Bioresource Technology 166 (2014) 103–111

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Effect of phenol on the nitrogen removal performance and microbial community structure and composition of an anammox reactor

Alyne Duarte Pereira [a], Cíntia Dutra Leal [a], Marcela França Dias [a], Claudia Etchebehere [b], Carlos Augusto L. Chernicharo [a], Juliana Calabria de Araújo [a,*]

[a] Department of Sanitary and Environmental Engineering, Universidade Federal de Minas Gerais (UFMG), Av. Antonio Carlos 6627, 31270-901 Belo Horizonte, MG, Brazil
[b] Laboratorio de Ecología Microbiana, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay

HIGHLIGHTS

- Phenol at 300 mg L^{-1} decreased the ammonium–N removal efficiency by half.
- The anammox performance recovered after 45 days without phenol addition.
- Phenol changed considerably the microbial community structure and composition.
- The phenol addition reduced the anammox population abundance from 14.7% to 10.1%.
- Phenol selected for some phenol-degrading genera under denitrifying conditions.

ARTICLE INFO

Article history:
Received 17 March 2014
Received in revised form 1 May 2014
Accepted 7 May 2014
Available online 21 May 2014

Keywords:
Anaerobic ammonium oxidation (anammox)
Microbial community structure
Phenol
Sequencing batch reactor
Pyrosequencing

ABSTRACT

The effects of phenol on the nitrogen removal performance of a sequencing batch reactor (SBR) with anammox activity and on the microbial community within the reactor were evaluated. A phenol concentration of 300 mg L^{-1} reduced the ammonium–nitrogen removal efficiency of the SBR from 96.5% to 47%. The addition of phenol changed the microbial community structure and composition considerably, as shown by denaturing gradient gel electrophoresis and 454 pyrosequencing of 16S rRNA genes. Some phyla, such as *Proteobacteria*, *Verrucomicrobia*, and *Firmicutes*, increased in abundance, whereas others, such as *Acidobacteria*, *Chloroflexi*, *Planctomycetes*, *GN04*, *WS3*, and *NKB19*, decreased. The diversity of the anammox bacteria was also affected by phenol: sequences related to *Candidatus* Brocadia fulgida were no longer detected, whereas sequences related to *Ca.* Brocadia sp. 40 and *Ca.* Jettenia asiatica persisted. These results indicate that phenol adversely affects anammox metabolism and changes the bacterial community within the anammox reactor.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

The anaerobic oxidation of ammonium (anammox) is a very promising technology for the removal of nitrogen from wastewaters. The anammox process is catalyzed by chemolithoautotrophic bacteria belonging to the phylum *Planctomycetes*, which can directly oxidize ammonium under anoxic conditions using nitrite as a terminal electron acceptor, converting ammonium to dinitrogen gas in the absence of oxygen (Strous et al., 1998). The anammox process has advantages over the traditional nitrification–denitrification process for nitrogen removal because it uses up to 60% less oxygen and does not require the addition of external carbon. Furthermore, the process produces less sludge and no CO_2 or N_2O emissions (Gao and Tao, 2011). The main drawbacks of the process include the slow growth of the anammox bacteria, which have a high doubling time of approximately 11 days (Strous et al., 1998), and the need for strict control of the process, because factors such as toxic organic compounds, including phenols, can inhibit the anammox bacteria (Jin et al., 2012).

Phenolic compounds are frequently found in industrial wastewaters that also contain nitrogen, such as coke-oven wastewater (Toh and Ashbolt, 2002), wastewater from petrochemical industries (Yang and Jin, 2012), and resin-producing wastewater (Amor et al., 2005). Therefore, before using the anammox process to treat these kinds of wastewater, it is vital to study the effect of phenol on the anammox process and on the microbial community present in anammox reactors. The effects of phenol

* Corresponding author. Tel.: +55 31 3409 3667; fax: +55 31 3409 1879.
E-mail address: juliana@desa.ufmg.br (J.C. de Araújo).

http://dx.doi.org/10.1016/j.biortech.2014.05.043
0960-8524/© 2014 Elsevier Ltd. All rights reserved.

on anammox activity have been studied previously, but the microorganisms involved were not identified (Toh and Ashbolt, 2002; González-Blanco et al., 2012; Jin et al., 2013; Yang et al., 2013). Anammox bacteria were acclimated in phenol-containing wastewater at low nitrogen-loading rates over a long period (Toh and Ashbolt, 2002). In a recent study (Yang et al., 2013), the short- and long-term effects of phenol on anammox activity were determined. Most of these studies investigated the effect of phenol on the anammox activity and on high-rate anammox processes. However, the effect of phenol on the microbial community structure and composition in an anammox reactor has not been investigated. A better understanding of the microbial community composition of an anammox reactor before and after the disruption caused by a toxic compound such as phenol may help elucidate the processes that occur inside the reactors under stress conditions, which is important for improving processes used to treat industrial wastewaters that contain toxic compounds.

Because of their high coverage, second-generation sequencing technologies provide more comprehensive information about microbial communities than the traditional Sanger-based methods. Pyrosequencing can detect organisms at low abundance. This technique has not previously been used to study changes in the microbial community structure of an anammox reactor subjected to phenol stress. Therefore, this study aimed to investigate the following: (1) the effects of increasing concentrations of phenol on the nitrogen removal performance of an anammox enrichment reactor (without biomass acclimatization) and (2) the effect of phenol on the microbial community structure and composition in an anammox reactor.

To achieve these objectives, a sequencing batch reactor (SBR) seeded with activated sludge was used to produce the anammox biomass. The reactor was monitored for 591 days, and phenol was added after 335 days of operation under anammox conditions. The microbial community structure and composition in the reactor were investigated using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis and 454 pyrosequencing of bacterial 16S rRNA genes.

2. Methods

2.1. Seeding sludge and experimental set-up

Activated sludge from a municipal wastewater treatment plant located in Belo Horizonte (Brazil) was used as an inoculum to cultivate anammox bacteria because a previous study successfully enriched anammox bacteria from this sludge (Araújo et al., 2011). A 2.0-L glass SBR (Benchtop Fermentor & Bioreactor Bio Flo®/CelliGen® 115, New Brunswick Scientific Co., Enfield, CT, USA) was used for enrichment of anammox bacteria. The reactor was fitted with a fermentor lid containing a feed in-flow tube, a dissolved oxygen probe, a pH probe, acid and base in-flow tubes for pH control, a gas line, a sampling line, and an effluent-withdraw line. The temperature was controlled and maintained at 34–35 °C via a water jacket, and the pH was controlled at 7.5. Anaerobiosis was maintained by bubbling N_2 gas (99.99%) through the liquid. This gas was also flushed in the mineral medium (feed vessel) in order to maintain anaerobic conditions in the synthetic wastewater. The reactor was monitored for 591 days and was operated as a continuously fed SBR with two cycles, one of 8 h (short cycle) and the other of 16 h (long cycle). Each cycle had four phases: (i) continuous feeding period (180 min in both cycles), (ii) anaerobic reaction period (240 min in the short cycle and 720 min in the long cycle), (iii) settling period (30 min in both cycles), and (iv) withdrawal period (30 min in both cycles).

2.2. Autotrophic medium

The reactor was fed autotrophic medium with a composition similar to that described previously (Van de Graaf et al., 1996). The final concentrations of ammonium and nitrite in the medium were increased gradually according to the evolution of anammox activity in the reactor (as described in Section 3).

2.3. Addition of phenol to the SBR

From days 336 to 377, phenol was introduced into the SBR during the long cycle (16 h) through a septum located at the top of the reactor using 60-mL syringes. Varying amounts of a concentrated solution of phenol (1 g L^{-1}) were added to the reactor in order to achieve the desired final concentration. The phenol concentration in the reactor was gradually increased every week to 10 (1st week), 25 (2nd week), 50 (3rd week), 100 (4th week), 200 (5th week), and 300 (6th week) mg L^{-1}.

Statistical analysis was performed to assess whether the different concentrations of phenol added to the SBR altered the nitrite and ammonium removal efficiency and the stoichiometric ratio of ΔNO_2-N:ΔNH_4^+-N). The Kruskal–Wallis test ($\alpha = 5\%$) was performed, followed by a multiple comparisons test between medians ($\alpha = 5\%$), using the Statistica 8 software. Periods in which phenol was added to the reactor were compared to the previous period, without phenol addition (291–335 days), in which the operating conditions were similar (ammonium and nitrite influent concentrations of 100 mg L^{-1} and 118 mg L^{-1}, respectively; temperature of 35 °C; agitation equal to 70 rpm; and hydraulic retention time of 24 h).

2.4. Analytical procedures

The concentrations of NH_4^+-N and NO_2^--N were determined colorimetrically by the phenol-hypochlorite (measured at 630 nm) and sulfanilic acid (measured at 520 nm) methods, 4500 NH_3F and 4500 NO_2^-B, respectively, according to the Standard Methods of the Examination of Water and Wastewater (APHA, 2005).

2.5. Analysis of the microbial community in the biomass from the anammox reactor

2.5.1. PCR-DGGE

The biomass in the SBR was sampled at day 0 (inoculum), days 110, 190, 270, and 330 (before phenol addition), and day 370 (period after phenol addition), which represent different stages of the SBR operation. Samples (20 mL) for molecular analyses were taken from the reactor and centrifuged at 14,000 rpm for 10 min, and the pellet was used for further studies. DNA was extracted using the protocol described by Egli et al. (2003). PCR-DGGE was performed using the primer set 1055F and 1392R, with a GC clamp, as described previously (Ferris et al., 1996). DGGE was performed at 60 °C in 0.5× TAE buffer at 80 V for 17 h with a Bio-Rad DCode Universal Mutation Detection System (Hercules, CA, USA), using an 8% polyacrylamide gel with a 45–75% (M/V) gradient of urea formamide denaturant. Gels were stained with a SYBR gold solution and visualized under UV transillumination. Specific gel bands were excised, re-amplified, purified, and sequenced. The PCR products were sequenced using a genomic service (Macrogen Inc., Seoul, Korea). Sequences were compared with sequences from the National Center for Biotechnology Information database using the Basic Local Alignment Search Tool (Altschul et al., 1990).

DGGE patterns were analyzed using the BioNumerics software version 2.5 (Applied Maths, Austin, TX, USA). Hierarchical cluster comparisons were carried out to group similar profiles and to generate a binary matrix of band classes. Whole profiles were

compared using the Dice similarity coefficient (D_{sc}). The dendrogram was generated using the method of un-weighted pair group with mathematical averages (UPGMA) at a 1% position tolerance.

2.5.2. Analysis of the bacterial communities by 454 pyrosequencing

The pyrosequencing technique was used to analyze the microbial community composition of biomass sampled at days 110 (beginning of anammox activity), 270 (before phenol addition), and 370 (period after phenol addition), which represent different stages of the SBR operation. Genomic DNA was extracted according to Egli et al. (2003) and further purified using the Wizard® Genomic DNA Purification kit (Promega, Madison, WI, USA). The amount of DNA was estimated using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA was precipitated with 95% ethanol and dried at 50 °C. The dehydrated samples were sent to the Institute for Agrobiology Rosario (INDEAR, Rosario, Argentina), where the pyrosequencing analyses were performed using a Roche Genome Sequencer FLX Titanium system. Barcodes with 10 bp were used for each sample. The 16S rRNA genes were amplified with primers for the v4 region: 563f (CACGACGTTGTAAAACGACAYTGGGYDTAAAGNG), in which the tag was included, and 802r (CAGGAAACAGCTATGACC). The sequences were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) software (Caporaso et al., 2010). Reads less than 200 bases, quality coefficient less than 25, homopolymer size higher than 6, and ambiguous bases were removed. Operational taxonomic units (OTUs) were defined using the UCLUST algorithm (Edgar, 2010) based on 97% identity. Reads were classified using the Classifier tool from the Ribosomal Database Project (http://edp.cme.msu.edu/classifier/classifier.jps), with the suggested bootstrap cut-off setting at 50%.

The alpha diversity indices (Shannon-Wiener, Simpson's diversity, evenness, and dominance) and beta diversity indices (Bray-Curtis and Jaccard) of each sample were calculated using the PAST software (version 2.14b). Reads classified as belonging to the different anammox genera were selected and used to construct a phylogenetic tree using MEGA version 4.0 (Tamura et al., 2007) by applying the neighbor-joining method and Kimura's two-parameter model of sequence evolution. Bootstrap re-sampling analysis for 1000 replicates was performed to estimate the confidence of the tree.

3. Results and discussion

3.1. Nitrogen removal by the SBR before and after the addition of phenol

The concentrations of nitrogenous compounds in the influent and effluent of the SBR over 591 days are shown in Fig. 1 (panels a, b, and c) and in Supplemental Table S1. To understand the effect of phenol on the anammox process in the reactor, the ammonium and nitrite concentration profiles were divided into three phases: the anammox enrichment phase (P_1), the phenol addition phase (P_2), and the recovery phase (P_3).

3.1.1. Anammox cultivation and enrichment phase (P_1)

The first 335 days of operation corresponded to the anammox enrichment and cultivation phase (phase 1). In the first 50 days of operation, denitrification was the favored process (anoxic environment and presence of nitrite), eliminating the organic matter in the medium that originated from the lysis of aerobic bacteria. This view is consistent with the initial consumption of nitrite, which was accompanied by an increase in the effluent ammonia concentration relative to the influent concentration (Fig. 1a), attributable to the death and lysis of the aerobic bacteria initially present in the seed sludge, which caused the release and breakdown of organic nitrogen to ammonia. From days 50 to 90, there was a propagation period in which the denitrifying activity decreased and then stopped. This probably allowed the anammox bacteria in the SBR to develop, because these two groups of microorganisms, anammox and denitrifying bacteria, compete for the same electron acceptor (nitrite). Anammox activity was evident in the reactor from day 90 onward, based on the simultaneous consumption of ammonium and nitrite, resulting in high nitrogen removal efficiency values (Fig. 1b). From days 90 to 335, as ammonium and nitrite were consumed, their influent concentrations gradually increased and promoted anammox enrichment. This resulted in high average removal efficiency values for ammonium (79.6% ± 18.4%, median = 87.2%) and nitrite (93.7% ± 14.5%, median = 100%) during this period (Fig. 1b and Table S1). However, it is important to mention that, for a short time during this phase (from days 200 to 250), during which the influent concentrations of nitrogenous compounds reached high levels (175 mg L^{-1} of NH_4^+-N and 150 mg L^{-1} of NO_2^--N), the ammonium and nitrite removal efficiencies in the reactor were low (Fig. 1b). This suggests that the high concentration of nitrite applied to the reactor disturbed the anammox process. Previous studies showed that nitrite concentrations ranging from 185 to 350 mg L^{-1} inhibited the anammox process (Dapena-Mora et al., 2007; Egli et al., 2001). To prevent the complete inhibition of the anammox process, the influent ammonium and nitrite concentrations were reduced to 100 mg L^{-1}. Thereafter, activity in the SBR re-established, and the nitrogen removal efficiency was high. The stoichiometric coefficient ΔN-NO_2^-/ΔN-NH_4^+, which remained near 1.1 ± 0.2 (median value = 1.1), close to the ratio reported in the literature for the anammox reaction, was further evidence of the stability of the anammox process in this period (days 90–335) (Fig. 1c and Table S1).

3.1.2. Phenol addition phase (P_2)

For 42 days (phase 2), from days 336 to 377, increasing concentrations of phenol were added to the reactor without biomass acclimatization (Fig. 1a). Phenol concentrations up to 200 mg L^{-1} did not influence nitrogen removal by the anammox reactor. However, when the phenol concentration increased to 300 mg L^{-1}, the reactor performance suddenly deteriorated, and the ammonium and nitrite removal efficiency values dropped (Fig. 1b), indicating that phenol had an adverse effect on anammox metabolism. When the phenol concentrations varied from 0 to 200 mg L^{-1}, the median values of nitrite removal efficiency were close to 99% (Supplementary Fig. S1). However, when the phenol concentration increased to 300 mg L^{-1}, the nitrite removal efficiency decreased to 85%. No statistical differences between these values were observed when they were compared using the nonparametric Kruskal–Wallis test (α = 5%; p-value = 0.1060). However, for the ammonium removal efficiency (Fig. S2), statistical differences between the median values were observed (p-value = 0.0193) with the Kruskal–Wallis test (α = 5%). The median values for ammonium removal efficiency remained near 96% when the phenol concentration ranged from 0 to 200 mg L^{-1} (Supplementary Fig. S2). Nevertheless, when the phenol concentration increased to 300 mg L^{-1}, the median ammonium removal efficiency decreased to 47% (Fig. S2). For multiple comparisons between median values, the Kruskal–Wallis test (α = 5%) was performed, and statistical differences were observed between periods with phenol concentrations of 300 and 25 mg L^{-1} (p-value = 0.026851), 300 and 50 mg L^{-1} (p-value = 0.001065), and 300 and 200 mg L^{-1} (p-value = 0.024134). Thus, the addition of phenol affected the ammonium removal efficiency.

In addition, the consumption of nitrite increased relative to the depletion of ammonium at the end of phase 2, resulting in higher stoichiometric coefficient values than in previous periods (Fig. 1c

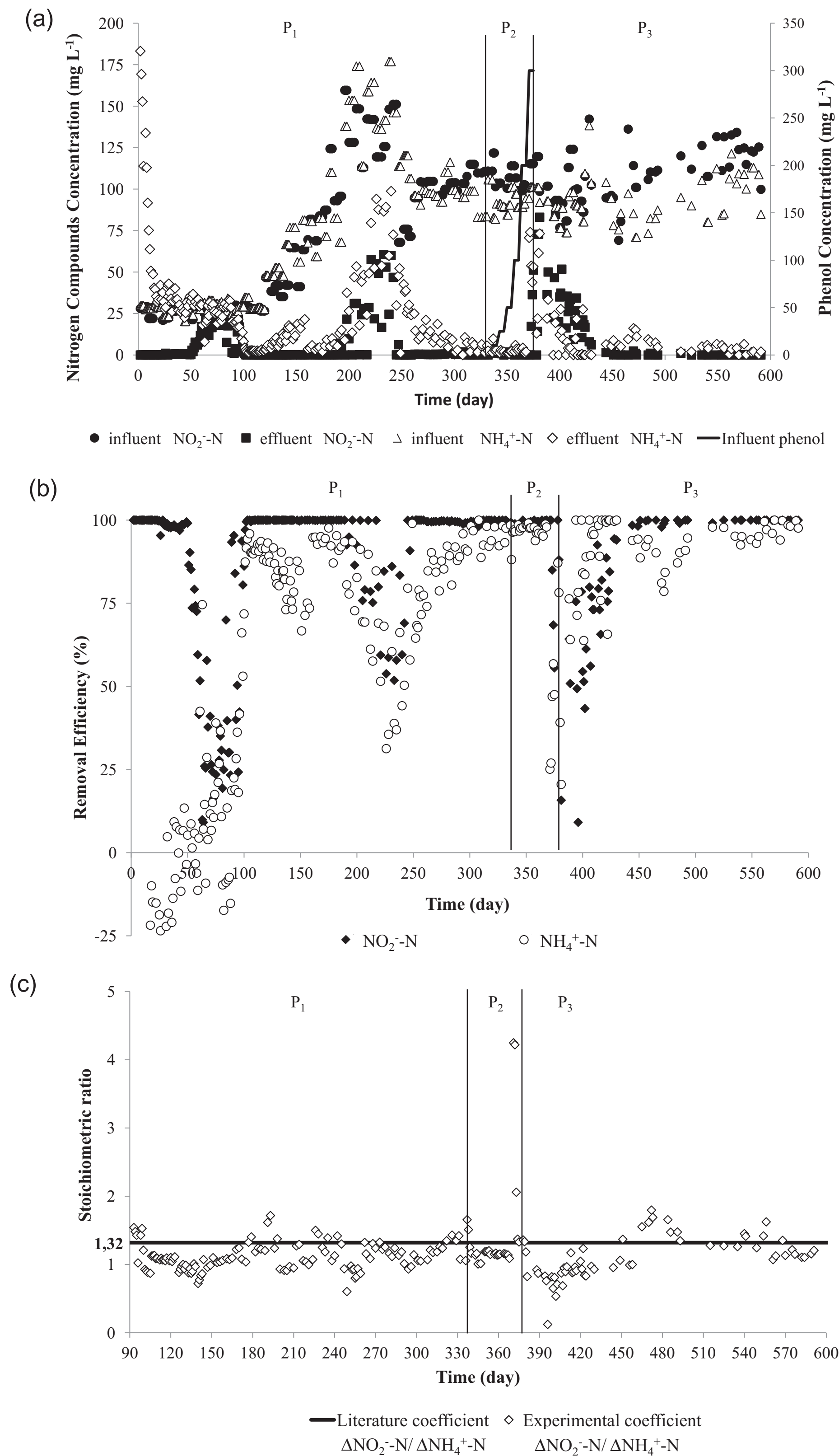

Fig. 1. Performance of the sequencing batch reactor (SBR). (a) Ammonium and nitrite concentrations in the influent and effluent during SBR operation, before and after the phenol feeding period. (b) Ammonium and nitrite removal efficiencies. (c) Changes in the stoichiometric ratio $\Delta N\text{-}NO_2^-/\Delta N\text{-}NH_4^+$ after the detection of anammox activity in the reactor (from 90 days onward).

and Supplementary Fig. S3). The stoichiometric coefficient median values throughout the phenol addition period were approximately 1.1–1.3 (Fig. S3), except when the influent phenol concentration was 300 mg L^{-1}, when the rate increased to 2.1. The nonparametric Kruskal–Wallis test ($\alpha = 5\%$) was performed, and statistical differences between the median values of the stoichiometric

coefficient (p-value = 0.0098) were observed. Moreover, statistically significant differences were observed between the stoichiometric coefficient values at concentrations of 300 mg L^{-1} and 25 mg L^{-1} (p-value = 0.012088). Thus, the addition of phenol affected nitrite consumption in the reactor. Toh and Ashbolt (2002) also observed that the addition of phenol changed the stoichiometric characteristics of the anammox process. These results suggest that the presence of organic matter (phenol) helps the metabolism of heterotrophic denitrifying bacteria. Therefore, more nitrite than ammonium was consumed during this period. The nitrite was probably used as an electron acceptor by heterotrophic bacteria for the oxidation of organic matter present in the SBR.

3.1.3. Recovery of anammox activity phase (P_3)

To eliminate the stress induced by phenol, its addition to the SBR was stopped (phase 3). During the first 45 days of this third phase, the reactor continued to suffer from the effects of phenol, as evidenced by reduced ammonium and nitrite removal efficiency values (Fig. 1b). However, the anammox activity recovered after 45 days without phenol, as shown by the high nitrogen removal efficiency values (around 94–98% for ammonium and nitrite) (Fig. 1b). The stoichiometric coefficient of nitrite consumption to ammonium depletion also recovered to a value closer to that reported in the literature for the anammox reaction (Fig. 1c).

3.2. The bacterial community in the SBR before and after phenol addition

3.2.1. Molecular fingerprint of the bacterial community determined by PCR-DGGE

DGGE fingerprints and band patterns from biomass sampled at day 0 (inoculum), day 110 (beginning of anammox activity), days 190 and 270 (high anammox activity), day 330 (before phenol addition), and day 370 (after phenol addition) were compared and evaluated. The patterns indicated that the anammox process in the SBR was dynamic (Fig. 2a). The bacterial community structure changed at different times during anammox enrichment as nitrogen concentrations increased and after the addition of phenol to the SBR (Fig. 2a).

The strongest bands in the DGGE gel were excised, and the DNA was sequenced to identify the microorganisms dominant in each period (0, 110, 190, 270, 330, and 370 days) (Table 1). The DGGE band pattern showed that band B, with a sequence closely related to the anammox bacterium *Candidatus* Brocadia sp. 40 (AM285341), appeared in the biomass sampled at day 110 and remained throughout the reactor operation period, even after the addition of phenol to the SBR (biomass sampled at day 370) (Fig. 2a and Table 1). In addition, band C, with a sequence related to *Candidatus* Brocadia fulgida (DQ459989), appeared in the biomass sampled at day 270, at which time the nitrogen removal performance of the SBR was excellent.

Sequences with high homology to sequences from denitrifying bacteria were also identified by DGGE in all samples taken from the SBR, indicating that denitrifying bacteria were present throughout the operation period. The sequence from band D, present only in the inoculum sample, had high homology to a sequence from *Acidovorax ebreus* (HQ149701). Band I, which appeared in all samples (before and after the addition of phenol), had a sequence closely related to *Acidovorax* sp. (AM084039). Organisms belonging to this genus are frequently found in activated sludge systems and play an important role in the degradation of aromatic compounds and the bioremediation of contaminated environments (Heylen et al., 2006).

One band (band H), with sequence homology to *Nitrosomonadaceae*, an ammonia-oxidizing bacterium (AOB), was detected in the inoculum sample as well as in the other samples, indicating that

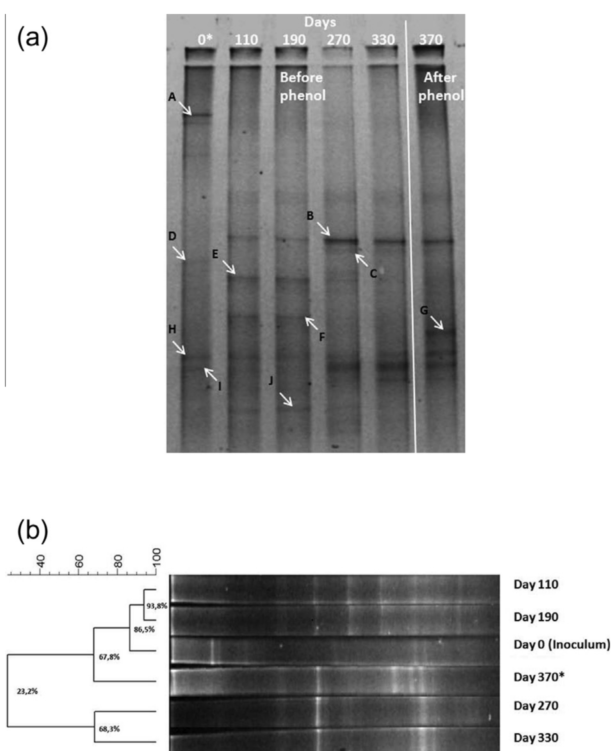

Fig. 2. Bacterial community analysis by denaturing gradient gel electrophoresis (DGGE). (a) The DGGE profile of the bacterial community in biomass sampled at day 0 (inoculum), days 110, 190, 270, and 330 (before phenol addition), and day 370 (after phenol addition), based on 16S rRNA partial gene sequences. (b) A dendrogram generated from the DGGE band profiles from biomass sampled at day 0 (inoculum), days 110, 190, 270, and 330 (before phenol addition), and day 370 (*period after phenol stress). The comparison of the DGGE profiles was generated with the BioNumerics software package using the un-weighted pair group with the mathematical averages method, as described in Section 2.5.1.

AOB remained in the SBR even during the anoxic conditions applied to the reactor during anammox enrichment. The co-existence of anammox bacteria, nitrifiers, and denitrifiers in anammox reactors has been reported (Xiao et al., 2009; Costa et al., 2014). The importance of denitrifying bacteria in a nitritation–anammox process was related to the overall nitrogen removal efficiency and contributed to nitrate removal by the anammox process (Desloover et al., 2011).

After the addition of phenol to the SBR (biomass sampled at day 370), a new band (band G) appeared with high sequence similarity to a sequence from *Pseudomonas putida* (JQ832762) (Fig. 2a and Table 1). This bacterium is associated with the degradation of aromatic compounds and is widely used in wastewater treatment systems for phenol degradation (Ullhyan and Ghosh, 2012).

The presence of anammox bacteria and bacteria capable of degrading phenolic compounds, such as *P. putida* and other denitrifying bacteria, in the same reactor can be beneficial when the goal is to treat wastewater containing nitrogen and organic compounds such as phenols, as is the case with many industrial wastewaters. In the present study, both bacterial groups were observed after feeding the reactor with phenol, indicating that the biomass that developed in the SBR has the potential to treat this kind of industrial wastewater.

The anaerobic degradation of phenol has been studied, and its association with denitrification processes has been described in other studies (Berinstain-Cardoso et al., 2009). Denitrifying bacteria were found in the SBR investigated in this study. These bacteria could play an important role in the use of phenol because they can use the compound as an additional carbon source, thereby decreasing its toxic effects on anammox bacteria.

Table 1
DGGE bands associated with 16S rRNA genes in biomass samples collected from an anammox SBR before and after phenol addition (see Fig. 2a).

Band	Phylum	Closest class or order	Acc. No.	16S rRNA gene	Similarity[a]
A	TM7	–	AF269027	Uncultured bacterium clone: NoosaAW40	92%
B	*Planctomycetes*	*Brocadiales*	AM285341	*Candidatus* Brocadia sp. 40	99%
C	*Planctomycetes*	*Brocadiales*	DQ459989	*Candidatus* Brocadia fulgida	90%
D	*Proteobacteria*	*Burkholderiales*	HQ149701	*Acidovorax ebreus* UTM FZZ15	94%
E	*Proteobacteria*	*Rhodocyclaceae*	HQ085996	Uncultured bacterium clone: F5OHPNU07HZUC0	93%
F	*Proteobacteria*	*Hydrogenophilaceae*	NR_074417	Thiobacillus denitrificans ATCC25259	99%
G	*Proteobacteria*	*Pseudomonadaceae*	JQ832762	*Pseudomonas putida* p105_F05	87%
H	*Proteobacteria*	*Nitrosomonadaceae*	HQ834716	Uncultured bacterium clone: HKY02-06	90%
I	*Proteobacteria*	*Burkholderiales*	AM084039	*Acidovorax* sp. R-25052	99%
J	*Proteobacteria*	*Myxococcales*	FJ889284	Uncultured bacterium clone: Plot 18_G03	94%

[a] Percentages indicate the similarity between the DGGE band sequence and the closest matched sequences in GenBank.

The DGGE band patterns from biomass sampled at day 0 (inoculum), days 110, 190, 270, and 330 (before phenol addition), and day 370 (period after phenol stress) were analyzed for similarities by digital image analysis using the BioNumerics version 2.5 software package. The similarities between the DGGE patterns for each sample were calculated and visualized as a clustered tree to evaluate bacterial community dynamics during the SBR operation (Fig. 2b). Two major and distinct groups (with only 23.2% similarity) were identified (Fig. 2b).

Clear differences in bacterial community structure were observed when the DGGE profiles from biomass sampled at days 110 and 190 (low nitrogen concentrations) were compared with biomass samples collected in subsequent periods (day 270 and day 330, high nitrogen concentrations), indicating that the cultivation conditions (with increasing nitrogen concentrations) were the driving force for bacterial selection and adaptation. Moreover, the addition of phenol changed the bacterial community profile. The DGGE profile from biomass sampled at day 370 (after phenol stress) was more similar to the inoculum profile than to the other profiles (Fig. 2b). This was expected, because the addition of phenol (an organic compound) favored heterotrophic metabolism, which was likely the dominant type of metabolism in the seed sludge.

3.2.2. Microbial community composition determined by 454 pyrosequencing

Biomass sampled at days 110, 270, and 370, which represented the three different SBR phases (low ammonium and nitrite concentrations in the influent, high ammonium and nitrite concentrations in the influent before phenol addition, and after phenol stress, respectively) were chosen for deep sequencing analysis. Pyrosequencing of these samples yielded 1603, 6193, and 6525 high quality sequences, respectively. Most of the sequences were 200–220 bp. These reads were grouped into 392 OTUs after removing the singletons. Among the total OTUs analyzed, 87 OTUs (85.2%) were common to the three samples, and 22, 82, and 110 OTUs were unique to the samples collected at days 110, 270, and 370, respectively.

The most abundant phyla in the three samples were *Proteobacteria* (accounting for 24.6–40.8% of the total reads), *Planctomycetes* (11.6–17.8%), *GN04* (11.9–15.3%), and *Chloroflexi* (9.1–15.7%). These four phyla accounted for more than 70% of the total reads in the three samples analyzed. Other dominant phyla accounted for 2–7% of the total reads, including *Acidobacteria*, *Firmicutes*, *NKB19*, *Verrucomicrobia*, and *WS3* (Fig. 3). *Nitrospirae* was detected in the three samples but at very low abundance (from 0.1% to 0.4%), suggesting the occurrence of nitrite oxidation by nitrite-oxidizing bacteria (NOB). The presence of *Nitrospirae* in anammox reactors has been reported (Egli et al., 2003; Costa et al., 2014). It was suggested that these organisms benefit anammox bacteria by removing dissolved oxygen as well as by consuming excess nitrite that could inhibit the anammox process.

The three samples analyzed presented similar diversity indices. The Shannon-Wiener, Simpson's diversity, dominance, and evenness indices obtained for each library (biomass sampled at days 110, 270, and 370) showed similar values (Supplementary Table S2). Thus, these results indicate that, although phenol favored some microbial groups over others and changed the abundance of some microbial groups, it did not change the diversity of the taxa in the reactor (Supplementary Table S2).

However, differences in the microbial community composition were observed when the Jaccard and Bray-Curtis indices were calculated to estimate the similarity amongst the three samples

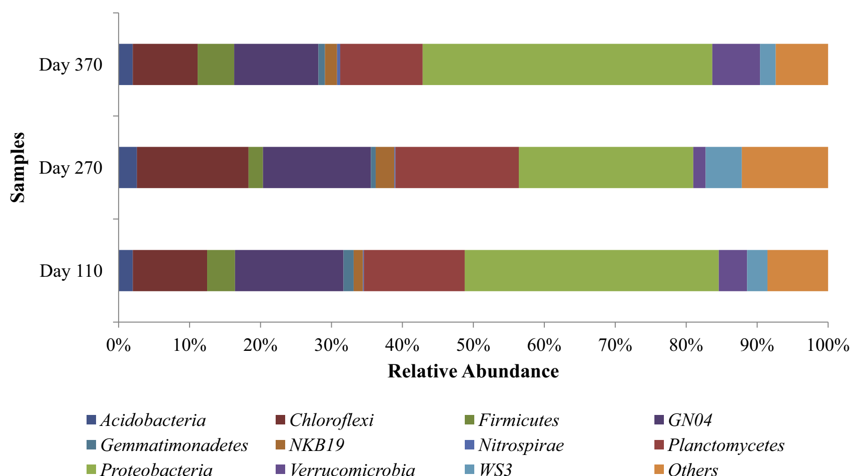

Fig. 3. Bacterial community composition at the phylum level. Biomass was sampled at day 110 (low nitrogen concentration), day 270 (high nitrogen concentration, before phenol addition), and day 370 (after phenol stress period) of operation. The abundance is presented as the percentage of the different phyla among the total reads in a sample.

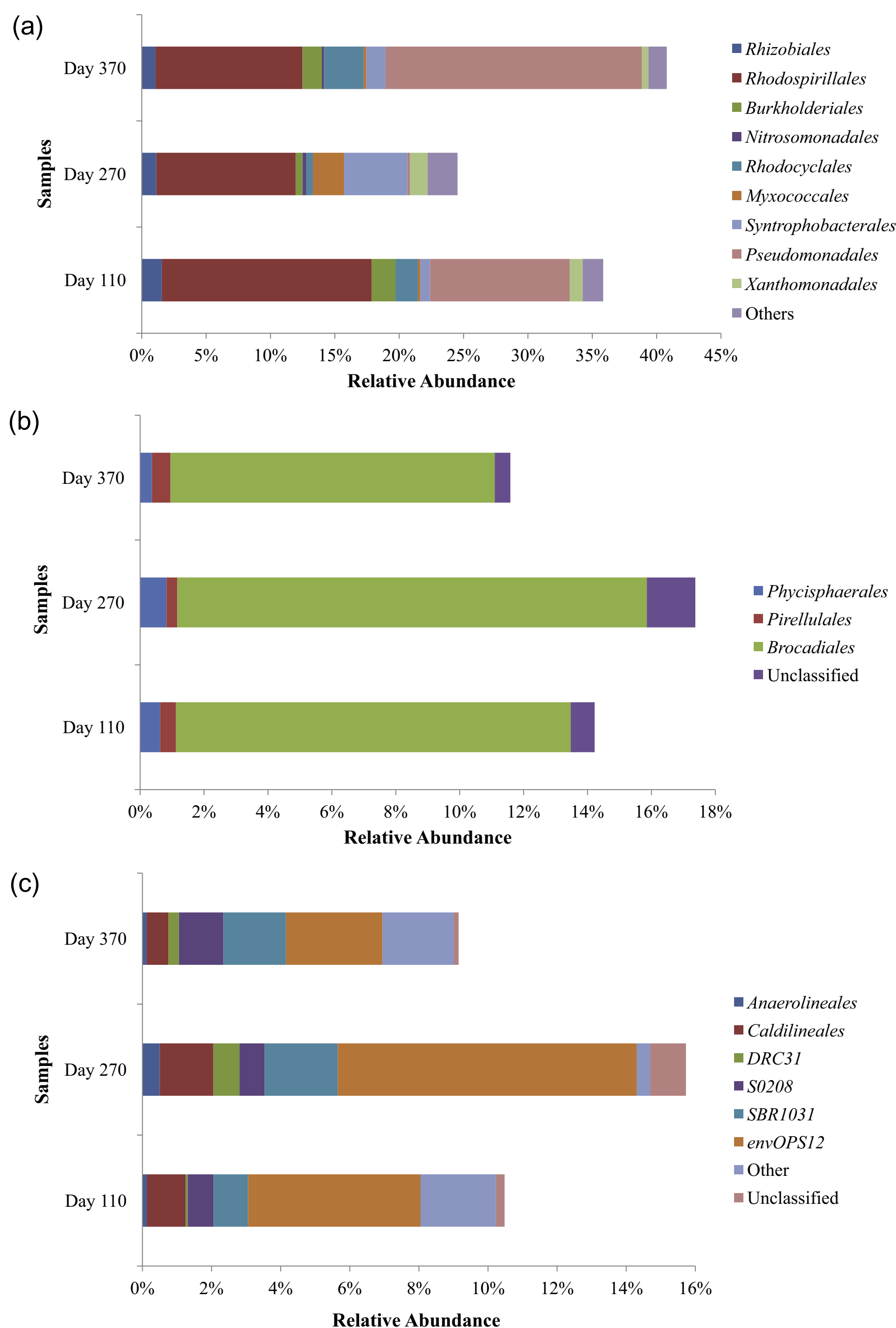

Fig. 4. Relative abundance of orders within the phyla *Proteobacteria*, *Planctomycetes*, and *Chloroflexi*. Biomass was sampled at days 110 and 270 (before phenol addition) and day 370 (period after phenol stress). The relative abundance is presented as the percentage of a given order among the reads classified as *Proteobacteria* (a), *Planctomycetes* (b), or *Chloroflexi* (c).

(Supplementary Table S3). These indices yielded low values (from 0.32 to 0.57), indicating low similarity among the microbial communities. Thus, considering that samples were taken from the same reactor, but at different operating conditions (day 110 at low nitrogen concentration, day 270 at high nitrogen concentration before phenol addition, and day 370 after phenol stress), it appears that the cultivation conditions had an important effect on the microbial community, selecting some bacterial groups over others (as shown in Fig. 3).

3.2.2.1. Proteobacteria. *Proteobacteria* was the most abundant phylum in the three samples. However, the relative abundance of this phylum decreased from 36% (in sample 110) to 25% (in sample 270) as ammonium and nitrite concentrations in the influent increased, but increased after the phenol stress period, indicating that some groups related to phenol degradation were favored in the reactor. In fact, the relative abundance of sequences affiliated with members of *Rhodospirillales*, *Burkholderiales*, *Rhodocyclales*, and *Pseudomonadales* increased after phenol stress (Fig. 4a), suggesting that heterotrophic denitrification was the favored process after phenol addition. The relative abundance of sequences affiliated with orders harboring genera with known denitrification capabilities increased (from 14.5% to 37.5%) after the phenol stress period.

Of the *Proteobacteria* detected in the samples, one of the most dominant orders was *Rhodospirillales*, which comprises acetic acid

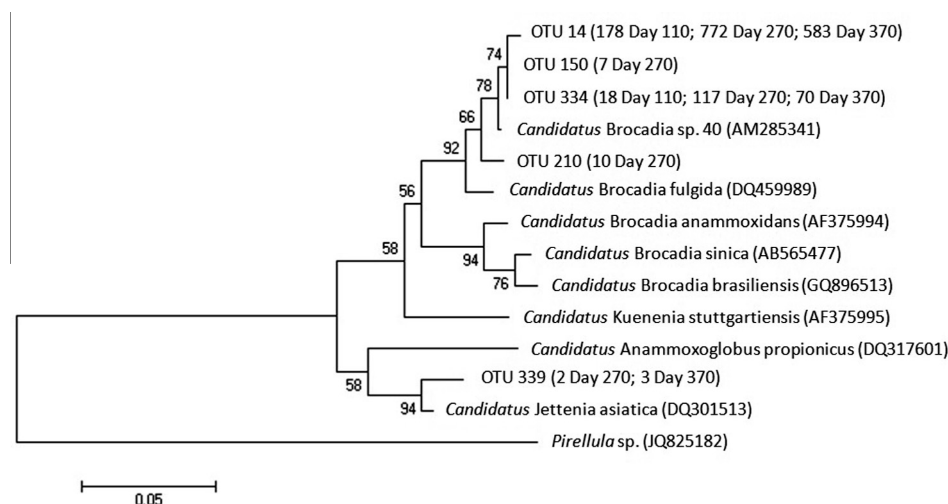

Fig. 5. Phylogenetic tree of the operational taxonomic units (OTUs) related to sequences from anammox bacteria. Biomass samples were collected from the sequencing batch reactor at days 110, 270, and 370. Sequences were obtained by pyrosequencing analysis. For each OTU, the number of reads classified as *Candidatus* Brocadia and *Candidatus* Jettenia are indicated in parentheses for each sample. The scale bar represents a 5% sequence divergence. The *Pirellula* sp. sequence served as the out-group for rooting the tree. Numbers at the nodes indicate the percentage recovery of relevant branch points in a 1000 bootstrap re-sampling analysis. Bootstrap values (>50%) are indicated at the branch points. GenBank accession numbers are shown.

bacteria and purple non-sulfur bacteria. The latter can grow anaerobically in the absence of light or in photoheterotrophic conditions, using light as an energy source and organic matter, including aromatic compounds such as phenols, as a carbon source.

Members of the orders *Burkholderiales* and *Rhodocyclales* are also denitrifying bacteria and are capable of degrading phenolic compounds. These groups were detected in oil-refinery wastewater that contained phenol, and the degradation of this compound was mainly attributed to these two groups (Silva et al., 2013). The order *Pseudomonadales* also harbors species, such as *P. putida*, that are able to degrade phenol under aerobic conditions (Ullhyan and Ghosh, 2012). Nevertheless, phenol degradation by *Pseudomonas* under denitrifying conditions (with nitrate as electron acceptor) has been described (Sueoka et al., 2009).

Unlike the groups mentioned above, the proportion of *Myxococcales*, *Syntrophobacterales*, and *Xanthomonadales* decreased after phenol stress (Fig. 4a), suggesting that phenol was toxic to these groups. The order *Nitrosomonadales* was also detected in the SBR at very low abundance, from 0.27% to 0.17% (Fig. 4a). These results indicate that AOB were present in the SBR, confirming the results obtained by the DGGE.

3.2.2.2. Planctomycetes. Anammox bacteria are distributed in the phylum *Planctomycetes*. In the three samples, *Planctomycetes* accounted for 11.6–17.4% of the total reads. The proportion of this group increased as the concentrations of ammonium and nitrite in the influent increased, but decreased after the phenol stress period (Fig. 3). The dominant order (accounting for 84% of the total *Planctomycetes* reads) was *Brocadiales* (Fig. 4b), of which *Candidatus* Brocadia dominated, confirming the results obtained by DGGE. Nevertheless, *Candidatus* Jettenia was detected at very low abundance (0.5% of the total *Brocadiales* reads). The relative abundance of *Brocadiales* members relative to the total reads was 12.4%, 14.7%, and 10.1% for sample days 110, 270, and 370, respectively (Fig. 4b). Thus, after 34 days of phenol addition to the reactor, the relative abundance decreased from 14.7% to 10.1% (i.e., by 31%), indicating that phenol negatively affected this group, which is consistent with the decrease in the nitrogen removal performance of the SBR after the addition of a high concentration of phenol (300 mg L^{-1}).

The relative abundance and composition of the phylum *Chloroflexi* were also affected by the addition of phenol to the reactor (Fig. 4c). The presence of organisms affiliated with *Chloroflexi* in anammox reactors has been demonstrated (Cho et al., 2010; Costa et al., 2014). Previous studies postulated that these microorganisms live in symbiosis with anammox bacteria (Zhang et al., 2012) by utilizing cellular compounds derived from dead biomass and metabolites from anammox bacteria (Kindaichi et al., 2012). This is in accordance with the results of the present study, because phenol reduced the abundance of anammox bacteria and affected the proportion and composition of *Chloroflexi*, with *Anaerolineales*, *Caldilineales*, and envOPS12 most affected (Fig. 4c).

Of the 392 OTUs obtained, five were affiliated with anammox bacteria: four were closely related to *Candidatus* Brocadia sp. 40 (OTUs 14, 150, 210, and 334), one was related to *Candidatus* Brocadia fulgida (OTU 210), and one was related to *Candidatus* Jettenia asiatica (OTU 339) (Fig. 5). The addition of phenol affected the diversity of the anammox community inside the reactor. During anammox enrichment (sample day 270), two anammox populations were selected for in the reactor: one dominant (*Ca.* Brocadia sp. 40) and one minor (*Ca.* Brocadia fulgida). After the phenol stress period, sequences related to *Ca.* Brocadia fulgida (OTU 210) were no longer detected (Fig 5). These results suggest that *Ca.* Brocadia sp. 40 has a higher tolerance for phenol than *Ca.* Brocadia fulgida and is preferable for use in anammox reactors that treat industrial wastewaters containing nitrogen and phenol. These results agree with previous studies that identified *Ca.* Brocadia as the most abundant anammox species in wastewater treatment plants where organic compounds, together with ammonium, nitrite, and nitrate, were present (Hu et al., 2010; Kartal et al., 2008). Moreover, a recent study described the prevalence of Brocadia in a sample of contaminated groundwater, which suggests that these bacteria have a broad metabolic and environmental niche (Moore et al., 2011).

The effects of phenol on anammox activity have been studied, but the dynamics of the microbial community were not described, and the microorganisms involved were not identified (Toh and Ashbolt, 2002; González-Blanco et al., 2012; Jin et al., 2013; Yang et al., 2013). Anammox bacteria were acclimated in phenol-containing wastewater at low nitrogen-loading rates over a long period (Toh and Ashbolt, 2002). Other authors verified that an influent phenol concentration of 50 mg L^{-1} suppressed anammox performance (Yang et al., 2013) and that this inhibition was reversible (Jin et al., 2013).

The results of previous studies and the results of this study demonstrate that phenol negatively affects the nitrogen removal performance of anammox reactors, as evidenced by the reduction in the nitrogen removal efficiency and by the change in the stoichiometric ratio of nitrite consumption to ammonium depletion. However, as demonstrated in the present study, the presence of phenol did not eliminate the anammox bacteria. Instead, the proportion of anammox bacteria decreased and the abundance of denitrifying bacteria capable of degrading aromatic compounds increased. Thus, the presence of denitrifying bacteria might reduce the inhibitory effect of phenolic compounds and gradually enhance the tolerance of anammox bacteria for these compounds.

4. Conclusions

High concentrations of phenol ($300\ mg\ L^{-1}$) decreased the ammonium removal efficiency of the anammox SBR by half. The microbial community composition changed after the phenol stress period, and organisms within the orders *Rhodospirillales*, *Burkholderiales*, *Rhodocyclales*, and *Pseudomonadales*, which harbor species capable of degrading phenol under denitrifying conditions, became dominant. The abundance and diversity of anammox bacteria were also affected by phenol. Thus, phenol adversely affected anammox metabolism and changed the bacterial community. However, anammox activity and performance recovered within 45 days after the removal of the phenol from the influent.

Acknowledgements

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil (CNPq), Fundação de Amparo a Pesquisa de Minas Gerais (FAPEMIG), and Coordenação de Aperfeiçoamento de Pessoal de nível Superior (CAPES). We wish to thank the team of the Laboratory of Genetics of Microorganisms (at the UFMG) for assistance with the analysis using the BioNumerics software 2.5.

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.biortech.2014.05.043.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 2015, 403–410.

American Public Health Association (APHA), American Water Works Association (AWWA), Water Environment Federation (AEF), 2005. Standard Methods for the Examination of Water and Wastewater, 21st ed. Washington, DC, USA.

Amor, L., Eiroa, M., Kennes, C., Veiga, M.C., 2005. Phenol biodegradation and its effect on the nitrification process. Water Res. 39, 2915–2920.

Araújo, J.C., Campos, A.P., Correa, M.M., Silva, E.C., Matté, M.H., Matté, G.R., Von Sperling, M., Chernicharo, C.A.L., 2011. Anammox bacteria enrichment and characterization from municipal activated sludge. Water Sci. Technol. 64, 1428–1434.

Berinstain-Cardoso, R., Texier, A., Alúche-Sólis, A., Gómez, J., Razo-Flores, E., 2009. Phenol and sulphide oxidation in a denitrifying reactor and its microbial community analysis. Process Biochem. 44, 23–28.

Caporaso, G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F., Costello, E., Fierer, N., Pena, A., Goodrich, J., Gordon, J., Huttley, Y.G., Kelley, S., Knights, D., Koenig, J., Ley, R., Lozupone, C., Mc Donald, D., Muegge, B., Pirrung, M., Reeder, J., Sevinsky, J., Turnbaugh, P., Walters, W., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336.

Cho, S., Takahashi, Y., Fujii, N., Yamada, Y., Satoh, H., Okabe, S., 2010. Nitrogen removal performance and microbial community analysis of an anaerobic up-flow granular bed anammox reactor. Chemosphere 78, 1129–1135.

Costa, M.C., Carvalho, L., Leal, C.D., Dias, M.F., Martins, K.L., Garcia, G.B., Mancuelo, I.D., Hipólito, T., Mac Conell, E.A., Okada, D., Etchebehere, C., Chernicharo, C.A., Araujo, 2014. Impact of inocula and operating conditions on the microbial community structure of two anammox reactors. Environ. Technol. 35, 1–12.

Dapena-Mora, A., Fernandéz, I., Campos, J.L., Mosquera-Corral, A., Méndez, R., Jetten, M.S.M., 2007. Evaluation of activity and inhibition effects on anammox process by batch tests based on the nitrogen gas production. Enzyme Microb. Technol. 40, 859–865.

Desloover, J., De Clippeleir, H., Boeckx, P., DuLaing, G., Colsen, J., Verstraete, W., Vlaeminck, S.E., 2011. Floc-based sequential partial nitritation and anammox at full scale with contrasting N_2O emissions. Water Res. 45, 2811–2821.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460–2461.

Egli, K., Fangers, U., Alvarez, P.J.J., Siegrist, H., Van der Meer, J.R., Zehnder, A.J.B., 2001. Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate. Archiv. Microbiol. 175, 198–207.

Egli, K., Bosshard, F., Werlen, C., Lais, P., Siegrist, H., Zehnder, A.J.B., Van Der Meer, J.R., 2003. Microbial composition and structure of a rotating biological contactor biofilm treating ammonium-rich wastewater without organic carbon. Microb. Ecol. 45, 419–432.

Ferris, M.J., Muyzer, G., Ward, D.M., 1996. Denaturing gradient gel electrophoresis profiles of 16 rRNA-defined populations inhabiting a hot spring microbial mat community. Appl. Environ. Microbiol. 62, 340–346.

Gao, D., Tao, Y., 2011. Versatility and application of anaerobic ammonium-oxidizing bacteria. Appl. Microbiol. Biotechnol. 91, 887–894.

González-Blanco, G., Beristain-Cardoso, R., Cuervo-López, F., Cervantes, F.J., Gómez, J., 2012. Simultaneous oxidation of ammonium and *p*-cresol linked to nitrite reduction by denitrifying sludge. Bioresour. Technol. 103, 48–55.

Heylen, K., Vanparys, B., Wittebolle, L., Verstraete, W., Boon, N., De Vos, P., 2006. Cultivation of denitrifying bacteria: optimization of isolation conditions and diversity study. Appl. Environ. Microbiol. 72, 2637–2643.

Hu, B.L., Zheng, P., Tang, C.J., Chen, J.W., van der Biezen, E., Zhang, L., Ni, B.J., Jetten, M.S.M., Yan, J., Hu, H.Q., Kartal, B., 2010. Identification and quantification of anammox bacteria in eight nitrogen removal reactors. Water Res. 44, 5014–5020.

Jin, R., Yang, G., Yu, J., Zheng, P., 2012. The inhibition of anammox process: a review. Chem. Eng. J. 197, 67–79.

Jin, R., Zhang, Q., Yang, G., Xing, B., Ji, Y., Chen, H., 2013. Evaluating the recovery performance of the anammox process following inhibition by phenol and sulphide. Bioresour. Technol. 142, 162–170.

Kartal, B., Van Niftrik, L., Rattray, J., Van de Vossenberg, J.L.C.M., Schmid, M., Damsté, J.S., Jetten, M.S.M., Strous, M., 2008. *Candidatus* 'Brocadia fulgida': an autofluorescent anaerobic ammonium oxidizing bacterium. FEMS Microbiol. Ecol. 63, 46–55.

Kindaichi, T., Yuri, S., Ozaki, N., Ohashi, A., 2012. Ecophysiological role and function of uncultured *Chloroflexi* in an anammox reactor. Water Sci. Technol. 66, 2556–2561.

Moore, T.A., Xing, Y., Lazenby, B., Lynch, M.D.J., Schiff, S., Robertson, W.D., Timlin, R., Lanza, S., Ryan, M.C., Aravena, R., Fortin, D., Clark, I.D., Neufeld, J.D., 2011. Prevalence of anaerobic ammonium-oxidizing bacteria in contaminated groundwater. Environ. Sci. Technol. 45, 7217–7225.

Silva, C.C., Hayden, H., Sawbridge, T., Mele, P., De Paula, S.O., Silva, L.C.F., Vidigal, P.M.P., Vicentini, R., Sousa, M.P., Torres, A.P.R., Santiago, V.M.J., Oliveira, V.M., 2013. Identification of genes and pathways related to phenol degradation in metagenomic libraries from petroleum refinery wastewater. PLoS One. 8, 1–11.

Strous, M., Heijnen, J.J., Kuenen, J.G., Jetten, M.S.M., 1998. The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. Appl. Microbiol. Biotechnol. 50, 589–596.

Sueoka, K., Satoh, H., Onuki, M., Mino, T., 2009. Microorganisms involved in anaerobic phenol degradation in the treatment of synthetic coke-oven wastewater detected by RNA stable-isotope probe. FEMS Microbiol. Lett. 291, 169–174.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA 4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.

Toh, S.K., Ashbolt, N.J., 2002. Adaptation of anaerobic ammonium-oxidising consortium to synthetic coke-ovens wastewater. Appl. Microbiol. Biotechnol. 59, 344–352.

Ullhyan, A., Ghosh, U.K., 2012. Biodegradation of phenol with immobilized *Pseudomonas putida* activated carbon packed bio-filter power. Afr. J. Biotechnol. 11, 15160–15167.

Van De Graaf, A.A., Bruijn, P., Robertson, L.A., Jetten, M.S.M., Kuenen, J.G., 1996. Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor. Microbiology 142, 2187–2196.

Xiao, Y., Zeng, G.M., Yang, Z.H., Liu, Y.S., Ma, Y.H., Yang, L., Wang, R.J., Xu, Z.Y., 2009. Coexistence of nitrifiers, denitrifiers and anammox bacteria in a sequencing batch biofilm reactor as revealed by PCR-DGGE. J. Appl. Microbiol. 106, 496–505.

Yang, G., Jin, R., 2012. The joint inhibitory effects of phenol, copper (II), oxytetracycline (OTC) and sulphide on anammox activity. Bioresour. Technol. 126, 187–192.

Yang, G., Guo, X., Chen, S., Liu, J., Jin, R., 2013. The evolution of anammox performance and granular sludge characteristics under the stress of phenol. Bioresour. Technol. 137, 332–339.

Zhang, J., Zhang, Y., Li, Y., Zhang, L., Qiao, S., Yang, F., Quan, X., 2012. Enhancement of nitrogen removal in a novel anammox reactor packed with Fe electrode. Bioresour. Technol. 114, 102–108.