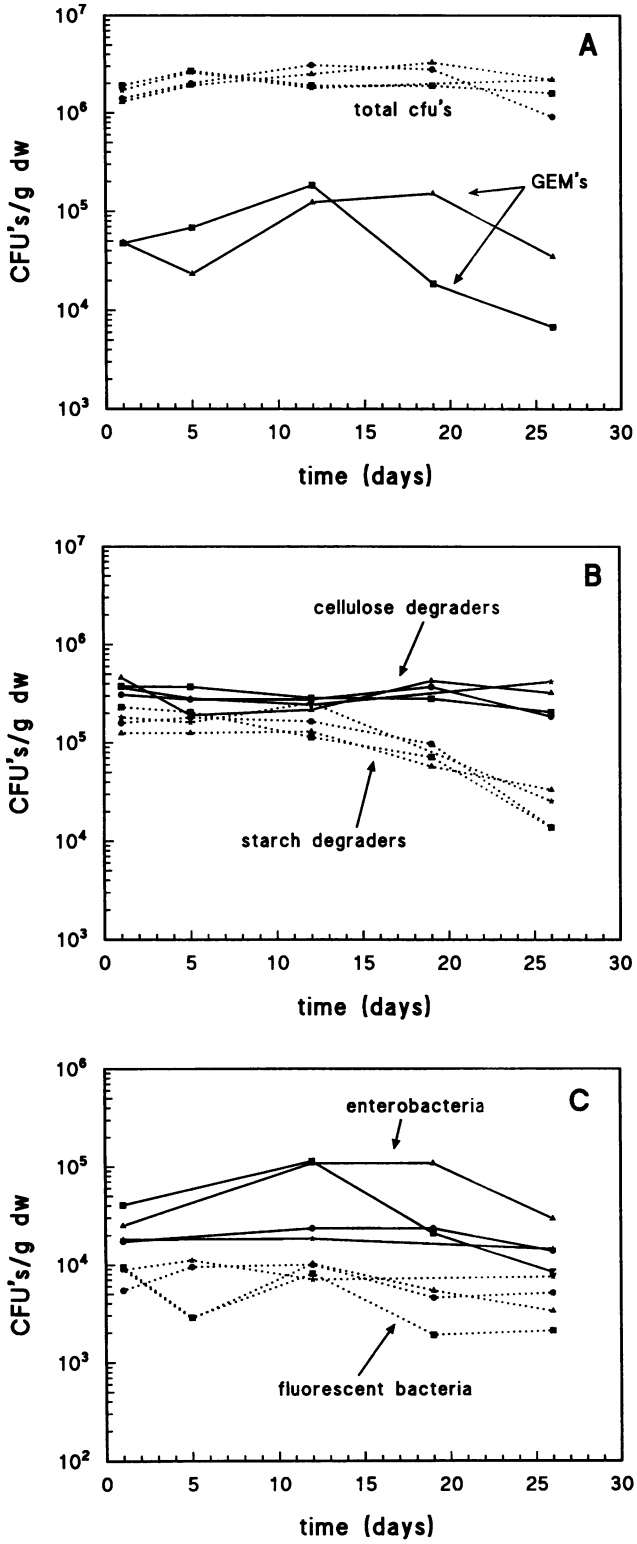

FIG. 6. Effect of the GEM on densities of functional groups of bacteria in the top layer of Rhine river sediment microcosms. Symbols reflect treatment of cores: ▲, GEM added; ■, GEM plus 3CB and 4MB added; ●, 3CB and 4MB added (control for effect of chemicals); *, untreated control. (A) Total CFU and GEMs. (B) Cellulose degraders and starch degraders. (C) Enterobacteria and fluorescent bacteria. dw, dry weight.

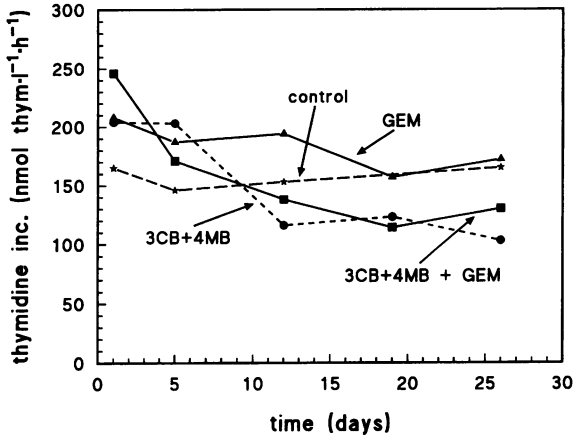

FIG. 7. Effect of the GEM and substituted aromatics on the incorporation rate of [^{3}H]thymidine in the top layer of Rhine river sediment microcosms.

was used as an indicator of photosynthetic potential. The community structure of heterotrophic bacteria was chosen because shifts in densities of important functional groups of bacteria (e.g., starch and cellulose degraders, nitrate reducers, and pseudomonads) are useful for assessing the effect of added pollutants and microorganisms, whereas total direct counts alone are probably not sensitive enough (29). Enzymatic activities were not measured, because emphasis was placed on structural responses of the ecosystem. Measurement of the bacterial community structure was based on culturing of bacteria, which neglects nonculturable microorganisms. Therefore, [^{3}H]thymidine incorporation rates were measured, since this parameter corresponds to overall bacterial growth and production and does not require culturing of bacteria. Since calculation of bacterial growth and production from rates of incorporation of [^{3}H]thymidine depends strongly on the values of the constants used for calculation, this transformation was not made and the uptake of thymidine was directly used as an ecosystem parameter.

The stability of ecosystem parameters in the laboratory microcosms reflected the stability of the lake sediment. In Grumbacher Teich the ecosystem parameters tested remained basically unchanged throughout the experiment. The experiment was carried out during the period of summer stagnation, characterized by a stable stratification of the lake, slow temperature changes (from 10 to 12°C immediately above the sediment), and stable rates of primary and secondary production (46).

Several differences between the laboratory and the lake samples appeared in weeks 3 and 4 and may be a result of confinement of the sediment in a tube and the resultant perturbation of the flow of matter and energy in the laboratory microcosm, i.e., the bottle effect. It became apparent in several ways. (i) Thymidine incorporation rates decreased. A decrease of bacterial production, which can be calculated from thymidine incorporation rates, is often observed in sediment cores and is thought to be the result of nutrient limitation (45a). In our case, however, nitrate and phosphate were not limiting, which indicated that other nutrients were not sufficiently augmented by the continuous input of site water at the selected flow rate. A higher flow rate of site water would possibly reduce the problem. (ii) Densities of pseudomonads decreased, and densities of denitrifiers increased. Since pseudomonads can use nitrate as a final

electron acceptor and some denitrifying bacteria can also use oxygen, a correlation between the density of those bacteria and the amount of oxygen or nitrate available is hard to establish. In future studies it may be useful to concentrate on bacterial genera which are obligate nitrate reducers or strict aerobes, since these may be better bioindicators of the condition of the ecosystem with respect to these parameters. (iii) The penetration depth of oxygen increased in the cores kept in the laboratory. It is well known that the penetration depth of oxygen in sediments is controlled largely by the activity of burrowing invertebrates (10). Therefore the increase in the oxygen penetration depth in the laboratory microcosms could have resulted from stronger bioturbation by invertebrates, caused by disturbance during sampling and handling, from nutrient limitation, or from mechanical stimulation on the Plexiglas wall.

Microcosm cores kept in the laboratory diverged more strongly than the lake sediment in terms of population densities of starch degraders, cellulose degraders, enterobacteria, and pseudomonads. The reason could be small differences in starting conditions for mechanisms controlling population densities, mainly competition and predation, caused by the patchiness of the environment. During the incubation period of 4 weeks, these differences were enlarged in the closed microcosms and thus resulted in various shifts in bacterial population densities.

How well can the results of the validation experiment, which was performed at a specific time of year and with a specific sediment, be transferred to other sites and other times of year? The validation experiment was carried out with samples from a fairly unpolluted lake during a period of high stability in the environment. During turnover periods for the lake, where changes in the environment are more drastic, stability in the microcosms would probably be similar to that found in the experiment reported here, but would therefore deviate more from processes in the environment. At much higher temperatures or with sediments having much higher heterotrophic activity, the processes described here may be accelerated. Conversely, during periods of low temperature, e.g., in winter, or with sediments having a much lower heterotrophic activity, stability in the microcosms and concordance with the natural environment would probably last longer than 4 weeks.

Lack of effect of a GEM on some ecosystem parameters. The introduction of ca. 10^6 GEMs per ml into the Rhine river microcosms did not affect the ecosystem parameters measured here (photosynthetic potential, thymidine incorporation rates, community structure of heterotrophic bacteria, and oxygen microgradients). For a sediment microbial community, 10^6 bacteria per ml (i.e., ca. 10^5 bacteria per g [dry weight] of sediment) is only a small fraction (ca. 0.01 to 0.001%) of the total bacterial numbers (8, 16). However, it is estimated that only about 10% of the sediment bacteria are actually metabolically active, whereas the others are dormant (28). Therefore, in terms of enzymatic activity the addition of 10^6 fully active bacteria per ml could be significant. The introduced GEMs were physiologically active at the beginning of the experiment, but could then have adopted a dormant state after the added aromatics had been used up or after they were transported to anaerobic sediment layers. Since *Pseudomonas* sp. strain B13 FR1(pFRC20P) is dependent on molecular oxygen as an electron acceptor, it is expected to function only in the oxygenized upper few millimeters of the sediment. However, this part of the sediment interacts strongly with the anaerobic zones below via diffusion and bioturbation by benthic invertebrates. As a result, the GEM was transported to deeper sediment layers (at least to a depth of 15 mm), where it remained viable during the 4-week incubation period (data not shown).

Although the addition of the GEM to the Rhine sediment microcosms had no effect, the system showed two effects related to the incubation of the sediment in the laboratory, namely a decrease in the density of starch degraders and of thymidine incorporation rates. The latter had also occurred in the validation experiment with sediment microcosms from Grumbacher Teich and had been of the same order of magnitude. Shifts in community structure had also been observed in the Grumbacher Teich sediment microcosms, although they were associated with other functional groups of bacteria. However, a decrease in the thymidine incorporation rate and minor shifts in the bacterial community structure are apparently typical for the described sediment microcosm system.

The possible effects of introducing bacteria into an ecosystem can be analyzed from two principal points of view. First, there will be observable changes of the ecosystem in terms of parameters such as biomass, enzymatic activity, predator-prey relationships, and respiratory activity. Detection of these changes depends on the appropriate resolution in space and time as well as the necessary measuring sensitivity of the parameter in question, and, most importantly, of significance. The second view relates to the assessment of possible undesirable effects associated with the introduction of a GEM into an ecosystem. These must be defined prior to analysis. Presently we rely on the fairly broad consensus that gross ecosystem processes, e.g., the flow of carbon and energy and the cycles of major nutrients, should remain unaffected (15, 23, 42–44). This was apparently the case in the present experiment. To our knowledge, only in one case has a negative influence of the introduction of a GEM into a model ecosystem been observed (39). This was due to the accumulation of toxic intermediates, which were produced because of lack of regulation under environmental conditions of an engineered catabolic pathway.

The sediment microcosm system described here appears to be a useful tool to study the ecology of biodegradation and the mechanisms governing the fate and effects of introduced GEMs prior to greenhouse or small-scale field experiments. Although the described microcosms are not identical to nature, their deviation from nature is small, can be described well, and depends on the incubation time and temperature and the ecological characteristics of the specific sediment. The system is especially suited to studying basic ecological mechanisms related to the environmental introduction of specialized strains of bacteria (these include survival, regulation in the environment of biodegradation activity, grazing by protozoa, competition with the indigenous fauna, and influence of selective pressure). Analysis of these mechanisms requires systematic manipulation of the ecological parameters in question, a condition which cannot be met in a field experiment.

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