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Bacterial community dynamics in a rotating biological contactor treating 2-fluorophenol-containing wastewater

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Abstract One of the main factors affecting the performance of rotating biological contactors (RBC) is the biofilm characteristics. Therefore, a deep understanding of the microbial population dynamics and structure of the biofilm is mandatory if optimization of organic matter and nutrients removal is targeted. This study focused on the effects of organic shock loads of 2-fluorophenol (2-FP) on the microbial diversity present in an RBC biofilm. The RBC was seeded with activated sludge from a conventional wastewater treatment plant and was operated during 496 days. During the first 126 days, the RBC was subjected to intermittent 2-FP shocks of 25 mg l^{-1} and no degradation occurred. Therefore, the reactor was subsequently augmented with a 2-FP-degrading strain (FP1). Afterwards, the RBC had a stable performance when subjected to 2-FP shocks up to 50 mg l^{-1} and to a starvation period, as indicated by removal of the compound. Denaturing gradient gel electrophoresis (DGGE) revealed large shifts in microbial communities present in the first and fifth stages, although no clear relation between the sample collection time and spatial factor was found. Phylogenetic affiliation of some predominant members was assessed by direct sequencing of correspondent DGGE bands. Affiliations to α-, β- and δ-Proteobacteria were found. Several bacterial strains isolated from the reactor showed capacity for 2-FP degradation. Strain FP1 was successfully recovered from the biofilm by plating and by DGGE, reinforcing that bioaugmentation was successfully achieved.

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Keywords Rotating biological contactor (RBC) · 2-Fluorophenol (2-FP) · Microbial dynamics · Denaturing gradient gel electrophoresis (DGGE)

Introduction

Halogenated phenols, such as 2-fluorophenol (2-FP), are relevant xenobiotics commonly used in the production of pharmaceuticals, chemicals, herbicides and pesticides, and other industrial applications [35]. Fluorinated molecules have been found to accumulate in the environment and in human blood samples [18], even though they occur at low concentrations and discontinuously. Their stability makes them potentially significant environmental contaminants by causing persistence.

The use of biofilm reactors for wastewater treatment is a promising technology due to their advantages, such as higher concentration of relevant organisms, small reactor space, high efficiency and lower sludge production [17]. Rotating biological contactors (RBC), which are biofilm reactors, are an effective method for wastewater treatment [7, 27]. Among others, RBCs have been successfully applied to wastewaters containing organopollutants, such as chlorophenols and trichloroethylene [2, 22, 30]. The system is based on the metabolic activities of complex microbial communities forming a biofilm attached to the discs' surface [3]. In local microenvironments, some conditions, such as dense microbial populations and substrate concentration gradients, may affect the microbial population dynamics and, consequently, change the microbial community architecture. Furthermore, the rate and location of detachment of excess biomass also have a significant influence on the microbial ecology within the biofilm [25]. Therefore, the study of the microbial population

dynamics is of great importance to understand biofilm ecology.

An RBC was operated under dynamic conditions, including 2-FP shock loadings and a starvation period, and its efficiency to remove the target pollutant has been reported [8]. Here we report on the effect of 2-FP shock loadings on the dynamics of the microbial community present on the biofilm established in the RBC. Bacterial strain isolation and denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene were used for this purpose.

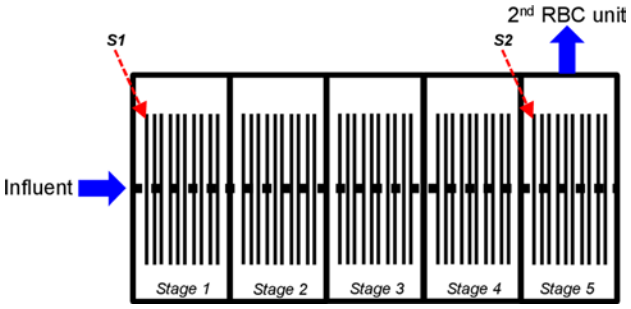

Fig. 1 Schematic representation of the first RBC unit. *S1* and *S2* are biofilm sampling points located in the first disc of the first and fifth stages, respectively

Materials and methods

RBC operation and analytical methodology

A laboratory-scale RBC composed of two units each containing five stages with 10 PVC discs (3 mm thick; diameter 16.5 cm; spaced 1 cm apart; 40 % of disc submergence), with a working volume of 10 l per unit, was set up and operated as described by Duque et al. [8]. The reactor was inoculated with activated sludge from the return sludge flow of a conventional wastewater treatment plant in Portugal for biofilm formation and on day 127 was bioaugmented with a specialized strain able to degrade 2-FP, *Rhodococcus* sp. strain FP1, previously isolated in our laboratories [10]. The reactor was inoculated with 2 l of an FP1 pure culture with an optical density at 600 nm (OD_{600}) of 0.4 and fed with 50 mg l^{-1} of 2-fluorophenol (2-FP) (Sigma-Aldrich Chemie, Steinheim, Germany). The hydraulic loading rate (HLR) was 0.25 dm^3 m^{-2} h^{-1}, the hydraulic retention time (HRT) was 18.6 h and the rotational speed of the discs was 12 rpm. The RBC was fed with 200 mg l^{-1} of acetate and with 2-FP, at concentrations ranging from 25 to 200 mg l^{-1}, during 496 days of operation length, divided into 30 different phases [8]. The composition of the concentrated influent media was as described by Duque et al. [8]. The concentration of 2-FP and fluoride ions was measured as described by Duque et al. [10].

Biofilm sampling

Representative samples of the biofilm present in the first disc of the first and fifth stages of the RBC were scrapped with a scalpel from the surface of the discs, at different locations within the disc, and were homogenized (Fig. 1) The biofilm was then subjected to sonication for a total time of 1 min (2 cycles of 30 s) at maximum intensity (97 % of 20 kHz), using a SONOPLUS ultrasonic homogenizer HD 2070 (BANDELIN electronic, Berlin, Germany). The resulting bacterial suspensions were used for plating, bacterial identification and DNA extraction for DGGE analysis.

2-FP-degrading bacteria isolation and identification

Bacterial isolation, DNA extraction and DNA sequencing analysis

Serial dilutions of bacterial suspensions from the first stage of the RBC were made in saline solution (0.85 % w/v NaCl) and 0.1 ml of each dilution was spread onto nutrient agar (NA) (LABM, UK). Plates were incubated at 25 °C for 3 days. On the basis of size, morphology and pigmentation, different bacterial colonies were isolated from NA plates using the streak-plate procedure. Isolated strains recovered from the NA plates were tested for their capacity to degrade 2-FP. The isolates were inoculated into 50 ml of sterile mineral salts medium (MM) [5] containing 2FP, as the sole carbon and energy sources, at a concentration of 50 mg l^{-1} in 250-ml flasks. Cultures were incubated on an orbital shaker (100 rpm) at 25 °C. When growth was observed, indicated by an increase in the optical density at 600 nm and by fluoride release, measured with a fluoride electrode [9], the culture was plated onto NA plates to verify its purity. DNA extraction and DNA sequencing analysis of the 2-FP-degrading strains were performed as described by Duque et al. [9].

Biofilm bacterial community analysis

DNA extraction

Genomic DNA from disrupted biofilm samples was extracted using the UltraClean microbial DNA isolation kit (MO BIO Laboratories, Inc., USA) according to the manufacturer's instructions. The extracted DNA was kept at −20 °C until its use for DGGE.

16S rRNA polymerase chain reaction (PCR) conditions

The V3 region of bacterial 16S rRNA gene fragments was amplified using the primers 338F-GC and 518R [26]. The

amplification was performed in 35-µl reaction mixtures containing 1× PCR buffer (Promega, US), 3 mM $MgCl_2$, 5 % dimethylsulfoxide, 200 µM of each nucleotide, 21 pmol of each primer, 1.4 U *Taq* polymerase (Promega, US) and 1–20 ng of purified DNA. The PCR temperature profile was as described by Henriques et al. [15], except the final extension step, which was of 30 min at 72 °C. The reactions were carried out in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Richmond, CA, USA).

DGGE

DGGE was performed using a DCode™ universal mutation detection system (Bio-Rad Laboratories, Richmond, CA, USA). Samples containing approximately equal amounts of the PCR product (ca. 300 ng) were loaded onto 8 % (w/v) polyacrylamide gels in 0.5× TAE buffer (20 mM Tris–acetate, pH 7.4, 10 mM sodium acetate, 0.5 mM Na_2EDTA) using a denaturing gradient ranging from 35 to 70 % (100 % denaturant contains 7 M urea and 40 % formamide). Electrophoresis was performed at 60 °C in 1× TAE buffer, initially at 20 V (15 min) and then at 75 V (960 min). The gels were stained in a 10× GelGreen nucleic acid stain solution (Biotium Inc., USA) in 0.1 M NaCl. Images were acquired using a Safe Imager™ blue-light transilluminator (Invitrogen™, USA) and a microDOC gel documentation system (Cleaver Scientific Ltd, UK).

The gel images were analysed using GelCompar® II software (version 4.6; Applied Maths, Sin-Martens-Latem, Belgium). Dendrograms were generated using the unweight pair group mean average (UPGMA) method. DGGE patterns were examined using the diversity (*H*) [33] and equitability (*E*) [28] indexes. Every gel contained three lanes with a standard of six bands for internal and external normalization and as an indication of the quality of the analysis. Bacterial species presence/absence in each DGGE gel was ordinated by principal component analysis (PCA) using PC-ORD (version 5, MJM Software) [16]. Monte Carlo randomization test (with 1,000 interactions as default) was used to evaluate the statistical significance of the PCA axis.

DGGE bands sequencing

Selected DGGE bands were excised with a sterile scalpel and eluted in 50 µl of sterile Tris–HCl buffer (10 mM Tris–HCl, pH 8.00), over 2 days at 4 °C. Two microlitres of the supernatant was used for re-amplification with the original primer set, but without the GC clamp attached to the forward primer (338F).

For sequencing analysis, PCR products were purified using illustra GFX™ PCR DNA and gel band purification kit (GE Healthcare, USA), according to the manufacturer's instructions. DNA sequencing was performed under BigDye™ terminator cycling conditions, using an automatic sequencer 3730xl (GATC Biotech, Konstanz, Germany). Band sequences were compared using the BLAST software available from the National Center of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) in order to determine their closest phylogenetic relatives.

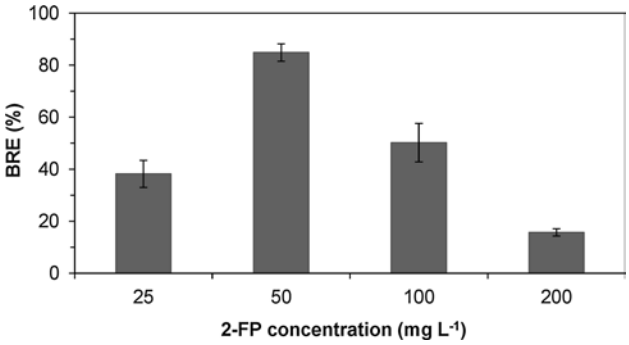

Fig. 2 Biological removal efficiency (BRE) of 2-FP after bioaugmentation with strain FP1. Values are mean ± standard error of the mean (SEM)

Nucleotide sequence accession numbers

The 16S rRNA gene sequences determined in the present study have been deposited in the GenBank database under accession numbers JF775489–JF775499.

Results and discussion

Reactor performance

The performance of the RBC treating 2-FP-containing wastewater was described by Duque et al. [8]. Before bioaugmentation with strain FP1, no biodegradation of the target compound was observed, indicating that unacclimated biomass was not able to remove the toxic compound. Figure 2 summarizes the 2-FP biological removal efficiency (BRE) after the bioaugmentation phase, derived from that study. During the initial 41 days after bioaugmentation, the reactor was fed with 25 mg l^{-1} of 2-FP and, after that, concentrations of 50, 100 and 200 mg l^{-1} were used. During the intermittent feeding of 25 mg l^{-1} of 2-FP, mimicking scenarios of industrial wastewaters where these micropollutants may occur discontinuously, the average BRE obtained was of ca. 38 %. Straight after bioaugmentation, it is possible that part of the inoculum, which might not have been attached to the biofilm, was washed out from the reactor. Therefore, the concentration of strain FP1 was most likely low and not enough to biodegrade all

Table 1 List of 16S rRNA gene analysis of 2-FP-degrading isolates enriched from the RBC

Isolate	Accession no.	Phylogenetic affiliation	Closest relative (accession no.)	Similarity (%)	Isolation source
Day 249					
RBC1	JF775489	γ-Proteobacteria	*Acinetobacter* sp. Z21 (EU236748)	99	Alpine grassland, 2,970 m elevation
Day 300					
RBC2	JF775490	γ-Proteobacteria	*Acinetobacter johnsonii* strain WAB1932 (AM184271)	98	River water
S2	JF775491	Actinobacteria	*Rhodococcus qingshengii* strain ZJB-09153 (HQ439600)	99	Soil of drain outlet near chemical plants
Day 374					
RBC4	JF775492	α-Proteobacteria	*Sinorhizobium* sp. BRAZ11 (GQ867232)	99	ND
RBC5	JF775493	Actinobacteria	*Microbacterium* sp. M449 (AB461787)	99	Soybean stem
RBC6	JF775494	Actinobacteria	*Pimelobacter simplex* strain CL-9.11b (HQ113209)	99	Swine waste biotreatment

ND not determined

of the 25 mg l^{-1} of 2-FP. However, biodegradation indicated that bioaugmentation was successfully achieved, although strain FP1 was still adapting to the RBC operating conditions. Later on in the operation, with a 2-FP continuous feeding concentration of 50 mg l^{-1}, the BRE values reached ca. 85 %, on average, indicating that strain FP1 attached to the biofilm established on the discs or/and gene transfer occurred. Recent studies suggest that biofilms are specially suited for horizontal gene transfer [24, 34]. Even though plasmids have been found in nearly all bacterial species studied to date, namely in the genus *Rhodococcus* to which strain FP1 belongs [23], a thorough investigation would be needed to ascertain the possibility of horizontal gene transfer within the biofilm.

Generally, the RBC showed a lower performance when fed with the highest 2-FP concentrations, 100 and 200 mg l^{-1}. When 100 and 200 mg l^{-1} of 2-FP were fed to the RBC, the BRE decreased to ca. 50 and 16 %, respectively, most probably because of 2-FP high toxicity.

2-FP-degrading bacteria recovered from the RBC

Cultivation-based methods, such as isolation, represent a conventional approach to identify the microbial populations in a microbial ecosystem. In the present study, after bioaugmentation, plating and isolation allowed one to find other microorganisms with the capability to degrade 2-FP. Apart from strain FP1, six bacterial isolates extracted from the RBC biofilm showed degradation activity towards 2-FP. Previous studies reported the isolation of several bacterial strains able to degrade 2-fluorobenzoate from an upflow bed reactor after being inoculated with a specialized strain, FB2 [12]. Rapid acclimatization to highly recalcitrant compounds, such as fluorinated organics, is unlikely to occur. However, in the present study we did not explore whether this ability was achieved by natural enrichment or by other mechanisms.

The isolates were characterized through sequencing of the 16sRNA gene and the resulting sequences were submitted to GenBank. According to BLAST results, two strains were affiliated with γ-Proteobacteria, three with Actinobacteria and one with α-Proteobacteria (Table 1). Some of these bacterial species, including *Acinetobacter* sp., *Rhodococcus* sp., *Sinorhizobium* sp. and *Microbacterium* sp. have previously been shown to degrade other aromatic compounds [1, 14, 23, 31].

Bacterial community in the RBC

The analysis of bacterial communities from biofilm samples, collected along the RBC operation, was performed by PCR-DGGE. The first and fifth stages were chosen to study the bacterial community as the first stage was receiving the initial 2-FP shock loadings and from the fifth stage 2-FP was not detected in the reactor. The DGGE profiles obtained for the biofilm samples from the first and fifth stages, along the RBC operation, are shown in Fig. 3. Each gel presents the variability within each stage at different sampling periods along the 496 days of reactor operation. The profiles were highly reproducible between gels and PCRs. The total number of band positions detected in the two gels was 216 and the number of DGGE bands per lane varied between 8 and 29. It is clear that there was a high variability between the bacterial assemblages, although some bands are common to several sampling days and to both stages. Pynaert et al. [29], who investigated the

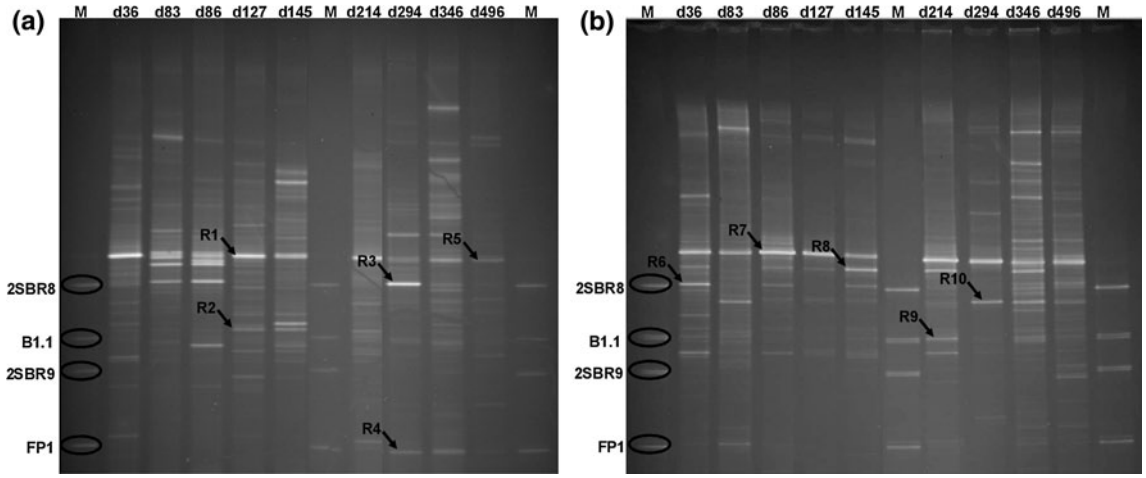

Fig. 3 DGGE analysis of 16S rRNA gene fragments of total population from samples of the biofilm from the first stage (**a**) and from the fifth stage (**b**) of the RBC. Gel lanes contain samples collected during RBC operation (days are indicated on the *top* of the *lanes*). *Lane M* DNA marker constructed using 2-FP-degrading strains. Bands that were excised for sequence analysis, indicated with an *arrow*, are labeled with the same number as in Table 3

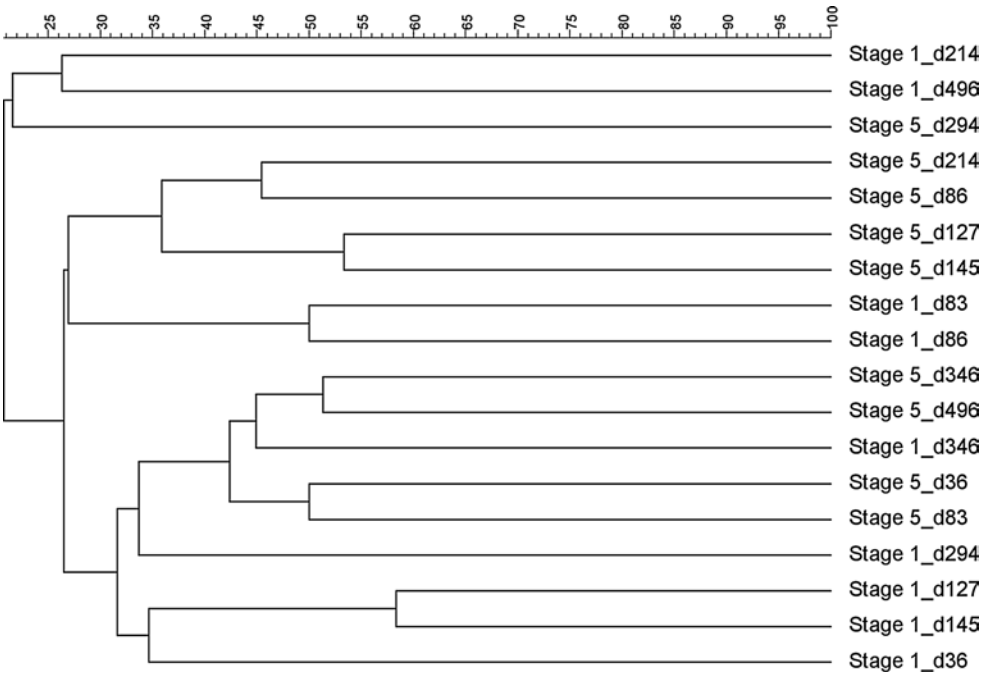

Fig. 4 Cluster analysis of bacterial communities based upon DGGE profiles. Similarities were calculated using the Bray–Curtis measure. The dendrogram presents the similarity, in percentage, between samples

start-up of an oxygen-limited autotrophic nitrification–denitrification (OLAND) process in an RBC, also found a diverse bacterial DGGE profile.

Cluster analysis was performed in order to assess the relatedness of phylogenetic profiles representing the communities of the first and fifth stages, at each sampling day (Fig. 4). Three clusters of samples could be identified and all clusters included samples from the first and fifth stages. However, the difference between the bacterial assemblages of each cluster and the sample dispersion within each cluster is evident, with low similarities (<58 %). Despite the fact that one of the clusters included samples collected from both stages at the start-up of the reactor (day 36) and after the starvation period (day 346), there was no clear relation between the sample collection time and the spatial factor (first stage or fifth stage). Additionally, principal component analysis (PCA) was performed in order to evaluate bacterial species distribution on the different stages of the reactor (data not shown). The groups formed corroborate with the cluster analysis dendrogram (Fig. 4).

Significant changes in the microbial communities have been reported both in studies when only one compound

was fed continuously [32] and when several substrates were fed continuously [20] and in an alternating mode [4, 12] to bioreactors. However, stable microbial communities during biodegradation of xenobiotic compounds, such as 1,2 dichloroethane and fluorobenzene, have also been described [6, 11]. Fernandez et al. [13] stated that higher species diversity or community stability is not directly related to functional stable reactors. The present study showed high species diversity and stable reactor performance, as indicated by continuous removal of 2-FP [8].

DGGE banding data were used to estimate diversity (*H*) [33], and equitability (*E*) [28] (Table 2) The diversity of bacterial communities, Shannon diversity index (*H*), ranged from 0.86 to 1.26 for the first stage and from 0.58 to 1.35 for the fifth stage. The highest *H* values obtained for each stage are mainly related to periods where 2-FP was not fed to the reactor, with acetate being the sole source of carbon and energy.

Table 2 Shannon diversity (*H*) and equitability (*E*) indexes, calculated for the first and fifth stages of the RBC

Samples	First stage		Fifth stage	
	H	*E*	*H*	*E*
Day 36	1.03	0.82	1.12	0.84
Day 83	0.97	0.87	1.05	0.81
Day 86	0.98	0.85	0.92	0.83
Day 127	1.08	0.86	0.58	0.64
Day 145	1.16	0.89	0.96	0.81
Day 214	1.00	0.85	1.05	0.82
Day 294	0.86	0.77	0.75	0.72
Day 346	1.26	0.94	1.35	0.92
Day 496	0.37	0.38	0.88	0.61

The xenobiotic nature of the compounds is a limiting factor of the number of microorganisms capable of tolerating their presence [12]. Furthermore, easily degradable substrates can lead to highly variable microbial communities [13]. Therefore, it was expected that microbial population diversity would increase when 2-FP was absent in the inlet feeding. The *E* index can range from near 0 (pronounced dominance) to 1 (complete evenness) [28]. The *E* index was on average 0.80 ± 0.06 for the first stage and 0.78 ± 0.03 for the fifth stage, showing an almost equal abundance of all species.

The phylogenetic affiliation of each sequenced DGGE band, representing different microorganisms, was determined by excision and sequencing of ten DGGE bands (Table 3). The DGGE bands selection was based on the intensity of the bands that likely indicate bacterial strains that are predominating, combined with the persistence and appearance of bands over time. All the bands were directly sequenced. According to BLAST results, four sequences affiliated with α-Proteobacteria, two with β-Proteobacteria, one with Actinobacteria and one with δ-Proteobacteria were identified. Other studies on the characterization of the microbial structure and diversity of RBC biofilms have reported bacterial clones with similarities to α-, β- and δ-Proteobacteria [19, 21, 37]. Two of the sequences, R9 and R10, were similar to 16S rDNA sequences reported from uncultured organisms from environmental samples (Table 3). The same

Table 3 Phylogenetic affiliation of DGGE band DNA sequences

Band no.	Sample		Phylogenetic affiliation	Closest relative (accession no.)	Similarity (%)	Isolation source
	Stage	Day				
R1	First	127	α-Proteobacteria	*Meganema perideroedes* strain Gr28 (AY170120)	100	Activated sludge
R2	First	127	α-Proteobacteria	Uncultured *Bradyrhizobium* sp. clone MWM2-53 (HQ674796)	96	Variably weathered outcrop
R3	First	294	β-Proteobacteria	*Leptothrix* sp. OTSz_A003 (FM886835)	92	Floodplain
R4	First	294	Actinobacteria	*Rhodococcus* sp. FP1 (HM210775)	99	Soil
R5	First	496	α-Proteobacteria	*Meganema perideroedes* strain Gr28 (AY170120)	100	Activated sludge
R6	Fifth	36	β-Proteobacteria	*Leptothrix* sp. OTSz_A003 (FM886835)	98	Floodplain
R7	Fifth	86	α-Proteobacteria	*Meganema perideroedes* strain Gr28 (AY170120)	100	Activated sludge
R8	Fifth	145	δ-Proteobacteria	*Chondromyces lanuginosus* strain KYC2904 (FJ176774)	92	Soil
R9	Fifth	214	Unknown	Uncultured bacterium clone BP087B169 (HM444863)	98	Yellow microbial mat from lava tube walls
R10	Fifth	294	Unknown	Uncultured bacterium clone EPR3967-O2-Bc49 (EU491796)	94	Seafloor lavas from the East Pacific Rise

nucleotide sequence was obtained for the bands R1, R5 and R7, which were found in several samples in the same position on the gel, being 100 % identical to *Meganema perideroedes* strain Gr28 isolated from activated sludge [36]. The bands R3 and R6 were also confirmed to belong to the same phylotypes, namely β-Proteobacteria, being most closely related to *Leptothrix* sp. OTSz_A003 retrieved from a floodplain, Germany. As expected, band R4 was closely related to *Rhodococcus* sp. strain FP1, which was the inoculated strain, showing 99 % similarity, corroborating the success of the bioaugmentation. This band was present in all samples from the first and fifth stages from day 145 (after bioaugmentation), although the intensity of the band varied over time. Therefore, although several strains were found to degrade 2-FP, the inoculated strain FP1 most likely played a key role in the 2-FP degradation.

In conclusion, the dynamics of the microbial community of the biofilm present in the first and fifth stages of an RBC treating 2-FP-containing wastewater was investigated. The clustering analysis together with the *H* and *E* indexes indicated that a diverse and distinct bacterial community was present in the first and fifth stages of the RBC, although an even distribution of species was observed within each stage. The bioaugmentation effect in terms of 2-FP degradation was successful when compared to the phase where there was no inoculation of the degrading strain. Recovery of strain FP1 corroborates the fact that bioaugmentation was successfully achieved.

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