Effect of salinity on nitrification efficiency and structure of ammonia-oxidizing bacterial communities in a submerged fixed bed bioreactor

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Effect of salinity on nitrification efficiency and structure of ammonia-oxidizing bacterial communities in a submerged fixed bed bioreactor

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HIGHLIGHTS

- The effect of salt (NaCl) on biological nitrogen removal was studied.
- Nitrification process is inhibited at high salt concentrations (⩾24.1 g NaCl/L).
- Ammonia oxidizing bacterial communities were studied by 454-pyrosequencing.
- Only 5 OTUs of 42 OTUs were found at all salinities tested.
- *amoA* sequences related to *Nitrosospira* disappeared at high salinity.

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ABSTRACT

The effect of salt (NaCl) on biological nitrogen removal and community structure of ammonia-oxidizing bacteria (AOB) was investigated in a submerged fixed bed bioreactor (SFBBR). Influent wastewater was supplemented with NaCl at 0 (control), 3.7, 24.1 and 44.1 g/L, and the rate of ammonia removal efficiency was measured by ion chromatography. The structure of the AOB community was profiled by 454-pyrosequencing, based on the amplification of partial ammonia-monooxygenase subunit A (*amoA*) genes. Salinity did not inhibit nitrification at 3.7 g/L, while ammonia oxidation activity significantly decreased and nitrite was consequently accumulated in the SFBBR when the salt concentration was ⩾24.1 g/L. The sequencing of *amoA* genes revealed that many of the OTUs found in the control experiment were still present at the full range of NaCl studied, while concentrations of 24.1 and 44.1 g of NaCl/L promoted the emergence of new OTUs phylogenetically related to AOB described in saline environments.

1. Introduction

Nitrogen, one of the main compounds in wastewater, causes serious environmental problems. Eutrophication, excessive growth of algae that causes the death of other organisms such as fishes, and oxygen depletion are some of the adverse environmental impacts associated with excess N [1]. Because of their negative effects, nitrogen compounds should be removed from the wastewater before it is discharged to the environment.

Nitrification is a key process in biological nitrogen removal. It occurs in two separate reactions, in the first one (phase I) ammonia-oxidizing bacteria (AOB) oxidize ammonia to nitrite and in the second one (phase II) nitrite is oxidized to nitrate by nitrite-oxidizing bacteria (NOB) [2]. Generally, nitrification is considered as the rate-limiting step of the overall biological wastewater treatment process due to the low growth rate of the organisms involved [3].

Salt is considered a common stress factor able to destabilise the microbial communities in wastewater treatment plants (WWTPs) [4]. It is well known that osmotic stress in wastewater reduces bacterial metabolic activities [5]. Nitrification is particularly susceptible to inhibition by salt [6,7], although AOB and NOB are thought to respond differently to changes of environmental conditions such as varying salinity. High sensitivity of AOB to increasing salt concen-

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Table 1
Operational parameters of experiments conducted in the submerged fixed bed reactor during the experiments E0, E3.7, E24.1 and E44.1. Average values marked with the same letter are not significantly different, according to the least significant difference (LSD), test ($p < 0.05$).

	E0	E3.7	E24.1	E44.1	LSD
Influent					
COD (mgO_2/l)	980.26 ± 233.05[a]	877.13 ± 129.82[a,b]	971.17 ± 209.74[a,b]	915.37 ± 160.25[b]	135.48
BOD_5 (mgO_2/l)	425 ± 97.83[a]	418 ± 72.46[a]	370 ± 78.04[a]	416 ± 65.05[a]	57.34
TSS (mg/l)	622.43 ± 398.95[a]	746.21 ± 207.54[a]	467.32 ± 423.31[a]	661 ± 298.43[a]	139.67
VSS (mg/l)	520.48 ± 153.24[a]	640.71 ± 165.12[a]	370.32 ± 83.65[a]	541.65 ± 94.03[a]	65.79
pH	7.55 ± 0.21[a]	8.20 ± 0.28[b]	7.42 ± 0.18[c]	7.23 ± 0.17[d]	0.16
Effluent					
COD (mgO_2/l)	78.47 ± 28.64[a]	89.50 ± 19.59[a]	312.76 ± 34.21[b]	553.14 ± 54.04[c]	23.87
BOD_5 (mgO_2/l)	17 ± 6.51[a]	163 ± 42.22[b]	247.33 ± 75.42[c]	358.66 ± 65.26[d]	48.12
TSS (mg/l)	19.48 ± 7.61[a]	24.31 ± 8.62[c]	16.05 ± 4.83[a]	22.54 ± 7.96[b]	0.12
VSS (mg/l)	13.10 ± 6.14[ab]	14.62 ± 5.84[c]	10.91 ± 4.58[a]	16.53 ± 6.32[bc]	4.05
pH	7.46 ± 0.26[ab]	7.78 ± 0.31[ab]	7.39 ± 0.27[a]	7.62 ± 0.11[b]	3.86

trations has been reported either for pure cultures [8] or for non-adapted and adapted enrichment cultures [6]. Notwithstanding, AOB seem to present a lower sensitivity to high salt concentrations than NOB [9].

The submerged fixed bed biofilm reactor (SFBBR) for wastewater treatment is an alternative to the traditional activated sludge system [10]. Submerged fixed film technology has many advantages, for instance 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 [11]. Other advantages are the simplest control and maintenance, low-energy requirements, low-operating costs and minimized odors and noise [12–14]. Intensive research in the field of biological wastewater treatment showed that biofilms are often more efficient for water purification than conventional suspended activated sludge for the removal of organic matter and nitrogen from wastewater through the biological process of nitrification–denitrification [15].

A limited number of studies are available in the literature addressing nitrifying microbial communities in submerged fixed bed bioreactors (SFBBRs) treating saline wastewater.

In order to understand the AOB communities of biological wastewater treatments, molecular methods based on the sequencing of partial 16S rRNA genes amplified from DNA extracted from environmental samples have been used to reveal intrinsic genetic biodiversity [14]. Furthermore, in recent years new technologies have been developed, such as the second-generation high-throughput sequencing, which can elucidate the characters of microbial community more completely and accurately [16,17]. Pyrosequencing, developed by Roche Life Science, was the first second-generation DNA sequencing platform to be commercially available [18] and it has been used in recent years to elucidate the biodiversity of microbiota involved in important wastewater processes such as nitrogen transformations [19,20]. The aim of this work was to evaluate the effect of saline wastewater on the nitrogen removal process and the community structure of nitrifying bacteria in biofilms developed in a SFBBR, using a pyrosequencing approach targeting the *amoA* gene encoding ammonia monooxygenase.

2. Materials and methods

2.1. Descriptions of the pilot-scale experimental plant and operating conditions

The SFBBR used in this study and the operational conditions were described in full detail in a previous work [12]. Briefly, the SFBBR consisted of a cylindrical methacrylate bioreactor of 0.15 m internal diameter and 0.65 m height, packed with porous plastic carriers, Bioflow 9® (RVT Company, Knoxville, TN, USA), with a surface of 800 m^2/m^3 and a bulk density of 145 kg/m^3 was used as support material for the formation of the biofilm. Air was supplied by a diffuser placed on the bottom of the reactor to achieve a concentration of 6 mg O_2/L. The bioreactor was operated with urban sewage water collected from the primary settling tank of the municipal wastewater treatment plant "EDAR SUR" (EMASAGRA S.A., Granada, Spain).

Four different working salt concentrations were used: i.e., the influent was unamended (named experiment E0) or amended with NaCl (3.7, 24.1 and 44.1 g/L; named experiments E3.7, E24.1 and E44.1, respectively) in order to cover a broad range of salt concentration, as performed by other authors [12,22,23]. The final conductivity of the influents assayed was as follow: 1.5 mS for experiment E0; 12 mS for experiment E3.7; 24 mS for experiment E24.1; and 48 mS for experiment E44.1. These four different experimental conditions were maintained for 45 days each, divided in 3 cycles of 15 days. At the end of each cycle, a backwashing of the biofilter was required due to clogging.

All experiments were carried out with the same inflow rate (50 mL/min), HRT (3.8 h), temperature (20 °C) and air flow rate, according to a previous study [12].

2.2. Physic-chemical analysis

Biological oxygen demand at 5 days (BOD_5), chemical oxygen demand (COD), total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to standard methods for the examination of waste and wastewater [21]. Chloride interference in chemical analysis was avoided by means of silver nitrate according to Ramos et al. [22].

The pH value was monitored using a Crison pH 25 pH-meter (Crison instruments S.A., Barcelona, Spain). Influent and effluent water samples were obtained daily for analytical studies and all the measurements were taken in triplicate.

Table 1 summarizes the characterization of the influents and effluents of the four experiments conducted in the SFBBR (average values of BOD_5, COD, TSS, VSS and pH). As previously reported [12], increasing salinity in the influent significantly reduced the efficiency of organic matter removal (COD and BOD_5) by the SFBBR.

2.3. Monitoring of ammonium, nitrate and nitrite concentrations

For ammonium, nitrate and nitrite analyses, water samples were filtered through 0.22 μm membrane filters (HAWP; Millipore Massachusetts, USA) and subsequently quantified by ion chromatography (IC) [22] using conductivity detection (Dionex® DX-300; Dionex Corporation, Sunnyvale, USA). A Metrosep ASUPP5 column

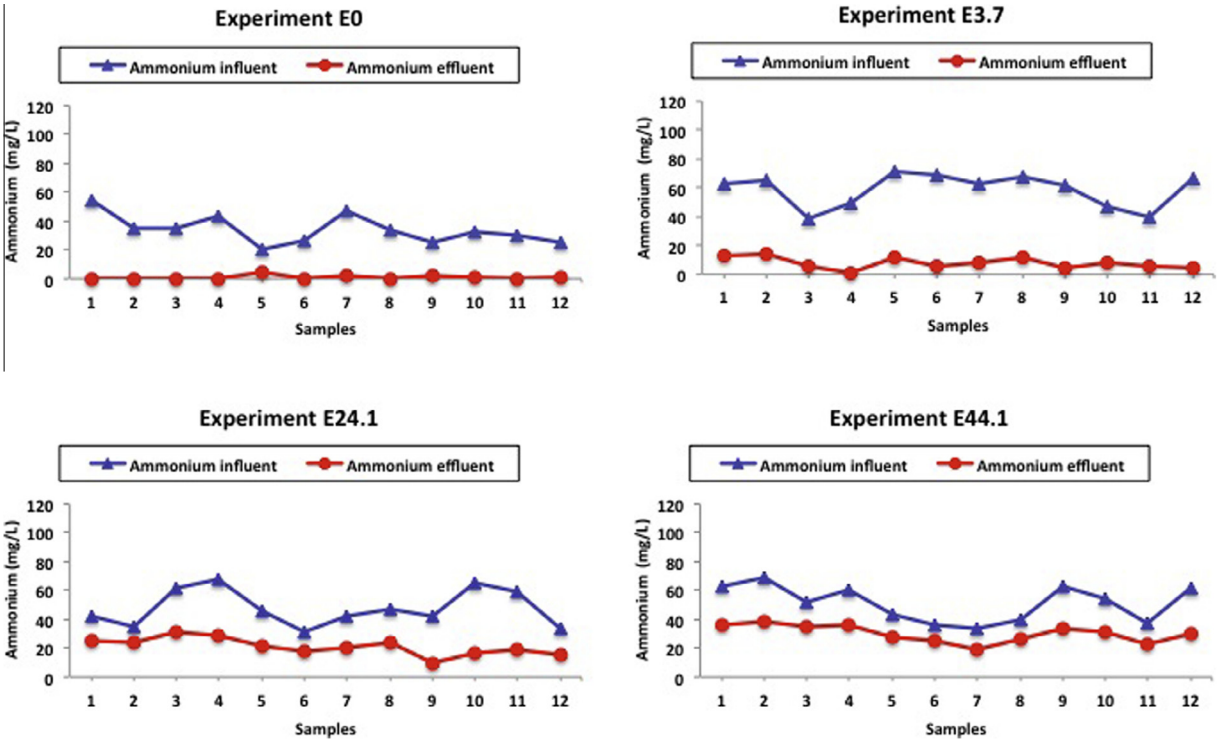

Fig. 1. Evolution of ammonia concentration (mg/L) in the influent and the effluent in the 4 experiments: E0, control unamended with NaCl, 1.5 mS; E3.7 wastewater amended with 3.7 g/L of NaCl, 12 mS; E24.1, wastewater amended with 24.1 g/L of NaCl, 24 mS; E44.1 wastewater amended with 44.1 g/L of NaCl, 48 mS.

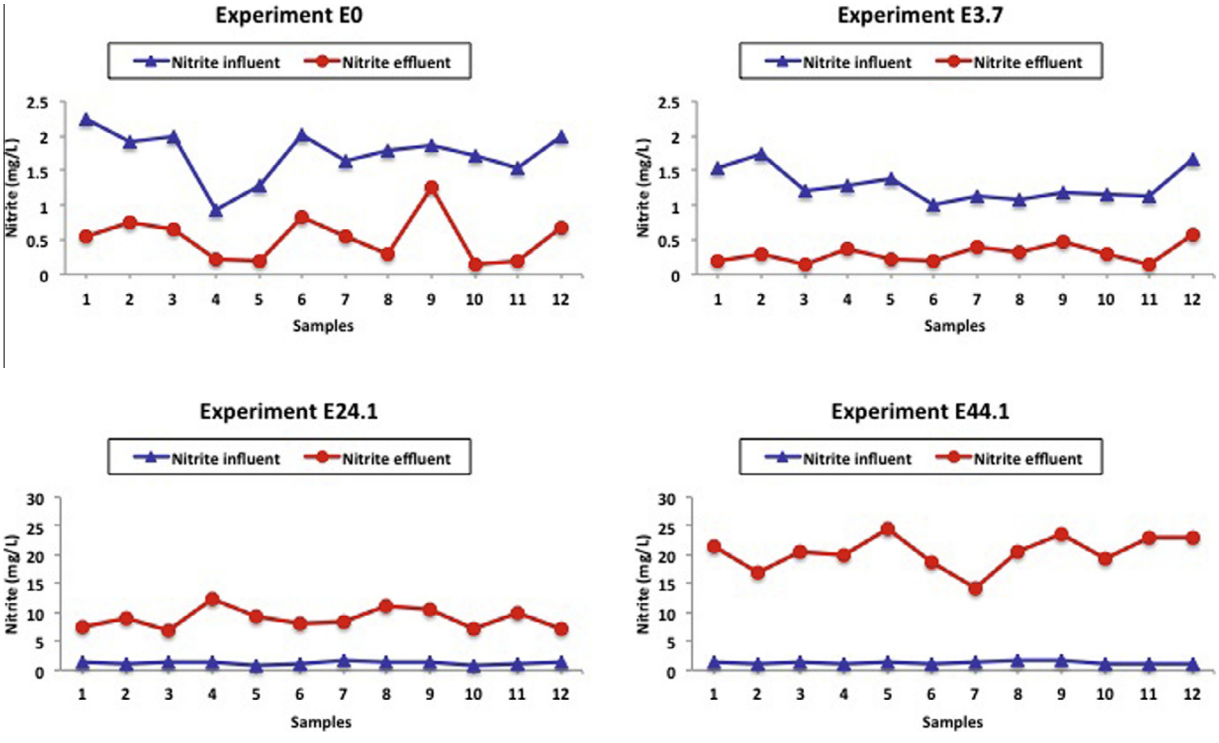

Fig. 2. Evolution of nitrite concentration (mg/L) in the influent and the effluent in the 4 experiments: E0, control unamended with NaCl, 1.5 mS; E3.7 wastewater amended with 3.7 g/L of NaCl, 12 mS; E24.1, wastewater amended with 24.1 g/L of NaCl, 24 mS; E44.1 wastewater amended with 44.1 g/L of NaCl, 48 mS.

with carbonate/bicarbonate as eluent was used to detect the anions and a Metrosep CO2150 column with tartaric acid/dipicolinic as eluent was used to detect the cations.

Before measurement, the filtered samples were diluted to obtain ammonium, nitrate, and nitrite concentrations lower than 10 mg/L. Before the IC analysis, samples were again filtered using a silver-impregnated filter (Dionex®, Sunnyvale, CA) to eliminate chloride ion interference with nitrite measurement.

Influent and effluent samples were collected at day 2, 5, 9 and 12 of every cycle in order to measure the rate analysis ammonium

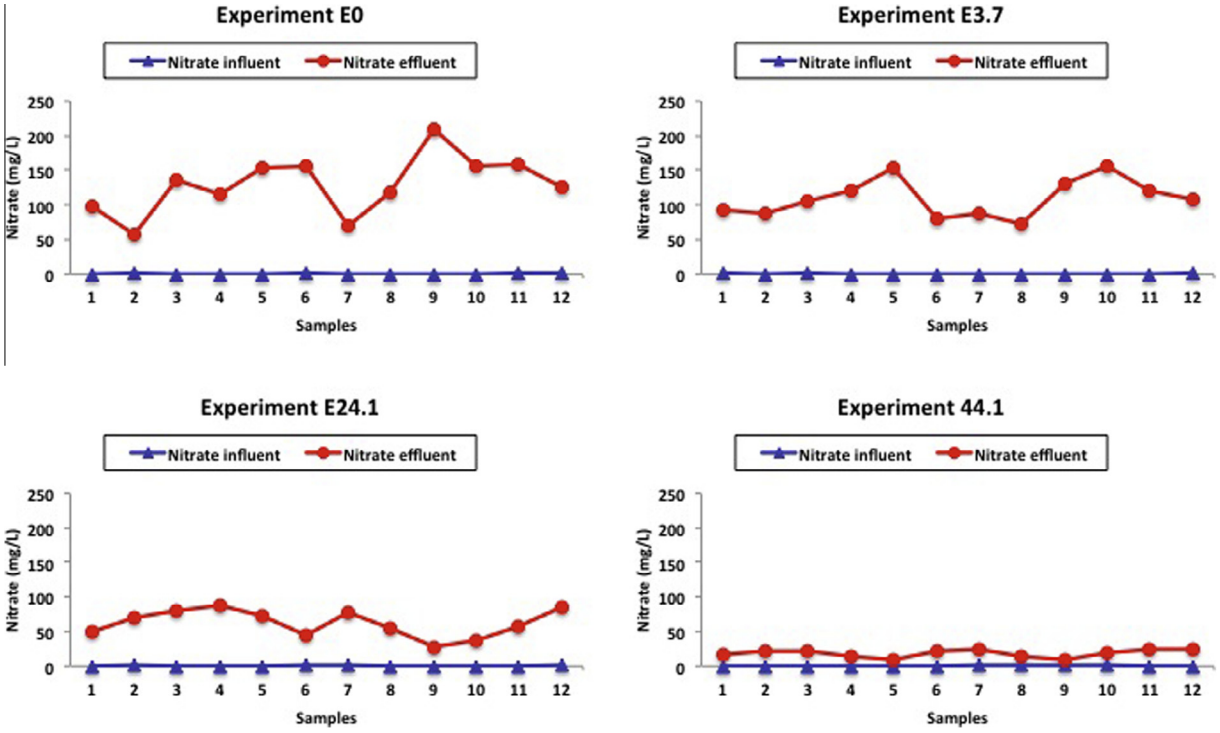

Fig. 3. Evolution of nitrate concentration (mg/L) in the influent and the effluent in the 4 experiments: E0, control unamended with NaCl, 1.5 mS; E3.7 wastewater amended with 3.7 g/L of NaCl, 12 mS; E24.1, wastewater amended with 24.1 g/L of NaCl, 24 mS; E44.1 wastewater amended with 44.1 g/L of NaCl, 48 mS.

Table 2
Ammonium, nitrate and nitrite concentrations in the submerged fixed bed reactor (SFBBR) during the four experiments. Average values marked with the same letter are not significantly different, according to the least significant difference (LSD) test ($p < 0.05$).

Experiment	Ammonium (mg/L) influent water	Nitrite (mg/L) influent water	Nitrate (mg/L) influent water
E0	33.90 ± 9.97[a]	1.74 ± 0.36[b]	0.37 ± 0.52[a]
E3.7	58.45 ± 11.63[b]	1.29 ± 0.24[b]	0.31 ± 0.37[a]
E24.1	47.74 ± 12.56[c]	1.26 ± 0.25[b]	0.35 ± 0.36[a]
E44.1	51.00 ± 12.42[bc]	1.29 ± 0.26[b]	0.31 ± 0.33[a]
LSD[a]	9.62	0.23	0.33
Experiment	Ammonium (mg/L) effluent water	Nitrite (mg/L) effluent water	Nitrate (mg/L) effluent water
E0	0.80 ± 1.21[a]	0.53 ± 0.33[a]	129.57 ± 41.62[d]
E3.7	7.73 ± 3.93[b]	0.33 ± 0.13[a]	100.74 ± 33.24[c]
E24.1	20.90 ± 5.97[c]	8.99 ± 1.74[b]	55.58 ± 25.44[b]
E44.1	29.94 ± 5.97[d]	20.48 ± 2.65[c]	19.20 ± 5.87[a]
LSD[a]	3.85	1.42	24.40

[a] LSD, least significant difference.

removal efficiency. Samples from the first cycle of experiment E0 were named 1, 2, 3 and 4; samples from the second cycle of experiment E0 were named 5, 6, 7 and 8; samples from the third cycle of experiment E0 were named 9, 10, 11 and 12, and accordingly for the remaining cycles in experiments E3.7, E24.1 and E44.1.

The effect of salt concentration on the parameters studied was evaluated by either one-way or multifactor analysis of variance (ANOVA), using the software package STATGRAPHICS 5.0 (STSC Inc., Rockville, MD, USA) to identify significant differences between measurements. A significance level of 95% ($P < 0.05$) was selected. All measurements were taken in triplicate.

2.4. Biofilm samples, DNA extraction and 454-pyrosequencing

One representative biofilm sample was selected at day 9 of the third cycle from each of the four experiments (stability condition of the SFBBR system). The samples were named as follows: AmoAE0 from experiment E0, AmoAE3.7 from experiment E3.7, AmoAE24.1 from experiment E24.1 and AmoAE44.1 from experiment E44.1. Biofilm samples were recovered from the support material as already described by Cortés-Lorenzo et al. [12]. The pellets were immediately used for DNA extraction, using the FastDNA kit (MP-BIO, Germany) and the FastPrep24 apparatus (MP-BIO, Germany). DNA was checked by electrophoresis in a 0.8 (w/v) agarose gel, and Nanodrop 2000c (Thermo Scientific, Wilmington, DE, USA) was used to assay the DNA purity. The DNA isolated was 1:10 diluted in nuclease-free water (Promega Benelux, Leiden, the Netherlands).

For high-throughput 454-pyrosequencing, DNA was amplified with a set of *amoA* primers. The *amoA* forward primer consisted of linker A, a barcode, and the sequence of the *amoA* F primer described by Rotthauwe et al. [24] (5′-CCATCTCATCCCTGCGTGTC TCCGACTCAG-"barcode"-GGGGTTTCTACTGGTGGT-3′). The reverse *amoA* primer contained the linker B and the sequence of the *amoA* R primer described by Rotthauwe et al. [24] (5′-CCTATCCCC TGTGTGCCTTGGCAGTCTCAG-CCCCTCKGSAAAGCCTTCTTC-3′). The barcodes were unique 8-nucleotides sequences attached to the forward primer to identify multiplexed samples. PCR was run according to the following procedure: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 40 s, and extension at 72 °C for 1 min, followed by another extension of 10 min at 72 °C. The 454-pyrosequencing method was performed using the standard Roche 454 FLX Titanium protocols at the Research and Testing laboratory (RTL, Lubbock, USA).

Purification of the pooled DNA was done by removing small fragments and primer dimers with the RapidTip PCR purification tips and Ampure beads (Beckman Coulter, Brea, CA).

2.5. Sequence data analysis and phylogenetic classification

Sequence data analysis was carried out using the QIIME pipeline [25]. Similar *amoA* gene sequences were grouped into Operational

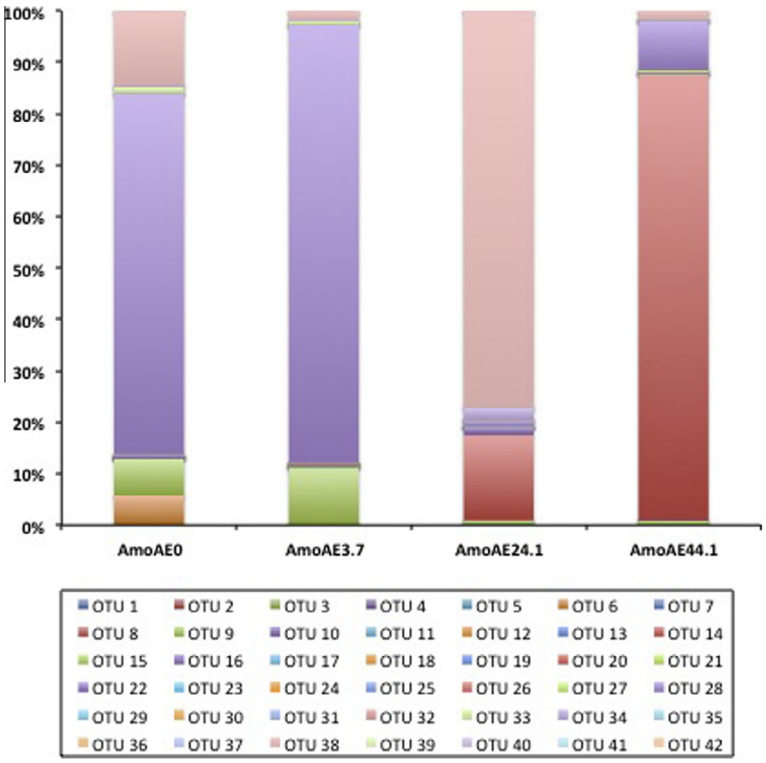

Fig. 4. Relative abundance of OTUs in the submerged fixed bed bioreactor (SFBBR) under different salt concentrations in the influent.

Table 3
amoA gene bacterial diversity indices from the four biofilm samples.

Experiment	No. of sequences[a]	Richness[b]	Chao1[c]	H[d]
E0	6.254	13	14.000	1.42
E3.7	10.188	19	23.178	0.81
E24.1	30.849	15	14.567	1.12
E44.1	69.165	28	15.267	0.74

[a] Number of sequences obtained after quality control.
[b] Number of OTUs obtained.
[c] Chao1 index.
[d] Shannon–Wiener index.

Taxonomic Units (OTUs) using UCLUST software [26] at 6% of cut-off level. We selected a 6% cut-off to cluster closely related phylotypes of the *amoA* gene without losing potentially valuable information [19]. Rarefaction curves, species richness estimators (Chao1 index) and community diversity index (Shannon-Wiener index (H') were calculated as a measure for α-diversity.

All the effective *amoA* gene bacterial representative sequences per OTU obtained by pyrosequencing in this study were transformed into protein sequences and compared in the sequence database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), using their BLASTp tool. Sequences were left out when they had no matches or matches were no *amoA* proteins. The filtered DNA sequences obtained in this study and other *amoA* sequences from validated species and environmental samples of AOB were then aligned using the MUSCLE algorithm [27] provided by the web page of the European Bioinformatics Institute (EMBL-EBI). Phylogenetic and molecular evolutionary analyses were conducted using MEGA 6 [28]. Evolutionary relationships of taxa were inferred using three different methods: A Neighbour-Joining evolutionary tree using the *p*-distance method [29]; a Maximum Likelihood evolutionary tree based on the Tamura–Nei model [30]; and a Maximum Parsimony evolutionary tree using the Subtree-Pruning–Regrafting algorithm [28]. For the three methods, a bootstrap test was conducted to infer the reliability of branch order [31].

3. Results and discussion

3.1. Effect of salt on the nitrification process

Nitrate, nitrite, and ammonium concentrations were determined by IC in the influent and effluent water during the experiments E0, E3.7, E24.1 and E44.1 to elucidate the effect of different salt concentrations on the nitrification process. Time course evolution of nitrate, nitrite and ammonium concentrations in influent and effluent water at the different salt concentrations assayed are shown in Figs. 1–3, and the average values are summarized in Table 2. The increase of salt from experiment E0 to experiment E3.7 in the SFBBR did not have a significant effect on the ammonia oxidation activity or resulted in nitrification inhibition. The opposite outcome was observed for experiments E24.1 and E44.1, where the rate of ammonia oxidation decreased approximately 50% and nitrite accumulated in the system. These results demonstrate that the nitrification process is inhibited at high salt concentrations ($\geqslant$24.1 g NaCl/L) and imply that nitrite-oxidizing bacteria (NOB) are more affected by salinity than ammonium oxidizing bacteria (AOB).

Our results correlate well with other studies which found that the ammonium removal rate was not significantly affected by the salt content of influent water when its concentration was less than 10 g NaCl/L [3,32], but a significant decrease in the rate of oxidation of ammonia with increasing salt concentrations was observed [3,6]. Other studies also showed that AOB are more resistant to the effects of higher salt concentrations than NOB [6,33]. In recent years there have been considerable advances in submerged fixed-biofilm bioreactor systems for ammonium removal. Aslan and

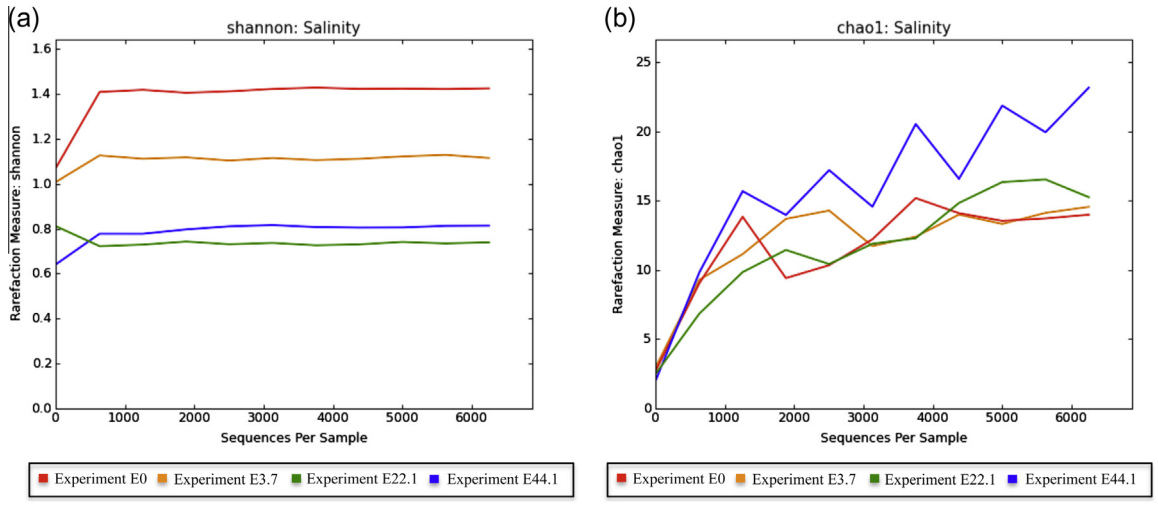

Fig. 5. Rarefaction curves of: (a) Shannon diversity index (H'); (b) Chao1 among 4 experiments.

Simsek [4] indicated that low concentration of NaCl (1 g/L) positively affects the nitrification process, while a concentration of 15 g NaCl/L deteriorate the nitrification activity in a SFBBR.

3.2. The effect of salt on AOB amoA gene diversity

Biofilm samples (AmoAE0, AmoAE3.7, AmoAE24.1 and AmoAE44.1) were analyzed by 454-pyrosequencing. A total of 116,456 high quality partial *amoA* sequences were obtained from all the experiments. The clustering of these reads based on the 94% similarity cut-off resulted in a total of 42 OTUs from which only 5 were shared between all the samples (OTUs 9, 16, 28, 34 and 38). The relative abundance of each OTU in each experiment is presented in Fig. 4 and Supplementary Table S1. Twelve of the 42 OTUs had no match or were similar to proteins other than *amoA* when compared as protein sequences and were disregarded.

As a common hypothesis, diversity may affect the performance of the biofilm process. As shown in Fig. 4, OTU 28 was the most abundant OTU in AmoAE0 and AmoAE3.7 samples, retrieved from biofilms at low salinity (experiments E0 and E3.7), accounting for approximately 70.35% and 85.13% of the reads, respectively. However, in experiments E24.1 and E44.1, the most abundant OTUs were 38 and 14 at a percentage of 77.13% and 86.87%, respectively. To our knowledge, an accurate estimation of OTUs in SFBBR biofilm samples under saline conditions, based on DNA pyrosequencing, has not been conducted before. Our results show that OTU 28 probably plays an important role in ammonium removal in the SFBBR.

In order to compare the AOB species richness and community diversity among these four experiments, the Shannon diversity index (H'), Chao1 index and rarefaction curves were calculated (Table 3). Plots of rarefaction measure: Chao1 index and Shannon diversity index, versus sequence number per sample (the rarefaction curves) are shown in Fig. 5. On the basis of the Chao1 index (Fig. 5b), the AmoAE3.7 sample from experiment E3.7 had the richest AOB species diversity, whereas the three samples from experiments E0, E24.1 and E44.1 displayed considerably less richness (Table 3).

The diversity of the ammonia-oxidizing bacterial community revealed by 454-pyrosequencing of the *amoA* gene was low for all experiments [34]. The nitrifying bacterial phylotype richness levels revealed that the biofilm community sampled in E0 had the highest AOB diversity and E24.1 had moderate richness, while E3.7 and E44.1 had the lowest richness. Although the AOB diversity in E0 was higher than in the other experiments, no trend along the salinity gradient was observed according to the Shannon diversity index (Fig. 5a). In agreement with our results, Aslan and Simsek [4] reported that biofilms showed greater density and abundance of AOB in an SFBBR when the wastewater was salt-free than after the addition of NaCl.

The phylogenetic analysis of the *amoA* sequences representative of the 30 OTUs identified in the SFBBR biofilms showed that they grouped in three different clusters. These clusters (named A, B and C) were retrieved almost identically by the three methods used (Fig. 6, and Supplementary Figs. S1 and S2) except for cluster A, for which only 3 of the 5 sequences were included in the group of the Maximum Parsimony method and its bootstrap was only reliable in the Neighbour-Joining method. Cluster A encompassed sequences obtained in experiments E0 and E3.7, sequences from bioreactors filed in the EMBL database and sequences from known species of *Nitrosomonas* and *Nitrosospira*. The presence of these nitrifying bacteria was in agreement with several studies [35,36]. Pyrosequencing results showed that *Nitrosospira* presented at a higher population during experiment E3.7, disappearing when the salt concentration in the influent was $\geqslant$24.1 g NaCl/L. These results suggest that *Nitrosospira* may be susceptible to high saline conditions. Although AOB belonging to the *Nitrosospira* cluster are not commonly reported as relevant AOB in WWTP [37,38] and this group of AOB has not been usually associated with nitrification in wastewater treatment plants [39], their presence may contribute to the higher efficiency in ammonium removal observed for experiments E0 and E3.7.

Cluster B encompassed sequences obtained in experiments E24.1 and E44.1, one sequence from experiment E3.7 and several other environmental sequences (many of them retrieved from saline environments). Cluster C encompassed sequences obtained in all experiments and other sequences from validated and non-validated species of *Nitrosomonas* and *Nitrosococcus* as well as sequences retrieved from different WWTPs. Sequences from cluster B could be regarded as belonging to AOB adapted to highly saline environments, being OTU 14 remarkably abundant. The sequence of OTU 14 was found to be phylogenetically related to those of the *Nitrosococcus mobilis* sp. Nc2 and interestingly this OTU was the most abundant in experiment E44.1. Remarkably, *N. mobilis* have also been described as dominant aerobic ammonia oxidizing Betaproteobacteria in sewage treatment plants. *N. mobilis*-like ammonia oxidizers were originally isolated from brackish

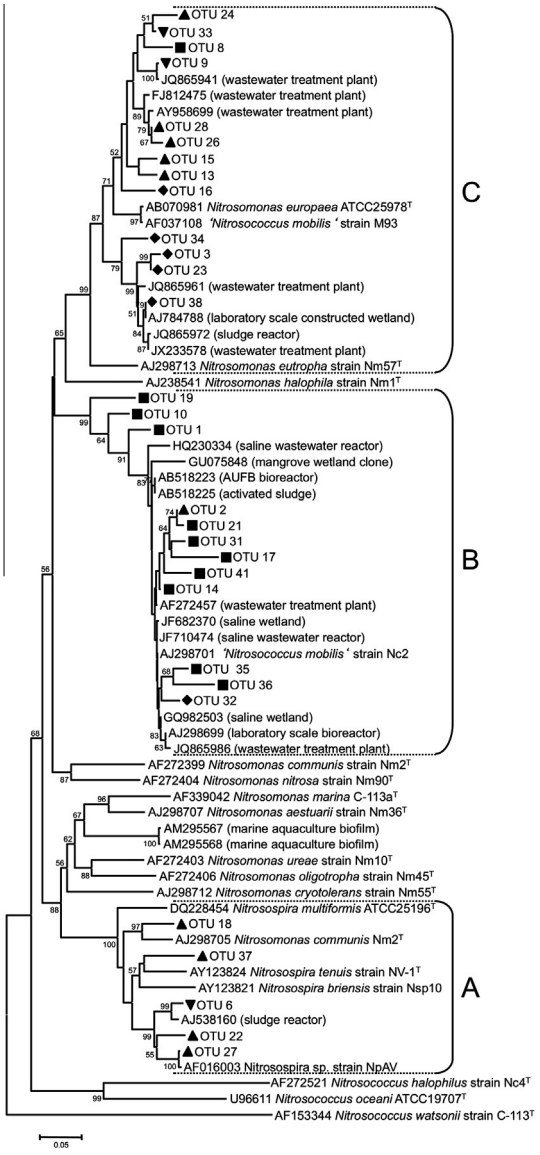

Fig. 6. Neighbour-Joining phylogenetic tree based on the *p*-distance method, performed for the *amoA* sequences obtained in this study and other sequences from environmental samples, validated and non-validated AOB species. Numbers at the nodes of branches are the result for a bootstrap test with a round of 1000 reassemblings. Only values >50% are shown. Biofilm sampled (down triangles for experiment E0, up triangles for experiment E3.7, diamonds for experiment E24.1 and squares for experiment E44.1).

water [40] and had not been reported to contribute to nitrification in wastewater treatments until the observation by Juretschko et al. [37].

Sequences from cluster C show that most of the OTUs retrieved in experiments E0 and E3.7 are related to AOBs also capable of metabolism in high salinity conditions. For instance, OTU 28 was found in all experiments, being the most abundant OTU at low salinity and relatively abundant at high salinity. A similar pattern was encountered for OTU 9. As expected, within cluster C, OTU 28 is related closely to organisms within the *Nitrosomonas europaea/'Nitrosococcus mobilis'* lineage. Members of this lineage have been most frequently detected in WWTPs [38,41]. On the other hand, several reports showed that in ammonium-rich systems like biofilm reactors [42,43], the dominant ammonia oxidizers were members of the genus *Nitrosomonas*. *N. europaea,* a species of AOB, performs the first step in the nitrification process and is critical to the removal of ammonia from WWTP [44]. The phylogenetic analysis showed that populations related to *N. europaea* were responsible for ammonia oxidation and are important for the removal of nitrogen in the SFBBR during experiment E0 and experiment E3.7.

For OTU 38, a concentration of 24.1 g NaCl/L seemed to be optimal as it represented more than 77% of the *amoA* reads, while at other salinities it was present, but did not represent the most abundant OTU. The same effect but in lower relative abundance was observed for OTUs 16 and 34 (also in cluster C).

Several halo-tolerant nitrifying and denitrifying bacteria have been isolated and identified in hypersaline wastewaters [14,22]. The diversity of AOB might be an indicator for the stability of the ammonia oxidation process. Consequently, linking functional changes to changes in community structure in biofilms is certainly important in microbial ecology. Therefore, this study contributed to better understanding of the microbiology of the nitrification process and detection of ammonia oxidizing bacteria in the environment, which should enable us to learn more about systems with different salinity and will help to improve the modeling, design, and operation of nitrifying wastewater treatment plants under saline conditions.

4. Conclusions

The nitrification process in the SFBBR was not significantly affected by NaCl concentrations $\leqslant$3.7 g/L. Concentrations of NaCl $\geqslant$24.1 g/L in the influent water resulted in a remarkable decrease of the ammonium oxidation capacity of the system and a shift in AOB species present in the biofilm, as well as the inhibition of the nitrification process. On the basis of the phylogenetic analysis, we infer that the increment of certain OTUs (cluster C) and the emergence of new OTUs (cluster B) at high salinity substantiate the persistent ammonia-oxidation activity in the SFBBR in experiments E24.1 and E44.1.

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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.cej.2014.12.083.

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