Journal of Applied & Environmental Microbiology, 2014, Vol. 2, No. 4, 185-193
Available online at http://pubs.sciepub.com/jaem/2/4/12

DOI:10.12691/jaem-2-4-12

Exploring the Efficacy of Bioaugmentation Strategy in Microbial Degradation of Chloroaniline

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Received June 03, 2014; Revised June 15, 2014; Accepted June 16, 2014

Abstract *Pseudomonas spp*. was isolated from activated-sludge and found to be able to mineralize 3-chloroaniline (3-CA). This strain was tested for its ability to clean wastewater containing 3-CA upon inoculation in activated-sludge. To monitor its survival, the strain was chromosomally marked with the *gfp* gene and designated A*gfp*. After inoculation into a lab-scale semi continuous activated-sludge (SCAS) system, the inoculated strain maintained itself in the sludge at least 45 days and was present in the sludge flocs. After an initial adaptation period of 6 days, complete degradation of 3-CA was obtained during two weeks, while no degradation at all occurred in the non-inoculated control reactors. Upon further operation of the SCAS system, only 50 % 3-CA removal was observed. Denaturing Gradient Gel Electrophoresis (DGGE) of 16S rRNA genes revealed a dynamic change in the microbial community structure of the activated-sludge. The DGGE patterns of the non inoculated and the inoculated reactors evolved after 7 days to different clusters, which suggests an effect of strain inoculation on the microbial community structure. The results indicate that bioaugmentation, even with a strain originating from that ecosystem and able to effectively grow on a selective substrate, is not permanent and will probably require regular resupplementation.

Keywords: *Pseudomonas, gfp, chloroaniline, DGGE*

Cite This Article: M. Shah, "Exploring the Efficacy of Bioaugmentation Strategy in Microbial Degradation of Chloroaniline." *Journal of Applied & Environmental Microbiology*, vol. 2, no. 4 (2014): 185-193. doi: 10.12691/jaem-2-4-12.

1. Introduction

Bioaugmentation is the accelerated removal of undesired compounds from contaminated hazardous waste sites or bioreactors by using indigenous or allochthonous wild type or genetically modified organisms (Van Limbergen et al., 1998). Biological wastewater treatment is, in essence, an endeavor in microbial ecological engineering. The goal of this endeavor is to manage microbial communities for the good of society by promoting degradation of oxygen-depleting organics, transformation of toxic substances, and removal of nutrients from water; thus, a firm understanding of the microbial ecology of wastewater treatment bioreactors is essential (Rittmann et al., 2006). Performance changes observed at the treatment plant scale (hundreds of cubic meters) are the emergent properties of an incredibly diverse assembly of w1018 individual microbial cells (1e2 cubic micrometers) (Curtis et al., 2003). Many of the critical process failures (i.e. unacceptable reduction in final effluent quality) that arise during biological wastewater treatment are likely attributable to variations in the relative abundance or activity of these cells (Graham and Smith, 2004). Such variations in microbial community structure are thought to be influenced by a combination of deterministic (reactor design, environmental and operational variables) and stochastic (probability of microbial dispersal into or out of a reactor) properties (Curtis and Sloan, 2006). Critical questions important to rational design and operation of these systems remain unanswered (Rittmann et al., 2006): What community structures, and what range of community dynamics, are optimal for different applications? What environmental conditions trigger optimal community assembly with its desired function? Are the conditions and outcomes predictable, reproducible, and controllable? Understanding, and eventually predicting, dynamics in community composition and its relationship to ecosystem function is one of the key engineering problems that might be solved through a more quantitative understanding of the microbial ecology of wastewater treatment processes (Gentile et al., 2007; Graham and Smith, 2004). Removal of COD is catalyzed in biological wastewater treatment plants (WWTPs) by activated sludge communities composed of a diverse assemblage of chemoorganoheterotrophic microorganisms. Numerous studies in recent years have characterized activated sludge microbial community structure via culture-independent studies (Wagner et al., 2002; Xia et al., 2010), and others have investigated succession and dynamics in these communities, particularly within specific microbial subpopulations such as nitrifiers (Wells et al., 2009), phosphorus-accumulating organisms (Slater et al., 2010), denitrifiers (Gentile et al., 2007), and methanogens

(Fernandez et al., 1999).Many of these studies have occurred in lab-scale systems, however, where selective pressures likely differ dramatically from those in full-scale plants (Seviour and Nielsen, 2010). Indeed, fewstudies have directly characterized long-term, finescale microbial population dynamics in activated sludge from full-scale WWTPs. Moreover, we lack a quantitative understanding of the relative influence of specific environmental or operational drivers of community dynamics in full-scale systems, and it is not clear whether these dynamics are random or predictable. Activated sludge bioreactors are excellent test beds for fundamental questions in microbial ecology (Daims et al., 2006). A longstanding area of inquiry in ecology as a whole focuses on patterns of generation and maintenance of species diversity through time and space. In macroecology, spatial patterns in species diversity have long been recognized to follow a SpecieseArea Relationship (SAR) of the form S ¼ cAz, where S is species richness, A is the spatial scale of observation, c is an empirically derived constant, and z is a scaling exponent that reflects species turnover. Accumulation of microbial taxa richness with increasing spatial scales follow a similar pattern (Horner-Devine et al., 2004). Conversely, the speciesetime relationship (herein referred to as the taxaetime relationship [TTR]) has received comparatively little attention. First proposed by Preston (1960), the TTR describes the increase in observed taxa richness, S, for increasing time of observation, T, by a power law model, similar in form to the SAR: S ¼ cTw, where the scaling exponent w is a reflection of species turnover. The TTR has been documented in a rapidly expanding literature in macroecology (White et al., 2006), and has very recently been tested in a limited number of microbial systems (Redford and Fierer, 2009; van der Gast et al., 2008). The aims of this work was to investigate eventual enhanced 3-CA degradation by the activated-sludge after inoculation with a specialized 3-CA metabolizing strain. In addition, the effect of inoculation on the microbial community structure of the sludge was examined.

2. Material and Methods

2.1. Media and Culture Conditions

The mineral medium MMN (mineral medium without nitrogen and carbon) is derived from MMO mineral medium (280) by eliminating all nitrogen. The MMN medium contained 1419.6 mg Na2HPO4, 1360.9 mg KH2PO4, 98.5 mg MgSO4, 5.88 mg CaCl2.2H2O, 1.16 mg H3BO4, 2.78 mg FeSO4.7H2O, 1.15 mg ZnSO4.7H2O, 1.69 mg MnSO4.H2O, 0.38 mg CuSO4.5H2O, 0.24 mg CoCl2.6H2O, 0.10 mg MoO3 and 3.2 mg EDTA in 1 liter of distilled water. The liquid mineral media were supplemented with 150 to 250 mg aniline (Sigma- Aldrich Chemie, Germany) or 3-CA (Fluka AG Chemische Fabrik, Switzerland) per liter, while for the solidified media, aniline and 3-CA were supplemented at a concentration of 500 mg/liter. Luria Broth (LB) medium containing 10 g Bacto Peptone (Difco, Detroit, Mich., USA), 5 g Bacto yeast extract (Difco), and 5 g NaCl in 1 liter of distilled water was used as a rich medium. These media were solidified with 2 % agar for plate growth. Cultures were incubated on a rotary shaker under aerobic conditions at 28 °C. Growth was monitored by measuring the turbidity at 600 nm. Two hundred microliters of an overnight-grown LB culture of strain I2, washed twice in saline (0.85 % NaCl), was inoculated in 200 ml of MMN medium with 3-CA (150 mg/L) (0.1 % inoculation) to monitor the transformation of 3-CA.

2.2. Marking with *gfp*

Pseudomonas spp. A was isolated from a effluent treatment plant. Strain A can mineralize 3-CA completely and is rifampin resistant (100 µg/ml) and on LB-agar plates, the strain A showed a phenotypic instability, resulting in two types of colonies with different morphology. The strain *Escherichia coli* S17-1 λpir (pUT-miniTn*5 gfp*Km) (Herrero, 1990 ; Tomboline, 1997) was obtained by transformation (Chung, 1989). The pUTplasmid, was used for insertion of the *gfp* gene into the chromosome of strain A. Biparental mating between the donor strain *E. coli* S17-1 λpir (Herrero, 1990) and the recipient strain A with selection on LB plates with rifampin (100 mg/liter) and kanamycin (50 mg/liter) resulted in A*gfp* derivatives with the *nptII* and *gfp* genes inserted in the chromosome. This was confirmed by PCR with *gfp*-primers (see below).

2.3. SCAS Reactors

The experiments were conducted in duplicate with sludge freshly collected from industrial effluent treatment plant. The total count of the sludge was 4.4 10^8 bacteria/ml, determined with the Live/Dead Bacterial Viability Kit (L-13152, Molecular Probes, Eugene) as described by Boulos et al (Boulos, 1999). The reactors (2 liters total volume), with an active volume of 1.1 liters, were operated according to the semi continuous activated-sludge (SCAS) procedure at room temperature (ca. 21 °C). The reactors were fed every day after wastage of excess sludge and settling The SCAS reactors were operated at a volumetric loading rate of 1 g COD/liter· day, with a hydraulic retention time of 4 days and a sludge retention time of 11 days. All four reactors had a loading rate of 40 mg of 3-CA/liter· day, added as a daily single dose. One liter of the mixed liquor was subjected to a half-hour period of settling in an Imhoff cone to analyze the sludge volume (SV) (Greenber, 1992). On days 1, 3 and 5 of each week, the settling was followed by a decantation of 400 ml of the supernatant and the addition of 500 ml of fresh influent. The wasted sludge was used for analyze as follows: at day 1 of the week, a DNA-extraction of the sludge was performed and pH, oxygen uptake rate (OUR), and concentration of strain A *gfp* were determined; daily, an HPLC-sample was taken and the suspended solids (SS) and sludge volume index (SVI) were measured (Greenberg, 1992). Two duplicate reactors were inoculated with *Pseudomonas spp.* A *gfp* (reactors A), and two duplicate reactors were used as noninoculated control (reactors B). No important differences could be observed between the two duplicate reactors. Hence, unless otherwise indicated, the data reported are averages of both duplicates. The reactors were operated without 3-CA for 12 days to allow the microbial community to adapt to the changed environment and growth conditions. After this period, reactors A were inoculated with *Pseudomonas spp.*

A *gfp* to a final concentration of 3 x 10^6 cells/ml. The cells were pregrown overnight in LB medium, containing 100 mg 3-CA /L, washed twice with saline, and finally resuspended in saline.

2.4. Respirometric Activity Measurements

The metabolic activity of the activated sludge in general was expressed as OUR. Therefore, activated-sludge samples (200 ml) were transferred to the vessel and saturated with oxygen by means of bubbling air with a pump. Once oxygen saturation (ca. 8 mg O2/L) was reached, the aeration was ceased and the oxygen electrode was placed in such a way that the opening of the vessel was barely closed. Sodium acetate was added to a final concentration of 50 mg/liter. Samples were mixed with a magnetic stirrer during measurements. The method was further performed as described by Surmacz-Gorska et al. (Surmacz Gorska, 1995), calculating the activity from the constant slope of oxygen concentration over time. The activity measurements resulted in the OUR (grams of O2 per liter per day).

2.5. Analytical Methods

The supernatants of cultures were analyzed for 3-CA content by reversed-phase HPLC after centrifugation of the cells at 5,000 x g for 10 min. The HPLC system consisted of a Kontron liquid chromatograph with a DEGASYS DG-1310 system to degas the mobile phase, three Kontron 325 high-pressure pumps, a Kontron MSI 660 injector with a 20-µl loop, a Kontron DAD 495 diode array detector, and a 450 MT2/DAD software system. An Alltima C18 reversed-phase column (250 mm x 8 mm [inner diameter], 5-µm particle size; Alltech, Deerfield, Ill., USA) was used. The mobile phase consisted of CH3OH/NH4H2PO4 (0.1 M, pH 3.8)-H2O (70:25:5), with a flow rate of 0.75 ml/min. The UV-detector was used at 210 nm. Quantitative data for 3-CA were obtained by comparing the peak areas of unknown concentrations with the peak areas of standards of known concentrations.

2.6. DNA Extraction and Purification

Total DNA was extracted from the sludge samples by a method based on the protocols described previously (El Fantroussi, 1997; 1999). This protocol was modified as follows. Two milliliters of sludge was added to a 14-ml polypropylene round bottom tube (Falcon). To this 3 g of beads (0.10-0.11 mm) and 4 ml of 10 mM Tris/HCl (pH 9) were added. The mixture was beaten three times for 90 s using a bead beater at 2000 rpm. Then, 2 ml of 4 mg of lysozyme per ml in 10 mM Tris-/HCl (pH 9) were added, followed by incubation of the samples for 15 min at 28°C on a rotary shaker. Subsequently, 300 µl of 20% SDS was added and samples were slowly mixed for 5 to 10 min. After this, 1 ml of 8 M ammonium acetate was added. The supernatant was collected after centrifugation at 7,000 x g for 15 min at 4°C. A chloroformisoamyl alcohol (24:1) purification was done, followed by a centrifugation at 7,000 x g for 15 min at 4°C. The aqueous phase was transferred to a new tube, and 0.8 times volume of isopropanol was added. The precipitation was performed for 1 h at -20°C. Alternatively, 2.5 volumes of ethanol (100 %) were added for an overnight precipitation. The pellet (crude extract) was obtained by centrifugation at 12,000 x g for 25 min and was re-suspended in 250 µl of distilled water. A 100 µl aliquot of the crude extract was further purified using Wizard PCR preps (Promega, Madison, Wis., USA), and the purified DNA was finally recovered in 50 µl of sterile distilled water. For axenic cultures, the template for PCR-amplification was obtained by suspending a colony in 200 µl sterile distilled water, boiling for 15 min and storing at –20 °C. Two microliters of the lysed cells was used in the PCR reaction.

2.7. PCR Conditions

Two microliters of the extracted DNA was amplified by PCR with a 9600 thermal cycler (Perkin-Elmer, Norwalk, CT, USA). The PCR mixture used contained 0.5 µM each primer, 200 µM each deoxynucleoside triphosphate, 1.5 mM MgCl2, 10 µl of Thermophilic DNA Polymerase 10X Reaction Buffer (MgCl2-free), 2.5 U of *Taq* DNA Polymerase (Promega), 400 ng of bovine serum albumin (Boehringer) per µl, and Dnase- and Rnase-free filter-sterilized water (Sigma-Aldrich Chemie, Germany) to a final volume of 100 µl. The *gfp*-gene was amplified by PCR with a set of primers, based on specific regions of the *gfp*-sequence. The set consisted of the primer gfpF (5'-CCA-TGG-CCA-ACA-CTT-GTC-AC-3' [forward]) and gfpR (5'-CTT-TCG-AAAGGG-CAG-ATT-GT-3' [reverse]). The 16S rRNA genes from sludge microbial communities were amplified by PCR as suggested by El Fantroussi et al. (1999), using the forward primer P63f (5'-CAG-GCC-TAACAC-ATG-CAA-GTC-3'-forward) and the reverse primer P518r (5'-ATT-ACC-GCG-GCTGCT-GG-3'-reverse) (208, 221). A GC-clamp of 40 bp (208, 221) was added to the forward primer. The length of the expected amplified fragment with GC-clamp was 530 bp.

2.8. DGGE

Denaturing Gradient Gel Electrophoresis (DGGE) based on the protocol of Muyzer et al. (208) was performed using the D Gene System (Bio-Rad, USA). PCR samples were loaded onto 6% (wt/vol) polyacrylamide gels in 1xTAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4). The polyacrylamide gels were made with denaturing gradient ranging from 40 to 60 % (where 100% denaturant contains 7 M urea and 40% formamide). The electrophoresis was run for 16 hours at 60°C and 50 V. After the electrophoresis, the gels were soaked for 5 min in fixation buffer (10% ethanol, 0.5 % acetic acid), and subsequently for 10 min in SYBR GreenI nucleic acid gel stain (1:10,000 dilution; FMC BioProducts, USA). The stained gel was immediately photographed on an UV transillumination table with a video camera module (Vilbert Lourmat, Marne-la vallé, France).

2.9. Analysis of DGGE Patterns

The statistical comparison of the DGGE patterns on the same gel was done with the GelCompar software 4.1 (Applied Maths, Kortrijk, Belgium). The calculation of the matrix of similarities is based on the Pearson product-moment correlation coefficient. The clustering algorithm of Ward (1963) was used to calculate dendrograms.

3. Results

3.1. Survival and Activity of *Pseudomonas spp.* A *gfp* in the SCAS-reactors

In order to monitor the survival of strain A in activated sludge, it was chromosomally marked with the *gfp* gene, which was expressed constitutively by a P*psbA*-promotor (Tomboline, 1997). The insertion of the *gfp* gene in several transconjugants was confirmed by PCR with the *gfp*specific primers gfpF and gfpR. The *gfp*-marked strain A *gfp* showed the same degradation characteristics as the original strain in MMN medium (data not shown). Before strain A *gfp* was inoculated into the sludge reactors (reactors A) and before 3-CA was added, the sludge was adapted for 12 days to the operating SCAS system. At this point (day 0 in Figure 3.1), the daily supplementation with 3-CA started. During the first three days, no degradation was observed in any of the reactors. In the inoculated reactors A, enhanced degradation was observed from day 4 until day 7, and during the next 12 days complete degradation of 3-CA was achieved. After three weeks, however, the concentration of 3-CA increased and stabilized at a level corresponding with ca. 50 % degradation. A mass balance calculation of 3-CA in a reactor (with 0 % degradation) showed that the concentration of 3-CA fluctuated weekly, based on the daily addition and the washout. No significant removal of 3-CA occurred in the control reactors B during the first 30 days. However, from day 30, enhanced degradation was observed in reactor B1, comparable to that in the inoculated reactors. The other control duplicate, B2, did not show any enhanced degradation. The survival and behavior of *Pseudomonas spp.* A *gfp* were monitored by different methods. The most sensitive way was by plating on LB medium with kanamycin and rifampin (detection limit, 10 CFU/ml). In case of background growth by indigenous bacteria, the inoculated strains could be recognized by green fluorescence protein autofluorescence under a long-wave-length UV lamp. During the first week, the concentration of A*gfp* cells did not differ much from the initial value, and during week 2 and 3, the concentration of strain A*gfp* even increased (Figure 3.1). After 30 days, the inoculum size stabilized at ca. 5 x 10^5 CFU/ml. PCR with *gfp* primers was tested as an alternative technique to monitor the survival in sludge. The detection limit to obtain an amplification signal was ca. 5 x 10^5 CFU/ml, however, which was higher than for the plating method. In the control reactors no amplification was observed. The distribution in the cell flocs was studied by epifluorescence microscopy. After a few days of inoculation, *Pseudomonas spp.* A*gfp* cells were visible as green cells under UV-light, although the sludge microbial community had background fluorescence. The inoculated bacteria were not randomly distributed but were observed as clusters within the sludge flocs.

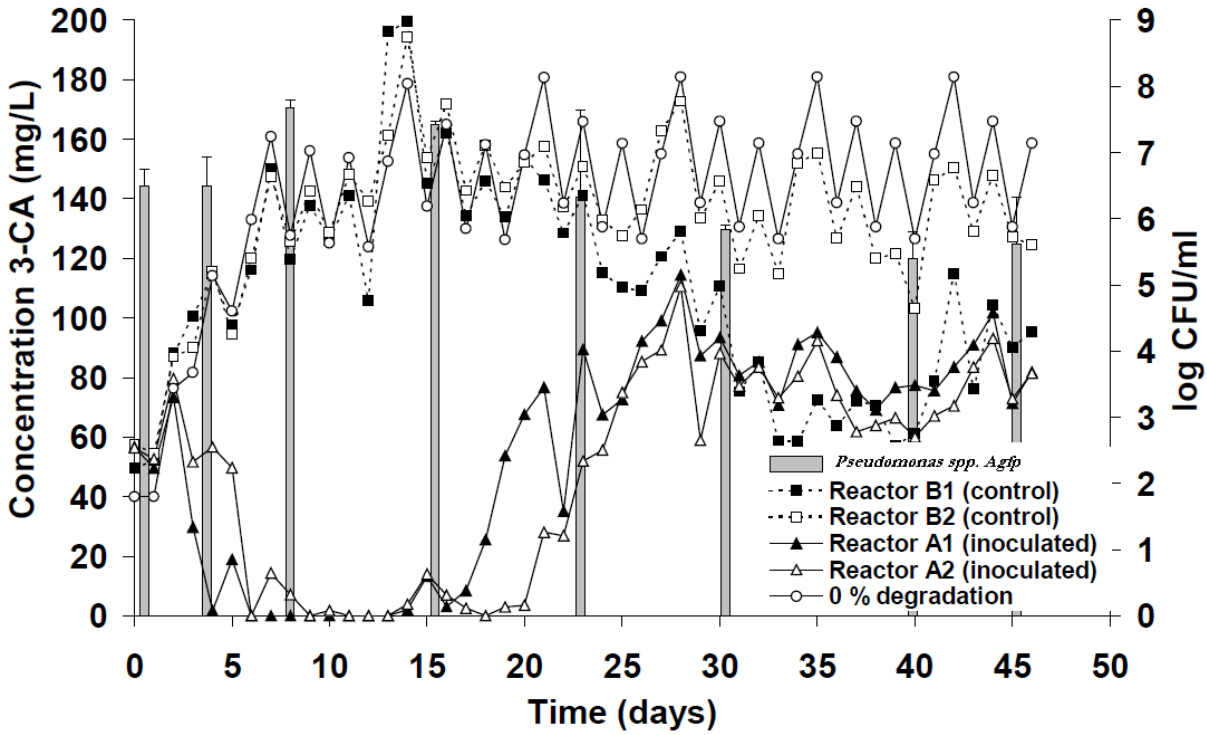

Figure 3.1. Concentration of 3-CA in the inoculated reactors A1 (▲) and A2 (△) and the control reactors B1 (■) and B2 (), together with a simulation of the 3-CA concentration if no degradation occurred (0% degradation) (), and survival of *Pseudomonas spp. Agfp* in reactors A1 and A2 (bars)

3.2. Reactor Performance Characteristics

At the beginning of the 3-CA supplementation, all reactors showed the same performance parameters (OUR= 74 mg O2/L.h; SS = 4.2 g/L; SV =250 ml/L; SVI = 60 ml/L; pH = 7.6). The OUR and pH of both reactor sets did not differ much from each other during the experiment (Figure 3.2). The OUR variability of different days of one week was rather high, but the values were in the same range when the same day of different weeks were compared. After a week the concentration of suspended solids (SS) of the control reactors B decreased about 0.6 g/liter in comparison to that in reactors A. The sludge volume (SV) after 30 minutes of both reactors was

significantly different after ca. one week (two-tailed t-test; $\alpha = 0.05$). During the first week, the SV of reactors A increased from 300 ml to 500 ml, and remained 500 ml for the rest of the experiment. The SV of the control reactors B was stable at 300 ml during the first 40 days and it increased only during the last week. This resulted in a significant difference between the SVI-values of both reactors for the period before 40 days (two-tailed t-test; $\alpha = 0.05$) (Figure 3.3).

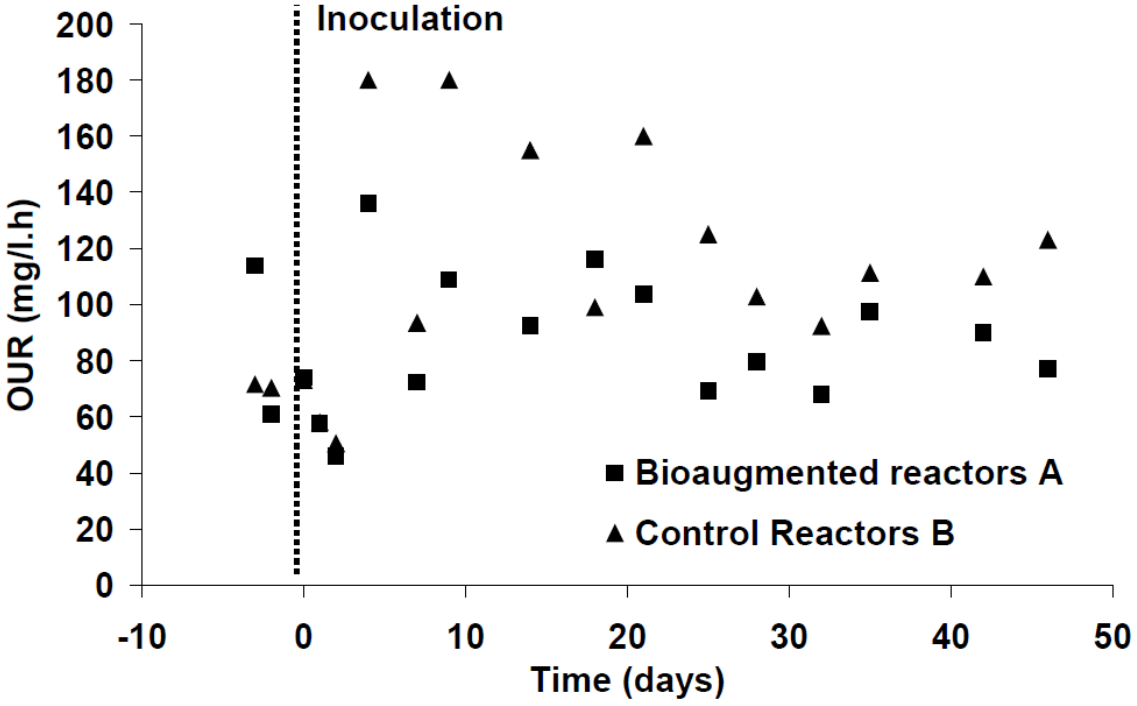

Figure 3.2. Oxygen uptake rates (OUR) of the inoculated reactors A and the control reactors B; the dotted line represents the start of 3-CA supplementation time of inoculation

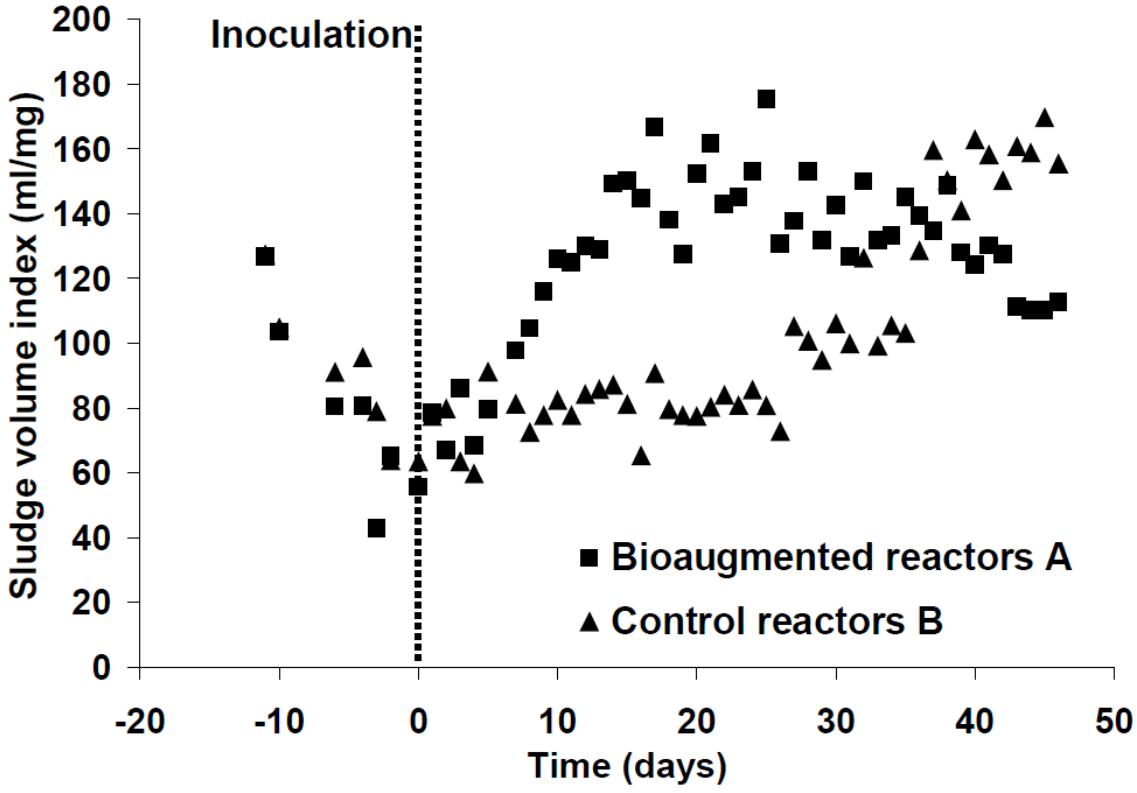

Figure 3.3. Sludge volume index (SVI) of the inoculated reactors A and the control reactors B; the dotted line represents the start of 3-CA supplementation time of inoculation

3.3. DGGE

In order to monitor the changes within the microbial community of the SCAS reactors, the diversity of a 16S rRNA fragment was examined. Each week, a sample was

taken from the four reactors and, after DNA extraction and purification, the PCR-amplified product was analyzed on a DGGE-gel (Figure 3.4A and B). The patterns of the four reactors were compared with each other after normalization. To determine the information content of the banding patterns in terms of structural diversity, they were analyzed by clustering (Figure 3.4C). The cluster analysis revealed three major groups. The fingerprints showed several very strong bands, some bands of lower intensity, and an additional number of weak bands, resulting in a smear. Before the adaptation period started (day -12) and at the day of the inoculation and feeding with 3-CA (day 0), the DGGE patterns of both reactors of both times clustered together. After the first week (day 7), each reactor series began to develop a different microbial community, which was clearly separated from that of the other reactor series and from the earlier days of the experiment. On some bands, corresponding with a bacterial species, the applied treatment and the inoculation had no influence. Some bands became dominant in the reactors, while other bands vanished. The intensity of some fragments seems to be enhanced by the presence of strain A*gfp*, while other species were disfavored by the inoculation.

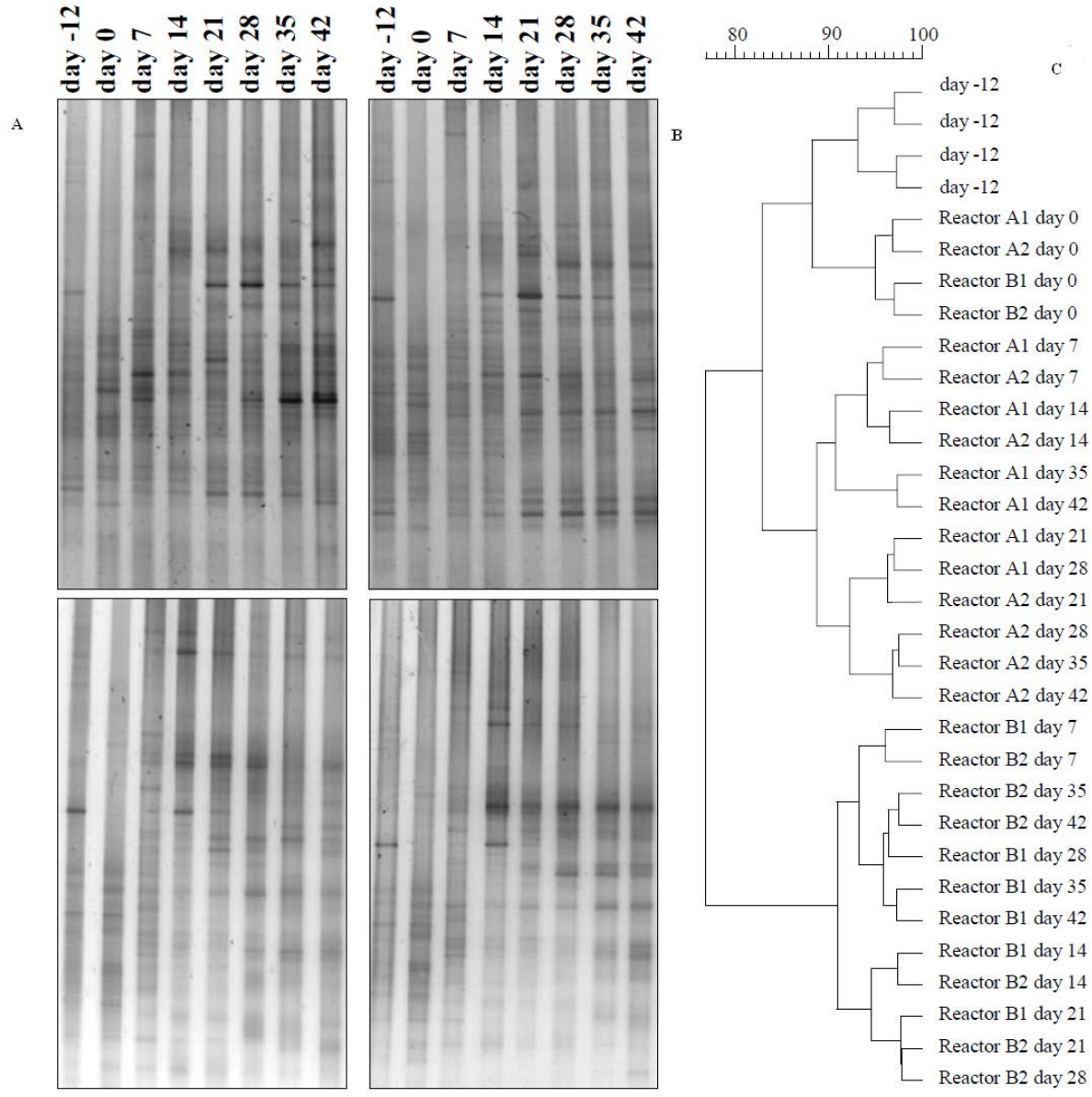

Figure 3.4. Analysis of the DGGE profiles of the different reactors at different times; using partial 16S rRNA gene fragments. (A) DGGE gel of the inoculated reactors A1 and A2; (B) DGGE gel of the control reactors B1 and B2; (C) dendrogram of all reactors, clustered by use of the Ward method (326)

4. Discussion

In this paper, the 3-CA degrading indigenous activated-sludge bacterium *Pseudomonas spp.* A was used to accelerate the removal of 3-CA from wastewater by reinoculating the strain at high density into the same sludge system. The initial sludge microbial community was not able to effectively degrade 3-CA during the first weeks of the experiment, although strain A was probably present, since it was isolated from the same activated-sludge plant. Apparently the natural level of the indigenous strain *Pseudomonas spp.* A was too low to affect the degradation of 3-CA. To distinguish the inoculated strain from identical or similar indigenous sludge bacteria, the strain was chromosomally marked

with the *gfp* gene. The plating method, combined with the *gfp* visualization with UV light, allowed sensitive and reliable monitoring of the survival of the inoculated strain and was preferred over PCR amplification of the *gfp*-gene, which was not as reliable and sensitive. Similar difficulties with PCR-based strain detection were described by Tchelet et al (1999), who used specific primers for the 16S rRNA gene and chlorobenzene degradation (*tcb*) genes to monitor an inoculated *Pseudomonas* strain in activated sludge. Their study and ours thus show that plating can still be a reliable method when the strain has natural or inserted (*gfp*-Km) specific phenotypes. It is not known, however, if this culturable fraction (CFU of A*gfp*) resembles the total viable count of A*gfp* in the sludge. Successful bioaugmentation mainly depends on the behavior of the inoculated strain in the environment where it is introduced. Therefore, a first criterion is good survival and retention of the strain in the system. The growth rate of the organism may be slower than washout (Watanabe, 1998) and the rate of predation, for example, by protozoa (Goldstein, 1985). In our experiment, an equilibrium seemed to be reached between washout, predation, and growth rate after three weeks with an inoculum cell densities of ca. 5×10^5 CFU of strain A*gfp* per ml. The origin and the type of inoculated strain also can play an important role in the survival of the strain. Tchelet et al. (1999) used *Pseudomonas* sp. P51, originally isolated from sediments, for a bioaugmentation experiment in a soil column and sewage sludge. The survival and activity of strain P51 in the soil column were successful, but the strain was not able to maintain itself in the sludge reactors and thus no degradation was observed. McClure et al. (1991) showed that a sludge isolate, AS2, was able to reach a stable level after inoculation, in contrast to other inocula tested. Strain AS2 had a characteristic flocculation, which may have been an important factor in the survival. *Pseudomonas spp.* A *gfp*, used in this study, also maintained a stable population in the activated-sludge system. The original strain A, also isolated from a sludge environment, tends to form clusters within the sludge flocs. This observation, together with the unstable colony morphology on agar plates, suggests the formation of exopolysaccharide production, which was described by Bossier and Verstraete (1996). The possibility that strain A*gfp* changes phenotype under unfavorable conditions may be an important factor in the maintenance of a stable population in the SCAS-reactor. The second criterion for successful bioaugmentation is the activity of the inoculum. In our experiment, after an initial adaptation period of 6 days, complete degradation of 3-CA was obtained during two weeks, while no degradation at all occurred in the noninoculated control reactor. Upon further operation of the SCAS system, 50 % 3-CA removal was observed. The lower 3-CA removal of the inoculated reactors can be due to the declining population density of *Pseudomonas spp.* A *gfp* or due to a decreased metabolic activity of the A*gfp* cells. Although McClure et al. (1991) could establish a stable population of the introduced strain in the sludge environment, no enhanced degradation of chlorobenzoate was observed. Different authors proposed that the inability of the inoculated strains to degrade the xenobiotics may have been due to the availability of alternative substrates (Blumenroth, 1998; Goldstein, 1985; McClure, 1991; McClure, 1989; Schmidt, 1985; Swindoll, 1988). However *Pseudomonas spp.* A *gfp* received daily only 40 mg 3-CA/liter together with 1 g of COD per liter (diluted milk powder) and performed its specific activity within 2 weeks. Two preliminary SCAS experiments with the same strain A*gfp* showed a very similar positive effect on 3-CA degradation during at least two weeks (data not shown). This observation was corroborated by the findings that when the pure culture was grown in LB medium supplemented with 100 mg 3-CA/liter, 3-CA was not detectable after one day (data not shown). The degradation of 3-CA by strain A*gfp* therefore is not repressed by additional nutrients. Compared with the calculated 0 % degradation curve, no degradation of 3-CA was observed in either noninoculated control reactor B within 4 weeks. However, during the fifth week, enhanced degradation was observed in one of the reactors, probably due to the enrichment of indigenous bacteria with degradative capacities. It has been reported that in some cases indigenous bacteria become capable of removing xenobiotics after a long exposure time, either by metabolism or co-metabolism (McClure, 1989; Nüßlein, 1992; van der Meer, 1998**;** Weber, 1994). However, in our parallel SCAS reactors, the differences in degradation rates between the inoculated and control reactor were striking and stable over a prolonged time period, suggesting that the bioaugmentation was effective and not ephemeral. The microbial community structure of the SCAS-reactors was monitored by DGGE of 16S rRNA genes. The changes of the patterns over time suggest that the structure of the microbial communities was not static but rather dynamic. After 7 days, the microbial communities in both series of reactors evolved into separate clusters. The inoculated strain could not be seen in the DGGE patterns, most probably because its proportion of the total bacterial cell count was too small and a DGGE pattern only reveals the numerically dominant populations. Eichner et al. (1999) investigated the bioprotection of activated sludge from pollutant shocks by the related method TGGE (Temperature Gradient Gel Electrophoresis). Those authors observed subtle shifts in community structure during adaptation to laboratory conditions. In their tests, the microbiota of the non-inoculated control reactor collapsed after the shock load of xenobiotics, resulting in both a lower OUR and a decrease in bands in the TGGE-pattern. In our studies, the diversity of bands in the pattern of the control reactor did not visibly decrease, and no drastic changes in the reactor performance were observed during the experiment. This was confirmed by the OUR- measurements, where only small differences could be observed with the inoculated reactor. In contrast to shock load, applied by Eichner et al. (1999), the continuous supplementation of low concentrations of 3-CA in our experiment gave the sludge time to adapt. Remarkably, the DGGE-technique combined with clustering analysis revealed subtle responses to the inoculation of strain A*gfp*. Indeed some species seemed to be enriched after the inoculation, while others tended to be less abundant. This work indicates that bioaugmentation of activated-sludge systems for specific trace organics, such as 3-CA, can be achieved successfully. Moreover, this work corroborates what is often experienced in the use of activated sludge systems, i.e., that inoculation with a specific strain generally has only a transient effect. The fact that even an indigenous strain is

only temporarily effective in activated-sludge communities substantiates the experience that biological supplements for such systems have to be added on a regular basis in order to assure continuous treatment efficacy. Further research will be performed to try to prolong the period of efficient degradation by the inoculum.

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