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# Biological treatment of propanil and 3,4-dichloroaniline: Kinetic and microbiological characterisation

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ABSTRACT

Propanil (3,4-dichloropropionanilide) is a widely used herbicide, applied worldwide in rice paddies. Propanil is primarily transformed in nature to 3,4-dichloroaniline (DCA), which is more slowly biodegradable. Both compounds have adverse health and ecotoxicity effects. This work investigated the microbial ecology and kinetics of propanil-degrading enrichments obtained from soil in a sequencing batch reactor (SBR) operated with different feeding strategies, aiming at the enhanced biological removal of propanil and DCA from contaminated waters.

During SBR operation with a dump feeding strategy, a high propanil concentration led to DCA accumulation, which was only fully degraded after 5 days, likely due to DCA inhibition. For this reason, the operational mode was changed to fed-batch operation with lower initial propanil concentrations, which resulted in faster propanil and DCA biodegradation. Thus a fed-batch operation seems more appropriate for the acclimatisation of an effective propanil- and DCA-degrading population.

The changes in performance were accompanied by a shift in the microbial population structure, as determined by DGGE of the 16S rRNA gene, particularly after a feed of DCA as the sole carbon source. Isolates obtained from the acclimatised population included members of the genera *Enterococcus* and *Rhodococcus*, as well as *Brevundimonas*, which displayed >90% propanil biodegradation efficiency.

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## 1. Introduction

The intensive use of herbicides in today's agricultural practices is a matter of worldwide concern. Most of the compounds used to prevent undesirable grass and weed growth are xenobiotics, i.e. man-made and foreign to natural biological systems. Thus, the biological mechanisms necessary to fully biodegrade these molecules are often not readily available in nature, and they tend to accumulate intact or only partially transformed. Designed to have an effect on target organisms, most herbicides (and often also their metabolites) can represent a health and ecotoxicity hazard and should be removed from contaminated waters and soils.

Propanil, or 3,4-dichloropropionanilide, is a post-emergent contact herbicide, classified as an acylanilide. It is an extensively used herbicide worldwide, and it is often applied in the cultivation of rice to control the growth of broadleaf weeds (Silva et al., 2006; Primel et al., 2007; Marchesan et al., 2007).

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Propanil is applied to flooded rice paddies, thus contaminating large amounts of irrigation water, which can overflow from paddy fields or infiltrate through the soil and contaminate surface and ground water resources (Dabrowski et al., 2002). Propanil has been detected in concentrations between 0.1 and 3600 μg $L^{-1}$ in irrigation water (Primel et al., 2007), whereas the maximum allowed concentration for discharge into the aquatic environment is 0.1 μg $L^{-1}$ (Pesticides Framework Directive 2009/128/EC).

Propanil is primarily converted into 3,4-dichloroaniline (DCA) and propionate through enzymatic hydrolysis (Pothuluri et al., 1991), but it can also undergo chemical hydrolysis at pH below or above the 3–9 range, and photodegradation under direct sunlight with a half-life of 12 h (Dahchour et al., 1986). DCA is also a metabolite of the microbial transformation of other herbicides, such as diuron and linuron (Pothuluri et al., 1991; Livingston and Willacy, 1991; Widehem et al., 2002; Sørensen et al., 2008). In irrigation waters, DCA has been found at levels up to 568 μg $L^{-1}$ (Primel et al., 2007), and a similar concentration (470 μg $L^{-1}$) was observed in the Ebro river delta area (Santos et al., 2000). Indeed, DCA is known to have low biodegradability (Pothuluri et al., 1991) and to accumulate in the environment. DCA and, to a lower extent the propanil itself, have been shown to produce toxic effects on mammals and fish, and to affect the human immune system (Pothuluri et al., 1991; Salazar et al., 2008). Moreover, DCA can be converted into 3,3′,4,4′-tetrachloroazobenzene (TCAB), which is a known carcinogen and a potential genotoxin (Pothuluri et al., 1991). DCA biodegradation in soil seems to compete with binding and polymerisation reactions, which increase its recalcitrance in the environment (You and Bartha, 1982). Therefore, these compounds should be removed from irrigation waters before entering the natural aquatic systems and reaching water supply resources or accumulating in the soil.

Biological treatment can be an inexpensive and sustainable solution to remove propanil and DCA from contaminated waters and soils. The hydrolysis of propanil has been observed using pure cultures, including various species of *Pseudomonas* (Dahchour et al., 1986; Pothuluri et al., 1991; Zablotowicz et al., 2001) and some species of the fungus *Fusarium* (Lanzilotta and Pramer, 1970; Reichela et al., 1991). However, to date there is no report of a single culture able to totally mineralise propanil, although this has been achieved by a co-culture of *Pseudomonas putida* and *Streptococcus* (now *Enterococcus*) *avium* (Dahchour et al., 1986). You and Bartha (1982) identified a *P. putida* strain as able to mineralise DCA, but only as co-metabolism of its unchlorinated analogue, aniline. Since then, several other DCA-degrading bacteria have been isolated, such as *Pseudomonas* (now *Brevundimonas*) *diminuta* and *Paracoccus denitrificans*, which were isolated from soils contaminated with a propanil spill (Surovtseva et al., 1985; Bakhaeva et al., 2001), or *Variovorax* sp., *Delftia acidovorans* and *Arthrobacter* sp., isolated from cultures degrading linuron or diuron, herbicides with a chemical structure similar to propanil (Dejonghe et al., 2003; Breugelmans et al., 2007; Sørensen et al., 2008). In these studies, it was also found that complete herbicide biodegradation was better performed by bacterial consortia rather than by individual isolates. These studies suggest that the synergistic interactions occurring in mixed cultures enhance propanil mineralisation since different activity niches (e.g., a range of propanil or DCA concentrations) are naturally covered, which might not be the case with a consortium of only a few selected isolates. Such a mixed culture could be useful for *in situ* treatment of contaminated waters, bioaugmentation of industrial wastewater treatment plants or soil bioremediation. However, propanil biodegradation has not yet been studied using mixed culture enrichments and the best operational conditions for the enrichment of a propanil- and DCA-degrading consortium have not yet been elucidated.

In this study, different SBR operational modes were compared to enrich mixed microbial populations able to degrade propanil and DCA. The objective is to understand the conditions that enhance the selection of the appropriate communities and improve their performance. The efficiency of each process was assessed through a kinetic characterisation carried out with different propanil concentrations. Simultaneously, the structure and composition of the microbial community enriched in the SBR at different operating conditions was assessed through 16S rRNA gene-based denaturing gradient gel electrophoresis (DGGE).

## 2. Material and methods

### 2.1. Microbial enrichments

Microbial enrichments were initiated from a mixture of soil contaminated with several herbicides, including propanil, and soil from organic rice agriculture. Twenty grams of soil was added to 45 mL of mineral medium B (Barreiros et al., 2003), supplemented with 4 mM of $(NH_4)_2SO_4$ and 0.5 mM of propanil. The cultures were incubated at 30 °C with an agitation of 120 rpm. When propanil and DCA concentrations were very low, the culture was settled, decanted and 5 mL of the solids was used to inoculate the next culture medium. After eliminating the soil residues through this process, the culture was centrifuged at 8000 rpm during 15 min and resuspended in propanil-containing media (0.45 mM). This process was repeated for 60 days.

### 2.2. SBR operation

The pre-enriched mixed culture was used to inoculate a sequencing batch reactor (SBR), which was initially operated with dump feeding (5 min), followed by 24–96 h of aerated reaction, 1 h of settling and 0.25 h of decanting. During this stage, the biomass was fed mostly with a propanil concentration of 0.5 mM. Changes in the concentration fed or in the feeding frequency took place when batch tests were conducted (see Section 2.3) or when the culture failed in completely degrading the propanil and DCA. After 76 days, the SBR operational mode was changed to fed-batch, where the reactor was fed twice per 24 h-cycle with a propanil concentration of 0.15 mM in each feed. The second feeding was carried out 11 h after the beginning of the cycle, with 1 h of settling/decanting at the end of each cycle. The reactor was operated during 64 days with these conditions. While the hydraulic retention time (HRT) during the dump feed operational phase was variable

due to DCA accumulation, during the fed-batch operational mode the HRT was maintained at 46 h.

The SBR had a working volume of 0.5 L, it was completely mixed during the feeding and reaction periods, and aerated through a ceramic diffuser (>1 L $min^{-1}$). The pH was controlled at 7.20 ± 0.1 using NaOH (1 M) and the temperature was maintained at 28 ± 1 °C.

### 2.3. Batch tests

The batch tests were carried out in the same reactor used for culture development. During dump feed operation, batch tests were carried out with propanil concentrations of 0.075, 0.59 and 0.30 mM on days 15, 55 and 63, respectively. After the introduction of the fed-batch operational mode, a set of batch tests was carried out with concentrations of 0.075, 0.15 and 0.45 mM of propanil on days 87, 89 and 91 to characterise the first days of acclimatisation to this feeding strategy. Later, new assays with 0.075, 0.30 and 0.59 mM of propanil were performed at days 107, 112 and 115 respectively to characterise the acclimatised biomass. Two final batch tests were conducted on days 123 and 129 to study the biodegradation of DCA as the sole carbon source with concentrations of 0.25 and 0.59 mM, respectively.

### 2.4. Analytical procedures

Microbial density was determined by measuring the optical density (OD) at 610 nm, which was converted to total suspended solids (TSS) and volatile suspended solids (VSS) through calibration curves between OD values of 0.01 and 0.28. TSS and VSS were determined according to Standard Methods (APHA, 1995).

The samples were centrifuged at 10 000 rpm at 4 °C for 15 min and the supernatant and pellet were stored separately at −20 °C. Propanil and DCA were analysed by high performance liquid chromatography (HPLC) with a reverse-phase column (BDS Hypersil C18 100 × 4.6 mm, 3 μm, column, Thermo), using a 70% methanol:30% water degassed mobile phase acidified to pH 3 with orthophosphoric acid, at 0.6 mL $min^{-1}$ flow rate, with a total run time of 12 min. HPLC was carried out in a Waters system coupled with a pump and controller (Waters 600), an in-line degasser (X-Act-4 channels, Jour Research), an autosampler (Waters 717 plus) and a photodiode array detector (DAD, Waters 996) from 200 to 400 nm. All samples were analysed in duplicate. Limits of detection and quantification were 0.88 and 1.28 μM for propanil, and 2.93 and 4.25 μM for DCA, respectively.

Propionate was analysed by HPLC with an Aminex HPX-87H column (Bio-Rad) at 50 °C coupled to an ultraviolet detector at 210 nm. The mobile phase was $H_2SO_4$ 0.01 N with a flow rate of 0.6 mL $min^{-1}$. The limit of quantification was 13 μM.

The accumulation of other degradation products in the SBR effluent was checked by gas chromatography–mass spectrometry (GC–MS). Two hundred mL of decanted effluent was centrifuged (10000 rpm, 15 min). The pH of the supernatant was adjusted to 3 with orthophosphoric acid. Solid-phase extraction was performed with a flow rate of approximately 5 mL $min^{-1}$ using Waters RP-C18 cartridges (500 mg, 50 μm, Waters, Milfort, U.S.), which were pre-conditioned with 1 × 3 mL dichloromethane, followed by 1 × 3 mL water (pH 7) and 1 × 3 mL water (pH 3). The solid-phase material was dried with a continuous nitrogen stream for 1 h, before elution with 2 × 1 mL dichloromethane.

GC–MS analysis was performed using an Agilent 6850 GC fitted with a 5975 VL MSD (Triple Axis Detector) Agilent mass spectrometric detector. The injection port was operated in splitless mode, during 3 min. A DB-5MS 5% Phenyl-/95% dimethylpolysiloxane capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) from Agilent was used, with helium as carrier gas at a flow rate of 1 mL $min^{-1}$. The injection port temperature was 250 °C. The ion source, the quadrupole and the transference line were kept at 230, 150 and 280 °C respectively. The oven temperature was maintained at 40 °C for 4 min, programmed to 120 °C at 10 °C $min^{-1}$, then programmed to 300 °C at 20 °C $min^{-1}$, and held 10 min. The MS spectrum was obtained with electron energy 70 eV, mass range m/z 40–650 and using MSD ChemStation software (Agilent).

### 2.5. PCR-DGGE of the 16S rRNA gene

The SBR bacterial population structure was analysed through DGGE after 63 days of operation under dump feed conditions (sample DF), on day 112, i.e. after 39 days of operation under fed-batch conditions (FB), and on day 129, after the batch tests with DCA as the sole carbon source (FB-DCA). Four mL of SBR biomass was filtered through 0.2 μm-pore-size sterile polycarbonate membranes (Whatman, Kent, United Kingdom) and their total DNA was extracted from the filters using the PowerSoil™ DNA Isolation Kit (MOBIO Laboratories Inc., Carlsbad, CA, United States) with an additional incubation period of 15 min at 65 °C, after cell lysis. The 16S rRNA gene fragments were amplified from 2.2 to 4.8 μg $mL^{-1}$ DNA template (Qubit™ Fluorometer, Invitrogen, United States) using the primers 338F_GC and 518R (Muyzer et al., 1993), as described by Barreiros et al. (2008). Amplified bacterial 16S rRNA gene fragments (~75 ng of DNA) were separated in a double gradient poly-acrylamide gel containing 6–9% acrylamide, to improve band resolution (Cremonesi et al., 1997) and a gradient of 30–58% of denaturant (100% denaturant corresponds to 7 M urea and 40% (v/v) formamide), using a D-code System (Bio-Rad). The run was performed in 1× TAE buffer at 60 °C at 200 V for 6 h, preceded by a pre-run at 60 V for 30 min. The DGGE gels were silver-stained according to Heuer et al. (1997).

### 2.6. Statistical analyses

The DGGE profiles were compared using the Bionumerics software (version 6.0, Applied Maths, Belgium) after normalisation with a standard reference. Ordination techniques based on DGGE fingerprints performed using PC-ORD (version 5, MJM software, Gleneden, USA) were used to analyse the differences in bacterial community composition (band intensity). An initial Detrended Correspondence Analysis of the data demonstrated that it was homogeneous, therefore a linearization method (ter Braak, 1994), principal component analysis (PCA), was carried out.

DGGE banding data were also used to estimate the Shannon index of diversity ($H = -\sum(n_i/N)\log(n_i/N)$; Shannon and

Weaver, 1963) and the evenness index ($E = H/\log S$; Pielou, 1966). Each band was treated as an individual operational taxonomic unit (OTU). The number of DGGE bands was used to indicate the number of species ($S$). The relative surface intensity of each band, expressed as peak height in the densitometric curve ($n_i$), and the sum of all peak heights in the curve of a given sample ($N$) were used as estimates of species abundance (Fromin et al., 2002). The diversity indices ($H$ and $E$) values of the samples were compared using a two-way analysis of variance (ANOVA).

### 2.7. Sequence analysis of the DGGE bands

Major DGGE bands were excised from the gels, eluted with 20 μL of ultrapure water, re-amplified with the same primers, and cloned using the InsTAclone™ PCR cloning kit (Fermentas, Canada) according to the manufacturer's instructions. DNA inserts of at least three different clones matching the original band in the respective DGGE pattern were subsequently sequenced with the primer M13PUCF (InsTAclone™ PCR cloning kit). Nucleotide sequences were determined using a ABI 3700 DNA Analyser (Applied Biosystems, California, USA) and their quality was checked manually using the BioEdit software (http://bioedit.software.informer.com/). Sequences were compared to the GenBank nucleotide data library using the BLAST software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) in order to infer their closest phylogenetic relatives.

### 2.8. Isolation, identification and characterisation of propanil- and DCA-degrading organisms

In order to identify cultivable members of the propanil- and DCA-degrading biomass, sample FB-DCA was serially diluted in sterile saline solution (NaCl 0.85%, w/v), spread on Luria Bertani Agar and incubated at 30 °C for 48 h. Individual colonies with distinct morphologies were purified by sub-culturing on the same medium. After a preliminary characterisation (Gram-staining, presence of catalase and cytochrome $c$ oxidase), 7 cultivable organisms (EPL1 to EPL7), were identified. Sequencing of the 16S rRNA gene of the isolates was determined after PCR amplification using primers 27f and 1492r (Lane, 1991), as described by Ferreira da Silva et al. (2007).

In order to assess the presence of each isolate in the SBR biomass, they were analysed by PCR-DGGE simultaneously with the SBR samples.

The ability of each isolate to degrade propanil and/or DCA was assessed in resting cell assays, performed in 54 mM phosphate buffer, pH 7.2, with 0.25 mM propanil or DCA and cell densities corresponding to 2.5 mg $mL^{-1}$ cells (dry weight). The suspensions were incubated at 30 °C and shaken at 150 rpm for 18 h, after which the percent removal of each compound was determined.

## 3. Results and discussion

Photodegradation by exposure to sunlight and adsorption to the biomass were assessed in abiotic tests, which showed that these processes had a negligible effect on propanil and DCA removal (data not shown). These results also showed that the propanil was not volatilised from the media under the working conditions of this study. Thus, the removal of propanil and DCA observed in the SBR system was due to biological activity.

### 3.1. Dump feeding strategy

#### 3.1.1. SBR performance during dump feed operation

The reactor was initially operated with a dump feeding strategy, where the propanil-containing mineral medium was added once per cycle. During the first two weeks of this stage, the propanil concentration in the reactor after dump feed varied between 0.2 and 0.5 mM. In order to promote biomass growth, the propanil concentration was increased to close to its solubility limit (0.59 mM, the propanil solubility at 25 °C) after day 17. The enrichments responded to this increase with a rapid biomass growth until day 30 (Fig. 1a). After that, the biomass concentration was stable for approximately 10 days and then started to decrease. This decrease was probably related to the accumulation of DCA (and also propanil) during this period (Fig. 1b). Indeed, with a dump feed strategy, a high concentration of propanil was added to the reactor and rapidly converted into DCA, which was degraded at a much slower rate and thus accumulated in the reactor up to 1.29 mM. At this concentration, DCA was possibly not only inhibitory to its own degradation, but also to the degradation of propanil, as suggested by the accumulation of the herbicide after day 35. In view of this effect, the addition of propanil was interrupted between days 35 and 45 in an attempt to prevent additional DCA accumulation and allow the removal of the accumulated compounds. After this, the feeding of propanil was more spaced out, resulting in longer starvation periods inbetween feedings and, consequently, to a dramatic decrease in biomass concentration.

#### 3.1.2. Kinetics of propanil and DCA degradation during dump feed operation

Three batch tests were carried out in the reactor during dump feed operation with different propanil concentrations (0.075 mM, 0.30 mM and 0.59 mM, Fig. 2) in order to study the biodegradation kinetics of propanil and its metabolites.

Transitory accumulation of propionate during propanil biodegradation was observed only in the batch test carried out with the highest concentration of herbicide (0.59 mM, Fig. 2c). In contrast, transitory accumulation of the intermediate DCA was observed in all of the batch tests. In fact, the maximum propanil degradation rates (7.9–12.4 mmol $gVSS^{-1}$ $d^{-1}$) were always higher than those of DCA (1.4–5.6 mmol $gVSS^{-1}$ $d^{-1}$) (closed symbols on Fig. 3), which decreased with increasing propanil (and therefore increasing DCA) concentration.

The preferential consumption of propionate as compared to the DCA is expectable due to its higher biodegradability. In fact, in previous studies focussed on propanil biodegradability, DCA is often considered recalcitrant or very slowly biodegradable (Dahchour et al., 1986; Pothuluri et al., 1991; Correa and Steen, 1995), whereas propionate is an easily biodegradable volatile fatty acid.

In the two first assays, the DCA was fully degraded shortly after the propanil was no longer detected (Fig. 2). However, the

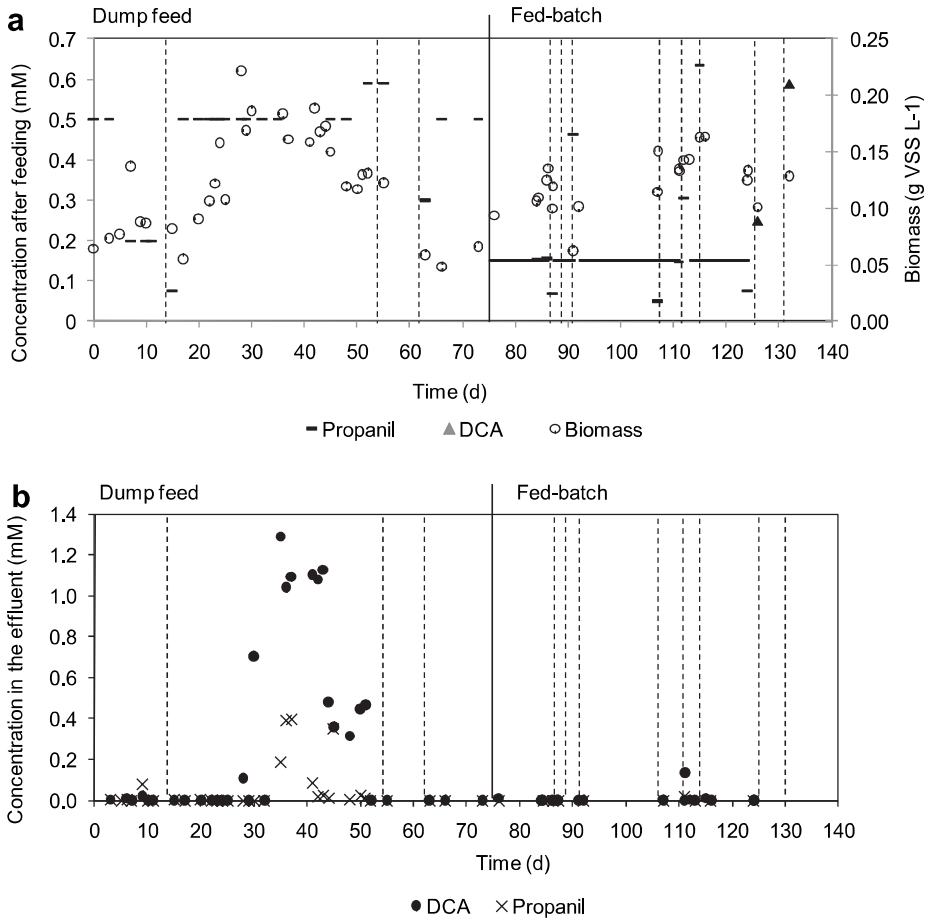


**Fig. 1 – a) Biomass concentration and substrate concentration fed to the reactor throughout the study. b) Propanil and DCA concentrations accumulated at the end of each SBR cycle. Batch tests carried out in the reactor are marked with broken lines.**

batch test carried out with the highest concentration of propanil (0.59 mM, Fig. 2c), resulted in the accumulation of DCA up to 0.53 mM. Little DCA degradation was observed between days 1 and 3.5 of this batch test, despite the fact that propanil was completely hydrolysed during the first day. After day 3.5, a significant increase in the DCA degradation rate occurred. This increased degradation rate could be due to acclimatisation to the higher concentration of DCA, which may have been inhibitory to the biomass in the first days of the test. A low DCA degradation rate, resulting in a lag phase in the DCA degradation profile, was also observed by Livingston and Willacy (1991), who reported DCA inhibition at high DCA concentrations in a study with mixed microbial cultures.

The accumulation of propionate in the batch test fed with 0.59 mM propanil was not likely due to DCA inhibition as accumulation was observed since the beginning of this batch test, when the concentration of DCA was still low. Propionate more likely accumulated due to inhibition by the high concentration of propanil fed (0.59 mM). Indeed, also the degradation rate of propanil was lower for the test fed with 0.59 mM of propanil (closed symbols on Fig. 3a), suggesting some substrate inhibition on the degradation of propanil itself.

### 3.2. *Fed-batch strategy*

#### 3.2.1. *Kinetics of propanil and DCA degradation during fed-batch operation*

Due to the substrate inhibition observed during the dump feed stage, the reactor was subjected to long starvation periods, as the accumulated DCA was slowly degraded. Therefore, it was decided to change the operational mode to a fed-batch strategy, with a lower initial propanil concentration (0.15 mM in the reactor after each feed), where the reactor was fed twice per 24 h-cycle. As a result, the accumulation of either DCA or propanil was no longer detected at the end of the cycle (see Fig. 1) and the biomass concentration gradually increased. No other metabolites were detected through GC–MS analysis of the SBR supernatant (results not shown), suggesting that propanil, DCA and propionate were fully degraded during the SBR cycle.

Two series of batch tests were carried out to characterise the kinetics during the fed-batch operation stage, approximately 10 days and 30 days after switching to this feeding strategy. Fig. 4 shows the batch tests performed after acclimatisation to the new operational mode (31–39 days after the change to fed-batch operation), where an increase in performance was clear as compared to the dump feed stage (Fig. 2).

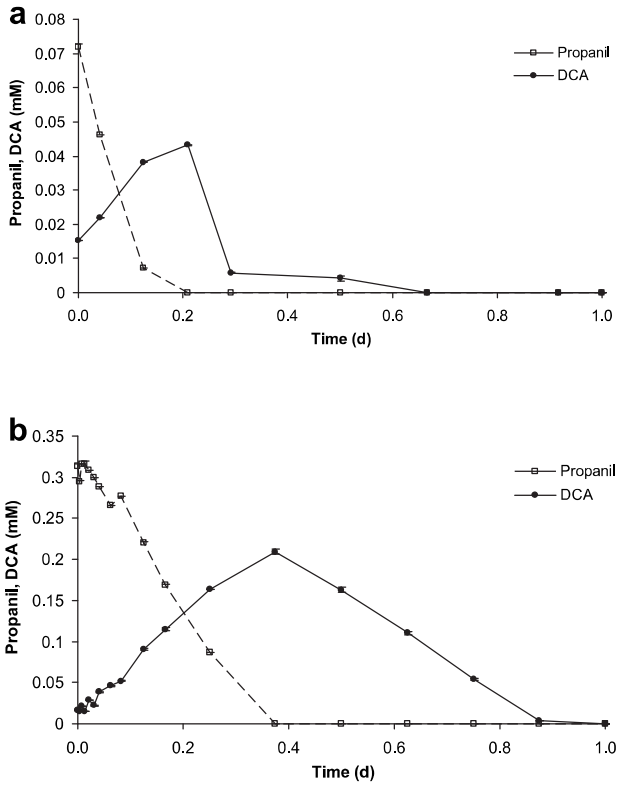


**Fig. 2 – Propanil and DCA degradation profiles obtained from batch tests carried out during SBR operation with a dump feeding strategy and initial propanil concentrations of (a) 0.075 mM, (b) 0.30 mM and (c) 0.59 mM.**

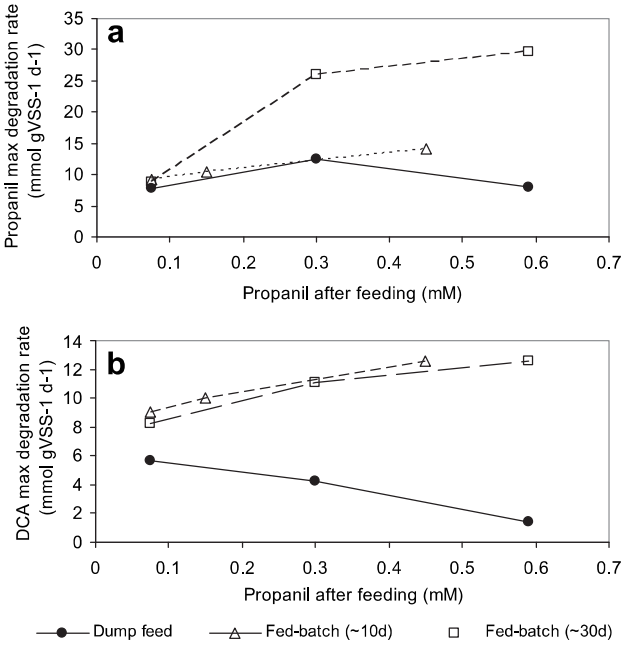


**Fig. 3 – Maximum degradation rates of (a) propanil and (b) DCA determined from batch tests carried out during dump feed operation, and ~10 days and ~30 days after the change to fed-batch operation.**

This could be related to a shift in the microbial population composition. In fact, the multivariate analysis of the DGGE profiles of the biomass operated under dump feed and fed-batch conditions showed some changes in the bacterial community structure (see Section 3.3). However, it is also expected that the fact that the DCA concentration was kept below inhibitory levels (through feeding lower propanil concentrations) simply led to increased synthesis or activity of propanil- and DCA-degrading enzymes. Additionally, it is possible that these enzymes remained active for longer periods, since the fed-batch strategy prevented the need to impose the long starvation periods resulting from DCA accumulation.

The accumulation of propionate was not detected in any of the tests carried out after the introduction of the fed-batch strategy, which further demonstrates the increase in performance with this operation mode. This was validated by the analysis of the propanil and DCA degradation rates in the two series of batch tests during fed-batch operation (see open symbols on Fig. 3). All degradation rates increased as compared to the dump feeding strategy period, except for the batch tests fed with a propanil concentration of 0.075 mM (Fig. 3a), likely because the low initial concentration was already limiting the degradation rate.

The propanil degradation rates remained similar in the series of batch tests that were carried out during dump feed and ~10 days after switching to fed-batch conditions (Fig. 3a). Interestingly, the maximum propanil degradation rates substantially increased in the second series of batch tests carried out one month later. This may be explained by an increase in the microbial populations responsible for the primary degradation of propanil, reflected in the shifts observed in biomass bacterial composition (see Section 3.3). Fig. 3b shows that the maximum DCA degradation rate substantially increased in the batch tests performed immediately following the change in operational mode, including the 0.075 mM test. This was likely because DCA no longer accumulated to inhibitory levels. Nevertheless, the DCA degradation rate did not show any further increase one month after operation in fed-batch conditions, as occurred with the propanil degradation rate. While the underlying reason for this result is unclear, it could be due to the low selective

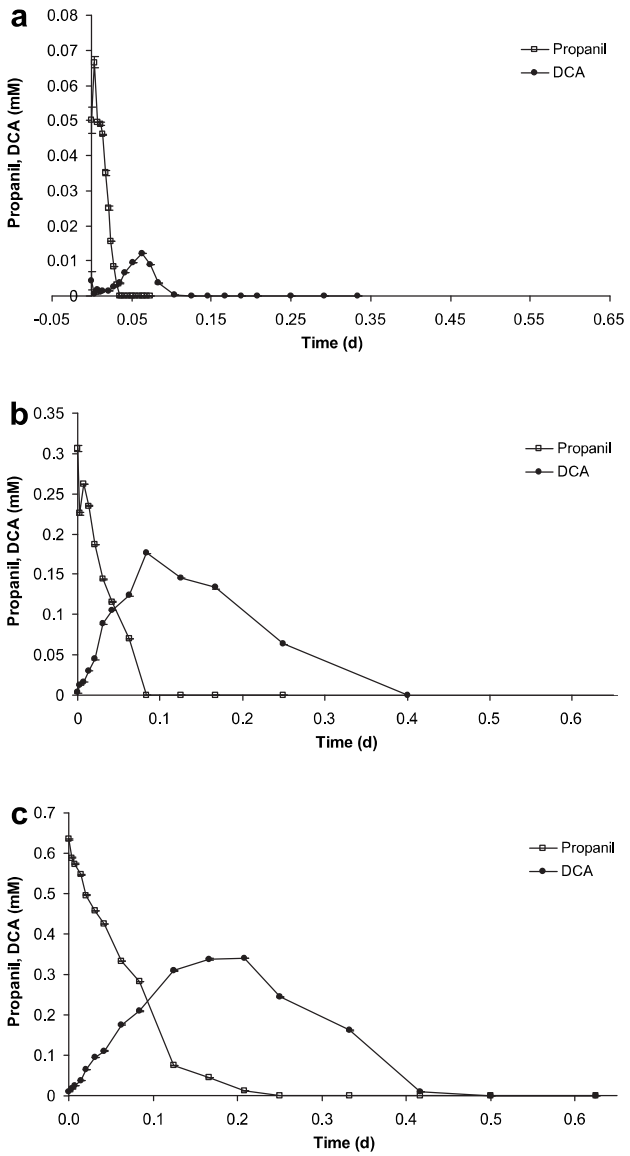


**Fig. 4 – Propanil and DCA degradation profiles obtained from batch tests carried out during SBR operation with a fed-batch strategy and initial propanil concentrations of (a) 0.075 mM, (b) 0.30 mM and (c) 0.59 mM.**

pressure towards the enrichment of additional DCA degraders, since DCA was always present in low concentrations during fed-batch mode. Further, it is possible that the DCA degraders had a slower growth rate than the organisms responsible for propanil degradation.

#### 3.2.2. *Performance with DCA feeding*

After imposition of the fed-batch operational mode, additional batch tests were carried out where DCA was the only carbon source in order to investigate the potential for DCA inhibition to the biomass acclimatised with fed-batch operation mode.

The first test was performed with a DCA concentration of 0.25 mM, similar to the maximum observed in the tests carried out with propanil during fed-batch operation, except that no other substrates (propanil, propionate) were present. The results obtained (Fig. 5a) show that this amount of DCA was removed with a maximum degradation rate of 4.5 mmol $gVSS^{-1}$ $d^{-1}$, whereas the biomass fed with propanil (Fig. 4c) removed the 0.30 mM of DCA accumulated from propanil degradation with a maximum degradation rate of 12.5 mmol $gVSS^{-1}$ $d^{-1}$ (Fig. 3b). This lower DCA degradation rate when DCA was fed may be related to the time required to synthesise the necessary enzymes (possibly including a dioxygenase and a pyrochatechase (You and Bartha, 1982; Breugelmans et al., 2010)) to eliminate the amino group and open the dichloroaniline ring. Under propanil feeding, DCA is accumulated gradually, potentially allowing sufficient time for enzyme induction. Another explanation for the reduced DCA degradation rate is the re-structuring of the SBR bacterial community. In fact, a shift in the microbial population was observed after these batch assays (see Section 3.3). In the second batch test performed with a higher DCA concentration (0.59 mM), an initial lag phase (1 day) occurred prior to the commencement of DCA degradation (6.2 mmol $gVSS^{-1}$ $d^{-1}$), which may have been necessary to increase the number and/or activity of organisms able to degrade DCA. Indeed, 0.59 mM of DCA can be considered a shock load to the biomass in this system, likely necessitating this lag phase prior to DCA degradation due to the inhibitory and hardly biodegradable nature of DCA.

#### 3.2.3. *Implications of the results on propanil and DCA removal*

The results suggest that the use of a fed-batch operational strategy, where each feeding step provided the sludge with a low concentration of propanil (0.15 mM), enriched an efficient microbial population capable of removing propanil (and the resulting DCA) with higher maximum degradation rates, as compared to dump feeding. This strategy enabled the addition of a higher amount of propanil to the SBR during the same period of time (since there was no need to impose starvation periods for the elimination of accumulated DCA), which contributed to the enrichment of a propanil- and DCA-degrading biomass with faster kinetic rates. Additionally, the initial inhibitory effect that this metabolite (DCA) imposed on the biomass was substantially reduced with acclimatisation. Nevertheless, a lag phase in the degradation of DCA should still be expected when high concentrations of this compound (e.g., 0.59 mM) occur in the reactor, even with acclimatised sludge. However, this effect is unlikely to take place as a result of propanil degradation, even at the maximum soluble propanil concentration (0.59 mM). Indeed, the results showed that, simultaneously to propanil removal, the adapted microbial populations were able to induce the necessary enzymes for DCA degradation, preventing the accumulation of this intermediate to values above 0.30 mM.

### 3.3. *Bacterial population analysis*

#### 3.3.1. *Population dynamics analysis through DGGE*

Modifications in the composition of the SBR bacterial community due to alterations in the operating conditions were assessed through DGGE profiling of samples collected at the end of the dump feed (DF) and fed-batch (FB) periods, and

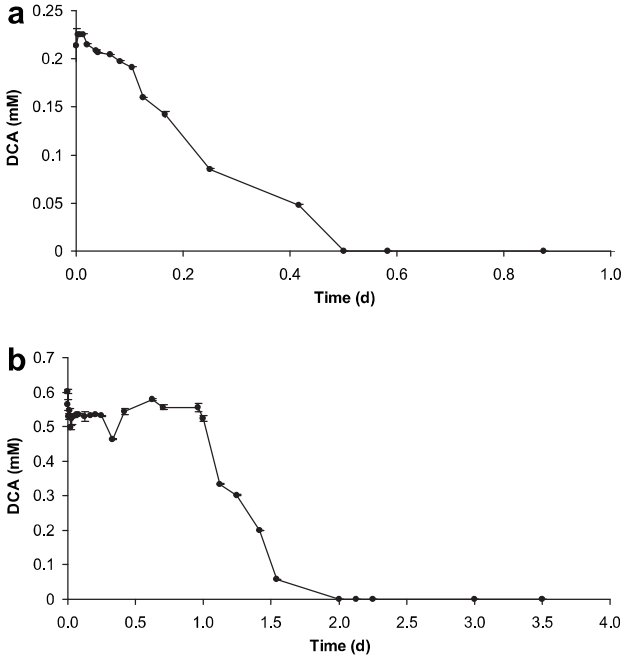


**Fig. 5 – DCA degradation observed in batch tests carried out during SBR operation with a fed-batch strategy and initial DCA concentrations of (a) 0.25 mM and (b) 0.59 mM.**

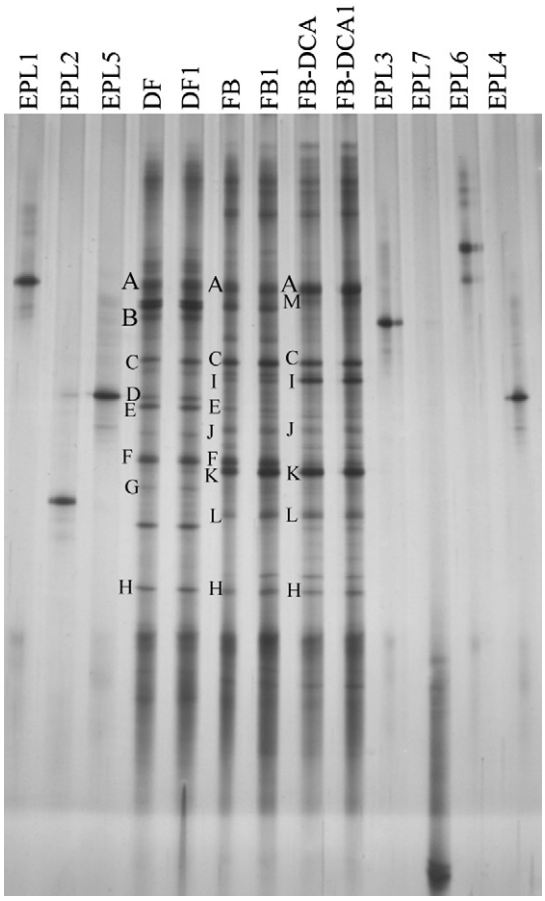


**Fig. 6 – DGGE band pattern of the SBR biomass at different operating conditions (DF, FB and FB-DCA) and of each of the isolates. Duplicates of each of the mixed culture samples are presented. Sequenced bands are indicated by letters.**

after the batch tests carried out in the SBR with DCA feed (FB-DCA). Considering all the DGGE profiles analysed, a total of 21 different bands were detected and assigned, with 14 bands in each profile. Only five bands were observed simultaneously in all the analysed profiles and the majority varied both in presence and intensity (Fig. 6).

The switch from dump feed to fed-batch operational mode did not influence the SBR bacterial diversity and evenness, even after a 40 day period of acclimatisation to this operation mode, as the Shannon and evenness indices presented similar average values ($H = 1.08 \pm 0.01$ and $1.07 \pm 0.01$, and $E = 0.94 \pm 0.01$ and $0.93 \pm 0.01$ for dump feed and fed-batch, respectively). However, after the two batch tests with a DCA feed, both diversity and evenness decreased significantly ($p < 0.001$) ($H = 1.02 \pm 0.01$ and $E = 0.89 \pm 0.01$) in only 8 days, suggesting an enrichment of the DCA-degrading organisms.

The multivariate analysis of the DGGE profiles showed variations in the bacterial community structure due to changes in the SBR operating conditions. The first two principal components of the PCA could explain 89.8% of the observed variation. Three distinct groups could be distinguished over axis 1, corresponding to DGGE profiles of each of the SBR operating conditions (DF, FB, and FB-DCA) in the biplot (Fig. 7). This analysis suggests that the bacterial community was restructured after the operational change from dump feed to fed-batch mode, which is consistent with the changes observed in propanil and DCA degradation performance (Fig. 3), although no impact was observed on the overall microbial diversity and evenness. After the DCA-fed batch tests, further population changes occurred, likely related to the simultaneous withdrawal of propanil from the feed and the addition of high DCA concentrations as the sole carbon and energy source, which probably had a selective effect on the bacterial population.

The closest neighbours of the nucleotide sequences of the DGGE bands of resident organisms (those found in all the profiles) and of those that most contributed to the observed variations belonged either to the phylum *Proteobacteria* or *Bacteroidetes* (Table 1). Among the sequenced bands, A, C and H, related to uncultured *Bacteroidetes*, *Burkholderia hospita* (*Betaproteobacteria*) and an uncultured *Sphingomonas* (*Alphaproteobacteria*), respectively, were present in all the analysed profiles. The presence of these organisms in the SBR independently from the SBR operating conditions suggests that these organisms were not affected by the changes in the feeding strategy or by the DCA concentration.

Bands D, E, G, I, J, K and L were those showing the highest Eigen values for the PC1 axis (which captured 63.6% of the variance) (Table 1), and thus, those that most contributed to the observed variation in the SBR bacterial community. Bands D, E and G, were present under dump feed conditions (DF) but were either not detected (D, G) or showed lower intensity (E) under fed-batch conditions (FB), disappearing after the DCA-fed batch tests (FB-DCA) (Fig. 6). In contrast, bands I, J, K and L, which appeared after the operation switched to fed-batch

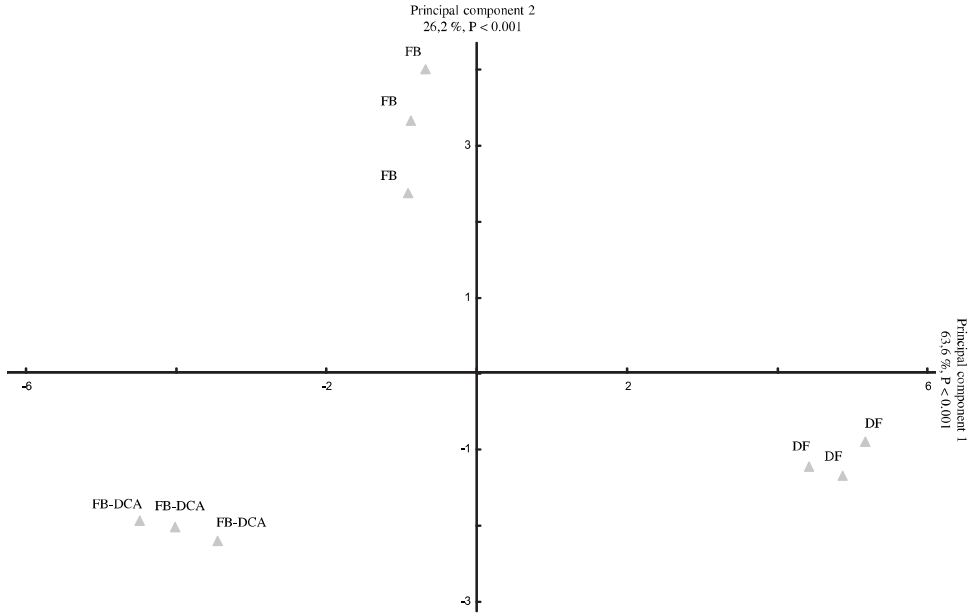


**Fig. 7 — Principal component analysis biplot of the SBR biomass DGGE profiles at different operating conditions. This analysis was based on triplicate DGGE profiles of each sample run in the same gel.**

(and also when DCA was fed), were faint or not detected under dump feed conditions. These results show the impact of fed-batch conditions on the overall bacterial community, leading to shifts in the microbial population present in the SBR. Examples of the organisms no longer detected after approximately 40 days of operation under fed-batch conditions are those related to *Stenotrophomonas* sp. (*Gammaproteobacteria*, band D), *Pedobacter* sp. (*Bacteroidetes*, band E) and uncultured *Burkolderiales* (*Betaproteobacteria*, band G). Indeed, two *Stenotrophomonas* strains isolated from this culture showed only limited propanil and DCA removal abilities (Table 2). The fed-batch operational mode seemed to have enhanced the

**Table 1 — Closest relatives of the most relevant DGGE bands, characterised by principal component analysis (PCA).**

| Eigen values | | DGGE band | DGGE profile | % Similarity | Closest relative | Acession number | Phylogenetic afiliation |
|---|---|---|---|---|---|---|---|
| PC 1 | PC 2 | | | | | | |
| −0.2077 | 0.0406 | A | DF, FB, FB-DCA | 97 | Uncultured bacterium | AJ318155 | *Bacteroidetes* |
| 0.2201 | −0.2480 | B | DF | 97 | Uncultured bacterium | AY154391 | *Proteobacteria* (*Epsilonproteobacteria*) |
| −0.1462 | 0.3015 | C | DF, FB, FB-DCA | 99 | *Burkholderia hospita* | AY949187 | *Proteobacteria* (*Betaproteobacteria*) |
| 0.2493 | −0.1417 | D | DF | 100 | *Stenotrophomonas* sp. N46 | AB480332 | *Proteobacteria* (*Gammaproteobacteria*) |
| 0.2707 | −0.0469 | E | DF, FB | 95 | *Pedobacter* sp. AKB-2008-IJ6 | AM988945 | *Bacteroidetes* (*Sphingobacteria*) |
| 0.2100 | 0.2698 | F | DF, FB | 99 | *Kaistella koreensis* | NR_028815 | *Bacteroidetes* (*Flavobacteria*) |
| 0.2506 | −0.1415 | G | DF | 99 | Uncultured *Burkholderiales* bacterium | EU572427 | *Proteobacteria* (*Betaproteobacteria*) |
| −0.1920 | −0.1223 | H | DF, FB, FB-DCA | 99 | Uncultured *Sphingomonas* sp. | FJ037465 | *Proteobacteria* (*Alphaproteobacteria*) |
| −0.2628 | −0.1112 | I | FB, FB-DCA | 98 | *Variovorax* sp. PBL-E5 | AM502921 | *Proteobacteria* (*Betaproteobacteria*) |
| −0.2505 | 0.1570 | J | FB, FB-DCA | 96 | Uncultured bacterium | DQ450747 | *Bacteroidetes* |
| −0.2513 | 0.1644 | K | FB, FB-DCA | 98 | Uncultured *Rhizobium* sp. | FJ712855 | *Proteobacteria* (*Alphaproteobacteria*) |
| −0.2649 | 0.0534 | L | FB, FB-DCA | 100 | *Mesorhizobium* sp. | EU652176 | *Proteobacteria* (*Alphaproteobacteria*) |
| −0.2073 | −0.2646 | M | FB-DCA | 100 | Uncultured *Flavobacteria* bacterium | EU043595 | *Bacteroidetes* |

**Table 2 — Phylogenetic affiliation of SBR isolates and their ability to degrade propanil and DCA, observed 18 h after the addition of the herbicide or its metabolite.**

| Isolate | % Similarity | Type strain | Accession number | Phylogenetic affiliation | % Removal | |
|---|---|---|---|---|---|---|
| | | | | | DCA | Propanil |
| EPL1 | 98 | *Pseudomonas aeruginosa* | X06684 | *Proteobacteria* (*Gammaproteobacteria*) | 3 | 33 |
| EPL2 | 99 | *Brevundimonas vesicularis* | AJ227780 | *Proteobacteria* (*Alphaproteobacteria*) | 3 | 97 |
| EPL3 | 98 | *Enterococcus faecium* | AJ301830 | *Firmicutes* (*Bacilli*) | 22 | 90 |
| EPL4 | 99 | *Stenotrophomonas nitritireducens* | AJ012229 | *Proteobacteria* (*Gammaproteobacteria*) | 29 | 13 |
| EPL5 | 99 | *Stenotrophomonas nitritireducens* | AJ012229 | *Proteobacteria* (*Gammaproteobacteria*) | 14 | 32 |
| EPL6 | 96/96 | *Alicycliphilus denitrificans*/ *Acidovorax avenae* | AJ418042/AF508114 | *Proteobacteria* (*Betaproteobacteria*) | 51 | 37 |
| EPL7 | 99 | *Rhodococcus erythropolis* | X79289 | *Actinobacteria* (Actinobacteridae) | 13 | 98 |
| Abiotic control | – | – | – | – | 0 | 0 |

enrichment of the SBR biomass in organisms apparently better adapted to the imposed conditions, which possessed higher propanil and DCA degradation rates (Fig. 3). Some of the organisms only detected through DGGE after acclimatisation to fed-batch conditions were related to *Variovorax* sp. (*Betaproteobacteria*, band I), uncultured *Rhizobium* sp. (*Alphaproteobacteria*, band K), or *Bacteroidetes* (band J), and *Mesorhizobium* sp. (*Alphaproteobacteria*, band L). The increase of organisms related to rhizosphere microbiota in the SBR after acclimatisation is not surprising given their ability to encode aryl acylamidases, one of the key enzymes needed to degrade propanil (Hoagland et al., 1994).

Interestingly, band I, corresponding to an organism related to *Variovorax* sp., became particularly intense after the DCA feedings, suggesting its association with the degradation of this compound. Also Dejonghe et al. (2003) and Sørensen et al. (2008) reported the isolation of two different strains of *Variovorax* sp. (WDL1 and SRS16, respectively), which were able to mineralise phenylurea herbicides as the sole carbon, nitrogen and energy source with a transient accumulation of DCA. Recent studies further showed that *Variovorax* sp. WDL1 encodes proteins with high amino acid sequence similarity to different components of the multicomponent aniline dioxygenase of aniline degraders, which may be involved in DCA degradation (Breugelmans et al., 2010).

### 3.3.2. *Characterisation of propanil and DCA degrading isolates*

None of the isolates (EPL 1-7) co-migrated with bands of the SBR DGGE profiles, suggesting that they were present in low numbers in the reactor, irrespectively of the operating conditions. The isolation procedure did not allow the cultivation of organisms related to the putative SBR community members identified by DGGE and observed, by the same method, to prevail among the acclimatised biomass. Specifically, organisms corresponding to bands I, J, K and L, which most contributed to distinguishing the SBR community after acclimatisation (see Eigen values in Table 1), could not be isolated. The high content in readily metabolisable nutrients and absence of xenobiotics in the isolation medium possibly enhanced the growth in the plates of other organisms rather than those that better thrived in the SBR. In fact, it is known that culture-based methods favour fast growing organisms, and may underestimate the microbial community composition (Scow et al., 2001; Kirk et al., 2004).

Additionally, a possible bias introduced by the DNA extraction-PCR-DGGE methodology may have led to an underestimation of Gram-positive bacteria, as those of the genera *Enterococcus* and *Rhodococcus*. Isolates belonging to these genera, along with a *Brevundimonas vesicularis* (EPL2, 3 and 7), presented high removal percentages of the herbicide, suggesting their relevant role in the propanil-fed SBR (Table 2). To the best of our knowledge, organisms belonging to these same taxonomic groups had never been described as able to degrade propanil without accumulation of DCA in axenic cultures. However, *Enterococcus avium* was described as able to mineralise propanil in co-culture with *P. putida* (Dahchour et al., 1986). *Rhodococcus erythropolis* has been described as exhibiting aryl acylamidase activity (Vaughan et al., 1990) and *Rhodococcus* spp. to be a DCA-metabolising organism (Travkin and Golovleva, 2003). The involvement of organisms affiliated to the genus *Brevundimonas* in the degradation of dichloroanilines was also previously reported (Surovtseva et al., 1985).

While some of the isolates seemed to efficiently biodegrade propanil and DCA, others (EPL4 and 6) were apparently more specialised on DCA degradation, as their removal efficiencies were higher for DCA than for propanil (Table 2). Particularly, the isolate EPL6, which could not be affiliated to any known genus, could degrade approximately 50% of the initial DCA concentration (0.25 mM). Similar results were obtained when these cultures were grown in mineral medium with higher concentration (0.5 mM) of each compound (data not shown). Interestingly, DCA accumulation was never observed during the propanil degradation tests, even though some of the isolates presented very low removal efficiencies when supplied with DCA as the sole substrate, and none of the isolates was able to deplete DCA within the 18 h biodegradation tests. In organisms EPL2, 3 and 7, DCA degradation may require induction, as reported before for the degradation of hexamethyleneimine, which needs azepane-1-carboxylic acid induction during molinate degradation by *Gulosibacter molinativorax* ON4$^T$ (Barreiros et al., 2008). Such phenomenon may explain the time delay observed in DCA degradation in DCA-fed batch tests, when compared with propanil-fed batch tests (Section 3.2.2). In fact, organisms such as isolates EPL2, 3 and 7, may have not contributed to the DCA degradation in the SBR observed during the DCA-fed batch tests in the absence of propanil.

In summary, although it was possible to isolate organisms able to degrade propanil without accumulation of DCA, the

SBR bacterial community proved to be very complex. Thus, besides the cultivable organisms, many others present in the SBR acclimatised biomass may be involved in propanil degradation, and it is likely that the high degradation performance observed could only be achieved by the microbial activity of the whole consortium.

## 4. Conclusions

- A microbial enrichment able to degrade propanil, as well as its intermediates DCA and propionate, was developed from an inoculum obtained from a mixture of propanil contaminated and non-contaminated soils.
- With a dump feed operational mode, the enrichment was able to degrade propanil and DCA, but higher concentrations of propanil (0.59 mM) led to the accumulation of inhibitory concentrations of DCA.
- A fed-batch operational mode proved to be an effective strategy towards avoiding DCA inhibition, thus enabling the enrichment of a propanil- and DCA-degrading biomass with higher degradation rates.
- The bacterial community analysis showed that the increased performance in propanil and DCA degradation of the acclimatised enrichment culture from dump feed to fed-batch operational mode was accompanied by shifts in bacterial community structure. The addition of DCA as the sole carbon source induced further changes in the microbial population structure, with reduction in the population diversity and evenness.
- Although it was possible to isolate organisms able to degrade propanil without accumulation of DCA, none was detected in the SBR DGGE profiles, suggesting that other non-cultivable organisms may be involved in the improved degradation performance observed after acclimatisation, or that it was the result of the combined activity of several members of the consortium.
- Overall, this work defined, based on the understanding of biodegradation kinetics, an effective reactor operation strategy to obtain a propanil- and DCA-degrading mixed culture that can be used for bioaugmentation or bioremediation purposes. The culture stability and performance when applied to real contaminated waters and soils is an important issue which deserves future research.

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