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Characterization of the bacterial communities of aerobic granules in a 2-fluorophenol degrading process

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ABSTRACT

Aerobic granular sludge constitutes a novel technology for wastewater treatment. This study focused on the effect of 2-fluorophenol (2-FP) shock loadings on the microbial community diversity present in aerobic granules before and after inoculation with a bacterial strain able to degrade 2-FP, *Rhodococcus* sp. strain FP1. After bioaugmentation, apart from strain FP1, five culturable bacteria were isolated from the 2-FP degrading granules, belonging to the following genera: *Serratia*, *Chryseobacterium*, *Xanthomonas*, *Pimelobacter* and *Rhodococcus*. The latter two isolates are able to degrade 2-FP. Changes in the aerobic granules' bacterial communities related to 2-FP shock loadings were examined using denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene pool. Numerical analysis of the DGGE profiles showed high diversity with an even distribution of species. Based on cluster analysis of the DGGE profiles, the bacterial communities present in the aerobic granules changes were related to the sampling time and the 2-FP concentration fed.

1. Introduction

Fluorinated compounds, such as 2-fluorophenol, have been found to accumulate in the environment due to their widespread application as agrochemicals, pharmaceuticals and in other industrial processes [22,37]. Most fluoroaromatics are recalcitrant and cause acute toxicity to various life forms [22], although they occur discontinuously and at low concentrations.

Aerobic granulation is an innovative microbial self-immobilization strategy for biological wastewater treatment. Aerobic granules have a strong, dense, smooth and spherical structure, excellent settling properties, high biomass retention, ability to withstand at high organic loadings and tolerance to toxicity [2,10]. The aggregation of microorganisms into compact aerobic granules allows protection against predation and resistance to chemical toxicity [21,41,42]. Therefore, aerobic granular sludge has been successfully used to treat high strength toxic organic wastewater and synthetic wastewater containing pharmaceuticals, namely fluoroquinolones [2,5,25]. The granules have a diverse microbial community, a complex spatial structure, coordinated physiological functions and specific temporal changes [20,39,40,46,47,48]. The microbial diversity of aerobic granules, which has been scarcely studied, has been related to the structure of the aerobic granules and the composition of the culture media in which they were developed [2,25]. Most recently, aerobic granular sludge microbial diversity has been correlated with reactor characteristics and to fluctuations in operation conditions [14,47,48]. However, to our knowledge, the effect of toxic compounds on the microbial community composition dynamics of aerobic granular sludge was not reported yet. Thus, a better understanding of the microbial composition, structure and stability of the microbial community will help to further understand and optimize granule formation in the presence of toxic micropollutants.

It is known that culture-dependent methods have limitations for studying natural microbial community composition, because only a small part of bacteria in environmental samples are culturable under laboratory conditions [4,17,31]. DNA-based molecular techniques, which are culture-independent methods, such as denaturing gradient gel electrophoresis (DGGE), provide a more comprehensive, rapid and concise characterization of the bacterial population diversity in biological wastewater treatment systems. However, culture-dependent methods cannot be totally supplanted, as bacterial isolates are needed to better understand their physiology and function. Population diversity alone does not drive ecosystem stability; hence, gaining an understanding of the functions of microbial communities is important [8].

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The aim of this work was to investigate the dynamics of the microbial community present on aerobic granules subject to 2-FP shock loadings, before and after bioaugmentation of a granular sludge sequencing batch reactor (SBR) with a 2-FP degrading strain. Bacterial strains isolation and denaturing gradient gel electrophoresis (DGGE) analysis of the 16S rRNA gene were used for this purpose.

2. Materials and methods

2.1. Granular sludge SBR set-up

A laboratory scale sequencing batch reactor (SBR) with a working volume of 2.5 L was set up (110 cm height and an internal diameter of 6.5 cm) and was operated during 444 days [12] as described in Table 1. Briefly, the volume exchange ratio per cycle was ca. 40% and the settling time was chosen such that only particles with a settling velocity larger than 6 m h^{-1} were effectively retained in the reactor. This set up was previously reported to successfully conduct lab scale experiments with aerobic granular sludge [7,10,29,47]. The reactor was inoculated with granular sludge removing phosphate biologically (500 mL wet granules) from an aerobic granular sludge pilot plant treating sewage in The Netherlands (Epe wastewater treatment plant) and was bioaugmented with a specialized strain able to degrade 2-FP previously isolated in our laboratories [13], a *Rhodococcus* sp. strain FP1 (LMG 26251; DSM 45581), on day 210. Aeration was supplied at the bottom of the reactor at an airflow rate of 4 L min^{-1}. The experiment was conducted with no oxygen control (dissolved oxygen (DO) 100%) and the pH was maintained at 7.0 ± 0.8 by dosing 1 M NaOH or 1 M HCl.

The composition of the SBR influent media was as described in Duque et al. [12].

2.2. Analytical methods

Samples were collected from the influent (after 60 min influent feeding) and from the effluent of the SBR. The physico-chemical parameters were determined as follows: NH_4^+, NO_3^- and NO_2^- were measured by sequential injection analysis (SIA) as described by Segundo et al. [34] and Mesquita et al. [27], respectively; PO_4^- concentration of filtered samples was determined by flow injection analysis (FIA) according to Torres et al. [45]; 2-FP was analyzed by high performance liquid chromatography (HPLC) and fluoride concentration was measured with an ion-selective combination electrode as previously described [12].

The DO concentration in the reactor was measured online with a DO-sensor (InPro 6820, Mettler-Toledo) as percentage of the oxygen saturation concentration (100% = 9.1 mg L^{-1}) and the pH was monitored online using a pH-electrode (InPro 3030, Mettler-Toledo).

2.3. Sampling of aerobic granular sludge

Aerobic granular sludge samples (about 5 g of granules) were collected during the aeration phase in order to achieve a representative sample of the biomass present in the reactor. The granules were crushed, using a pottering tube and a pestle as described by Weissbrodt et al. [47]. The resulting bacterial suspensions were used for plating, bacterial identification and DNA extraction for DGGE analysis.

2.4. Isolation and identification of 2-FP degrading strains from the SBR

Granules characterization was done by plating serial dilutions of bacterial suspensions of crushed granules in saline solution (0.85% w/v NaCl) onto nutrient agar (NA) (LABM, UK). A volume of 0.1 mL of each dilution was spread onto the NA plates. Plates were incubated at 25 °C for 3 days. Based on size, morphology and pigmentation, different bacterial colonies were isolated from NA plates using the streak–plate procedure. The isolated strains recovered from the NA plates were tested for their capacity to degrade 2-FP, using 250 mL flasks containing 50 mL of sterile mineral salts medium (MM) [9] with 50 mg L^{-1} of 2-FP, as the sole carbon and energy source. In wastewaters pollutants can be found within the ppm range and *Rhodococcus* sp. strain FP1 has been shown to withstand up to 50 ppm of the compound in earlier studies [13]. Cultures were incubated on an orbital shaker at 100 rpm and 25 °C. When growth was observed, indicated by an increase in the optical density and by fluoride release, 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. [12].

2.5. Aerobic granular sludge microbial community analysis

2.5.1. DNA extraction

The genomic DNA extraction of crushed aerobic granules samples was performed using the UltraClean Microbial DNA

Table 1
Summary of the operating conditions tested in the SBR and performance.

Operating conditions	Phase						
	Before bioaugmentation		After bioaugmentation				
	I	II	III	IV	V	VI	VII
Length of operation (days)	0–99	100–209	210–222	223–229	230–266	267–400	401–444
Cycle time (h)	3	3	12	12	8	4	4
Influent feeding (min)	60	60	60	60	60	60	60
Aeration (min)	112	112	652	652	412	172	172
Settling (min)	3	3	3	3	3	3	3
Effluent withdrawal (min)	5	5	5	5	5	5	5
HRT (h)	7.9	7.9	31.6	31.6	21.1	10.5	10.5
Influent $COD_{acetate}$ ($kg\,d^{-1}\,m^{-3}$)[a]	1.0	1.0	0.3	0.3	0.4	0.8	0.8
Inlet 2-FP concentration (mM)[b]	0	0.08	0.08	0.17	0.17	0.17	0.08
2-FP biodegraded (mM)[c]	NA	0	0.056 ± 0.009	0.104 ± 0.008	0.117 ± 0.010	0.106 ± 0.005	0.100 ± 0.009

NA: not applicable.
[a] $COD_{acetate}$: chemical oxygen demand based on acetate.
[b] 2-FP concentration inside the bioreactor.
[c] Values are means ± standard error of the mean (SEM).

Isolation Kit (MO BIO Laboratories, Inc., USA) according to the manufacturer's instructions. The extracted DNA was kept at −20 °C until it was used as template for PCR amplifications.

2.5.2. 16S rRNA polymerase chain reaction (PCR) conditions

The primers 338F-GC and 518R were used for the amplification of the highly variable V3 region of bacterial 16S rRNA gene fragments [32]. The PCR amplification was carried out in 50 μL reaction mixtures containing 1× PCR buffer (Promega, US), 3 mM $MgCl_2$, 5% dimethylsulfoxide, 200 μM of each nucleotide, 30 pmol of each primer, 2 U *Taq* polymerase (Promega, US), and 1–20 ng of purified DNA. The PCR temperature profile was as described by Henriques et al. [18], changing the final extension step to 30 min at 72 °C. The reactions were performed in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Richmond, CA, USA).

2.5.3. Denaturing gradient gel electrophoresis (DGGE)

PCR-amplified 16S rRNA gene fragments were separated by DGGE using a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Richmond, CA, USA). The PCR products containing ca. 300 ng of DNA were loaded onto 8% (w/v) polyacrylamide gels (37.5:1, acrylamide:bisacrylamide) in 0.5× TAE buffer (20 mM Tris–acetate, pH 7.4, 10 mM sodium acetate, 0.5 mM Na_2 EDTA) 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. The DGGE images were acquired using a Safe Imager™ Blue-Light Transilluminator (Invitrogen™, USA) and a microDOC gel documentation system (Cleaver Scientific Ltd., UK). Other adaptation of this method was successfully applied to aerobic granular sludge by Winkler et al. [48].

DGGE profiles 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. Every gel contained 3 lanes with a standard of 6 bands for internal and external normalization and as an indication of the quality of the analysis. The standard was prepared with bacterial strains existing in the lab and was used just for normalization procedures. DGGE patterns were examined using the diversity, *H* [35] and equitability, *E* [33] indices. Bacterial species presence/absence in each DGGE gel was ordinated by Principal Correspondence Analysis (PCA) using PC-ORD (version 5, MJM Software) [19]. Monte Carlo randomization test (with 1000 interactions as default) was used to evaluate the statistical significance of the PCA axes.

2.5.4. Sequencing of DNA from DGGE bands

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). After 2 days at 4 °C, 2 μL 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 manufacturers' 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 at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) for identification and phylogenetic classification.

2.6. Nucleotide sequence accession numbers

The partial 16S rRNA gene sequences determined in the present study have been deposited in the GenBank database under accession numbers: JF775495–JF775499 and HM210775.

3. Results and discussion

3.1. Performance of the granular sludge SBR

A granular sludge sequencing batch reactor (SBR) was fed with acetate supplemented with 0.08 mM of 2-fluorophenol (2-FP). During the first 109 days no 2-FP degradation occurred, suggesting that the microbial community present in the aerobic granules was not able to remove this xenobiotic compound. Concerning nitrogen, phosphate and carbon removal, an in depth discussion of the performance of the granular sludge SBR treating synthetic wastewater containing 2-FP is provided in Duque et al. [12]. When the granular sludge SBR was inoculated with *Rhodococcus* sp. strain FP1 (a 2-FP degrader described by Duque et al. [13]), degradation of the target compound took place when it was added to the influent at concentrations ranging from 0.08 to 0.17 mM (average removal 80%). This revealed adaptation of the aerobic granules to the toxic compound and/or incorporation of strain FP1 in the granules.

3.2. Capacity of bacterial isolates recovered from the granular sludge SBR to degrade 2-FP

The microbial community present in the aerobic granules was investigated by culture-dependent methods, namely by plating on nutrient agar (NA), and the isolates were subsequently tested for their capacity to degrade 2-FP. Six bacterial isolates were obtained and were further characterized through partial sequencing of the 16S rRNA encoding gene. According to BLAST results, 2 strains were affiliated with γ-Proteobacteria, 1 with Flavobacteria and 3 with Actinobacteria (Table 2). Previous studies, which used culture-based methods and culture-independent methods to study the microbial population present in aerobic granules, showed that Proteobacteria and Actinobacteria constitute a large fraction of the bacteria in aerobic granular sludge [38,21]. Actually, some bacterial

Table 2
Phylogenetic affiliation of bacterial isolates extracted from the aerobic granules.

Isolate	Accession no.	Phylogenetic affiliation	Closest relative (accession no.)	Similarity (%)	Origin	2-FP degradation
SBR1	JF775495	γ-Proteobacteria	*Serratia marcescens* strain W2.3 (JF317349)	99	Dead freshwater fish	No
SBR2	JF775496	Flavobacteria	*Chryseobacterium* sp. PB2 (AM232811)	99	Experimental biofilter	No
SBR3	JF775497	γ-Proteobacteria	*Xanthomonas* sp. shui13-10 (HQ436442)	100	13 m water layer of Hugangyan Maar Lake in summer	No
SBR4	JF775498	Actinobacteria	*Pimelobacter simplex* strain CL-9.11b (HQ113209)	99	Swine waste biotreatment	Yes
SBR5	JF775499	Actinobacteria	*Rhodococcus erythropolis* strain SBUG 107 (FR745420)	100	Gasoline station	Yes
SBR6	HM210775	Actinobacteria	*Rhodococcus* sp. FP1 (HM210775)	99	Isolated from soil	Yes

populations, such as *Xanthomonadaceae,* which are denitrifyers and produce exopolysaccharides [11,44,3], were previously described as having a key role in the aerobic granules' microbiomes [47]. Therefore, their presence in the aerobic granules in this study could be expected.

All of the bacterial isolates shared similarities between 99% and 100% with sequences deposited in GenBank and were closely related to environmental isolates. Two of the bacterial isolates, namely *Pimelobacter* sp. and *Rhodococcus* sp., extracted from the aerobic granules, apart from strain FP1, were able to degrade 2-FP. Bacterial strains affiliated to these genera have been previously reported as being able to degrade other aromatic compounds [15,26].

Before bioaugmentation, the aerobic granules were not able to degrade 2-FP due to the high recalcitrance of the compound. Members of the genus *Rhodococcus*, such as strain FP1, are known to harbor large linear and circular plasmids [26]. These can, in biofilm systems, be effectively transferred by horizontal gene transfer (HGT) as reported by Molin and Tolker-Nielsen [28], Wuertz et al. [49] and Sorensen et al. [36]. However, in the present study it was not explored whether the ability to degrade 2-FP was achieved by natural enrichment or by other mechanisms, such as HGT.

3.3. Microbial community in the aerobic granules

The denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene was performed to investigate bacterial community changes in aerobic granules along granular sludge SBR operation. The DGGE profiles obtained for each sampling day are shown in Fig. 1. The number of DGGE bands per lane varied between 7 and 19, indicating a wide bacterial diversity within the granules, as previously reported [43,48,50,51]. Some bands were common in almost all samples (e.g., bands S1 and S3), while others were only present in some sampling days (e.g., bands S4 and S5).

Cluster analysis was performed aiming at characterizing the similarity between the DGGE profiles (Fig. 2). The dendrograms showed that the bacterial communities remained fairly stable during operation of the granular sludge SBR. Previous studies on the microbial community of chloroanilines-, methyl *tert*-butyl ether- and *tert*-butyl alcohol-degrading aerobic granules have also reported stable microbial community [43,50–52]. Emanuelsson et al. [15] stated that xenobiotic substrates are responsible for the stability of the microbial community, as they may reduce the diversity of a given microbial community by exerting a selective pressure. The largest shifts in bacterial assemblage were identified between samples collected at day 0 and day 100 (ca. 20% similarity), when the reactor started to be fed with 2-FP, and between day 301 and day 408 (<40% similarity), when the 2-FP inlet concentration was decreased to 0.08 mM (Fig. 2). Less significant shifts were also observed between day 100 and day 114 (ca. 40% similarity), when 2-FP was already being fed to the SBR for 14 days, between day 114 and day 210 (ca. 74% similarity), when the 2-FP degrading strain was added to the SBR and between day 217 and day 227 (<50% similarity), when the 2-FP inlet concentration was increased to 0.17 mM (Fig. 2 and Table 1). Microbial shifts in phenol-cultured aerobic granules when pyridine was present were also observed [1]. The significant impact of organic loading condition on species selection during aerobic granulation has been previously reported [23]. The cluster analysis showed a clear relation between the sample collection time and 2-FP concentration fed to the SBR.

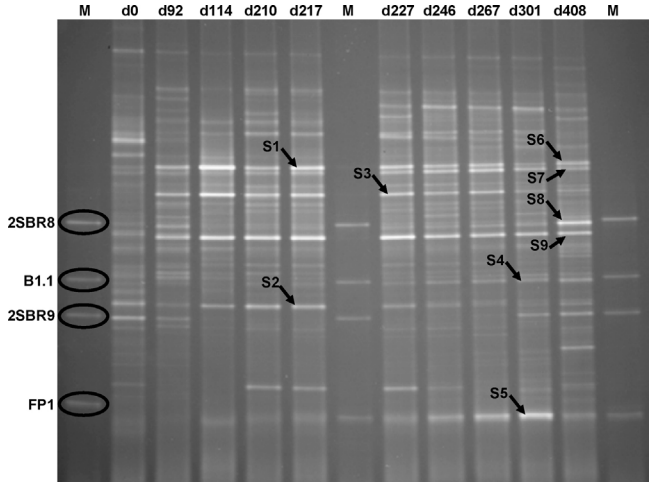

Fig. 1. DGGE community fingerprint of the SBR 16S rRNA gene population. Gel lanes contain samples collected during SBR operation (days are indicated on the top of the lanes). Lane M: DNA marker constructed using 2-FP degrading bacteria. Bands that were excised for sequence analysis, indicated with an arrow, are labeled with the same number as in Table 3.

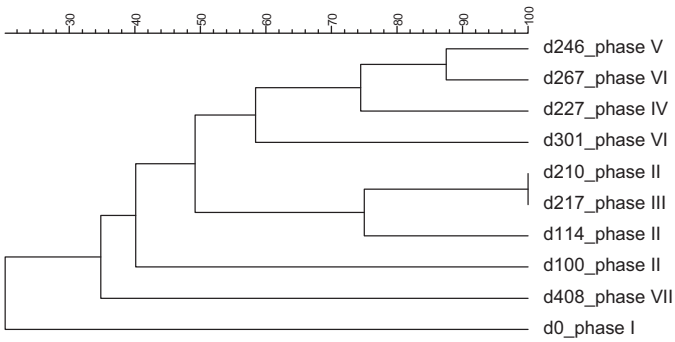

Fig. 2. Cluster analysis of bacterial communities based upon DGGE profiles. Similarities were calculated using the Bray–Curtis measure.

Principal component analysis (PCA) was performed in order to ordinate bacterial species in the DGGE gel, using gel bands presence and absence and the variable time (before and after bioaugmentation) (Fig. 3). Similarly to the cluster analysis dendrogram, samples collected at days 227 and 226 and days 217 and 210 grouped together in the PCA. However, contrarily to the cluster analysis dendrogram, samples collected at days 301 and 408 grouped together in the PCA. Indeed, this was expected as these samples were subjected to the same conditions, such as time, cycle length and 2-FP concentration in the feed. PCA diagram shows that the microbial community change along the sampling time through axis 1 (eigenvalue 13.6), corroborating the cluster analysis dendrogram.

The use of indices based on relative band intensities provides more information about community composition than simply the number of species [30]. Therefore, numerical analysis of DGGE profiles was performed using two indices, diversity (H), used to calculate diversity of bacterial communities [35], and equitability (E), which can range from 0, indicating pronounced dominance, to 1, indicating equal abundance of all species [33]. The Shannon diversity index (H) ranged from 0.72 to 1.12. Similarly, Li et al. [23] have reported H values below 1.2 for aerobic granules subjected to different loading rates. The highest H values were obtained in days 227, 246 and 267, when the 2-FP inlet concentration was 0.17 mM, indicating species richness and higher relative abundance in those phases. The E index, which ranged from 0.73 to 0.96, indicated that the distribution of species is even. Other research conducted on aerobic granular sludge reached similar E index results [48].

Selected DGGE bands (Fig. 1) were excised for direct PCR amplification followed by DNA sequencing. The selection was

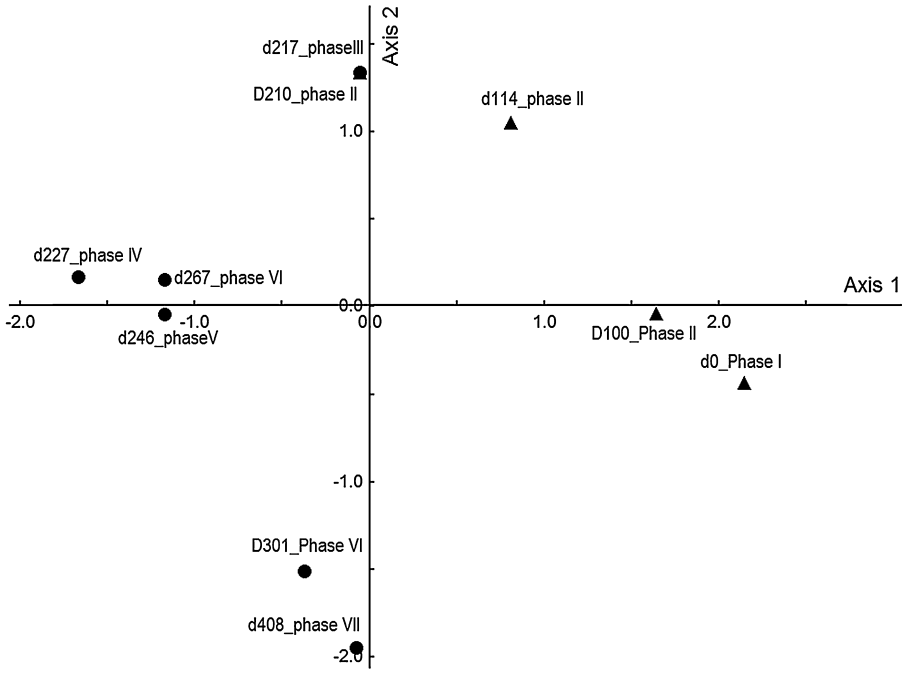

Fig. 3. Ordination biplot for principal correspondence analysis (PCA) of DGGE banding patterns (samples indicated using the day and operational phase of sampling). Axis 1 explains 29.9% and axis 2 explains 24.1% of the observed variance ($p < 0.01$).

Table 3
Phylogenetic affiliation of DGGE band DNA sequences.

Band no.	Sample day	Phylogenetic affiliation	Closest relative (accession no.)	Similarity (%)	Origin
S1	217	γ-Proteobacteria	Uncultured *Pseudoxanthomonas* sp. clone R7–15 (JF808749)	100	Activated sludge in a membrane bioreactor
S2	217	Unknown	Uncultured bacterium clone F1Q32TO05F4L3V (GU486262)	99	Guri wastewater (suspended biomass)
S3	227	γ-Proteobacteria	*Xanthomonas* sp. SBR3 (JF775497)	99	Aerobic granular sludge reactor
S4	301	α-Proteobacteria	Uncultured Rhizobiales bacterium clone PSB-8 (JF731367)	95	Fluvo-aquic soil of Yellow River
S5	301	Actinobacteria	*Rhodococcus* sp. FP1 (HM210775)	99	Inoculated strain
S6	408	β-Proteobacteria	*Acidovorax delafieldii* strain THWCSN42 (GQ284426)	92	Sediment sample of natural spring
S7	408	γ-Proteobacteria	*Dokdonella* sp. PYM5–8 (AM981202)	94	Drinking water network
S8	408	Nitrospirae	Uncultured *Nitrospira* sp. clone ND_11_NTSPA (HM486344)	95	Trickling filter/solids contact basin from full-scale wastewater treatment plant
S9	408	Unknown	Uncultured bacterium clone F1Q32TO06G2588 (GU508443)	95	Guri wastewater (attached biomass)

based on the intensity of the bands, which likely indicate bacterial strains that are predominating, combined with the persistence and appearance of bands along time. The obtained partial 16S rRNA gene sequences were compared with nucleotide sequences from GenBank and results of their closest relative are shown in Table 3. Many of the sequences were similar to 16S rDNA sequences reported for uncultured organisms obtained from environmental samples, such as activated sludge, wastewater and soil, reinforcing the importance of culture-independent methods for the study of microbial communities. According to BLAST results, bacterial communities were composed of organisms affiliated with Actinobacteria (1/9), Nitrospirae (1/9), but mainly belonged to the Proteobacteria group (5/9), subclasses γ-, α- and β-Proteobacteria (3/9, 1/9, 1/9, respectively). Sequences belonging to this group have previously been retrieved from aerobic granules degrading chloroanilines, methyl *tert*-butyl ether, *tert*-butyl alcohol and simple carbon sources such as glucose, acetate and propionate [6,16,23,24,43,47,50–52]. Particularly, *Pseudoxanthomonas* sp., *Xanthomonas* sp., *Rhizobales* sp., *Acidovorax* sp., and *Nitrospira* sp., which were the closest relatives of the sequences from bands S1, S3, S4, S6, and S8, respectively, have been previously found in aerobic granules [3,16,24,46–48,52]. Interestingly, these species were identified in the aerobic granules as being involved on volatile fatty acids and amino acids uptake, on exopolysaccharides production and on the nitrification and denitrification processes [3,47]. Therefore, it was not unexpected to detect them in the present study, as they have an important role on aerobic granules ecosystem.

From day 210, band S5 was present in all samples, albeit the intensity of the band varied over time, and, as expected, was closely related to *Rhodococcus* sp. strain FP1, which was the bioaugmented strain. The success of the bioaugmentation was thus demonstrated.

4. Conclusions

The microbial diversity of aerobic granular sludge treating 2-FP containing wastewater was assessed. After inoculation of strain FP1, two other bacterial strains with the capacity for biodegradation of 2-FP were recovered. Despite the fact that two bacterial

isolates able to degrade 2-FP were extracted from the reactor, the inoculated strain FP1 remained present in the SBR throughout its operation, most probably playing a key role in the 2-FP degradation. The 16s rRNA gene-targeted DGGE results showed that bacterial community remained fairly stable during operation of the SBR, although a wide bacterial diversity was observed. However, concerning the small shifts observed in the bacterial population, 2-FP seemed to have the biggest effect on the dynamics of 16S bacterial communities, rather than the bioaugmentation. Overall, 2-FP did not affect the bacterial communities responsible for granule formation, stability and those involved on the main processes of nutrients removal, namely nitrification, denitrification and carbon sources uptake. Aerobic granular sludge is a promising technology for wastewater containing toxic compounds treatment.

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References

[1] S.S. Adav, D.-J. Lee, N.Q. Ren, Biodegradation of pyridine using aerobic granules in the presence of phenol, Water Res. 41 (2007) 2903–2910.
[2] S.S. Adav, D.-J. Lee, K.Y. Show, J.H. Tay, Aerobic granular sludge: recent advances, Biotechnol. Adv. 26 (2008) 411–423.
[3] S.S. Adav, D.-J. Lee, J.Y. Lai, Microbial community of acetate utilizing denitrifiers in aerobic granules, Appl. Microbiol. Biotechnol. 85 (2010) 753–762.
[4] R. Amann, W. Ludwig, K.H. Schleifer, Phylogenetic identification and in situ detection of individual microbial cells without cultivation, Microbiol. Rev. 59 (1995) 143–169.
[5] C.L. Amorim, A.S. Maia, R.B.R. Mesquita, A.O.S.S. Rangel, M.C.M. van Loosdrecht, M.E. Tiritan, P.M.L. Castro, Performance of aerobis granular sludge in a sequencing batch bioreactor exposed to ofloxacin, Water Res. 50 (2014) 101–113.
[6] S. Bathe, T.V.K. Mohan, S. Wuertz, M. Hausner, Bioaugmentation of a sequencing batch biofilm reactor by horizontal gene transfer, Water Sci. Technol. 49 (2004) 337–344.
[7] J.J. Beun, A. Hendriks, M.C.M. van Loosdrecht, E. Morgenroth, P.A. Wilderer, J.J. Heijnen, Aerobic granulation in a sequencing batch reactor, Water Res. 33 (1999) 2283–2290.
[8] A. Briones, L. Raskin, Diversity and dynamics of microbial communities in engineered environments and their implications for process stability, Curr. Opin. Biotechnol. 14 (2003) 270–276.
[9] M. Caldeira, S.C. Heald, M.F. Carvalho, I. Vasconcelos, A.T. Bull, P.M.L. Castro, 4-Chlorophenol degradation by a bacterial consortium: development of a granular activated carbon biofilm reactor, Appl. Microbiol. Biotechnol. 52 (1999) 722–729.
[10] M.K. De Kreuk, J.J. Heijnen, M.C.M. van Loosdrecht, Simultaneous COD nitrogen and phosphate removal by aerobic granular sludge, Biotechnol. Bioeng. 90 (2005) 761–776.
[11] J.M. Dow, L. Crossman, K. Findlay, Y.Q. He, J.X. Feng, J.L. Tang, Biofilm dispersal in *Xanthomonas campestris* is controlled by cell–cell signaling and is required for full virulence to plants, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 10995–11000.
[12] A.F. Duque, V.S. Bessa, M.F. Carvalho, M.K. de Kreuk, M.C.M. van Loosdrecht, P. M.L. Castro, 2-Fluorophenol degradation by aerobic granular sludge in a sequencing batch reactor, Water Res. 45 (2011) 6745–6752.
[13] A.F. Duque, S.A. Hasan, V.S. Bessa, M.F. Carvalho, G. Samin, D.B. Janssen, P.M.L. Castro, Isolation and characterization of a *Rhodococcus* strain able to degrade 2-fluorophenol, Appl. Microbiol. Biotechnol. 95 (2012) 511–520.
[14] S. Ebrahimi, S. Gabus, E. Rohrbach-Brandt, M. Hosseini, P. Rossi, J. Maillard, C. Holliger, Performance and microbial community composition dynamics of aerobic granular sludge from sequencing batch bubble column reactors operated at 20 °C, 30 °C and 35 °C, Appl. Microbiol. Biotechnol. 87 (2010) 1555–1568.
[15] M.A.E. Emanuelsson, O.M. Begoña, J. Sipma, P.M.L. Castro, Treatment of halogenated organic compounds and monitoring of microbial dynamics in up-flow fixed bed reactors under sequentially alternating pollutant scenarios, Biotechnol. Bioeng. 99 (2008) 800–810.
[16] G. Gonzalez-Gil, C. Holliger, Dynamics of microbial community structure of and enhanced biological phosphorus removal by aerobic granules cultivated on propionate or acetate, Appl. Environ. Microbiol. 77 (2011) 8041–8051.
[17] I.M. Head, J.R. Saunders, R.W. Pickup, Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms, Microb. Ecol. 35 (1998) 1–21.
[18] I.S. Henriques, A. Alves, M. Tacão, A. Almeida, Â. Cunha, A. Correia, Seasonal and spatial variability of free-living bacterial community composition along na estuarine gradient (Ria de Aveiro, Portugal), Estuar. Coast Shelf Sci. 68 (2006) 136–148.
[19] M.O. Hill, H.G. Gauch Jr., Detrended correspondence analysis: an improved ordination technique, Vegetation 42 (1980) 47–58.
[20] V. Ivanov, J.-H. Tay, Q.-S. Liu, X.-H. Wang, Z.-W. Wang, B.A.M. Maszenan, S. Yi, W.-Q. Zhuang, Y.-Q. Liu, S. Pan, S.T.-L. Tay, Microstructural optimization of wastewater treatment by aerobic granular sludge, in: S. Bathe, M.K. de Kreuk, B. McSwain, N. Schwarzenbeck (Eds.), Aerobic Granular Sludge, IWA Publishing, London, 2005, pp. 43–52.
[21] H.L. Jiang, J.H. Tay, S.T.L. Tay, Bacterial diversity and function of aerobic granules engineered in a sequencing batch reactor for phenol degradation, Appl. Environ. Microbiol. 70 (2004) 6767–6775.
[22] B. Key, R. Howell, C. Criddle, Fluorinated organics in the biosphere, Environ. Sci. Technol. 31 (1997) 2445–2454.
[23] A.J. Li, S.F. Yang, X.Y. Li, J.D. Gu, Microbial population dynamics during aerobic sludge granulation at different organic loading rates, Water Res. 42 (2008) 3552–3560.
[24] A.J. Li, T. Zhang, X.Y. Li, Fate of aerobic bacterial granules with fungal contamination under different organic loading conditions, Chemosphere 78 (2010) 500–509.
[25] Y. Liu, J.H. Tay, State of the art of biogranulation technology for wastewater treatment, Biotechnol. Adv. 22 (2004) 533–563.
[26] L. Martínková, B. Uhnáková, M. Pátek, J. Nesvera, V. Kren, Biodegradation potential of the genus *Rhodococcus*, Environ. Int. 35 (2009) 162–177.
[27] R.B.R. Mesquita, M.T.S.O.B. Ferreira, R.L.A. Segundo, C.F.C.P. Teixeira, A.A. Bordalo, A.O.S.S. Rangel, Development of a sequential injection system for the determination of nitrite and nitrate in waters with different salinity: application to estuaries in NW Portugal, Anal. Methods 1 (2009) 195–202.
[28] S. Molin, T. Tolker-Nielsen, Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure, Curr. Opin. Biotechnol. 14 (2003) 255–261.
[29] A. Mosquera-Corral, M.K. de Kreuk, J.J. Heijnen, M.C.M. van Loosdrecht, Effects of oxygen concentration on N-removal in an aerobic granular sludge reactor, Water Res. 39 (2005) 2676–2686.
[30] A. Moura, M. Tacão, I. Henriques, J. Dias, P. Ferreira, A. Correia, Characterization of bacterial diversity in two aerated lagoons of a wastewater treatment plant using PCR-DGGE analysis, Microbiol. Res. 164 (2009) 560–569.
[31] C.L. Moyer, F.C. Dobbs, D.M. Karl, Estimation of diversity and community structure through restriction fragment length polymorphism distribution analysis of bacterial 16S rRNA genes from a microbial mat at an active hydrothermal vent system Loihi Seamount, Hawaii, Appl. Environ. Microbiol. 60 (1994) 871–879.
[32] G. Muyzer, E.C. Dewaal, A.G. Uitterlinden, Profiling of complex microbial populations by denaturing gradient gel electrophoresis of polymerase chain reaction-amplified genes coding for 16S rRNA, Appl. Environ. Microbiol. 59 (1993) 695–700.
[33] E.C. Pielou, Ecological Diversity, Wiley, New York, 1975.
[34] R.L.A. Segundo, R.B.R. Mesquita, M.T.S.O.B. Ferreira, C.F.C.P. Teixeira, A.A. Bordalo, A.O.S.S. Rangel, Development of a sequential injection gas diffusion system for the determination of ammonium in transitional and coastal waters, Anal. Methods 3 (2011) 2049–2055.
[35] C.E. Shannon, W. Weaver, The Mathematical Theory of Communication, University of Illinois Press, Urbana, IL, 1963.
[36] S.J. Sorensen, M. Bailey, L.H. Hansen, N. Kroer, S. Wuertz, Studying plasmid horizontal transfer *in situ*: a critical review, Nat. Rev. Microbiol. 3 (2005) 700–710.
[37] S.J. Tavener, J.H. Clark, Can fluorine chemistry be green chemistry? J. Fluorine Chem. 123 (2003) 31–36.
[38] S.T.L. Tay, V. Ivanov, S. Yi, W.Q. Zhuang, J.H. Tay, Presence of anaerobic *Bacteroides* in aerobically grown microbial granules, Microbial Ecol. 44 (2002) 278–285.
[39] J.H. Tay, S. Pan, S.T.L. Tay, V. Ivanov, Y. Liu, The effect of organic loading rate on the aerobic granulation: the development of shear force theory, Water Sci. Technol. 47 (2003) 235–240.
[40] J.H. Tay, S.T.L. Tay, V. Ivanov, S. Pan, H.L. Jiang, Q.S. Liu, Biomass and porosity profiles in microbial granules used for aerobic wastewater treatment, Lett. Appl. Microbiol. 36 (2003) 297–301.
[41] S.T.L. Tay, B.Y.P. Moy, H.L. Jiang, J.H. Tay, Rapid cultivation of stable phenol degrading granules using acetate fed granules as microbial seed, J. Biotechnol. 115 (2005) 387–395.

[42] S.T.L. Tay, B.Y.P. Moy, A.M. Maszenan, J.H. Tay, Comparing activated sludge and aerobic granules as microbial inocula for phenol biodegradation, Appl. Microbiol. Biotechnol. 67 (2005) 708–713.
[43] S.T.L. Tay, W.Q. Zhuang, J.H. Tay, Star-up, microbial community analysis and formation of aerobic granules in a *tert*-butyl alcohol degrading sequencing batch reactor, Environ. Sci. Technol. 39 (2005) 5774–5780.
[44] S. Thierry, H. Macarie, T. Iizuka, W. Geissdörfer, E.A. Assih, M. Spanevello, F. Verhe, P. Thomas, R. Fudou, O. Monroy, M. Labat, A.S. Ouattara, *Pseudoxanthomonas mexicana* sp. nov. and *Pseudoxanthomonas japonensis* sp. nov. isolated from diverse environments and emended descriptions of the genus *Pseudoxanthomonas* Finkmann et al., 2000 and of its type species, Int. J. Syst. Evol. Microbiol. 54 (2004) 2245–2255.
[45] A.F. Torres, P.A.R. Mesquita, F.M. Campos, J.A. Couto, I.V. Tóth, A.O.S.S. Rangel, T. A. Hogg, Development of a flow injection method for monitoring cell membrane damage of wine lactic acid bacteria, Microchim. Acta 159 (2007) 87–93.
[46] D.G. Weissbrodt, T.R. Neu, U. Kuhlicke, Y. Rappaz, C. Holliger, Assessment of bacterial and structural dynamics in aerobic granular biofilms, Front. Microbiol. 4 (2013) 175–192.
[47] D.G. Weissbrodt, N. Shani, C. Holliger, Linking bacterial population dynamics and nutrient removal in the granular sludge biofilm ecosystem engineered for wastewater treatment, FEMS Microbiol. Ecol. 88 (2014) 579–595.
[48] M.-K.H. Winkler, R. Kleerebezem, L.M.M. de Bruin, P.J.T. Verheijen, B. Abbas, J. Habermacher, M.C.M. van Loosdrecht, Microbial diversity differences within aerobic granular sludge and activated sludge flocs, Appl. Microbiol. Biotechnol. 97 (2013) 7447–7458.
[49] S. Wuertz, S. Okabe, M. Hausner, Microbial communities and their interactions in biofilm systems: an overview, Water Sci. Technol. 49 (2004) 327–336.
[50] L.L. Zhang, R.Y. Zhu, J.M. Chen, W.M. Cai, Biodegradation of methyl *tert*-butyl ether as a sole carbon source by aerobic granules cultivated in a sequencing batch reactor, Bioprocess Biosyst. Eng. 31 (2008) 527–534.
[51] L.L. Zhang, J.M. Chen, F. Fang, Biodegradation of methyl *tert*-butyl ether by aerobic granules under a cosubstrate condition, Appl. Microbiol. Biotechnol. 78 (2008) 43–55.
[52] L. Zhu, X. Xu, W. Luo, D. Cao, Y. Yang, Formation and microbial community analysis of chloroanilines-degrading aerobic granules in the sequencing airlift bioreactor, J. Appl. Microbiol. 104 (2008) 152–160.