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ARTICLE *in* ENVIRONMENTAL SCIENCE AND TECHNOLOGY · DECEMBER 2002

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Environ. Sci. Technol. **2002**, *36,* 4698−4704

Bioaugmenting Bioreactors for the Continuous Removal of 3-Chloroaniline by a Slow Release Approach

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The survival and activity of microbial degradative inoculants in bioreactors is critical to obtain successful biodegradation of non- or slowly degradable pollutants. Achieving this in industrial wastewater reactors is technically challenging. We evaluated a strategy to obtain complete and stable bioaugmentation of activated sludge, which is used to treat a 3-chloroaniline (3-CA) contaminated wastewater in a lab-scale semi-continuous activated sludge system. A 3-CA metabolizing bacterium, *Comamonas testosteroni* strain I2, was mixed with molten agar and encapsulated in 4 mm diameter open-ended silicone tubes of 3 cm long. The tubes containing the immobilized bacteria represented about 1% of the volume of the mixed liquor. The bioaugmentation activity of a reactor containing the immobilized cells was compared with a reactor with suspended I2*gfp* cells. From day 25−30 after inoculation, the reactor with only suspended cells failed to completely degrade 3-CA because of a decrease in metabolic activity. In the reactors with immobilized cells, however, 3-CA continued to be removed. A mass balance indicated that ca. 10% of the degradation activity was due to the immobilized cells. Slow release of the growing embedded cells from the agar into the activated sludge medium, resulting in a higher number of active 3-CA-degrading I2 cells, was responsible for ca. 90% of the degradation. Our results demonstrate that this simple immobilization procedure was effective to maintain a 3-CA-degrading population within the activated sludge community.

Introduction

Xenobiotics transiently present in sewage persist for extended periods of time and disrupt reactor function (*1*). Because of slow microbial adaptation and growth, there is seldom sufficient metabolic capacity to protect reactors from these toxicants that only rarely appear. The removal of these chemicals is often enhanced by inoculating specialized xenobiotic degrading bacteria (*2*). These laboratory-cultured inocula, which can transform the xenobiotica very efficiently in pure cultures, are however usually of little effectiveness once they are inoculated into an established microbial community (*3*). It is hypothesized that bioaugmentation failures arise because (i) the contaminant concentration is too low to support microbial metabolism; (ii) microbial inhibitors are present in the environment; (iii) the growth rate of the degrading organism may be slower than the rate of cell removal, for example, by predation or wash-out; (iv) the inoculum may use substrates other than the pollutant whose destruction is desired; (v) the organism may physically fail to reach the pollutant; or (vi) the pollutant may not be bioavailable due to sorption (*3*).

As a result of these practical problems, few successful cases of lab-scale activated sludge bioaugmentation have been described (*4*−*8*), and most of these report only partial success. These studies are limited to the inoculation of suspended cells into the mixed liquor. After the introduction, cell densities decline (*4*, *7*, *9*, *10*), and there is relatively minor influence on reactor functions (*9*, *11*). To increase the success rate of bioaugmentation strategies, investigators use carrier materials such as alginate, agarose, polyurethane (*12*), Lentikats (*13*), or hollow fibers (*14*) to provide a temporary protective environment to the inoculum (*15*). However, there is no clear understanding of the mechanism by which these encapsulation processes protect bacteria, and there is no information available on how these protective measures influence the kinetics of biodegradation processes in activated sludge.

3-Chloroaniline (3-CA) is a chemical used in industry during the production of polyurethanes, rubber, azo dyes, drugs, photographic chemicals, varnishes, and pesticides (*16*, *17*). Chloroanilines are often transiently detected in wastewaters (*18*, *19*) at concentrations ranging from 12 (*19*) to 230 mg/L (*20*), and they can cause reactor failure when shock loads of 250 mg/L are applied (*21*). Recently, we reported successful removal of 3-CA in activated sludge by inoculating the strain *Comamonas testosteroni* I2*gfp* (*7*). During 14 days, the 3-CA was completely degraded, after which the degradation rate declined. This resulted in accumulation of 3-CA in the reactor, and only 50% 3-CA removal was achieved after these 2 weeks. The decreased 3-CA removal rate correlated with declining *C. testosteroni* I2*gfp* concentrations in the reactor. Regular re-inoculation of the strain seemed to be the logical solution to prevent this problem. However, repeated inoculations can themselves disrupt reactor function and, thus, can cause the very problem that the inoculums are designed to prevent (*22*).

Faced with the same difficulties encountered by other investigators, we evaluated a novel encapsulation method, i.e., silicone tubes (5.4 mm o.d., 4.0 mm i.d., 3 cm in length) as a means to protect the degradative activity. These tubes were open at their ends, so that release of cells from inside the tubes would occur. We estimated the 3-CA removal rate to elucidate how encapsulation alters biodegradation kinetics in semi-continuous activated sludge (SCAS) reactors. To differentiate between encapsulated and suspended bacterial cells in the sludge liquor, we used two marker genes that encode two different fluorescent proteins, wild-type GFP and DsRed (RFP) (*23*, *24*). This enabled us to count the inoculants as fluorescent colonies after plating.

Experimental Section

Chemicals. 3-CA is obtained from Fluka AG (Chemische Fabrik, Buchs, Switzerland; 99% pure). The octanol−water partition coefficient is 1.89 ($\log K_{OW}$), and the Henry coefficient is 1.5×10^{-6} (atm·m^3)/mol (*25*). The antibiotics rifampin and kanamycine were obtained from Ducheva (Haarlem, The Netherlands).

Bacterial Strains. The chromosomally *gfp*-marked strain *C. testosteroni* I2*gfp* originates from strain *C. testosteroni* I2,

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10.1021/es020076q CCC: $22.00
Published on Web 10/04/2002

isolated from a municipal wastewater treatment plant (*7*, *26*). Strain I2*gfp* mineralizes 3-CA completely and is rifampin-resistant (100 μg/mL). Because of the marking with pUT-miniTn*5 gfp*Km by means of the pUTgfp delivery vector (*27*), the strain is also kanamycin-resistant (50 μg/mL) and fluoresces green (excitation at 396/476 nm, emission at 508 nm) under UV light (TLD 18W/08, Philips, The Netherlands).

The genes for *dsRed* fluorochrome (Red Fluorescent Protein or RFP) and kanamycin resistance were inserted into the chromosome of *C. testosteroni* I2 by using the *rrnB*P1-RBSII-*dsRed*-T0-T1 cassette, which was located on a mini-Tn*5* cassette and inserted in the chromosome by means of the pUT delivery plasmid (*24*). The delivery plasmid was mobilized from *Escherichia coli* CC118 _pir to the recipients using the helper strain *E. coli* HB101(RK600) as previously described (*28*). The obtained RFP marked I2*rfp* cells were selected on LB plates supplemented with kanamycin (50 μg/mL) and rifampin (100 μg/mL). RFP fluorescence (excitation at 558 nm, emission at 583 nm) was visualized by a Dark Reader illuminator (Clare Chemical Research, Denver, CO) and by epifluorescence microscopy.

Strain I2, I2*gfp*, and I2*rfp* are deposited in the BCCM/LMG Bacteria Collection (Ghent, Belgium) under the numbers LMG 19554, LMG 21409, and LMG 21402, respectively.

Semi-Continuous Activated Sludge (SCAS) Reactors. SCAS reactors have been used previously as a model for activated sludge systems, and they allow us to examine different reactor setups simultaneously in a reliable and reproducible way (*7*, *29*, *30*). The experiments were conducted in duplicate with sludge freshly collected from a domestic wastewater treatment plant (Bourgoyen-Ossemeersen, Ghent, Belgium), according to a slightly modified SCAS procedure as described previously (*7*). In brief, the reactors (2-L plastic Erlenmeyer flasks) had an active volume of 1.2 L and were mixed and aerated on a rotary shaker at 140 rpm. The tests were conducted with synthetic influent (skimmed milk powder (Gloria, Nestlé); COD/N/P ratio equal to 100/6/1) at room temperature (ca. 21 °C). The reactors were fed every other day after wastage of excess sludge and settling. The SCAS reactors were operated at a volumetric loading rate of 1 g of COD/(L·d), with a hydraulic retention time (HRT) of 4 days, a sludge retention time (SRT) of 12 days, and ca. 5 g/L suspended solids (SS). The volatile suspended solids (VSS) made up 77% of the SS. All three reactors received a loading rate of 37 mg of 3-CA/(L·d), added as a single dose every other day. Every 2 days, 1 L of the mixed liquor was subjected to a 0.5-h period of settling in an Imhoff cone to analyze the sludge volume (SV) (*31*). The other 200 mL of mixed liquor was used for analysis and discharged. The settling was followed by a decantation of 400 mL of the supernatant and the addition of 600 mL of fresh influent. The wasted sludge was used for analyses. Unless otherwise indicated, the data reported are averages of the duplicate reactors. During the startup, the reactors were operated without 3-CA for at least 8 days to allow the microbial community to adapt to the changed environment and growth conditions.

Bacterial Counts. At different time intervals, the densities of strain I2*gfp* or I2*rfp* were determined. Sludge flocs were dispersed by purging a 1-mL sample 20 times through a sterile 1-mL syringe with a sterile needle (1.2 × 40 mm). LB agar medium, supplemented with rifampin (100 μg/L) and kanamycin (50 μg/L), was used to count I2*gfp*.

Cell dry weight (CDW) of *C. testosteroni* was determined as follows ($n = 2$). Glass tubes were dried overnight at 105 °C, and the weight was measured. An overnight grown culture of 100 mL in LB broth was concentrated 10 times by centrifugation (5000*g* for 15 min), resuspended in saline, and plated to determine the amount of colony-forming units (cfu)/mL. The remaining suspension was transferred to the glass tubes and dried for 24 h at 105 °C. After being cooled, the glass tubes were weighed again. After correction for the saline in the medium, the CDW of the I2 cells was determined to be 1.14 g of CDW/10^{12} cells.

Inoculum Preparation. The inocula were grown overnight at 28 °C in 5 mL of LB medium (1 L contains 5 g of NaCl, 10 g of trypton, and 5 g of yeast extract) containing 100 mg of 3-CA/L. Subsequently, these 5-mL cultures were used to inoculate 500 mL of LB medium + 3-CA (100 mg/L). After the cultures were shaken overnight at 28 °C, they were washed twice with saline (0.85% NaCl) and resuspended finally in 50 mL of saline.

The I2*gfp* or I2*rfp* containing silicone tubes (product code 990.0040.007, bore size 4 mm, wall size 0.7 mm; Wadson-Marlow, Cornwall, England) were prepared as follows: an overnight grown culture of I2*gfp* or I2*rfp* cells was centrifuged, resuspended in saline, and mixed with liquid LB agar (for 1 L: 5 g of NaCl, 10 g of peptone, 5 g of yeast extract, 40 g of agar) at 40 °C (ratio 1:1). Subsequently, while the mixture was still fluid, it was injected into the sterile silicone tubes. When the mixture was solidified, the tubes were cut into pieces of 3 cm long and placed in the reactors (100 cm, 12.7 g or 12.5 mL of agar/reactor); the silicone pieces remained open at both ends. The surface area represented by the tubes is 15% of that present in the reactor. The amount of extra nutrients present in the agar amounts to 166 mg/L and represents less than 0.5% of the total amount added during the overall run (45 g/L). After being introduced in the reactors, the tubes settled to the bottom and tumbled around due to the rotation of the shaker.

First Experimental Run. In the first experimental run, the use of a carrier system (i.e., agar in silicone tubes for enhanced 3-CA degradation) was assessed. One reactor was used as noninoculated control (A1). Duplicate reactors ($n = 2$) were inoculated with either *C. testosteroni* I2*gfp* in suspension (B1) or with immobilized as well as suspended *C. testosteroni* I2*gfp* (C1). The final concentration of *C. testosteroni* I2*gfp* was 9.0×10^7 and 1.2×10^7 cells/mL of mixed liquor in reactors B1 and C1, respectively. The final concentration of I2*gfp* cells in the agar mixture was ca. 5.0×10^8 cfu/mL agar mixture corresponding with 6.3×10^7 cfu/cm tube. The absolute total number of cells added via the silicone tubes was 5.2×10^6 cfu/mL of mixed liquor.

Second Experimental Run. In the second experimental run, we used an RFP marked variant of I2 as immobilized cells (I2*rfp*) to differentiate them from the cells that were inoculated as suspended cells (I2*gfp*). Additionally, an experiment was set up to evaluate the bioaugmentation potential of immobilized I2*rfp* cells alone. Duplicate reactors ($n = 2$) were inoculated with either *C. testosteroni* I2*gfp* in suspension (B2), with *C. testosteroni* I2*gfp* in suspension as well as immobilized in silicone tubes (C2), or with *C. testosteroni* I2*rfp* immobilized in silicone tubes (D2). Reactors B2 and C2 were inoculated with *C. testosteroni* I2*gfp* to a final concentration of 4.8×10^8 and 1.4×10^8 cells/mL, respectively. The final concentration of I2*rfp* cells in the agar mixture in reactors C2 and D2 was ca. 1.3×10^9 cfu/mL agar mixture corresponding to 1.6×10^8 cfu/cm silicone tube. The absolute total number of I2*rfp* cells added via the silicone tubes was 1.4×10^7 cfu/mL of mixed liquor or 18.5 mg of CDW_{I2rfp}/L of mixed liquor. The suspended solids of the different reactors was kept at 5 g/L, and no differences in function of time or treatment were observed.

The rate of I2*rfp* cell release was determined every 6 days as follows: one tube was transferred from the SCAS reactor to 100 mL of physiological solution (0.85% NaCl) for 1 h (shaking at 140 rpm). Subsequently, the amount of I2*rfp* cells was determined by plating on LB agar supplemented with 50 μg/mL kanamycin and 100 μg/mL rifampin. Theoretically, it is possible that the 3-CA absorbed on this tube was released in this side test. Thus, the tube when returned to the reactor

was calculated to potentially have released 0.1 mg of 3-CA (see below).

Absorption of 3-CA through Silicone Tubes. For the absorption test, 60 mL of sterile H_2O, containing 200 mg/L 3-CA, was incubated with different amounts of autoclaved silicone tubes filled with agar suspension (the inoculum was replaced by sterile saline), resulting in final concentrations of 0−170 g of tubes/L. The 3-CA concentration in the water was determined daily by HPLC.

Diffusion of 3-CA and Chloride Ions through Silicone Tubes. The diffusion of 3-CA was measured by bringing 1-mL stock solution of 3-CA (10 mg of 3-CA/mL of methanol; methanol was used because of the low solubility of 3-CA in water) in an empty autoclaved silicone tube, placed in a 100-mL sterile bottle filled with 50 mL of sterile H_2O, resulting in a final concentration of 200 mg/L. The ends of the tube were placed out of the flask so that the stock solution was not in direct contact with the water. A control for the diffusion test had the same setup, but 1 mL of the stock solution was directly added in the water. In case of total diffusion of 3-CA through the tubes, the 3-CA concentration in the diffusion experiment should be the same as the control test.

An identical setup was used to test if the chloride ions, released during the mineralization of 3-CA, could diffuse back into the medium. One milliliter of a 25 mM NaCl solution was injected inside the silicone tube and placed in 100 mL of distilled water. In a function of time, the chloride content was determined. In case of total diffusion, the chloride concentration should increase to 8.9 mg of Cl^-/mL.

Degradation of 3-CA by Suspended Cells. The degradation rate of suspended I2 cells was estimated by incubating I2 cells in a MMN mineral medium containing 75 or 100 mg of 3-CA/L. The different media were inoculated with 1.9×10^7 I2*gfp* cells/mL, and every 30 min a HPLC sample was taken. The degradation rate was calculated from the slope of the removal curve.

Degradation of 3-CA by Cells Contained within Silicone Tubes. Three different setups were examined in duplicate: one with I2*gfp* cells (1.3×10^9 cfu/mL), one with *E. coli* cells (3.5×10^9 cfu/mL), and one with sterile saline. A long tube was partially filled with different agar mixtures for a tube length of 8 cm, and the filled part was submerged in 100 mL of water containing 250 mg of 3-CA/L (same ratio tube length/mixed liquor as in the SCAS experiments). The ends of the tube were placed outside the flask so that the cells could not be in direct contact with the 3-CA in the water (Figure 3). The 3-CA and chloride concentration in the water was determined daily by HPLC and IC, respectively, and possible contamination of the water by the cells in the tubes was determined by plating the water on LB agar to count *E. coli* and on LB agar with 50 μg/mL kanamycin and 100 μg/mL rifampin to count the I2*gfp* cells. The flasks were incubated at 28 °C and shaken at 140 rpm.

Analytical Methods. The effluent was analyzed for 3-CA content by reversed-phase HPLC after centrifugation at 5000*g* for 10 min. The Summit HPLC system (Dionex, Wommelgem, Belgium) consisted of a Dionex pump series P580, a Dionex autosampler model ASI-100 (injection volume is 20 μL), a STH585 column oven (at 28 °C), a Dionex UV/VIS detector UVD 340S, and a Chromeleon software system version 6.10. A Hypersil Green Env column (150 mm × 8 mm i.d., 5 μm particle size; Alltech, Deerfield, IL) was used. The mobile phase consisted of CH_3OH/0.1% H_3PO_4 (ratio 70/30) with a flow rate of 0.8 mL/min. The UV detector was used at 210 nm.

Chloride concentrations were determined by ion chromatography (IC) after centrifugation of the samples at 5000*g* for 10 min and filtering through a 0.45-μm filter. The DX-600 system (Dionex, Wommelgem, Belgium) consisted of a Dionex pump GP50, a Dionex autosampler model AS50 (injection volume is 100 μL), a Dionex ED50 electrochemical detector, and a PeakNet 6 software system version 6.10. Ionpac AS9-HC (250 mm × 4 mm i.d., 9 μm particle size; Dionex) column and Ionpac CS12-HC (250 mm × 4 mm i.d., 8 μm particle size; Dionex) were used for anion separation. The mobile phase consisted of Na_2CO_3 (9 mM) and methanesulfonic acid (20 mM) with a flow rate of 1 mL/min. Quantitative data were obtained by comparing the peak areas of unknown concentrations with the peak areas of standards of known concentrations.

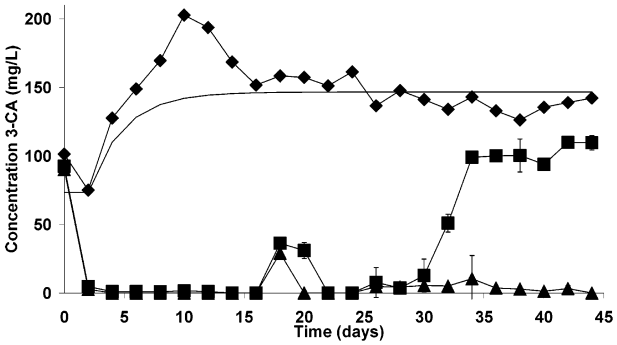

FIGURE 1. Concentration of 3-CA in the first experimental run for the control reactor A1 (◆); the reactors B1 (I2*gfp*) (■), and the reactors C1 (I2*gfp* and immobilized I2*gfp*) (▲). The solid line represents the theoretical 3-CA in case of 0% degradation concentration at the imposed loading rate of 37 mg of 3-CA/(L of mixed liquor·d). Values represent the mean ± error bars ($n = 2$); in some cases, the error bars were too small to be visible.

Microscopy. The GFP and RFP marked cells were observed by standard epifluorescence microscopy on a Zeiss Axioskop II microscope (Carl Zeiss, Jena, Germany). The microscope was equipped with a Peltier cooled single-chip digital color CCD camera (Hamamatsu Orca IIIm, Hamamatsu, Massy, Cedex, France) connected to a PC to obtain digital images.

Results

First Bioaugmentation Experiment. Encapsulation in silicone tubes increased the period of complete 3-CA removal by I2*gfp*-cells (Figure 1). Starting from day 2, complete removal of 3-CA was obtained in all inoculated reactors with a minimal removal rate of 37 mg of 3-CA/(L of mixed liquor·d). Because of technical problems on day 17, anoxic conditions were created for 12 h, and a small amount of 3-CA accumulated on day 18. Encapsulated cells recovered from this anoxic shock more quickly than suspended cells (in 2 days as compared to 4 days). By day 30, the 3-CA biodegradation rate in reactors without encapsulated cells decreased to 30% (12 mg of 3-CA/(L of mixed liquor·d)) of that of the encapsulated cells. The reduced biodegadation rate was accompanied by a 100-fold decrease in culturable I2*gfp* cells from their initial value of 10^7 to 10^5 cfu/mL of mixed liquor (data not shown). In contrast, the reactors with the encapsulated cells maintained its biodegradation rate of 37 mg of 3-CA/(L of mixed liquor·d) for the duration of the experiment (44 days in total), and culturable I2*gfp* cells remained quite stable. No significant removal of 3-CA was observed in the control reactor A1 during the first 25 days, and only a small amount of 3-CA was removed from day 25 until the end of the experiment with a biodegradation rate of 2.3 mg of 3-CA/(L of mixed liquor·d).

Diffusion and Sorption Experiments. The ability of 3-CA to diffuse through the walls of the silicone tubes was examined by adding a concentrated 3-CA solution inside the tube and by monitoring its concentration in the water outside the tubes as a function of time. In case of total diffusion, the concentration outside the tubes should have been 196 mg/L. 3-CA freely diffused through silicone tubes within 24

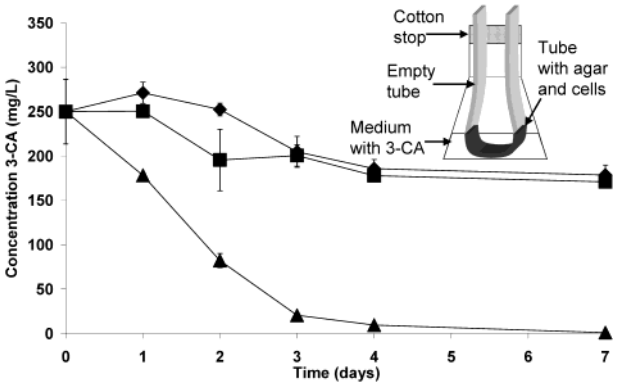

FIGURE 2. Concentration of 3-CA in the diffusion-mediated degradation experiment: control without cells (◆); agar-embedded I2*gfp* cells (▲); agar-embedded *E. coli* cells (■). Values represent the mean ± error bars ($n = 2$); in some cases, the standard deviations were too small to be visible. A representation of the experimental setup is shown beside the graph.

h with 93% (170 ± 13 mg of 3-CA/L) of the expected 3-CA found in the reactor liquor by 24 h. From day 2 on, the 3-CA concentrations stabilized at 107% of the level expected.

To estimate the amount of 3-CA that could be absorbed on the silicone tubes in reactors C1, the amount of 3-CA that disappeared from the solution in a separate absorption experiment was determined. Silicone tubes filled with agar (0, 33, 67, 100, 133, and 167 g/L) were incubated in 200 mg/L 3-CA containing water until maximal absorption occurred (75 h). Sorption by agar-filled silicone tubes was minimal with tubes absorbing ca. 0.27 mg of 3-CA/g within a 75-h period (linear sorption). This corresponds to 3.3 mg of 3-CA absorbing to tubes in the first reactor experiment.

The removal rate of free suspended cells was estimated by incubating pregrown cells in mineral medium. The average degradation rate was 64.6 mg of 3-CA/(L·h). This makes a specific removal rate for I2 of 71.9 mg of 3-CA/(mg of CDW_{I2gfp}·d). Cells contained within the silicone tubes were able to degrade the 3-CA present in the liquid phase (Figure 2). The linear degradation rate of encapsulated I2*gfp* cells was 77 mg of 3-CA/(L·d). This corresponded with a specific removal rate of 0.53 mg of 3-CA/(mg of CDW_{I2gfp}·d) [(0.1 L × 77 mg of 3-CA/(L·d))/(1.3 × 10^{10} cells × 1.14 mg of $CDW_{I2gfp}/10^9$ cells)]. This means that the degradation rate was 136 times higher for the free suspended cells than for the encapsulated cells. No I2*gfp* was detected in the medium by plate counts or optical density. Encapsulated *E. coli* and sterile agar displayed no 3-CA degradation and minor absorption. It should be noted that in this experiment the cells and 3-CA metabolites were contained in the silicone tubing. No increased chloride concentrations were measured in the water, indicating that the chloride released during the 3-CA mineralization remained inside the tubes doubling the chloride concentration to 9.7 mg of Cl^-/mL or 277 mM.

Second Bioaugmentation Experiment. In a second SCAS experiment, we differentiated between encapsulated and released cells by marking strain I2 with two different genes. Cells marked with the green fluorescent protein (I2*gfp*) were freely suspended, and cells marked with the red fluorescent protein (I2*rfp*) were encapsulated. Strain I2 marked with the *dsRed* gene showed the same 3-CA degradation characteristics as the wild-type strain (data not shown).

Similar to the first run, complete removal of 3-CA was obtained by day 2 in all inoculated reactors (Figure 3A). From day 26 on, 3-CA degradation decreased to only 24 mg/(L of mixed liquor·d) in the reactors inoculated with suspended I2*gfp* as compared to a rate of at least 37 mg/(L of mixed liquor·d) for the reactors with encapsulated cells for the duration of the experiment. Reactors containing encapsulated

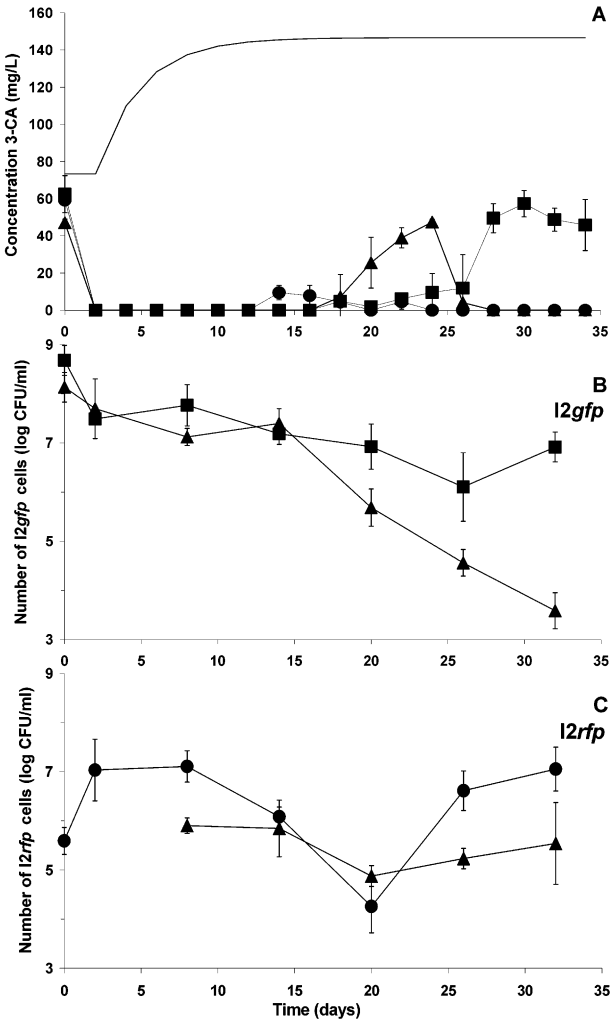

FIGURE 3. Effects of I2*gfp* and I2*rfp* cells in the mixed liquor on the amount of 3-CA in the effluent in the second experimental run. Concentration of 3-CA (A) and number of I2*gfp* (B) and I2*rfp* (C) cells in the reactors B2 (suspended I2*gfp*) (■), the reactors C2 (suspended I2*gfp* and immobilized I2*rfp*) (▲), and the reactors D2 (immobilized I2*rfp*) (●). The solid line represents the theoretical 3-CA concentration at the imposed loading rate of 37 mg of 3-CA/(L of mixed liquor·d). Values represent the mean ± error bars ($n = 2$); in some cases, the error bars were too small to be visible.

and suspended cells degraded 3-CA at a lower rate for a 4-day period from day 20 until day 24, but reactors containing only encapsulated cells continuously removed all 3-CA throughout the experiment.

Suspended *C. testosteroni* I2*gfp* inoculated in the reactors B2 remained quite constant at ca. 10^7 cfu/mL of mixed liquor throughout the study period despite the loss of 3-CA biodegradation activity (Figure 3B). In contrast, I2*gfp* in the presence of encapsulated I2*rfp* in reactors C2 only remained at 10^7−10^8 cfu/mL of mixed liquor until day 15 when the number of cells declined drastically to 4.0 × 10^3 cfu/mL of mixed liquor. The encapsulated I2*rfp* cells of reactors D2 remained quite stable at 10^7 cfu/mL of mixed liquor except for a brief period between day 15 and day 25. This corresponds to the observed lapse in 3-CA biodegradation activity. By transferring one tube out of the reactors to saline for 1 h and plating the amount of released cells, it was observed that the silicone tubes released ca. 10^6 cells/(h·silicone tube) via their open end. These measurements were repeated at regular intervals, and throughout the entire period of the SCAS tests this rate of release of cells essentially unchanged [average release rate over was log(6.0 ± 0.3 cfu/(tube·h))].

Bacterial Activity Estimates. On the basis of data from the second experimental run, bacterial kinetics were calculated, and the relative proportion of each mode of action was estimated. The calculations of the specific activity of cells present in the sludge for some time (aged cells) are based on reactors B2 (with only free suspended cells) and on the period where some 3-CA accumulation occurred. During this period (reference day 32), the removal rate for the suspended cells was 2.5 mg of 3-CA/(mg of CDW_{I2gfp}·d) [(24 mg of 3-CA/(L·d))/(8.3×10^9 cells/L of mixed liquor × 1.14 mg of $CDW_{I2gfp}/10^9$ cells)] (Figure 3). This was not sufficient to remove the daily dosed 37 mg of 3-CA/L, so accumulation occurred.

In the reactors D2 with only the immobilized I2*rfp* cells, four different modes of 3-CA removal are possible: absorption, diffusion with degradation by the immobilized cells inside the tubes, degradation by the freshly released cells in the mixed liquor, and degradation by the aged cells already present in the mixed liquor. The absorption effect is minimal since only 3.3 mg of 3-CA was able to bind to the tubes the first day and once the tubes are saturated they will no longer absorb 3-CA. Theoretically, it is possible that the 3-CA absorbed on the tube was released in the side test for the determination of the release rate of I2*rfp* cells from one tube. In theory, the tube when returned to the reactor was calculated to potentially release a negligible 0.1 mg of 3-CA.

The second effect of the tubes on the degradation rate is the 3-CA degradation by immobilized cells inside the tubes. This estimated biodegradation rate potential, mediated by diffusion through the silicone walls, was minor since the encapsulated cells in the batch experiment had the potential to degrade 9.8 mg/(L of mixed liquor·d) [(0.53 mg of 3-CA/(mg of CDW_{I2rfp}·d)) × (inside the tubes 18.5 mg of CDW_{I2rfp}/100 cm tube in 1.2 L of mixed liquor)].

A third fraction of the degradation can be considered to be due to the ongoing released I2*rfp* cells in the mixed liquor. On the basis of the 3-CA degradation rates of freshly released (within 24 h), active I2*rfp* cells in mineral medium (71.9 mg of 3-CA/(mg of CDW_{I2gfp}·d); see batch tests), the 3-CA removal rate of the freshly 6.6×10^8 released cells/(L of mixed liquor·d) [24 h × (10^6 I2*rfp* cells per tube) × 33 tubes in 1.2 L of mixed liquor] in reactor D2 was approximated at maximum 54.1 mg of 3-CA/(L of mixed liquor·d) [(71.9 mg of 3-CA/(mg of CDW_{I2gfp}·d)) × (6.6×10^8 freshly released I2*rfp* cells/L of mixed liquor) × (1.14 mg of $CDW_{I2rfp}/10^9$ I2*rfp* cells)]. The 12.9 mg of CDW_{I2rfp} aged cells/L of mixed liquor [(1.13×10^{10} aged I2*rfp* cells/L of mixed liquor) × (1.14 mg of $CDW_{I2rfp}/10^9$ I2*rfp* cells)] already present in the activated sludge (from earlier release) also counted for 33.0 mg of 3-CA/(L of mixed liquor·d) degradation [(12.9 mg of CDW_{I2rfp}/L of mixed liquor) × (2.5 mg of 3-CA/(mg of CDW_{I2gfp}·d))]. The 3-CA degradation rate of the aged suspended cells from reactor was assumed to be the same as for the aged suspended cells from reactor B2.

In conclusion, four different factors played a role in the 3-CA removal of reactors. The prolonged 3-CA degradative activity in reactor D may have been caused by a combination of minor adsorption on the silicone tubes (0.1%) and degradation activities by three different types of I2rfp cells: immobilized cells in the agar (estimated to be responsible for only 10% of the 3-CA removal), freshly released active cells (56% of the degradation capacity), and aged cells present in the sludge (34% of the degradation capacity).

I2*rfp* Cell Growth and Release from Silicone Tubes. During the whole experiment, about 40 mg of CDW_{I2rfp} was released [(10^6 cells/(h·tube)) × 33 tubes × 24 h × 44 d × (1.14 mg of $CDW_{I2rfp}/10^9$ I2*rfp* cells)]. Initially, only 18.5 mg of CDW_{I2rfp} was immobilized in the tubes [(1.3×10^6 cells/(h·tube)) × 33 tubes × 24 h × 45 d × (1.14 mg of $CDW_{I2rfp}/10^9$ I2*rfp* cells)]. Thus, growth of the I2*rfp* cells inside the tubes occurred, and they were released into the mixed liquor. The I2*rfp* cell growth rate (μ) and doubling time (t_D) were determined by

$$\mu = \ln(X_t/X_0)/t \quad (1)$$

$$t_D = \ln(2)/\mu \quad (2)$$

with X_t as the total biomass released and present in the tubes after 44 days (18.5 + 40 mg of CDW_{I2rfp}), assuming that the cell mass inside the tubes did not change over time; X_0 as the biomass present in the tubes at day 0 (18.5 CDW_{I2rfp}); and t as the duration of the experiment (44 days). The overall growth rate (0.026/day) and doubling time (26.5 days) were rather low.

The substrate necessary for growth was obtained from three sources. The first substrate source was the LB medium, which was mixed with the bacteria and inserted in the silicone tubes. One liter of LB medium contains as substrates 10 g of peptone/L and 5 g of yeast extract/L. This means that in the tubes 94 mg of substrate was present [6.25 mL of LB × (15 mg of substrate/L)]. A second source of substrate provision was obtained by the addition of 3-CA. During the whole experiment, 1936 mg of 3-CA [44 mg/(L·d) × 44 d] was added to the reactors, of which 6% or 119 mg was degraded by the immobilized cells (see previous section). The third substrate source could be the milk powder; however, it was not possible to determine how much substrate the immobilized cells received from this source. This means that for a yield factor of 40%, sufficient substrates were present to release 40 mg of CDW_{I2rfp} during the experiment. In conclusion, the cells immobilized in the silicone tube were growing slowly and were released into the mixed liquor. Substrates necessary for growth support were sufficiently provided by the LB medium present in the agar and by the 3-CA metabolism.

Discussion

Inoculation of a catabolic strain by immobilizing it in agar and surrounding it by open-ended silicone tubes significantly prolonged the bioaugmentation capacity of the strain in the SCAS system exposed to realistic 3-CA concentrations. Concentrations up to 230 mg of 2-chloroaniline/L in polymer industrial wastewaters (*20*) and a mixture of 4-chloroaniline (21.4 mg/L), 2-chloroaniline (1.1 mg/L), 2,5-dichloroaniline (3.1 mg/L), 2,3-dichloroaniline (99 mg/L), and 3,4-dichloroaniline (260 mg/L) in a 3,4-dichloroaniline manufacturing company (*32*) have been reported. The increase in bioaugmentation capacity was primarily due to degradation of 3-CA by the released suspended cells (56%), followed by the aged suspended cells (34%), and only a minor part by the encapsulated cells (10%).

The slow release concept is to our knowledge a new bioaugmentation strategy for bioreactors. In several studies where the use of carrier materials was examined, positive effects on survival and activity of inoculated strains have been reported (*15*). Loh et al. (*14*) used hollow fiber membranes to immobilize phenol-degrading *Pseudomonas putida* cells to treat high concentrations of phenol (1 g/L). Encapsulation of pentachlorophenol-degrading bacteria in polymeric material had no negative effect on the metabolic activity of the immobilized cells (*12*). Our work identifies an additional mechanism by which encapsulation improves biodegradation, namely, continuous but relatively low level release of "nonstarved" cells. In our setup in reactors D2, cell release could account directly or indirectly for 90% of the degradation occurring in a bioaugmented reactor due to the aged cells in the sludge flocs (34%) but also due to the freshly released cells (56%). The release of free suspended cells may become of critical importance for trace toxicants that need to be removed from industrial wastewater streams because

typically biodegradation rates at low concentrations are better in suspended cultures as compared to fixed cultures (*33*). Thus, a slow release bioaugmentation setup is at least as effective as the systems with complete immobilization or massive inoculation of specialized cells.

There was a significant difference between the capability of newly released or inoculated I2 cells to degrade 3-CA and aged I2*gfp* cells. While newly released cells continuously degraded 3-CA, the biodegradative activity of the original inoculum decreased substantially in time. In our previous work, lower degradation activity was also observed for I2*gfp* cells continuously exposed to 3-CA (*7*), resulting in an accumulation of 3-CA in the effluent. Bossier and Verstraete described a *C. testosteroni* strain, A20, that expressed a phenotypic shift when the strains were cultured under unfavorable conditions (*34*). The cells altered to the hydrophobic form and dense flocs were formed, providing cellular protection. The I2 cells also showed a similar phenotypic instability and formed flocs in the activated sludge once they were inoculated (*7*). We postulate that this phenotypic shift is detrimental to 3-CA degradation capacity. In a similar vein, starvation-induced flocculation by *Ralstonia eutropha* increased hydrophobicity and a complete loss of phenol-oxygenation activity (*10*). Thus, the continuous provision of new suspended I2*rfp* cells from the tubes could result in a higher metabolic rate since these cells are not yet starved and flocculated by the harsh environmental conditions.

The slow release strategy had no detrimental effects on the reactor functions. While the COD removal rate decreased slightly in the nonbioaugmented reactor during the first week, it remained constant in the bioaugmented reactor (data not shown). The suspended solids concentration remained the same for the different reactors. The sludge volume index of the reactors with the silicone tubes increased slightly as compared with the control reactors (data not shown). Also the use of regular reinoculation of metabolic active cells can be an option to maintain a high biodegradative capability. Repeated massive inoculation can however give rise to the disappearance of critical bacterial groups, such as nitrifiers due to the overgrowth of protozoa (*22*), so that the reactor is no longer achieving its desired function.

Over a 44-day period about 3.5×10^{10} I2*rfp* cells were released into the mixed liquor. The substrates, provided by the LB medium in the agar and by the 3-CA diffused into the silicone tubes, were sufficient to sustain growth of the immobilized cells, which were subsequently released. These slowly released I2*rfp* cells in reactors C2 survived better (3.5×10^5 cfu/mL of mixed liquor at day 32) as compared to the suspended I2*gfp* cells (3.9×10^3 cfu/mL of mixed liquor at day 32). Thus, I2*rfp* cells embedded in silicone tubes grew, and cells slowly diffused out of the silicone tubes into the mixed liquor where they rapidly degraded 3-CA. The dual labeling strategy allowed the monitoring of the replacement of the I2 cells that were inoculated as a suspension by the continuously released cells, as is shown in Figure 3. The newly released I2*rfp* cells thus seemed to be more competitive in the reactors than the aged I2*gfp* cells. In these experiments, the positive effect lasted for at least 45 days. Longer experimental setups are needed to verify how long the slow release seeding can be effective.

In most activated sludge bioaugmentation experiments, inoculation with a specific strain generally has no effect or only a transient one. This study has demonstrated that encapsulation of the inoculum improves biodegradation of 3-CA for an extended period of time in (industrial) wastewaters. The slow release of specialized, metabolic active cells was essential to maintain prolonged bioaugmentation, in contrast to their "aged" counterparts that lost biodegradation activity after 30 days in the reactor.

Acknowledgments

This work was supported by the project grant G.O.A. (1997−2002) of the "Ministerie van de Vlaamse Gemeenschap, Bestuur Wetenschappelijk Onderzoek" (Belgium) and by an equipment grant (epifluorescence microscope) of the Flemish Fund for Scientific Research (FWO-Vlaanderen) to E.M.T. We thank Søren Molin for providing the strains necessary for the construction of *C. testosteroni* I2*rfp*; Janus Haagensen for useful suggestions; Siska Maertens and Katrien Crul for technical assistance; and Tom Van de Wiele, Sunny Aiyuk, and Korneel Rabaey for helpful comments redrafting the manuscript.

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Received for review April 8, 2002. Revised manuscript received August 9, 2002. Accepted August 14, 2002.

ES020076Q