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ORIGINAL PAPER

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Biomonitoring of continuous microbial community adaptation towards more efficient phenol-degradation in a fed-batch bioreactor

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Abstract The anaerobic degradation of phenol was studied in a fed-batch culture. Nitrate was added as electron acceptor and phenol was provided three times, to a final concentration of 200 mg/l. Randomly amplified polymorphic DNA (RAPD) and terminal fraction fragment length polymorphism (T-RFLP) were used and compared in order to monitor the microbial succession in the reactor. Phenol degradation started after an initial lag phase of 14 days and was then completed within a few days. In addition, the duration of the lag phase was shortened and the degradation rate was increased after each phenol amendment. Nitrate reduction correlated with microbial growth and phenol depletion, confirming that the degradation was carried out anaerobically. Results from the DNA analysis showed that the structure of the microbial community changed after each phenol amendment. This study confirms the potential for anaerobic degradation of environmental pollutants and also confirms that microbial acclimation towards faster degradation rates occurred upon repeated substrate amendments. Furthermore, both of the DNA-based techniques described the phenol degradation-linked community shifts with similar general results. RAPD is a faster, simpler technique that gives a higher resolution and consequently reflects the shifts in the microbial community structure better, whereas T-RFLP is more suitable for phylogenetic studies.

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Introduction

Anaerobic treatment processes are based on the use of anaerobes to degrade environmental pollutants. They are attractive to implement in contaminated areas, such as deep sub-surface soil, groundwater, and sediments, where anaerobic conditions are likely to occur (Fathepure and Tiedje 1999). Anaerobic processes also limit the risk of volatilization of the hazardous pollutants, as there is no need for aeration (Bell et al. 1993; Johnston et al. 1998). Anaerobes able to degrade many environmental pollutants have been isolated and characterized during recent decades (Evans and Fuchs 1988; Coates et al. 1996; McNally et al. 1998). However, there is still a need to enlarge the known spectra of anaerobic degraders, especially since the lack of oxygen at contaminated sites often limits their natural remediation (Alexander 1994).

The isolation of a new pollutant-degrading strain often starts by exposing a microbial sample to the substance to be degraded. Indeed, when exposed to a new potential substrate, some microorganisms have the ability to undergo a series of changes in order to adapt and ultimately metabolize this compound. This adaptation phase is also called the acclimation phase; and the absence of acclimated, indigenous microbial populations at contaminated sites is often indicated as a cause of pollutant persistence (Mueller et al. 1989; Alexander 1994; Razo-Flores et al. 1996). Moreover, although it is generally accepted that the acclimation phase has a crucial effect on the results of biodegradability studies, little is known about the phenomena involved in this process (Alexander 1994; Buitrón and Capdeville 1995). Practically, the parameters strongly influencing the success of biodegradability include the mode of cultivation (batch, feed-batch or continuous), the presence or absence of a substrate other than the pollutant tested, both the type and size of the inoculum, and the type of electron acceptor used (Watson 1993; Razo-Flores et al. 1996; Zaidi et al. 1996; Hu et al. 1998).

In addition, the study of the microbial community's structure and dynamics during the biodegradation pro-

cess is an interesting and demanding field of research. The ability to investigate compositional changes in microbiotic systems will extend our comprehension of the ecology and may eventually lead to better control predictions and optimized operation of these processes. A number of different methodologies have been used in this field, each with specific advantages and limitations. An increasingly popular strategy is to use DNA as a source of community structure information. This overcomes some of the limitations attached to other approaches that are dependent on culturing or are affected by physiological changes. It is known that only a small proportion of the environmental microbiota is culturable; and this may preclude the usefulness of inherently culture-based methodologies, such as carbon-source utilization patterns (Biolog plates; Hackett and Griffiths 1997; Heuer and Smalla 1997a). The significance of methods dependent on the physiological state of the microorganisms, such as the analysis of signature lipid biomarkers (Tunlid and White 1992; Gao et al. 1996), may also be reduced due to the impact of changes in environmental conditions on the microbial physiology.

This study investigated the development of a non-adapted complex microbial community towards faster biodegradation rates of phenol in an anaerobic fed-batch culture system. In this context, two different methodologies, terminal restriction fragment length polymorphism (T-RFLP) and randomly amplified polymorphic DNA (RAPD), were used and compared for the monitoring of the microbial succession in the fed-batch reactor. Phenol was chosen as the model, being a well known and widely spread pollutant (Keith and Telliard 1979) that is easily degradable under various anaerobic conditions (Kuever et al. 1993; Fang et al. 1996; Boopathy 1997; van Schie and Young 1998; Tawfiki et al. 1999; Shinoda et al. 2000). Phenol can also cause the failure of anaerobic wastewater treatment plants (Bitton 1994). Therefore, the study of the adaptation of a mixed population to the presence of phenol could serve to simulate and aid understanding of the situation when high loads of organic contaminants reach an anaerobic wastewater plant.

Materials and methods

Cultivation medium

The mineral salts medium (MSM) was composed of (per liter of deionized water): 2 g KNO_3, 2 g $NaHCO_3$, 2.5 g Na_2HPO_3, 6.4 g KH_2PO_4, 0.5 g NH_4Cl, 0.05 g $MgSO_4$, and 0.02 g $CaCl_2 \cdot 2H_2O$. Carbonate was added, because it has been reported as being necessary to the anaerobic degradation of phenol (van Schie and Young 1998). Phenol was provided at a concentration of 200 mg/l. The anaerobic degradation of phenol with nitrate can be described by the following reaction (Wang and Wang 1996):

$$C_6H_6O + 0.5NH_4^+ + 3.6NO_3^- + 3.1H^+ \rightarrow 3.5CO_2 + 0.5C_5H_7O_2N + 1.8N_2 + 3.8H_2O$$

Thus, 3.6 mol of nitrate are required to degrade 1 mol of phenol. The nitrate concentration was set up in large excess with respect to this ratio, to fully cover the cost of phenol degradation.

Fed-batch cultivation

The reactor was made of a 4.5-liter glass cylinder, closed with a rubber stopper and equipped with several valves for gas- and liquid-sampling. The reactor was filled with 4 l of oxygen-free MSM, provided with phenol, and inoculated with a sample of an anaerobic, activated sludge from the wastewater treatment plant at Lund (Sweden). The pH was automatically maintained at 7.5 with 0.5 M HCl.

Phenol and nitrate were provided two more times, each time 1 week after the phenol was totally depleted and according to the following procedure: 2 l of reactor broth were saved in the reactor, the rest being discharged. The reactor broth volume was then readjusted to 4 l, using fresh oxygen-free MSM and 0.752 g of crystalline phenol. The nitrate concentration was also adjusted back to its initial value, using KNO_3. This procedure was performed under anaerobic conditions. Yeast extract was only added initially, to enhance microbial growth and to provide anaerobic conditions (Ljungdahl and Wiegel 1986).

Analysis

The dissolved oxygen was regularly measured using an OXI320 oxygen sensor (WTW, Weilheim, Germany). Gas samples from the reactor head-space (500 µl) were withdrawn on a daily basis during the first week of incubation and analysed using a gas chromatograph and thermal conductivity detector (150 °C).

Bacterial growth was estimated by spectrophotometry from the measurement of the optical density (OD) at 660 nm, using an Ultrospec 1000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden).

Three 0.5-ml samples for DNA extraction and a 20-ml sample for chemical analysis were periodically withdrawn from the reactor and stored at –17 °C. Nitrate and phenol concentrations were regularly measured, using colorimetric assays (Dr. Lange test tubes and a LP1W photometer; Düsseldorf, Germany). These analyses were based on the reaction of nitrate with 2,6-dimethylphenol and on the complexation of phenol with 4-nitroaniline.

DNA extraction

Triplet DNA extractions were performed at 17 different timepoints. The protocol used was a modified bead-beater method (Miller et al. 1999). Phosphate buffer (300 µl, 100 mM, pH 8), 300 µl of SDS solution (100 mM NaCl, 500 mM Tris, pH 8, 10% SDS, w/v), 300 µl chloroform:isoamylalcohol (24:1), and 1 g of zirconia/silica beads (0.1 mm; Biospec Products, Bartlesville, Okla.) were added to 500 µl of sample. The mix was shaken vigorously in a cell disrupter (FastPrep FP120, BIO 101/Savant, Vista, Calif.) at 4.0 m/s for 20 s. Separation of the aqueous phase from particles and chloroform was made after centrifugation at 10,000 *g* for 10 s. The aqueous phase containing nucleic acid was extracted once with an equal amount of phenol:chloroform:isoamylalcohol (25:24:1) and was then transferred to a new tube for 2-butanol volume reduction down to 100 µl. The extract was purified on a Microspin S-400 HR column (Pharmacia Biotech, Uppsala, Sweden) according to the manufacture's instructions; and the extracted and purified nucleic acids were stored at –20 °C prior to use.

RAPD procedure

A short (10-mer) non-specific primer, RAPD3 (5′-CGG CCT GCA T-3′), was selected for the RAPD reactions (Wikström et al. 1999). RAPD3 was labeled with a fluorescent dye (IRD-41; MWG-Biotech, Ebersberg, Germany) at the 5′ end, in order to enable subsequent detection of the fingerprints (FP) with an automatic sequencer (Licor, Lincoln, Nev.). The full RAPD protocol has been described elsewhere (Wikström et al. 1999). One RAPD reaction was performed on each sample, i.e., three RAPD reactions at 17 sampling occasions gave a total of 51 RAPD reactions.

Each PCR was performed in a total volume of 25 µl. Then, 20 µl of an RAPD cocktail [resulting in a final concentration of 1.5 mM $MgCl_2$, 650 µM dNTP, 10 mM Tris-HCl, pH 8.8, 0.1% Triton X-100, w/v, 850 µM dimethyl sulfoxide, 0.6 U DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland) and 1.2 µM RAPD3 primer] was added to 1 µl of extracted nucleic acid solution (approximately 10 ng DNA) and 4 µl H_2O and was placed in a Genius thermal cycler (Techne, Cambridge, England). The samples were denatured for 30 s prior to PCR cycling. The amplification cycle was as follows: 94 °C for 1 s, 30 °C for 1 s, and 72 °C for 35 s. The PCRs were run for 35 cycles, followed by a 7 min elongation at 72 °C after the last cycle.

T-RFLP procedure

A modified version of a previously reported T-RFLP protocol (Clement et al. 1998) was used for the amplification of the 16S rDNA gene, restriction endonuclease cutting of the amplified product, and the separation thereof. A 1-µl sample of the extracted nucleic acid was mixed into 4 µl H_2O and then placed in a Genius thermal cycler (Techne, Cambridge, England). A hot start was implemented by adding 20 µl of a PCR cocktail to each sample, resulting in a final concentration of 1.5 mM $MgCl_2$, 650 µM dNTP, 10 mM Tris-HCl, pH 8.8, 0.1% Triton X-100, w/v, 570 µM dimethyl sulfoxide, 0.6 U DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland), and 1.2 µM of each primer (K1F: 5′-AGA GTT TGT TCC TGG CTC AG-3′ and K2R: 5′-GTA TTA CCG CGG CTG CTG G-3′; Clement et al. 1998). K1F was labeled with a fluorescent dye (IRD-41; MWG-Biotech, Ebersberg, Germany) at the 5′ end in order to enable subsequent detection of the T-RFLP fragments with an automatic sequencer (Licor, Lincoln, Neb.). The amplification cycle was as follows: 94 °C for 30 s, 64 °C for 30 s, and 72 °C for 30 s. The PCRs were run for 35 cycles, followed by a 7 min elongation at 72 °C after the last cycle. The PCR product was ethanol-precipitated and dissolved in 40 µl of H_2O. Visual examination of the agarose gel electrophoresis plate showed a similar concentration of the resulting PCR product for all samples. The purified DNA was cut with two different restriction endonucleases: *Rsa*I (Boehringer Mannheim, Mannheim, Germany) and *Fnu*D II (New England Biolabs, Beverly, Mass.). Next, 5 µl of the amplified 16 S rDNA fragment solution, 2 µl of buffer L (Boehringer Mannheim, Mannheim, Germany), 13 µl of H_2O, and 3 U of *Rsa*I were mixed and incubated at 37 °C for 6 h. Then, 5 µl of the amplified 16 S rDNA sequence solution, 2 µl of buffer M (Boehringer Mannheim, Mannheim, Germany), 13 µl of H_2O, and 8 U of *Fnu*D II were mixed and incubated at 37 °C for 12 h. Loading buffer (Sambrook et al. 1989) was added (1:1) and each sample was loaded on a 41-cm long, 6% polyacrylamide gel (Sequagel XR, National Diagnostics, Atlanta, Ga.). The samples were flanked by sequence reactions of known sequence, to enable exact size-determination of the T-RFLP fragments. The gel was allowed to run for 10 h in a laser-equipped, automated sequencer (Licor, Lincoln, Neb.) according to the manufacturer's instructions.

RAPD and T-RFLP data analysis

The RAPD analysis has been previously reported (Wikström et al. 1999) and is basically a procedure where the samples are separated on a polyacrylamide gel and detected using a laser detector, followed by transformation of the computerized image to a data matrix. This data matrix consists of 51 rows (samples) and 1,174 columns (variables). The same procedure was used for the T-RFLP fragments.

The microbial community in the bioreactor was described by 1,174, 422, and 277 variables at each time-point, originating from the microorganisms' RAPD and T-RFLP profiles (*Rsa*I and *Fnu*D II), respectively. The differences in the number of variables were due to the different number of DNA fragments that were obtained from each technique. All variables were mean-centered, but remained unscaled prior to analysis. Principal component analysis (PCA) was performed on the microbial community data in order to visualize the majority of the information in the data matrix. Simca-P 8.0 (Umetrics, Umeå, Sweden) was used for multivariate data analysis.

Intra-triplet variance calculations

The projection variance within each triplet was calculated to compare the reproducibility between RAPD and T-RFLP by: (1) normalizing all principal components to enable comparisons between PCA models, (2) calculating the standard deviation for each triplet for both principal components (PC), and (3) calculating the average of PC 1 and PC 2 for all models.

T-RFLP fragment analysis

Analyses of T-RFLP patterns are facilitated by the Ribosomal database project II (RDP II; Marsh et al. 2000). This on-line tool lists theoretically calculated sizes of T-RFLP fragments based on, currently, >16,000 prokaryotic small subunit sequences, by entering the labeled primer sequence and the restriction enzyme (*Fnu*D II and *Rsa*I in this study). Size comparisons between the listed and the observed T-RFLP fragments were performed.

Results

Anaerobic conditions

Analysis of gas samples from the reactor headspace was performed during the first week of cultivation and did not reveal the presence of oxygen: only argon, nitrogen, and carbon dioxide were detected. The dissolved oxygen concentration was also found to be constantly below 0.1 mg/l, except for just after the three phenol amendments (being 0.2, 0.6 and 0.5 mg/l, respectively).

Microbial growth, nitrate, and phenol depletion

Figure 1 shows the changes in OD, the total amount of nitrate consumed and the remaining phenol concentration throughout the experiment. Phenol and nitrate concentrations were readjusted on days 33 and 49. A drop in OD followed these readjustments, as the reactor broth was diluted with fresh medium.

Four phases can be distinguished during the first stage of cultivation. A rapid growth was observed on days 0–2 and the OD then stabilized at approximately 0.3, until day 7, when it started to drop, until day 14. A new increase was then observed until day 25, when the OD started to decrease again, until day 33. The nitrate concentration dropped rapidly during the first days of cultivation, stabilizing at approx. 11.5 mM on day 6. Nitrate consumption then started again up, until day 26, when the nitrate concentration stabilized at approx. 4 mM, until the phenol concentration was amended again. The phenol concentration remained constant until day 12. It then started to decrease exponentially, until day 26.

The second phenol amendment was directly followed by rapid microbial growth over days 33–42. From then

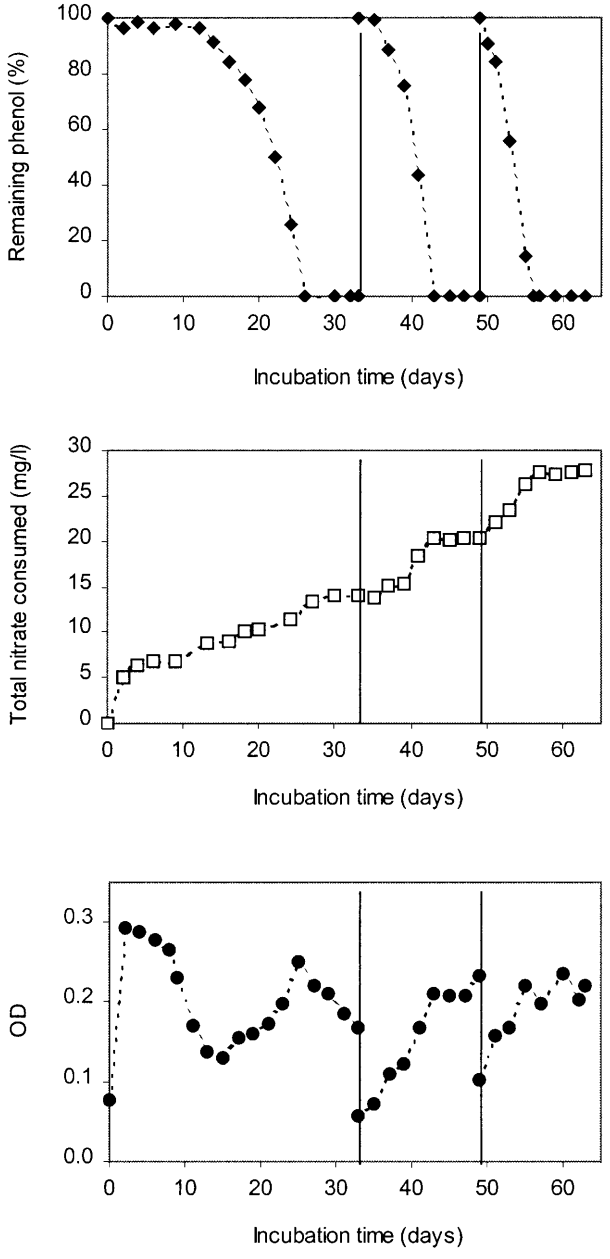

Fig. 1 Remaining phenol (◆), total nitrate consumed (□), and microbial growth (●) as a function of incubation time in the fed-batch reactor. Phenol and nitrate amendments performed at days 0, 33, and 49 are represented by a *vertical line*. *OD* Optical density

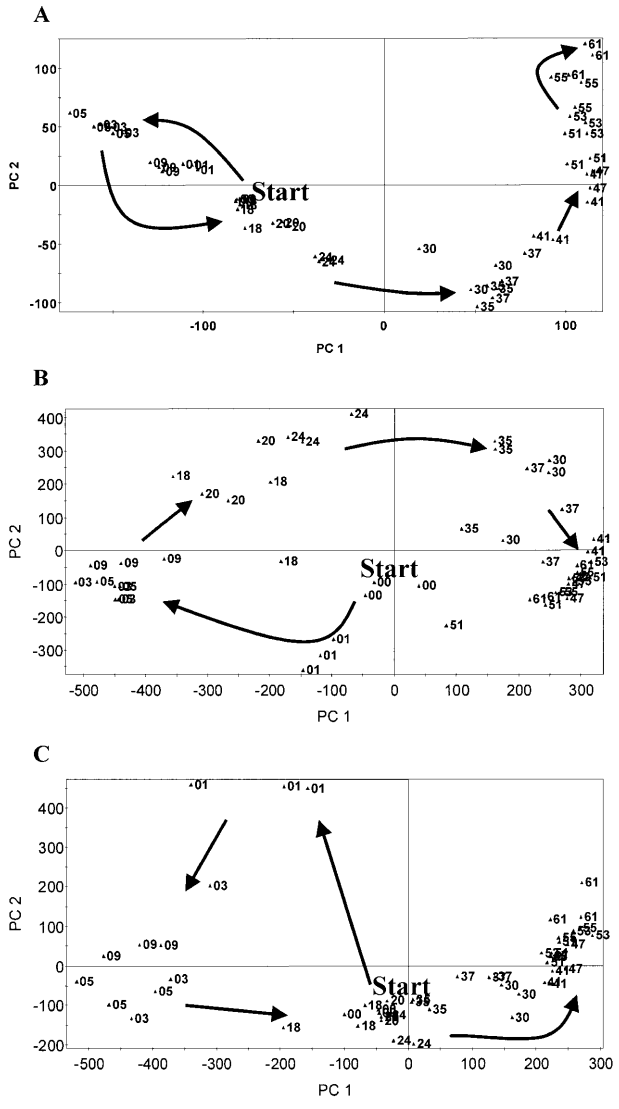

Fig. 2A–C Scoreplot from principal component (PC) analysis on 17 occasions for 3 replicates. **A** Randomly amplified polymorphic DNA (RAPD) profiles. The first and second principal components (*PC*) explained 50% and 16% of the total variation in data. **B** Terminal fraction fragment length polymorphism (T-RFLP) profiles (*Rsa*I). *PC 1*: 43% and *PC 2*: 18%. **C** T-RFLP profiles (*Fnu*D II). *PC 1*: 43% and *PC 2*: 16%. Days included were: 0, 1, 3, 5, 9, 18, 20, 24, 30, 35, 37, 41, 47, 51, 53, 55, and 61. The *arrows* indicate the time aspect

on, microbial growth only increased slowly, until day 49, when phenol was added for a third time. Phenol depletion started 2 days after this third amendment and was fully completed in 8 days. Nitrate consumption started on day 36, 3 days after the second phenol amendment. Nitrate concentration gradually decreased from 16.5 mg/l to approximately 10.1 mg/l, until day 43, when it stabilized.

Similar patterns in microbial growth, phenol, and nitrate concentrations were observed after the third phenol amendment. However, in that case, the phenol and nitrate consumptions were not delayed, but occurred immediately after amendment. Phenol removal was completed in 7 days and required 7.3 mM of nitrate. The OD increased from 0.1 on day 49 to 0.22 on day 55 and seemed to stabilize around this value until the end of the experiment.

RAPD and T-RFLP analysis

The scoreplots from the PCA of the RAPD and T-RFLP data revealed that the structure of the microbial community changed over time (Fig. 2A–C) and that shifts in the

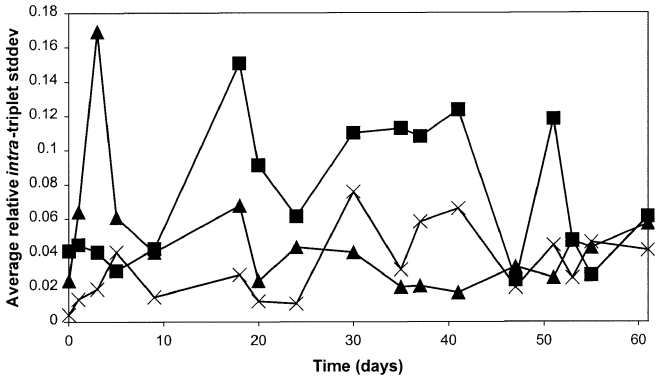

Fig. 3 Diagram showing the relative average standard deviation within each triplet, based on the first two principal components from three different PC analysis models over time. RAPD (×), T-RFLP[*Rsa*I] (■), and T-RFLP[*Fnu*D II] (▲)

microbial community generally correlated with the phenol amendment and degradation. A comparison of the reproducibility between RAPD and T-RFLP was also performed by calculating the average of the standard deviation in both PCs for each triplet over time (Fig. 3). The RAPD triplet variation was found to be at a relatively low level throughout the study, whereas the T-RFLP triplet variation differed, depending on the restriction endonuclease used. T-RFLP based on *Rsa*I-digestion resulted in higher variation than that based on *Fnu*D II-digestion. Taken together, the overall reproducibility between the triplets was better for RAPD than for T-RFLP analysis.

Changes measured by RAPD and the two T-RLFP analyses showed a change over days 0–3. After day 3, a steady state was reached, with a concomitant clustering of data points over days 3–9. However, the RAPD analysis resolved a change over days 5–9, which was not resolved in the two T-RFLP analyses. Over days 10–15, a transition was found to occur and both RAPD and T-RFLP analysis recorded this shift in the microbial community structure. Following this, there was a continuous change until the depletion of phenol (days 26–30). Next, a point cluster, containing days 30, 35, and 37, was observed in the scoreplots on RAPD and *Rsa*I-digested T-RFLP, indicating that the microbial community was relatively stable at these points. However, the FP generated by *Fnu*D II-digested T-RFLP was not coherent with this pattern.

A second amendment of phenol was made on day 33. The shift in the microbial community structure was not immediate, but delayed by at least 4 days. The PCA results from the RAPD and T-RFLP analyses differed somewhat beyond day 41. The T-RFLP profiles (Fig. 2B, C) beyond day 41 showed a high degree of clustering, whereas an extensive development of the microbial community was detected over days 41–61 by RAPD analysis (Fig. 2A). Consequently, the third phenol amendment on day 49 was undetected by T-RFLP analysis. In contrast, RAPD resolved the microbial community change after the third phenol amendment.

T-RFLP fragment analysis

A total of 27 and 23 T-RFLP fragments of different sizes appeared from all 17 time points, as a result of the *Fnu*D II and *Rsa*I digestion, respectively. For both digestions, approximately 50% of the fragments were decreasing in intensity or had disappeared after a few days. Ten of 27 (*Fnu*D II) and 11 of 23 fragments (*Rsa*I) were increasing in intensity or had appeared after a few days. The remaining T-RFLP fragments showed no clear trend. Five of the 27 *Fnu*D II and 6 of the 23 *Rsa*I fragments were not represented by any organism in the RDB II database. The T-RFLP on-line analysis resulted in a total of 360 and 66 candidate organisms from the remaining 22 *Fnu*D II and 17 *Rsa*I fragments, i.e., there were, on average, 16 (*Fnu*D II) and 4 (*Rsa*I) candidate organisms for each T-RFLP fragment. Fragments exhibiting similar appearance trends in both digestions did not in any case correlate with the same organisms in the RDP II database.

Discussion

The correlation observed between phenol removal, consumption of nitrate and microbial growth clearly prove that phenol was anaerobically degraded. Moreover, anaerobic conditions were maintained throughout the entire experiment and phenol was provided as sole carbon source in the system. The initial phase of microbial growth and nitrate depletion was probably caused by the use of yeast-extract and/or the chemical oxygen demand (COD) added from the inoculum. This was also confirmed quantitatively, in that the molecular ratios of nitrate to phenol depletion were estimated to be 4.2, 3.5, and 4.2 after the first, second, and third phenol amendments, respectively. These values, although they differ, are still close to the theoretical ratio of 3.6 found in similar experiments (van Schie et al. 1998). This emphasizes another advantage of anaerobic digestion: it is easier to control and supply with a sufficient amount of electron acceptor. This study also suggests that a novel acclimation methodology based on the use of nitrate or NOx sensors, combined with fed-batch culture, could be developed (Arvidsson et al. 1998), being the anaerobic equivalent to the aerobic respirometric techniques described by various authors (Buitrón and Capdeville 1995). Pollutant volatilization was also avoided, as the phenol concentration remained constant during the first 12 days of cultivation, before it started to be degraded.

Reactor performances were found to improve with the number of phenol amendments, thus showing that microbial adaptation was occurring. The length of the lag-phase significantly decreased after each phenol amendment, from initially being 12 days to being 2 days and then 1 day. Likewise, the phenol degradation rate (per day) increased from 0.02 to 0.05 and then 0.07 mmol/l after the first, second, and third phenol amendments, respectively. These results confirm that the use of a fed-batch reactor system with an activated sludge sample as

an inoculum is an efficient procedure for the isolation and acclimation of suitable degraders (Watson 1993; Razo-Flores et al. 1996).

The microbial succession in the fed-batch reactor was still developing after three phenol amendments. The largest microbial community composition alteration occurred over days 0–37, coinciding with the transition from the use of remaining COD and/or the yeast extract to phenol as the primary growth substrate (Fig. 2A–C). A smaller shift occurred from day 41, as consequence of the second phenol amendment. Moreover, the RAPD analysis showed that a continuation of the microbial selection occurred even after the third phenol amendment (Fig. 2A). This change was not resolved by the T-RFLP analysis. After the second and third phenol amendments, a lag period of approximately 4 days passed before any change in the microbial community could be detected. An immediate change was not expected, since the analyses were based on DNA, implying that only template concentration changes, i.e., differential growth, were being detected. After each phenol amendment, the phenol biodegradation rate was increased, corroborating the RAPD results, which showed a continuous adaptation of the microbial community, even after the third phenol amendment. This shows that the microbial population had not yet stabilized and that the phenol degradation rates could be further improved. In comparison, in a similar experiment conducted on an aerobic degradation of phenol, a stable activity was achieved in the reactor only after ten phenol amendments (Buitrón and Capdeville 1995). Likewise, two different denitrifying phenol degraders were isolated from the same enrichment culture after 1 year and 3 years of cultivation (Shinoda et al. 2000). Those authors also showed that the more recently isolated strain was better adapted to the iron-limited medium used in their experiment and, therefore, microbial acclimation was still occurring, even after this rather long period of cultivation.

T-RFLP is a three-step procedure reflecting the variation in the 16S rDNA composition (Clement at al. 1998; Marsh 1999). Therefore, it can indicate the replacement of strains within a microbial community. This was observed in this experiment, as various DNA fragments disappeared whilst others became visible during the incubation (data not shown). By comparison, RAPD is a DNA-fingerprinting method based on a one-step, random PCR-amplification of total chromosomal DNA originating from the microbial community (Franklin et al. 1999; Wikström et al. 1999). Hence, RAPD does not resolve whether the fluctuations observed are caused by the replacement of populations or by the adaptation of established organisms.

The comparison of RAPD and T-RFLP analysis for monitoring the microbial community structure over time exhibited a general common scheme (Fig. 2A–C). However, the intra-sample variance was smaller overall for RAPD, compared to T-RFLP (Fig. 3). The T-RFLP profiles obtained from *Fnu*D II digestion had a comparable, low intra-triplet variance level after day 20. The reproducibility of T-RFLP is apparently very dependent on the choice of restriction enzyme, since *Rsa*I-generated T- RFLP profiles exhibited the highest intra-triplet variance. In contrast, the major drawback with RAPD analysis is the low inter-laboratory reproducibility (Gao et al. 1996; McEwan et al. 1998). One reason for the higher intra-triplet reproducibility of RAPD analysis could be the high number of individual amplification products that were generated (~100), compared to T-RFLP, which resulted in approximately ten bands on the gels. The loss or appearance of one new band would affect the variability more for T-RFLP than for RAPD.

The higher information content of RAPD analyses compared to 16S rRNA based methods such as T-RFLP and DGGE (Heuer and Smalla 1997b; Liu et al. 1997; Marsh 1999) corroborated with the herein observed higher resolution, in detecting smaller structural changes in a microbial community. RAPD may also reflect physiological inferences better than analyses based on 16S rRNA, because bacteria closely related by 16S rRNA criteria can display widely different biodegradative capabilities. Moreover, additional limitations of the 16S rRNA-based strategies include the occurrence of chimeric by-products (Kopczynski et al. 1994; Robinson-Cox et al. 1995; Wang and Wang 1996) during the PCR amplification and primer-dependent exclusion of potentially important microorganisms.

The occurrence of chimeric PCR by-products is believed to be dependent on a high sequence homology of the PCR templates. Since the amplicons of the RAPD-PCR should have a low chance of being homologous, due to the short and randomly designed primers, the impact of this problem should be comparably small. Also, because of the primer design, no DNA species should be considerably biased for amplification over any other. In agreement with this, it has previously been reported that RAPD is a reproducible and quantitative tool for the monitoring of complex microbial communities (Frostegård et al. 1997; Larsson et al. 1999; Wikström et al. 1999).

However, the 16S rRNA techniques such as T-RFLP have the advantage that sequences can be compared with the extensive database of 16S rRNA sequences that is at hand (Ribosomal Database Project at Michigan State University, (http://www.cmc.msu.edu/RDP/html) and may permit sequence identification and a linkage to phylogeny. This is in contrast to RAPD analysis, where no such database is available. The analyses of the T-RFLP data, however, demonstrate the ambiguity of 16 S rRNA sequences. The first bias lies in the fact that no 16S rRNA primers are truly universal. Only a fraction of the published 16S rRNA sequences can be targeted with primers; and the published sequences are already most likely in a minority for the total number of microorganisms. Secondly, a large number of candidate organisms (in our study and average of 4 and 16, depending on restriction enzyme) have the same digestion pattern. This implies that, in complex microbial communities, it is almost impossible to determine the organism identity, based on the T-RFLP fragment length. Fragment isola-

tion from a high-resolution gel, purification, cloning, and sequencing of the 16S rRNA sequence would increase the resolution, but the process suffers from uncertainty, since there might be more than one fragment of the same size. It would require sequencing of a representative subset of clones from each ligation to determine a reliable result.

In conclusion, the fed-batch culture method has been proven to be an efficient method for the selection and acclimation of an anaerobic phenol-degrading bacterial consortium. Increasing biodegradation rates could be observed upon repeated phenol amendments and it was shown that the acclimation of the inoculum undergoes series of structural changes that could efficiently be observed with genotypic-fingerprinting techniques, such as RAPD and T-RFLP, analysed by PCA. However, RAPD was found to have a comparable or better reproducibility than T-RFLP for the monitoring of the microbial community structure in this study. RAPD analysis was also shown to have higher resolution for detecting the microbial succession. Such studies are fundamental for soil and groundwater remediation through microbial inoculation or bioaugmentation.

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