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Research article

Performance and microbial features of the partial nitritation-anammox process treating fish canning wastewater with variable salt concentrations

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ABSTRACT

The partial nitritation-anammox (PN-AMX) process applied to wastewaters with high NaCl concentration was studied until now using simulated media, without considering the effect of organic matter concentration and the shift in microbial populations. This research work presents results on the application of this process to the treatment of saline industrial wastewater. Obtained results indicated that the PN-AMX process has the capability to recover its initial activity after a sudden/acute salt inhibition event (up to 16 g NaCl/L). With a progressive salt concentration increase for 150 days, the PN-AMX process was able to remove the 80% of the nitrogen at 7–9 g NaCl/L. The microbiological data indicated that NaCl and ammonia concentrations and temperature are important factors shaping PN-AMX communities. Thus, the NOB abundance (*Nitrospira*) decreases with the increase of the salt concentration, while heterotrophic denitrifiers are able to outcompete anammox after a peak of organic matter in the feeding.

1. Introduction

The fish canning industry represents a very important economic sector in the Northwest of Spain (Galicia), with more than 65 factories, located mainly in coastal areas. As a consequence of this industrial activity large wastewater volumes are produced, which contain high solids, organic matter and nitrogen concentrations. Therefore, these effluents need to be efficiently handled previous discharge to avoid pressures over the marine environment. These effluents are normally treated by means of anaerobic treatment technologies, with the main purpose of reducing the chemical oxygen demand (COD) concentration of the wastewater and produce energy as biogas. Nevertheless, the effluents coming from the anaerobic digester (AD) contain high concentrations of nitrogen, mainly from proteins, which has to be removed in a subsequent step to adjust to disposal limits. Conventionally, the combined nitrification-denitrification process is applied to the removal of nitrogen. This process requires much energy to create aerobic conditions for bacterial nitrification, as well as the availability of organic carbon to remove nitrate by heterotrophic denitrifying organisms (Kartal et al., 2010). If a complete autotrophic process is applied the organic matter can be saved to produce more energy in the AD.

For this reason, most of recent research efforts have been focused on other alternatives for nitrogen removal, such as the combination of partial nitritation and anammox (PN-AMX) processes. Its application is suitable for wastewater streams with high nitrogen but low organic matter concentrations, like the supernatant from anaerobic digesters (Lackner et al., 2014). In the PN-AMX process, half of the ammonium present is oxidized following the nitritation pathway, combined with the subsequent biological reaction of the produced nitrite and the remaining ammonium to produce nitrogen gas according to the anammox reaction. However,

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the presence of certain inhibitory compounds in the wastewater may hinder the application of the anammox based processes at industrial scale (Jin et al., 2012). For example, anammox bacteria are very sensitive to environmental parameters such as salinity (Scaglione et al., 2017), that is one of the main components of the fish canning effluents (Cristóvão et al., 2016).

The occurrence of high saline concentrations in fish canning effluents, which is mainly caused by a high NaCl concentration, may induce salt stress to the microorganisms involved in the biological treatments, with the subsequent inhibition of many enzymes, decrease in the cell activity and eventually plasmolysis (Jin et al., 2012). The salt content is strongly related to the seasonality in the fish canning industry, which may generate sudden saline shock loads or unexpected variations in the salt concentration depending on the changes in the raw material processed in the factory, with concentration that varies from 2 to 35 g NaCl/L (Cristóvão et al., 2016).

Although, there are several research works that report on the effect of salt concentrations on the anammox bacteria activity, most of them show results about batch activity tests and/or the continuous operation of only the anammox process fed with a synthetic medium (Scaglione et al., 2017). Windey et al. (2005) performed the first research work with the PN-AMX process at long term operation increasing the NaCl concentration, although they used a synthetic medium. More recently, Malovanyy et al. (2015) tackled the adaptation of one-step PN-AMX biomass to increasing salt concentrations from 0 to 10–15 g NaCl/L in 160 days. In their study, the feeding consisted in reject water from an anaerobic sludge digester, while the salinity as NaCl was synthetically added.

As the inoculum available to start-up a PN-AMX process is normally not adapted to salinity, an important aspect to consider is the possible shift in the microbial populations. Despite the advancements of next generation sequencing platforms in microbial ecology, the previous studies about the influence of salinity in the PN-AMX process had paid little attention to this aspect. Moreover, only few studies have analysed the PN-AMX reactor communities using this cutting-edge technology (Agrawal et al., 2017; Wang et al., 2017b). For example, Wang et al. (2017b) studied the microbiological shift in a PN-AMX process with the progressive increase in the salt concentration from 0 to 20 g NaCl/L. However, their research work was performed with a synthetic medium ignoring the microorganisms that can be present in industrial effluents and the perturbations in composition of wastewaters, such as changes in organic matter compounds.

In the present research work the combined PN-AMX process is applied for the treatment of industrial saline wastewater produced in a fish cannery. The effect of salt concentrations on the performance of a PN-AMX granular sludge reactor was evaluated as: (1) sudden salt shock loads, up to 16 g NaCl/L, and (2) progressive adaptation to concentrations up to 10 g NaCl/L. The evolution of the main microbial populations present in the biomass from the reactor was determined to prove the effects of salinity and organic matter concentration on the PN-AMX performance.

2. Materials and methods

2.1. Experimental set-up

A laboratory sequencing batch reactor (SBR) with a working volume of 1.5 L was used for the experiments. The aeration system consisted in a diaphragm pump (Laboport N86, KNF) for the air supply and an air diffuser located at the bottom of the reactor. This system provided good mixture inside the reactor and the dissolved oxygen (DO) concentration necessary for the partial nitritation process. The DO concentration was periodically measured with a DO probe (Hach Lange, model HQ40d Portable Meter) and was manually regulated by changing the closing degree of an air valve located in the gas inlet conduction.

The operational cycles were of 180 min and distributed as follows: 5 min of batch feeding, 160 min of aeration, 10 min of settling and 5 min of effluent withdrawal. The hydraulic retention time (HRT) varied between 0.9 and 1.3 days for all the operational period (Table 1). The industrial wastewater was periodically collected (every 1–2 months) after the AD in operation in a fish canning industry (Catoira, Pontevedra) and stored at 4 °C. The industrial wastewater was characterized by a high variability in its composition, especially in terms of NaCl and organic matter concentrations (Table 1). The fluctuations on the industrial wastewater composition could not be controlled or predicted, and they were mainly due to changes in the processed product at the industrial facility (tuna, mussels, sardines, etc.), as well as the performance of the anaerobic digester, which worsened when the industrial wastewater contained high salt concentrations.

2.2. Operational conditions

Two different experiments were assessed to study the influence of salinity over the PN-AMX process treating industrial wastewater.

The first experiment served to test the performance of the PN-AMX process at moderate salt concentrations and its posterior recovery after a shock of salinity took place. It lasted 150 days and the SBR was operated at laboratory room temperature (24 ± 2 °C). The operation was divided in three stages according to the different salt concentration of the fed industrial wastewater (Table 1). The wastewater produced by the industry in this period was characterized by a moderate salt concentration (between 1.7 and 4.3 g

Table 1
Operational conditions and characteristics of the wastewater fed to the reactor in the different operational stages.

	First experiment			Second experiment		
	Stage I	Stage II	Stage III	Stage IV	Stage V	Stage VI
Days	0–73	74–127	128–155	0–64	65–220	221–445
Temperature (°C)	24.0 ± 0.7	23.7 ± 1.1	25.6 ± 1.3	29.3 ± 0.9	30.3 ± 1.5	31.0 ± 2.1
HRT (d)	1.3 ± 0.2	1.1 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.3 ± 0.1
DO (mg O_2/L)	2.7 ± 0.5	3.1 ± 1.1	4.0 ± 0.3	1.4 ± 0.5	1.1 ± 0.4	0.5–3.5
NaCl (g/L)	3.5 ± 0.2 (15.4 ± 0.8[a])	1.7 ± 0.3	4.3 ± 0.4	3.4–12.2	2.8–8.3	8.6 ± 0.9
NH_4^+ (mg N/L)	291 ± 51	206 ± 22	216 ± 15	262 ± 29	250 ± 44	220 ± 35
TOC (mg C/L)	54 ± 10	47 ± 10	49 ± 7	50 ± 9	50 ± 9	45–200[b]
IC (mg C/L)	345 ± 30	285 ± 35	349 ± 16	324 ± 38	299 ± 33	350 ± 63
PO_4^{3} (mg/L)	124 ± 16	97 ± 21	48 ± 12	17–85	96 ± 14	13–704
SO_4^{2} (mg/L)	82 ± 45	76 ± 18	156 ± 46	89 ± 46	111 ± 27	0–284
pH	7.8 ± 0.1	7.6 ± 0.1	7.5 ± 0.2	7.6 ± 0.2	7.6 ± 0.1	7.6 ± 0.3

[a] Punctual salt peak in days 32–36.
[b] Total organic carbon (TOC) concentration very variable.

NaCl/L) with a sudden peak of high salinity (15.4 ± 0.8 g NaCl/L) for a short period of time (Table 1). The inoculum was PN-AMX granular sludge from a pilot plant of 200 L (ELAN® process) treating the supernatant of a sludge AD in operation in an urban WWTP (Morales et al., 2015). The system was inoculated with 7.5 g VSS/L of ELAN® granular sludge, characterized by a specific anammox activity (SAA) of 0.356 ± 0.025 g N/(g VSS·d) at 30 °C. This first experiment was stopped due to a failure of the AD, and the posterior stop of the cannery industrial activity during the summer period (two months), which provoked the unavailability of wastewater.

In the second experiment the available fish canning wastewater contained higher salinity than in the previous experiment (>10 g NaCl/L), which could inhibit the anammox process if it were used directly (based on the results of the first experiment). Therefore, in this second experiment the progressive adaptation of the PN-AMX process to increasing salt concentrations was studied. The operational period was divided in three stages: Stage IV corresponded to an increase of salt concentration from 3.4 to 12.2 g NaCl/L in 41 days; in Stage V the adaptation from 2.8 to 8.3 g NaCl/L was performed in 150 days; finally, in Stage VI the salt concentration was maintained at 8.6 ± 0.9 g NaCl/L, corresponding to the industrial wastewater (Table 1). The industrial wastewater was mixed with the supernatant of an anaerobic sludge digester (Morales et al., 2015) in Stages IV and V to have increasing concentrations of NaCl without the need to dilute the nitrogen concentration of the feeding. The supernatant of the anaerobic sludge digester was used due to its low NaCl concentration (<1 g NaCl/L) and high ammonia concentration (approximately 1000 mg NH_4^+-N/L), as well as the fact that this was the effluent treated by the biomass used as inoculum. The proportion of anaerobic sludge digester supernatant in the feeding was of 35% and 60% at the beginning of Stages IV and V, respectively. Then, it was progressively decreased until having in the feeding only industrial wastewater. The characteristics of the wastewater fed to the SBR in each operational period are presented in Table 1. The inoculum for this second experiment consisted in a mixture of ELAN® sludge (65% as VSS) with sludge from the first experiment (35% as VSS), resulting in a solids concentration of 8.0 g VSS/L with a SAA of 0.252 ± 0.002 g N/(g VSS·d) at 30 °C. In this second experiment the operational temperature was controlled by a thermostatic bath and set at 30 ± 1 °C.

2.3. Analytical methods

Analytical determination of ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), pH, total suspended solids (TSS) and volatile suspended solids (VSS) was carried out according to the standard methods (APHA-AWWA-WPCF, 2005). Total Organic Carbon (TOC) content was determined by a Shimadzu analyser (TOC-L, automatic sample injector Shimazdu ASI-L) as the difference between the Total Carbon (TC) and the Inorganic Carbon (IC) concentrations. Cation and anion concentrations were determined by ion chromatography with an Advanced Compact IC system (861, Metrohm). The morphology and size distribution of the granules were measured by using an image analysis procedure (Tijhuis et al., 1994). Images of the granular sludge were taken with a digital camera (Coolsnap, Roper Scientific Photometrics) combined with a stereomicroscope (Stemi 2000-C, Zeiss). For digital image analysis the programme Image ProPlus® was used. The qualitative composition of the granules surface was assessed with SEM (scanning electron microscope) technique, following the procedure of Figueroa et al. (2008). The specific anammox activity (SAA) was determined by batch assays following the methodology described by Dapena-Mora et al. (2007). The SAA tests were carried out in closed vials of 25 mL by triplicate at 30 °C and 150 rpm. The maximum SAA (expressed as mg N/(g VSS·d)) was determined from the slope of the curve described by the cumulative N_2 production with the time and related to the biomass concentration in the vials.

2.4. Rate calculations

Ammonia and nitrite oxidation rates (AOR and NOR, respectively) as well as nitrogen removal rate (NRR) were estimated based on nitrogen balances and the anammox process stoichiometry and expressed as g N/(L·d), according to Morales et al. (2016).

The maximum nitrogen removal percentage by a possible heterotrophic denitrification process (*% HD*) was determined based on organic matter balance according to equation (1).

$$\%\,HD = \frac{\left(TOC_{inf} - TOC_{eff}\right)\cdot 0.933}{TN_{inf}}\cdot 100 \quad (1)$$

where TOC_{inf} and TOC_{eff} are the concentrations of total organic carbon (as mg C/L) in the influent and in the effluent, respectively. TN_{inf} is the total nitrogen present in the feeding (as mg N/L) and 0.933 is the stoichiometric coefficient (as g N-NO_3^-/g C) that relates nitrate and organic carbon consumption in the heterotrophic denitrification process considering acetic acid as the source of organic carbon (Giustinianovich et al., 2018). Note that part of the organic matter could be consumed for growth and/or aerobically due to the presence of oxygen, for this reason equation (1) considers only the "maximum potential" value for the heterotrophic denitrification, not the actual.

2.5. Molecular techniques and sequences analysis

Biomass samples from the two inoculums and from the reactor at different operational days (first experiment: 30, 65 and 154 days; second experiment: 55, 183, 386 and 442 days) were analysed. The samples were obtained by carefully homogenizing a large volume and taking 2 mL aliquots that were stored at −20 °C until further analysis. Total genomic DNA was extracted using the phenol—-chloroform protocol (Alonso-Gutiérrez et al., 2009), quantified in a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and checked for size and integrity by standard electrophoresis. To prepare the 16S rRNA gene amplicon libraries, the V4 hypervariable region of the 16S rRNA bacterial gene was amplified using the 515F-806R primer pair (Gilbert et al., 2010) as described previously by Regueiro et al. (2014). DNA libraries were checked for size quality and integrity using a Bioanalyzer (Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA). After determining DNA concentrations by quantitative PCR, libraries were pooled in equimolar amounts and sequenced at the genomics unit of the Parque Científico de Madrid (Spain) on an Illumina MiSeq System (Illumina) using MiSeq Reagent Kit v3 (Illumina). Initial sequence quality-filtering and analysis was performed as previously described Regueiro et al. (2014). To determine the alpha diversity of the bacterial community, observed species, Simpson, Chao1, Shannon and Simpson evenness indices were calculated using 100 rarefactions of 116,728 sequences in QIIME v.1.9.1 (quantitative insight into microbial ecology) (Caporaso et al., 2010). Beta diversity, the degree of community differentiation between samples, was measured with the Bray-Curtis dissimilarities using log(x+1) and Hellinger transformed operational taxonomic unit (OTU) relative abundances. Community structure variation was visualized using Principal Component Analysis (tbPCA) to which the correlations between microbial community composition and operational parameters were fitted. Operational parameters were transformed as follows:

log(x+1) transformation was applied to temperature, influent ammonium, TOC and sulfate concentrations whereas squared root transformation was applied to dissolved oxygen concentration. The p-values were adjusted for multiple comparisons using Bonferroni correction with 999 permutations with a significance threshold of 0.05. Statistical analyses were performed in R (R-Core-Team, 2016) using the vegan package (Oksanen et al., 2016).

3. Results and discussion

3.1. First experiment: salinity shock

In the first experiment, until day 30 of operation (Stage I) the fed wastewater had a salt concentration of approximately 3.5 ± 0.2 g NaCl/L. The achieved NRR and AOR had average values of 0.156 ± 0.009 g N/(L·d) and 0.103 ± 0.005 g N/(L·d), respectively (Fig. 1a). The total nitrogen and ammonium removal percentages were approximately 80 and 90%, respectively. After day 31, a new batch of feeding collected in the industry presented a higher salt concentration. Consequently, the salt concentration inside the reactor increased from 3.5 ± 0.2 to 15.4 ± 0.8 g NaCl/L in only 35 h (one HRT), and it was maintained in this high value for 4 operational days. This salt shock caused a strong decrease over the NRR (values close to zero) while the AOR remained practically constant (Fig. 1a). Therefore, the ammonium and nitrite concentrations in the effluent increased to values above 100 mg N/L, being the nitrite concentration as high as 200 mg NO_2^--N/L on day 36 (Fig. 1b). These results indicate that the shock of salt inhibited anammox bacteria, while the ammonium oxidizing activity was not affected. After this brief episode of salt shock, the salt concentration of the wastewater returned to the previous values (approximately 3.3 g NaCl/L), and the nitrogen removal efficiency was recovered in only 4 days to values of approximately 80%.

In Stages II and III, with a salt concentration at an average value of 1.7 ± 0.3 and 4.3 ± 0.4 g NaCl/L, respectively, the nitrogen removal efficiency was maintained at approximately 80%. The NRR was between 0.105 and 0.203 g N/(L·d) and varied according to the different ammonium concentration of the fed wastewater (Fig. 1). Regarding the concentration of the nitrogen species in the effluent, the ammonium and nitrite concentrations were low, while the nitrate fitted the expected values for the anammox stoichiometry (Fig. 1b). Only in some days of Stage II the nitrate concentration was higher than the stoichiometric value, which corresponded with detectable nitrite oxidizing bacteria (NOB) activity (NOR in the range 0.020–0.030 g N/(L·d)). If the profiles of NOR are compared to the evolution of NaCl concentration (Fig. 1b) a slight NOB activity was detected in Stage II, which correlated with the lower salt concentration, while it was insignificant during the rest of the operational period.

The results from this first experiment confirm the suitability and stability of the PN-AMX process to remove nitrogen from fish canning wastewater with moderate salt concentrations (<5 g NaCl/L), as well as the good capability to quick restore (4 days) after a sudden/acute salt inhibition.

3.2. Second experiment: salinity adaptation

In this second experiment (Stages IV, V and VI) the possibility to adapt the PN-AMX biomass to treat industrial wastewater with high salt concentrations was tested. In Stage IV an increase in the salt concentration from 3.4 g NaCl/L to 12.2 g NaCl/L was progressively performed during 41 days. However, the time of adaptation was not enough for anammox bacteria. The NRR decreased from values of 0.189 ± 0.035 g N/(L·d) to 0.048 ± 0.008 g N/(L·d), while the AOR was maintained at approximately 0.154 ± 0.022 g N/(L·d) (Fig. 2a). The loss of anammox activity provoked the increase of ammonium and nitrite concentrations in the effluent up to values of approximately 60 mg NH_4^+-N/L and 120 mg NO_2^--N/L (Fig. 2b). The total nitrogen removal efficiency decreased from 80% to 20%. For this reason, at the end of Stage IV the salt concentration was restored to values of approximately 4 g NaCl/L.

In Stage V a new progressive adaptation was performed to increasing salt concentrations from 2.8 to 8.3 g NaCl/L throughout 150 days. Following this strategy, the PN-AMX process was able to

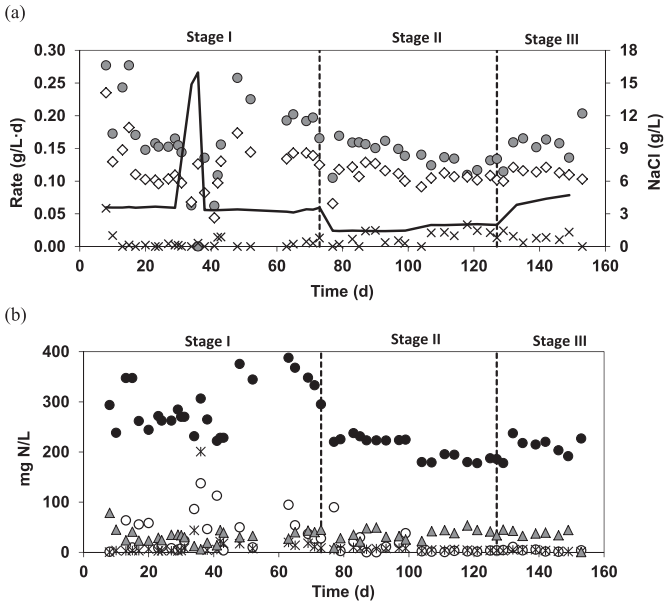

Fig. 1. Data from the first experiment: (a) Nitrogen removal rate (NRR, ●), ammonium oxidation rate (AOR, ◇), nitrite oxidation rate (NOR, ×) and NaCl concentration (–); (b) Concentration of NH_4^+ in the influent (●); NH_4^+ (○), NO_2^- (✱) and NO_3^- (▲) in the effluent.

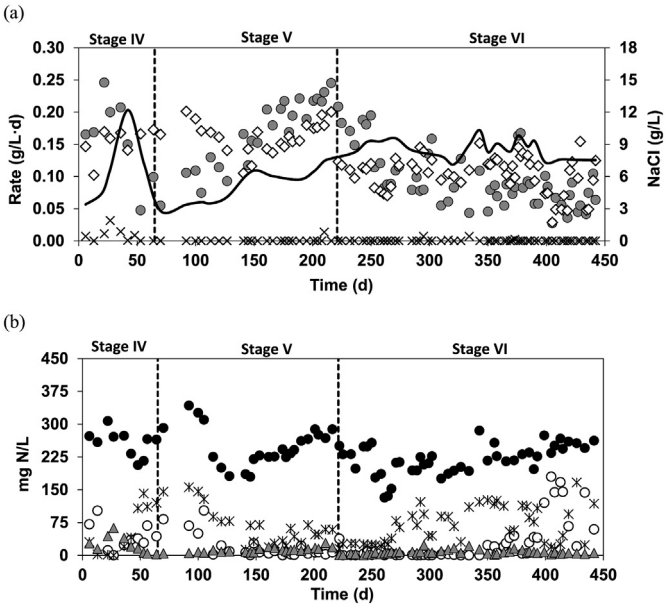

Fig. 2. Data from the second experiment: (a) Nitrogen removal rate (NRR, ●), ammonium oxidation rate (AOR, ◇), nitrite oxidation rate (NOR, ×) and NaCl concentration (–); (b) Concentration of NH_4^+ in the influent (●); NH_4^+ (○), NO_2^- (✱) and NO_3^- (▲) in the effluent.

recover from the salt inhibition episode of previous stage. The NRR increased from 0.055 g N/(L·d) on day 70, to a maximum value of 0.250 g N/(L·d) on day 216 at a salt concentration of 7.7 g NaCl/L (Fig. 2a). In terms of specific activity, the SAA increased from 0.018 g N/g VSS·d (day 63) up to 0.272 g N/g VSS·d (day 156).

In Stage VI the salt concentration of the feeding varied between 7 and 10 g NaCl/L, due to the variability of the industrial wastewater collected. At the beginning, days 221–260, the nitrite concentration in the effluent decreased from approximately 60 to 25 mg NO_2^--N/L, probably due to the higher concentration of organic matter in the fed wastewater, with TOC concentrations of 140 ± 43 mg C/L, much higher than those measured in previous stages of 40–50 mg C/L (Table 1), which can promote the heterotrophic denitrification. On these days the achieved NRR was of 0.180 ± 0.020 g N/(L·d) for a salt concentration of 9.2 g NaCl/L, with a total nitrogen removal efficiency of 87%.

The achieved NRR values (Stages V and beginning of Stage VI) are comparable and even higher than the ones obtained by Malovanyy et al. (2015) in a moving bed biofilm reactor. These authors studied the performance of the PN-AMX process at increasing salt concentrations fed with anaerobic reject water supplemented artificially with NaCl. They obtained, after an adaptation period of 70 days, a NRR of 0.078 g N/L·d at 10 g NaCl/L. However, in comparison with PN-AMX systems using saline synthetic medium the achieved NRR values were lower. For example, Windey et al. (2005) increasing the NaCl concentration in a rotating biological contactor with the PN-AMX process fed with synthetic medium achieved a NRR of 0.609 g N/(L·d) at 30 g NaCl/L after 172 days of adaptation. Furthermore, in these previous studies (Malovanyy et al., 2015; Windey et al., 2005), the authors observed instability of the PN-AMX process due to the inhibition of ammonia oxidation bacteria (AOB) at high salt concentrations (>10 g NaCl/L), with the subsequent accumulation of free ammonia.

From day 260 onwards the NRR was variable and under 0.1 g N/(L·d) the most part of the operational days. The AOR was more stable and its fluctuations were due to the regulation of the DO concentration required to avoid the accumulation of nitrite. Furthermore, a progressive decrease of the total nitrogen removal from 87% to 50% was observed. The increase of nitrite concentration beyond 75 mg NO_2^--N/L from day 270 suggested a decline in the anammox activity, confirmed by batch assays. On day 273 the SAA value fell to 0.028 g N/(g VSS·d). As it will be discussed later, this deterioration of the anammox activity might be caused by the presence of high concentrations of organic matter and related to heterotrophic denitrification processes, favouring a significant shift in the involved microbial populations.

Thus, the results obtained in this second experiment indicated that the anammox bacteria are able to operate at high salt concentrations (up to 9 g NaCl/L) if enough adaptation time is provided (approximately 150 days in this research work). However, the stability of the PN-AMX process is limited and the presence of organic matter concentrations as high as 200 mg C/L (Table 1) in the industrial wastewater affect the process negatively.

3.3. Granular sludge characteristics

In the first experiment, coinciding with the sudden shock of salinity, the concentration of biomass inside the reactor decreased from 6.1 ± 0.7 g VSS/L, on day 28, to 3.5 ± 0.8 g VSS/L, on day 42. Afterwards, the biomass content inside the reactor progressively increased, reaching a concentration of 7.9 ± 0.4 g VSS/L on day 114. The solid concentration in the effluent was lower than 50 mg VSS/L in Stage I. Although, values up to 130 ± 7 mg VSS/L were punctually obtained around day 65, due to the flotation of some granules. This flotation event was presumably provoked by the entrapment of nitrogen gas in the internal core of the granules. Then, the concentration of solids in the effluent was lower than 25 mg VSS/L in Stages II and III, confirming the appropriated biomass retention inside the reactor. The average diameter of the granules increased during the experimental time from 2.5 ± 0.3 (day 7) to 3.8 ± 0.2 mm (day 146).

In the second experiment the solid concentration inside the reactor was maintained stable with an average value of 7.3 ± 0.6 g VSS/L. The concentration of solids in the effluent was of 100 ± 30 mg VSS/L. Although, values up to 200 ± 17 mg VSS/L were punctually obtained on day 273, again due to granule flotation episodes. The average diameter of granules in this experiment was of 3.3 ± 0.4 mm.

To understand the importance of floatation events SAA tests were performed for floating and settling granules on day 273, resulting in values of 0.061 ± 0.011 and 0.028 ± 0.001 g N/(g VSS·d), respectively. These results indicate that the floating granules had two-fold the activity of the settling ones, and emphasize the importance of avoiding the wash out of this fraction of the biomass. Campos et al. (2017) stated that the floatation of anammox granular biomass occurs when the nitrogen gas remains entrapped inside the granule due to the overloading of the system. These authors stated that the accumulation of nitrite in the liquid phase and the increase of the granules size are factors that promote the granules floatation. As both factors occurred in the present research work, they probably were responsibly of floatation events of the granular biomass.

Another event that took place with the biomass in the second experiment was the appearance of a whitish layer of precipitates on the surface of some granules (Supplementary material, Figure S1). This fact was observed for the first time on day 292. An elemental analysis of the granules surface using SEM was performed in zones with and without such precipitates. The results obtained were only qualitative, but indicated that the main difference between both zones was a high presence of elemental sulphur in the precipitates zone. The formation of these surface precipitates may suggest a partial explanation to the observed loss in the anammox activity, due to the appearance of substrate transfer limitations between the liquid media and the inner layers of the granules, where anammox are located. Dapena-Mora et al. (2010) observed the formation of precipitates over granular anammox biomass when treating high saline wastewater up to 30 g NaCl/L, being $Ca_3(PO_4)_2$ the main compound justifying these precipitates.

3.4. Bacterial community of PN-AMX reactor

To investigate the bacterial diversity in the PN-AMX, 16S rRNA bacterial gene libraries were constructed and sequenced at different times throughout the entire study. More than 1.3 M high-quality sequences (148,905 ± 19,039 per sample) were obtained and clustered into OTUs at 3% cutoff (Supplementary material, Table S1). The bacterial diversity and richness remained stable (Table 2) despite the changes on operational conditions, indicating the presence of a salinity adapted community in the reactor. Previous studies in similar systems observed a diversity loss with increasing salinity levels due to the selective pressure of salinity in non-adapted communities (Wang et al., 2017a); but also a diversity recovery once the community became adapted (Wang et al., 2017b). The PN-AMX reactor microbial community is very complex (estimated species: 1069 ± 56; Simpson diversity, the higher index the more diverse community: 0.97 ± 0.01). However, the community is markedly uneven (Simpson evenness, the higher the index the more even community: 0.034 ± 0.009) indicating the low evenness of the community, that was dominated by few organisms and maintained numerous low abundant bacteria (rare bacteria).

Table 2
Bacterial alpha diversity, indices of richness, diversity and evenness.

	First experiment				Second experiment				
	Stage I			Stage III	Stage IV		Stage V	Stage IV	
Day	0	30	65	154	0	55	183	386	442
Observed Species	1150	1088	1124	1007	1039	1067	972	1097	1074
Simpson diversity	0.967	0.971	0.980	0.960	0.967	0.983	0.966	0.974	0.973
Shannon	6.626	6.508	6.882	6.094	6.389	6.909	6.195	6.543	6.440
Chao1	1488	1335	1296	1245	1279	1313	1200	1370	1369
Evenness	0.026	0.032	0.044	0.025	0.030	0.055	0.031	0.035	0.034

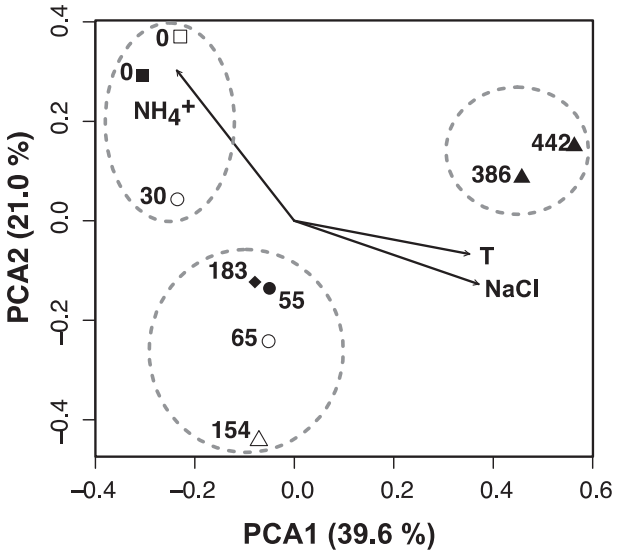

Fig. 3. Bacterial community structure at OTU level and its relationship with the operational parameters: temperature (T), salt (NaCl) and ammonia (NH_4^+) concentrations. The tbPCA analysis of the Bray-Curtis dissimilarities and the operational parameters that are significantly correlated with the community structure (p-value < 0.05). The % of community variance explained by each axis is indicated. Groups of samples with dissimilarities below 0.4 are indicated with grey dashed circles. Labels indicate operational day. Opened symbols indicate the first experiment samples: Inoculum 1 (□), Stage I (○), Stage III (△). Closed symbols indicate second experiment samples: Inoculum 2 (■), Stage IV (●), Stage V (◆), Stage IV (▲).

The bacterial community similarity at OTU level across different time points was evaluated using Bray-Curtis dissimilarities (Fig. 3; Supplementary material, Figure S2). The results indicated that community structure was dynamic throughout time, shifting during the different stages. Despite belonging to different experiments, samples from both inoculums and the reactor before suffering any NaCl peak (Stage I at day 30) were similar, and were clearly separated from the communities during the mid-stages. But the most distinct communities were those from the late days of the last experimental stage when sustained salinity concentrations were the highest. The tbPCA analysis confirmed that these changes of the community structure were correlated with variations in the influent concentration of NaCl and NH_4^+ and temperature of operation (Fig. 3; Supplementary material, Figure S3). All these three parameters are well known to influence anammox and nitrifying microorganisms activities (Agrawal et al., 2017; Gonzalez-Martinez et al., 2015; Lotti et al., 2015; Ma et al., 2016; Wang et al., 2017a, 2017b).

The bacteria taxonomic diversity was unequally distributed across 26 phyla, where the 10 most abundant taxa represented over the 90% of the bacterial community (Fig. 4a). The microbial community was dominated by organisms of the *Proteobacteria* phylum (average relative abundance: 52.6 ± 11.5%), especially from the *Beta* (20.2 ± 1.5%), *Alpha* (13.3 ± 4.7%) and *Gammaproteobacteria* (10.5 ± 3.9%) classes. Other major phyla included *Firmicutes* (9.3 ± 5.2%), *Bacteroidetes* (9.0 ± 3.4%), *Planctomyces* (6.2 ± 3.9%), *Actinobacteria* (3.6 ± 1.3%) and *Chlorobi* (2.1 ± 1.3%). These taxa are commonly found in partial nitritation and/or anammox reactors (Agrawal et al., 2017; Dosta et al., 2015; Gonzalez-Martinez et al., 2015; Wang et al., 2017b). The low relative abundance of *Planctomycetes* despite the high anammox activity is in accordance with previous observations (Dosta et al., 2015; Gonzalez-Martinez et al., 2015). *Chloroflexi*, known to co-exists with anammox bacteria (Yamagishi et al., 2013; Yamamoto et al., 2011), is thought to scavenge organic matter from the dead biomass (Kindaichi et al., 2012) and support granules formation due to its filamentous characteristics (Ni et al., 2011).

The largest fraction of the community was characterized by heterotrophic bacteria. Many of the predominant families (Fig. 4b) were heterotrophic bacteria such as *Rhodocyclaceae, Rhodobacteraceae*, *Comamonadaceae, Xanthomonadaceae, Burkholderiaceae, Ignavibacteriaceae*, *Hyphomicrobiaceae* or *Flavobacteriaceae*.

Previous analyses of anammox reactors have already shown that heterotrophic bacteria represent a large fraction of the community (Agrawal et al., 2017; Costa et al., 2014; Dosta et al., 2015; Garcia Costas et al., 2012; Kindaichi et al., 2007; Langone et al., 2014; Laureni et al., 2015; Ni et al., 2012; Persson et al., 2017). Speth et al. (2016) hypothesized that the existence of variable macro- and micro-environments allows the coexistence of such large diversity of heterotrophic bacteria in the reactors. Many of these families also contain known complete or partial denitrifiers and denitrification intermediate reducers (Liu et al., 2012; Ni et al., 2011).

Many of the major OTUs (average relative abundance > 0.5%) seemed to be influenced by the ammonium, salinity and TOC concentrations of the feeding and the temperature of the operation (Fig. 5a; for the influence of parameters at phyla level see Supplementary material Figure S3). High salinity and temperature conditions were correlated with the presence of heterotrophic denitrifiers *Thauera*, *Paracoccus*, *Thiotrix* when TOC was high. Increasing *Thauera* abundances with salinity has been already reported in a simultaneous nitrification, denitrification and organic matter removal process (Wang et al., 2017a). High levels of ammonium and TOC favored many heterotrophs like *Cohnella*, *Bacteroidetes*, *Firmicutes*, *Chitinophaga*. Anammox bacteria abundances were not correlated by any of the parameters studied.

Two anammox bacteria were found within the major genus (Supplementary material, Figure S4): Candidatus *Brocadia* (4.6 ± 3.0) and Candidatus *Scalindua* (1.2 ± 0.8), both with similar temporal tendencies. Previous studies determined that C. *Brocadia* predominate in reactor communities under high nitrite concentrations and COD/N ratios (Jenni et al., 2014; Laureni et al., 2015). *Nitrospira* was the most abundant NOB detected, although the genera average relative abundances were lower than 0.6%. *Nitrospira* has been previously reported as the main NOB of a PN-AMX system under high salinity conditions (Wang et al., 2017b). *Nitrobacter,* and some AOBs (*Nitrosospira*, *Nitrosovibrio* and *Nitrosococcus*) were also detected within the rare bacteria (<0.01%).

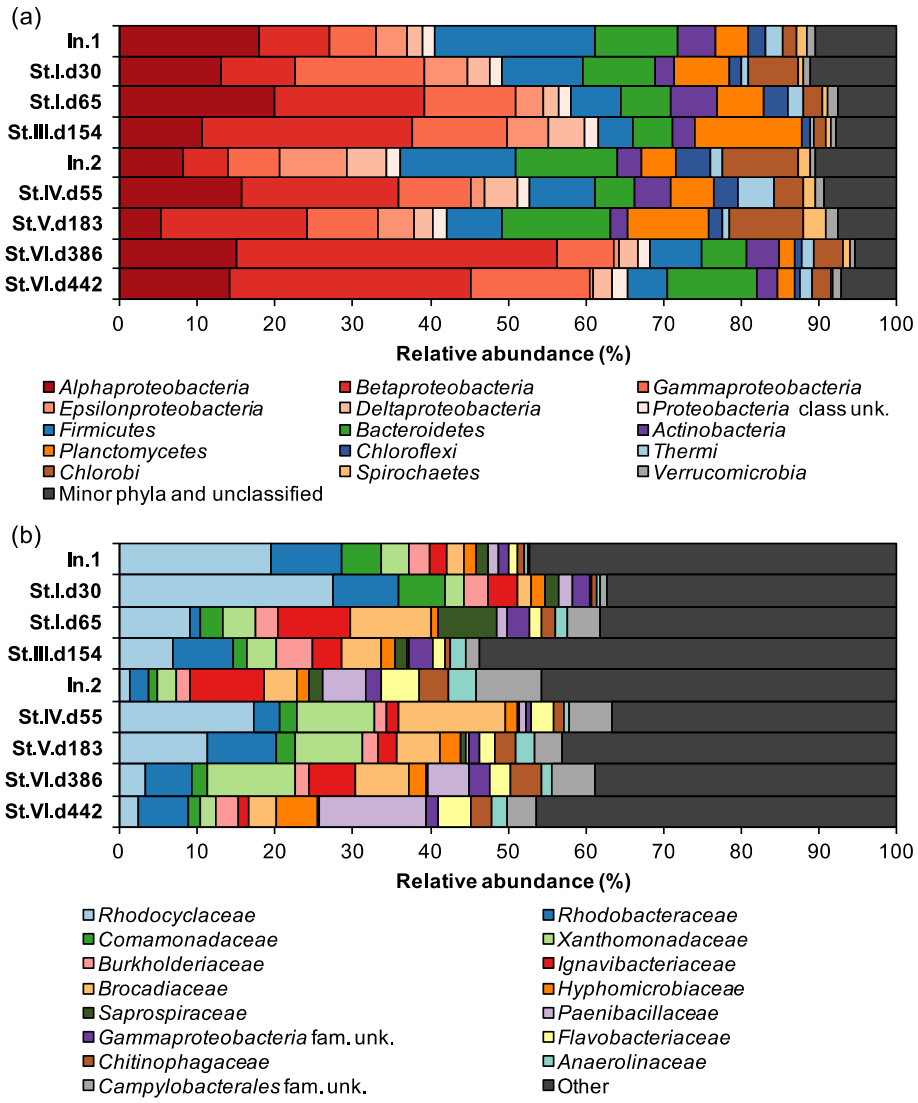

Fig. 4. Taxonomic profile of the inoculum and the PN-AMX reactor bacterial community at different times. (a) Most abundant phyla. For *Proteobacteria* phylum, the different classes are shown. (b) Most abundant families. Sample labels indicate: inoculum of first experiment (In.1) or second experiment (In.2) or the reactor Stages (St.I-VI) followed by the operational day.

Previous studies have reported low fractions of AOBs and NOBs in PN-AMX communities (Agrawal et al., 2017; Almstrand et al., 2014). In the present research work however no AOBs were detected among the abundant bacteria. One plausible explanation could be the presence of Ammonia Oxidizing *Archaea* (AOA). Unfortunately, Archaea was not explored in this study, although we have observed the enrichment of *Nitrosopumilus* in the *Archaea* fraction of other anammox reactors in our lab (unpublished data). AOA group has been detected as the dominant nitrifying community in a marine aquarium biofilter and appears to be widespread in WWTPs (Junier et al., 2010) and that AOA can outcompete AOB as they are less sensitive to salinity (Wang et al., 2017a).

Temporal tendencies of the major genera of anammox bacteria (C. *Brocadia*, C. *Scalindua*), (partial) denitrifiers (*Thauera*, *Paracoccus*, *Burkholderia*, *Comamonas*, *Thiobacillus* and *Pedobacter*) and NOB (*Nitrospira*) are indicated in Fig. 5b (see Supplementary material Figure S4 for individual trends). Presence of NOB was expected when NOR activity was higher and NaCl concentrations were relatively low. In the first experiment, *Nitrospira* remained at low relative abundances for Stage I. Afterwards, *Nitrospira* abundance presumably increased during Stage II (no 16S data available) because the rise of NOR. This fact can justify the presence of *Nitrospira* at day 154 (Stage III). As a result, the relative abundances of *Nitrospira* during Stage III were higher than at Stage I, but probably lower than during Stage II. In the second experiment, *Nitrospira* was low in all samples except at day 55 in Stage IV. In this case, *Nitrospira* probably grew during days 20–23, when NOR increased and the salinity was still moderated, and although it was likely inhibited by the salt increment of days 30–50 the relative abundances measured at day 55 were still reflecting that increment. Then, when NaCl was kept over 6 g/L, *Nitrospira* dropped in abundance, in agreement with the NOR data; and remained as rare bacteria as other NOBs (*Nitrobacter*).

Anammox bacteria increased almost steadily from Stage I to Stage III. The small drop observed between days 30 and 65 was probably related with the inhibition during the salt peak. The data during Stage IV and V showed an enrichment of anammox. However, anammox decreased during Stage VI when the NRR dropped. In this stage, the denitrifying bacteria noticeably increased their importance in the community. The temporal trends of anammox and denitrifying bacteria are opposite, probably reflecting the competition for resources.

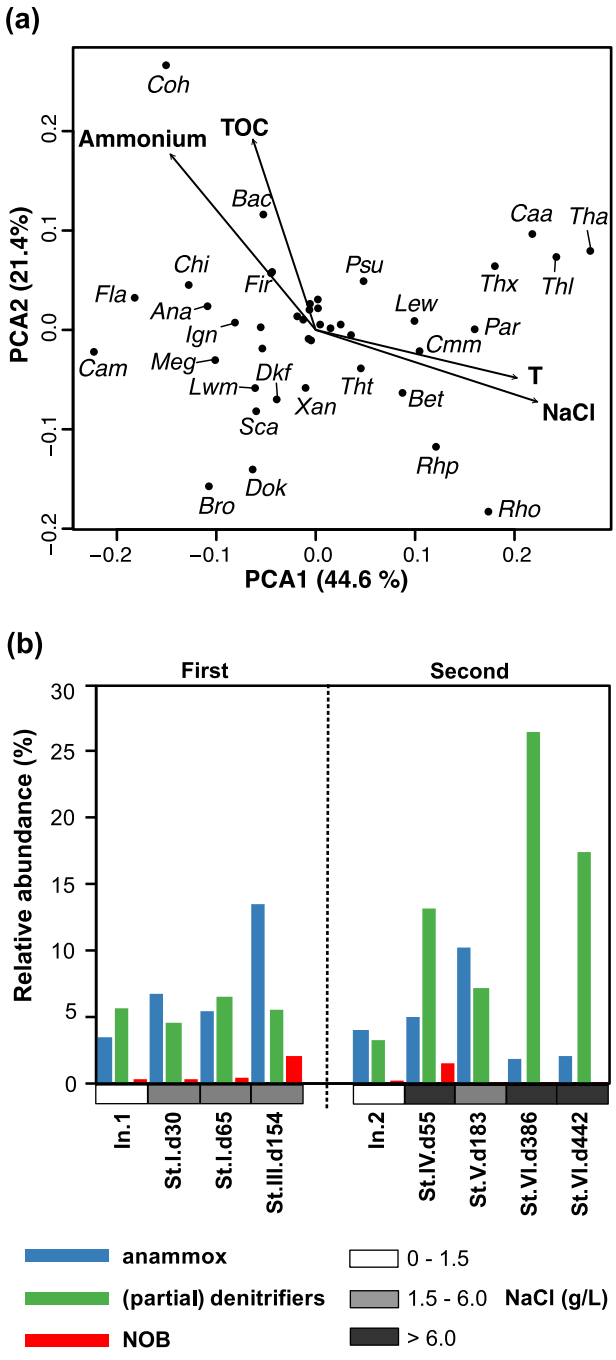

Fig. 5. a) The tbPCA analysis of major OTUs relative abundances and the operational parameters (p-value < 0.05). The % of community variance explained by each axis is indicated. T: temperature; *Ana: Anaerolineaceae, Bac: Bacteroidetes, Bet: Betaproteobacteria, Bro:* Candidatus *Brocadia, Caa:* Candidatus *Amoebophilus asiaticus, Cam: Campylobacterales, Chi: Chitinophaga soli, Cmm: Comamonadaceae, Coh: Cohnella soli, Dkf: Dokdonella fugitiva, Dok: Dokdonella* sp., *Fir: Firmicutes, Fla: Flavobacteriaceae, Ign: Ignavibacterium, Lew: Lewinella* sp., *Lwm: Lewinella marina, Meg: Megasphaera hominis, Par: Paracoccus* sp., *Psu: Paracoccus sulfuroxidans, Rho: Rhodociclaceae, Rhp: Rhodocyclus purpureous, Sca:* Candidatus *Scalindua brodae, Tha: Thauera* sp., *Thl: Thauera linaloolentis, Tht: Thauera terpenica, Thx: Thiotrix* sp., *Xan: Xanthomonadaceae.* (b) Temporal changes of the major anammox bacteria, (partial) denitrifiers and NOB. The NaCl range of each day is indicated in the grey horizontal bar. Notice the opposite trends between anammox bacteria and denitrifiers. For sample label description see Fig. 4 caption.

3.5. Nitrite oxidizing bacteria (NOB) activity

The presence of significant NOB activity is one of the most common problems that reduce the nitrogen removal efficiency in a PN-AMX system (Lackner et al., 2014). Among the different parameters that can be used to suppress the NOB activity, several studies have confirmed the salt stress as a good strategy, because NOB are more sensitive than AOB to saline conditions (She et al., 2016). Therefore, the treatment of saline effluents by the application of PN-AMX process is considered as a suitable option (Liu et al., 2008). Also this salt stress may be used as a technical approach to inhibit the NOB activity during the start-up of a PN-AMX process for industrial saline wastewaters, as other authors found working with synthetic mineral medium (Zhang et al., 2010). Nevertheless, care must be taken in this case and a salt increasing strategy long enough to ensure the adaptation of anammox biomass to high salt conditions must be applied.

The results obtained in this research work showed that the NOR was only significant during the first days of both experiments and in Stage II (Fig. 1a and 1b). In the first experiment the NOR decreased from 0.058 ± 0.002 g N/(L·d) (day 8) to zero, in only 5 days with a salt concentration of 3.6 g NaCl/L. Then, in Stage II at a salt concentration of 1.7 g NaCl/L, the NOR had an average value of 0.014 ± 0.010 g N/(L·d). The increase of the NOR in Stage II can be correlated with the decrease of the salt concentration (Fig. 1a). In the second experiment the NOR decreased from 0.032 ± 0.001 g N/(L·d) (day 27) to zero in 15 days. In this period, the salt concentration increased from 3.4 to 12.2 g NaCl/L.

These results indicate that the saline conditions helped to maintain a low NOB activity inside the reactor. However, the salt concentrations tested were not enough to completely wash out the NOB. *Nitrospira*, as well as other NOB bacteria, could subsist as part of the rare members of the bacteria community when the salinity was high. If the conditions become favourable (low salt concentration) they can develop significant activity in only few days. The number of reports showing the growth of rare members of the community to become part of abundant fraction with the adequate conditions is increasing. It has been hypothesized that the rare biosphere serves as a reservoir of diversity that increases the plasticity and adaptability of the communities (Shade and Gilbert, 2015). This phenomenon in an environment such as the PN-AMX can be an inconvenient as NOBs are not totally wash-out of the system.

3.6. Influence of organic matter and heterotrophic denitrification

The process of heterotrophic denitrification is known to occur simultaneously with the PN-AMX process when biodegradable organic matter is available. This heterotrophic denitrification facilitates the removal of the nitrate produced due to the anammox bacteria activity and, consequently, nitrogen removal efficiencies higher than 89% can be achieved (Giustinianovich et al., 2016). However, the development of heterotrophic denitrification might bring a shift in the microbial populations behaviour and compete with anammox bacteria for the nitrite (Jenni et al., 2014).

In the first experiment and until day 220 of the second one, the total organic carbon to nitrogen (TOC/N) ratio in the feeding was lower than 0.25 g TOC/g N. The estimated maximum nitrogen removal percentage due to a possible heterotrophic denitrification process was calculated for each operational period with Equation (1) and resulted to be lower than 12% (Fig. 6). Furthermore, the molar ratio of nitrate-N production to ammonium-N consumption was close to the stoichiometry value for anammox process (0.11 g NO_3^--$N_{produced}$/g NH_4^+-$N_{consumed}$), with average values of 0.11 ± 0.04, 0.18 ± 0.06, 0.16 ± 0.02 and 0.15 ± 0.05 g NO_3^--$N_{produced}$/g NH_4^+-$N_{consumed}$ for Stages I, II, III and IV, respectively. Only in Stage V this ratio was lower than the expected (average value of 0.05 ± 0.01 g NO_3^--$N_{produced}$/g NH_4^+-$N_{consumed}$), which could indicate a possible nitrate consumption by the denitrification process. In fact, some

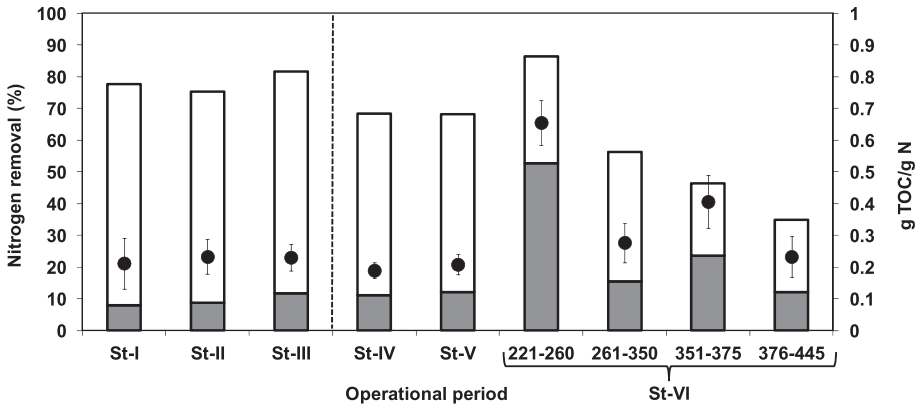

Fig. 6. Percentages of nitrogen removal (in overlapping bars): total nitrogen (TN, □) and maximum possible heterotrophic denitrification (HD, ■). TOC/N ratio in the industrial feeding (●).

putative autotrophic denitrifying bacteria are present during those stages. Therefore, in these operational stages the PN-AMX process was the main mechanism of nitrogen removal and anammox bacteria outcompeted denitrifying microorganisms.

In the second experiment, from day 221 on (Stage VI), the TOC/N ratio in the feeding was variable and in some operational periods higher than 0.30 g TOC/g N, which provoked a competition between anammox and denitrifying bacteria. Between days 221 and 260 a high organic matter peak was detected in the feeding due to a new industrial batch of wastewater, with a TOC/N ratio of 0.66 ± 0.07 g TOC/g N. On these days, the estimated maximum nitrogen removal percentage due to a possible denitrification process was calculated to be as high as 53% (Fig. 6). Furthermore, the ratio of nitrate-N production to ammonium-N consumption was very low (<0.006 g NO_3^--$N_{produced}$/g NH_4^+-$N_{consumed}$), which indicates a possible consumption of nitrate by heterotrophic denitrification. In this period (days 221–260) the total nitrogen removal efficiency had the higher values of the experiment (approximately 87%). Thus, the improvement in the nitrogen removal in this case was not due to an improvement in the anammox activity, but to a rise in the heterotrophic denitrification process driven by the organic matter content. When the TOC/N ratio decreased in the following days, the total nitrogen removal efficiency was not maintained following a decreasing trend (Fig. 6), despite that another relevant peak of organic matter was detected between days 351–375. These observations can be attributed to the proliferation of heterotrophic microorganisms between days 221–260 and 351–375. Heterotrophic bacteria can displace anammox competing for nitrite due to their high growth rates (Liang et al., 2014; Zhang et al., 2012). The changes in the TOC/N ratio produced a large shift in the community composition during Stage VI by stimulating the growth of heterotrophic denitrifiers (i.e: *Thauera*, *Paracoccus*) and the drop of anammox bacteria.

Overall, the organic matter content can act as a supplementary aid for the nitrogen removal process, as previously reported in literature (Giustinianovich et al., 2016; Jenni et al., 2014). However, the occurrence of sudden increases in the TOC/N ratio of the fed wastewater may end up shifting the competition between heterotrophic denitrifiers and anammox bacteria.

4. Conclusions

The study of the PN-AMX process with industrial saline wastewater indicated that after a short salt shock (4 days at 16 g NaCl/L) the anammox bacteria can quick restore their activity in few days. With enough adaptation time (150 days) the PN-AMX process showed a good total nitrogen removal efficiency (80%) and NRR (0.2 g N/L·d) at 7–9 g NaCl/L. However, the presence of organic matter in the industrial wastewater, at concentrations as high as 200 mg TOC/L, destabilized the process. The microbiological data confirmed that high NaCl concentrations decreased the NOB abundance, while the presence of organic matter favours that heterotrophs displace anammox bacteria.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jenvman.2017.12.007.

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