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**Stream Microcosm for Investigating GEM Impact on the Indigenous Bacterial
Community in River Water and Sediment**

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Abstract

A flow-through stream microcosm is presented which was used to investigate the impact of the mercury reducing, genetically engineered microorganism (GEM) *Pseudomonas putida* KT2442::mer73 on the bacterial community in Elbe River sediments and water. To simulate the discharge of an industrial bioreactor into the environment, GEMs were inoculated continuously at low density into the microcosm. Experiments were carried out in three parallel microcosms of which one remained uninoculated, one was inoculated with the GEM and one with its non-recombinant parent strain *Ps. putida* KT2440. Survival of the GEM in water was low, even after cell shock with 2×10^6 cells ml⁻¹. In sediments a slight tendency towards increasing cell numbers could be observed. Community profiles based on thermogradient gel electrophoresis (TGGE) of 16S rDNA genes amplified from total community DNA showed a highly complex pattern in the sediments and a declining number of bands in the water column over the experimental period independent of the presence of GEMs. There was hardly any variation between the three treatments studied. The stream microcosm is a good laboratory representation of a river and well suitable for investigations of stream bacterial ecology. Changes in bacterial community composition in water or sediment could not be ascribed to the GEM.

Introduction

Genetically engineered microorganisms may be advantageous to wildtype isolates in industrial applications or bioremediation if they are provided with capabilities specialized for the problem to be tackled (e.g. (1-3). For the remediation of mercury polluted environments, biosorption has been explored (4-7). Recently, a technology has been developed which is based on the active enzymatic detoxification of ionic mercury by mercury resistant bacteria (8-11). A technical scale pilot bioreactor was operated continuously at European chloralkali factories and the changes in the composition of the microbial biofilm community inside the bioreactor were studied (12,12).

In an attempt to construct by genetic engineering a strain which would be both safe in the environment and more efficient with respect to mercury transformation, the mercury resistance genes (*mer* operon) were introduced into the non pathogenic safety strain *Pseudomonas putida* KT2440 by transposon mutagenesis. The expression of the mercuric resistance operon in gram-negative bacteria is induced by the *merR* gene product, which is a DNA binding protein acting as a negative regulator in the absence of the inducer [Hg^{2+}] and as an activator in the presence of Hg^{2+} (13). Since constitutive expression of the *mer* operon could be critical for cells in bioremediation applications if mercury concentrations are fluctuating between near zero and very high and prompt mercury reduction is essential for survival, the regulatory *merR* gene was excised prior to transposon mutagenesis. The resulting recombinant strains lacked the regulatory *merR* gene and carried a constitutive mercury resistance cassette stably inserted into the chromosome, thus minimizing the chance of gene transfer (14). The strain best able to cope with high concentrations of inorganic mercury was the rifampicin resistant *Ps. putida* KT2442::*mer73* (maximum concentration of 80 ppm phenyl mercuric acetate or 45 ppm mercury chloride that still permitted growth in a casamino

acids containing medium) which was selected to be studied here for possible industrial application in the bioreactor.

The field application of GEMs requires the evaluation of their possible ecological impact in case of an accidental or deliberate release into the environment. Several types of stream microcosms have been used for ecological research, mainly to study the effects of toxic chemicals (heavy metals, insecticides) or of predation on lotic or benthic macroinvertebrate communities (15-17). Rarely have stream microcosms been used in microbiology research (18). However, investigations involving stream bacteria have been carried out in stirred beakers containing water, sediment and leaves (19,20). Hill *et al.* (21) used a recirculating stream microcosm to study the persistence and mobilization of the catabolic recombinant plasmid pD10 in the epilithon. In this work, a stream microcosm was designed and characterised to be employed in experiments regarding the ecological impact of the mercury reducing GEM *Ps. putida* KT2442::mer73. If the GEM was used in a bioreactor that discharged its effluents into a river, low densities of the GEM would continuously be released. A second feasible scenario is the breakdown of the bioreactor with the sudden release of its total contents into the river, thus supplying shock loads of GEMs. The microcosm experiments reported here take both of these two scenarios into account. The liberated microbes would quickly be diluted by the water current; however, some would be able to settle down in the sediment and use it as a substrate for colonization. A supposed influence of the GEM on the indigenous bacterial community would most likely be seen here. Hence, in this work stream microcosms with river sediment were used to study the survival of the GEM and its influence on the indigenous bacterial community in sediment and water as represented by 16S rDNA community profiles on thermogradient gels.

Methods

Microcosm design. Stream microcosm design was adapted from Vogel and LaBarbera (22) and Beyers and Odum (23). The microcosms (Fig. 1) consisted of acrylic troughs (700 mm × 150 mm × 250 mm) with a loose lid. Upstream and downstream collimators were placed 75 mm from each end and were made of 20 mm thick plexiglass plates with 2000 holes (Ø 3mm) drilled into each plate. Inflow and outflow of water were directed through funnels at each head side of the microcosm. The funnels possessed an opening with a diameter of 15 mm on one end, and opened into the microcosm with a diameter of 75 mm over a length of 100 mm. For the recycling and water inflow PharMed® 6485 tubing was used with an inner diameter of 7.9 mm and 3 mm walls. Water was pumped by an L/S® (Laboratory Standard) Masterflex® Console Drive with a capacity of 6-600 revolutions per min. The drive was provided with an eight channel pump head that contained four cassettes for tubing. Water was recycled through the system at 1000 ml min⁻¹. Drainage of superfluous water was regulated by water level. The water was drained through three 15 mm holes at the downstream head of the microcosm. Pressurised air was pumped into the pre-chamber through a 25 mm × 25 mm × 25 mm aquarium aeration stone that was connected with an aquarium pump (WISA 300, Germany; 300 l h⁻¹) through silicon tubing. The aeration stone also helped distributing the inflowing water over the area of the collimator.

To determine water flow characteristics, one Pasteur pipette full (2 ml) of a saturated crystal violet solution was added directly into the inflow of water in the pre-chamber, the distribution of the dye was recorded by camera and the velocity calculated. Afterwards and before use of the microcosms in the GEM impact experiment, they were thoroughly cleaned to remove all traces of the dye. The Reynolds number which describes the turbulence of a moving liquid was calculated to be 463, indicating laminar flow. However, regions with transitional flow could be observed in the main chamber due to differences in local flow patterns.

The microcosms were filled with ca. 12.5 l of Elbe River water that was continuously replaced with tap water at a refreshment rate of 15 ml min⁻¹ (which translates into a complete exchange of water within 14 h). Tap water was non-chlorinated, non-sterilised drinking water from a reservoir in the Harz mountains in Germany. For the whole of the experiment a total volume of 3400 l of water was needed to feed the three microcosms for 53 days (duration of the experiment). Transportation of this amount of water from the Elbe River to Braunschweig, even if making several tours, was beyond our possibilities. The main chamber was filled with 4 cm of Elbe River sediment (appr. 3 L, 3.4 kg wet weight or 1.2 kg dry weight). With these settings (9 l over 50 cm at 1.03 l min⁻¹) current velocity was calculated to be 5.6 cm min⁻¹ (or $9.3 \times 10^{-4} \text{ m s}^{-1}$).

The microcosms were kept at room temperature. Water temperature was constant at 22°C, pH was 7 as determined with pH-indicator strips (Merck, Darmstadt, Germany; pH 0-14). To control primary production, the side walls of the troughs were darkened with paper cartons, and a tubular fluorescent plant growth lamp (Lampi-1915-1, 50 cm, 15W) was mounted at appr. 1.0 m above the microcosms to give a 12:12h light:dark cycle.

Inoculation with bacteria was accomplished by inserting silicon tubing from the chemostat with a long needle (Braun, Melsungen, Germany, Sterican, 0.90 × 70 mm, 20 G × 2 4/5" Luer Lock) into the microcosm through the middle of the three 15 mm diameter rubber stoppered holes at the upstream head side.

Elbe River sampling and recycling period. Water and sediment were collected in July 2002 from the Elbe River near Magdeburg, Germany, at the measurement platform at km 320. Water temperature was 17.9 °C, water pH 6.7, and oxygen content 12.9 mg L⁻¹. The composition of Elbe water is regularly monitored by the “Arbeitsgemeinschaft zur Reinhaltung der Elbe” (Working group for the cleaning of the Elbe) (<http://www.arge-elbe.de>) and ecologically important pollutant data and flow velocities are also summarized in Brümmer *et al.* (24). Surface water velocity was determined at the measuring station

approximately 10 m from the river bank by dropping Styrofoam crumbs and measuring the time they needed to travel 10 m down the river. The surface water flow velocity was 0.45 m sec⁻¹. The sediment was scraped off the bottom of the Elbe River about 3-5 m into the river off the shore, at a water depth of about 1.0 m. The sediment appeared smooth and of dark brown colour (silt). Black sediment was avoided as it was assumed to be mainly anaerobic. The sediment was sieved through a 5 mm sieve before addition to the microcosm. The water was recycled for ten days without addition of fresh water or inoculants so that the disturbed bacterial community could re-establish itself. Samples were taken regularly during this period.

Inoculation of microcosms. Two of the microcosms were inoculated from continuous cultures of *Ps. putida* KT2440 or *Ps. putida* KT2442::mer73, respectively. The chemostats consisted of 1 l Schott bottles with an exit at the bottom of the flask for withdrawal of culture suspension and another outlet above the 1 l mark for implementation of a sterile filter. They were fed with M9 minimal medium (25) amended with 10 mM sodium benzoate at 30 ml h⁻¹. A stirrer provided good mixing and aeration of the culture. Medium was added via a sterile steel needle through a silicon seal matching the Schott PBT (Polybutylene Terephthalate) lids. Cell density in the chemostats was approximately 10⁷ cells ml⁻¹. The sterile filter allowed for sterile air inflow. Bubble traps were used to prevent contamination of the medium from the chemostat and contamination of the chemostat from the microcosm.

Microcosm sampling and sample treatment. Water samples of 10 ml were taken with a glass pipette and transferred into a 50 ml tube (Greiner, Frickenhausen, Germany). After the addition of 1 ml sodium-acetate (3 M, pH 5.8) and 20 ml absolute ethanol to destroy bacterial cell membranes they were stored at -20°C.

Total DNA was extracted by centrifuging the cold sample (4°C, 20 000×g, 30 min). The supernatant was discarded and the pellet re-suspended in 1.5 ml of PB Buffer (Qiagen, Hilden, Germany). The DNA was loaded to a NucleoSpin Extract column (Macherey & Nagel GmbH & Co. KG, Düren, Germany) and centrifuged (10000×g, 1 min). Then, the DNA was

washed twice with 500 µl B5 Buffer (Macherey & Nagel) and the flow-through discarded. In order to remove residual B5 Buffer completely the column was centrifuged for an additional 2 min. The DNA was eluted with 100 µl of 70°C pre-warmed BE Buffer (Macherey & Nagel). Sediment samples were taken with a sterile 5 ml plastic pipette equipped with a blunt end. The pipette was inserted into the sediment at the centre of the microcosm and 5 ml of sediment were withdrawn in and transferred into a 50 ml tube (Greiner, Frickenhausen, Germany). From this sample aliquots of 0.5 g were transferred into a 1.5 ml reaction tube for DNA extraction with the FastDNA® SPIN Kit for soil (BIO 101, Carlsbad, CA, USA) following the manufacturer's instructions and eluting the DNA with 100 µl of sterile water. The remaining sediment was weighed (wet), mixed with Phosphate Buffer (PBS: 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄ ad 1 L, pH 7.3), in a 1:1 (w(g):v(ml)) ratio, vortexed for 1 min, sonicated in a water bath (Bandelin Sonorex Super RK510H, Berlin, Germany) for 2 min and again vortexed for 1 min. When the sediment had settled (after 15-30 min), 1.5 ml of the supernatant were transferred into a reaction tube and a dilution series was prepared with 1% NaCl for determination of colony forming units (cfu).

Enumeration of bacteria. Aliquots (100 µl) from three serial dilutions (in 1% NaCl) of fresh water or sediment samples from the microcosms were spread in triplicate on solid R2A (Fluka Chemie, Buchs, Switzerland) agar medium for the determination of total cfu. For the determination of mercury-resistant bacterial numbers, samples were spread as described above on solid R2A agar containing 5 ppm Hg (HgCl₂). GEM numbers were followed by spreading three serial dilutions of the samples in triplicate on solid M9 medium containing 10 mM sodium benzoate (Fluka, Buchs, Switzerland) as sole carbon source, 100 µg ml⁻¹ rifampicin and 1 ppm Hg²⁺ (HgCl₂). Spreading of water or sediment samples not containing the GEM did not result in colonies on the selective plates. The detection limit was at 10 cfu ml⁻¹.

Statistical analysis of cell densities. Each data point (Fig. 2A-D) represents the average from triplicate platings. Although samples from three serial dilutions were spread, the data including the standard deviation shown for the different sampling dates represent the same dilution (10^{-3}) to ensure identical treatment of the samples. To test if the sum of the differences between two curves differed statistically significantly from zero, i.e. if the curves from two of the three microcosms were essentially the same or not, the t-test for paired observations was performed. This test is applicable if the samples are dependent, e.g. if they are derived from the same original sample but have been treated differently in the course of the experiment as in this case. Since three curves had to be compared with each other, this test was repeated for all three combinations (microcosm 1 vs. microcosm 2; microcosm 1 vs. microcosm 3; microcosm 2 vs. microcosm 3). The Null hypotheses were stated as: $H_{0-1}: \mu_1 - \mu_2 = 0$; $H_{0-2}: \mu_1 - \mu_3 = 0$; $H_{0-3}: \mu_2 - \mu_3 = 0$, μ representing the cfu average over the course of the experiment for a given microcosm, and all three t-values were compared against the critical t-value for a two-sided t-test at a significance level of $\alpha = 0.05$ (Table 1), with 15 (total bacteria in water) or 16 (all other curves) degrees of freedom ($df = n-1$). The t-value was calculated

$$\text{with } t = \frac{\sum_{i=1}^n (\bar{x}_i - \bar{y}_i) / n}{\sqrt{\frac{\sum_{i=1}^n (\bar{x}_i - \bar{y}_i)^2 - (\sum_{i=1}^n \bar{x}_i - \bar{y}_i)^2 / n}{n(n-1)}}}, \text{ where } n \text{ is the number of data pairs and } \bar{x}_i \text{ and } \bar{y}_i$$

are the mean data points from triplicate for two specific microcosms at a specific sampling day i.

16S rDNA gene amplification. PCR for TGGE was carried out with AmpliTaq® DNA Polymerase Stoffel Fragment (Perkin Elmer, Rodgau-Jügesheim, Germany), a recombinant polymerase with no associated 3' to 5' nuclease activity and enhanced thermal stability. The reaction mixture (20 μ l) contained 1 $\times$ Stoffel Buffer (Perkin Elmer), 3 mM $MgCl_2$, 1/20 volume DMSO, 0.2 mM dNTPs, 100 nM of each primer (U968-GC1: 5'-

CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG-

AACGCGAAGAACCTTAC-3', R-1401: 5'-CGGTGTGTACAAGGCCC-3'; (26), 5 units of Stoffel Fragment and between 0.5 to 2 µl of community DNA template. The 16S rDNA was amplified using a 7 min denaturation step at 94°C, 35 cycles of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C. The final extension step was performed for 10 min at 72°C. One Microliter of PCR products were visualized in an 0.8% agarose-gel.

TGGE. For separation 5 µl of the PCR products were loaded on a 0.8 mm polyacrylamide gel (6% w/v acrylamide, 0.1% w/v bis-acrylamide, 8M urea, 20% formamide, 2% glycerol) with 1× MOPS buffer (20 mM 3-morpholinopropanesulfonic acid, 1 mM EDTA, pH 8.0) and exposed to a 37°C to 47°C (sediment samples) or 35°C to 45°C (water samples) thermal gradient with a fixed voltage of 350 mV (ca. 28 mA) in a Qiagen TGGE system (Qiagen, Hilden, Germany). After the run, the gel was fixed for 3 min in 10% ethanol/ 0.5% acetic acid and then stained with 0.2% AgNO₃ (dissolved in 10% ethanol/ 0.5% acetic acid). The gel was developed in 3% NaOH with 1/200 volume formaldehyde.

TGGE Standard. The TGGE standard was composed of a mixture of PCR amplified 16S rDNA gene fragments of various soil bacteria (27). As templates served the genomic DNAs of *Erwinia carotovora* subsp. *carotovaora*, *Agrobacterium tumefaciens*, *Erwinia herbicola*, *Burkholderia gladioli*, *Streptomyces aureofaciens*, *Actinomyces* sp. strain QMB-814, *Clostridium pasteurianum*, *Rhizobium leguminosum*, *Actinosynnema mirum*, *Actinoplanes auranticolor*, and *Pseudomonas fluorescens* R2f.

Results

Survival of the GEM. The microcosms were allowed to stabilize for 10 days prior to inoculation (recycling period) with a re-circulation of water at 1000 ml min^{-1} . For comparison, Eichner *et al.* (28) operated laboratory scale sewage plants which they allowed to equilibrate for 1 week before they started with their experiments. On day 10, the tap water inflow was opened and fresh water was added to the microcosms at 15 ml min^{-1} . *Pseudomonas putida* KT2442::mer73 was inoculated continuously into the system from day 10 on to yield GEM numbers in the water between 10^1 and 10^2 cfu ml^{-1} (Fig. 2 A&B). After the first full exchange of water (after 14 h) steady state conditions with an equilibrium between bacterial wash-out and growth were established. Re-circulation in our model was the best possible way to maintain a stable bacterial community without access to natural river water and also permitted much faster flow velocities. Two weeks after the start of inoculation, a cell shock was performed by inoculating with the GEM to yield $2 \times 10^6 \text{ cfu ml}^{-1}$ to simulate a bioreactor breakdown. In the water, the GEM did not grow to higher cell numbers than those introduced, neither by continuous inoculation of low cell numbers, nor through introduction of large cell numbers. In the sediment, a tenfold increase in cfu of the GEM could be observed over the duration of the experiment. However, cell numbers were fluctuating and a longer observation period would have been necessary to confirm this trend.

Effect of the GEM on total and mercury resistant colony forming bacteria. To determine an influence of the GEM on cultivatable total or mercury resistant bacterial numbers, colony forming units (cfu) were investigated over the course of the experiment (Fig. 2). Total bacterial densities declined over time by one order of a magnitude, both in the sediment and in the water. However, the same trend could be observed in all three microcosms and thus cannot be ascribed to the GEM. Mercury resistant bacterial densities

remained stable after inoculation for about five weeks both in the water and in the sediment before declining 5-10fold.

During the recycling period total bacterial densities remained stable in the sediment and increased 5fold in the water. In addition, mercury resistant bacterial densities increased in the water from 3×10^2 to approximately 10^4 cfu ml⁻¹ during this time period, i.e. before the GEM was introduced. At the same time they decreased by one order of magnitude in the sediment. These effects may have been caused by population shifts as a result of the new constraints of the microcosm (“artefacts of enclosure”, “succession and self-organisation”, “bottle effect”):(25,29,30). After the cell shock on day 24, a brief increase of total and mercury resistant bacterial densities in the water could be observed in the GEM inoculated microcosm. Mercury resistant bacterial densities were similar to those determined for the GEM, and after 8 days they had declined to densities found in the control microcosms. The same effect was seen for total bacterial densities in the water. In the sediment, densities of total and mercury-resistant bacteria remained unaffected by the cell shock.

Statistical analysis (t-test for paired observations) of total and mercury resistant bacterial densities in the water and sediment supported the conclusion that there was no significant difference between the two curves at a significance level of $\alpha = 0.05$ (Table 1).

Influence of the GEM on the indigenous bacterial community. To determine whether the GEM disturbed the indigenous microbial community in the microcosms, the 16S rRNA genes were amplified from the total DNA of sediment and water samples and separated on thermogradient polyacrylamide gels to generate community fingerprints (Fig. 3).

Sediment samples yielded an extremely complex profile (Fig. 3A). More than 50 bands could be detected with the naked eye. For comparison, Eichner *et al.* (28) could find a maximum of 18 bands in activated sludge samples. The TGGE 16S rDNA profile from soil presented by Engelen *et al.* (26) contained ca. 20-30 bands, and Rasmussen and Sørensen (31) could visualise 25 vs. 50 16S rDNA bands for mercury contaminated soil vs. untreated soil.

A small community shift could be observed as the sediment was transferred from the Elbe River to the microcosms (confinement effect, see above). Small differences between the microcosms could further be observed in the beginning before inoculation ($d = 4$), which might well be within the experimental error since they often occurred in all three samples from one date. Later, there were no detectable differences between the microcosms.

Fewer bands could be amplified from the water samples (Fig. 3B). This was probably caused by differences in diversity, as discussed by Torsvik *et al.* (32). A drastic decline in the number of bands after transfer of the samples to the laboratory could be observed, with more than 30 bands for the sample “Elbe $d = 0$ ”, representing the undisturbed Elbe river water community, and approximately 20 bands, some of which became very dominant, for microcosm samples from day 21 to day 42. Differences between the three microcosms were undetectable on day 4 (before inoculation) and on days 21-42 (after inoculation). Pronounced differences were, however, observed on day 14 (after inoculation). This was also the time of a reduction in diversity from 30 to 20 bands, caused by growth of some phylotypes and disappearance of others. Thus, differences in community profiles may have been caused by differences in the succession process, although the profiles eventually converged on day 21. The 16S rDNA band from a pure culture of the GEM ran at the same height as a community band which was present in all three microcosms before inoculation. It may have originated from indigenous *Pseudomonas* bacteria, which are widespread in natural soils, sediments, and water, but this would need to be confirmed by sequencing.

Discussion

Genetically engineered microorganisms possess a great potential and economical value for research and industry. However, concern has arisen about their safety and their influence on the environment if released either on purpose or by accident and the issue has entered political discussion, e.g. by the United Nations Environmental Programme (33) and legislature (34).

Microcosm design. In nature flow varies appreciably within the cross-section of a river. While it reaches greatest velocity at the water surface, current is reduced to near zero at the bottom of the river. Flow patterns and velocity control the availability of nutrients and the forces applied on organisms living within the stream and at the water-sediment interface (35-37). The surface velocity of the Elbe River at the time of sampling was 0.45 m s^{-1} . Even with seasonally fluctuating discharge volumes (24) this speed should not vary much, since mean current speed at a particular station along a river only doubles for every tenfold increase in discharge (37). The Elbe River velocity was 136fold higher than the visually determined speed in the microcosm. However, from the water surface to the riverbed flow decreases significantly and the depth of the Elbe River exceeded microcosm depth by far. The flow very near the sediment layer was visually determined to be very slow in our microcosms. Therefore, near bed velocity under natural river conditions was reflected well by the microcosms. In the environment laminar flow (shallow, very slowly moving water) is rarely found and turbulence mostly the rule. Flow in our microcosms was not highly turbulent, however it showed turbulence to some degree (transitional flow), thus resembling real environmental conditions well.

The microcosm as a model river. Rivers are extremely complicated ecosystems which are comprised of many different habitats (e.g. water, sediments, biofilms) that are intricately interconnected (19,23,38). Bacteria form biofilms on plant material surfaces immersed in water or in sediment but may be washed off and carried downstream with the water phase.

During floods flow is elevated and more turbulent, sediments are re-suspended and transported downstream, resulting in a higher release of bacteria by abrasion. Rates for attachment, detachment and mortality are important determinants for the distance a bacterium is carried down the length of a river (19). Flow rate can be considered the most important abiotic force in streams. This means that bacterial dissemination will be mainly controlled by current speed. In addition, rivers run for several hundreds of kilometres, facing tributaries, waste water influents, changing vegetation, geology and agricultural use (35). Thus, it is impossible to consider all possible parameters that influence the survival of a GEM in a river. Sayler and Ripp (39,40) emphasized the need for GEM field release experiments for environmental impact studies and gave a rare example for such an investigation. If a field release is not feasible, maximum authenticity in the laboratory can be achieved by the closest possible connectivity to the environment that is being investigated. For example, had our laboratory been closer to the Elbe River, we could have continuously added fresh river water and not have had to improvise by using water from the tap. The operating expense and expenditure of our experimental set-up would, however, have increased immensely. The addition of tap water instead of fresh river water (1) probably introduced different bacteria to the microcosm and (2) did not exactly resemble the river water. Our results might have qualitatively been affected by this (e.g. by obtaining a different TGGE pattern), however, so they would have been, had we sampled the Elbe River some kilometres downstream or upstream or had we sampled the river at a different time of the year and so on. The main conclusion from our results, namely that there was no difference between the microcosms, regardless of whether they had been inoculated with the GEM, the non-recombinant parent strain, or if they were not inoculated at all, remained the same. Thus, we have tried to provide a system for GEM safety studies that would more realistically represent a river environment than previous studies have attempted. This microcosm was consequently used for studies regarding the influence of a GEM that was constructed for the bioremediation of mercury-

contaminated waste water. Nevertheless, the microcosms can be universally applied to investigate stream bacterial ecology (41).

Survival of the GEM. Survival of introduced bacteria depends on biotic and abiotic factors. Nutrients as well as predation are major determinants, and temperature, pH, oxygen demand, and water activity play additional roles in the adaptation and survival process. Liang *et al.* (42) suggest “that some species persist in environments in which they are not indigenous because they tolerate abiotic stresses, do not lose viability readily when starved, and coexist with antagonists. The species that fails to survive need only be affected by one of these factors”. So far, GEM survival in rivers has mainly been studied by inoculation with large cell numbers of the strain at the beginning of the experiments and following the die-off (19,43-45). Here, we investigated an alternative scenarios, e.g. leakage of the GEM-containing system and subsequent escape of relatively small numbers of bacteria over a considerable time period. Despite rapid dilution of the GEM by the current, establishment of the strain would be conceivable due to continuous re-inoculation from the leakage. However, we could not observe increasing cell numbers in the water and only a slight increase in the sediment. *Ps. putida* is wide spread in the environment. With a number of closely related competitors and no selective pressure offering an advantage for the GEM, increased growth would be difficult to achieve. Nevertheless, persistence below the detection limit of 10 cfu ml⁻¹ could not be determined here.

Impact of the GEM on the microbial community. TGGE/DGGE analyses have proven to be powerful tools to investigate the composition of complex microbial communities based solely on the 16S rDNA sequences of the organisms in the sample, thus avoiding cultivation-derived artefacts (46,47). Separation of the 16S rDNA gene fragments on a polyacrylamide gel and the application of a thermogradient (or chemical gradient, as in DGGE) allow discrimination of sequences of the same length differing in only one base (48-50). However, when studying microbial communities, the TGGE banding patterns are subject to bias owing

to selectivity during DNA extraction and PCR (e.g. preferential amplification of some sequences and chimera formation) (51). Moreover, TGGE/DGGE can only show the predominant species present, i.e. those that comprise 1 % or more of the total community (47), leaving a large number of phylotypes undetected. However, TGGE/DGGE investigations are well suited to monitor changes in the diversity of complex bacterial communities caused by disturbances (e.g. GEM influence, pollutants, temperature, grazing, pH). Eichner *et al.* (28) have used TGGE analysis to determine changes in community patterns after a pollutant shock with and without the presence of a pollutant-degrading GEM. For soil, the effect of root exudates contaminated with different levels of heavy metals as well as the impact of mercury and herbicides on a bacterial soil community have been investigated using TGGE/DGGE (26,31,52). Here, the effect of the genetically engineered *Ps. putida* KT2442::mer73 on sediment and water communities was examined. Moreover, one microcosm was inoculated with the non-recombinant *Ps. putida* KT2440 parent strain to exclude an effect of the inoculation of a *Ps. putida* strain on the microbial community. The resulting 16S rDNA patterns were highly complex for the water samples and even more so for the sediment samples, forming a solid basis for interpretation. However, an effect of inoculation could not be detected.

Conclusions. In this study, the ecological impact of a genetically engineered strain was studied which was constructed for maximum safety in the environment. Thus, a plasmid-less *Ps. putida* safety strain was chosen as the parent organism. The introduced trait, e.g. mercury resistance, was stably inserted into the chromosome, and no antibiotic resistance gene was introduced (14). Both host strain and mercury resistance are common in soils and sediments (53). If the GEM were released, the environment would face a ubiquitous strain, carrying a gene that is equally wide spread in nature. In the absence of mercury, the constitutive expression of the *mer* structural genes might even represent a competitive disadvantage to the

GEM. However, although negative impacts of the GEM are not to be expected based on these theoretical considerations, experimental evidence is needed prior to release.

An influence of the GEM *Pseudomonas putida* KT2442::mer73 on the indigenous microbial communities in river water and sediment microcosms could not be observed by TGGE, nor did the numbers of total bacteria or mercury resistant bacteria change except for a brief increase in the water just after the cell shock. A change in total bacterial densities as well as a decline in the number of TGGE bands in the community fingerprints occurred in all three microcosms and can be attributed to the effect of confinement.

However, for a more complete assessment of the strain's environmental impact additional parameters would need to be studied, especially the behaviour of the GEM under mercury selective pressure as well as protozoan and viral influence on survival. Experiments conducted to investigate the genetic stability of the mercury resistance genes in *Ps. putida* KT2442::mer73 are presented elsewhere (54). Laboratory simulations of the environment will always remain imperfect. However, the laboratory offers the possibility to test different parameters step by step and determine their contribution to the question under investigation. This study contributes a model system for research on stream microbial ecology and gives an application.

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Figure Legends

Fig. 1 Schematic view of the stream microcosm. The GEM and the parent strain *Ps. putida* KT2440 (“wildtype” = wt) were cultivated in separate chemostats with benzoate as sole carbon source and added continuously to the respective microcosms. Water and the inoculant strain were mixed in the pre-chamber by bubbling air in through an aeration stone. Re-circulation of water enabled faster flow velocities and allowed the establishment of a stable bacterial community.

Fig. 2 Total bacterial densities (A,C) and densities of mercury resistant bacteria (B,D) in the water (A,B) and sediment (C,D) of three parallel microcosms: [---◆---] microcosm (1) inoculated with the GEM; [.....▲.....] microcosm (2) inoculated with the parental wild type strain; [—□—] uninoculated control microcosm (3); and [—×—] GEM. Elbe river water was recycled through the microcosms for 10 days before continuous addition of tap water and inoculation were started. At day 24 shock loads of the GEM were added yielding 2×10^6 cells ml⁻¹. For every data point the bacterial densities were determined in triplicate. The standard deviation is indicated and if not visible it was smaller than the data point symbol.

Fig. 3 Community profiles based on thermogradient gel electrophoresis (TGGE) of 16S rDNA gene fragments amplified from total community DNA of sediment (A) and water (B) over the time course of the microcosm experiment. A 16S rDNA marker (lanes 1, 9, 18, 25; see text for composition of the marker) and the pure GEM (lanes 10 & 17) were included in the gel for reference. Of the three parallel microcosms, one remained uninoculated (1), one was inoculated with the non recombinant parent strain *Pseudomonas putida* KT2440 (2) and one was inoculated with the GEM, *Pseudomonas putida* KT2442::mer73 (3). The days of sampling are indicated above the sample triplets. Although changing banding patterns can be

observed for different sampling dates, differences between microcosms remain minimal (see text).

Table 1 Statistical analysis of the significance of differences between the three microcosms for densities of total and mercury resistant bacteria. The t-values for the statistical analysis of the cfu data obtained for total and mercury reducing bacteria in the water and in the sediment are given. The critical t-value for a two sided t-test at a significance level of $\alpha = 0.05$ and 15 degrees of freedom (total cfu in water) was 2.131, for all other curve pairs it was 2.120 ($\alpha = 0.05$, $df = 16$).

Table 1

t-values	Microcosm I vs. Microcosm II (+GEM/ +parent strain)	Microcosm I vs. Microcosm III (+ GEM/ uninoculated)	Microcosm II vs. Microcosm III (+parent strain/ uninoculated)
Total cfu in water	0,357	0,719	0,635
Hg-resistant cfu in water	0,231	1,619	1,478
Total cfu in sediment	-1,094	-1,159	0,021
Hg-resistant cfu in sediment	-0,883	1,018	2,012

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Figure 1

Fig. 2 A, B

Figures 2C, D

Fig. 4