# Long-Term Performance of Bioreactors Cleaning Mercury-Contaminated Wastewater and Their Response to Temperature and Mercury Stress and Mechanical Perturbation

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**Abstract:** The long-term performance of bioreactors retaining mercury from contaminated industrial wastewater was analyzed at the laboratory scale, and its response to mechanical perturbations (gas bubbles and shaking) as well as to physical (increased temperature and hydraulic load) and chemical stresses (increased mercury concentration) likely to occur during on site operation was studied. Two packed-bed bioreactors with 80-cm$^3$ lava chips as biofilm carrier were inoculated with nine Hg(II)-resistant natural isolates of $\alpha$- and $\gamma$-proteobacteria. Chloralkali wastewater containing ionic mercury (3.0 to 9.7 mg/L $Hg^{2+}$), amended with sucrose and yeast extract, flowed through the bioreactors at 160 mL/h. During the 16-month investigation the bioreactors showed no sign of depleted performance in terms of mercury-retaining capacity. After 16 months, both bioreactors still retained 96% of the mercury load. The performance of the bioreactors was sensitive to mechanical perturbations (e.g., sheer forces of gas bubbles). Shifts to higher $Hg^{2+}$ inflow concentrations initially decreased the mercury retention efficacy slightly. However, the bioreactors could adapt to $Hg^{2+}$ concentrations of up to 7.6 mg/L within several days. Old biofilms were less affected than the younger ones. The performance of the bioreactors was not affected by an increase in temperature up to 41°C and an increased volumetric load (up to 240 mL/h). The bioreactors regained activity spontaneously after the stress had stopped. Recovery could be accelerated by increased nutrient concentration, although this may lead to blocking of the packed bed. © 2001 John Wiley & Sons, Inc. *Biotechnol Bioeng* **74**: 212–219, 2001.

**Keywords:** bioreactors; mercury-contaminated wastewater; mercury stress; mechanical perturbation

## INTRODUCTION

Mercury and mercury compounds are extremely toxic (Langford and Ferner, 1999), yet they continue to be used widely in industry. Industrial dumping of mercury into rivers, the combustion of coal, and solid waste incineration have led to significant mercury pollution of the environment (Bryan and Langston, 1992; Zilloux et al., 1993). The efficient elimination of the dilute mercury from large volumes of contaminated waste streams (e.g., chloralkali electrolysis wastewater) by conventional physical or chemical methods is technically difficult and expensive. Therefore, a biotechnological approach was pursued, which is based on the active enzymatic reduction of ionic mercury ($Hg^{2+}$) to metallic mercury ($Hg^0$), a transformation carried out by mercury-resistant microorganisms as a detoxification mechanism (Izaki et al., 1974; Robinson and Tuovinen, 1984; Summers, 1986). It is encoded by the microbial *mer* operon, and involves regulatory proteins, transport proteins, and the enzyme mercuric reductase, encoded by the *mer*A gene (Hobman and Brown, 1997; Silver and Misra, 1988; Silver and Phung, 1996).

The removal of mercury from wastewater by Hg(II)-reducing biofilms was first demonstrated by Brunke et al. (1993) using synthetic Hg(II) solutions. We showed that this process is also applicable to industrial wastewater of the chloralkali industry (von Canstein et al., 1999). The process took place in laboratory-scale packed-bed bioreactors, where the bacteria were immobilized as active biofilms on carrier chips and fed continuously with wastewater amended with carbon sources, because mercury reduction is a process that consumes metabolic energy. The bacteria import the ionic mercury into the cytoplasm in an active manner. Subsequently, the mercury is reduced by the cytosolic enzyme mercuric reductase to Hg(0), whereby Nicotinamide Adenine Dinucleotide Phosphate (NADPH) is consumed (Silver and Misra, 1988). The metallic mercury atoms diffuse out of the cells where they stick together, resulting in steadily increasing mercury droplets of several microns in diameter, which remain in the matrix of the bioreactor (Brunke et al., 1993).

Previous fixed-bed column experiments have focused on the performance of communities and pure cultures of natu-

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ral mercury-resistant isolates for Hg(II) retention from synthetic $HgCl_2$ solutions. The community retained 82% to 99% of the load of 1 to 10 mg/L Hg(II) (Frischmuth et al., 1993). Bioreactors inoculated with a pure culture of *Aeromonas hydrophila* HGS2 were run for up to 100 days with a maximum retention rate of 99% at 10 mg/L Hg(II). The retention efficacy remained unaffected by an increased Hg(II) concentration or hydraulic load of up to 4 bed volumes per hour (Brunke et al., 1993). A bioreactor with a pure culture of *Pseudomonas putida* Spi 3 was not inhibited by an increase in sodium chloride concentration of up to 24 g/L and $HgCl_2$ concentrations of up to 7 mg/L Hg(II), and obtained mercury retention rates of 95% to 99%. A partial breakthrough occurred, however, after 1 day at 9 mg/L Hg(II) (von Canstein et al., 1999).

The process was then applied to the cleaning of industrial chloralkali wastewater from three different factories in Europe and maximum retention rates of 97%, 98%, and 98.5%, respectively, were obtained (von Canstein et al., 1999). The development of a process for remediation of chloralkali wastewater based on microbial mercury reduction required answers for questions arising from on site operation of a bioreactor on a technical scale. In this work we use laboratory-scale bioreactors and focus on the long-term stability of the process, the expected lifetime of the bioreactors, and the response to on site–specific stresses like fluctuations in Hg(II) concentration, high temperatures (caused by the continuous neutralization of the acidic or alkalic wastewater), and accidental mechanical perturbations caused by mixing, gas bubbles, or pump failures.

## MATERIALS AND METHODS

### Bioreactor Setup

The bioreactors had double-glass walls for cooling or heating by water, an internal diameter of 5.5 cm, and a height of 13.5 cm (W. O. Schmidt, Braunschweig, Germany). The columns were filled with 80 $cm^3$ of lava chips (diameter 1.0 to 1.5 cm). A bottom grid ensured an even inflow distribution. Columns and tubing were sterilized by autoclaving (121°C, 20 min). Nonsterile, neutralized, oxygen-saturated chloralkali electrolysis wastewater was pumped into the bioreactors in upflow mode using a peristaltic pump. Sterile medium was added with a second peristaltic pump. The total volume entering the column was 160 mL/h (unless indicated otherwise), consisting of nutrient medium (8 mL/h) and wastewater (152 mL/h). A bubble trap was used to prevent medium contamination and also to prevent the transport of gas bubbles into the columns. For inoculation, a bypass was used. Most of the time (383 of 486 days) the nutrient amendment resulted in a final concentration of 0.1 g/L sucrose and 0.02 g/L yeast extract in the wastewater inflow. During the first 5 days, nutrient supply was increased to allow growth of the biofilm on the carrier material (days 0 to 3 sucrose and yeast extract 1.0 g/L each; days 3 to 5 sucrose and yeast extract 0.5 g/L each). To correct decreased bioreactor performance, the nutrient concentration was temporarily increased. Nutrients were dissolved in a salt solution (10 g/L NaCl) until day 25; thereafter, they were dissolved in aliquots of the actual wastewater batch.

### Wastewater

The wastewater used was process wastewater from a chloralkali plant (Elektrochemie Ibbenbüren [ECI], Ibbenbüren, Germany), provided in batches of 800 L in polyethylene containers. The wastewater had pH levels of 2.1 to 2.6 and was neutralized by addition of NaOH (5 *M*). Oxygen saturation was obtained by bubbling with compressed air for 2 to 5 h. The wastewater batches differed with respect to mercury concentration (3.0 to 9.7 mg/L, average 6.2 mg/L) and chloride concentration (8.8 to 32.5 g/L, average 19.5 g/L).

### Strains

The strains *Pseudomonas putida* (*Pp*) Spi 3, *Pp* Spi 4, *Klebsiella* sp. (*K*sp) Spi 5, *Sphingomonas* sp. (*S*sp) Spi 7, and *Pseudomonas fulva* (*Pf*) Spi 11 were isolated from sediments of the Spittelwasser River, a tributary of the Elbe River. Strains *Pp* Elb 2 and *Pf* Elb 5 were isolated from sediments of the Elbe River. Strains *Citrobacter freundii* (*Cf*) Tin 2 and *Pp* Kon 12 were isolated from the outflow of mercury-reducing bioreactors run previously. The strains were identified by the German Culture Collection of Microorganisms and Cell Cultures (DSMZ) using partial 16S rDNA sequence analysis, physiological tests, ribotyping, and fatty acid methylester (FAME) analysis.

### Inoculation

The bioreactors were inoculated with a mixture of nine cultures of strains Elb 2, Elb 5, Kon 12, Spi 3, Spi 4, Spi 5, Spi 7, Spi 11, and Tin 2. For cultivation of the inoculum, one colony of each strain was picked from a plate [sucrose 5 g/L, yeast extract 5 g/L, Hg(II) 1 mg/L] and suspended in 100 mL of neutralized, nutrient-amended chloralkali wastewater [Hg(II) 4.0 mg/L, $Cl^-$ 8.8 g/L, sucrose 4 g/L, yeast extract 2 g/L], and grown at 30°C with a rotary shaker for 2 days. Thereafter, the nine separate cultures were combined. Two hundred forty milliliters of the mixed culture inoculum was pumped through each sterilized bioreactor at 80 mL/h for 3 h. At day 61, after 6 days of low mercury retention, the bioreactors were reinoculated as described earlier to regain an active biofilm.

### Determination of Mercury and Chloride Concentration

The reactor effluent samples were collected in vials containing 1% of the end-volume $HNO_3$ (65%) to stabilize solved mercury.

Total mercury was determined by flameless cold vapor absorption spectroscopy using a flow injection system (FIAS 200, Perkin-Elmer, Überlingen, Germany) that was linked to an atomic absorption spectrophotometer (AAS 2100, Perkin-Elmer). For determination of total mercury, samples (1 mL) were oxidized with 150 μL $KMNO_4$ (5%), 10 μL $H_2SO_4$ (96%), and 10 μL $HNO_3$ (65%). After 2 min, 100 μL $K_2S_2O_8$ (potassium peroxodisulfate, 4%) was added and mixed. After 10 min, 230 μL $H_3NOHCl$ (hydroxyl ammonium chloride, 10%) was added and mixed until decolorization of the sample occurred. By addition of deionized water the sample was diluted to Hg concentrations of <100 μg/L (maximum value of calibration). Subsequently, the sample was injected and ionic mercury was reduced with $NaBH_4$ (4 g/L) to metallic mercury, which was volatilized by the carrier gas argon and detected at 253.7 nm by the AAS. Chloride concentrations were determined photometrically with kits from Dr. Lange (Dr. Lange, Düsseldorf, Germany).

### Determination of Culturable Cell Number

Densities of culturable cells were determined in the outflow of the bioreactors by counting the colony forming units (cfu) on complex, Hg-free medium. A representative sample (50 mL) was collected within approximately 20 min. The sample was mixed well and an aliquot (100 μL) was diluted in tenfold dilution steps with a sterile salt solution (NaCl 15 g/L). Fifty microliters of the expected appropriate dilution (minimum 10, maximum 100 cfu/plate) was plated in triplicate on agar plates (NaCl 15 g/L, sucrose 4 g/L, and yeast extract 2 g/L) immediately after sampling. Plates were incubated at room temperature for 7 days before counting.

## RESULTS

### Long-Term Performance of Mercury-Reducing Bioreactors

Figure 1 shows the mercury concentration in the inflow and in the outflow of bioreactors A1 and A2 for the investigated time of 480 days. The bioreactors were run with a hydraulic load of 2 bed volumes (bv) per hour, except for days 142 to 160 (1 bv/h) and days 181 to 195 (3 bv/h). Until day 330, both bioreactors were run in parallel at room temperature of approximately 22°C. Bioreator A1 was then stepwise warmed to 30°, 35°, 38°, and 41°C to simulate the high wastewater temperatures on site at a chloralkali plant. Bioreactor A2 remained as a control at room temperature until day 480.

During the observed time of 480 days we found no sign of age-dependent reduced performance. At day 21, both bioreactors retained 94% of 5.5 mg/L Hg(II) inflow, similar to day 480 with 6.9 mg/L Hg(II) inflow. The maximum mercury retention efficacy reached 97% at day 379 [inflow concentration of 7.6 mg/L Hg(II)]. The minimum Hg outflow concentration was 140 μg/L (days 352 and 436). At optimum performance, for instance, from day 440 to 460, an average outflow mercury concentration of 278 μg/L was obtained. In response to stress situations, partial and total mercury breakthrough was observed on several occasions. However, the bioreactors spontaneously regained activity within a few days, accelerated by nutrients or by dilution of the inflow wastewater. After 480 days, 1835 L of CAE wastewater with an average mercury concentration of 6.2 mg/L Hg(II) had passed through each bioreactor. During this time of massive physical and chemical stresses to the

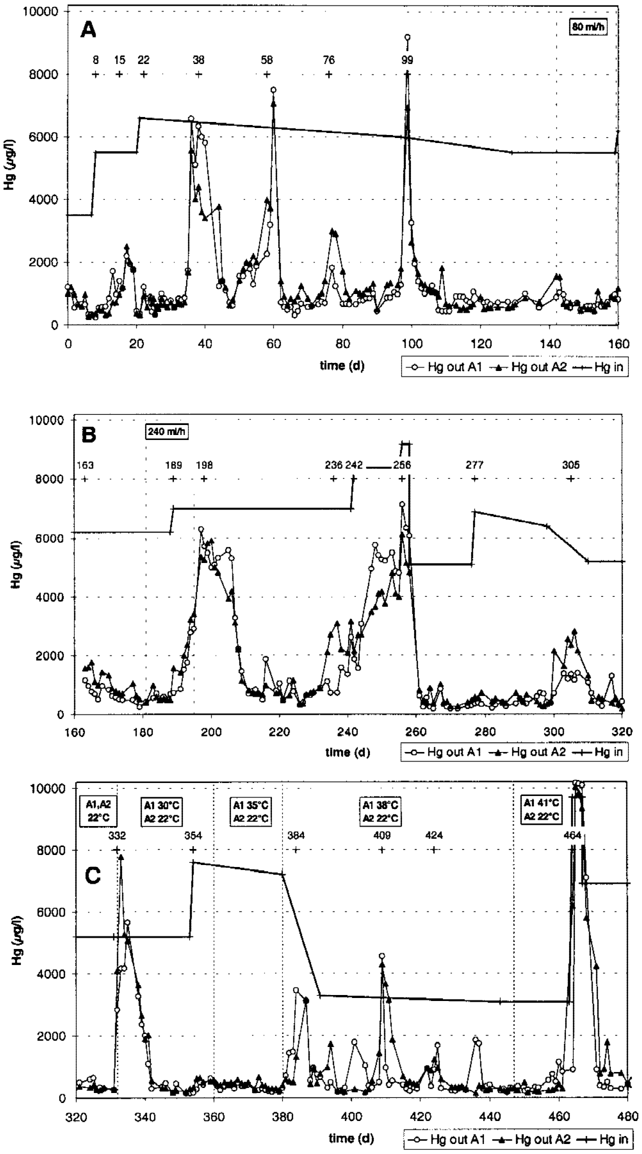

**Figure 1.** Performance of two mercury-retaining packed-bed bioreactors (A1 and A2) operated in parallel during 480 days with different batches of chloralkali wastewater. The hydraulic load was 2 bed volumes/hour and temperature was approximately 22°C, unless noted otherwise. The numbers in the top line indicate the days of mercury inflow shifts and peaks of mercury outflow concentration referred to in the text. (+) Mercury inflow concentration; (○) mercury outflow concentration of bioreactor A1; (▲) mercury outflow concentration of bioreactor A2. (A) Days 0 to 160. (B) Days 160 to 320. (C) Days 320 to 480.

biofilm, the overall retention rates of the bioreactors A1 and A2 were 79% and 78%, respectively, which corresponded to 9.1 and 9.0 g retained mercury.

## Chronology of Perturbations and Stresses During Long-Term Operation

During 480 days of operation, both bioreactors were subjected to perturbations and stresses, resulting in mercury outflow peaks, as indicated by their date in Figure 1, and are highlighted chronologically as follows. The first peak of Hg outflow concentration around day 15 occurred after a shift in Hg inflow concentration from 3.5 to 5.5 mg/L at day 8. Because the wastewater was amended only with sucrose from days 5 to 18, the detoxifying effect of yeast extract (Farrell et al., 1993) was missing. The bioreactors regained activity after increasing the concentration of yeast extract (0.1 g/L) at day 18.

At day 21, the Hg inflow concentration was increased from 5.5 to 6.6 mg/L, followed by only a minor increase in Hg outflow concentration at day 22. Because the wastewater was still amended with yeast extract (0.1 g/L), the Hg shift was less severe at this timepoint.

At around day 38, the Hg outflow concentration peaked again, caused by gas bubbles passing the bubble trap and entering the bioreactors. The sheer forces of the gas bubbles disrupted the biofilm and large portions were subsequently washed out, resulting in muddy outflow samples. The Hg outflow concentration increased drastically for two reasons: first, the washed-out biofilm fragments already contained large amounts of metallic mercury; and, second, only parts of the Hg-retaining biofilms remained in the bioreactors. The bioreactors recovered completely in the next 10 days, due to the increased nutrient concentration (0.5 g/L sucrose and 0.5 g/L yeast extract) at day 40.

During the following days, blocking of the bioreactors occurred as a result of the high nutrient inflow concentrations. The peak at around day 58 was the result of massive mixing of the bioreactors by repeated inversion at day 55, followed by a flushing of the packed bed with a NaCl solution (20 g/L) at 1000 mL/h for 45 min. At day 61, the bioreactors were reinoculated and the nutrient concentration slightly increased for 4 days (sucrose and yeast extract 0.1 g/L each). Performance was restored within 1 day.

The peak around day 76 was again caused by gas bubbles entering the bioreactor. The bioreactors regained their former performance spontaneously within 4 days. At day 98, the pumps failed and neither nutrients nor wastewater were pumped through the bioreactors until day 99. The bioreactors recovered their Hg(II)-retaining activity within a few days after restart of the pumps.

At day 160, the hydraulic load was increased from 1 to 2 bv/h and the Hg inflow concentration was enhanced slightly. Furthermore, at day 163, some bubbles entered the bioreactors, resulting a peak at day 163.

At day 189, the Hg inflow concentration was shifted from 6.2 to 7.0 mg/L, resulting in an outflow peak at around day 198. Accelerated by increased nutrient amendment from day 207 on (sucrose and yeast extract 0.1 g/L each) the bioreactors regained their former performance within approximately 10 days. The reason for the peak at around day 236 remains unclear, but may have been due to gas bubbles once again.

At day 242, mercury inflow concentration was increased to 8.4 mg/L Hg(II). In the following days performance decreased significantly, despite the increased nutrient concentration from day 250 onward. At day 256, an additional Hg inflow shift to 9.2 mg/L further reduced the retention efficiency. The dilution of the wastewater to 5.1 mg/L Hg(II) at day 258 resulted in complete recovery within 3 days. At day 277, the Hg inflow concentration was raised from 5.1 to 6.9 mg/L Hg(II), but bioreactor performance remained completely unaffected.

Gas bubbles are the most probable cause for the outflow mercury peak at around day 305. Activity was regained spontaneously, supported by slightly increased nutrient concentration at day 307 (sucrose 0.1 g/L, yeast extract 0.05 g/L).

At day 332, the experimental setup was modified to connect A1 to a water thermostat. The mechanical disturbances resulted in a mercury outflow peak. During the following 10 days the bioreactors regained their former performance spontaneously. At day 354, the Hg(II) inflow concentration was increased to 7.6 mg/L. In the following days, the Hg outflow concentration increased slightly, but recovered automatically.

The outflow peaks around day 384 and day 424 were most probably caused by spontaneous or gas-bubble-induced disruption of the aged biofilm. The peak around day 409 was caused by a lack of medium and the import of gas bubbles instead. A1 regained the former retention efficacy spontaneously within 1 day, whereas A2 needed a few additional days.

At day 464, a wastewater batch with a mercury concentration of 9.7 mg/L Hg(II) was entered into the bioreactors, together with gas bubbles. The mercury load broke through totally. However, after diluting the wastewater to a mercury inflow concentration of 6.9 mg/L at day 467 the bioreactors recovered their previous performance within 5 days.

## Culturable Cell Number in Outflow of Mercury-Reducing Bioreactors

In Figure 2 the numbers of culturable cells in the outflow of bioreactors A1 and A2 are shown during the investigated time period of 480 days. The average number of culturable cells in the outflows were $4.6 \cdot 10^5$ cfu/mL in A1 and $5.1 \cdot 10^5$ cfu/mL in A2. At times of high mercury retention efficacy the outflow usually contained more than $5 \cdot 10^5$ cfu/mL (e.g., days 420 to 460). Culturable cell numbers below $10^4$ cfu/mL occurred only at times of low mercury retention efficacy (e.g., around day 200). Mechanical and

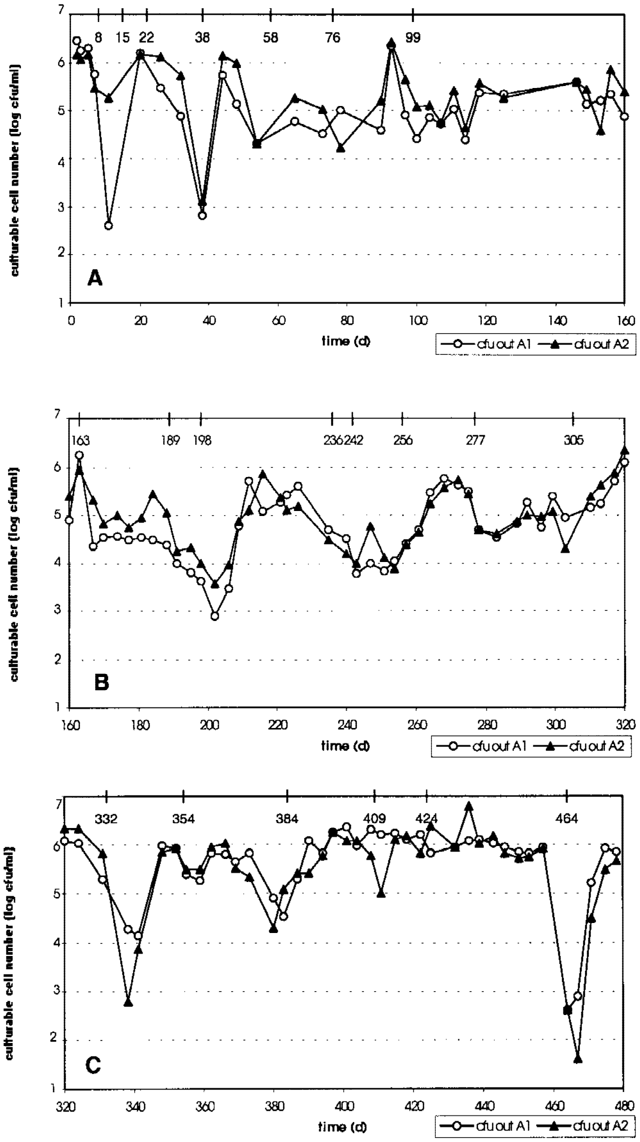

**Figure 2.** Culturable cell number (colony forming units) in the outflow of two mercury-retaining packed-bed bioreactors (A1 and A2) operated in parallel during 480 days with different batches of chloralkali wastewater. Details as in Figure 1. (○) Culturable cell numbers in the outflow of bioreactor A1; (▲) culturable cell numbers in the outflow of bioreactor A2. (A) Days 0 to 160. (B) Days 160 to 320. (C) Days 320 to 480.

chemical stresses resulted in reduced densities of culturable cells in the bioreactor outflow. For example, after the shifts to higher mercury inflow concentrations at days 8, 189, and 277, the number of culturable cells in the outflow decreased by more than one order of magnitude, after the shifts at days 160 and 354 by half an order of magnitude. At day 38, several hours after biofilm disruption by gas bubbles, the culturable cell number decreased by approximately two orders of magnitude, and at day 464 it dropped to $<10^3$ cfu/mL as a result of mercury inflow concentrations of 9.7 mg/L. By contrast, directly after bubble disruption at day 163, the culturable cell number in the outflow increased, probably due to biofilm fragments containing culturable cells.

## Effect of Stresses on Performance of Mercury-Reducing Biofilms

### *Mechanical Perturbation*

Mechanical disturbances (e.g., gas bubbles, vigorous mixing of the bioreactors, changes in the experimental setup, and pump failures) disrupted the biofilms and represented the most severe threat to performance, as can be seen around days 38 (bubbles), 58 (mixing), 332 (rearrangement of the bioreactor setup), 98, and 409 (pump failure). The bioreactors responded instantly with partial or total breakthrough of the mercury load. In the following days, decreased numbers of culturable cells in the outflow were detected until the biofilms had recovered.

### *Mercury Toxicity*

The change of wastewater batches was usually accompanied by the entrance of gas bubbles into the bioreactors. Shifts to higher mercury inflow concentrations in combination with gas bubbles resulted in an increased mercury outflow concentration and a decreased number of culturable cells in the outflow. It is likely that the higher mercury inflow concentration directly caused the inactivation of viable biofilm cells due to the toxicity of mercury. At up to 7.6 mg/L Hg(II), the bioreactors were again able to regain their optimal performance automatically. An increase of mercury concentrations to 9.2 mg/L (day 256) or 9.7 mg/L (day 464) resulted in complete breakthrough of the mercury and a decrease in outflow culturable cell density by up to four orders of magnitude (day 464).

### *Volumetric Load*

An increase in flow rate resulted in the combined stresses of increased mercury load and higher flow velocity of the wastewater. The shift from 160 to 240 mL/h at a mercury inflow concentration of 6.2 mg/L on day 181 initially resulted in a slightly increased mercury outflow concentration. However, during the following days, the Hg(II) outflow concentration returned to the former level, corresponding to an increased amount of retained mercury per time. The number of culturable cells in the outflow remained unaffected.

### *Temperature*

While bioreactor A1 was warmed in a stepwise manner from room temperature (approximately 22°C) up to 41°C, A2 remained as a control at room temperature. Both bioreactors showed essentially the same performance and number of culturable cells in the outflow. No stress effect of temperatures up to 41°C was observed.

### Stress Recovery of Mercury-Reducing Biofilms

During the first 317 days of model reactor operation, nutrient concentrations in the inflow were increased slightly following stresses to support restoration of full bioreactor performance. From day 317 onward, recovery occurred without increased nutrient concentration. The spontaneous recovery of old biofilms took approximately the same amount of time (4 to 10 days) as the nutrient-enhanced recovery of the younger biofilms. Recovery from mercury inflow concentrations of >7.6 mg/L (e.g., days 256 and 464) was achieved by diluting the wastewater to <7.0 mg/L.

## DISCUSSION

### Long-Term Performance of Mercury-Reducing Bioreactors

So far, the maximum run time of a mercury-reducing packed-bed bioreactor was 102 days (Brunke et al., 1993). We previously treated chloralkali electrolysis wastewater effectively for up to 22 days (von Canstein et al., 1999). Here, we showed that such bioreactors can run for at least 480 days without loss of performance. The best performance of 97% was similar to the data obtained in an earlier study (von Canstein et al., 1999). In theory, the lifetime of the bioreactor's lifespan is infinite, because the bacteria serve as biocatalysts and the mercury accumulates outside the cells. In practice, however, the lifetime of a bioreactor is determined by its capacity to accumulate mercury without blocking. In 480 days, we observed no blocking by metallic mercury deposits. The bioreactors ran best when amended with a limited amount of nutrients as carbon and energy source. Large amounts of nutrients may lead to excess growth and blocking, which occurred once during this run. In contrast to the biodegradation of organic waste, the reduction of ionic mercury consumes metabolic energy. Hence, stationary-phase cells can also reduce the ionic mercury. The amendment of wastewater with nutrients is necessary only for Hg(II) detoxification and for replacement of inactivated and washed-out cells.

Even in periods of stable, undisturbed performance, mercury outflow concentration was always >140 μg/L. The solubility of metallic mercury in water is approximately 60 μg/L (Elvers et al., 1990) and depends strongly on accompanying ions. Therefore, we assume that a fraction of the mercury in the chloralkali wastewater was complexed and not bioavailable. When applying the process to the cleanup of industrial wastewater, the discharge limit of mercury must be taken into account. In Germany, the European discharge limit of 50 μg/L has to be met, and some local authorities even demand 10 μg/L. Therefore, a second filter is necessary to reduce the mercury outflow concentrations to levels that are reliably below the discharge limit. This second filter could consist of activated carbon or of bacteria with a high affinity to mercury and an intercellular accumulating capacity for Hg(II), as described by Chen et al. (1998). Another approach would be to separate the wastewater stream from the immobilized bacteria by membranes that are permeable for Hg(II) but not for Hg(0).

### Response of Mercury-Reducing Bioreactors to Stresses and Mechanical Perturbations

#### *Mercury Toxicity*

Previous fixed-bed column experiments have shown that they could cope with up to 10 mg/L Hg(II) in synthetic mercury solutions (Brunke et al., 1993; Frischmuth et al., 1993), without an increase in outflow mercury concentration. The insensitivity to a Hg(II) shift from 1 to 10 mg/L may have been caused by the presence of immobilized yeast cells in the packed bed, as it was demonstrated that yeast extract decreases the amount of free Hg(II) (Chang et al., 1993) and thus the toxicity of Hg(II) (Farrell et al., 1993). In this study we observed a breakthrough of mercury at inflow concentrations of >7.6 mg/L in the chloralkali wastewater. Inactivation of mercury-resistant cells may be caused by several factors: Medium components can serve as co-ions of Hg(II), and can thereby enhance or decrease its toxicity (Barkay et al., 1997; Farrell et al., 1990, 1993). The amount of inactivated cells depends strongly on the cell density; that is, the higher the cell density, the higher the percentage of surviving cells (Chang and Hong, 1995). Furthermore, different strains exhibit different levels of resistance (Brunke et al., 1993). Therefore, it remains unclear if the differences in the higher mercury concentration treatable by the microbial biofilm just reported were caused by differences in the number of active cells, the resistance level of the bacteria, or by components present in the chloralkali wastewater.

#### *Mechanical Perturbation*

The most severe perturbation observed in our experiments was mechanical disruption of the biofilms on the carrier material, mainly caused by the entrance of gas bubbles. The bioreactors were designed for an instant response to perturbations. They were constructed with a comparatively small active layer with no buffering capacity. Washout of disrupted biofilms can be avoided by a top layer sedimentation zone of low current, and biofilm disruption can be avoided by implementation of sufficient bubble traps.

#### *Temperature*

Little is known about the effect of temperature on the biotransformation of ionic mercury. Schneider (1994) determined the dependence of the activity of purified mercuric reductase enzyme on temperature and found maximum activity at 37°C. At 30°C and 55°C, 55% of the activity was maintained, whereas at 20°C it was reduced to 20%. This suggests a better performance of the enzyme at temperatures increasing from 20°C to 41°C. However, most of the inoculant strains were members of the genus *Pseudomonas*, which has a growth optimum of 30°C. None of the inoculum strains showed growth at >40°C (data not shown). On the other hand, the solubility of metallic mercury in water increases with temperature, even though we found neither an increase nor a decrease in mercury retention efficiency of the bioreactors between 22°C and 41°C, indicating that the contrasting effects just described must have been compensated by the buffering capacity of the bioreactors.

### Culturable Cell Number and Recovery of Bioreactor Performance

*Pseudomonas* sp. biofilms consist of a fraction of sessile and a large fraction of planctonic cells (Tolker-Nielsen et al., 2000). In flow-through systems, a part of the population is therefore continuously washed out, and this fraction is dependent on the growth rate of the whole biofilm population. Metabolic activity of biofilms has been measured over a wide range of organisms and experimental systems. However, activity assays have often given conflicting results (Wimpenny et al., 1993). Therefore, we used the number of culturable cells in the efflux of the bioreactors as a noninvasive way to determine the metabolic activity of the biofilms. Bioreactors A1 and A2 were run in parallel and generally showed similar culturable cell numbers, indicating that changes in outflow culturable cell numbers were not random, but caused by factors affecting both bioreactors in a similar way.

A decrease in the number of culturable cells in the bioreactor outflow usually correlated with a decreasing mercury retention efficiency, and vice versa. Yeast extract had an important function in restoring bioreactor activity after stresses. It not only acts as a growth substrate, but, in addition, binds Hg(II) and thereby decreases the acute toxicity of high mercury concentrations (Chang et al., 1993; Farrell et al., 1993). We found that by increasing the yeast extract concentration in the wastewater (e.g., at days 18, 38, and 207) the bioreactors quickly regained their earlier performance. A possible approach to maintain stable performance of the bioreactor would be stress-dependent feeding, where the amount of yeast extract added to the wastewater correlates with the mercury inflow concentration. After the stress has ended, or after the biofilm has adapted, the feeding must soon be decreased to prevent blocking.

## CONCLUSIONS

The retention of ionic mercury from chloralkali wastewater streams by mercury-reducing microbial biofilms in a packed-bed bioreactor was shown to be a stable process of long-term activity of ≥480 days. The maximum mercury retention efficacy was 97% [7.6 mg/L Hg(II) inflow]. The minimum mercury outflow concentration was 140 μg/L. The performance of the bioreactors decreased several times as a result of different stresses. The bioreactors regained full retention efficiency spontaneously within a few days, but recovery was enhanced by increased feeding. The process was insensitive to changes in flow rate between 1 and 3 bv/h and to a stepwise increase of the temperature up to 41°C. The process responded to mechanical stress with decreased performance. The bioreactors could adapt to initially toxic Hg(II) concentrations of up to 7.6 mg/L in a spontaneous manner. For technical application, a bioreactor must not be allowed to undergo mercury shock loads of ≥8 mg/L Hg(II) (e.g., by an automated dilution device) or excessive mechanical stress (e.g., by bubble traps). A second filter after the bioreactor is required to reduce the mercury outflow concentration to safely below official discharge limits.

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