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Simultaneous nitrification and phosphate removal by bioaugmented aerobic granules treating a fluoroorganic compound

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ABSTRACT

The presence of toxic compounds in wastewater can cause problems for organic matter and nutrient removal. In this study, the long-term effect of a model xenobiotic, 2-fluorophenol (2-FP), on ammonia-oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB) and phosphate accumulating organisms (PAO) in aerobic granular sludge was investigated. Phosphate (P) and ammonium (N) removal efficiencies were high (>93%) and, after bioaugmentation with 2-FP degrading strain FP1, 2-FP was completely degraded. Neither N nor P removal were affected by 50 mg L^{-1} of 2-FP in the feed stream. Changes in the aerobic granule bacterial communities were followed. Numerical analysis of the denaturing gradient gel electrophoresis (DGGE) profiles showed low diversity for the ammonia monooxygenase (*amoA*) gene with an even distribution of species. PAOs, including denitrifying PAO (dPAO), and AOB were present in the 2-FP degrading granules, although dPAO population decreased throughout the 444 days reactor operation. The results demonstrated that the aerobic granules bioaugmented with FP1 strain successfully removed N, P and 2-FP simultaneously.

Key words | 2-fluorophenol (2-FP), aerobic granular sludge, ammonia-oxidizing bacteria (AOB), microbial population dynamics, nitrite oxidizing bacteria (NOB), phosphate accumulating organisms (PAO)

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HIGHLIGHTS

- Inoculation with strain FP1 led to 2-FP removal within the aerobic phase.
- Nitrifiers and PAOs activities were not affected by 2-fluorophenol.
- Bioaugmented granules with FP1 were able to simultaneously remove N, P and 2-FP.
- Granular sludge is promising for the treatment of wastewaters containing toxics.

INTRODUCTION

Aerobic granular sludge is a novel technology for the biological treatment of wastewater. It has several advantages over activated sludge, such as excellent settling properties, high biomass retention and the ability to deal with high organic loading rates and to perform simultaneously diverse biological processes, such as chemical oxygen demand (COD), N and P removal (Winkler *et al.* 2018; Nancharaiah & Sarvajith 2019). Aerobic granulation has been applied for the treatment of a wide variety of wastewaters, including domestic and industrial wastewaters (Amorim *et al.* 2017; Nancharaiah & Sarvajith 2019).

Several industries are dealing with a variety of toxic compounds in their wastewater. Xenobiotics can inhibit the

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biological processes of the plants treating these industrial wastewaters, especially nitrification and phosphate removal. Nitrification can be affected by several environmental factors like pH, temperature, dissolved oxygen concentration, toxic compounds, available substrate, and product inhibition (Antoniou *et al.* 1990). Previous research showed that microbial granules seem to be more resistant to wastewater containing toxic compounds than suspended microbial sludge (Liu *et al.* 2005, 2009). There are a few studies on the influence of phenol on nitrification by microbial granules. Liu *et al.* (2005) has reported that for phenol concentrations up to 10 mg L^{-1}, the nitrifying activity of aerobic granules was recovered after phenol degradation. Furthermore, Liu *et al.* (2009) proposed that the granular structure could beneficially protect microbial cells from phenol toxicity, mainly at a longer exposure time. Generally, the latter studies report that nitrifying bacteria embedded in microbial granules might have a better ability to resist the shock of toxic compounds in wastewaters than suspended nitrifying bacteria (Fang 2000; Liu *et al.* 2005, 2009).

As for nitrification, the biological phosphate removal process is also affected by some environmental and operating factors, namely temperature, dissolved oxygen, carbon sources, pH, among other parameters (Mulkerrins *et al.* 2004). To date, several explanations for the instability of the biological phosphate removal process have been reported, namely: (i) competition with glycogen accumulating organisms (GAO) (López-Vázquez *et al.* 2008), (ii) intrusion of nitrite and/or nitrate into anaerobic phase (Puig *et al.* 2007), and (iii) excessive aeration (Lopez *et al.* 2006). The effect of highly recalcitrant compounds on simultaneous biological phosphate removal and nitrification processes by granular sludge has not been yet reported. Thus, it is of practical interest to investigate the effect of xenobiotic compounds on aerobic granule performance. Fluorinated compounds have significant biological effects as enzyme inhibitors, modifiers of cell–cell communication, and they may disrupt membrane transport and processes for energy generation (Key *et al.* 1997). They are widely used in pharmaceutical and agricultural and other industrial applications, such as polymers and liquid crystals (Clark *et al.* 1996; Natarajan *et al.* 2005). The stability that makes fluorinated organics interesting for commercial use also makes them ubiquitous environmental contaminants due to their recalcitrance, tending to persist and accumulate in the environment (Key *et al.* 1997; Moody & Field 2000; McCulloch 2003). A granular sludge sequencing batch reactor (SBR) bioaugmented with a specialized strain capable of degrading 2-FP as sole source of carbon and energy, named *Rhodococcus* sp. strain FP1, was previously shown to perform COD removal and 2FP degradation when treating a synthetic wastewater, as previously described by Duque *et al.* (2011). Moreover, Ramos *et al.* (2017) have demonstrated simultaneous partial nitrification and 2FP biodegradation using aerobic granules bioaugmented with *Rhodococcus* sp. Strain FP1. In this study, the simultaneous nitrification, phosphate and 2-FP removal processes by bioaugmented aerobic granular sludge, as well as the microbial community involved in such processes, was investigated. The dynamics of the nitrifying and phosphate and glycogen accumulating microbial community present on aerobic granules was investigated using fluorescence *in situ* hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE) analysis of the ammonia monooxygenase (*amoA*) gene.

MATERIALS AND METHODS

Reactor set-up and operation

A laboratory-scale SBR with a working volume of 2.5 L, 110 cm height and an internal diameter of 6.5 cm was established and operated (Duque *et al.* 2011). Air was introduced at the bottom of the reactor (4 L min^{-1}). The dissolved oxygen (DO) was measured as percentage of the oxygen saturation concentration (100% = 9.1 mg L^{-1}). The experiment was conducted with no oxygen control (DO 100%). The pH was maintained at 7.0 ± 0.8 by dosing 1 M NaOH or 1 M HCl. Biological phosphate removing granular sludge (500 ml wet granules) collected from a pilot plant treating sewage in the Netherlands (Epe wastewater treatment plant) was used to inoculate the reactor (Figure 1).

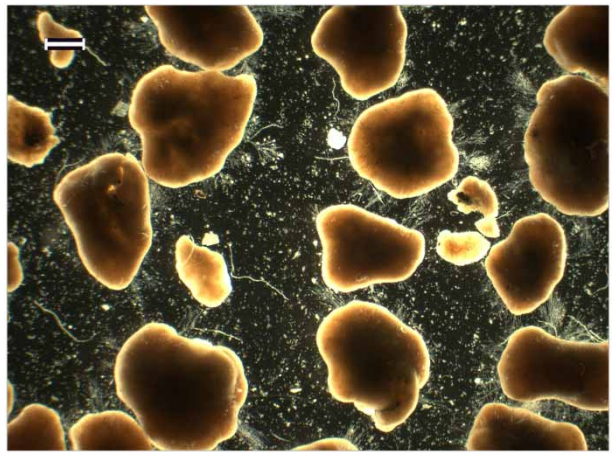

Figure 1 | Aerobic granular sludge inoculum used for the start-up of the SBR. The bar size is 1 mm.

The SBR operation was divided in seven different phases as previously described by Duque *et al.* (2011) and here summarized in Table 1.

Briefly, the reactor was operated in successive cycles of 3 h (phases I and II) or 4 h (phases VI and VII) or 8 h (phase V) or 12 h (phases III and IV). Each cycle consisted of 60 min influent feeding (which was introduced in the bottom of the reactor), 112 (phases I and II) or 172 (phases VI and VII) or 412 (phase V) or 652 (phases III and IV) min aeration, 3 min settling and 5 min effluent withdrawal. The volume exchange ratio per cycle was ca. 40% (2-FP concentration inside the bioreactor was diluted to a concentration of 19 or 38 mg L^{-1}). Acetate was used as the growth substrate at a volumetric loading rate of 3.9 g L^{-1} d^{-1}. 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. Excess sludge (including accumulated cell internally stored phosphate) was removed at the same time as effluent discharge occurred. The solid retention time (SRT) was calculated based on the suspended solids concentration in the discharged effluent and the sludge content of the reactor. The calculated SRT in the reactor was 30 days on average. Between phases II and III, the reactor was bioaugmented with a specialized bacterial strain able to degrade 2-FP previously isolated in our laboratories (Duque *et al.* 2012), a *Rhodococcus* sp. strain FP1 (LMG 26251; DSM 45581). As previously described by Duque *et al.* (2011), the reactor was inoculated with 1.25 L of suspended FP1 pure culture with an optical density at 600 nm of 0.8. As bioaugmentation with the specialized strain proved successful in terms of 2-FP removal, simultaneous removal of N and P was subsequently investigated (Phases VI and VII).

SBR synthetic industrial wastewater

As previously described by Duque *et al.* (2011), the SBR synthetic industrial wastewater consisted of two different media with the following compositions: (A) 63 mM sodium acetate, 3.6 mM magnesium sulphate, and 4.7 mM potassium chloride; and (B) 35.4 mM ammonium chloride, 4.2 mM disodium hydrogen phosphate, 2.1 mM potassium dihydrogen phosphate, and 10 mL L^{-1} trace element solution according to Vishniac & Santer (1957). During fluoroorganic shock loadings, 2-FP was added to medium A (2.38 mM in phases II, III and VII and 4.76 mM in phases IV to VI). In each cycle, 89 mL of each medium were dosed together with 772 mL of tap water to the SBR.

Analytical methods

Ammonium, nitrate and nitrite concentrations were assessed by sequential injection analysis (SIA) as described by Segundo *et al.* (2011) and Mesquita *et al.* (2009), respectively. Phosphate concentration of filtered samples was determined by flow injection analysis (FIA) as described by Torres *et al.* (2007).

Total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to Standard Method 2540 (APHA 1998).

The DO concentration in the reactor was measured online with a DO-sensor (InPro 6820, Mettler-Toledo) as percentage of the oxygen saturation concentration

Table 1 | Operation conditions applied to the SBR

Parameter	I	II		III	IV	V	VI	VII
Days of operation	99	109		12	6	36	133	43
Cycle length (h)	3	3		12	12	8	4	4
HRT[a]	7.9	7.9		31.6	31.6	21.1	10.5	10.5
COD[b] (mg L^{-1})	330	374		374	418	418	418	374
2-FP OLR[c] (kg m^{-3} d^{-1})	–	0.075[f]	FP1 addition[g]	0.019	0.037	0.056	0.112	0.056
NH_4^+-N VLR[d] (g L^{-1} d^{-1})	0.37	0.37		0.09	0.09	0.14	0.28	0.28
PO_4^{3-}-P VLR[e] (g L^{-1} d^{-1})	0.15	0.15		0.04	0.04	0.05	0.11	0.11

[a]HRT – hydraulic residence time.
[b]COD – Theoretical influent chemical oxygen demand values per cycle.
[c]2-FP OLR – 2-fluorophenol organic loading rate.
[d]NH_4^+-N VLR – ammonia-nitrogen volumetric loading rate.
[e]PO_4^{3-}-P VLR – phosphate-phosphorous volumetric loading rate.
[f]Organic shocks loadings with 2-FP applied 1 cycle/2 days.
[g]Bioaugmentation with *Rhodococcus* sp. strain FP1.

(100% = 9.1 mg L^{-1}). The pH was monitored online using a pH-electrode (InPro 3030, Mettler-Toledo).

2-FP was analyzed by high performance liquid chromatography (HPLC), on a System Gold 126 (Beckman Coulter, Fullerton, USA) with a LiChroCART 25-4 LiChrospher 100 RP-18 reversed-phase column, 5 μm particle size (Merck, Darmstadt, Germany) as described by Duque *et al.* (2011).

Calculations

The theoretical NH_4^+-N and PO_4^{3-}-P concentrations related to the beginning of the SBR cycle (time 0) were calculated by dividing the amount of phosphate or nitrogen present in the influent media (mg PO_4^{3-}-P or mg NH_4^+-N) by the reactor working volume (2.5 L).

Aerobic granular sludge microbial community analysis

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. The resulting bacterial suspensions were used for DNA extraction for DGGE analysis. The samples for DGGE analysis were selected considering whole reactor operation, representing each operating phase (Table 2).

DNA extraction

The genomic DNA extraction of crushed aerobic granules was performed 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 used for denaturing gradient gel electrophoresis (DGGE).

Table 2 | Sample selection criteria for DGGE analysis

Sampling day (d)	Operating phase
d100	II – Before the first feeding with 0.22 mM 2-FP (end phase I)
d114	II – After intermittent 0.22 mM 2-FP organic shocks
d210	II – Before bioaugmentation with FP1 (end phase II)
d217	III – End of feeding with 0.22 mM 2-FP (end phase III)
d227	IV – End of feeding with 0.44 mM 2-FP (end phase IV)
d246	V – Feeding with 0.44 mM 2-FP, 8 h cycles
d267	VI – End of 8 h cycles (end phase V)
d301	VI – Feeding with 0.44 mM 2-FP, 4 h cycles
d408	VII – Feeding with 0.22 mM 2-FP, 4 h cycles

Polymerase chain reaction (PCR) of the *amoA* gene fragment

The gene encoding the active site of ammonia monooxygenase (*amoA*) fragments was amplified using the primer set *amoA*-1F-GC and *amoA*-2R (Rotthauwe *et al.* 1997). Amplification was performed in 50 μl reaction mixtures using a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Richmond, CA, USA). Each PCR reaction mixture contained 1× PCR buffer (Promega, USA), 2 mM $MgCl_2$, 0.3 mg L^{-1} bovine serum albumin (BSA), 200 μM of each nucleotide, 50 pmol of each primer, 2 U *Taq* polymerase (Promega, USA), and 1–20 ng of purified DNA. The PCR conditions were as described previously (Hornek *et al.* 2006). Double PCR was performed using as the template 2 μl of the DNA amplicon obtained after the first amplification round and using the same primers and conditions applied in the first PCR amplification.

DGGE

PCR-amplified *amoA* 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 6% (w/v) polyacrylamide gels (37.5:1, acrylamide:bisacrylamide) in 0.5× Tris-acetate-EDTA (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 20% to 80% (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).

DGGE profiles were analyzed using GelCompar® II software (VERSION 4.6; Applied Maths, Sin-Martens-Latem, Belgium). Dendrograms were generated using the unweighted pair group method with arithmetic mean (UPGMA). Every gel contained three lanes with a standard of four bands for internal and external normalization and as an indication of the quality of the analysis. Numerical analysis of DGGE

profiles was performed using two indexes, diversity (H) (Shannon & Weaver 1963), and equitability (E) (Pielou 1975).

Fluorescence *in situ* hybridization (FISH)

In order to identify the distribution of the microbial population, FISH was performed on crushed granules. FISH was performed as described in Amann (1995). The EUBMIX probe (mixed probe of EUB338, EUB338-II and EUB338-III) was applied to target almost all bacteria (Daims *et al.* 1999). The PAOMIX probe (mixed probe of PAO462, PAO651 and PAO846) was applied to target *Accumulibacter* (phosphate accumulating organisms (PAO)) (Crocetti *et al.* 2000). PAO probes ACC-444-I and ACC-444-II were used to target denitrifying *Accumulibacter* clade IA (denitrifying PAO (dPAO)) and clade IIa (Flowers *et al.* 2009) and the GAOMIX probe (mixed probe of GAOQ431 and GAOQ989) was applied to target *Competibacter* GAO (Crocetti *et al.* 2002). Ammonia-oxidizers belonging to β-Proteobacteria were detected with the use of NSO190 and NEU653 (Wagner *et al.* 1995; Mobarry *et al.* 1996). FISH was performed using hybridization and washing buffers as described by Manz *et al.* (1992). The hybridized samples were analyzed using a Zeiss Axioplan2 Imaging Epifluorescence microscope. Images were taken with a Zeiss Axiocam MRm Black and White CCD camera. Zeiss Axiovision software was used to acquire, to color and compose different multichannel images.

RESULTS AND DISCUSSION

Long-term effect of 2-FP on nitrification and phosphate removal

The SBR overall performance after 5 months of 2-FP feeding at a concentration of 50 mg L^{-1} (phases VI and VII) is shown in Table 3 and the average typical patterns of phosphate and nitrogen concentrations during a cycle are presented in Figure 2. After start-up (phase I), the phosphate and ammonium removal was on average 93 and 99%, respectively, and the nitrite and nitrate concentrations found in the effluent were on average 0.03 and 2.4 mgN L^{-1}. respectively (Table 3). Acetate was fully consumed and stored during the anaerobic period, and therefore was not present in the bulk liquid during aeration. Duque *et al.* (2011) described that there was no 2-FP removal observed in phases I and II, indicating that the initial microbial population before bioaugmentation was not capable of 2-FP degradation, neither under anaerobic nor aerobic conditions. Nitrite did not accumulate, and all the ammonium and phosphate were removed, therefore it can be assumed that NOB, AOB and PAO were not inhibited by the presence of 19 mg L^{-1} of 2-FP. Liu *et al.* (2009), who have studied the toxicity effect of phenol on aerobic granules, have shown that aerobic granules are more resistant to toxic effects of phenol than flocculated sludge, supposedly mainly because of the compact and shielding structure of the granules. In the layered structure of granules, PAO are present more inside the granule and the nitrifiers grow in the outer layer (De Kreuk *et al.* 2005). Therefore, PAO are potentially not exposed to the same high concentration as present in the wastewater, being less susceptible to toxic compounds (Fang 2000; Liu & Tay 2004).

Due to batch feeding and since 2-FP was not degraded during the feeding phase, within each cycle the aeration phase starts with a high 2-FP concentration. Therefore, AOB and NOB adaptation to 2-FP can be due either to the presence of the 2-FP degrading bacteria that are removing the toxicity via degradation of the compound or to a

Table 3 | Summary of SBR treatment performance after being fed with 2-FP for 5 months

Parameter	Beginning of cycle[a]	End of anaerobic feeding[b]	Effluent[b]
PO_4^{3-}-P (mg L^{-1})	6.94	32.8 ± 1.9	2.49 ± 0.65
NH_4^+-N (mg L^{-1})	17.65	8.15 ± 1.94	0.07 ± 0.04
NO_2^--N (mg L^{-1})	–	0.02 ± 0.01	0.027 ± 0.009
NO_3^--N (mg L^{-1})	–	2.11 ± 0.54	2.38 ± 0.49

[a]Theoretical values.
[b]Values are means ± standard error of the mean (SEM).

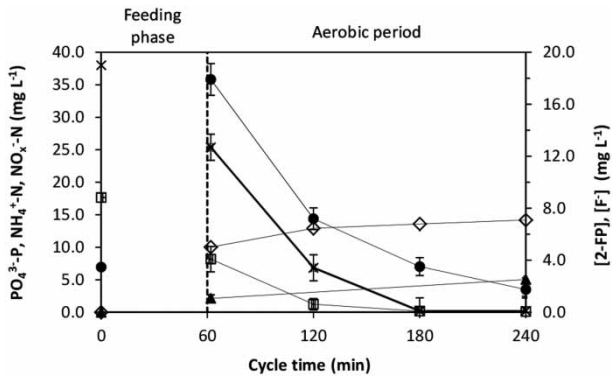

Figure 2 | Concentration profiles of phosphate (•), ammonium (□), nitrite plus nitrate (NO_x^-, ▲), 2-FP (×) and F^- (◇) in the granular SBR during a cycle. Values refer to 1 month operation after being fed with 2-FP for 5 months and are means ± standard error of the mean (SEM). The concentrations related to time 0 are theoretical values.

protection provided by the granular structure (Tay *et al.* 2005a, 2005b; Carucci *et al.* 2008, 2009). Liu *et al.* (2009), who have studied the toxicity effect of phenol on aerobic granules, have shown that aerobic granules are more resistant to the toxic effects of phenol than flocculated sludge, supposedly mainly because of the compact and shielding structure of the granules. Ammonium started to be consumed after the 60 min anaerobic feeding phase of the SBR cycle (during the aeration phase) (Figure 2). 2-FP accumulated in this period since it was not degraded anaerobically. This indicates that the 2-FP degrading bacteria were not playing an important role in removing the toxicity during that period. Therefore, these results demonstrate that most likely the nitrifying bacteria in the granular matrix can adapt to the presence of toxic compounds, shielding the bacteria inside the granules against these toxic effects, corroborating the results obtained by Jiang *et al.* (2004). Liu *et al.* (2005), who have studied the effect of phenol on nitrifying granular sludge, reported that nitrifying bacteria in microbial granules might have a better capacity to resist shocks from toxic compounds in wastewater than suspended nitrifying bacteria. In fact, it is known that microorganisms can regulate extracellular polymers (EPS) synthesis and modify their properties as a response towards the effects of antimicrobial agents (Allison *et al.* 2000; Amorim *et al.* 2017). Wei *et al.* (2015) observed that the presence of 4-chlorophenol induced EPS production by the aerobic granules, as the polysaccharides and proteins levels increased. The presence of some functional groups in EPS, such as carboxyl, phosphoric, sulfhydryl and hydroxyl groups, also seems to be an important defense strategy as it can promote the adsorption of toxics to the EPS matrix (Amorim *et al.* 2017). Moreover, the layered structure of the granules can act as a diffusion limitation barrier by the development of a concentration gradient within the granules, preventing toxicants to reach the inner biomass (Amorim *et al.* 2017).

Microbial community in the aerobic granules

DGGE analysis of *amoA* gene

A full overview of the organisms present in the aerobic granules and the effect of 2-FP on the full community was described by Duque *et al.* (2015). The later study demonstrated that, generally, the 16S bacterial communities were not affected by the presence of 2-FP. Moreover, a wide bacterial diversity was observed, hindering the identification of low abundant bacteria, such as AOB. Therefore, DGGE analysis was performed to assess the AOB population present in the aerobic granules. The gene encoding the active site of ammonia monooxygenase (*amoA*), one of the enzymes responsible for the conversion of ammonia into nitrite, was selected for DGGE as it has been defined as a molecular marker for AOB diversity (Hornek *et al.* 2006). DGGE was chosen over 16S rRNA-Next Generation Sequencing in order to easily monitor the possible disappearance of the low abundant *amoA* communities due to 2-FP dosage. In total, 24 band positions were detected in the *amoA* gel and the number of bands per lane ranged between 2 and 4 (Figure 3).

The DGGE patterns obtained were very similar between all samples, although two clusters could be identified (Figure 3). One of the clusters included samples collected at day 100 and day 267 and was clearly separated from the cluster that included samples collected at day 301 and day 408. Therefore, a major shift in bacterial assemblage was identified between days 267 and 301, when the hydraulic residence time (HRT) decreased from 21.1 h to 10.5 h concomitant with an increased loading rate. Within the cluster that included samples collected between day 100 and day 267, a less significant shift was identified between the beginning of the feeding with 2-FP (day 100) and continuous feeding of 2-FP (day 114) (<80% similarity), indicating that the presence of 2-FP had a slight influence on AOB population present in the aerobic granules. Previous studies on bioaugmentation as a tool to protect activated sludge nitrifying bacterial community against 3-chloroaniline shocks, demonstrated that toxic shocks caused changes in the structure of the AOB community (Boon *et al.* 2003). However, in the present study, nitrification was not affected by the presence of 2-FP, suggesting that 2-FP affected the AOB population diversity present in the aerobic granules, but not the reactor performance. Corroborating the present study, Emanuelsson *et al.* (2008) showed that an operationally stable reactor does not imply a stable microbial community and vice versa. Shannon's diversity index (H), used to calculate diversity of bacterial communities (Shannon & Weaver 1963), was on average 0.49 ± 0.04 and the equitability index (E), which can range from 0, indicating pronounced dominance, to 1, indicating equal abundance of all species (Pielou 1975), was on average 0.95 ± 0.01. Thus, the calculation of H and E indexes based on *amoA* gene DGGE profiles showed that the AOB community presented low diversity, low species richness and complete evenness.

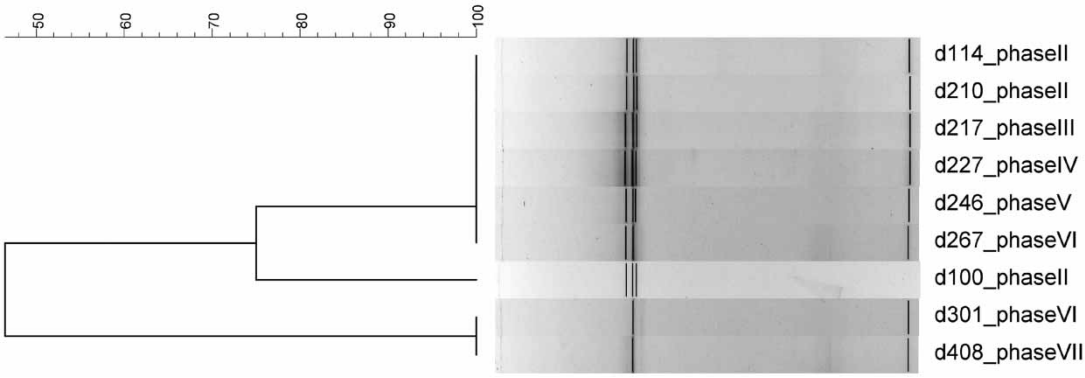

Figure 3 | Cluster analysis of the AOB community present in the aerobic granules based upon DGGE profiles of *amoA* gene amplification using the total genomic DNA extracted from the aerobic granules. Similarities were calculated using the Bray–Curtis measure. The dendrogram presents the similarity, in percentage, between samples. 'd____' label refers to the day of the sample.

Analysis of nitrifying bacteria and phosphate and glycogen accumulating organisms in aerobic granules based on FISH

The FISH analysis for the nitrifiers (AOB), PAO (including dPAO) and GAO populations was carried out to assess the population dynamics. Figure 4 presents FISH images of crushed granules from two sampling days, day 210 and day 301, stained with specific probes for PAO, dPAO, nitrifiers and eubacteria.

Nitrifiers, particularly AOB, were present in low numbers in all SBR samples, corroborating *amoA* gene DGGE

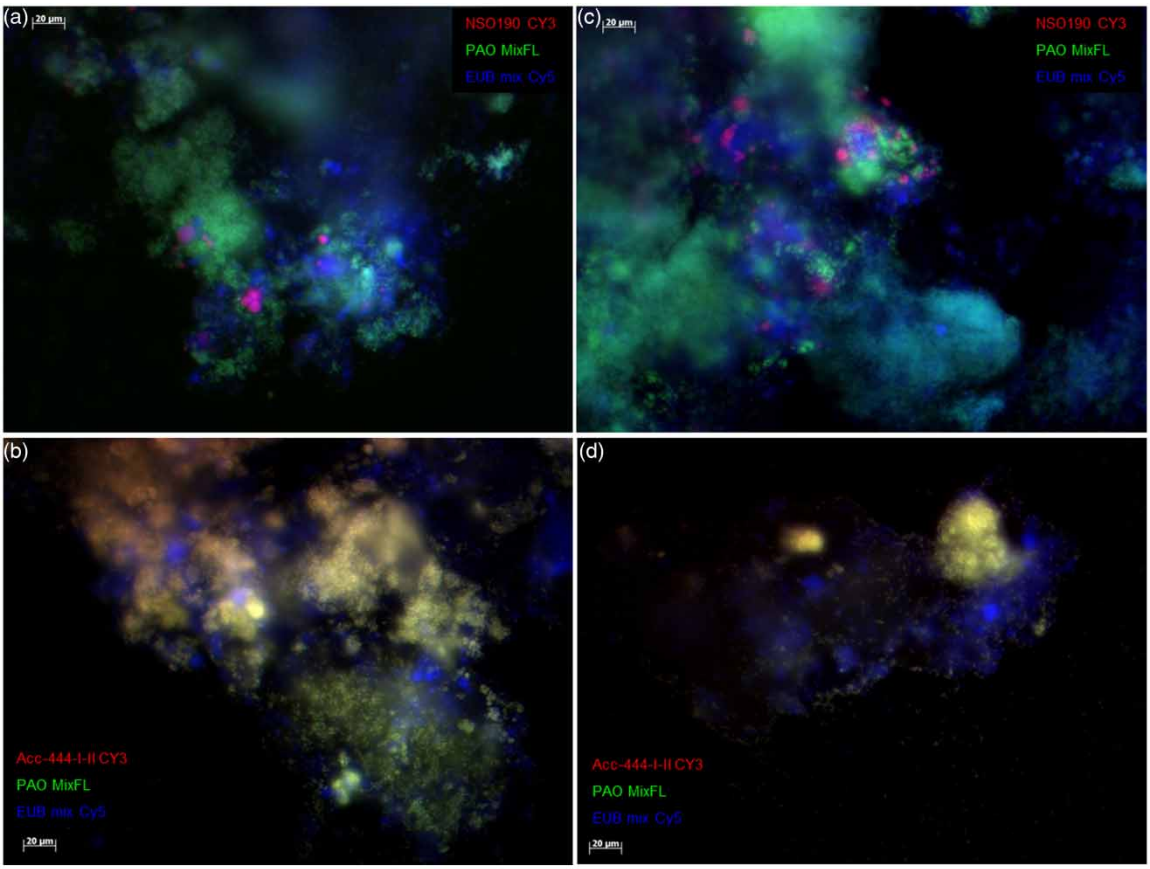

Figure 4 | FISH analysis of crushed granules from day 210 (a and b) and from day 301 (c and d). The bar size is 20 µm. (Blue: Eubacteria; Green: Phosphate accumulating organisms; Red: Ammonia-oxidizing bacteria belonging to β-Proteobacteria group (a and c) and denitrifying *Accumulibacter* (b and d). The full colour version of this figure is available in the online version of this paper, at http://dx.doi.org/10.2166/wst.2021.142.

results. The low numbers are due to the low growth yield of this autotrophic organism. Heterotrophic PAO were present in high numbers (Figure 4(a) and 4(c)). A mixture of PAOI and PAOII showed full overlap with the PAO mix, suggesting that all PAO were PAOI and/or PAOII (Figure 4(b) and 4(d)). According to De Kreuk *et al.* (2005), in the layered structure of granules, PAO are present more inside the granule and the nitrifiers grow in the outer layer. Therefore, PAO are potentially not exposed to the same high concentration as present in the wastewater, being less susceptible to toxic compounds (Fang 2000; Liu & Tay 2004). On day 210, PAOI were still present and towards the end of the experiment, there was not much of both PAOI and PAOII remaining. This result is in agreement with the observed nitrate accumulation in the effluent towards the end of 2-FP continuous feeding, suggesting that denitrification was most probably inhibited by the long period of continuous 2-FP feeding and/or by high DO concentration (ca. 100%) during the aeration phase. In fact, the granules size decreased along the experiment (Duque *et al.* 2011), which could have resulted in a change in oxygen penetration depth by diffusion. Hence, operational conditions, such as DO concentration, could be optimized to achieve simultaneous removal of N, P, COD and toxic compounds, such as 2-FP.

GAO were almost absent in all analyzed samples, which was expected, as phosphate was being completely removed from the wastewater. The applied operational conditions were favourable for enhanced biological phosphorus removal (EBPR), providing a selective advantage to PAO over their competitors, GAO, as reported by Oehmen *et al.* (2004) and Puig *et al.* (2007).

CONCLUSIONS

The microbial population of aerobic granules involved in nitrification and phosphate removal are capable of adapting to the presence of pollutants. In this study there was no indication of direct interaction between the different metabolic groups, 2-FP degrading bacteria and nitrification and phosphate conversion bacteria. The AOB population was stable, presenting low diversity, low species richness and complete evenness. PAO, including dPAO, were dominant in the 2-FP degrading aerobic granules, but numbers decreased, suggesting that operational conditions could be optimized. The bioaugmented granules with capacity to remove 2-FP were also able to simultaneously remove N and P. Granular sludge is promising for N and P removal from wastewaters containing toxic compounds.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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