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Treatment of industrial effluent with high ammonium concentration using new autotrophic nitrification/denitrification biological techniques

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El doctorando Alejandro González Martínez y los directores de la tesis Francisco Osorio Robles y Ernesto Hontoria García Garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

Granada , abril 2014

Doctorando

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Hoy, sí, hoy, que después de tanto tiempo y esfuerzo empiezo a escribir mi tesis doctoral, siempre mirando hacia delante, siempre esperando ese ansiado reactivo que nunca llega, ese dato que falta para completar un trabajo, esa respuesta de una revista que se demora, esa dichosa autorización que se hace desear, y así sucesivamente hasta hoy. ¿Qué es la ciencia sino mirar hacia delante?. Sin embargo, ahora ha llegado el momento de parar durante un tiempo y mirar hacia atrás. Y creedme que esto es lo que más me cuesta, parar. Estoy seguro que todos los que han pasado y los que lo pasarán cuando lean esto me entenderán. Es el momento de mirar lo que he sido estos cuatro años, y gracias a este tiempo de reflexión sé que hay muchas que anclar para poder volver a mirar hacia delante. Y esto no podría conseguirlo sin agradecerle a todas aquellas personas que me han permitido convertirme en lo que soy hoy, porque en la vida hay que valorar a aquellas personas que te dedican su tiempo, porque te están dando algo que nunca recuperaran.

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"Eres maestro de lo que has vivido,

artesano de lo que estás viviendo

y aprendiz de lo que vivirás."

Richard Bach

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Summary

This doctoral thesis is made up of several published studies based on the effect of different operational conditions and the structure of microbial communities through submerged-bed partial nitritation technology constructed at bench scale. Different hydraulic retention times (HRTs) were used to achieve the perfect ammonium-nitrite mixture, converting 50% of the ammonium to nitrite. Moreover, to find the perfect HRT, several bench-scale submerged-bed partial nitritation bioreactors were built to determine several physico-chemical parameters, such as ammonium, nitrite, and nitrate concentrations, over time.

Molecular biology techniques were used to compare the microbial communities growing in the different bioreactors under different HRTs. Scanning electron microscopy was used to determine the most accurate position and growth rates of the biofim in the carrier (Bioflow 9). On the other hand, molecular biology techniques were used to study the microbial population (16S rRNA genes) and specific bacterial groups involved in the nitrification process, such as ammonium-oxidising bacteria (CTO) and nitrite-oxidising bacteria (nxrA), using a cultivation-independent approach based on PCR-TGGE fingerprinting.

Further, once the results of the optimum HRT were obtained, four bench-scale submerged-bed partial nitritation bioreactors were constructed to evaluate the influence of different concentrations of an emerging contaminant (ciprofloxacin) on nitrogen removal performance and microbial population of submerged-bed partial nitritation bioreactors.

Finally, to establish an objective correlation between physico-chemical parameters and microbial communities and reveal the relationships between the structure of nitrifying microbial communities and a set of variables (ammonium, nitrite, time, antibiotic concentration, and HRT) related to the operating parameters, multivariate analysis by CANOCO and 3D surface polynomial fitting redundancy analysis (RDA) was performed.

Introduction

Partial nitritation process

Today, one of the most important ecological problems in the world is the proliferation of wastewater. The presence of nitrogen in urban wastewater in the form of urine, synthetic nitrogen fertilisers, and industrial wastewater is evidently a significant environmental risk that must be eliminated before discharge of urban wastewater into natural waters. Traditionally, nitrogen has been removed from wastewater through complete nitrification-denitrification processes (Table 1). New regulations approved during this time, such as the European Union (EU) Water Frame Directive 91/271/EEC, impose more restrictive standards on nitrogen contamination in the effluent of wastewater treatment plants(WWTPs). To achieve these standards in a cost-effective way, autotrophic nitrogen removal technologies such as partial nitritation/anammox, DEMON, ANITAMOX, OLAND, and CANON technologies have been developed (Mosquera-Corral et al., 2005; Figure 1). Among them, the partial nitritation/anammox system has been developed as a two-step autotrophic nitrogen removal process. The first step involves the oxidation of ammonium to nitrite under aerobic conditions in such a way that roughly 50% of the ammonium is oxidised to nitrite. In the second step, autotrophic anammox bacteria convert ammonium and nitrite directly into dinitrogen (Jetten et al., 2001). The first step is achieved by partial nitritation technology.

Figure 1. Full-scale partial nitritation (left) and anammox (right) bioreactors in Dokhaven wastewater treatment plant.

Partial nitritation systems have several advantages over conventional nitrification-denitrification technologies, such as 25% savings in aeration, 30% reduction of biomass generation, with biomass yield of about 0.15 g biomass (g NH_4^+-N)$^{-1}$ (Gut et al., 2007), and 20% less CO_2 emission (Sri Shalini & Joseph, 2012). Partial nitritation bioreactors have been reported to successfully treat nitrogen pollution of several wastewater types, such as food and agriculture industry wastewater (Strous et al., 1997; Van Dongen et al., 2001; Fux et al., 2002), anaerobic sludge digesters (Vazquez-Padin et al., 2009), and slaughterhouse wastewater or piggery wastewater (Hwang et al., 2005; Reginatto et al., 2005; Waki et al., 2007) at pilot-plant scale.

Table 1. Comparison of economic aspects of conventional nitrification/denitrification and partial nitritation/anammox processes

	Conventional nitrification/ denitrificación	Partial nitritation/ anammox	
Energy requeriment	**3–5**	**1–2**	**kWh/kg N**
Methanol	**2.5–3.5**	**0**	**kg/kg N**
Sludge production	**0.6–1.0**	**0.1**	**kg VSS/kg N**
CO_2 production	**>4.1**	**0.69**	**kg/kg N**
Total Cost	**3–5**	**1–2**	**€/kg N**

With a partial nitritation reactor, the main performance is aerobic oxidation of ammonium to nitrite and, therefore, nitrite accumulation in the system. Although nitrite has been thought not to accumulate in ecosystems, some reports show that it can accumulate in natural and engineered environments, such as soils, sediments, and

wastewater treatment plants (Paredes et al., 2007). This is achieved by metabolism of ammonium-oxidising bacteria (AOB). Conventional nitrication/denitrification and partial nitritation/anammox are achieved by different stoichiometry formulae (Table 2).

AOB use ammonium mono-oxidase (AMO) to oxidise ammonium to hydroxylamine (NH_2OH), using oxygen as an electron acceptor. Following this reaction, hydroxylamine is oxidised to nitrite with mediation of hydroxylamine oxidoreductase (HAO), with hydrazine as an intermediate (Peng & Zhu, 2006; Sri Shalini & Joseph, 2012).

AOB are autotrophic microorganisms, so they utilise inorganic carbon as a carbon source. If insufficient carbon is found in the partial nitritation reactor influent, bicarbonate can be added to obtain the required inorganic carbon concentration, thus reaching oxidation of the desired ammonium fraction (Ganigué et al., 2012). AOB communities belong to the β-Proteobacteria class, with species like *Nitrosomonas* spp., *Nitrosospira* spp., *Nitrosolobus* spp., and *Nitrosovibrio* spp., among others. It has also been found that *Nitrosomonas* and *Nitrosospira* are the most popular genera among partial nitritation reactors, with *Nitrosospira* dominating under high-ammonium conditions (Okabe, 2011).

Two different types of AOB have been differentiated so far: fast-growing AOB and slow-growing AOB. The difference between these two groups resides in their affinity for ammonium, which is higher in slow-growing AOB. Slow- growing AOB, k-strategists, dominate in environments with ammonium limitation. In partial nitritation reactors, the ammonium concentration is high. Therefore, fast- growing AOB, r-strategists, dominate these systems (Zhang et al., 2004). Even though it is known that species of AOB such as *Nitrosomonas* can carry out denitrifying metabolism that reduces nitrite to nitric oxide, nitrous oxide, and dinitrogen (Bagchi et al., 2012), it is thought that AOB only perform ammonium oxidation in partial nitritation reactors.

Table 2. Stoichiometry of different nitrification-denitrification processes in the nitrogen cycle

Technology	Stoichiometry	Process	Bacteria
Conventional Nitrification/ Denitrification	$NH4+ + 1.5O_2 + 2HCO_3^- \rightarrow NO_2^- + 2CO_2 + 3H_2O$	Nitritation	AOB
	$NO_2^- + 0.5O_2 \rightarrow NO_3^-$	Nitratation	NOB
	$5C + 2H_2O + 4NO_3^- \rightarrow 2N_2 + 4HCO_3^- + CO_2$	Denitrification	DB
Partial Nitritation/ Anammox	$2NH_4^+ + 1.5O_2 + 2HCO_3^- \rightarrow NH_4^+ + NO_2^- + 2CO_2 + 3H_2O$	Partial nitritation	AOB
	$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03\ H_2O$	Anammox process	Anammox

Nevertheless, AOB have to compete with other microbial communities inside a partial nitritation reactor. The main competitors of AOB are nitrite-oxidising bacteria (NOB), which utilise oxygen for the oxidation of nitrite to nitrate. As NOB metabolism utilises nitrite as the metabolic substrate under aerobic conditions, partial nitritation systems represent viable environments for NOB to grow. The NOB species most commonly isolated from activated sludge systems are *Rubrivivax*, *Rhodobacter*, and *Pseudomonas* (Martienssen & Schöps, 1999; Etchebehere et al., 2001). NOB population growth is the major problem related to partial nitritation operational performance. It is thought that the key for the achievement of desired performance of partial nitrification systems is related to the understanding of AOB and NOB community structure and the effect of operational conditions on AOB and NOB community dynamics (Liang & Liu, 2007). If NOB communities are uncontrolled, nitrate will appear due to complete nitritation of ammonium when ammonium loading declines below 0.5 kg N m^{-3} day^{-1} (Okabe, 2011).

Also, when partial nitritation systems are placed before an anammox bioreactor, elimination of nitrite from the system will make anammox bacteria not find enough of it to achieve proper nitrogen removal (Sri Shalini & Joseph, 2012). Therefore, controlling the NOB population is needed in order to achieve the desired nitrogen removal (Okabe,

2011). The different characteristics of AOB and NOB have been studied in order to develop strategies for NOB control in partial nitritation systems. These are based on temperature, dissolved oxygen, hydraulic retention time (HRT) and solids retention time (SRT), and free ammonia (FA) and free nitrous acid (FNA) concentrations, among others (Van Dongen et al., 2001; Liang & Lu, 2007; Ahn, 2006; Posmanik et al., 2014). When 50% of the ammonium is oxidised under steady-state conditions, partial nitritation reactors have a relative abundance of 64% AOB and less than 5% NOB (Liang & Lu, 2007). The most common AOB can be seen in Table 3.

Table 3. The main aerobic ammonium oxidation (AOB) bacteria reported in the nitrifying process in wastewater treatment plants

Genus	Phylogenetic group	DNA (mol %GC)	Characteristics	Reference
Nitrosomonas	**Beta**	**45–53**	**Gram-negative short to long rods, motile (polar flagella) or nonmotile, peripheral membrane systems**	**Schmidt et al. (1997)**
Nitrosococcus	**Gamma**	**49–50**	**Large cocci, motile, vesicular or peripheral membranes**	**Ward et al. (2002)**
Nitrosospira	**Beta**	**54**	**Spirals, motile (peritrichous flagella), no obvious membrane system**	**Maixner et al. (2006)**
Nitrosovibrio	**Beta**	**59–65**	**Slender, curved rods, motile, by means of a polar-to-subpolar flagellum**	**Ida et al. (2004)**
Nitrosolobus	**Beta**	**54**	**Pleomorphic, lobular, compartmented cells, motile (peritrichous flagella)**	**Watson et al. (1971)**

Effect of influent characteristics on the partial nitritation process

Partial nitritation reactors have been developed for the treatment of wastewater with low organic matter content and high concentrations of ammonium, such as landfill leachate (Table 4). The effects of different substances in the wastewater such as ammonium and organic matter loading on these systems have been extensively investigated.

Table 4. Standard composition of a landfill leachate

Parameters	Levels in landfill leachate (kg/m^3)	Units
SS	0.056	(kg/m^3)
BOD	0.023	(kg/m^3)
COD	0.081	(kg/m^3)
Total Nitrogen	1.1-2.1	(kg/m^3)
Total P	0.027	(kg/m^3)
HCO_3^-	4.1	(kg/m^3)

The ammonia loading rate (ALR) has been shown to affect chemical composition of the effluent generated in a partial nitrification reactor. At an ALR of 3.1 mM/h to 5.4 mM/h, the composition of the influent or effluent is stable and dominated by nitrite and ammonia, at a ratio of about 1.2:1, with a small fraction of nitrogen present as nitrate. A similar trend is observed in a biofilm reactor configuration at the range of 6.4 to 12.1 mM/H. This effluent composition is suitable for further anammox treatment for nitrogen elimination. Lower ALRs lead to an excess of nitrate in the effluent for this purpose, and higher ALRs achieve an excess of ammonia in the effluent (Daalkhaijav & Nemati, 2014). The ALR has been proposed as a practical way to control the performance of

partial nitritation reactors and has been claimed to be more practical than other control strategies, such as oxigen demand (DO) control (Daalkhaijav & Nemati, 2015).

Ammonium concentration has been shown to affect the performance of partial nitritation reactors due to production of FA and FNA. Both AOB and NOB can be inhibited by their metabolic substrates and/or by-products. It has been found that FA and FNA can inhibit AOB and NOB. In any case, NOB is much more sensitive than AOB. FNA has been shown to inhibit NOB at concentrations from 0.26 mg HNO_2-N L^{-1} which is lower than the inhibitory concentration for AOB (0.49 mg HNO_2-N L^{-1}; Ganigué et al., 2012). Inhibition by FNA is related to the donation of a proton to the electron transport chain, which impedes the transmembrane pH gradient for synthesis of ATP (Peng & Zhu, 2006). NOB shows inhibition by FA at concentrations ranging from 1–7 mg NH_3-N L^{-1}, while AOB start to be inhibited at 150 mg-NH_3-N L^{-1} (Sri Shalini & Joseph, 2012). Other authors have proposed different thresholds for FA inhibition of NOB (1.75 mg NH_3-N L^{-1}; Weismann, 1994) and AOB (605 mg NH_3-N L^{-1}; Ganigué et al., 2012). Inhibition of NOB by FA is thought to be due to competitive inhibition of nitrite oxide reductase (NOR), an NOB enzyme, by FA (Peng & Zhu, 2006). It has been stated that AOB becomes inhibited by FA and FNA when nitrogen loading rates become higher than 1.5 kg m^{-3} day^{-1} (Ganigué et al., 2012). Regardless of this, some *Nitrobacter* spp. strains, typical NOB in partial nitritation reactors, have been found to resist up to 40 mg NH_3-N L^{-1} FA (Sri Shalini & Joseph, 2012). Adaptation of NOB to FA concentration has been discussed by some authors (Villaverde, 2000; Fux et al., 2004). In addition, AOB communities have been acclimated to FA concentrations of 122–224 mg L^{-1} (Liang & Liu, 2007). Therefore, control based on FA concentrations might not be an efficient practical tool for assessment of the performance of partial nitrification systems.

Hydroxylamine, an intermediate of the oxidation of ammonium to nitrite, was reported to have the capability of stabilising a partial nitritation system operating at a high COD/N ratio, low temperature, and high DO concentration, due to its inhibitory effect on NOB populations (Xu et al., 2012). Hydroxylamine has an inhibitory effect in NOB communities at 250 µM and on AOB populations at 2000 µM (Sri Shalini & Joseph, 2012).

A certain amount of organic matter can enter a partial nitritation reactor with the influent. For the purpose of an anammox treatment train, partial nitritation following an anammox reactor is recommended when the influent contains a considerable amount of organic matter (Van Hulle et al., 2005). Organic matter entering a partial nitrification reactor affects its performance. One of the reasons is that organic matter favours the development of heterotrophs, which have a shorter duplication time than AOB and, therefore, they could outcompete AOB for oxygen inside the bioreactor (Poth et al., 1985). It has been reported that the stability of the partial nitrification process is disturbed by high COD/N ratios due to the promotion of heterotrophic bacteria in the system (Wei et al., 2014). It has been shown that the C/N ratio does not affect the performance of the partial nitritation process at ammonium volumetric loading rates of 0.5 kg $N\text{-}NH_4^+$ m^{-3} day^{-1}. At higher ammonium volumetric loading rates, higher C/N ratios require higher DO concentrations if the same ammonium oxidation efficiency is desired (Liang & Liu, 2007).

In spite of promotion of heterotrophic growth, the impact of TOC concentration on a partial nitrification system has been found to depend on the carbon concentration. It has been reported that a TOC concentration of 0.2 g TOC L^{-1} as acetate stimulates ammonium oxidation in partial nitrification reactors, but also that 0.3 g TOC L^{-1} as acetate decreases ammonium conversion by 10% in these systems (Mosquera-Corral et al., 2005).Therefore, addition of organic matter leads to lower conversion rates of ammonium to nitrate (Ganigué et al., 2012).

In spite of this fact, it has been proved that elimination of organic matter can be achieved in partial nitritation reactors at the same time as ammonium oxidation when carbon loading rates do not exceed 2 kg m^{-3} day^{-1}. Nevertheless, it has been found that recovery of partial nitritation reactors after excessive loading of organic matter is a long process (Wei et al., 2014).

Salinity affects the performance of partial nitritation systems. It has been found that 85 mM NaCl increases ammonium conversion by up to 30%. As NaCl concentration rises, the system loses stimulation and tends to similar values of those in the no-salinity scenario. Nevertheless, at NaCl concentrations of 256 mM and higher, the system loses the capacity for ammonium oxidation. At 342 mM NaCl, there is 70% less ammonium

oxidation in the system compared with no-salinity operation (Mosquera-Corral et al., 2005). Sensitivity of AOB was observed after short-term exposure to salinity (Moussa et al., 2006), but adaptation of *Nitrosomonas europaea* strains to high salinity conditions has been observed with utilisation of targeted oligonucleotide probes (Tal et al., 2003; Moussa et al., 2006).

Addition of certain organic and inorganic compounds could play an important role in partial nitritation processes. It has been found that fulvic acid affects nitrite accumulation in partial nitritation reactors, impeding ammonium oxidation when its concentration is below 0.002 mg L^{-1} or over 0.07 mg L^{-1}. It has also been reported that NOB are more sensitive than AOB to orthocresol, aniline, and phenol. ClO_2^- has been proved to inhibit NOB activity at 3 mM; thus, chlorine could be used to control NOB population growth (Peng & Zhu, 2006).

Effect of temperature on the partial nitritation process

Temperature shows a clear relationship with ammonium oxidation in partial nitritation systems. It has been reported that, at a constant ammonium volumetric loading rate and DO concentration, higher temperatures, up to 35°C, lead to higher ammonium oxidation. Beyond this point, higher temperatures lead to FA formation and activity of AOB becomes inhibited (Liang & Liu, 2007). In similar studies, it was found that at 25°C, ammonium oxidation reached values of up to 60% of those at 28–39°C. At 41°C, bacterial activity stopped and thus ammonium oxidation did not take place. Also, it has been observed that ammonium uptake rate in partial nitritation reactors is maximum in the temperature range of 33–37°C (López-Palau et al., 2013).

The effect of temperature on the performance of a partial nitritation reactor has been explained by its influence on the growth rate of AOB and NOB and formation of FA and FNA. It has been found that NOB has slower growth rates than AOB when temperatures go above 24°C (Fux et al., 2002; Peng & Zhu, 2006). Nevertheless, at temperatures as low as 15°C, NOB are dominant over AOB in partial nitritation reactors (Peng & Zhu, 2006). It was reported that temperatures above 30°C led to the prevalence

of AOB over NOB due to the faster growth rate of the former (Mosquera-Corral et al., 2005). Thus, when operating at temperatures higher than 24°C, the environment will select for AOB.

Also, temperature affects concentrations of FA and FNA inside partial nitritation reactors. While FA concentration increases with temperature, from about 20 mg/L at 25°C to 120 mg/L at 35°C, FNA shows the opposite behaviour, with 0.5 mg/L at 25°C and 0.1 mg/L at 35°C. Inhibition of AOB activity by FA has been widely reported (Ganigué et al., 2012). Thus, at low temperatures, FNA is the main inhibitor of AOB, while at high temperatures it is FA, combined with FNA, which inhibits ammonium oxidation.

Normally, partial nitritation reactors are operated at the temperature range of 30–35°C to ensure AOB outcompetite NOB (Gabarró et al., 2012). However, even though partial nitritation reactors have been widely operated at 35ºC, there is not much difference in practical operation between 25°C and 35°C in terms of growth of AOB and NOB; in practical operation, 25°C is considered enough for the purpose of NOB control (Peng & Zhu, 2006). The bacterial community structure of partial nitritation reactors seems not to be influenced by temperature. Therefore, a difference in microbial activity due to temperature has been proposed as an explanation for differences in performance of the system at different temperatures (Kim & Lee, 2011).

Effect of pH and DO on the partial nitritation process

The pH in a partial nitritation reactor has an impact on the performance of these systems. In fact, pH has been reported as a key parameter affecting influent quality in models for laboratory-scale partial nitritation bioreactors (Magrí et al., 2007). It has been proposed that the influence of pH on ammonium oxidation in partial nitritation reactors is driven by three processes: activation-deactivation of nitrifying enzymes (Quinlan, 1984); changes in inorganic carbon concentrations; and changes in FA and FNA concentrations (Paredes et al., 2007). At higher pH, carbonate and bicarbonate are present at higher concentrations; thus, the buffer capacity of the system increases. On

the contrary, as pH drops below 7.7, the equilibrium tends to carbon dioxide, leading to a loss of buffer capacity (Ganigué et al., 2012). High pH has been related to the formation of FA, which is the primary substrate of AOB, and has been related as well to a decrease in FNA concentration, which is the primary substrate of NOB communities (Daalkhaijav & Nemati, 2014). It has been demonstrated that nitrification does not occur below pH 6 (Paredes et al., 2007; Sri Shalini & Joseph, 2012). Therefore, control of pH in partial nitritation systems can select for AOB and inhibit NOB due to the formation of FA and limitation of FNA (Villaverde, 2000).

The optimal pH for *Nitrosomonas*-like microorganisms, typical AOB, ranges between 7.9 and 8.2, and for *Nitrobacter*-like microbes, typical NOB, it ranges between 7.2 and 7.6 (Sri Shalini & Joseph, 2012; Daalkhaijav & Nemati, 2014). It has been found that the optimal pH for operation of partial nitritation reactors ranges between 7.0 and 8.0 (Sri Shalini & Joseph, 2012). The higher tolerance of AOB to low pH is thought to result from their ability to develop thick EPS layers (Manipura et al., 2005).

DO concentrations have an impact on the performance of a partial nitritation reactor. It has been found that ammonium oxidation increases with DO concentration, regardless of the C/N ratio, for the same ammonium volumetric loading rate (Liang & Liu, 2007). Half saturation constant values for oxygen of AOB and NOB are reported to be 0.2–0.4 mg L^{-1} and 1.2–1.5 mg L^{-1}, respectively (Peng & Zhu, 2006), which supports the hypothesis of the lower affinity for oxygen of NOB than AOB (Sri Shalini & Joseph, 2012).

Accumulation of nitrite can be controlled in a short time by setting DO concentration to 0.4–0.8 mg L^{-1}. Also, at DO concentrations of 2 mg L^{-1} or higher, substantial accumulation of nitrate occurs in partial nitritation reactors (Wei et al., 2014). It has to be borne in mind that low DO concentrations are related to increased NO and N_2O emissions (Sri Shalini & Joseph, 2012). Some authors have used an aeration of partial nitritation bioreactor lower than 0.1 m_{air}^3 day^{-1}/kg N m^{-3} day^{-1} (Okabe, 2011).

Thus, oxygen limitation inside partial nitritation reactors is an efficient way to control NOB development. Some reports state that the growth rate of AOB is faster than that of NOB when DO concentrations drop below 1 mg L^{-1} (Okabe, 2011). Therefore, DO

concentrations lower than 1 mg L^{-1} are used to control NOB in partial nitritation reactors (Tokutomi, 2004; Sinha & Annachatre, 2006).

Effect of HRT and SRT on the partial nitritation process

It has been reported that *Nitrosomonas* spp., typical AOB, have a maximum growth rate of 0.54 ± 0.09 day^{-1}, while *Nitrobacter* spp., typical NOB, have a maximum growth rate of 0.67 ± 0.03 day^{-1} (Blackburne et al., 2008). Other authors have reported similar values for minimum doubling time of AOB, 7–8 hours, and NOB, 10–13 hours (Peng & Zhu, 2006). Given the lower growth rate of AOB compared with NOB, control of NOB populations can be achieved with utilisation of the HRT/SRT. SRT should be set longer than the AOB growth rate but shorter than the NOB growth rate. In addition to this, partial nitritation bioreactors avoid sludge retention, given that the recycling of biomass makes NOB persist in the system and therefore develop in it. For this reason, partial nitritation reactors operate without sludge retention (Jetten et al., 2001). Conventionally, HRT and SRT are set up to the same time in partial nitrification reactors. However, the development of non-coupled HRT and SRT partial nitrification bioreactors has been attempted, in the form of biofilter partial nitrification reactors.

The impact of HRT on partial nitritation reactors has been evaluated by several authors. It was found that higher HRTs, at the same SRTs, led to higher oxidation of ammonium to nitrite (Liang & Liu, 2007). Differences in ammonium oxidation at different HRTs result from differences in microbial community structure inside the bioreactor at these HRTs. SRT also has an influence on bacterial communities in partial nitritation reactors. As a main control of NOB in the system, the SRT should be set lower than the duplication time of NOB, thus ensuring washout of these bacteria from the reactor (Jetten et al., 2001). Nevertheless, a short SRT also leads to the loss of AOB biomass. In this way, partial nitritation reactors have been conventionally operated as suspended growth processes (Sliekers et al., 2003; Ganigué et al., 2012; Li et al., 2013).

On the other hand, it has been confirmed that attached growth partial nitrification processes with attached/granular biomass have advantages over suspended growth partial nitrification processes, such as enhanced AOB biofilm formation (Wik, 2003).

Effect of antibiotics on the partial nitritation process

Antibiotics are present in urban and industrial wastewater treatment systems all around the world (Table 5). Today, it is well known that only a small amount of antibiotics in wastewater is removed at wastewater treatment plants. Obviously, the presence of antibiotics in wastewater is a significant environmental risk that must be eliminated before discharge of the wastewater into natural waters. The sorption behaviour of pharmaceuticals can be very complex and difficult to assess(Jelic et al., 2011). These compounds can absorb onto bacterial lipid structures and the fat fraction of sewage sludge through hydrophobic interactions, and often adsorb onto negatively charged polysaccharide structures on the outside of bacterial cells through electrostatic interactions, and/or chemically bind to bacterial proteins and nucleic acids (Meakins et al.,1994). Antibiotic-resistant strains are more numerous downstream than upstream of wastewater treatment plants (Zhang et al., 2004). In these environments, antibiotic-resistant microorganisms become selected under antibiotic concentrations. Then, a high density of microbial biomass helps to transfer genetic information that allows the bacteria to become resistant (Zhang et al., 2004). During wastewater treatment, quinolones are drastically removed from the water stream (N80%), but their fate is associated with sewage. Moreover, the sensitivity of nitrification processes to antibiotics has been recorded (Sáez et al., 2003). In this sense, the effect of antibiotics on partial nitritation systems must be studied to understand the effect of these emerging contaminats (ECs) on the microbial population and nitrogen removal.

Table 5. Different antibiotic concentrations reported in wastewater and sludge at different wastewater treatment plants (WWTPs) (Watkinson et al., 2007; Zorita et al., 2009; Ghosh et al., 2009, García-Galán et al., 2011; Jelic et al., 2011; Verlicchi et al., 2012)

Pharmaceutical compound	Chemical formula	Molecular weight (g/mol)	Concentration in wastewater (ng/L)	Concentration in sludge (ng/L)
Amoxicillin	$C_{16}H_{19}N_3O_5S$	365	24–40	nd
Azithromycin	$C_{38}H_{72}N_2O_{12}$	749	40–100	18–160
Chlortetracycline	$C_{22}H_{23}ClN_2O_8$	479	5–10	nd
Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	331	40–5000	45–2300
Clarithromycin	$C_{38}H_{69}NO_{13}$	748	300–1300	10–90
Clindamycin	$C_{18}H_{33}ClN_2O_5S$	425	4–10	nd
Cloxacillin	$C_{19}H_{18}ClN_3O_5S$	436	12–16	nd
Doxycycline	$C_{22}H_{24}N_2O_8$	463	5–70	nd
Enrofloxacin	$C_{19}H_{22}FN_3O_3$	359	5–10	nd
Erythromycin	$C_{37}H_{67}NO_{13}$	734	100–1800	10–90
Lincomycin	$C_{18}H_{34}N_2O_6S$	407	7–12	nd
Norfloxacin	$C_{16}H_{18}FN_3O_3$	319	10–30	10–2200
Penicillin G	$C_{16}H_{18}N_2O_4S$	334	2–4	nd
Penicillin V	$C_{16}H_{18}N_2O_5S$	350	0–1	nd
Roxithromycin	$C_{41}H_{76}N_2O_{15}$	837	50–1500	15–130
Sulphadiazine	$C_{10}H_{10}N_4O_2S$	250	3000–5000	nd
Sulphadimethoxine	$C_{12}H_{14}N_4O_4S$	310	80–100	nd
Sulphamethazine	$C_{12}H_{14}N_4O_2S$	278	16–20	nd
Sulphamethoxazole	$C_{10}H_{11}N_3O_3S$	253	40–1000	1–50
Sulphapyridine	$C_{11}H_{11}N_3O_2S$	249	200–3000	nd
Sulphasalazine	$C_{18}H_{14}N_4O_5S$	398	20–40	nd
Sulphathiazole	$C_9H_9N_3O_2S_2$	255	10–100	nd
Tetracycline	$C_{22}H_{24}N_2O_8$	444	30–200	45–175
Trimethoprim	$C_{14}H_{18}N_4O_3$	290	70–700	10–70
Tylosin	$C_{46}H_{77}NO_{17}$	916	50–60	nd

Partial nitritation technologies

For a long time, partial nitritation has been used in different technologies. However, this technology became more and more important in the last decade, with the appearance of autotrophic nitrogen removal systems. In fact, there are two main partial nitritation configurations, but also other technologies such as DEMON, CANON, and ANITA Mox processes for partial nitritation (Table 6).

Table 6. Different partial nitritation technologies

Technology	Fluidised-bed partial nitritation	Submerged-bed partial nitritation	Demon™ process	CANON™ process	ANITA™ Mox process
Conditions	Aerobic	Aerobic	Oxic and Anoxic	Aerobic	Aerobic
DO requirements (g O_2/g N)	1.5	1.5	0–3	1.5	1.5
Alkalinity (g $CaCO_3$/g N)	3.57	3.57	3.75	2.75	2.75
Performance	50%/50% ammonium/nitrite	50%/50% ammonium/nitrite	50%/50% ammonium/nitrite	50%/50% ammonium/nitrite	50%/50% ammonium/nitrite
Bacterial growth	Active sludge	Carriers	Active sludge	Granules	Carriers
COD requirements	no	no	no	no	no
Temperature (°C)	15–30	15–30	30	12–30	30
HRT	24	7	12	8	8

In all the technologies, the partial nitritation is achieved by AOB. However, the growth position in each one is different. In submerged-bed partial nitritation, the AOB have a bigger growth-specific area due to the carriers (Figure 4), in comparison with the fluidised-bed and DEMON bioreactors where the AOB form flocs. However, in the

other technologies, ANITA Mox and CANON, AOB need to grow on the external surface of the biofilm in the carriers and the granules, respectively. In these two technologies, this situation arises from the need to maintain autotrophic denitrification (anaerobic ammonium oxidation, the anammox process) in the core of the granules and the carriers due to the requirement for anaerobic conditions (Figure 2). In fact, there are six examples of full-scale partial nitritation plants in The Netherlands and one in the USA (Bagchi et al., 2012).

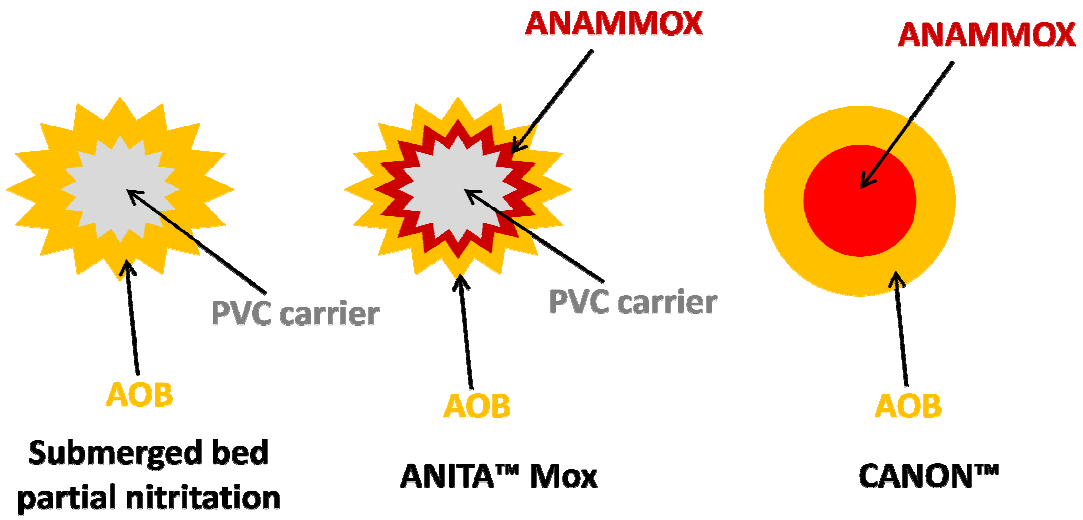

Figure 2. Biomass structure in different partial nitritation technologies.

Future trends in partial nitritation technologies

Future development of partial nitritation systems has to solve the main problem that these processes have: the development of NOB communities inside the bioreactor. Many steps can be taken to avoid the growth of NOB species in partial nitrification reactors. One of these steps is to turn partial nitritation processes into fully anaerobic processes. This can be done by taking into account the anaerobic nature of autotrophic ammonium oxidation (Bagchi et al., 2012). Strains of *Nitrosomonas eutropha* were reported to perform anaerobic ammonium oxidation with mediation of nitrogen dioxide as an electron acceptor (Schmidt & Bock, 1997). Further investigation has shown that dinitrogen tetraoxide can also be utilised as a final electron acceptor for anaerobic autotrophic ammonium oxidation by *N. eutropha*. Later, Schmidt et al. (2001) proposed

a new pathway for anaerobic, autrotrophic ammonium oxidation. Here, dinitrogen tetraoxide oxidises ammonium to hydroxylamine and nitrous oxide with mediation of the enzyme AMO. In this way, for anaerobic ammonium oxidation by *N. eutropha*, the presence of oxygen just causes nitrous oxide to become oxidised to nitrogen dioxide (Schmidt et al., 2001). Also, study of the nitrogen cycle in oxygen-depleted marine areas suggests that anaerobic ammonium oxidation is linked to Mn, Fe, and S reduction (Zehr & Ward, 2002; Clement et al., 2005).

If AOB can perform anaerobic ammonium oxidation, partial nitrification reactors could avoid the growth of NOB by setting anaerobic conditions in the system. On the other hand, several authors have reported that autotrophic nitrogen removal technologies are able to operate at low temperatures (Vazquez-Padin et al., 2011; Hu et al., 2013; Hendrickx et al., 2012). As a result, the introduction of these technologies in the water line of the WWTPs is in progress, and the technologies will provide low-cost processes for nitrogen removal.

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Objectives

Currently, one of the most important environmental problems is the discharge of urban and industrial wastewaters. These waters contain a large amount of nitrogen and represent a big risk of eutrophication in freshwater. This problem is caused by intense population growth as well as an ever-widening range of industrial processes. Therefore, in the last decade some autotrophic nitrogen removal technologies have been developed for high-performance and low-cost nitrogen removal.

In this context, the main objective of this study was to evaluate the effect of several different operating conditions on nitrogen removal efficiency and the microbial population, in order to achieve the best performance for submerged-bed partial nitritation.

Specifically, to achieve this primary objective, the following sub-objectives were developed:

1. Conduct an overview of the partial nitritation process and its importance in wastewater treatment technologies.

2. Evaluate the effect of HRT on a partial nitritation bioreactor constucted as a submerged biofilter.

3. Study nitrifying communities in a partial nitritation biorreactor.

4. Evaluate the effect of different concentrations of antibiotic (ciprofloxacin) on the partial-nitritation process and bacterial community structure of a submerged biofilter.

5. Apply predictive mathematical models and multivariate analysis to evaluate objectively the correlation between physicochemical parameters and microbial communities in partial nitritation bioreactors.

Results

Chapter 1

Treatment of Effluents Polluted by Nitrogen with New Biological Technologies Based on Autotrophic Nitrification-Denitrification Processes

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Treatment of Effluents Polluted by Nitrogen with New Biological Technologies Based on Autotrophic Nitrification-Denitrification Processes

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Abstract: In recent years, various technologies have been developed for the removal of nitrogen from wastewater, that is rich in nitrogen but poor in organic carbon, such as the effluents from anaerobic digesters and from certain industries. These technologies have resulted in several patents. The core of these technologies is some of the processes and patents described in this paper: Aerobic denitrification, Sharon, Anammox, OLAND, CANON, NO_x process, DEMON. More specifically, one of the first innovative options described for removing nitrogen include partial nitrification under aerobic conditions (partial Sharon process) followed by autotrophic anaerobic oxidation (Anammox process). The partial Sharon-Anammox process can be performed under alternating oxic and anoxic conditions in the same bioreactor or in two steps in two separate bioreactors. This overview focuses on the technical and biological aspects of these new types of treatment system, and compares them to other technologies. Given the fact that nitrification is a sensitive process, special attention is paid to conditions such as temperature, dissolved oxygen, hydraulic retention time, free ammonia, nitrous acid concentration, and pH. A discussion of the pros and cons of such treatment systems is also included since autotrophic nitrogen removal has advantages as well as drawbacks. The paper concludes with a discussion of future research that could improve these systems by enhancing performance and reducing costs.

Keywords: Wastewater, sludge, biological nutrient removal, nitrogen removal, deammonification, sharon process, partial ni-tritation/ anammox system.

1. INTRODUCTION

One of the most serious ecological problems in the world today is the proliferation of wastewater. This problem is caused by intense population growth as well as an ever-widening range of industrial processes. Unfortunately, it is impossible to determine the typical chemical composition of these wastes because human activities vary so greatly throughout the world. However, the presence of nitrogen in urban and industrial wastewaters in the form of urine, syn-thetic nitrogen fertilizers, and specific industrial pollutants is evidently a significant environmental risk that must be eliminated before their discharge into natural waters. Re-search has shown that wastewater discharges containing high concentrations of nitrogen can be toxic to aquatic life, as well as cause oxygen depletion and eutrophication [1].

The aim of Council Directive 91/271/EEC concerning urban wastewater treatment (see Table **1**) is to "protect the environment from any adverse affects due to discharge of (untreated) urban and industrial waters". The requirements for discharges from domestic wastewater treatment plants (WWTPs) into sensitive areas subject to eutrophication, as specified in Directive 91/271/EEC, were subsequently amended by Commission Directive 98/15/EC. This high-lights the fact that the development of new biotechnological processes for water management solutions is a high priority for stakeholders and citizens. The selection of wastewater treatment systems currently depends on various factors. Be-cause biological nitrogen removal is considered more effec-tive and is relatively inexpensive, it is generally preferred to physicochemical processes [2].

In conventional WWTPs, nitrogen is often removed by biological processes of nitrification and denitrification. Nitrification involves the oxidation of ammonia to nitrite and the subsequent oxidation of nitrite to nitrate under aerobic conditions. The nitrate generated is then denitrified to nitrite in the presence of an organic carbon source to dinitrogen [3]. The advantages of this process are its high potential removal efficiency, high process stability and reliability, relatively easy control, and small space requirements [4]. However, the N cycle and particularly nitrification-denitrification processes have been recently re-evaluated see Fig. (**1**). The application of new nitrogen-removal biotechnologies (i.e. partial Sharon-Anammox, OLAND, and NOx processes) should also be regarded as innovative and cost-efficient. This paper reviews these new systems from a biological and technical viewpoint and compares them.

2. CONVENTIONAL NITRIFICATION/ DENITRIFICATION

Conventional WWTP nitrogen, which is normally am-monium and organic substances, is removed by biological processes of nitrification/denitrification (mainly in wastewa-ters rich in nitrogen but poor in organic carbon) or by deni-trification/nitrification (mainly in urban wastewaters). The fact that different conditions are required for the bacteria

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Table 1. European Directive 91/271/EEC Requirements in Matter of Nutrients

Parameter	Concentration	Mínimum Percentage of Reduction[1]	Reference Method of Measurement
Total Phosphorus	2mg/L (10,000-100,000 p.e) 1mg/L (more than 100,000 p.e)	80	Molecular Absorption Spectrophotometry
Total Nitrogen [2]	15mg/L (10,000-100,000 p.e) [3] 10mg/L (more than 100,000 p.e) [3]	70-80	Molecular Absorption Spectrophotometry

[1]Reduction in relation to the load of the influent.

[2]Total nitrogen means the sum of total Kjeldahl nitrogen (organic and ammoniacal nitrogen), nitrate-nitrogen and nitrite-nitrogen.

[3]These values for concentration are annual means as referred to in Annex I, paragraph D.4(c). However, the requirements for nitrogen may be checked using daily averages when it is proved, in accordance with Annex I, paragraph D.1, that the same level of protection is obtained. In this case, the daily average must not exceed 20 mg/l of total nitrogen for all the samples when the temperature from the effluent in the biological reactor is superior or equal to 12°C. The con-ditions concerning temperature could be replaced by a limitation on the time of operation to take account of regional climatic conditions.

performing these biological processes means that separate reactors must be designed for each process.

Most of these biological treatments involve a combina-tion of two separate reactors under aerobic and anoxic condi-tions. The integration of this biological process is possible in two different configurations: pre-denitrification and post-denitrification.

Pre-denitrication system is the combination of an anoxic process followed by an aerobic process without addition of organic carbon and with internal recirculation of treated ef-fluent, in the other hand, the post-denitrification is an aero-bic-anoxic process [5] without internal recirculation but with requirement of external carbon source. Pre-denitrification is more frequently applied, although post-denitrification can be used too as alternative technology.

The best known bacteria that perform ammonia oxidation are the following: (i) bacteria containing strains of *Nitroso-coccus oceanus* and *Nitrosococcus halophilus* in the _-proteobacteria [6]; (ii) bacteria composed of species of *Ni-trosomonas* and *Nitrosospira* (which include strains of _-proteobacteria) [7]. They are able to oxidize ammonium to nitrite by using ammonium as an energy source and oxygen as an electron acceptor.

In nitrite oxidation, the most common genera are *Nitro-bacter, Nitrospira, Nitrococcus* and *Nitrospina* [8]. During this stage, nitrite is converted to nitrate by using nitrite as an energy source and oxygen as an electron acceptor. In the nitrification process, carbon dioxide is the carbon source because it is an autotrophic process. The conversion of am-monium to nitrite and the subsequent oxidation of nitrite to nitrate are reflected in the following formulas [9, 10]:

$$NH_4^+ + 1\_ O_2 + 2\ HCO_3^- \rightarrow NO_2^- + 2\ CO_2 + 3\ H_2O$$

nitritation $NO_2^- + \_\ O_2 \rightarrow NO_3^-$ nitratation

In nitritation, 7.07 mg of $CaCO_3$ is required per mg of ammonia nitrogen oxidized. The pH of the bioreactor de-creases during the nitrification process when the alkalinity in the wastewater approaches depletion because of the nitrite produced. If the bioreactor is not buffered to maintain a pH higher than 7.0, the process is not as effective.

In the second step, nitrate is reduced to gaseous dinitro-gen by heterotrophic microorganisms (denitrification reac-tion) under anaerobic conditions. In this process, the carbon and energy source is organic matter, and nitrate is the final electron acceptor. The most common denitrifying bacteria are *Pseudomonas, Alcaligenes* (Gram-negative) and *Bacillus* (Gram-positive) [11]. A few halophilic *Archaea* (such as *Halobacterium*) are also able to denitrify [12]. This denitrifi-cation process is represented by the following formula [9, 10]:

$$5\ C + 2\ H_2O + 4\ NO_3^- \rightarrow 2\ N_2 + 4\ HCO_3^- + CO_2$$ Denitrification

An efficient anoxic denitrification demands a variety of electron donors, such as ethanol, acetate, methanol, lactate, or glucose [10]. In this context, methanol (CH_3OH) is the compound most frequently used. Its popularity is due to the fact that it is relatively inexpensive [13].

Finally, in conventional nitrification-denitrification tech-nology, the nitrification process needs significant amounts of oxygen, whereas the denitrification process requires the ad-dition of an external source of carbon. Thus, this system de-mands considerable resources, namely, 4.57g $O_2\ g^{-1}$ N and approximately 4 g COD g^{-1} N [14]. Obviously, this increases the costs of the technology.

3. AEROBIC DENITRIFICATION

In consonance with the conventional concept of nitrogen removal, nitrification requires the presence of oxygen whereas denitrification must take place in anoxic conditions. However, according to Zumft [12] with certain exceptions, denitrification need not obligatorily occur in anaerobic condi-tions. Some of the first denitrifying bacteria observed under aerobic conditions were *Thiomicrospira denitrificans* in a wastewater treatment pilot plant [15] and *Paracoccus deni-trificans* [16]. Afterwards *Pseudomonas stutzeri* was discov-ered, when researchers observed that it was able to reduce nitrate to gaseous dinitrogen under aerobic conditions [17].

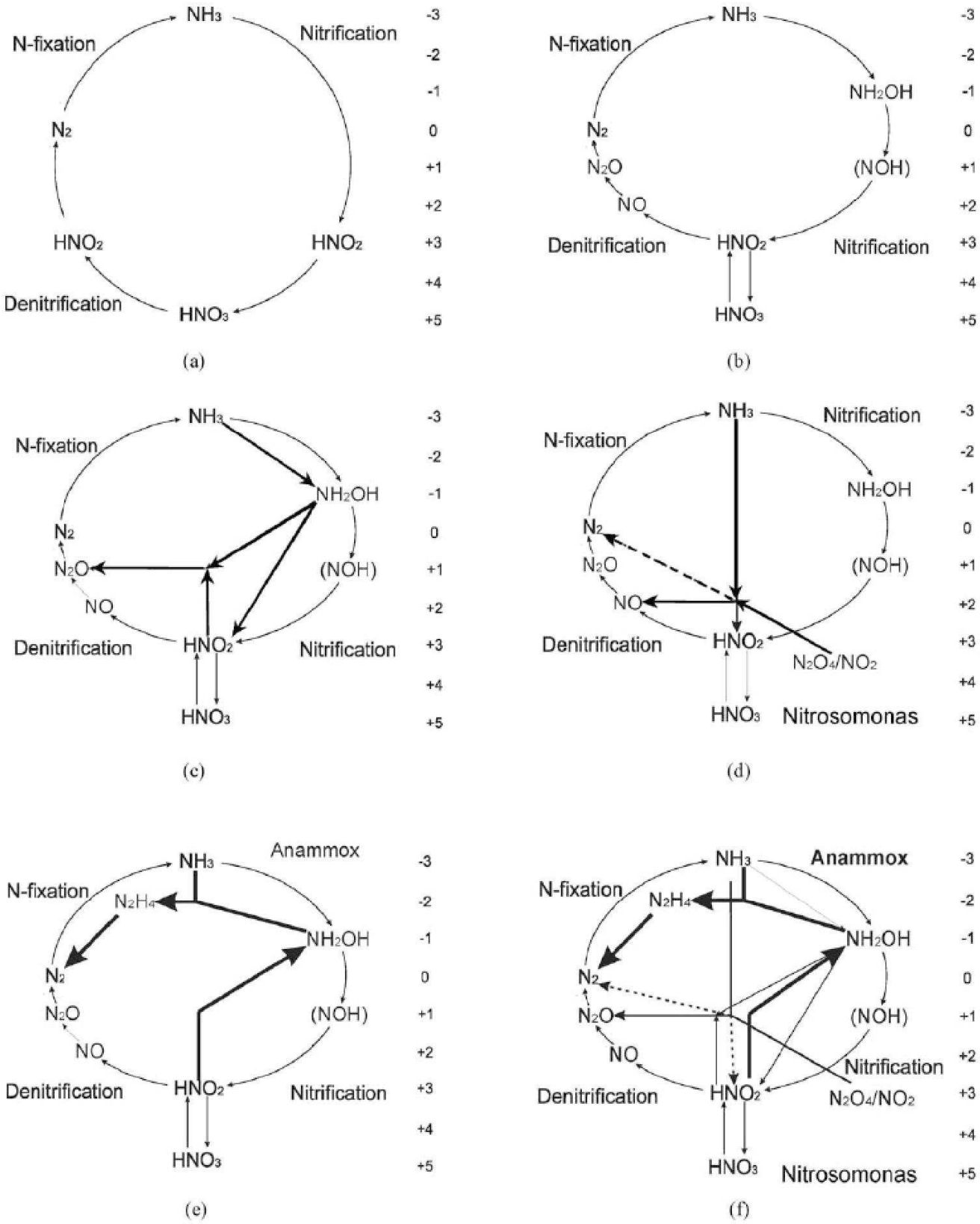

Fig. (1). Nitrogen cycle according to Van Loosdrecht [4]. (**a**) Classical N-cycle, (**b**) Sharon process, (**c**) Nitrosomonas aerobic denitrification or aerobic deammonification, (**d**) Nitrosomonas denitrification, (**e**) anaerobic ammonium oxidation and (**f**) overall nitrogen web.

Aerobic denitrification has been described in wastewater treatment technologies, such as full-scale continuous proc-esses [18], submerged biofilter [19] and bench-scale se-quencing batch reactor (SBR) systems [20]. In this context, aerobic denitrification may have several advantages over the previously described nitrification/denitrification processes. In this technology, nitrification and denitrification can be performed in one bioreactor, and a second (anoxic) tank is thus not required because nitrification/denitrification occur simultaneously under the same conditions. Obviously, this saves money in the construction of WWTPs. Moreover, since working conditions are constant in this system, the process is easier to control.

Finally, phytoremediation technologies has been applied to nitrogen removal. In this context different authors [21] has reported the importance of this biotechnology as an alterna-tive system to the conventional nitrogen removal systems. Phytoremediation can be considered as a low cost technol-ogy useful for urban wastewater treatment in small towns. Moreover this technology is able to reduced the concentra-tion of nitrogen in values up to 80% [21].

4. AUTOTROPHIC NITROGEN REMOVAL SYS-TEMS

4.1. SHARON Process

The patented SHARON process [22] (Single reactor sys-tem for High-activity Ammonia Removal Over Nitrite) is described in detail by Hellinga *et al.* [23]. This technological process was developed for the removal of ammonia via the so-called ‘‘nitrite route’’ [24]. Accordingly, this system in-volves the oxidation of ammonia to gaseous dinitrogen by using nitrite as an electron donor without the need for high concentrations of organic matter [25]. For these reasons, it was designed to reduce the concentration of rich streams in ammonium. The Sharon process is described by the follow-ing formula [4, 26]:

$2\ NH_4^+ + 3\ O_2\ 2\ NO_2^- + 2\ CO_2 + 4\ H_2O + 4\ H^+$ Nitritation 2

NO_2^- + 4.8 g COD + 2 H^+ N_2 + 1.8g sludge Denitritation

WWTPs using the Sharon process have lower exploita-tion costs than conventional WWTPs that use nitrifica-tion/denitrification technologies. In fact, studies have shown that the Sharon process significantly reduces the organic matter (40%) and oxygen (25%) needed for ammonia re-moval as compared to traditional nitrification/denitrification processes [27]. The Sharon process uses a single bioreactor in which it combines a partial nitrification under aerobic conditions followed by a conventional denitrification under anaerobic conditions. In order to obtain a partial nitrification in the Sharon process, it is necessary to bear in mind that nitrite-oxidizing bacteria are more sensitive than ammonium-oxidizing bacteria to different environmental parameters, such as pH, dissolved oxygen concentration, free ammonia (FA, NH_3), free nitrous acid (FNA, HNO_2) concentration, temperature, and hydraulic retention time (HRT). Conse-quently, an effective application of this biotechnological process requires a strict control of these parameters in order to obtain a high efficiency in ammonia removal.

Studies have reported temperatures of 30-40 ºC that stimulate the growth of ammonia-oxidizing bacteria [28]. However, under such conditions, the cell growth and bio-logical activity of nitrite-oxidizing bacteria are strongly in-hibited. Consequently, even though these temperature ranges facilitate the partial nitrification process, they prevent or at least significantly reduce the transformation of nitrite to ni-trate.

Free ammonia (NH_3) and free nitrous acid (HNO_2) con-centrations have a strong influence since these uncharged nitrogen forms are the actual substrate/inhibitor for ammo-nium and nitrite oxidation instead of ammonium (NH_4^+) and nitrite (NO_2^-) [29]. This had been confirmed by Van Hulle *et al.* [30] for ammonium oxidizers that are active in a Sharon reactor [31]. Regarding inhibition, NH_3 is the main inhibitor of nitrification at a high pH (>8), whereas HNO_2 is the main inhibitor at a low pH (<7.5). Recently, Hawkins *et al.* [8] state that free ammonia has only a limited impact on the in-hibition of nitrite oxidation. They found that pH changes and ammonia oxidizing activity had the greatest influence on nitrite-oxidizing activity.

Hydraulic retention time (HRT) is a working parameter that affects the performance of a Sharon bioreactor as dem-onstrated by Dijkman [32]. Research has shown that by ad-justing the HRT, the ammonium-oxidizing bacteria are re-tained in the bioreactor whereas the nitrite-oxidizing bacteria are washed away [33]. It is relatively simple to limit the sludge retention time in such a way that ammonium is oxi-dized to nitrite, but is not oxidized to nitrate.

The overall optimum pH for nitrifying bacteria is 7-8. In this sense, the pH must be kept at near optimum values be-cause this directly affects the process [30]. When the pH is lower than 6.5, the nitrification rate decreases since carbon limitations because of CO_2 stripping occur [34]. Therefore the bioreactors must be buffered because this parameter in the Sharon system decreases during the partial nitrification process. This can be prevented by adding alkaline chemicals such as carbonate or bicarbonate to keep the pH value above 6.5.

Philips *et al.* [35] highlight the importance of the dis-solved oxygen (DO) concentration for nitrifying bacteria. More specifically, when there is a low oxygen concentration, the activity of ammonium oxidizers remains constant, but nitrite oxidizers are inhibited. This biological effect is due to the higher oxygen affinity of ammonium-oxidizing microbi-ota in comparison to nitrite-oxidizing bacteria [36]. In con-clusion, an oxygen deficiency in the Sharon process stem-ming from low dissolved oxygen concentrations can affect the activity of nitrite oxidizers more significantly than that of ammonium oxidizers [35]. Consequently it is important to maintain the DO below 2.5 mg O_2/L in order to optimize the partial nitrification process.

The most common bacteria in a Sharon process that are able to transform ammonium into nitrite (ammonium oxidiz-ers) are *Nitrosomonas europaea* [37], *Nitrosomonas eutro-pha* [38], and *Nitrosospira sp.*

This system has been tested in many full-scale plants all over the world. Examples of Sharon WWTPs can be found

in Zwolle, Rotterdam, Den Haag (all in the Netherlands) and New York (USA) [38]. This is indicative of the viability of this process.

4.2. Anaerobic Ammonium Oxidation (Anammox)

Traditional WWTPs using conventional nitrification-denitrification processes, generate ammonium-rich side-streams. These streams are basically produced during the anaerobic digestion of the sludges (digestion supernatant). These supernatants have been found to possess a nitrogen concentration greater than 2 kg N m^{-3} [40]. Generally speaking, these supernatants are recirculated in the WWTP, which increases the total nitrogen in the plants by 20% [41]. However, as is well-known, high concentrations of ammonia can be toxic for nitrifying bacteria [42]. Consequently, the development of new systems for the treatment of these digester supernatants is evidently regarded as useful technology.

In the 1970s, Broda [43] predicted that the anaerobic ammonium oxidation (Anammox) process was possible when based on thermodynamic calculations. Twenty years later, in a fluidized bed bioreactor, Mulder *et al.* [44] ob-served the disappearance of ammonium with the removal of nitrate along with a corresponding increase in gaseous dini-trogen. In this system, ammonium is converted to dinitrogen gas under anaerobic conditions with nitrite as the electron acceptor [45].

The Anammox process [46] is a low- cost option for removing nitrogen from wastewater rich in nitrogen but poor in organic matter. The main bacteria that perform the Anammox process are *phylum Planctomycetales* [47]. Cur-rently, four genera of Anammox bacteria have been defined: *Brocadia, Kuenenia, Scalindua* and *Anammoxoglobus* (see Table **2**).

Today it is well known that the Anammox process occurs naturally in many different habitats all over the world, such as oceans, lakes, and soil [56]. In addition, this biological process has been reported in anaerobic digesters used in wastewater treatments [57]. Jetten [58] found that Anammox bacteria have a very slow growth rate. Thus, for an optimal performance in the Anammox process, a sludge reactor must be activated for a time period longer than 100 days [28]. This is evidently one of the principal disadvantages of this sys-tem. The Anammox process is represented by the following formula [25]:

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- \ 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O$$

During the Anammox biological process, dinitrogen and nitrate are produced at concentrations of 90% and 10%, re-spectively. The overall nitrogen balance has an ammonium-to-nitrite conversion ratio of 1:1.32, and a nitrite conversion to nitrate production ratio of 1:0.22 [59].

Several studies have reported that the Anammox process can be influenced by different environmental factors [2, 60]. For example, the ammonium-nitrite mixture must be com-posed of 50% ammonium and 50% nitrite. Moreover, pa-rameters, such as temperature, DO, pH, and organic carbon, should be strictly controlled. Strous *et al.* [60] demonstrated that the Anammox process requires anoxic conditions be-cause Anammox bacteria are strictly anaerobic. Conse-quently, they are inhibited by dissolved oxygen. However, inhibition produced by high oxygen concentrations can be reversible [61].

The optimum pH for the growth of the Anammox bacte-ria is between 6.7 and 8.3 [62], whereas the optimum temperature range is 30-40 ºC and the O_2-concentration being kept between 0.2 mg/l and 0.4 mg/l [63]. However, certain Anammox bioreactors are actually able to operate at 20 ºC with satisfactory results [64].

The Anammox process is designed to treat streams with a low carbon/nitrogen ratio and a high ammonia concentration, such as the supernatants from anaerobic digesters or the effluents of certain industries. In these streams, the heterotro-phic denitrifying populations are inhibited because they need high concentrations of organic matter. However, autotrophic Anammox bacteria that do not require organic carbon can grow, and consequently their populations can be increased [46]. Furthermore, research [65, 66] has shown that organic matter has a negative impact on the Anammox process be-cause it cannot compete for nitrite with the heterotrophic denitrifying populations.

Table 2. Microbial Biodiversity of Autotrophic Denitrifying and Anaerobic Ammonium Oxidation (Annamox) Bacterial Species

Genus	Species	Source	Reference
Brocadia	*Brocadia anammoxidans*	Wastewater	[48]
	Brocadia fulgida	Wastewater	[49]
Scalindua	*Scalindua brodae*	Wastewater	[50]
	Scalindua wagneri	Wastewater	[50]
	Scalindua sorokinii	Marine water	[51]
	Scalindua Arabica	Marine water	[52]
Kuenenia	*Candidatus Kuenenia stuttgartiensis*	Wastewater	[53]
Anammoxoglobus	*Anammoxoglobus propionicus*	Wastewater	[54]
Jettenia	*Jettenia asiatica*	Not reported	[55]

4.3. Combined Partial Sharon/Anammox Process in Two Bioreactors

The partial Sharon/Anammox process removes nitrogen in two steps. The Anammox process requires a nitrite-ammonium mixture to obtain an optimal performance. For this reason, this system has to be preceded by a partial Sharon (partial nitrification). This combined process makes the technology more flexible and is conducive to a more sta-ble performance of the system since each step can be con-trolled separately [67].

The partial Sharon can be regarded as a modification of the traditional Sharon, which transforms 100% of the ammo-nium into nitrite. In contrast, the partial Sharon process, as its name implies, involves a partial nitritation, and only transforms 50% of the ammonium into nitrite. Although in an effective Anammox process, the optimum ratio of ammo-nium-nitrite should be 1:1.32 [68], in combined partial Sharon-Anammox systems, the ratio is closer to 1:1 to pre-vent the inhibition of ammonia-oxidizing bacteria by the nitrite. Under these conditions, Van Dongen *et al.* [28] tested a partial Sharon-Anammox process for the treatment of am-monium-rich wastewaters, and obtained excellent results. The following formula designates the partial Sharon/ Anammox process:

$NH_4^+ + \_\ O_2 + HCO_3^- \_\ NH_4^+ + \_\ NO_2^- + CO_2 + 1\_\ H_2O$ partial Sharon (bioreactor 1)

$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^-\ 1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O$ Anammox (bioreactor 2)

The partial Sharon process is most effective when pa-rameters, such as temperature, pH, DO, and HRT, are strictly controlled. Temperatures of approximately 35 ºC and pH values of 7.0-8.0 have proven to be the most favorable envi-ronmental conditions for this biological process [2, 31]. When pH values are lower than 6.0, there is an increase in the concentration of free ammonia and nitrous acid, and this has an inhibitory effect on the ammonium-oxidizing bacteria [69]. According to Peng and Zhu [70], an optimum partial nitritation will only occur when factors other than free am-monia and free nitrous acid are regulated. For this reason, pH values in the partial Sharon process must be kept above 6.5.

As previously stated, the DO concentration significantly affects the biological activity of the nitrifying bacteria. As a result, higher oxygen concentrations (up to 3.0 mg O_2 /L) have a positive impact on the growth of nitrite oxidizers. It is thus crucial to maintain the DO below 2.5 mg O_2 /L in order to inhibit the growth of nitrite-oxidizing microbiota and stimulate the growth of ammonia-oxidizing bacteria.

The HRT is one of the most important working parame-ters affecting partial nitrification. In fact, research has shown that the optimal HRT for partial nitritation is approximately one day [4, 28, 33]. However, the hydraulic retention time must change in order to vary the influent load. Moreover, anaerobic ammonium oxidation can be performed in the anammox bioreactor with the ammonium-nitrite mixture (50% ammonium-50% nitrite) in anoxic conditions

This technology transforms roughly 90% of the ammo-nium into dinitrogen and 10% of the ammonium into nitrate.

The main advantage of this system is that it is a low-cost system for nitrogen removal. According to Fux [71], the overall cost is 2.5_ per kg of nitrogen removed for partial nitritification/anammox, and 3.0–4.0_ per kg of nitrogen removed for the nitrification/denitrification alternative. The partial Sharon/Anammox process is even more economical for the following reasons: (i) aeration only requires 50% of the nitrite [72]; (ii) no external carbon source (autotrophic process) is needed. Nevertheless, the fact that two bioreac-tors are required raises construction costs [69].

4.4. A Combined Partial Sharon/Anammox Process in a Single Bioreactor

A combined partial Sharon/Anammox process in a single bioreactor has a higher nitrogen removal rate and lower con-struction costs [73]. This system is characterized by the co-existence of aerobic and anaerobic ammonium-oxidizing bacteria with alternating aerobic and anaerobic conditions or with a limited oxygen supply in order to avoid the combined process. Some examples of this technology are described in the sections that follow.

4.4.1. OLAND Process

The OLAND process (Oxygen Limited Autotrophic Ni-trification and Denitrification) [74] is a biotechnology where the ammonia-oxidizing bacteria are able to convert ammo-nium into gaseous dinitrogen [75].). The OLAND system transforms the ammonium to nitrite as shown in the follow-ing formula [76]:

$NH_4^+ + \_\ O_2 + HCO_3^- \_\ NH_4^+ + \_\ NO_2^- + CO_2 +$

$H^+ \_\ NH_4^+ + \_\ NO_2^- \_\ N_2 + 2\ H_2O$

$NH_4^+ + \_\ O_2 \quad \_\ N_2 + H^+ + 1\_\ H_2O$

This system can be constructed with one or two reactors. A rotating biological contactor (RBC) reactor is generally used in a one-stage system, in which partial nitrification and Anammox simultaneously take place in one reactor under limited oxygen conditions [77]. Alternatively, in a two-stage system, a membrane-assisted bioreactor (MBR) is used in which partial nitrification and Anammox take place in sepa-rate reactors [73]. One of the main advantages of OLAND is that it operates under low temperature conditions (22~30 ºC).

The environmental conditions of the OLAND process produce a growth inhibition of the nitrite- oxidizing microbi-ota and a stimulation of the ammonia oxidizer. Research has thus confirmed the presence of anaerobic ammonium oxidiz-ers (40%) and aerobic ammonia oxidizers (45%) in OLAND bioreactors [78]. The generally low performance of this sys-tem is regarded as the main disadvantage of the OLAND process, In Sliekers [78], the nitrogen removal rate in an SBR system was only 0.064 $kgNm^{-3}\ d^{-1}$.

4.4.2. CANON Process

The CANON process (Completely Autotrophic Nitrogen removal Over Nitrite) [79] is a new system where a co-culture of aerobic and anaerobic ammonium-oxidizing bacte-ria can be performed under oxygen-limited conditions (< 0.5 % air saturation) [80]. The Canon process is described by the following formula [81]:

$NH_3 + 0.85\ O_2$ $0.11\ NO_3^- + 0.44\ N_2 + 0.14\ H^+ + 1.43\ H_2O$

This biotechnology is thus based on the concept of simultaneous nitrification and denitrification (SND) in a single reactor vessel at constant operating conditions [82, 83, 84]. The simultaneous nitrification and denitrification process is possible because the ammonium oxidizers are able to convert ammonia into nitrite, consume oxygen, and create the anoxic conditions required by the Anammox process. This system had been described [85] as an economic and efficient option for wastewater treatment with a maximal nitrogen removal rate of 0.075–1.5 $kgNm^{-3}\ d^{-1}$ and an overall optimum tem-perature of 30–35 _C.

In regards to the Canon process, Schmidt el al. [86] assessed the harmonious and balanced interaction between *Nitrosomonas* (ammonium oxidizers) and *Brocadia anammoxidans* (anammox bacteria*).* The nitrite requirement for *Brocadia* is supplied by *Nitrosomonas* in the oxic-anoxic biofilm interface. Even though ammonium is the substrate of these bacteria, cooperation between ammonium oxidizers and anammox bacteria is possible. In the Canon bioreactor, the ammonium oxidizers (*Nitrosomonas*) limit the Anammox process due to the fact that they prevent the diffusion of oxygen into the deeper layers, and also supply nitrite to the Anammox bacteria [87].

4.4.3. NOx Process

In the 1980s, Poth and Focht [37] found that *Nitrosomonas* strains were able to obtain energy during nitrification or during aerobic and anaerobic ammonia oxidation by using hydrogen or organic compounds as electron donors. A decade later, in the 1990s, research showed that nitrogen dioxide (NO_2) could be used by *Nitrosomonas* to produce anaerobic ammonia oxidation [88].

The NOx process is a nitrogen removal biotechnology that involves the control and stimulation of denitrification activity of *Nitrosomonas* strains by adding nitrogen oxides [89, 90]. Obviously, this biological process can be applied to wastewater treatments since *Nitrosomonas* consume ammo-nium and NO_2 with a ratio of 1:1 [88]. The NOx process can be described by the following formula [91]:

$3\ NH_4^+ + 3\ O_2\ N_2 + NO_2^- + 4\ H^+ + 4$

$H_2O\ NO_2^- + H^+ + 3\ [H]\ \_\ N_2 + 2\ H_2O$

The performance of this NOx system is about 67%. However, it should be underlined that in this biotechnology, the nitrification process is more efficient than the denitrifica-tion process [89].

4.4.4. DEMON Process

The term aerobic/anoxic deammonification or DEMON was first used when significant losses of inorganic nitrogen of up to 90% were observed in the nitrification step of a ro-tating biological contactor (RBC) treating ammonium-rich landfill leachate under low oxygen Conditions. This Deam-monification process consists in two subsequent processes, a partial nitritation of ammonia and the subsequent anaerobic oxidation of the residual ammonia by nitrite to nitrogen gas [92], according to the following formula:

$NH_4^+ + 1.5\ O_2\ NO_2^- + H_2O + 2\ H^+$ Partial nitritation (55-60% of ammonium to nitrite)

$NH_4^+ + NO_2^-$ $N_2 + 2\ H_2O$ Anammox (nitrogen removal)

Both process steps are catalysed by different consortia of organisms. Such as, a population of aerobic autotrophic am-monia oxidizers and a consortium of anaerobic autotrophic ammonia oxidizers, DEMON process achieves better treatment than existing conventional technologies, is more energy efficient and No carbon dosage is required due to the autotrophic nature of the process. Currently full-scale plants are in operation in for example Strass (Austria) and Zurich (Switzerland). The plant in Strass treats the wastewater of 200,000 population equivalents, and is equipped with a 500m3 sequencing batch reactor (SBR) for deammonifica-tion of reject-water originating from digested sludge dewa-tering [93].

5. COMPARISON OF THE DIFFERENT SYSTEMS

The comparison of these processes is given in Table **3.** This table shows that traditional nitrogen removal processes (conventional nitrification/denitrification) perform well, but have a very high cost in comparison to autotrophic nitrogen removal systems. For this reason, anaerobic ammonium oxi-dation is regarded as a new and promising option for waste-water treatment [46], especially for treatment of ammonium-rich sidestreams from anaerobic digesters. Biotechnologies coupled with partial nitritation processes have several advan-tages such as a reduction in the oxygen and chemical re-quirements, a lower nitrite and nitrate production, addition of organic compounds as a carbon source, and a low sludge production. In this sense, Fux [97] state that the overall costs for the combined partial nitritation/Anammox process are 1.4 times lower than for the traditional nitrification/ denitrifica-tion process.

The main disadvantage of the anaerobic ammonium oxi-dation system is its long start-up time caused by the slow growth rate of anammox bacteria. To mitigate this problem, different laboratory cultures of anammox microorganisms can be re-used to inoculate the bioreactor because the accu-mulation of a sufficiently large anammox biomass is needed for a faster start-up.

The Canon process has a nitrogen removal rate that is much lower than the Anammox process, but even so, it can be regarded as a low-cost and efficient biotechnology. Nevertheless, only a few researchers have studied this process because of the complexity of the system [79, 80, 85]. The OLAND system does not require a direct supply of nitrite, and can directly treat ammonium-rich wastewater. Neverthe-less, system capacity is not as yet sufficiently high, and needs to be improved.

Finally the DEMON process has been implemented in a full scale plant, for example in Strass (Austria), with start-up problems, such as, low growth rate and hard control of the process, however when the sludge reactor are activate (50 days approximately) the bioreactor can be regarded as an optimal nitrogen removal technology

6. CURRENT & FUTURE DEVELOPMENTS

Nitrogen compounds can be removed from urban and industrial waters by a variety of physicochemical and biological processes. The biotechnological processes for

Table 3. Comparison of Different Nitrogen Removal Systems According to [13,91,94,95,96]

	Traditional Nitrification/ Denitrification	Sharon Process	Partial Sharon / Anammox Process	OLAND Process	CANON Process	DEMON Process
Number of bioreactors	2	1	1 (or 2)	1 (or 2)	1	1
Inocula	Activated sludge	Activated sludge	Denitrifying or Anammox bacteria	Nitrifying bacteria	Anammox (80%)+ Nitrifier (20%)	Anammox50%+ nitrifier 50%
Oxygen conditions	Oxic/anoxic	Oxic/anoxic	Oxic/anoxic	Oxygen limited	Oxygen limited	Oxygen limited
Dissolved oxygen requirement (g O2/g N)	High 4.57	Medium 3.43	Low 1.71	Low 1.94	Low 1.90	Very low 0.3
Alkalinity (g CaCO3/g N)	7.14	7.14	3.57	3.75	3.75	3,75
Performance (Nitrogen removal) (%)	95	90	90	85	90	90
Bacterial growth	Biofilm/ Suspension	Suspension/ Biofilm	Suspension/ Biofilm/ Granules	Biofilm	Biofilm	Biofilm
COD requirement	Yes	Yes	No	No	No	No
Energetic requirement	High	Low	Very Low	Low	Low	Very low
Sludge production	High	Low	Very Low	Very Low	Very Low	Very low
Construction Cost	High	Low	Low	Very Low	Very Low	Very low
Nitrogen removal Cost	High	Medium	Very Low	Very Low	Very Low	Very low
Types of Bacteria	NH4 and NO2 oxidizers, Heterotrophic bacteria	Aerobic NH4 oxidizers, N. eutropha and Heterotrophic bacteria	*Planctomycetales* such as *Scalindua brodae, Wagneri and sorokinii Brocadia anammoxidans, and Kuenenia stuttgartiensis*	Autotrophic nitrifiers	Planctomycetales and Aerobic ammonium oxidizers	Planctomycetales and Aerobic ammonium oxidizers

nitrogen removal are more effective and relatively less expensive than physicochemical systems. However, the cost of nitrogen removal has progressively increased over the years given the fact that traditional WWTPs based on conventional nitrification/denitrification processes require significant amounts of energy and organic matter. Thus, alternative bio-technologies for nitrogen removal with sustainable, and envi-ronmentally-friendly processes are urgently needed. In this context, recent scientific advances have focused on basic biological and technological studies that can be applied in this field.

One of the most promising alternatives to conventional nitrification/denitrification processes is the application of innovative technologies based on autotrophic microorgan-isms, such as anammox microbiota. However, little is known about the habitat, nutritional requirements, metabolic activity, and physiological characteristics of this microbial group. Similarly, the genetic characteristics of these microorgan-isms are still somewhat of a mystery and in urgent need of further research.

New nitrogen removal options such as Sharon, Anam-mox, CANON, OLAND and NOx are microbial technolo-gies that have a better performance as well as a lower cost than conventional nitrification/denitrification systems. How-ever, the main drawbacks of these systems are their slow start-up and the general lack of scientific knowledge about how they work. Therefore, future research should be carried out with a view to discovering faster start-up methods and sustainable operational control methods to reduce the cost and enhance the performance of autotrophic systems. Moreover, this new approach to nitrogen removal must be economically competitive and technically viable. Evidently,

as described in this paper, more scientific knowledge about these microbial processes is necessary so that these tech-nologies can be widely applied in the near future.

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ABBREVIATIONS

SHARON	=	Single high ammonia removal over nitrite
ANAMMOX	=	Anaerobic Ammonium oxidation
EU	=	European Union
WWTP	=	Wastewater treatment plant
FA	=	Free ammonia demand
HRT	=	Hydraulic retention time
OLAND	=	Oxygen Limited Autotrophic Nitrification and Denitrification
CANON	=	Completely Autotrophic Nitrogen removal Over Nitrite
SBR	=	Sequencing batch reactor
DO	=	Dissolved oxygen
MBR	=	Membrane-assisted bioreactor
SND	=	Simultaneous nitrification and denitrifica-tion

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Chapter 2

Biological and technical study of a partial-SHARON reactor at laboratory scale: effect of hydraulic retention time

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ORIGINAL PAPER

Biological and technical study of a partial-SHARON reactor at laboratory scale: effect of hydraulic retention time

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Abstract This study was on the technical and biological characteristics of a partial-SHARON submerged-filter bioreactor of 3 L. The main focus was the influence of the hydraulic retention time (HRT) on biofilms. For this purpose, we used molecular tools based on the partial 16S rRNA genes. The results showed that the HRT may affect the nitrification processes of a bioreactor using synthetic wastewater containing 600 mg/L of ammonia. It was found that an HRT of 0.5 day transformed 100 % of the ammonium into nitrite. However, when the HRT was decreased to 0.4 day, there was a significant reduction (35 %) in the quantity of ammonia transformed, which confirmed the complexity of the system operation. Moreover, a PCR-TGGE approach highlighted the differences observed. The results obtained showed that an HRT of 0.5 day reduced bacterial biodiversity in the biofilms, which were mainly formed by Nitrosomonas and Diaphorobacter. In contrast, an HRT of 0.4 day facilitated the formation of heterogeneous biofilms formed by nitrifying bacteria, such as Nitrosomonas sp., Nitrosospira sp., and Nitrosovibrio sp.).

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Keywords SHARON process Partial nitrification Hydraulic retention time (HRT) Wastewater treatment Submerged biofilter Nitrogen removal

Introduction

In the last 10 years, soaring population levels as well as a corresponding growth in industrial activity have led to increased amounts of wastewater in densely populated areas. This surfeit of waste is having an extremely negative impact on the environment. For example, high concentrations of nitrogen, one of the main compounds in wastewater, cause serious environmental problems such as oxygen depletion and eutrophication [1]. The EU Water Framework Directive 91/271/EEC clearly requires EU member states to protect the environment from any adverse effects due to the discharge of (untreated) urban and industrial waters. In this context, new technologies, such as the partial-SHARON/Anammox process, provide a cost-effective way to treat highly contaminated effluent [1, 2]. This combined process is an excellent alternative to conventional nitrification-denitrification processes since it reduces the organic matter (40 %) and oxygen (25 %) required for ammonia removal in comparison to more conventional technologies [3].

In order to fully understand the biodiversity of biological wastewater treatments, it is first necessary to identify the microbiota present and analyze their numerical significance. Culture-dependent methods have sometimes been regarded as inadequate for the analysis of microbial communities in natural environments because of the high numbers of unculturable bacteria. Furthermore, in recent years, molecular methods, based on the sequencing of PCR-amplified the partial 16S rRNA genes from DNA extracted from environmental samples, have been widely used to reveal intrinsic

genetic biodiversity [4]. In particular, denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) approaches yield large quantities of data regarding the diversity of microorganisms in their natural habitats. This has the advantage of permitting the taxonomic classification of community members [5].

The single reactor system for high-activity ammonia removal over nitrite (SHARON) process was described in detail by Hellinga et al. [6], who proposed the partial-SHARON technology. The partial-SHARON process is a modification of the traditional SHARON process, in which 100 % of the ammonium is converted into nitrite. In contrast, the partial-SHARON process, as its name implies, consists of a partial nitritation. More specifically, only 50 % of the ammonium is converted to nitrite. This process was developed for the elimination of ammonium by the "nitrite route" [7]. When the partial-SHARON process is used in combination with the Anammox process, nitrogen removal takes place in two steps. According to Van Dongen et al. [8], the Anammox process achieves an optimal performance with an ammonium–nitrite mixture of 50 % ammonium and 50 % nitrite. For this reason, the Anammox process has to be preceded by a partial-SHARON process involving a partial nitrification.

Molecular techniques have been used to provide a broader vision of the different biotechnological systems in wastewater treatment as shown in recent studies (e.g., [4]). These techniques have been used to obtain a wide range of data regarding microbiota in their habitats. In fact, they facilitate the study of non-cultivable bacteria by specifying the microbial populations that carry out these processes [9, 10]. For this reason, this research analyzed the following: (1) the hydraulic retention time (HRT) in a partial-SHARON reactor in which submerged filters were used to remove nitrogen; (2) the effect of the HRT on the structure of the bacterial community. In our study, molecular fingerprinting tools (PCR-TGGE) and scanning electron microscope (SEM) were used to evaluate the structure of the bacterial community.

Materials and methods

The SHARON bioreactor: bench-scale plant

The bench-scale plant used in our experiments consisted of a plastic SHARON bioreactor with a volume of 3 L. It was constructed as a submerged biofilter with PVC carriers (BioFlow 9). A schematic diagram of the experimental plant is shown in Fig. 1. The bioreactor received synthetic wastewater [2] from a peristaltic pump, and was operated in continuous flow.

The operating conditions in the bioreactor (i.e., HRT, pH, dissolved oxygen concentration, and temperature) were monitored every 24 h in order to verify that they remained stable. Four 15-cm air diffusers at the bottom of the vessel supplied oxygen from an air pump to ensure that the oxygen concentration in the bioreactor was maintained at 2 mg/L. All of the experimental work was performed at a pH of 7.5 and a temperature of 35 C [11, 12], thanks to an adjustable thermostat.

Inoculation of the pilot plant

The partial-SHARON bioreactor was inoculated with mixed liquor from an aerobic reactor located in the Los Vados urban wastewater treatment plant (Granada, Spain). The mixed liquor was recirculated for 3 days until a biofilm formed on the surface of the plastic carriers used in the construction of the submerged biofilter. After inoculation, synthetic wastewater was fed into the bioreactor.

Synthetic wastewater

The synthetic wastewater [2] used in our study simulated the leachate from an anaerobic digester, since it contained a high concentration of ammonium and was low in organic matter (see Table 1).

To prepare the synthetic wastewater, 24 L of distilled water was poured inside the 60-L tank along with the exact quantity of the chemical compounds that made up the synthetic sewage medium. All components were then mixed and dissolved. The influent was continuously fed into the bioreactor by a peristaltic pump (Watson Marlow s-520) that pumped the synthetic wastewater at different flow rates.

Physico-chemical parameters

The physico-chemical parameters analyzed in our study were the following: pH, dissolved oxygen concentration, temperature, and nitrogen concentration in its various

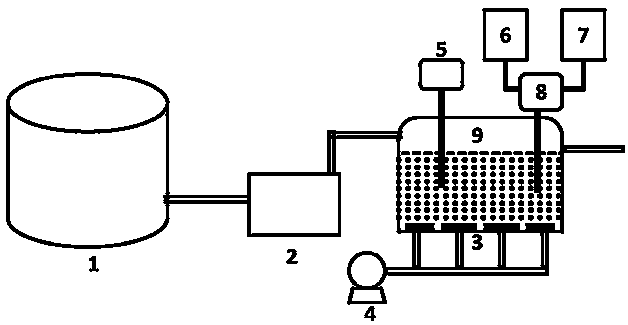

Fig. 1 Diagram of the pilot-scale partial-SHARON bioreactor used in the experiments. 1 Synthetic wastewater tank; 2 peristaltic pump; 3 oxygen diffusers (porous plates); 4 air pump; 5 thermostat; 6 tank of NaOH 0.1 M for pH control; 7 tank of H_2SO_4 0.1 M for pH control; 8 pH meter; 9 partial-SHARON bioreactor stuffed with carriers

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Table 1 Composition of the synthetic wastewater in g/L used in the experiments

Chemical	g/L
$(NH_4)_2SO_4$	2.35
$NaHCO_3$	3.25
$CaCl_2$	0.30
KH_2PO_4	0.07
$MgSO_4$	0.02
$FeSO_4$ $7H_2O$	0.009
H_2SO_4	0.005

Table 2 Conditions of the partial-SHARON bioreactor in experiments 1 and 2

Parameter	Experiment 1	Experiment 2	References
Oxygen demand (mg/L)	1.5	1.5	[13]
pH	7.5	7.5	[14]
Temperature (C)	35	35	[3]
HRT (days)	0.5	0.4	

inorganic forms (ammonium, nitrite, and nitrate). Samples were taken every 24 h because of the slow growth of ammonia-oxidizing bacteria [8].

In constant pH, oxygen, and temperature conditions, two experiments were performed at different HRTs (0.4 and 0.5 day) with a view to analyzing the evolution of inorganic nitrogen concentration in the bioreactor and also the microbial diversity in the biofilm. Table 2 shows the conditions of both experiments.

pH

The pH was measured directly in the bioreactor at 8-h intervals, using a pH meter (Crison GLP 91) [15]. The equipment was adjusted daily with buffer solutions of pH 4.0 and 7.0.

Dissolved oxygen concentration

The dissolved oxygen concentration in the bioreactor was determined by means of a pulse oximeter (CRUCIBLE OXI320), which was calibrated according to the manufacturer's instructions.

Determination of ammonium, nitrite, and nitrate

Concentrations of the various inorganic forms of nitrogen (nitrite, nitrates and ammonium) were measured daily at the entry and exit points of the partial-SHARON bioreactor with an ionic chromatograph Metrohm. Nitrite and nitrate levels were measured with an anion column Metrosep A supp-4-250, and ammonium levels, with a cation column Metrosep C 2-150. A carbonate/bicarbonate solution was used as an eluent. Calibration curves of known concentrations of ammonium, nitrite, and nitrate (10, 500 and 1,000 mg/L) were also analyzed daily.

DNA extraction and PCR amplification of partial bacterial 16S rRNA genes

DNA was extracted from the biofilm that formed in the submerged biofilter. This was done by vortexing approximately 200 mL of plastic carriers from the biofilters with a saline solution, and then centrifuging them to obtain the biofilm fraction. Samples (approx. 200 mg) from the biofilm were collected with the FastDNA Kit and the FastPrep24 apparatus (MP-BIO, Germany).

Polymerase chain reaction (PCR) amplification was performed in two steps, following other research on TGGE and DGGE fingerprinting [4, 9]. One microliter (2–5 ng) of the DNA extracted was used as a template for all the PCRs. At the first PCR, the template was diluted 1:10. High-performance liquid chromatography (HPLC)-purified oligonucleotides were purchased from Sigma. AmpliTaq Gold polymerase (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) was used for all PCRs, which were performed in an Eppendorf Master Cycler (Eppendorf, Hamburg, Germany). Primers and conditions for each of the PCR reactions were those described in Molina-Muñoz et al. [9]. The final PCR products were cleaned and/or concentrated (when required) using Amicon Ultra-0.5 mL Centrifugal Filters (Eppendorf, Hamburg, Germany). Ten microliters (60–100 ng DNA) were loaded into each well for TGGE.

TGGE analysis

TGGE was performed using a TGGE Maxi system (Whatman-Biometra, Goettingen, Germany). The denaturing gels (6 % polyacrylamide [37.5:1 acrylamide:bisacrylamide], 20 % deionized formamide, 2 % glycerol, and 8 M urea) were prepared and run with 2 Tris–acetate-EDTA buffer. All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). The temperature gradient was optimized at 43–63 C [9]. The bands were visualized by silver staining with the Gel Code Silver Staining kit (Pierce, Thermo Fisher Scientific, Rockford, IL, USA). Various PCR reactions were tested, and different TGGE gels were run to check the reproducibility of the results.

Analysis of TGGE fingerprints

The band patterns generated by TGGE were normalized, compared, and clustered by using the Gel Compar II v. 5.101 software (Applied Maths, Belgium). For cluster

analysis, the TGGE profile was compared by means of a band assignment independent method (Pearson product-moment correlation coefficient) as well as a method based on band presence/absence (Dice coefficient). In reference to band assignment, a 1 % band position tolerance (relative to the total length of the gel) was applied [4]. Dendrograms relating band pattern similarities were automatically calculated with unweighted pair group method with arithmetic mean (UPGMA) algorithms. The significance of UPGMA clustering was estimated by calculating the cophenetic correlation coefficients.

Range-weighted richness indices (R_r), which estimate the level of microbial diversity in environmental samples, were calculated, based on the total number of bands in each TGGE pattern (N) and the temperature gradient (C) between the first and last band of each pattern (Tg), following Marzorati et al. [16]. The resulting values were divided by 100 [5] to keep an order of magnitude analogous to that of the R_r index, as originally described for DGGE in Marzorati et al. [16].

Pareto-Lorenz distribution curves rendered a graphical representation of the evenness of the bacterial communities in the different samples, based on the TGGE fingerprints [16]. The bands in each TGGE lane were ranked from highest to lowest based on intensity levels. The cumulative normalized band intensities for each TGGE lane were plotted against their respective cumulative normalized number of bands. The curves were numerically interpreted by the functional organization index (F_o), given by the horizontal y-axis projection on the intercept with the vertical 20 % x-axis line [16]. The calculation of the F_o indexes permitted the evaluation of the functional redundancy of the microbial communities analyzed by fingerprinting methods [16].

DNA reamplification and sequencing

Portions of individual bands on silver-stained TGGE gels were picked up with sterile pipette tips, placed in 10 1L of filtered autoclaved water, and 3 1L of the resulting DNA suspensions were used for reamplification with the appropriate primers. The PCR products were electrophoresed in agarose gels and purified with the Qiaex-II kit (Qiagen, Hamburg, Germany). The recovered DNA was directly used for automated sequencing in an ABI PRISM 3100 Avant Genetic Analyzer (Life Technologies, CA, USA).

Bacterial community analysis

The DNA sequences were analyzed and compared with the biocomputing tools provided online by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Sequence similarity analysis was performed with the BLASTn program [17]. ClustalX v. 2.0.3 software was used for the alignment of the DNA sequences. The graphical distribution of the main bacterial groups found is shown in this article.

Scanning electronic microscopy

The biofilm formed in the submerged biofilter was analyzed by scanning electron microscopy (SEM). Individual pieces of plastic carriers from the biofilter were fixed with glutaraldehyde (5 % v/v) in a 0.2 M sodium cacodylate buffer (pH 7.1), washed, and post-fixed in OsO_4, before being dehydrated with graded ethanol solutions (10, 30, 50, 70, 90, and 100 % ethanol). All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). The samples were transferred to fresh 100 % ethanol and critical point-dried from liquid carbon dioxide at 36.1 C and 7.37 Pa, using a Samdri 780B apparatus (Tousimis, Rockville, USA). Samples were coated with gold before being examined by variable pressure scanning electron microscopy (VP-SEM), model LEO 1430VP-SEM.

Results and discussion

Physico-chemical parameters at different HRT

Experiment 1: HRT of 0.5 day

The partial-SHARON bioreactor was fed with synthetic wastewater at a constant flow rate of 4.16 mL/min and an HRT of 0.5 day. The concentration of ammonium, nitrate, and nitrite was measured at the entry and exit points of the system. These results are shown in Fig. 2.

As can be observed in Fig. 2, after 5 days of operation, 100 % of the ammonium was converted to nitrite. After this period, the partial-SHARON bioreactor stabilized and maintained its high capacity for biotransformation. However, the higher nitrite concentration caused a sharp drop in the pH of the bioreactor. To correct this, it was necessary to add small amounts of NaOH 1 % (p/v), which kept the pH value at 7.5.

When the biotransformation capacity of ammonium into nitrite in submerged biofilters was compared with that of other systems such as conventional partial-SHARON bioreactors [8, 12], the results showed that submerged biofilters have higher levels (three times higher) of biotransformation. The high transformation capacity of submerged-biofilter systems should be regarded as an important operational factor for the development and future design of partial-SHARON/Anammox systems, which can be applied to the treatment of effluents with high nitrogen content such as landfill leachate [18].

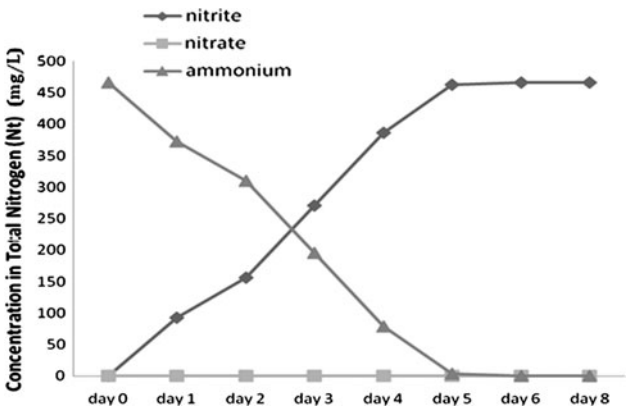

Fig. 2 Values of ammonium and nitrite expressed as total nitrogen detected in the effluent of a partial-SHARON bioreactor over time with an HRT of 0.5 day

Experiment 2: HRT of 0.4 day

Experiment 2 was performed at an HRT of 0.4 day and a constant flow rate of 5.20 mL/min of synthetic wastewater. In the same way as in experiment 1, the concentration of ammonium, nitrate, and nitrite was measured at the entry and exit point of the partial-SHARON bioreactor. The results are shown in Fig. 3.

As can be observed in Fig. 3, the transformation of ammonium into nitrite reached 60 % after 5 days of operation. After this period, the partial-SHARON bioreactor stabilized, and its capacity for the biotransformation of ammonia to nitrite remained constant. The increased nitrite concentration caused a sharp drop in the pH level of the bioreactor. To correct this, it was necessary to add small amounts of NaOH 1 % (p/v) to maintain the pH value at 7.5.

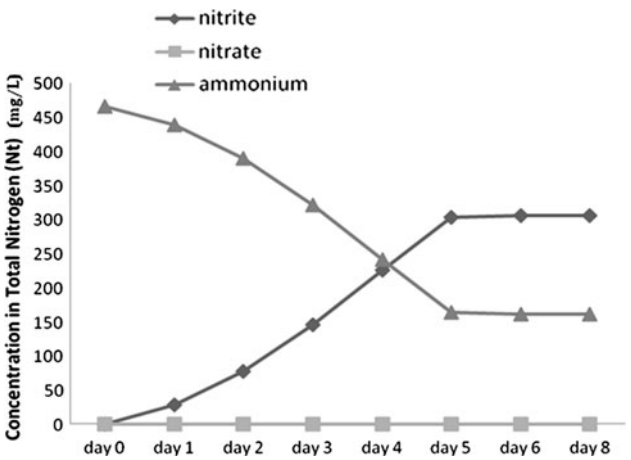

Fig. 3 Values of ammonium and nitrite expressed as total nitrogen detected in the effluent of a partial-SHARON bioreactor over time with an HRT of 0.4 day

The results obtained in the submerged-biofilter partial-SHARON system showed that working at experimental conditions of temperature (35 C), oxygen concentration (1.5 mg/L), pH (7.5), and HRT from 0.5 to 0.4 days, an evident reduction in the biotransformation of ammonium to nitrite was observed when the HRT was decreased. When the bioreactor was operating at an HRT of 0.5 day, 100 % of the ammonium was converted to nitrites, whereas when the bioreactor was operating at an HRT of 0.4 day, only 60 % of the ammonium was converted to nitrites. However, undetectable amounts of nitrates were produced at the exit point of the partial-SHARON bioreactor. This low capacity of transformation of ammonium to nitrate in the bioreactor can be due to the operational conditions of the system that increase the biological activity of the ammonium-oxidizing bacteria and decrease the biological activity of the nitrite-oxidizing bacteria. In this sense, according to the bacterial community analysis obtained in our study (described below), the use of an HRT of 0.5 days, determined the production of highly specialized biofilms mainly integrated by Nitrosomonas sp., which are very effective in the oxidation of ammonium into nitrite.

According to Van Dongen et al. [8], the optimal ammonium and nitrite ratio in the effluents in partial-SHARON systems for their combination with Anammox bioreactors is 50 % ammonium and 50 % nitrite. In this context, our data suggest that in submerged-biofilter partial-SHARON systems, the ammonium–nitrite ratio can be modified by the HRT. Moreover, the results obtained in our experiments show that the submerged-filter technology applied to partial-SHARON processes increased the transformation of ammonium into nitrite and decreased the time required for the start-up of the bioreactors. This is evident when the data obtained in submerged-biofilter systems are compared with other technologies [12, 18, 19].

Study of the bacterial diversity in the partial-SHARON bioreactor

The structure of bacterial communities was analyzed by TGGE fingerprinting. The prevalent TGGE bands indicated the phylogenetic groups. The sequencing of the TGGE bands revealed that the prevalent bacteria populations were developmentally close to Proteobacteria and specifically to Alphaproteobacteria, Betaroteobacteria, Gammaproteobacteria, and Deltaproteobacteria. The bacteria populations in the partial-SHARON bioreactor varied, depending on operational conditions. Accordingly, the PCR-TGGE method showed significant differences in the structure of the bacteria community at HRTs of 0.5 and 0.4 day (see Fig. 4). The Pearson coefficient-based analysis permitted the identification of four clusters corresponding to the different treatments analyzed. On the other hand, the Dice

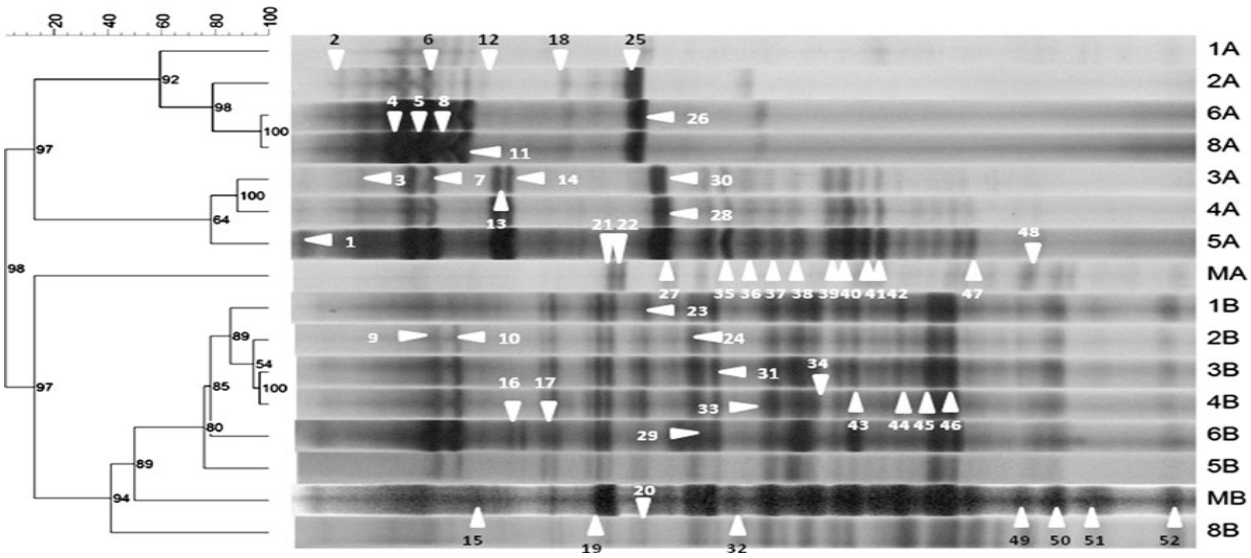

Fig. 4 Pearson coefficient-based analysis of the band patterns generated from 52 samples analyzed in the partial-SHARON bioreactor. Samples named with letter A corresponds to the first experiment and letter B corresponds to the second experiment. The numbers indicate the days on which samples were extracted from the bioreactor. MA and MB samples were taken at the beginning of the first and second experiment, respectively, related to zero time

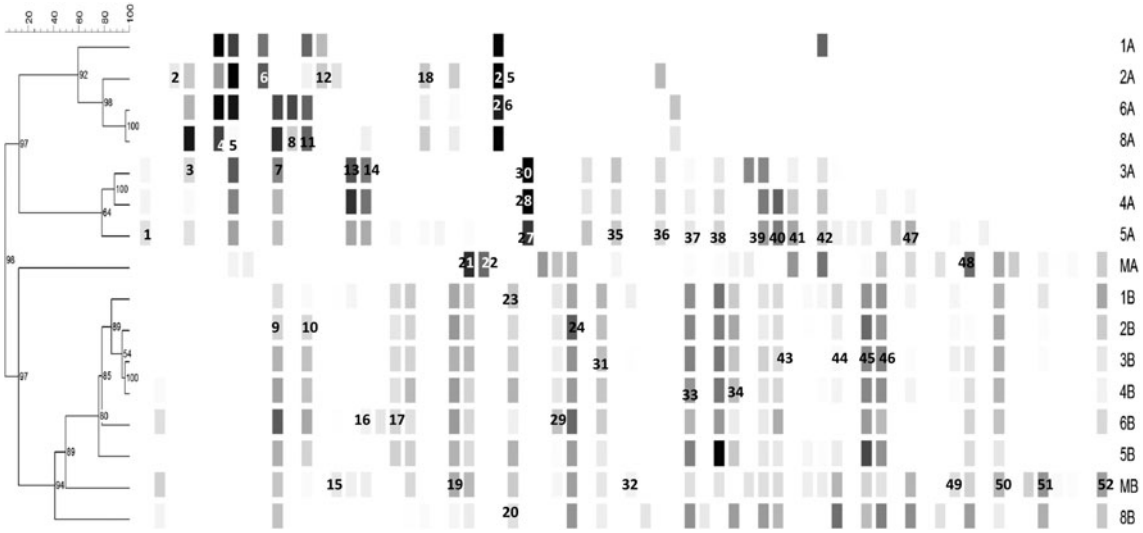

Fig. 5 Dice coefficient-based analysis of band patterns generated from all samples analyzed with a presence/absence matrix

coefficient was used to obtain 66 unique band classes in both experiments (see Fig. 5).

Study of biofilms formed at an HRT of 0.5 day

The TGGE profiles demonstrated that when the partial-SHARON system operated at an HRT of 0.5 day, a significant number of bands disappeared 48 h after its start-up (Fig. 4). For example, bands 21 and 22 related to Pseudacidovorax sp. and Aquaspirillum sp., vanished completely. However, after 2 days of operation, new bands were detected in the TGGE gels. Finally, the PCR-TGGE studies showed how the bacteria populations of the biofilms in the partial-SHARON system began to stabilize after 4 days of operation. Moreover, some bands gained in intensity over time, such as bands 4 (Nitrosomonas

eutropha), 14 (Variovorax sp.), and 27 (Nitrosomonas europaea).

After the stabilization of the partial-SHARON bioreactor (5 days after the pilot plant start-up), when 100 % of the ammonium was converted into nitrite, there was a significant decrease in the bacterial biodiversity of the biofilms in the submerged biofilter. Consequently, bands 38, 39, 40, 41, 42 and 47 were no longer detected in the TGGE gels (Fig. 4). In contrast, certain bands, such as 4 and 11 (N. europaea and N. eutropha), gained in intensity. In addition, a new band (band 8) appeared that was related to Diaphorobacter sp.

According to Khan and Hiraishi [20], and Anshuman et al. [21], Diaphorobacter sp. is an interesting bacteria in nitrogen removal processes. Nevertheless, to our knowledge, this is the first time that these microorganisms have been observed in a partial-SHARON bioreactor. Furthermore, our data showed that N. europaea and N. eutropha, which have a great affinity for ammonium, were prevalent over the rest of the ammonium-oxidizing bacteria [22, 23].

Samples 1A and 2A were collected from the partial-SHARON bioreactor when the concentration of ammonium was high and the concentration of nitrite very low. They showed a balanced community with low microbial diversity (Fig. 4). Similar results were obtained in samples 6A and 8A, which were taken from the bioreactor when the concentration of ammonium was very low and the concentration of nitrite was high (Fig. 4). This reflects that in extreme environments of high concentrations of ammonium or nitrite, microbial diversity decreases in the biofilms in order to preserve its functionality in changing environmental conditions.

The Pearson coefficient-based analysis (Fig. 4) allowed for the identification of two separate clusters of Bacteria, which corresponded to samples taken at two different sampling times: the start-up point and the stabilization point of the bioreactor. The sample taken at the start-up point clustered at 60 % and the one taken at the stabilization point clustered at 80 %, which indicated a good relationship between the compositions of the two bacteria cluster communities. Cluster analysis based on the Dice coefficient (Fig. 5) showed the same results as the Pearson-based clustering.

A total of 24 bands selected from the TGGE fingerprints targeting bacteria were successfully amplified and sequenced from TGGE gels (Table 3), corresponding to the dominant bacteria populations in biofilms formed in the partial-SHARON bioreactor. A prevalence of Proteobacteria in the set of sequences analyzed was found in the sampling periods. The main group of identifiable TGGE bands was related to Betaproteobacteria (59 %), whereas in order of abundance, the second group was Alphaproteobacteria (36 %) and the third group was Gammaproteobacteria (5 %) (Fig. 6). Ten TGGE bands were reamplified and sequenced from the TGGE gels corresponding to the dominant Nitrosomonas populations in the partial-SHARON bioreactor (Table 3). Five of these were closest to N. eutropha and five to N. europaea. Three sequences were related to Diaphorobacter in the TGGE gels.

In conclusion, the results of this experiment demonstrated that Proteobacteria and members of the genus Nitrosomonas dominated the composition of the bacteria communities of the submerged-biofilter partial-SHARON bioreactor at an HRT of 0.5 day. However, nitrite-oxidizing bacteria such as Nitrobacter were not detected in the TGGE gels.

Study of biofilms formed at an HRT of 0.4 day

In the second experiment, which was performed in the partial-SHARON bioreactor at an HRT of 0.4 day, the Pearson coefficient (Fig. 4) showed only one cluster of bacteria, which clustered at 80 % similarity. This result clearly indicates that there was less variation in the samples over time. Interestingly, the bands belonging to bacteria, such as Roseobacter sp. (Band 32) or Burkholderia sp. (band 50) disappeared, depending on the operating time. However, other microorganisms such as Nitrosospira sp. (band 10), Nitrosomonas sp. (band 10) and Paracoccus sp. (band 46) increased in abundance.

According to Hiroaki and Hiroshi [24], Paracoccus sp. is a common bacterium in wastewater treatment bioreactors with an important role in nitrogen removal. On the other hand, several bands had a high intensity level. This was the case of bands 31 (Vibrio sp.), 45 (Rhodobacter sp.), and 46 (Catellibacterium sp.), among others. The increasingly high intensity of these bands indicates that the development of these bacteria was favored by these conditions [25–27].

A total of 14 bands selected from the TGGE fingerprints targeting bacteria were successfully amplified and sequenced (Table 4). These bands corresponded to the dominant bacteria populations in the partial-SHARON bioreactor. The main group of identifiable TGGE bands was related to Proteobacteria and specifically to Alphaproteobacteria (56 %), Betaproteobacteria (40 %) and Delta-Protobacteria (4 %) (Fig. 6). Four TGGE bands were reamplified and sequenced from the TGGE gels corresponding to the dominant ammonium-oxidizing bacteria populations (Nitrosospira, Nitrosomonas and Nitrosovibrio) in a partial-SHARON bioreactor (Table 4). In these experiments, Nitrosospira sp. and Nitrosovibrio sp. were detected as a normal microbiota in the bioreactor working at an HRT of 0.4 day. However, these microorganisms were not identified in the TGGE gels when the bioreactor was operating at an HRT of 0.5 day. In all likelihood, when the HRT of the partial-SHARON bioreactor was reduced

Table 3 Bacteria obtained from the NCBI database from the sequencing of the bands extracted in experiment 1 (HRT 0.5 day)

No. band identification	Identities (bp)	% similarity	Experiment 1: name sequence reference	Phylogenetic class
1	97	96	HQ113216.1 Hydrogenophaga sp. CL-9.06	Betaproteobacteria
		96	GU300152.1 Diaphorobacter oryzae strain 3R2-14	Betaproteobacteria
3	85	100	GQ284427.1 Acidovorax delafieldii strain THWCSN39	Betaproteobacteria
		100	U51105.1 Denitrifying Fe＼II ▛-oxidizing bacteria	Betaproteobacteria
4	83	100	M96402.1 Nitrosomonas eutropha	Betaproteobacteria
		100	HM446362.1 Nitrosomonas europaea strain PD60	Betaproteobacteria
5	109	100	M96402.1 Nitrosomonas eutropha 16S ribosomal RNA	Betaproteobacteria
		100	AY856378.1 Nitrosomonas sp. CNS332 16S ribosomal RNA	Betaproteobacteria
8	75	100	HQ183880.1 uncultured beta proteobacterium clone De385 16S	Betaproteobacteria
		98	GU300152.1 Diaphorobacter sp. 16 s ribosomal RNA	Betaproteobacteria
11	92	100	M96402.1 Nitrosomonas eutropha 16S ribosomal RNA	Betaproteobacteria
		100	HM446362.1 Nitrosomonas europaea strain PD60	Betaproteobacteria
12	83	100	HM921137.1 uncultured bacterium clone ar2e1016	Betaproteobacteria
		100	HM001269.1 Methylophilus glucoseoxidans strain B	Betaproteobacteria
13	91	100	HM124369.1 Rhodobacter sp. 16-62 16S ribosomal RNA	Alphaproteobacteria
		100	EU652478.1 Catellibacterium sp. JPB-2.07 16S ribosomal RNA	Alphaproteobacteria
14	107	100	HQ385754.1 Variovorax sp. 2C1-21 16S	Betaproteobacteria
		100	EF203908.1 Variovorax paradoxus isolate DB1	Betaproteobacteria
21	98	100	HQ259687.1 Pseudacidovorax sp. A14(2010)	Betaproteobacteria
		100	AF384190.1 Aquaspirillum sp. TG27	Betaproteobacteria
22	98	100	HQ259687.1 Pseudacidovorax sp. A14(2010)	Betaproteobacteria
		100	AF384190.1 Aquaspirillum sp. TG27	Betaproteobacteria
25	125	97	AJ245760 uncultured beta proteobacterium partial 16S rRNA	Betaproteobacteria
	124	97	JN217090 uncultured bacterium clone S252 16S ribosomal RNA	Betaproteobacteria
26	113	100	GU980069.1 uncultured bacterium clone HKTJ485	Betaproteobacteria
		100	EU542425.2 uncultured bacterium clone Er-MS-1	Betaproteobacteria
27	83	100	M96402.1 Nitrosomonas eutropha 16S ribosomal RNA	Betaproteobacteria
		100	HM446362.1 Nitrosomonas europaea strain PD60	Betaproteobacteria
28	97	100	HQ183880.1 uncultured beta proteobacterium clone De385	Betaproteobacteria
	96	98	GU300152.1 Diaphorobacter oryzae strain 3R2-14	Betaproteobacteria
30	96	100	M96402.1 Nitrosomonas eutropha 16S ribosomal RNA	Betaproteobacteria
		100	HM446362.1 Nitrosomonas europaea strain PD60	Betaproteobacteria
35	92	100	EU445263.1 Agrobacterium tumefaciens isolate EFLRI 121	Alphaproteobacteria
		100	AJ784210.1 Rhizobium sp. P033 partial 16S rRNA gene	Alphaproteobacteria
37	98	100	GU574708.1 Parvibaculum sp. EPR92	Alphaproteobacteria
		100	FJ528267.1 Rhizobium sp. Cs218	Alphaproteobacteria
38	95	95	GQ351376.1 uncultured bacterium isolate DGGE gel band	Alphaproteobacteria
39	75	100	X87274.1 B.diminuta 16S rRNA gene	Alphaproteobacteria
		100	U63935.1 Caulobacter sp. 16S ribosomal RNA gene	Alphaproteobacteria
40	76	100	GU949635.1 uncultured bacterium clone 4EU1038B12	Alphaproteobacteria
	73	100	EU256442.1 Mesorhizobium mediterraneum strain CCBAU	Alphaproteobacteria
41	84	100	GU420646.1 Defluvibacter lusatiensis clone AW171	Alphaproteobacteria
		100	GU415542.1 Ochrobactrum anthropi clone AW034	Alphaproteobacteria
42	99	100	EU635967.1 uncultured bacterium isolate DGGE band 13	Alphaproteobacteria
	98	98	FJ587218.1 Pseudoxanthobacter sp.	Alphaproteobacteria
47	90	100	HM629504.1 Escherichia coli strain BAB-286	Gammaproteobacteria
	89	98	HM629493.1 Salmonella enterica strain	Gammaproteobacteria

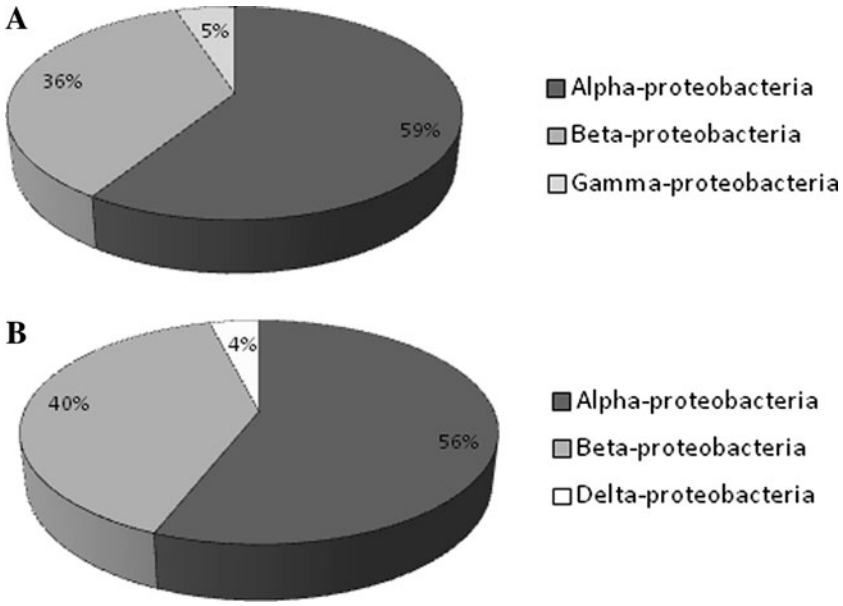

Fig. 6 Phylogenetic classes (%) detected in the partial-SHARON bioreactor at HRT of 0.5 day (a) and HRT of 0.4 day (b) analyzed by PCR-TGGE method

Table 4 Bacteria obtained from the NCBI database from the sequencing of the bands extracted in experiment 2 (HRT 0.4 day)

No. band identification	Identities (bp)	% similarity	Experiment 2: name sequence reference	Phylogenetic class
9	78	98	X84662.1 Nitrosospira sp. 16S rRNA gene	Betaproteobacteria
		98	M96405.1 Nitrosovibrio tenuis 16S ribosomal RNA	Betaproteobacteria
10	95	100	M96402.1 Nitrosomonas eutropha 16S ribosomal RNA	Betaproteobacteria
		100	HM446362.1 Nitrosomonas europaea strain PD60	Betaproteobacteria
15	99	100	FJ222605.1 Albidovulum sp. S1K1	Alphaproteobacteria
		100	HM705035.1 uncultured bacterium clone GB7N87002DSSDY	Alphaproteobacteria
16	65	100	GQ853528.1 uncultured Ochrobactrum sp.	Alphaproteobacteria
17	74	100	EF195167.1 Alcaligenes sp. RG-03/06 16S	Betaproteobacteria
24	70	100	HM001269.1 Methylophilus glucoseoxidans strain B	Betaproteobacteria
		100	GQ411499.1 Methylophilus methylotrophus strain NBCS15	Betaproteobacteria
31	98	100	EF079668.1 Thiobacillus sp. K6.2	Betaproteobacteria
	97	98	FM957479.1 Vibrio sp. MY-2008-U67	Betaproteobacteria
32	78	100	AM710422.1 uncultured bacterium partial 16S rRNA gene	Alphaproteobacteria
	79	98	AY576768.1 Roseobacter sp. 3X/A02/234	Alphaproteobacteria
33	87	95	AM922185.1 Sphingopyxis sp. Sulf-541	Alphaproteobacteria
34	94	98	HM687288.1 uncultured bacterium clone GB7N87001BDHL1	Alphaproteobacteria
	93	97	HQ596322.1 Bradyrhizobium sp. CNX333	Alphaproteobacteria
45	82	100	100 % HM124369.1 Rhodobacter sp. 16-62	Alphaproteobacteria
		100	100 % EU652478.1 Catellibacterium sp. JPB-2.07	Alphaproteobacteria
46	91	100	100 % HM124369.1 Rhodobacter sp. 16-62 16S ribosomal RNA gene	Alphaproteobacteria
52	52	100	100 % GQ183899.1 Geothermobacter sp.	Deltaproteobacteria

from 0.5 to 0.4 day, this caused the biodiversity of this specialized microbial group to increase.

In conclusion, the results of this experiment demonstrated that Proteobacteria and members of the genus Nitrosomonas, Nitrosospira, and Nitrovibrio dominated the composition of the bacterial community of the submerged-biofilter partial-SHARON bioreactor at an HRT of 0.4 day. However, as previously mentioned, nitrite-oxidizing bacteria such as Nitrobacter were not detected in the TGGE gels.

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Comparison of the bacterial diversity obtained in experiment 1 (HRT of 0.5 day) and experiment 2 (HRT of 0.4 day)

A comparison of the results of the two experiments seems to indicate that the modification of the HRT affected the bacterial diversity of the biofilms formed in a partial-SHARON system with a submerged filter. In the experiment performed at an HRT of 0.5 day, bacterial diversity was significantly reduced when the bioreactor operated in stable conditions (after 5 days). In fact, fewer than 10 bands were observed, probably as a consequence of the high level of specific ammonium-oxidizing bacteria. In these conditions, 100 % ammonium was converted into nitrite, and thus all the microbial population was obliged to compete for ammonium. However, in experiment 2, in which the HRT of the bioreactor was adjusted to 0.4 day, the microbial biodiversity was extremely heterogeneous. This result could explain the evenness observed in the bacterial community. Since 65.5 % of the ammonium was converted to nitrite, this led to a less specialized bacterial community (see Figs. 4, 5). These data are in consonance with the results reported in Logemann et al. [28], and Marzorati et al. [16].

Table 5 Average range-weighted richness (R_r) and functional organization (F_o) indices of the bacterial communities in partial-SHARON bioreactor samples from the two experiments in this study

Experiment	No. of samples	R_r	F_o	Statistic program
1	8	2*	49	ANOVA analysis
2	8	5*	43	ANOVA analysis

* Statistically significant difference (Student's t test, $p < 0.05$)

The comparison of the results obtained in the two experiments highlighted the similarity of different bands. This indicated the presence of certain microorganisms, such as Nitrosomonas sp., which were constant in both experiments (see Tables 3, 4). This fact is hardly surprising since this microbial group can be regarded as predominant in an extreme environment with a high dilution rate, a temperature of 36 C, and a high ammonia concentration [29]. These results suggest the significance of Nitrosomonas sp. in the biofilm formed in the submerged-filter partial-SHARON system and its important role in the biotransformation of ammonium into nitrite in this wastewater treatment biotechnology [22, 23].

Image analysis with Gel Compar II detected a total of 66 unique band classes in the TGGE fingerprints of bacteria among the 52 bands detected (Fig. 5). A total of 38 bands selected from the TGGE fingerprints targeting bacteria were successfully amplified and sequenced, representing the 73 % of the bands chosen for sequencing (Tables 3, 4).

The richness range-weighted (R_r) indices [14] showed significant differences with ANOVA analysis ($p < 0.05$) in both experiments (Table 5). The R_r of experiment 2 (HRT of 0.4 day) displayed higher average values than those of experiment 1 (HRT of 0.5 day). Construction of the Pareto-Lorenz curves of the bacterial community profiles in

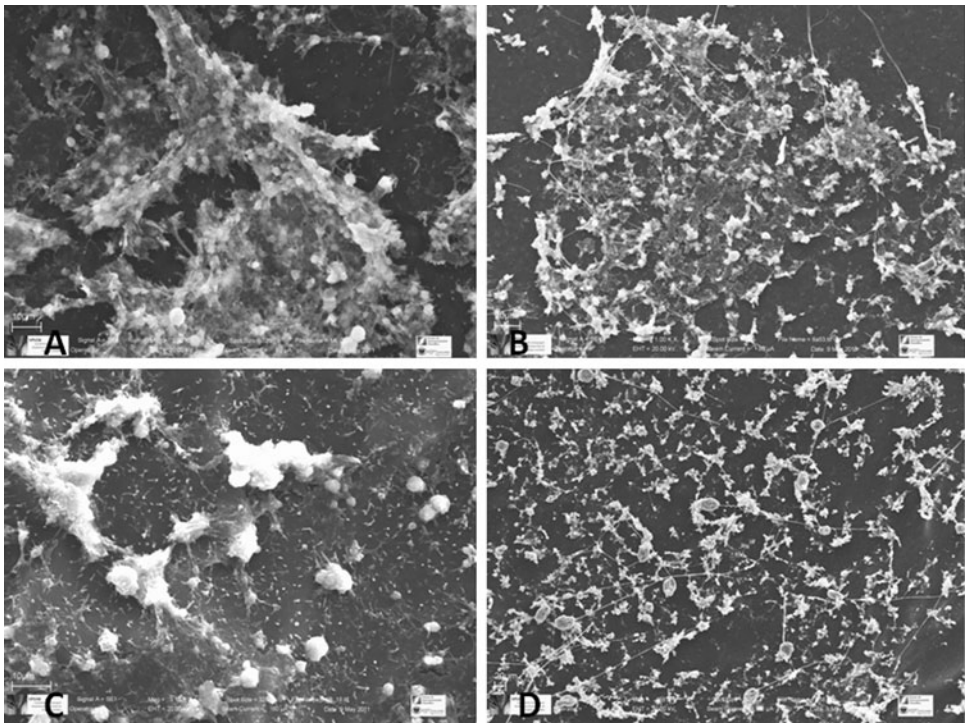

Fig. 7 SEM of the carriers used in a submerged-filter partial-SHARON bioreactor in different conditions. a Microscopic images of day 1 with an HRT of 0.5 (sample 1A); b microscopic images of day 8 with an HRT of 0.5 (sample 8A); c microscopic images of day 1 with an HRT of 0.4 (sample 1B); d microscopic images of day 8 with an HRT of 0.4 (sample 8B)

experiment 2 permitted the calculation of the functional organization (F_o) indices [14].

F_o indices of 49 and 43 % were obtained in the experiments 1 and 2, respectively. According to Marzorati et al. [16], F_o index values of around 45 % represent a balanced community, potentially able to preserve its functionality under changing environmental conditions. Obviously, these results reflect the higher specialization of the bacterial community in the submerged-filter partial-SHARON bioreactor and its capacity to adapt to different working conditions.

Scanning electronic microscopy

The colonization of the plastic carrier, used in the construction of the submerged-filter partial-SHARON bioreactor with an HRT of 0.5 and 0.4 day, was studied after 1 and 8 days (see Fig. 7). The results showed a rapid colonization of the carrier with the formation of a complex and heterogeneous biofilm. At the end of both experiments, the samples were found to contain a large number of different morphological types as well as filamentous bacteria. These results coincide with the data previously reported in this paper, which suggest the presence of complex microbiota in both experiments, independently of the HRT used in the wastewater system.

Conclusions

The results obtained in our study within the context of recent research in the field lead to the conclusion that the HRT affects the functioning of a partial-SHARON bioreactor built with submerged-filter technology. This is reflected in different levels of biotransformation of ammonium into nitrite. This signifies that the application of submerged-filter technology to the partial-SHARON system increases the biotransformation of ammonium into nitrite, in comparison to other technologies such as a fluidized bed. Moreover, our results show that the HRT affects the microbial diversity of biofilms formed in a partial-SHARON bioreactor, possibly as a result of the different nutritional conditions that arise when this variable is modified.

On the other hand, the use of an HRT of 0.5 day determines the formation of highly specialized biofilms (mainly by Nitrosomonas sp.), which are effective in the biotransformation of ammonium into nitrite. On the contrary, the use of an HRT of 0.4 day, determines the formation of more heterogeneous biofilms that allow a closer ammonium/nitrite ratio, which is more effective for the combined development of partial-SHARON/Anammox systems.

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Chapter 3

Study of nitrifying microbial communities in a partial-nitritation bioreactor

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Study of nitrifying microbial communities in a partial-nitritation bioreactor

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abstract

The present study focused on the technical and biological characteristics of a bench-scale partial-nitritation bioreactor and established its operating parameters. In this manner 2 bench-scale submerged-bed bioreactor of 3 L were operated under identical conditions of pH, oxygen concentration and temperature but under different hydraulic retention time (9 and 12 h). This made it possible to study the influence of the hydraulic retention time (HRT) on the nitrification processes and on the nitrifying microbiota of the biofilms. Moreover, specific bacterial groups involved in the nitrification process, such as ammonium oxidizing (CTO) and nitrite oxidizing (nxrA) were investigated using a cultivation-independent approach based on PCR-TGGE fingerprinting. The results showed that the HRT may affect the nitrification processes of a partial-nitritation bioreactor using a synthetic wastewater containing 600 mg/L of ammonia. It was found that HRT of 12 h transformed 100% of the ammonium to nitrite. However, when the HRT was 9 h there was a significant reduction (35%) in ammonia converted. Cluster analysis of PCR-TGGE fingerprints showed significant differences in the profiles depending on the different HRT applied, especially on the ammonia oxidizing bacteria. The importance of this factor was confirmed by multivariate analysis. Phylogenetic analysis of bands sequences showed that CTO and nxrA sequences presented similarity to those present in the database and grouped in specific clusters. Our results suggested that changes in HRT can affect significantly the nitrifying microbial community and the performance of the partial nitritation system.

1. Introduction

One of the most serious ecological problems in the world is the proliferation of wastewater, since human activities have greatly accelerated and extended the natural cycles of nitrogen in the soil, water and atmosphere. In conventional wastewater treatment plants (WWTPs), nitrogen is often removed by the biological processes of nitrification and denitrification. Nitrification involves the oxidation of ammonia to nitrite and the subsequent oxidation of nitrite to nitrate under aerobic conditions, requiring 2.5 mol of oxygen per mol of NH_4–N. The nitrate generated is then denitrified to NO_2 in the presence of an organic carbon source to dinitrogen (Mosquera-Corral et al., 2005). However, this conventional process is not suitable for the treatment of effluents such us dewatering concentrate stream due to the toxic effects that occur, even to those microorganisms able to degrade it (Van Hulle et al., 2005). New and sustainable technologies are needed to comply with the stringent discharge standards (Van Loosdrecht et al., 2004). Thus in recent years more efficient and cost effective alternative systems have been developed for the removal of this nutrient, such us partial-nitritation Anammox technology.

The partial-nitritation process, is a technique in which nitrification is achieved with nitrite as the intermediate under stable process conditions, where only 50% of the ammonium in the influent is converted into nitrite. This system was described in detail by Hellinga et al. (1998), and its combination with the Anammox (Anaerobic Ammonium Oxidation) process has led to the development of a new technology of great interest in the treatment of effluents with a high content of N.

Nowadays, biological wastewater treatments are considered more effective and relatively inexpensive and have been widely adopted instead of the physicochemical processes. Thus, the study of microbial diversity in biological systems utilised for the treatment of urban or industrial wastewater represents one of the best

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ways to improve the efficiency of biological processes in the different wastewater treatment technologies. For this purpose, questions about community structure, activity and population kinetics have to be answered by means of molecular monitoring tools, which allow identifying and quantifying the microorganisms present in the WWTP. One of the major advances in the field of microbial ecology is the introduction of molecular biology techniques based on the in situ detection of nucleic acids (Ji et al., 2013). Several methods are available for assessing the abundance or diversity of bacterial communities in ecosystems, such as fluorescence in situ hybridisation (FISH) and fingerprinting methods (Sun et al., 2012). Denaturing and temperature gradient gel electrophoresis (DGGE/TGGE) are fingerprinting methods which yield extensive information about the diversity of microorganisms in their habitats, also allowing the taxonomic identification of community members (Muyzer, 1999). These data allow the monitoring of variations in the community profiles due to external factors. Both techniques were often used in recent studies on the ecology of biological processes in WWTPs, providing interesting new data in this area (Molina-Muñoz et al., 2009).

The biofilm technologies for wastewater treatment are an alternative to the suspended growth activated sludge process (Schlegel and Koeser, 2007). Researchers have been trying to correlate the microbial community structures of biofilms with the performance of wastewater treatment, and reported the link between them and the efficiencies of nitrification, denitrification and phosphorus removal. Thus knowledge of the microbial community's composition involved in the biofilm processes and the influence of operating conditions on their structure is regarded as crucially important for the optimisation of nutrient removal rates on submerged fixed bed bioreactor systems and to implement control strategies. Determining the identity of microorganisms responsible for specific biotransformation processes in complex environments remains one of the major challenges in environmental microbiology and environmental engineering (Calderón et al., 2012). The aim of our study was to analyse the effect of HRT on the performance and community structure of ammonium oxidizing bacteria and nitrite oxidizing bacteria in a partial-nitritation bioreactor assembled as a fixed bed biofilm reactor.

2. Materials and methods

2.1. Operating conditions of the bench-scale partial-nitritation bioreactor

Two partial-nitritation bioreactors bench-scale plants were constructed as a submerged bed with PVC carriers (Bioflow 9®) with a volume of 3 L. The Bioflow 9® with filigree structure shows a very high specific surface area of 800 m^2/m^3. It is mostly used for wastewater with low organic load. A schematic diagram of the experimental plants is shown in Fig. 1.

The operating conditions in the bioreactors (i.e. HRT, pH, dissolved oxygen concentration and temperature) were monitored continuously during the whole operational period to ensure that they remained constant. Four 15 cm porous plates at the bottom of the vessel supplied oxygen from an air pump to maintain a constant concentration of 1.5 mg/L. All the experimental work was performed at pH 7.5 and temperature of 35 °C thanks to an adjustable thermostat.

The partial-nitritation bioreactors were inoculated with the same mixed liquor from an aerobic reactor located in the Los Vados urban wastewater treatment plant (Granada, Spain). The mixed liquor was recirculated for three days until the appearance of a biofilm on the surface of the plastic carriers and acclimated under defined laboratory conditions for 5 days (Kaewpipat and Grady, 2002). During acclimation the partial nitritation bioreactors were fed with synthetic wastewater.

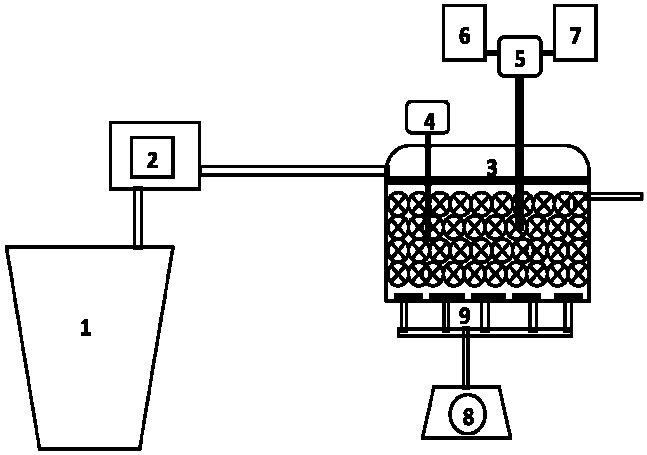

Fig. 1. Diagram of the bench-scale partial nitrification (Partial-SHARON) bioreactor used in the experiments.

The synthetic wastewater (Mosquera-Corral et al., 2005) used in this research simulated leachate from an anaerobic digester, since it contained a high concentration of ammonium and was low in organic matter. The chemical composition of the synthetic wastewater can be observed in Table 1.

To prepare the synthetic wastewater, 24 L of distilled water were poured inside a 60 L tank along with the exact quantity of chemical compounds that made up the synthetic sewage medium. Later others 25 L of distilled water were added and all components were then mixed and dissolved. The influent was continuously fed into the bioreactors by a peristaltic pump (Watson Marlow s-520), that pumped the synthetic wastewater at different flow rates.

2.2. Physico-chemical parameters

The physico-chemical parameters analysed were the following: pH, dissolved oxygen concentration, temperature and nitrogen concentration in its various forms (ammonium, nitrite, and nitrate). All samples for ammonium, nitrite and nitrate determination were taken every 24 h for 30 days.

Under constant conditions, two experiments in parallel were performed at different HRT (9 and 12 h) with a view to analysing the evolution of nitrogen concentration in the bioreactors and also the microbial diversity in the biofilms. During both experiments the pH was constant at 7.5 (Van Hulle et al., 2005; Tao et al., 2012). This parameter was continuously measured in the bioreactors using a pH meter (ORION). The equipment was adjusted automatically with buffer solutions of pH 4.0 and 7.0. On the other hand, the Oxygen was supplied by means of air gasification through the liquid phase using porous plates to obtain small air bubbles kept

Table 1
Chemical composition of the synthetic wastewater in g/L used in the experiments.

Chemical	g/L
$(NH_4)_2SO_4$	2.35
$NaHCO_3$	3.25
$CaCl_2$	0.30
KH_2PO_4	0.07
$MgSO_4$	0.02
$FeSO_4 \cdot 7H_2O$	0.009
H_2SO_4	0.005
EDTA	0.006

the oxygen concentration constant at 1.5 mg/L during the whole operational period (González-Martínez et al., 2012).

The concentration of the various forms of nitrogen (nitrite, nitrate and ammonium) was measured daily at the entrance and exit points of the partial-nitritation bioreactor with an ionic chromatograph (Metrohm). Nitrite and nitrate levels were measured with an anion column (Metrosep A supp-4-250), and ammonium levels with a cation column (Metrosep C 2-150). A carbonate/bicarbonate solution was used as the eluent. Calibration curves of known concentrations (10, 500 and 1000 mg/L) of ammonium, nitrite, and nitrate were also analysed daily.

2.3. DNA extraction and PCR amplification

To perform TGGE analysis, samples were taken every 24 h during acclimation inoculum process until the system achieved stabilisation (samples from 0A to 5A for the first experiment and from 0B to 5B for the second). After stabilisation, samples were taken on days 10, 15 and 30 (10A, 15A and 30A) for the first bioreactor and days 15 and 30 (15B and 30B) for the second bioreactor, both working in parallel. Total DNA was extracted from the biofilm formed in the bioreactors as follows: approximately 200 mL of plastic carriers obtained from different representative areas of each bioreactor were stirred by vortex with saline solution and then centrifuged to obtain the biofilm fraction. Samples obtained (approx. 200 mg) were used for DNA extraction by using the FastDNA Kit and FastPrep24 apparatus (MP-BIO, Germany) according to the manufacturer's instructions.

Two different genes were used as the target for the study of nitrifying the microbial communities present in the bioreactors: 16S rDNA gene (CTO) fragment for the ammonia-oxidizing bacteria and the nitrite -oxidoreductase gene nxrA for nitrite-oxidizing bacteria.

A two-step PCR (nested PCR) approach was selected for amplification of all target genes, as reported by various authors for TGGE or DGGE fingerprinting of each specific gene (Kowalchuk et al., 1997; Wertz et al., 2008). One microlitre (2–5 ng) of the DNA extracted was used as a template for all first step amplifications. All primers were purified by HPLC and purchased from Sigma Aldrich (St. Louis, MO, USA).

Amplification of the 16S rDNA gene was carried out with CTO189f and CTO654r primers, which are specific to the majority of -proteobacterial ammonia oxidizing bacteria as reported by Kowalchuk et al. (1997). PCR products were then diluted at 1:20 to prevent amplification of non-target sequences (Freitag et al., 2006), and used as the template for the second PCR using universal primers GC-P1 and P2. The program for the first PCR was: 7 min at 95 °C followed by 35 cycles of 95 °C for 30 s, annealing at 57 °C for 1 min and elongation at 68 °C for 45 s with terminal elongation at 68 °C for 5 min. The second PCR was carried out according to the method reported by Molina-Muñoz et al. (2009).

For nitrite -oxidoreductase gene nxrA amplification, first the PCR was conducted using nxrA primers F1 and R2 (Wertz et al., 2008). The second step was then carried out with 1 L of the first PCR product and the same primers containing a GC clamp added at the 5' end of the forward primer. Thermocycler conditions for each PCR reaction were as reported by Wertz et al. (2008), with only slight modifications as follows: 7 min at 94 °C followed by 35 cycles of 94 °C for 30 s, annealing at 60 °C for 45 s and elongation at 72 °C for 45 s with terminal elongation at 72 °C for 5 min.

All final PCR products were cleaned and/or concentrated (when required) using Amicon Ultra-0.5 mL Centrifugal Filters (Eppendorf, Hamburg, Germany). Ten microlitres (60–100 ng DNA) were then loaded into each well in the TGGE gel.

3. Analysis of TGGE fingerprinting

Runs were done on a TGGE Maxi system (Whatman-Biometra, GmbH, Germany). Denaturing gels (6% PAGE with 20% deionized formamide, 2% glycerol and 8 M urea) were run, with the TAE buffer, at 150 V for 18 h except for nxrA gel which was run at 125 V. All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). The optimal temperature gradient for efficient bands separation was 41–51 °C for nxrA and 34–51 °C for 16S rDNA CTO. Gel bands were visualised by silver staining using the Gel Code Silver Staining kit (Pierce, Thermo Fisher Scientific, Rockford, IL, USA), following the manufacturer's instructions. Stained gels were photographed with a Canon digital camera. TGGE band patterns were normalised compared and clustered using the Gel Compar II image analysis software (version 5.102, Applied Maths, Belgium). Bands were automatically detected and matched, and further corrections were applied manually. For cluster analysis, TGGE profiles were compared using the Dice band-based similarity coefficient: band-matching (band assignment) and identification of band classes were performed automatically by the program. A band class is defined as a group of bands present in different profiles and showing the same electrophoretic behaviour along the gradient. Dendrograms, concerning band pattern similarities, were automatically calculated with UPGMA algorithms (Unweighted pair group method with arithmetic mean). The significance of UPGMA clustering was estimated by calculating the cophenetic correlation coefficients.

3.1. DNA sequencing of TGGE-isolated bands, phylogenetic and molecular evolutionary analysis

Portions of prominent bands were collected with sterile pipette tips from silver stained gels, placed in 10 L of filtered (0.22 m) and autoclaved distilled water, and directly used for reamplification with the appropriate primers. PCR products were purified by agarose gel running and extracted with the Quiaex-II kit (Quiagen, Germany). Recovered DNA was used for sequencing in an ABI PRISM 3100 Avant genetic analyser. Sequences were analysed and compared with those present in the database with biocomputing tools provided online by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The BLASTn program was used for preliminary sequence similarity analysis, and ClustalX version 2.0.3 software was used for sequences aligning. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Kumar et al., 2008). A p-distance based evolutionary tree was inferred using the Neighbour-Joining algorithm.

3.2. Statistical multivariate analysis by Canoco

TGGE band patterns generated by GelCompar II were converted to a binary matrix by scoring bands as present, 1, or absent, 0. This matrix was preliminary evaluated, without transformation, by detrended correspondence analysis (DCA), showing a linear, rather than unimodal, response to the operating parameters (lengths of gradient ≤3) (Lepš and Šmilauer, 2003). Accordingly, redundancy analysis (RDA) was performed to reveal the relationships between the structure of nitrifying microbial communities and a set of variables (ammonium, nitrite and HRT) related to the operating parameters. All variables were transformed to log(X + 1). The Monte Carlo permutation test was used to assess statistical significance of the ordination axes. All the multivariate statistics were computed using the "Canoco for Windows" v. 4.5 software (ScientiaPro, Budapest, Hungary).

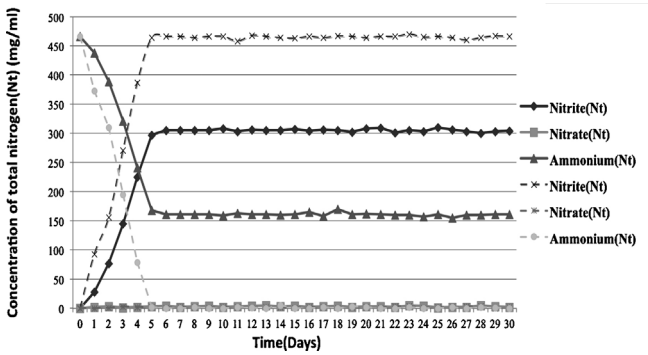

Fig. 2. Values of ammonium (,), nitrite (, X) and nitrate (, ✕) expressed as total nitrogen in the effluent of a partial nitrification bioreactor (Partial-Sharon) over time with a HRT of 9 h (- - - -) and 12 h (——).

4. Results and discussion

4.1. Physico-chemical parameters at different HRT

The first partial-nitritation bioreactor was operating at a constant flow rate of 4.16 mL/min and HRT of 12 h. After five days of operation (acclimation process of the inoculum to the experimental conditions) 100% of the ammonium was converted to nitrite. After this period, the partial-nitritation bioreactor showed stabilisation, maintaining its high capacity to transform ammonia into nitrite. These results (Fig. 2) suggested that the capacity of the partial-nitritation bioreactor to transform ammonium to nitrite with submerged fixed bed biofilm reactor could be considered as an alternative to the suspended growth activated sludge process, such as the conventional SHARON technology without carriers (Schlegel and Koeser, 2007; Van Dongen et al., 2001). The high transformation capacity of submerged-bed systems should be regarded as an important operational factor for the development and future design of partial-nitritation bioreactors, which can be applied to the treatment of effluents with high nitrogen content, such as landfill leachate (Van Loosdrecht et al., 2004). Moreover, fixed bed biofilm reactors are one of the extensively used systems in the removal of nutrient pollutants from wastewater because of their simple mechanical configuration, low-energy requirements and low-operating costs.

The second bioreactor was operated at a HRT of 9 h and a constant flow rate of 5.20 mL/min of synthetic wastewater. The methodology was the same as in the first bioreactor where the concentration of ammonium, nitrate and nitrite was measured at the entry and exit points of the partial-nitritation bioreactor.

When HRT was adjusted to 9 h the transformation of ammonium to nitrite reached 60% after five days of operation (acclimation process of the inoculum to the experimental conditions). After this period, the partial-nitritation bioreactor stabilised and its capacity for the biotransformation of ammonia to nitrite remained constant (Fig. 2). The addition of small amounts of NaOH 1% (p/v) was required due to the increased nitrite concentration which caused a sharp drop in the pH level of the bioreactor.

The results obtained in the partial nitritation system showed that at constant conditions of temperature (35 °C), oxygen concentration (1.5 mg/L) and pH (7.5) during the whole operational period, an evident reduction in the biotransformation of ammonium to nitrite was observed when the HRT was decreased from 12 to 9 h. Thus at 12 h of HRT the bioreactor converted the 100% of ammonium to nitrite, whereas when the bioreactor was operating at HRT of 9 h only 60% of the ammonium was converted. It is therefore evident that modifications in HRT can dramatically affect the functioning of the partial-nitritation technology. Morgenroth et al. (2002) stated that HRT appears to have an important influence on biofilm reactors since the retention time of the substrate can be smaller than the retention time of bacteria in the biofilm. It is important to note that during all the experiments the total nitrogen in the bioreactor was constant, thus in the partial-nitritation system the only biotransformation of the ammonium was via nitrite, except at some moments when very small quantities of nitrate were detected in both bioreactors. This can be due to the operating conditions of the system, such us pH, temperature and oxygen demand (DO), which increased the biological activity of the ammonium-oxidizing bacteria and decreased the biological activity of the nitrite-oxidizing bacteria.

According to Van Dongen et al. (2001), the optimal ammonium and nitrite ratio in the effluents in partial-nitritation systems for their combination with Anammox bioreactors can be estimated at 50% ammonium and 50% nitrite. In this context, our data suggest that in partial-nitritation systems the ammonium-nitrite ratio can be modified by changing the HRT (Fig. 2). Moreover, the results obtained in our experiments showed that the partial-nitritation bioreactor under submerged-bed technology increased the transformation of ammonium into nitrite and decreased the time required for the start-up of the bioreactors in comparison with other technologies (Mosquera-Corral et al., 2005; Van Loosdrecht et al., 2004).

4.2. Study of nitrifying communities present in the partial-nitritation bioreactor

TGGE fingerprinting analysis was used to investigate the structure of nitrifying microbial communities present in the bioreactor. Differences in the structure were detected by the PCR-TGGE method depending on working conditions.

4.2.1. Ammonia-oxidizing bacteria community structure (CTO) analysis and phylogeny

Results obtained in our studies on ammonia-oxidizing bacteria evidenced two different clusters according to the different conditions of the HRT applied to the system. Moreover, both bacterial clusters showed very low levels of similarity.

As for the 12 h HRT, it was evident from the clustering that throughout the whole experiment significant changes in the samples profiles occurred (samples 0A to 30A, Fig. 3). However, after acclimation inoculum period when all the ammonium was converted to nitrite (days 5 to 30, Fig. 2), the microbial populations seemed to stabilise. This result was confirmed by the 100% similarity that appeared in the samples after system stabilisation (sample profiles 10, 15 and 30A, Fig. 3).

In the 9 h HRT experiment (sample 0B to 30B, Fig. 3), it was evident that 2 days after the inoculum, a rapid change in the profiles of the ammonia-oxidizing bacteria occurred. This is evident in the clustering where 0B and 1B grouped away from all other samples with a similarity of only 60%. Again when the system achieved stability, the resulting sample profiles were identical during the next days (samples 15B and 30B, Fig. 3).

In the TGGE gel, a total of 14 different band classes were detected and 8 bands were re-amplified and sequenced in order to perform phylogenetic analysis. To obtain information on the species present in the community, a phylogenetic tree was developed (Fig. 4). Most of the sequences were grouped in the Nitrosomonas europaea/eutropha or in the Nitrosomonas marina/oligotropha groups. However sequences of Nitrosospira and Nitrosovibrio were also detected.

The ammonium oxidizing bacteria species present in the partial-nitritation bioreactors seemed clearly related to the working

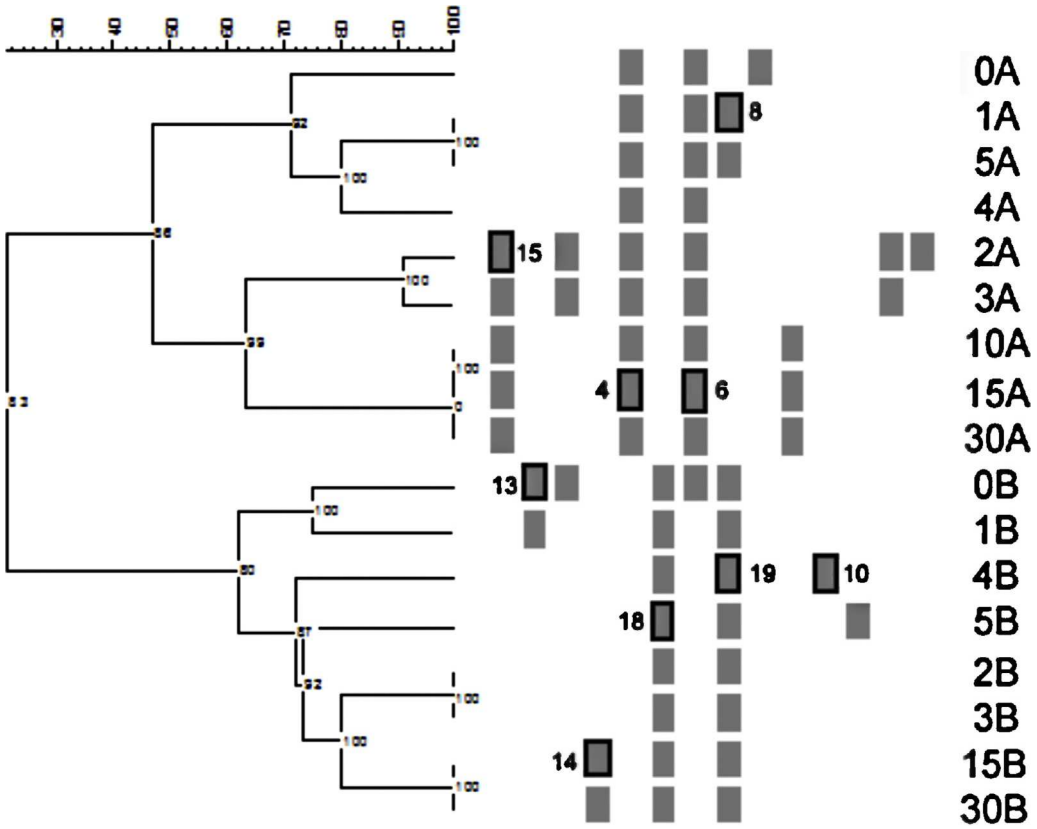

Fig. 3. CTO community in the partial nitrification bioreactor.

conditions. In the first bioreactor (HRT of 12 h), the resulting community was heterogeneous, showing a high number of bands (nine different classes of bands). As expected, the presence of the different band classes (different species) seemed to be related to the ratio of ammonium-nitrite. In fact, when the concentration of these two compounds was similar, the highest number of species (bands) appeared in the partial-nitritation bioreactor (samples 2A and 3A, Fig. 3). Nevertheless, at stability, the resulting bands in the profiles were unvarying (samples 10, 15 and 30A, Fig. 3). Species Nitrosomonas eutropha (band 6) was present throughout the whole experiment, while Nitrosospira sp. only appeared in two samples.

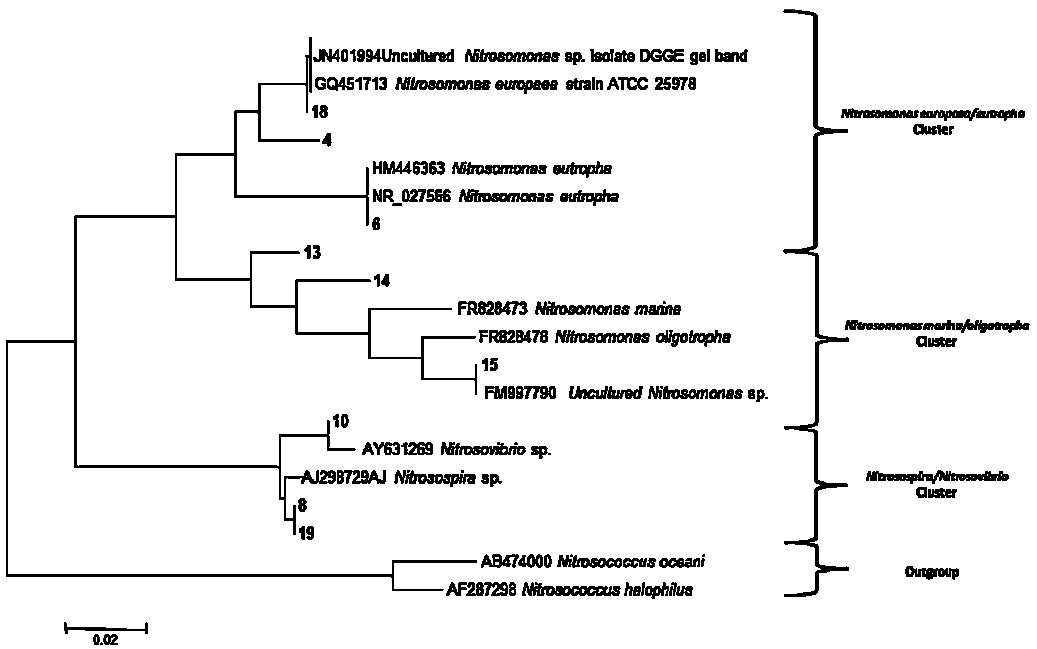

Fig. 4. Phylogenetic tree of CTO community.

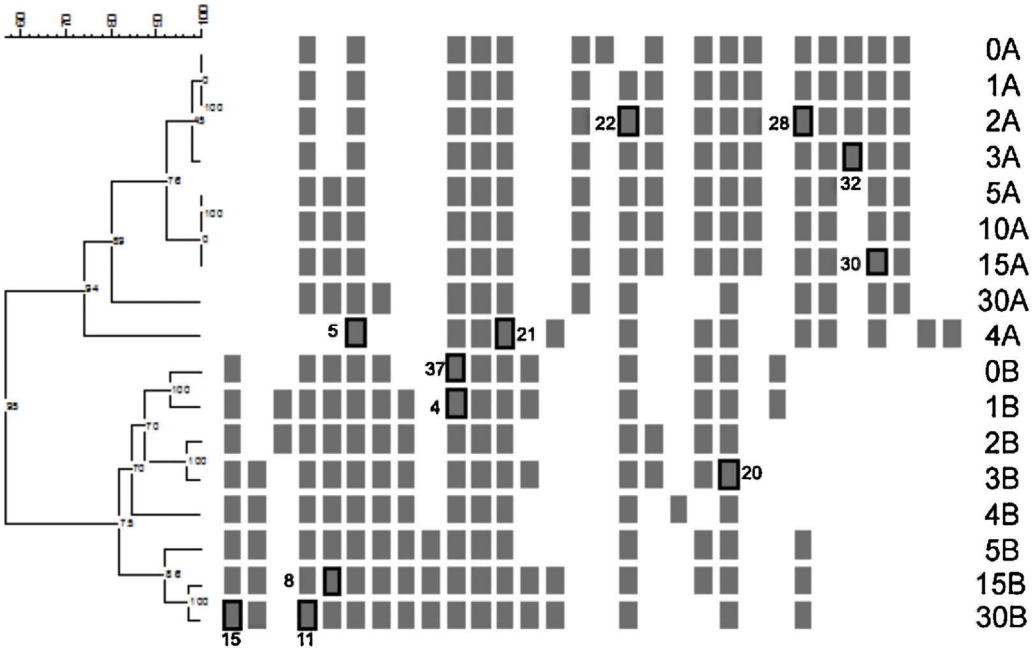

Fig. 5. NxrA community in the partial nitrification bioreactor: Dice coefficient-based analysis of band patterns generated from samples.

In the second bioreactor, the ammonia-oxidizing bacteria community resulted in more homogeneous and fewer bands compared to the first experiment. Species of Nitrosomonas europaea and Nitrosospira (bands 18 and 19, Fig. 3 and Fig. 4) were present during all experiments. Moreover, at the end of both experiments when the system achieved stabilisation, a species grouping in the Nitrosomonas marina/oligotropha cluster appeared (bands 14 and 15, Fig. 3 and Fig. 4).

The results obtained were not surprising in terms of the species present. The oxidation of ammonia is carried out predominantly by bacteria of the genera Nitrosomonas and Nitrosospira (Utåker et al., 1995; Posmanik et al., 2014). Furthermore, as reported by various authors (Dionisi et al., 2002, Vejmelkova et al., 2011) the Nitrosomonas oligotropha clusters prevail in environments with low ammonia concentrations, while most of the bacteria present in nitrifying bioreactors operating at high ammonium levels, such as our partial-nitritation bioreactor, belong to the Nitrosomonas europaea lineage. Also in our case, in both bioreactors working in parallel when the ammonia concentration was low the appearance of N. oligotropha was observed (bands 13, 14 and 15, Fig. 3 and Fig. 4). However, in general the dominant bacteria in both experiments were those belonging to the N. europaea–N. eutropha clusters, species that are well known for their great affinity for ammonium and most commonly recognised as the main species that carries out ammonia oxidation (Schmidt and Bock, 1997; González-Martínez et al., 2012; Wang et al., 2013). In this context, our results suggest that the different operating conditions seemed to influence the presence of each species. When the HRT was increased to 12 h, the prevalence of N. eutropha was evident during the whole experiment. Nevertheless, at a HRT of 9 h N. europaea prevailed. Operating conditions also seemed to have an influence on the presence of Nitrosospira. In fact when the experiment was performed at a HRT of 9 h, Nitrosospira spp. (band 19, Fig. 3 and Fig. 4) was present during the whole experiment, while at a HRT of 12 h its appearance was not detected.

Our results showed that in the partial-nitritation system constructed as a submerged bed reactor, the dominant ammonia oxidizers bacteria were members of the genus Nitrosomonas. These results are in agreement with those obtained in ammonium-rich systems like activated sludge (Pal et al., 2012). Obviously, our results have been obtained with CTO189f and CTO654r primers, which are specific to the majority of -proteobacterial ammonia oxidizing bacteria, although none of these sequences identify all the recognized species in the wastewater treatment environments (Purkhold et al., 2000).

4.2.2. Nitrite-oxidizing bacteria community structure (nxrA) analysis and phylogeny

Image analysis showed the presence of two big clusters, one that included all the samples taken with HRT of 12 h (bands 0A to 30A) and the other of samples taken with at HRT of 9 h (bands 0B to 30B); the similarity between these two clusters was around 60% (Fig. 5).

As for the HRT operating condition of 12 h (samples 0A to 30A, Fig. 5), image analysis revealed a total of 22 different band classes, while in the 9 h experiment (samples from 0B to 30B, Fig. 5) a total of 21 different bands appeared. Band sequencing was carried out in order to obtain a phylogenetic tree (Fig. 6). All resulting sequences were related to the typical nitrite-oxidizing species of genus Nitrobacter. It is well known that species of this genus are the key nitrite-oxidizing bacteria (NOB) in nitrifying wastewater treatment plants (Kim and Kim, 2006). The Nitrobacter genus currently consists of four valid species: Nitrobacter winogradsky, Nitrobacter hamburgensis, Nitrobacter vulgaris and Nitrobacter alkalicus (Vanparys et al., 2007). In our case all these species were present at both operating conditions and throughout the experiments. The only exception was N. hamburgensis that only appeared in the bioreactor at a HRT of 12 h (band 30, Fig. 5 and Fig. 6).

Although the community of nitrite oxidizing bacteria presented high number of different species, the samples structure showed low changes over the experiment and under the different operating conditions. Indeed, physico-chemical analysis showed that over time the transformation of nitrite to nitrate was not significant (Fig. 2). This low capacity of transformation can be due to the working conditions of the system, which increases the biological activity of the ammonium-oxidizing bacteria that can grow relatively fast at high temperatures and under high ammonia concentrations and

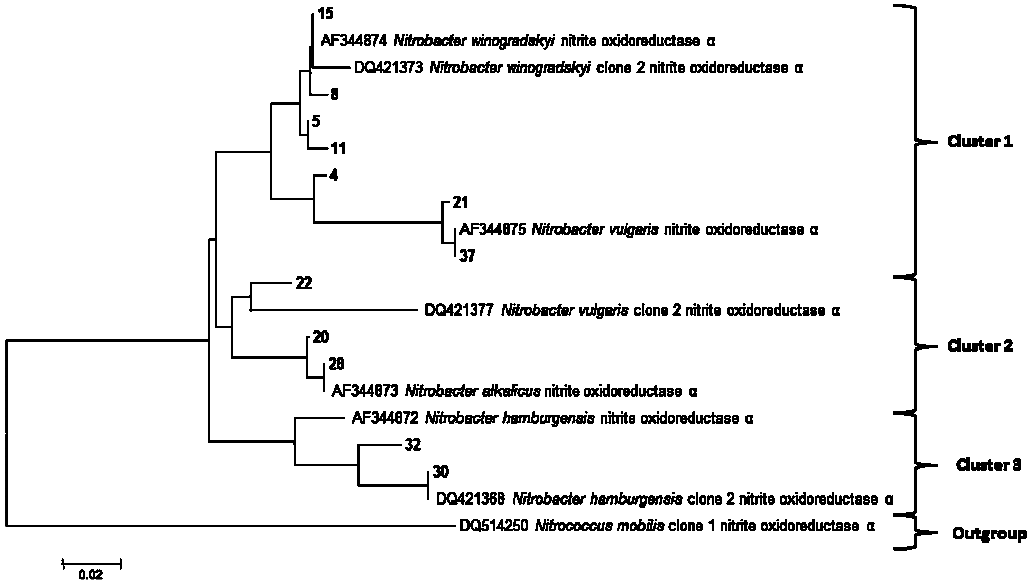

Fig. 6. Phylogenetic tree of nxrA community.

decrease that of the nitrite-oxidizing bacteria (González-Martínez et al., 2011). In fact nitrite-oxidizing bacteria are more sensitive than ammonium-oxidizing bacteria to different environmental parameters, such as pH, dissolved oxygen concentration, temperature and HRT. This means that probably, even if they are present and show a high level of biodiversity, nitrite-oxidizing bacteria cannot work at these operational settings and are probably in a lag phase. Actually, the partial-nitritation bioreactor was designed to prevent or at least significantly reduce the transformation of nitrite to nitrate and to obtain a high efficiency in ammonia removal (González-Martínez et al., 2011).

4.3. Statistical multivariate analysis (Redundancy analysis, RDA)

RDA showed that, according to the results of the Monte Carlo permutation test, the most significant factor explaining the variation in TGGE profiles related to the different specific bacterial groups was the operating parameter HRT, which explained the highest percentage of total sample variance of species data and of sample-environment relations.

In the case of the CTO gene profiles, HRT and ammonium concentration was significant ($p < 0.05$). HRT was correlated to the 1st ordination axis, which described 53.9% of the total sample variance of the species data (samples) and 81.8% of the sample-environment relation variance. Ammonium concentration was correlated to the 2nd ordination axis, which described 7.7% of the total sample variance of the species data (samples) and 11.6% of the sample-environment relation variance. No significant correlation with nitrite was recorded.

For the nxrA profiles, HRT and ammonium concentration were the significant variables ($p < 0.05$). Again HRT was correlated to the 1st ordination axis which described 53% of the total sample variance of the species data and 83.6% of the sample-environment relation variance, while ammonium was correlated to the 2nd ordination axis which described 5.8% of the total sample variance of the species data (samples) and 9.3% of the sample-environment relation variance. Nitrite was not significant.

In Fig. 7 the results of all the statistical multivariate analyses are reported. It is clear that the samples are grouped together in relation to the two different HRT. In fact in all the Canoco graphics, the samples (numbered circles) were positioned in the positive or negative quadrants of the graphic depending on the operating conditions of each experiment, and thus with respect to positive or negative relations with HRT (Fig. 7A an B).

According to the results of the Monte Carlo permutation test, HRT was the major factor explaining the variations in the biotransformation of ammonium to nitrite under the experimental working conditions. It has been reported (Nybroe et al., 1992) that temporal

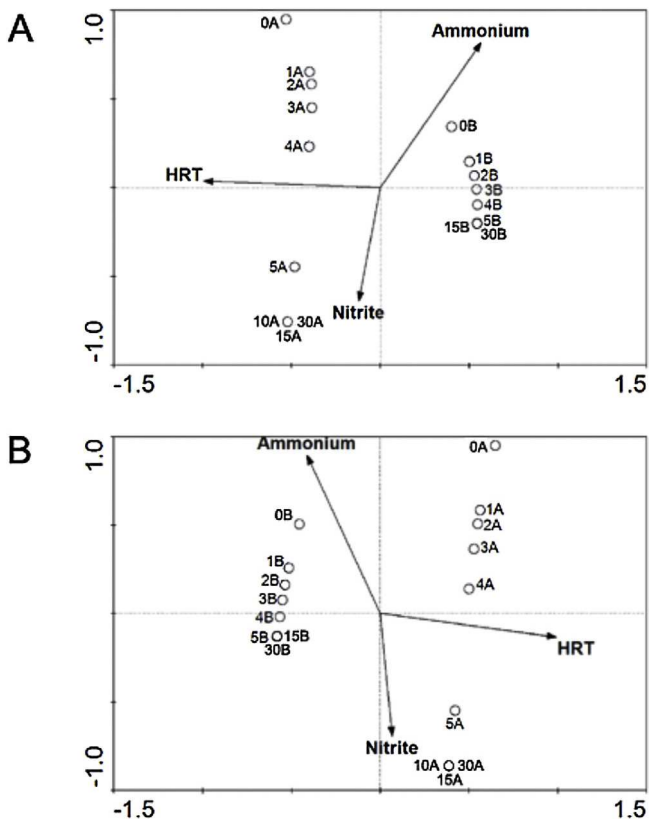

Fig. 7. Redundancy Analysis (RDA) ordination diagram (biplot) showing samples (numbered circles) and significant ($p < 0.05$) environmental parameters (straight arrows) using CANOCO. A = CTO community, B = nxrA community.

variations in biological activities may react to a bacterial regulatory mechanism in response to changing environmental conditions, e.g. the amount of substrate, the electron acceptor, the pH or temperature. This same trend has been generally observed in our study, i.e. when the HRT was increased in the bioreactor a significant increase in the biotransformation of ammonium to nitrite was detected. Thus changes in the operating conditions such as HRT may produce changes in the cellular physiology as well as changes at a community level. Consequently our results suggest that the performance of the biotransformation of ammonium to nitrite in a partial-nitritation bioreactor can be directly affected by the HRT, which can be crucial for the optimisation of nutrient removal rates and to implement control strategies.

5. Conclusions

Our study shows that in a partial-nitritation bench-scale bioreactor a change in the community structure was observed depending on the operating conditions. Specifically, a significant factor that produced modifications in the communities was HRT. This result was also confirmed by statistical multivariate analysis, and it was particularly evident for the ammonium oxidizing bacteria where different species appeared at the different HRTs tested. When the HRT was 12 h, the majority of bacteria present were Nitrosomonas eutropha, while with a HRT of 9 h Nitrosomonas europea and Nitrosospira were dominant. Also in the case of the other bacterial group analysed, nitrite-oxidizing bacteria, a modification in the structure of these communities was detected in response to the HRT used in each experiment. However, although the nitrite-oxidizing bacteria were present in both bioreactors, they showed no ability to oxidize nitrite under the operating conditions of the system. In this sense, nitrate was not detected and aerobic conditions were maintained throughout the process. Our data confirmed that the partial-nitritation system working parameters are able to offer an advantage to ammonium oxidizing bacteria over nitrite oxidizing bacteria, and that heterotrophic denitrification is not performed due to the absence of anoxic conditions. However these results have been performed with synthetic wastewater and new experiments with real wastewater are needed in order to confirm the present study. More researches are in progress.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ecoleng.2014.01.009.

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Supplementary Material

Denitrifying bacteria community structure (nosZ) analysis

In our research, nosZ gen (Throback et al. 2004), was investigated using a cultivation-independent approach based on PCR-TGGE fingerprinting, a nitrous oxide reductase gene typical in denitrifying bacteria was included in order to evaluated the presence or absence of this microbial community in partial nitrification system.

The amplification of the nitrous oxide reductase gene was performed as reported by Gómez-Villalba et al. (2006). Amplification conditions for both PCR reactions were slightly modified as follows: 7 min at 95 °C followed by 35 cycles of 94 °C for 30 s, annealing at 50 °C for 1 min and elongation at 72 °C for 1 min with terminal elongation at 72 °C for 10 min.

The optimal temperature gradient for efficient bands separation was 44–53°C for nosZ. Gel bands were visualised by silver staining using the Gel Code Silver Staining kit (Pierce, Thermo Fisher Scientific, Rockford, IL, USA), following the manufacturer's instructions.

Similarity calculated by the Dice coefficient again showed that the structure of the denitrifying bacteria community was influenced by the HRT. In this case, two clusters were observed with a similarity below 60% (Figure 8).

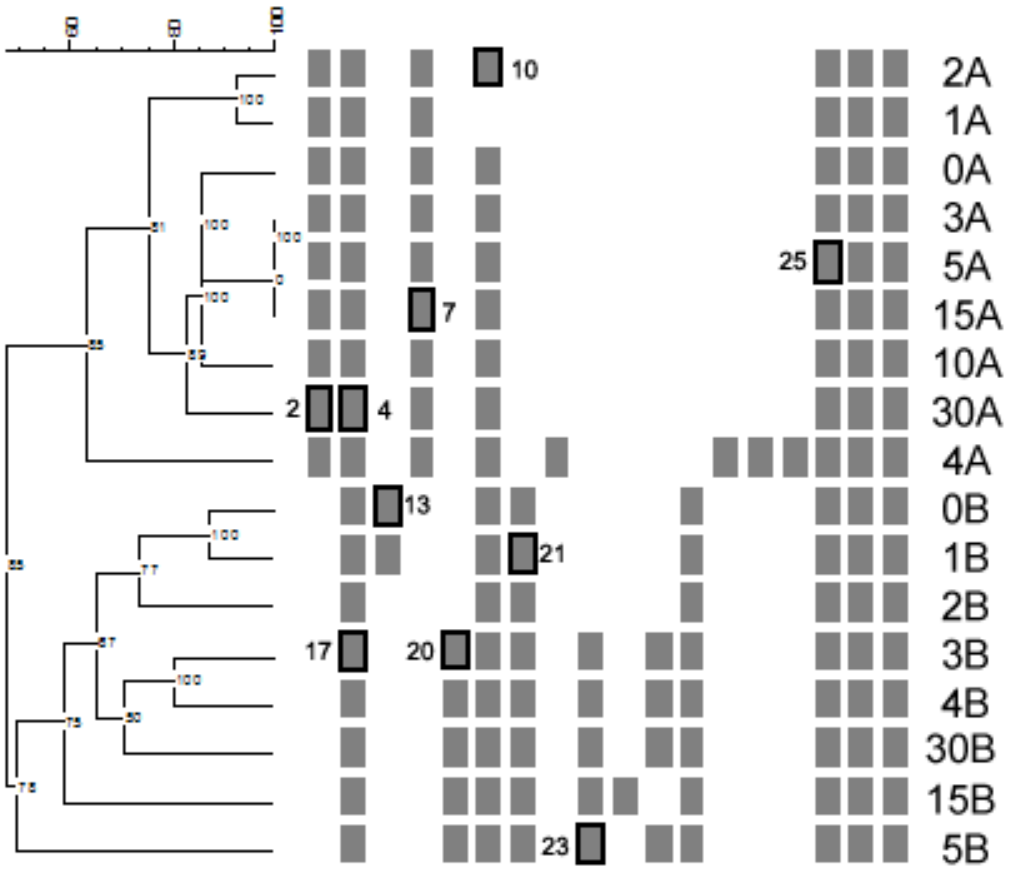

Fig.8.-NosZ community in the partial nitrification bioreactor:Dice coefficient-based analysis of band patterns generated from samples.

The total band classes detected were 11 in the first experiment and 12 in the second experiment. A phylogenetic tree was obtained by sequencing the most prominent bands. The phylogenetic tree presented 3 clusters, where most of the bands were related to some common denitrifying species (Figure 9). As for the first cluster (cluster 1, Figure 8), five bands (indicated as 10, 20, 21, 23) were mainly correlated with the denitrifying species *Alicycliphilus denitrificans*, which is common in mixed liquors and the biofilm of

wastewater treatment plants (Hou et al. 2012, Mechichi et al. 2003). It is worth noting that the majority of the bands did not show high levels of similarity with the sequences reported in the databases. The closest relative found for these sequences was *Diaphorobacter nitroreducens* (cluster 2, Figure 9). This species is common in wastewater treatment plants and it is an interesting bacterium in nitrogen removal processes (Khan and Hiraishi 2002). It is also well known for its ability to perform simultaneous nitrification and denitrification under aerobic conditions (Khardenavis et al. 2007). Finally, one band (indicated as 25) was grouped with a high similarity to *Paracoccus denitrificans.* This bacterium is also very common in wastewater treatment bioreactors (Uemoto and Saiki 1996). Although only one band was related to this species, it was detected for the two different operating conditions (HRT of 12 and 9 h) and throughout the entire experiment.

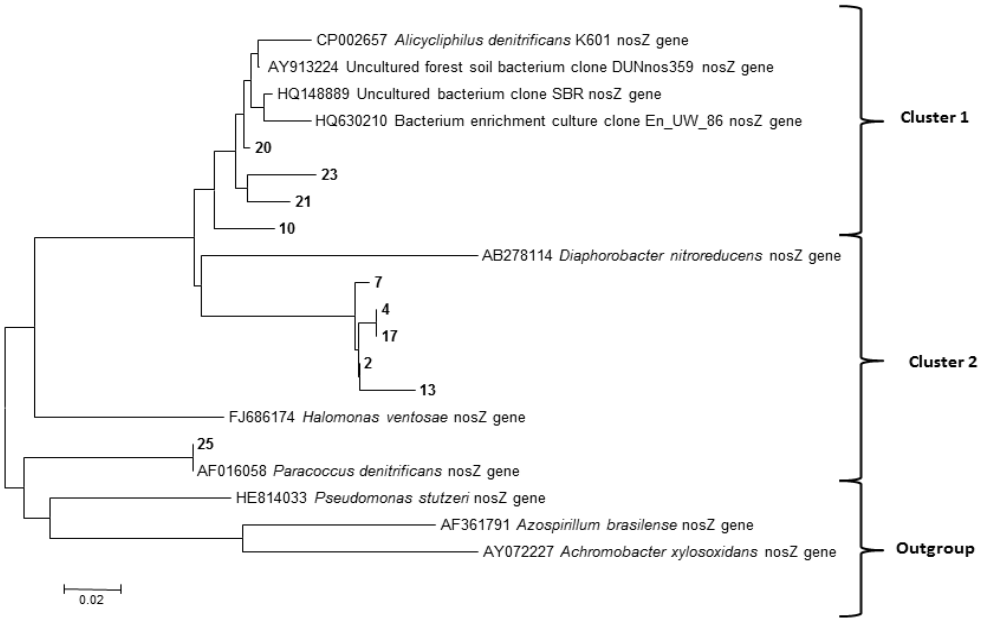

Fig.9. -Phylogenetic tree of nosZ community.

As for the band profiles, it was observed that in the first experiment the bands were almost the same in the different samples. This means that the species remained unvarying over time. This is not surprising since, even if available, these species are probably not active because of the operating parameters used in the bioreactor. The only exception could be the band related to the genus *Paracoccus*. The presence of this bacterium could be due to possible aerobic denitrification. Indeed, the *Paracoccus* species are the first species reported to carry out complete denitrification under aerobic conditions (Ahn 2006). However further studies are needed in order to confirm this hypothesis.

Even considering the above, in general terms denitrification is normally performed in anoxic conditions that are obviously not present in the partial-nitrification process (Dijkman and Strous 2002). Thus our results suggest that although denitrifying microbiota were present in the partial-nitrification bioreactor, these microbial populations were probably not biologically active under the experimental conditions

Statistical multivariate analysis (Redundancy analysis, RDA)

The nosZ genes only HRT was the apparent cause of changes in the communities' structures, since it was the only significant variable. Again, HRT was mainly correlated to the 1st ordination axis which described 66.2% of the total sample variance of the species data and 91.2% of the sample-environment relation variance. In this case the other two different operating parameters resulted in no significance. In Figure 10 the results of NosZ statistical multivariate analyses are reported.

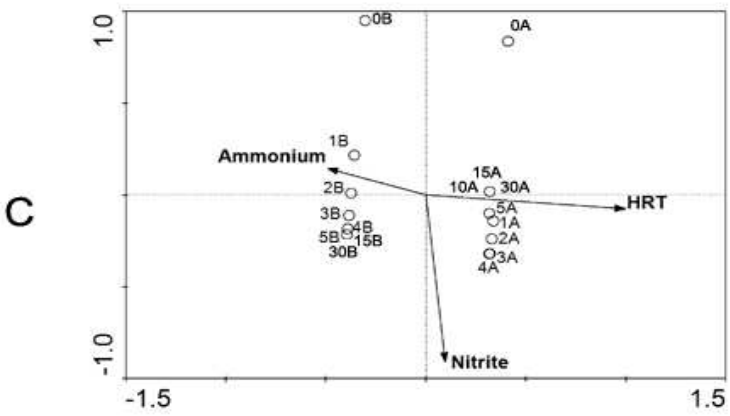

Fig.10. - Redundancy Analysis (RDA) ordination diagram (biplot) of NosZ community showing samples (numbered circles) and significant ($p < 0.05$) environmental parameters (straight arrows) using CANOCO.

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Chapter 4

Effect of ciprofloxacin antibiotic on the partial-nitritation process and bacterial community structure of a submerged biofilter

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Effect of ciprofloxacin antibiotic on the partial-nitritation process and bacterial community structure of a submerged biofilter

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HIGHLIGHTS

- The study was done in 4 bench-scale partial-nitrification submerged bed bioreactors.
- Changes in performance were studied under different antibiotic concentrations.
- We focused the study on the influence of ciprofloxacin on the microbial population.
- Ciprofloxacin effect in partial-nitritation was tested by multivariate analysis.

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abstract

A partial-nitritation bench-scale submerged biofilter was used for the treatment of synthetic wastewater con-taining a high concentration of ammonium in order to study the influence of the antibiotic ciprofloxacin on the partial-nitritation process and biodiversity of the bacterial community structure. The influence of ciproflox-acin was evaluated in four partial-nitritation bioreactors working in parallel, which received sterile synthetic wastewater amended with 350 ng/L of ciprofloxacin (Experiment 1), synthetic wastewater without ciprofloxa-cin (Experiment 2), synthetic wastewater amended with 100 ng/L of ciprofloxacin (Experiment 3) and synthetic wastewater amended with 350 ng/L of ciprofloxacin (Experiment 4). The concentration of 100 ng/L of antibiotics demonstrated that the partial-nitritation process, microbial biomass and bacterial structure generated by tag-pyrosequencing adapted progressively to the conditions in the bioreactor. However, high concentrations of cip-rofloxacin (350 ng/L) induced a decay of the partial-nitritation process, while the total microbial biomass was in-creased. Within the same experiment, the bacterial community experienced sequential shifts with a clear reduction of the ammonium oxidation bacteria (AOB) and an evident increase of Commamonas sp., which have been previously reported to be ciprofloxacin-resistant. Our study suggests the need for careful monitoring of the concentration of antibiotics such as ciprofloxacin in partial-nitritation bioreactors, in order to choose and maintain the most appropriate conditions for the proper operation of the system.

1. Introduction

One of the most important environmental problems is the in-crease in the volume of industrial and urban wastewater produced. In this way, different technologies have been used for the removal of different elements present in this water such as nitrogen, organic carbon and phosphorous. In this context, in the wastewater treatment plants (WWTPs), nitrogen is often removed by conventional biolog-ical processes such as nitrification and heterotrophic denitrification. However, in recent years, alternative biological systems that are more efficient and cost effective have been developed for the removal of this nutrient. In particular, the development of autotro-phic technologies for nitrogen removal (anammox systems) has been a truly novel alternative.

Different autotrophic removal systems have been described in the last decade. Thus, autotrophic systems of two bioreactors (i.e. Sharon/ anammox system) and autotrophic systems of a single bioreactor (i.e., Cannon system, Demon system, etc.) have been developed. However, in all of the autotrophic technologies' systems for nitrogen removal, a partial-nitritation is needed before the anammox process, where rough-ly 50% of the ammonium that is present in the influent is converted to nitrite. Finally, the remaining ammonium will be converted into gas-eous N_2 by autotrophic anammox bacteria, with nitrite as the final electron acceptor (Peng et al., 2006).

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Partial-nitritation systems were developed by the Delft University of Technology between 1996 and 1999; these have several advantages over total nitrification-based technologies, such as 25% savings in aeration, 30% reduction of biomass generation – with the biomass yield being about 0.15 g biomass (g NH^{+}_{4}-N)$^{-1}$ (Gut et al., 2007) – and 20% less CO_2 emissions (Sri Shalini & Joseph, 2012). Moreover, this system has been shown to perform efficient nitrogen removal with elim-ination rates ranging from 26 kg − $Nm^{-3}d^{-1}$ to 76 kg− $Nm^{-3}d^{-1}$ (Okabe et al., 2011). These technologies have different drawbacks, such as their slow start-up and strict operational conditions (Gonzalez- Martinez et al., 2011). Nevertheless, these systems have been expanded due to their higher performance and lower costs than traditional nitrogen-removal technologies, with some authors reporting up to a 90% saving in operational costs (Jetten et al., 2001).

Antimicrobial agents are among the most commonly used pharmaceuticals and are widely used. Beta-lactams, macrolides, sulphonamides, fluoroquinolones, and tetracyclines are the most important antibiotic groups used in both human and veterinary medicine. The high global consumption of up to 200,000 t per year and high percentage of antibiotics that may be excreted without undergoing metabolism (up to 90%) have resulted in their widespread presence in the environment (Jelic et al., 2011). In this context, in the last five years, the oral consumption of quinolone antibiotics such as ciprofloxacin has increased by 30%. Quinolones have been detected at levels of up to 36 ng/L and 450 ng/L in surface waters and urban wastewaters, respectively (Batt et al., 2007). In addition, this family of antibiotics raises concerns about their potential ecotoxicity and genotoxicity (Hartman et al., 1999), in particular, that caused by the last generations of quinolones. Ciprofloxacin is a fluoroquinolone that affects DNAgyrase and topoisomerase IV of many different Gram positive and Gram negative bacteria, thus preventing cell replication. Specifically, fluoroquinolones have been found to be effective against many pathogenic bacteria including Salmonella spp., Campylobacter spp., Escherichia coli, Klebsiella spp., Pseudomonas aeruginosa, Neisseria gonorrhoeae and Streptococcus pneumoniae (Harris et al., 2013). It is com-monly utilised for the treatment of diseases such as pharyngitis, sinusitis or earaches, as well as airway diseases, such as pneumonia or bronchitis (Coutu et al., 2013).

Although antibiotics are of great importance for human health, they can also be a great problem for the environment. Antibiotics can be re-leased into nature, where they spread throughout water bodies, pro-moting the antibiotic resistance of bacteria due to the selection of resistant strains after exposure to antibiotic concentrations (Fick et al., 2009). Since the concern about the discharge of pharmaceuticals (and other emerging contaminants, as well) into wastewater is relatively re-cent, it is not unexpected that they have not been covered by the cur-rently existing regulation (Jelic et al., 2011). As it is today, antibiotic release into the environment carries an intrinsic risk that is not yet fully comprehended (De Graaff et al., 2011). In this context, it has been reported that municipal wastewater treatment plants are the major source of antibiotics being released to the environment (Senta et al., 2013). This is due to the use of antibiotics by humans, which are then released into wastewater treatment plants through black waters in substantial concentrations (De Graaff et al., 2011). Also, due to the improper design of wastewater treatment plants for the removal of these compounds (Verlicchi et al., 2012; Senta et al., 2013), only small amounts of antibiotics are removed at wastewater treatment plants (De Graaff et al., 2011). In fact, antibiotic-resistant strains are more nu-merous downstream than upstream of the wastewater treatment plant (Zhang et al., 2009). In these environments, antibiotic-resistant micro-organisms become selected under antibiotic concentrations. Then, a high density of microbial biomass will help to transfer genetic informa-tion that allows bacteria to become resistant (Manaia et al., 2009; Zhang et al., 2009).

During wastewater treatment, quinolones are drastically removed from the water stream (N80%), but their fate is associated with sewage sludge of their strong sorption properties (Golet et al., 2003) and their poor degradation (Al-Ahmad et al., 1999). In this context, the biological processes of nitritation have been described as particularly sensitive to toxic substances such as pesticides and antibiotics (Sáez et al., 2003). Thus, the effect of fluoroquinolone antibiotics such as ciprofloxacin, which is a popular antibiotic that is widely found in municipal wastewa-ter, on the partial-nitritation process may be of undoubted interest, mainly because this has never been studied before. Thus, the objective of our study was to evaluate the influence of different concentrations of ciprofloxacin on the performance of a partial-nitritation bioreactor constructed at bench-scale under the configuration of a fixed-biofilm bioreactor. Also, the effects of this antibiotic on the microbial communi-ties growing in the bioreactor were studied using tag-pyrosequencing techniques.

2. Materials and methods

2.1. Operating conditions of the bench-scale partial-nitrification bioreactors

Four partial-nitrification bioreactor bench-scale plants were constructed as a submerged bed with a volume of 5 L. It was con-structed as a submerged biofilter with PVC carriers (BioFlow 9). The carrier BioFlow 9 has a diameter of 9 mm, a height of 7 mm, a density of 0.84 g/cm^3 and a specific surface area of 800 m^2/m^3 (Chai et al., 2013). A schematic diagram of the experimental plant is shown in Fig. 1. The bioreactor received synthetic wastewater from a peristaltic pump, and was operated at a continuous flow of 715 mL/h with a hydraulic retention time (HRT) of 7 h. The operat-ing conditions in the bioreactor (i.e., HRT, pH, dissolved oxygen con-centration, and temperature) were continuously monitored in order to verify that they remained stable. Four 15-cm air diffusers at the bottom of the vessel supplied oxygen from an air pump to ensure that the oxygen concentration in the bioreactor was maintained at 1.5 mg/L. All of the experimental works were performed at a pH of 7.5 and a temperature of 35 °C due to an adjustable thermostat (Liang & Liu, 2007; Vilar et al., 2010).

Before starting the experiment with different concentrations of cip-rofloxacin, three partial-nitrification bioreactors were inoculated with mixed liquor from the same aerobic reactor located in the Los Vados urban wastewater treatment plant (Granada, Spain). The mixed liquor was recirculated for three days until the appearance of a biofilm on the surface of the plastic carriers and acclimated under defined labora-tory conditions for 5 days (Kaewpipat and Grady, 2002). After inocula-tion, a standard synthetic wastewater (Mosquera-Corral et al., 2005) without ciprofloxacin was fed into the bioreactors for 30 days to obtain

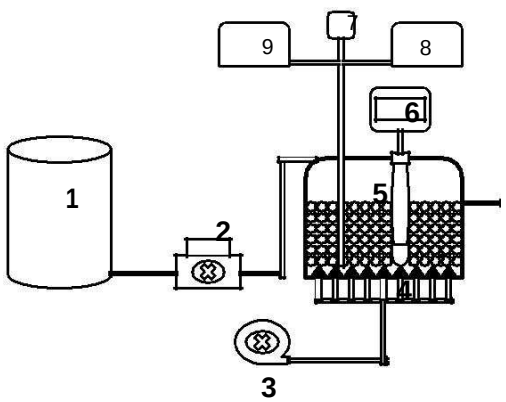


Fig. 1. Diagram of the pilot-scale partial-nitritation bioreactors used in the experiments. 1) Synthetic wastewater tank; 2) Peristaltic pump; 3) Air pump; 4) Oxygen diffusers (porous plates); 5) Partial-nitritation bioreactor stuffed with BioFlow 9 carriers; 6) Thermostat; 7) pH metre; 8) Tank of H_2SO_4 0.1 M for pH control; and 9) Tank of NaOH 0.1 M for pH con-trol. Four partial-nitritation bioreactors working in parallel were constructed in our study.

Table 1
Composition of the synthetic wastewater used to feed the four partial-nitritation bioreactors.

Chemical	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Unit
Ciprofloxacin($C_{17}H_{18}N_3FO_3$)	350	0	100	350	ng/L
$(NH_4)_2SO_4$	2.35	2.35	2.35	2.35	g/L
$NaHCO_3$	3.25	3.25	3.25	3.25	g/L
$CaCl_2$	0.30	0.30	0.30	0.30	g/L
KH_2PO_4	0.07	0.07	0.07	0.07	g/L
$MgSO_4$	0.02	0.02	0.02	0.02	g/L
$FeSO_4\_7H_2O$	0.009	0.009	0.009	0.009	g/L
H_2SO_4	0.005	0.005	0.005	0.005	g/L

a perfect partial-nitritation with the optimal operational conditions to obtain 50% ammonium and 50% nitrite (Van Dongen et al., 2001; González-Martínez et al., 2013). The fourth bioreactor was not inoculat-ed with mixed liquor and only received sterile synthetic wastewater for 35 days. Obviously, the non-inoculated bioreactor did not form biofilm, and was used in our experiment to determine the sorption properties of the support material (BioFlow 9).

After the period of acclimatisation of the microbiota in the inoculat-ed partial-bioreactors, different concentrations of ciprofloxacin (0, 100 and 350 ng/L) were added to the synthetic wastewater (see Table 1). These antibiotic concentrations were selected in agreement with previ-ous descriptions of ciprofloxacin in wastewater influents (Jelic et al., 2011; Dorival-García et al., 2013). The non-inoculated bioreactor was fed with sterile synthetic wastewater amended with 350 ng/L of cipro-floxacin (Globuce™ 500 mg, Sigma-Tau). Synthetic wastewater used in our study simulated the leachate from an anaerobic digester, since it contained a high concentration of ammonium and was low in organic matter. The influent was continuously fed into the bioreactor by a peri-staltic pump (Watson Marlow s-520) which pumped the synthetic wastewater at a constant flow rate.

2.2. Determination of ammonium, nitrite, nitrate and ciprofloxacin concentrations

Concentrations of the inorganic forms of nitrogen, ammonium, ni-trite and nitrate were measured daily at the influent and effluent points of the four partial-nitritation reactors. Determination of ammonium, ni-trite and nitrate concentrations was done by ionic chromatography (Metrohm) in agreement with González-Martínez et al. (2013). Ammo-nium concentration was measured utilising a Metrosep C 2–150 cation column, while nitrite and nitrate concentrations were measured with a Metrosep A supp-4-250 anion column. A carbonate/bicarbonate solu-tion was used as eluent. Calibration curves of known concentrations of ammonium, nitrite and nitrate (10, 500 and 1,000 mg/L) were also analysed on a daily basis.

Ciprofloxacin concentration in the influent and effluent from the partial-nitritation reactors was measured on a daily basis in all of the ex-periments, in agreement with Dorival-García et al. (2013). 100 mL of wastewater sample filtrates (0.45 μm membrane filter, Millipore), which had been acidified to pH 3.0 with 98% (v/v) formic acid, were passed through the Oasis HLB SPE cartridges. The analytes were then eluted with 8 mL of methanol, and the extracts were evaporated to dry-ness. 1 mL of the initial mobile phase was added to dissolve the residues prior to LC injection (Acquity™ Ultraperformance LC system) equipped with a BEH™C_{18} column (1.7 μm, 50 mm × 2.1 mm). Separations were performed using binary gradient mobile phases (Li et al., 2009).

2.3. Samples collection and DNA extraction

Biomass samples from the biofilm formed on the BioFlow 9 were col-lected from Experiments 2, 3 and 4 after 0, 7, 15 and 30 days. Tests on carrier samples were carried out in order to establish the amount of bio-mass attached to the carriers. The biofilm solids were determined using four carrier elements that were sampled. The carrier samples were son-icated for 3 min. The biomass attached to the carriers was separated by centrifugation at 3000 rpm/10 min and then washed off; the clean car-riers were weighed and the amount of biofilm attached to the four car-rier elements was calculated. The amount of biomass in the reactor could then be determined from the number of carrier elements per litre.

Samples collected for PCR amplification and further pyrosequencing were done by vortexing 200 mL of plastic carriers from biofilters in a sterile saline solution (0.95 NaCl), then centrifuging them to obtain the biofilm fraction. Biofilm samples were then subjected to DNA ex-traction using the FastDNA Kit and Fast-Prep24 apparatus (MP-BIO, Germany).

2.4. Pyrosequencing studies

PCR analysis using primers 28 F (5′-GAGTTTGATCNTGGCTCAG-3′) and 519R (5′-GTNTTACNGCGGCKGCTG-3′), which had been reported by Fan et al. (2012), was used for the collection of amplicons. This amplicons were then subjected to pyrosequencing. Samples for pyrose-quencing were stored at −20 °C and sent to the Research and Testing Laboratory, Lubbock, TX (http://www.researchandtesting.com/). Pyro-sequencing was done using a 454FLX instrument and 454 pyrotag methods following the report by Dowd et al. (2008). Analysis of bacte-rial 16S rRNA gene datasets for quality and chimaeras was performed with results from the pyrosequencing procedure. Genetic

Table 2
Ciprofloxacin concentration in the effluent for Experiment 1 (without biomass and a ciprofloxacin concentration of 350 ng/L^{-1}), Experiment 2 (without ciprofloxacin), Experiment 3 (Ciprofloxacin concentration of 100 ng/L^{-1}) and Experiment 4 (Ciprofloxacin concentration of 350 ng/L^{-1}).

Experiment 1			Experiment 2			Experiment 3			Experiment 4		
Date	Point of measure	Concentration (ng/L)	Date	Point of measure	Concentration (ng/L)	Date	Point of measure	Concentration (ng/L)	Date	Point of measure	Concentration (ng/L)
Day 0	Influent	331.715.1	Day 0	Influent	010	Day 0	Influent	97.812.6	Day 0	Influent	357.213.1
	Effluent	342.8 ± 1.9		Effluent	010		Effluent	0.210.01		Effluent	0.410.07
Day 7	Influent	366.513.6	Day 7	Influent	010	Day 7	Influent	107.119.0	Day 7	Influent	342.0 + 17.3
	Effluent	351.215.4		Effluent	010		Effluent	0.210.03		Effluent	0.710.3
Day 15	Influent	346.1 + 3.8	Day 15	Influent	0 + 0	Day 15	Influent	113.8 + 1.4	Day 15	Influent	386.8 + 6.7
	Effluent	327.512.2		Effluent	0 + 0		Effluent	0.310.1		Effluent	3.210.5
Day 30	Influent	348.3 13.5	Day 30	Influent	0 + 0	Day 30	Influent	68.710.5	Day 30	Influent	329.816.3
	Effluent	351.214.2		Effluent	0 + 0		Effluent	0.510.2		Effluent	0.210.06

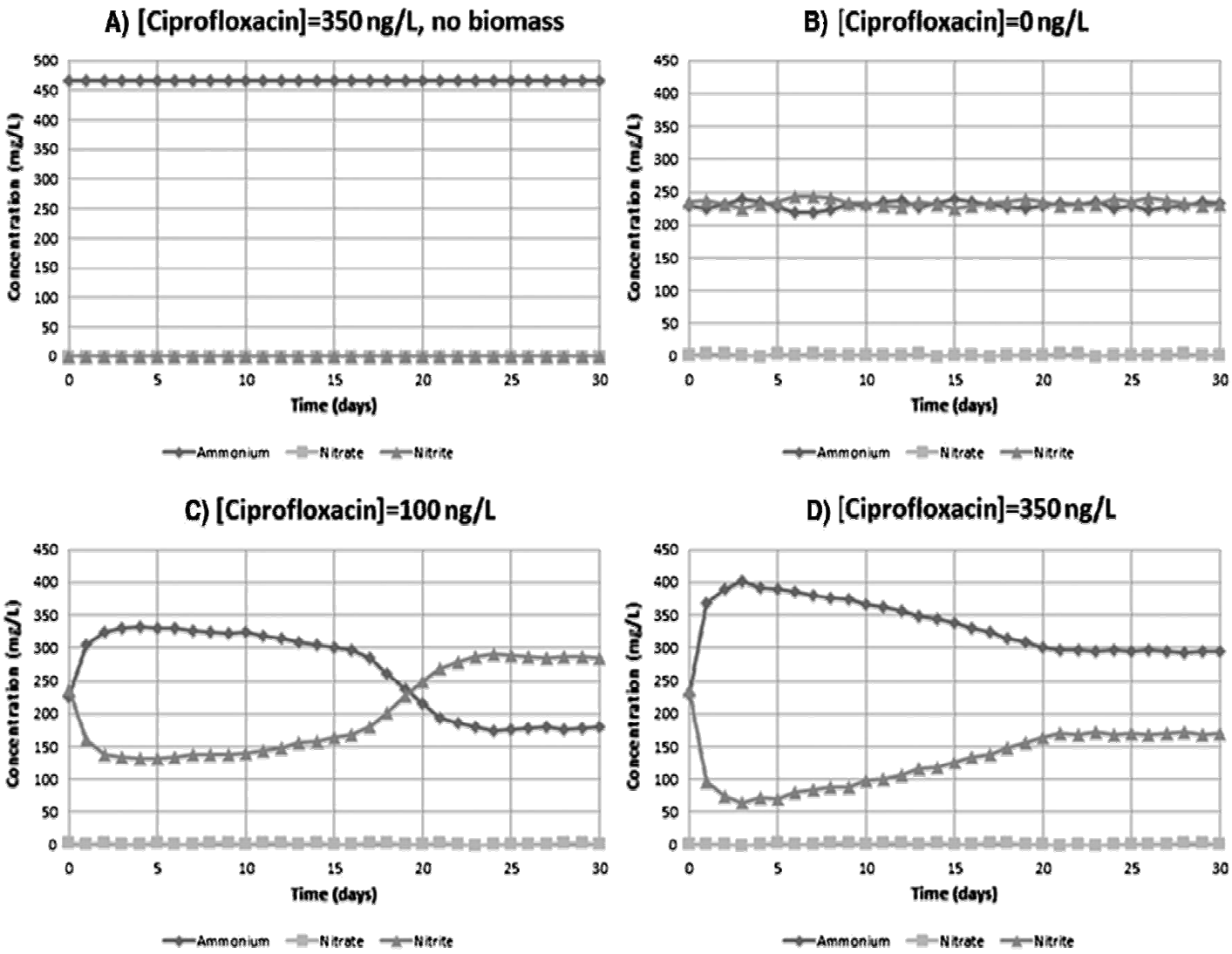


Fig. 2. Concentrations of ammonium, nitrite and nitrate through time in Experiments 1(A), 2(B), 3(C) and 4(D).

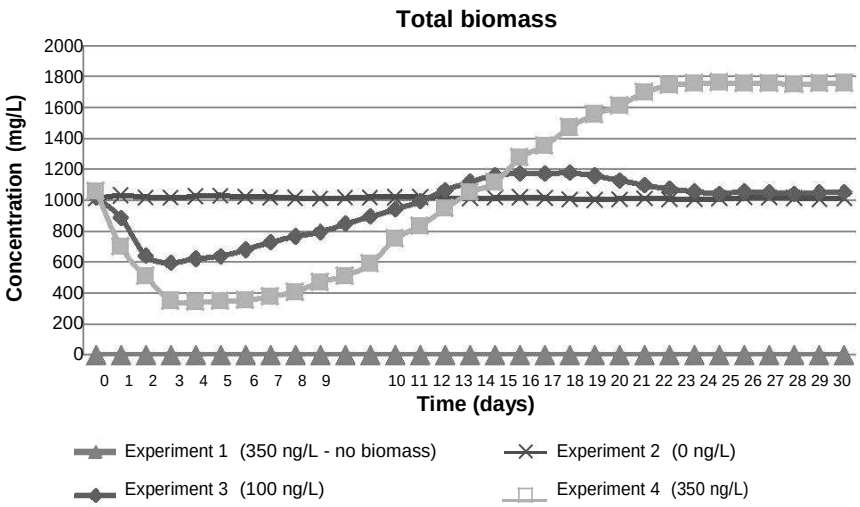


Fig. 3. Total biomass concentration inside the partial-nitritation reactor in Experiments 2, 3 and 4. Experiment 1 was performed in a partial-nitritation bioreactor fed with sterile synthetic wastewater containing 350 ng/L of ciprofloxacin.

characterisation was checked within several databases for reference and classification, such as Silva-compatible alignment database, mothur (Schloss et al., 2009) and Greengenes database. Finally, sequences were clustered into OTUs, and the abundance and diversity of these were checked with proper statistical analysis.

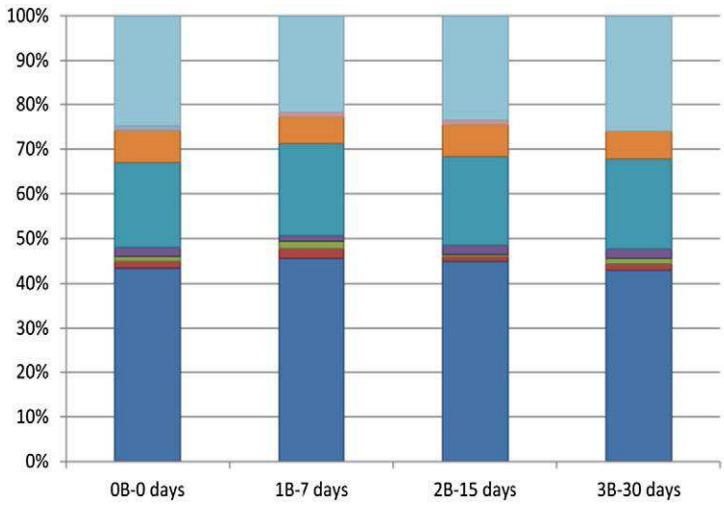
2.5. Phylogenetic trees

Phylogenetic trees for Experiments 2, 3, and 4 were developed using sequences generated in the pyrosequencing process. The Qiime U-Clust method using a genetic distance of 3% was used in order to construct all OTUs for each of the experiments. Generated OTUs were phylogeneti-cally related to the closest sequences using a BLAST search through the GenBank database. Phylogenetic trees for Experiments 1, 2 and 3 were developed using MEGA 5.2 software. Phylogenetic trees were cal-culated through the neighbour-joining statistical method. The test of phylogeny chosen was the bootstrap model of 1000 bootstrap replica-tions. The substitute model used was the Jukes–Cantor model.

2.6. Statistical analysis and curve fitting

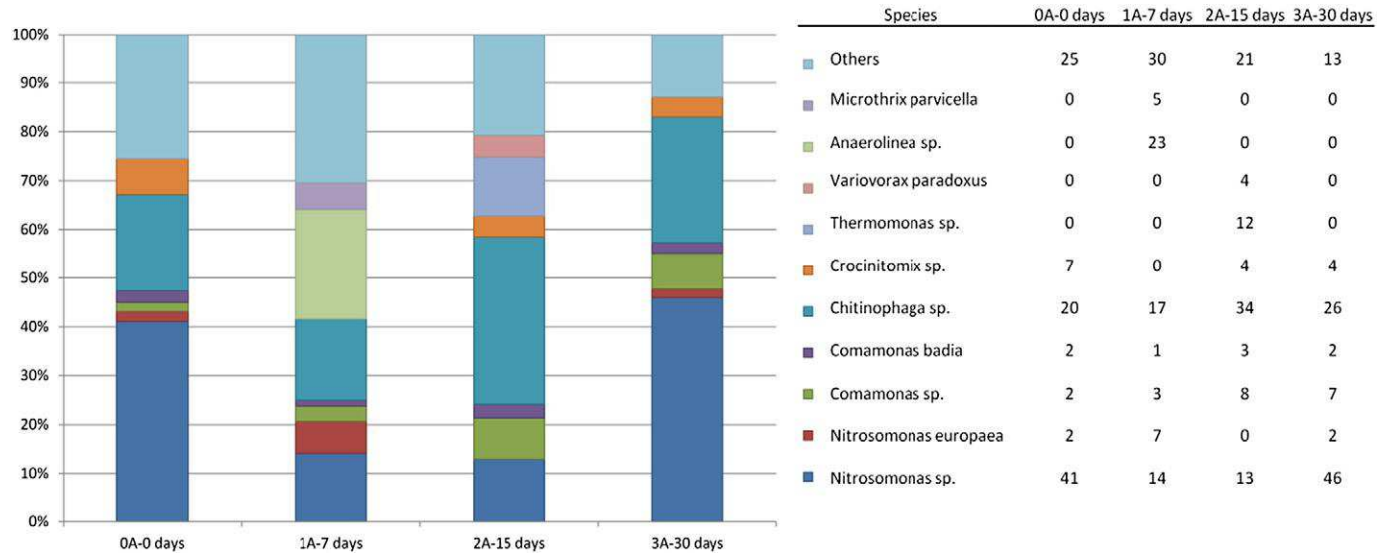
A principal component multivariant analysis between nitrite con-centration in the effluent, ciprofloxacin concentration in the influent and time of exposure to ciprofloxacin was developed. The objective was to determine the effect of ciprofloxacin addition over the perfor-mance of a partial-nitritation reactor. Data utilised for principal compo-nent analysis were collected from daily measurements of nitrite concentration in the effluent and ciprofloxacin concentration in the in-fluent for Experiment 2 (0 ng/L ciprofloxacin), Experiment 3 (100 ng/L ciprofloxacin) and Experiment 4 (350 ng/L ciprofloxacin), reaching a total population of 93 points. Principal component analysis was per-formed using the statistical software R-Project.

A mathematical model that explained nitrite concentration in the ef-fluent of the reactor through ciprofloxacin concentration in the influent and through time was developed. Ciprofloxacin concentration in the in-fluent and time of exposure to ciprofloxacin was taken as independent variables. Nitrite concentration in the effluent was taken as the depen-dent variable. Data used for correlation were taken from daily values



Species	0B-0 days	1B-7 days	2A-15 days	3B-30 days
Others	26	23	25	26
Myroides sp.	0	1	1	0
Dokdonella sp.	1	0	0	0
Crocinitomix sp.	7	6	7	6
Chitinophaga sp.	19	21	20	20
Comamonas badia	2	1	2	2
Comamonas sp.	1	2	0	1
Nitrosomonas europaea	1	2	1	1
Nitrosomonas sp.	43	45	45	43

Fig. 4. Bacterial community structure of biofilm at 0, 7, 15 and 30 days in a partial-nitritation bioreactor fed with synthetic wastewater without the addition of ciprofloxacin.



Species	0A-0 days	1A-7 days	2A-15 days	3A-30 days
Others	25	30	21	13
Microthrix parvicella	0	5	0	0
Anaerolinea sp.	0	23	0	0
Variovorax paradoxus	0	0	4	0
Thermomonas sp.	0	0	12	0
Crocinitomix sp.	7	0	4	4
Chitinophaga sp.	20	17	34	26
Comamonas badia	2	1	3	2
Comamonas sp.	2	3	8	7
Nitrosomonas europaea	2	7	0	2
Nitrosomonas sp.	41	14	13	46

Fig. 5. Bacterial community structure of biofilm at 0, 7, 15 and 30 days in a partial-nitritation bioreactor fed with synthetic wastewater amended with 100 ng/L of ciprofloxacin.

obtained through the determination of nitrite and ciprofloxacin in Experiments 2, 3 and 4; thus, regression fitted a population of 93 data points. Data were correlated using a polynomial fitting for the variables ciprofloxacin concentration and time. Curve fitting calculation was de-veloped using a Matlab R2013a Curve Fitting APP (Demuth and Beale, 2000).

3. Results and discussion

3.1. Ammonium, nitrite, nitrate, total biomass, ciprofloxacin evolution and dynamics of microbial communities within partial-nitritation reactors

3.1.1. Experiment 1

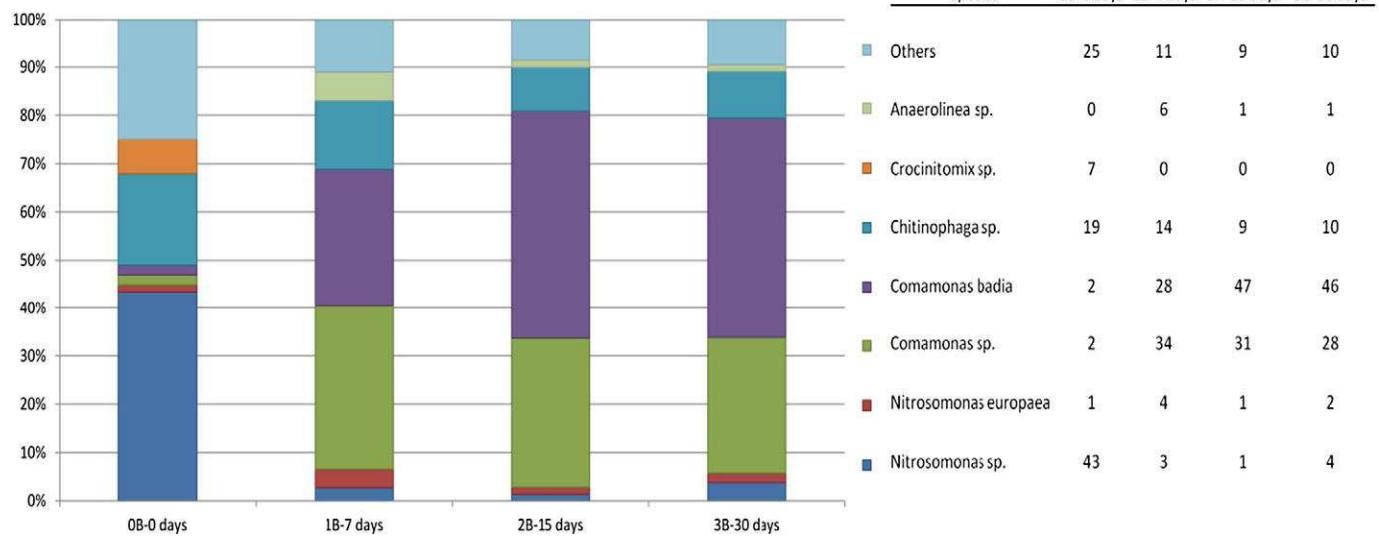
This experiment was conducted in a non-inoculated reactor fed with synthetic wastewater containing 350 ng/L of ciprofloxacin in order to confirm the sorption capacity of the carrier. The results clearly demon-strated that the antibiotic was not retained by the carrier BioFlow 9 (Table 2) and, consequently, the same amounts of ciprofloxacin were detected in the influent as in the effluent (350 ng/L), throughout the study period. Also, transformation of ammonium to nitrite or nitrate in the non-inoculated reactor was not detected (Fig. 2-A). Finally, no biofilm formation on the BioFlow 9 was microscopically observed throughout Experiment 1. Obviously, the non-inoculated bioreactor did not form biofilm and studies on the microbial communities were not performed.

3.1.2. Experiment 2

A partial-nitritation reactor was operated with no ciprofloxacin added to its influent. The evolution of ammonium, nitrite and nitrate concentrations in the effluent can be observed in Fig. 2-B. By day 0, the system was operating at a stable performance, characterised by an effluent of 50% ammonium-50% nitrite, which is in agreement with Van Dongen et al. (2001). Performance of the system acquired a stable value within the experiment time.

The evolution of total biomass concentration inside the partial-nitritation reactor subjected to no ciprofloxacin addition can be seen in Fig. 3. Total biomass concentration presents a steady value during the entire experimental period.

Operational conditions of the partial-nitritation reactor were not changed throughout the experiment time. This might have led to a



Species	0B-0 days	1B-7 days	2A-15 days	3B-30 days
Others	25	11	9	10
Anaerolinea sp.	0	6	1	1
Crocinitomix sp.	7	0	0	0
Chitinophaga sp.	19	14	9	10
Comamonas badia	2	28	47	46
Comamonas sp.	2	34	31	28
Nitrosomonas europaea	1	4	1	2
Nitrosomonas sp.	43	3	1	4

Fig. 6. Bacterial community structure of biofilm at 0, 7, 15 and 30 days in a partial-nitritation bioreactor fed with synthetic wastewater amended with 350 ng/L of ciprofloxacin.

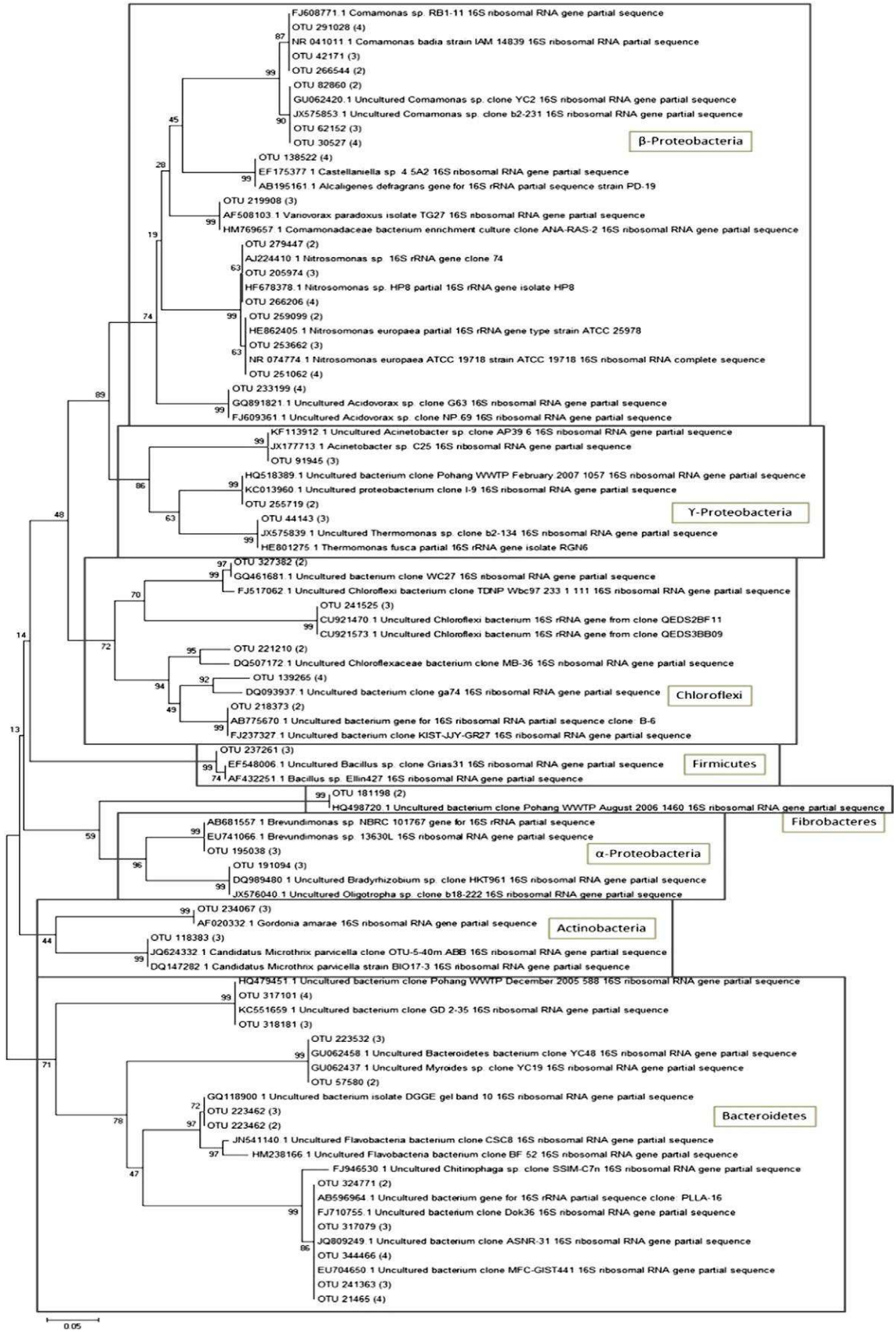


Fig. 7. Phylogenetic tree for Experiment 2, Experiment 3 and Experiment 4. OTUs followed by (2) were found in the Experiment 2 partial-nitritation reactor, OTUs followed by (3) were found in the Experiment 3 partial-nitritation reactor and OTUs followed by (4) were found in the Experiment 4 partial-nitritation reactor.

steady-state of the system, where neither performance nor total bio-mass of the system shifts with time. In this regard, total biomass concen-tration and ammonium conversion to nitrite are related.

The structure of the microbial community was stable during the entire experiment and was similar to previous reports (González-Martínez et al., 2013), where the dominance of Nitrosomonas sp. and Nitrosomonas europaea can be appreciated (Fig. 4). These bacteria develop autotrophic nitrification, utilising oxygen and ammonium to yield nitrite (Koops et al., 1991; Mußmann et al., 2013; Waheed et al., 2013). Nitrosomonas spp. are common bacteria found in partial-nitritation reactors, and carry out partial-nitritation reactions, along with other autotrophic nitrifying bacteria, such as Nitrosospira spp., Nitrosolobus spp. and Nitrosovibrio sp. (Logemann et al., 1998; Sri Shalini & Joseph, 2012; González-Martínez et al., 2013). However, in our experiment, it could be suggested that the partial-nitritation process is mainly due to the presence of Nitrosomonas spp. The microbial community structure also shows the moderate impor-tance of Chitinophaga sp., which is a heterotrophic and aerobic bacteria family (Sangkhobol & Skerman, 1981; Weon et al., 2009), containing some strains that are capable of N-acetylglucosamine utilisation (Del Rio et al., 2010), as well as Crocinitomix sp., which is a family of heterotro-phic, strictly anaerobic, N-acetylglucosamine utilising bacteria (Bowman et al., 2003). As is already known, N-acetylglucosamine is one of the major components of bacterial cell walls, and it is possible that N-acetylglucosamine utilisers inside the system are scavengers, developing metabolism with the utilisation of decaying bacterial biomass as a carbon source and nitrogen source (Rigali et al., 2006). The relatively low pres-ence of Comamonas sp. and Comamonas badia, which are aerobic and het-erotrophic bacteria (Gumaelius et al., 2001) has to be remarked. It has been found that Comamonas sp. and Comamonas badia are not able to uti-lise N-acetylglucosamine in their metabolism (Gumaelius et al., 2001; Young et al., 2008; Yu et al., 2011). However, it has been previously re-ported (Jung et al., 2009) that ciprofloxacin might be used as a carbon source by Comamonas sp. and Comamonas badia.

3.1.3. Experiment 3

The third experiment was conducted in a partial-nitritation bench-scale reactor fed with synthetic wastewater amended with 100 ng/L of ciprofloxacin. During this experiment, the complete removal of cipro-floxacin in the partial-nitritation bioreactor was observed (Table 2). Similar results have been reported in MBR systems exposed to several antibiotics (Dorival-García et al., 2013). It has been found that ciproflox-acin adheres strongly to biosolids due to its high octanol-water partition coefficient (Wu et al., 2009; Wunder et al., 2011). These facts could ex-plain the antibiotic removal in the bioreactor as a result of the sorption properties of the biomass, although a biotransformation process cannot be excluded.

Evolution of ammonium, nitrite and nitrate concentrations through-out the entire experiment is shown in Fig. 2-C. At time zero (previous to the addition of antibiotic), the system was balanced and the values of transformation of ammonium to nitrite were similar to those of the con-trol bioreactor fed with synthetic wastewater without ciprofloxacin (Fig. 2-B). However, after addition of the antibiotic, the transformation of ammonium to nitrite was drastically reduced. This inhibition process was extended for 15 days, detecting a decrease to 60% in relation to the control without the addition of ciprofloxacin. After this stage, ammonium transformation to nitrite experienced a stable phase of increase that started at day 15. After that time, the performance of the reactor was found to suffer a sharp increase that lasted up to day 24, detecting an increase of approximately 20% in relation to the control without the addition of antibiotics. However, from day 24 until the end of the experi-ment, it was observed that nitrite concentration in the bioreactor became stable with time.

As the generation of nitrite in the partial-nitritation bioreactor was coupled with ammonium transformation, no ammonium removal was observed without the proper generation of nitrite. Consequently, the concentration of nitrate in the bioreactor was negligible during Experi-ment 3.

The evolution of the biomass (mg/L of biofilm) in the partial-nitritation bioreactor subjected to 100 ng/L ciprofloxacin is recorded in Fig. 3. After addition of the antibiotic, the biomass was drastically reduced. This inhibition process was extended for 72 h, detecting a decrease to 40% in relation to the control without the addition of cipro-floxacin. However, microbial biomass grown on the surface of the BioFlow 9 carrier was slowly increased, reaching similar values to the control bioreactor without antibiotics after 15 days of operation time. From that moment, microbial biomass in the partial-nitritation bioreactor containing 100 ng/L of ciprofloxacin was stable in the system until the end of Experiment 3.

The results obtained in Experiment 3 suggest that there may be a re-lationship between the transformation of ammonium to nitrite and the microbial biomass concentration in the partial-nitritation bioreactor. In this sense, a decrease in microbial biomass during the first working days after having fed the system with synthetic wastewater supplemented with 100 ng/L of ciprofloxacin represents a decrease in the nitrite concentration in the bioreactor. However, after this lag period, an in-crease in the parameters microbial biomass attached to the BioFlow 9 carrier and nitrite concentration in the bioreactor was observed. At the end of Experiment 3, nitrite concentration and microbial biomass in the bioreactor fed with 100 ng/L of ciprofloxacin were 20% and 5% higher, respectively, than that in the control bioreactor fed without an-tibiotics. These data suggest that the presence of concentrations of 100 ng/L of ciprofloxacin produces a temporary inhibition of the ammo-nium oxidation process together with a temporary reduction of the microbial biomass. This ciprofloxacin effect over bacterial biomass de-tected in the partial-nitritation bioreactor has also been described by other authors (Wilson et al., 2003) for microorganisms in natural fresh-water, confirming the acclimatisation of the microbial communities at low concentrations of this antibiotic.

Samples of biofilm from the partial-nitritation bioreactor subjected to 100 ng/L ciprofloxacin concentration were collected at 0, 7, 15 and 30 days during the experiment. Samples were analysed using the tag-pyrosequencing technique, providing data about bacterial population dynamics within the partial-nitritation bioreactor. Evolution of bacterial community structure in the partial-nitritation bioreactor subjected to 100 ng/L ciprofloxacin can be seen in Fig. 5.

At the beginning of the experiment, the bacterial community structure resembles that of a typical partial-nitritation reactor fed with synthetic wastewaters without antibiotics (see also Experiment 2, Fig. 4). However, when the bioreactor was fed with synthetic wastewater treated with 100 ng/L of ciprofloxacin, the bacterial community structure was temporarily modified compared with the control bioreactor fed with synthetic wastewater without antibiotics. In particular, this modification was detected in the AOB bacteria (Nitrosomonas sp.) and showed values of 41, 14, 13 and 46%, after 0, 7, 15 and 30 days of operation, respectively. These results are consistent with the values of partial-nitritation observed in the bioreactor (see Fig. 2-C), suggesting that the presence of 100 ng/L of ciprofloxacin in the synthetic wastewater temporarily reduces the AOB microbial popu-lation, although this inhibitory effect disappears after long periods of operation. Consequently, the partial-nitritation bioreactor can be adapted to the presence of 100 ng/L of ciprofloxacin, probably due to the sorption capacity of the biofilm formed on the carrier (Dorival-García et al., 2013), although the presence of antibiotic de-grader microorganisms (i.e., Commamonas sp.) should be consid-ered for future studies.

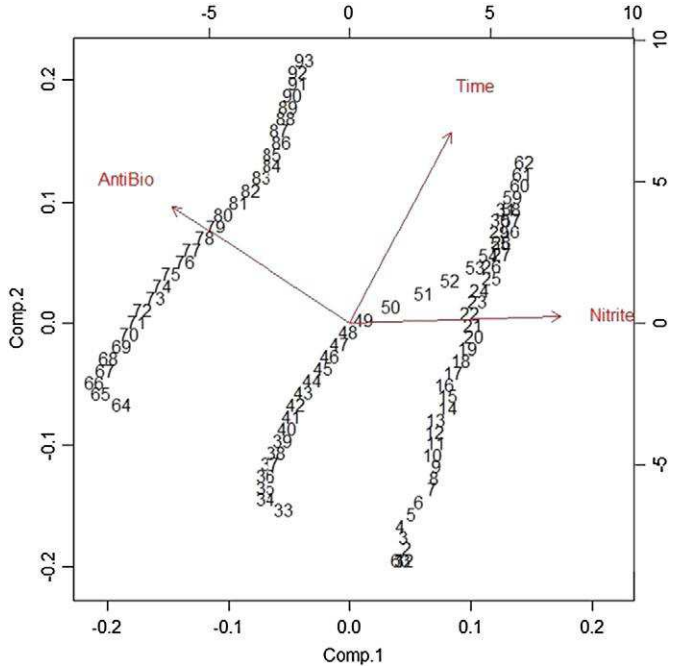


Fig. 8. Multivariant analysis obtained from 93 samples. From each experiment, 31 samples were taken which were represented as 1 to 31 for Experiment 2, 32 to 62 for Experiment 3 and 63 to 93 for Experiment 4. Variable parameters used in this multivariate analysis were nitrite concentration in the effluent, ciprofloxacin concentration in the influent and oper-ation time.

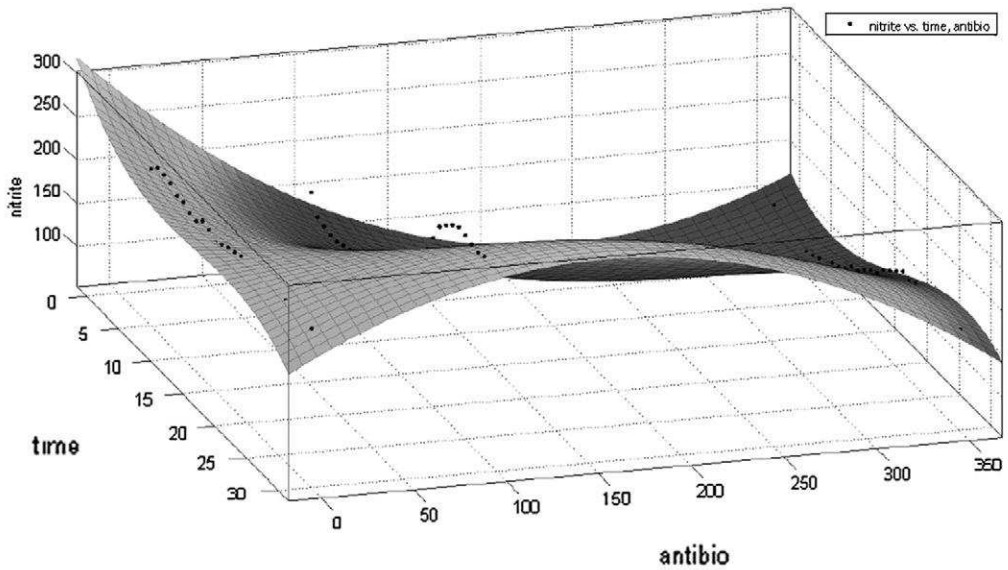
3.1.4. Experiment 4

A partial-nitritation bench-scale bioreactor was fed with synthetic wastewater supplemented with 350 ng/L of ciprofloxacin. As shown in Experiment 3, complete removal of ciprofloxacin in the effluent was observed (Table 2), suggesting that the antibiotic was retained or de-graded in the bioreactor.

The evolution of ammonium, nitrite and nitrate concentrations throughout the experiment is shown in Fig. 2-D. At time zero (prior to the addition of antibiotics), the system was balanced and the values for the transformation of ammonium to nitrite were similar to those of the control bioreactor fed with synthetic wastewater without cipro-floxacin (Fig. 2-B). However, after the addition of antibiotics, the trans-formation of ammonium to nitrite was reduced, showing a drastic reduction in relation to the control without ciprofloxacin. After 5 days of antibiotic entry into the bioreactor, the ammonium transformation to nitrite showed a phase increase that started at day 5 and continued up to day 21. However, the partial-nitritation bioreactor fed with 350 ng/L of antibiotic produced effluents with lower nitrite concentra-tions than those produced before the addition of antibiotics, or pro-duced in control bioreactors fed with synthetic wastewater that had not been treated with ciprofloxacin. From day 21 until the end of the ex-periment, it was observed that nitrite concentration in the bioreactor became stable through time. As detected in Experiments 2 and 3, the nitrate concentration in the effluent was negligible.

The microbial biomass (mg/L of biofilm) in the partial-nitritation bioreactor fed with synthetic wastewater containing 350 ng/L ciprofloxacin is shown in Fig. 3. After addition of the antibiotic, as report-ed in Experiment 3, microbial biomass was reduced at values of approx-imately 40% of those detected before the addition of ciprofloxacin. After 4 days, the microbial biomass growing on the surface of the BioFlow 9 carrier was continuously increased, reaching maximum values at
23 days of operation.

The results obtained in Experiment 4 suggest that the presence of higher concentrations of ciprofloxacin (350 ng/L) in synthetic wastewa-ter induces an inhibitory effect on the microbial communities (AOB) in the partial-nitritation bioreactor, which also affects the biotransforma-tion of ammonium to nitrite. An evident decrease in nitrite concentration was observed in the bioreactor, although an increase in the microbial at-tached biomass was also observed. These data suggest that the presence



$$[NO_2^-] = 266 - 10.94\ t - 1.739\ [Cipro] + 0.7849\ t^2 + 0.09502\ t\ [Cipro] + 0.003506\ [Cipro]^2 - 0.01539\ t^3 - 0.0002024\ t^2\ [Cipro] - 0.0002211\ t\ [Cipro]^2$$

R-square: 0.9567; Adjusted R-square: 0.9524

Fig. 9. 3D surface polynomial fitting comparing effluent nitrite concentration (nitrite, mg/L), influent antibiotic concentration (antibio, ng/L) and time of exposure (time, days) with the polynomial fitting that explains nitrite concentration in the effluent using time of exposure and ciprofloxacin concentration in the influent. $[NO^-_2]$ stands for nitrite concentration in the effluent measured in mg/L; t stands for time of exposure measured in days; [Cipro] stands for ciprofloxacin concentration in the influent measured in ng/L. Goodness of fit, expressed by R-square, is also indicated.

of high concentrations of ciprofloxacin produce an inhibition of the ammonium oxidation process without any reduction in the microbial bio-mass in partial-nitritation systems constructed as submerged fixed-bed biofilm reactor.

In the last 25 years, intensive research in the field of biological wastewater treatment has shown that fixed-biofilm systems are often more efficient for water purification than conventional suspended acti-vated sludge (Weber et al., 2007). Growth attached to the surface of car-rier materials has some advantages, such as a long sludge retention time, prevention of washout of biomass, and better process stability in terms of withstanding shock loadings or short-term disturbing effects (Sudarno et al., 2011). Moreover, biofilm also protects microorganisms in hostile environments, e.g. antimicrobial agents, UV light and other stressors (Simões et al., 2010; Lyon et al., 2008). However, our study suggests that a partial-nitritation bioreactor constructed as a fixed-biofilm system could adapt to certain concentrations (i.e., 100 ng/L) of ciprofloxacin, but an increase in the concentration (i.e., 350 ng/L) of antibiotics would finally determine an inhibition of the partial-nitritation process.

Biofilm samples from the partial-nitritation bioreactor fed with syn-thetic wastewater amended with 350 ng/L ciprofloxacin were collected at 0, 7, 15 and 30 days during the experiment, and the structures of the microbial communities were studied by tag-pyrosequencing techniques (Fig. 6).

At the beginning of the experiment, the bacterial community structure resembled that of a typical partial-nitritation reactor fed with synthetic wastewaters without antibiotics (see also Experiment 2, Fig. 4). However, after the addition of 350 ng/L of cip-rofloxacin into the system, drastic changes in the microbial commu-nity structure were observed (Fig. 6). In particular, Comamonas sp. and Comamonas badia experienced an evident increase from 2 to 28% and 2 to 46%, respectively. Comamonas badia is a heterotrophic gram-negative rod-shaped bacterium which has been isolated from activated sludge (Tago and Yakota, 2004). Also, Jung et al. (2009) reported that strains of Comamonas sp. and Comamonas badia can use ciprofloxacin as carbon and energy sources. In the same way, differ-ent strains of Commamonas have been reported to be resistant to this antibiotic (Almuzara et al., 2013; Taylor et al., 2013). Conse-quently, it might be suggested that the increase observed in the Commamonas populations in the partial-nitritation bioreactor fed with 350 ng/L of ciprofloxacin may be due to their resistance to this antibiotic and probably to the ability of these microorganisms to use ciprofloxacin as a carbon source. In this sense, our study sug-gests that the presence of high concentrations of ciprofloxacin in partial-nitritation bioreactors produces a selective enrichment of microorganisms of the genus Commamonas that are presumably re-sistant and/or degraders of this antibiotic, directly affecting the bio-logical activity in the partial-nitritation bioreactor. However, further experiments are needed in order to confirm these results and understand the environmental risks of the selection of resistant Commamonas sp. in wastewater treatment systems fed with ciprofloxacin.

Our results also showed that the addition of 350 ng/L of cipro-floxacin into a partial-nitritation bioreactor constructed as a fixed-biofilm system drastically affected the AOB populations (Fig. 6). Thus, the addition of this antibiotic decreased the Nitrosomonas pop-ulation from 43% to 3–4% of the total microbial community. Obvious-ly, AOB microorganisms were sensitive to this antibiotic at a concentration of 350 ng/L in the influent. Furthermore, these results were consistent with a reduction of ammonium oxidation to nitrite detected in the bioreactor after the addition of ciprofloxacin. Thus, our data suggest that while AOB microorganisms can adapt to low concentrations of ciprofloxacin in the wastewater (100 ng/L), when the antibiotic concentration is increased to values such as 350 ng/L, the AOB microorganisms are clearly reduced in the microbial com-munity of the bioreactor.

3.2. Phylogenetic study of bacterial OTUs

A phylogenetic tree for Experiments 2, 3 and 4 was constructed with the representative 16S rRNA partial sequences of bacterial OTUs generated through tag-pyrosequencing analysis (Fig. 7). OTUs belonging to α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Bacteroidetes, Chloroflexi, Firmicutes and Actinobacteria were found. The results show that most of the OTUs were evolutionally related to Proteobacteria; according to our data, β-Proteobacteria were identified as the major group. Beta-Proteobacteria are wide-spread components of the bacterial community in biofilm systems (Calderón et al., 2013), where members of the phylogenetic group are responsible for the processes of degradation and transformation of nutrients during wastewater treatment, and display an ability to become part of the heterogeneous biofilms formed under such conditions.

Among β-Proteobacteria, OTUs related to Nitrosomonas sp., Nitrosomonas europaea, Comamonas sp. and Comamonas badia were predominant in Experiments 2, 3 and 4. Obviously, the pres-ence of AOB microorganisms is expected in a partial-nitritation bio-reactor, while the presence of Comamonas could be influenced by the presence of ciprofloxacin in the bioreactor; however, these OTUs must be present in the mixed liquor used for the start-up of the bench-scale plants.

Beta-Proteobacteria related with Variovorax paradoxus were only found in Experiment 3, and two β-Proteobacteria OTUs were present only in Experiment 4; one of them was identified as Acidovorax sp. and the other related to both Castellaniella sp. and Alcaligenes defragans. Gamma-Proteobacteria only included two OTUs which were found in Experiment 3; one related to Thermomonas sp. and Thermomonas fusca, while the other was identified as Acinetobacter sp. α-Proteobacteria in-cluded two OTUs found in Experiment 3; one was related to Brevundimonas sp., while the other was closely related to both Bradyrhizobium sp. and Oligotropha sp. OTUs related to the Actinobacteria phylum were also found in Experiment 3. These OTUs were identified as
Microthrix parvicella and Gordonia amarae, respectively.

Bacteroidetes was the second major group of OTUs found in Experiments 2, 3 and 4. These microorganisms are widely found in urban wastewater and wastewater treatment systems. In our study, four OTUs affiliated with Chitinophaga sp. were found in all of the experi-ments and were related to Chitinophaga sp. and Flavisolibacter sp. Two OTUs identified with uncultured bacterium clones were identified as Haliscomenobacter sp. through the pyrosequencing process, and OTU 223462 was identified as Crocinitomix sp. and closely related to two un-cultured Flavobacteria clones. OTU 223532 was identified as Myroides sp.

Finally, OTUs related to Firmicutes and Chloroflexis phyla were also detected, although the presence of these groups was insignificant. Thus, Firmicutes-related sequences consisted of a single OTU affiliated with Bacillus sp. bacterium and the Chloroflexi phylum containing two OTUs, which were identified as Anaerolinea sp. (OTU 139265) and Anaerolinea sp. (OTU241525). Chloroflexi phylum contained one OTU from Experiment 3, which was identified as Anaerolinea sp. (241525), as well as one OTU from Experiment 4 (139265). Three Chloroflexi OTUs found in Experiment 2 were identified as Halochromatium sp. (327382), Chloroflexus sp. (221210) and Bellilinea sp.(218373).

3.3. Curve fitting

Principal component analysis of variable nitrite concentrations in the effluent, ciprofloxacin concentration in the influent and time of ex-posure to ciprofloxacin was performed using daily performance data from partial-nitritation reactors in Experiments 2, 3 and 4. Graphical representation of principal component analysis results can be seen in Fig. 8.

The data obtained from the multivariable analysis (Fig. 8) could be used to suggest that there is a negative correlation between ciprofloxa-cin concentration in the influent and the partial-nitritation process, expressed as nitrite concentration in the effluent independent of the operation time. However, the presence of a low concentration of antibiotics in the influent produced an initial reduction in the partial-nitritation activity, although this inhibitory effect was not detected after long periods of operation in the bioreactor. In conclusion, this multivariable analysis shows that the partial-nitritation activity is-clearly negatively correlated with the concentration of ciprofloxa-cin in a bioreactor constructed as a submerged biofilter.

A regression curve relating nitrite concentration in the effluent with ciprofloxacin concentration in the influent and time of exposure to cip-rofloxacin was developed using the Matlab Curve Fitting APP. Results obtained for a polynomial fitting of 3rd degree for time and 2nd degree for ciprofloxacin concentration can be seen in Fig. 9:

The data obtained from Fig. 9 confirm that the mathematical model (polynomial fitting) can be used for the prediction of nitrite concentra-tion in a partial-nitritation bioreactor fed with synthetic wastewater, amended with different concentrations of ciprofloxacin. Obviously, these data can be useful under our experimental conditions, but can be considered for future experiments at real scale. Experiments in this context are in progress in our laboratory in order to predict the effects of this group of antibiotics on the partial-nitritation process at the full scale.

4. Conclusions

Ciprofloxacin addition has an impact on the performance of a partial-nitritation reactor. Different concentrations of ciprofloxacin lead to dif-ferent steady-states of the partial-nitritation reactors, as well as to different microbial communities inside the bioreactors. Under the con-centration of 100 ng/L ciprofloxacin, the system acquired a more effi-cient removal of ammonium than the desired 50% ammonium-50% nitrite. Also, the microbial community structure suffered a period of adaptation with changes in respect to bioreactors without the addition of ciprofloxacin. With a concentration of 350 ng/L ciprofloxacin, the partial-nitritation reactor could no longer reach operation equilibrium with regard to ammonium removal. In addition, the microbial communi-ty structure experienced a deep change with a significant reduction of the AOB populations. Also, the presence of ciprofloxacin in the bioreactor increased Comamonas sp., which has been reported to be a family of ciprofloxacin-resistant bacteria. In this context, it could be suggested that the presence of antibiotics such as ciprofloxacin can seriously affect partial-nitritation systems, and select microorganisms with antibiotic re-sistance. This can produce problems in partial-nitritation reactors oper-ating in full scale wastewater treatment plants, and can also spread antibiotic-resistant strains that can endanger both human health and the environment alike. However, more research is needed in order to un-ravel the effect of other antibiotics and pharmaceutical compounds in partial-nitritation bioreactors.

Conflict of interest

There are no conflicts of interest with this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2014.01.012.

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General Discussion

As described in earlier sections, biological processes are widely used in wastewater treatment plants for several reasons, such as their high efficiency and relatively low cost in comparison with physicochemical treatment. For these reasons, it is important to improve knowledge about biological technologies. In this sense, establishment of an objective correlation between physicochemical parameters and microbial communities is needed for a better understanding of biological technologies such as partial nitritation systems.

Throughout this research, has tried treated to give a special emphasis on the effect of several different operating conditions on nitrogen removal efficiency and the effect of these parameters on the microbial population, trying to achieve the best performance out of submerged-bed partial nitritation. Thus, knowledge of the changes in the microbial population may have a significant impact on future optimisation of the system.

The first chapter of this thesis gives a general review of the different autotrophic nitrogen removal systems. It explains how these technologies are evolving rapidly, saving the cost and increasing the yield of the process of nitrogen removal. Thus, in autotrophic nitrogen removal technologies a shortcut in the nitrogen cycle can be used. In the second chapter, the effect of different HRTs was studied. When we started to work with partial nitritation, we saw that the fluidised bed configuration (SHARON process) operated without any biomass retention. This means that the SRT is the same as the HRT (24 h), as reported by Van Dongen et al. (2001). However, some full-scale granular anammox systems such as Rotterdam has an HRT of 9 h. In this sense, to try to decrease the HRT in the partial nitritation process, several bench-scale submerged-bed partial nitritation bioreactors fed with synthetic wastewater was built. The first step of this research was to find the perfect HRT to obtain an ammonium-nitrite mixture with conversion of 50% of the ammonium to nitrite.

In this way, HRTs of 9 h and 12 h were used. Using an HRT of 12 h, we observed that after 5 days of operation, all the ammonium was converted to nitrite. However, with an HRT of 9 hm the conversion of ammonium to nitrite reached 65% after 5 days of operation. After this period, the submerged-bed partial nitritation bioreactor was stabilised and its capacity for biotransformation of ammonia to nitrite remained

constant. Thus, in accordance with Mosquera-Corral et al. (2005), with these two HRTs the perfect ammonium-nitrite mixture was not achieved.

On the other hand, to understand the effect of these different HRTs on nitrogen removal performance, it was decided to evaluate the microbial population in both conditions, focusing on the general microbial population in the start-up (8 days) and the specific bacterial groups involved in the nitrification process, such as AOB (CTO), NOB (nxrA), and denitrifying bacteria (NosZ). The experiments were performed for 30 days using a cultivation-independent approach based on PCR-TGGE fingerprinting.

Comparing the TGGE results of the two experiments, it can be seen how the modification of the HRT directly affected the bacterial diversity of the biofilms formed in the start-up of a submerged-bed partial nitritation system. In the experiment performed with an HRT of 12 h, bacterial diversity was significantly reduced when the bioreactor operated in stable conditions (after 5 days). In fact, fewer than 10 bands were observed, probably as a consequence of the high level of specific ammonium-oxidising bacteria. However, with an HRT of 9 h, the microbial biodiversity was extremely heterogeneous. These data have been corroborated by the richness range-weighted (Rr) indices (Van Hulle et al., 2005), which were significantly different in the two experiments (analysis of variance; $p<0.05$). The Rr of the experiment with an HRT of 9 h displayed higher average values than those of the experiment with an HRT of 12 h. These data are in accordance with the results reported by Logemann et al. (1998) and Marzorati et al. (2008), and showed great differences in the microbial population in the start-up of the two experiments. However, several bands such as *Nitrosomonas* sp. were present in both experiments. The presence of *Nitrosomonas* sp. in these bioreactors is hardly surprising, since this microbial group has been reported by several authors as playing an important role in the conversion of ammonium to nitrite in this wastewater treatment biotechnology (Poth et al., 1985; Stehr et al., 1995; Schmidt & Bock, 1997).

In the start-up with an HRT of 12 h, some species, such as *Pseudacidovorax* sp. and *Aquaspirillum* sp., completely vanished over time. However, other species became more abundant, such as *N. eutropha*, *Variovorax* sp., and *N. europaea*. In order of abundance, the main group of identifiable TGGE bands was related to Betaproteobacteria (59%), followed by Alphaproteobacteria (36%) and Gammaproteobacteria (5%).

On the other hand, in the start-up with an HRT of 9 h, the Pearson coefficient showed only one cluster of bacteria, with 80% similarity. This result clearly indicates that there was less variation in the samples over time. Interestingly, the bands belonging to bacteria such as *Roseobacter* sp. (Band 32) and *Burkholderia* sp. (Band 50) disappeared, depending on the operating time. However, other microorganisms such as *Nitrosospira* sp., *Nitrosomonas* sp., and *Paracoccus* sp. incresed in number. Moreover, the presence of some AOB such as *Nitrosovibrio* sp. was detected as normal microbiota in the bioreactor operating with an HRT of 9 h. The main group shown in the TGGE bands was related to Proteobacteria and, specifically, to Alphaproteobacteria (56%), Betaproteobacteria (40%), and Deltaproteobacteria (4%), showing differences in the start-up in comparison with the bioreactor operating with an HRT of 12 h.

When we studied the specific AOB in all the experiments (30 days) using CTO primers, according to the HRT applied to the system, we obtained two clusters with a very low level of similarity between them. However, the results with these specific primers are in accordance with the AOB found with universal primers. In this sense, the results obtained in this experiment with an HRT of 12 h are not surprising in terms of the species found. The oxidation of ammonia is carried out predominantly by *Nitrosomonas* and *Nitrosospira* genera (Utåker et al., 1995; Posmanik et al., 2014). Furthermore, as reported by several authors (Dionisi et al., 2002, Vejmelkova et al., 2011), *Nitrosomonas oligotropha* clusters prevail in environments with low ammonium concentrations, while most of the bacteria present in nitrifying bioreactors operating at high ammonium levels, such as our partial nitritation bioreactor, belong to the *N. europaea* lineage. Moreover the presence of *N. eutropha* was evident during the whole experiment. Nevertheless, at an HRT of 9 h, *N. europaea* prevailed. Operating conditions also seemed to have an influence on the presence of *Nitrosospira*. In fact, when the experiment was performed with an HRT of 9 h, *Nitrosospira* spp. were present during the whole experiment, while with an HRT of 12 h, they were not detected.

On the other hand, as described in earlier sections, the conversion of nitrite to nitrate can be done if DO concentrations are not constant during the experiment. For this reason, the study of NOB community structure using NxrA primers was done. The NOB

study showed the typical nitrite-oxidising species of the genus *Nitrobacter*. It is well known that species of this genus are the main NOB in nitrifying wastewater treatment plants (Kim & Kim, 2006). The *Nitrobacter* genus currently consists of four valid species: *Nitrobacter winogradsky; Nitrobacter hamburgensis; Nitrobacter vulgaris;* and *Nitrobacter alkalicus* (Vanparys et al., 2007). In our case, all these species were present with both operating conditions and throughout the experiments. The only exception was *N. hamburgensis*, which only appeared in the bioreactor operating with an HRT of 12 h. However, the physicochemical parameters showed that nitrate conversion was negligible. This means that probably, even if they are present and show a high level of biodiversity, NOB cannot function with these operational settings and are probably in the lag phase.

Moreover, amplification of the nitrous oxide reductase (NosZ) gene was performed to determine the presence or absence of denitrifiying bacteria in a submerged-bed partial nitrification bioreactor. The similarity between these HRTs (9 h and 12 h) was assessed by Dice coefficient. This coefficient of similarity showed that the structure of the denitrifying bacteria community was influenced by the HRT. In this case, two clusters were observed, with a similarity below 60%. The total band classes detected were 11 (HRT of 12 h) and 12 (HRT of 9 h) in the experiments.

A phylogenetic tree was obtained from sequencing the most prominent bands. The results showed that some bands correlated with common denitrifying species, such as *Alicycliphilus denitrificans*, *Diaphorobacter nitroreducens,* and *Paracoccus denitrificans.* All of these denitrifying species are common in mixed liquors and the biofilm of wastewater treatment plants (Uemoto & Saiki, 1996; Khan & Hiraishi, 2002; Mechichi et al., 2003; Hou et al., 2012). *D. nitroreducens* is also well known for its ability to perform simultaneous nitrification and denitrification under aerobic conditions (Khardenavis et al., 2007). Although only one band was related to this species, it was detected for both operating conditions (HRT of 12 h and 9 h) and throughout the entire experiment. The only exception was the band related to the genus *Paracoccus*. The presence of this bacterium could be due to possible aerobic denitrification. Indeed, *Paracoccus* species are the first ones reported to carry out complete denitrification under aerobic conditions (Ahn, 2006). However, further studies are needed to confirm this hypothesis. These microbial populations were probably not biologically active

under the experimental conditions; however, the presence of these bacteria in the submerged-bed partial nitritation was shown.

Moreover, redundancy analysis (RDA; a multivariate statistical analysis) of these data showed that the samples were grouped together in relation to the two HRTs. In this sense, when the HRT was increased in the bioreactor a significant increase in the biotransformation of ammonium to nitrite was detected.

Thus, the RDA showed that changes in the operating conditions such as HRT and the concentration of ammonium might produce changes in the microbial population, with the exception of NxrA where only the HRT was significant. Consequently, our results suggest that the performance of the biotransformation of ammonium to nitrite in a submerged-bed partial nitritation bioreactor can be directly affected by the HRT, which can be crucial for the optimisation of performance rates and setting up control strategies for the system.

According to the mentioned results, a reduction in the HRT could be applied to obtain optimum performance in a submerged biofilter for partial nitritation. In this research, the reduction of the HRT to 7 h was done. After 7 days of operation, the system was operating with stable performance, with an ammonium-nitrite mixture of 50% of ammonium and 50% of nitrite. About this bench-scale bioreactor, it is important to emphasise the high nitrogen removal performance obtained.

Once these results of the optimum HRT were obtained, the influence of an antibiotic commonly found in urban wastewater (ciprofloxacin) was evaluated in four partial nitritation bioreactors working in parallel(Table 7), which received sterile synthetic wastewater with 466 mg/L of total nitrogen with addition of 350 ng/L of ciprofloxacin, synthetic wastewater without ciprofloxacin, synthetic wastewater with 100 ng/L of ciprofloxacin, and synthetic wastewater with 350 ng/L of ciprofloxacin. In each one, the influences of ciprofloxacin on the performance and on the microbial population were studied during 30 days.

Table 7. Different Synthetic wastewater composition in each antibiotics experiment

Chemical	Experiment A	Experiment B	Experiment C	Experiment D	Unit
Ciprofloxacin($C_{17}H_{18}N_3FO_3$)	**350**	**0**	**100**	**350**	**ng/L**
$(NH_4)_2SO_4$	**2.35**	**2.35**	**2.35**	**2.35**	**g/L**
$NaHCO_3$	**3.25**	**3.25**	**3.25**	**3.25**	**g/L**
$CaCl_2$	**0.30**	**0.30**	**0.30**	**0.30**	**g/L**
KH_2PO_4	**0.07**	**0.07**	**0.07**	**0.07**	**g/L**
$MgSO_4$	**0.02**	**0.02**	**0.02**	**0.02**	**g/L**
$FeSO_4\_7H_2O$	**0.009**	**0.009**	**0.009**	**0.009**	**g/L**
H_2SO_4	**0.005**	**0.005**	**0.005**	**0.005**	**g/L**

The first bioreactor was not inoculated with mixed liquor and only received sterile synthetic wastewater with 350 ng/L of ciprofloxacin. For these reasons, conversion did not take place; this set-up was used to determine the sorption properties of the support material. The results clearly demonstrated that the antibiotic was not retained by the carrier BioFlow 9. On the other hand, the second bioreactor was operated with no ciprofloxacin added to its influent. As has been described before, it operated with stable performance, producing an effluent of 50% ammonium-50% nitrite over the time.

The third submerged-bed partial nitritation bioreactor was fed synthetic wastewater with addition of 100 ng/L of ciprofloxacin. During this experiment, after addition of the antibiotic, the transformation of ammonium to nitrite was drastically reduced. This inhibition lasted 15 days, with a decrease of performance to 60% of that of the control without the addition of ciprofloxacin. After that time, the performance of the bioreactor showed a sharp increase that lasted up to day 24, with an increase of approximately 20% in relation to the control without the addition of antibiotic. However, from day 24 until the end of the experiment, the nitrite concentration in the bioreactor became stable.

Finally, the fourth bioreactor was fed synthetic wastewater with 350 ng/L of ciprofloxacin. The transformation of ammonium to nitrite was drastically reduced in comparison with the control without ciprofloxacin. After 5 days, the conversion of ammonium to nitrite showed a phase increase that started at day 5 and continued up to

day 21. From day 21 until the end of the experiment, it was observed that the nitrite concentration in the bioreactor became stable, with 15% lower performance in comparison with the control.

The effect of ciprofloxacin could be also seen in the total biomass. In the experiments with addiction of ciprofloxacin, the microbial biomass was reduced after some days of operation. Thus, in both experiments a relationship between the transformation of ammonium to nitrite and the microbial biomass concentration in the partial nitritation bioreactor was found. In this sense, the decrease in microbial biomass during the first working days represented a decrease in the nitrite concentration in the bioreactor. However, after this lag period, an increase in the microbial biomass attached to the BioFlow 9 carrier and nitrite concentration in the bioreactor was observed. These data suggest that high concentrations of ciprofloxacin inhibit the ammonium oxidation process without any reduction in the microbial biomass in partial nitritation systems constructed as submerged-bed partial nitritation bioreactors.

To understand the effect of the different ciprofloxacin concentrations on the performance of the submerged-bed partial nitritation bioreactor, a study of the microbial population in each experiment using 454-pirosequencing methods was done. Obviously, the non-inoculated bioreactor did not form a biofilm and studies on the microbial communities were not performed. In the bioreactor without ciprofloxacin, with the perfect conversion of ammonium to nitrite and perfect operational conditions in a submerged-bed partial nitritation bioreactor, the structure of the microbial community was stable during the entire experiment, with dominance of *Nitrosomonas* spp. and *N. europaea*. These bacteria perform autotrophic nitrification, utilising oxygen and ammonium to yield nitrite. The microbial community structure also showed moderate importance of *Chitinophaga* spp., which are a heterotrophic and aerobic bacteria family (Weon et al., 2009), containing some strains that are capable of utilising N-acetylglucosamine (Del Rio et al., 2010), as well as *Crocinitomix* spp., which are a family of heterotrophic, strictly anaerobic, N-acetylglucosamine-utilising bacteria (Bowman et al., 2003). As is already known, N-acetylglucosamine is one of the major components of bacterial cell walls, and it is possible that N-acetylglucosamine utilisers in the system are scavengers that use decaying bacterial biomass as a carbon source and nitrogen source (Rigali et al., 2006). The relatively low abundance of *Comamonas* spp.

(including *Comamonas badia*), which are aerobic and heterotrophic bacteria (Gumaelius et al., 2001) is noteworthy.

At the beginning of control experiments(Experiment B) without cirprofloxacin the bacterial community structure resembled that of a typical partial nitritation reactor fed synthetic wastewaters without antibiotics. However, when the bioreactor was fed synthetic wastewater containing 100 ng/L of ciprofloxacin(Experiment C), the bacterial community structure was temporarily modified compared with the control bioreactor fed synthetic wastewater without the antibiotic. In particular, this modification was detected in the AOB (*Nitrosomonas* spp.) and showed values of 41, 14, 13, and 46%, after 0, 7, 15, and 30 days of operation, respectively. The inhibitory effect of ciprofloxacin led to the reduction of AOB, although this inhibitory effect disappeared after long periods of operation.

After the addition of 350 ng/L of ciprofloxacin(Experiment D) to the system, drastic changes in the microbial community structure were observed. In particular, *Comamonas* spp. and *Comamonas badia* showed an evident increase from 2 to 28% and 2 to 46%, respectively. These data could be explained by the resistance of different strains of *Commamonas* to ciprofloxacin (Almuzara et al., 2013; Taylor et al., 2013). An inhibitory effect of the ciprofloxacin on AOB as observed. Furthermore, this inhibitory effect did not disappear and the low abundance of AOB was constant over time.

OTUs belonging to Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Bacteroidetes, Chloroflexi, Firmicutes, and Actinobacteria were found. The results showed that most of the OTUs were evolutionally related to Proteobacteria; according to our data, Betaproteobacteria were the major group. Betaproteobacteria are widespread components of the bacterial community in biofilm systems (Calderón et al., 2013). Among Betaproteobacteria, OTUs related to *Nitrosomonas* spp., *Nitrosomonas europaea*, *Comamonas* spp., and *Comamonas badia* were predominant in Experiments B, C, and D. Obviously, the presence of AOB is expected in a partial nitritation bioreactor, while the presence of *Comamonas* could be influenced by the presence of ciprofloxacin in the bioreactor; however, these OTUs must be present in the mixed liquor used for the start-up of the bench-scale plants.

Finally, to understand the correlation between ciprofloxacin concentration in the influent and the partial nitritation process, a multivariable analysis was done. The results showed that the partial nitritation activity was clearly negatively correlated with the concentration of ciprofloxacin in a bioreactor constructed as a submerged-bed partial nitritation. Moreover, a regression curve relating nitrite concentration in the effluent with ciprofloxacin concentration in the influent and time of exposure to ciprofloxacin was developed using Matlab. The regression curve can be used to predict the nitrite concentration in a partial nitritation bioreactor fed synthetic wastewater containing different concentrations of ciprofloxacin.

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Conclusions

Based on the results obtained, the following conclusions can be drawn:

1. The HRT affects the functioning and microbial community structure of bench-scale partial nitritation bioreactors built with submerged-filter technology.

2. The application of HRT of 7 h in submerged-bed partial nitritation technology increases the biotransformation of ammonium into nitrite.

3. The HRT in a partial nitritation bioreactor constructed as a submerged fixed-biofilm bioreactor affects the structure of the microbial community. With an HRT of 12 h, the majority of the bacteria were *N. eutropha*, while with an HRT of 9 h *N. europea* and *Nitrosospira* were dominant. Moreover, the abundance of other bacterial groups analysed, such as NOB, was also affected by the HRT used.

4. The presence of ciprofloxacin has an impact on the performance of partial nitritation bioreactors. With 100 ng/L of ciprofloxacin, the system showed more efficient removal of ammonium than the desired 50% ammonium-50% nitrite. However, with concentrations up to 350 ng/L of ciprofloxacin, the partial nitritation bioreactors could no longer reach operation equilibrium with regard to ammonium removal.

5. The presence of ciprofloxacin in wastewaters affects the microbial community structure in partial nitritation systems. In this context, the presence of low concentrations of antibiotic leads to a period of adaptation, different steady states of the partial nitritation reactor, as well as differences in microbial communities inside the bioreactor with respect to a bioreactor without the addition of ciprofloxacin.

6. In addition, with antibiotic concentrations of up 350 ng/L, microbial community structure experienced a deep change, with a significant reduction of the AOB populations. Finally, the presence of high concentrations of ciprofloxacin in urban wastewater produces an enrichment of resistant strains.

Conclusiones

En base a los resultados obtenidos, se han alcanzado diferentes conclusiones:

1. El tiempo de retención hidráulico(TRH) afecta al funcionamiento de un biorreactor de nitrificación parcial a escala piloto construido bajo una configuración de filtro sumergido y a la estructura de las poblaciones microbianas.

2. Mediante el uso de un biorreactor de nitrificación parcial con filtro sumergido puede alcanzarse un TRH de 7 horas incrementando la biotransformación de amonio a nitrito.

3. El TRH en un biorreactor de nitrificación parcial a escala piloto construido bajo una configuración de filtro sumergido afecta a la estructura de la comunidad microbiana existente en el. Asi, con un TRH de 12h, las bacterias mayoritarias pertenecen a *Nitrosomonas eutropha*, mientras que con un TRH de 9h las bacterias dominantes fueron *Nitrosomonas europea* y *Nitrosospira* sp. Además otros grupos bacterianos analizados , tales como, las bacterias oxidadoras de nitrito, también mostraron modificaciones en respuesta al cambio en cada experimento del TRH.

4. La presencia de ciprofloxacino tiene un impacto sobre el rendimiento de un biorreactor de nitrificación parcial. Bajo un concentración de 100ng/L de ciprofloxacino, el sistema adquiere una mayor eficiencia en la transformación de amonio que se pretende de 50% de amonio y 50% de nitrito. Sin embargo, concentraciones mayores de 350ng/L de ciprofloxacino el biorreactor de nitrificación parcial no es capaz de alcanzar ek equilibrio operacional de eliminación de amonio

5. La presencia de ciprofloxacino en las aguas residuales afecta a la estructura de las poblaciones microbianas en los sistemas de nitrificación parcial. En este contexto, la presencia de bajas concentración de antibiótico producen un periodo de adaptacion con cambios con respecto a los biorreactores sin adicción de ciprofloxacino, conduciendo a diferentes estados de equilibrio, ademas de a cambios de la estructura en las poblaciones microbianas dentro de los biorreactores.

6. Además, con concentración superiores a 350ng/L, la estructura de las poblaciones microbianas experimentan un cambio profundo con una reducción significativa de las bacterias oxidadoras de amonio. Finalmente, la presencia de altas concentraciones de ciprofloxacino en las aguas residuales producen un enriquecimiento de bacterias resistentes.