Microbial community shifts on an anammox reactor after a temperature shock using 454-pyrosequencing analysis

Eduardo Isanta[a], Tercia Bezerra[a], Isaac Fernández[a], María Eugenia Suárez-Ojeda[a], Julio Pérez[a,b], Julián Carrera[a]*

[a]GENOCOV Research Group, Department of Chemical Engineering, Escola d'Enginyeria, Universitat Autònoma de Barcelona, Spain

[b]Present Address: Department of Biotechnology, Delft University of Technology, The Netherlands

*Corresponding author. Phone: +34935812141; Fax: +34935812013

e-mail: julian.carrera@uab.cat

Abstract

To explore the changes in the microbial community structure during the recovery process of an anammox reactor after a temperature shock, the 454-pyrosequencing technique was used. The temperatures shock reduced the nitrogen removal rate up to 92% compared to that just before the temperature shock, and it took 70 days to recover a similar nitrogen removal rate to that before the temperature shock (ca. 0.30 g N L^{-1} d^{-1}). Pyrosequencing results indicated that microbial diversity in the reactor decreased as the reactor progressively recovered from the temperature shock. Anammox bacteria were accounted as 6%, 35% and 46% of total sequence reads in samples taken 13, 45 and 166 days after the temperature shock. These results were in agreement with N-removal performance results and anammox activity measured in the reactor during the recovery process. An anammox specific primer was used to precisely determine the anammox species in the biomass samples.

Keywords

454-pyrosequencing; *Brocadia anammoxidans*; *Candidatus Kuenenia*; Microbial diversity

1. Introduction

The anaerobic ammonium oxidation (anammox) is widely considered as the most economical and sustainable process for nitrogen removal from wastewater, due to its high nitrogen removal potential at reduced operating costs (Kartal et al., 2010; Lackner et al., 2014). Compared to conventional nitrification-denitrification process, the anammox process does not require organic matter for nitrogen removal, and oxygen requirements are reduced since only ca. half ammonium needs to be oxidized into nitrite in a previous partial nitritation step (Lackner et al., 2014).

The main disadvantage of the anammox process is, however, the very slow growth rate of the microbes responsible for this process, i.e. the "anammox bacteria". Due to this slow growth rate, anammox bacteria doubling times around 10-14 days have been typically reported at their optimum growth temperature, i.e. 35-40 ºC (Dosta et al., 2008; Strous et al., 1999). Anammox growth rate is reduced ca. 30-40% every 5 ºC of temperature decrease (Dosta et al., 2008; Strous et al., 1999). Perhaps for this reason, early anammox process implementations were generally at temperatures close to the optimum (Abma et al., 2010; van der Star et al., 2007), in order to minimize the slow growing characteristics of anammox bacteria. For some applications, anammox reactors are even heated to enhance the anammox growth, such as in some lab-scale rectors (Hu et al., 2013) or industrial reactors (Wett et al., 2013).

Although in natural environments, anammox growth has been reported to occur at temperatures as low as −2.5°C in sea ice (Rysgaard and Glud, 2004) and as high as 70°C in hot springs and hydrothermal vent areas (Byrne et al., 2009), in wastewater treatment applications anammox growth is limited to a maximum temperature of 43ºC (Strous et al., 1999). In fact, Toh et al. (2002) could not cultivate thermophilic anammox biomass at 55ºC. Dosta et al. (2008) observed that the exposure of anammox

biomass at a temperature of 45 ºC during a few hours resulted in a complete loss of activity and evidences of cell lysis. Similarly, Liu et al., (2015) found that an accidental overheating at 48 ºC for 1 h caused anammox bacteria damage and death in an irreversible process. After that short temperature shock, they needed two weeks to recover a measurable anammox activity. Accordingly, temperature in anammox reactors should be carefully controlled below ca. 40ºC, especially in those reactors using heaters for temperature control (as abovementioned), or in reactors used for the treatment of industrial hot effluents (Lopez-Vazquez et al., 2014), to avoid the risk of overpassing the maximum permissible temperature of anammox bacteria which would lead to an anammox reactor failure. However, to the best of the authors' knowledge, there is lack of information in the literature about the impact of a long term temperature shock over the performance of an anammox reactor.

Changes in environmental conditions, such as a temperature shock, have a direct impact in microbial communities. The study of these microbial communities and their changes provides valuable information to better understand the nutrient removal processes occurring in wastewater treatment ecosystems. Different molecular biology techniques, such as fluorescence in-situ hybridization (FISH) (Lotti et al., 2014), denaturing gradient gel electrophoresis (DGGE) (Park et al., 2010) or clone library (Hu et al., 2010) have been widely used for this purpose. The second generation of 454-pyrosequencing has been recently developed. This technique offers a high-throughput, fast and economical sequencing platform, with exceptional accuracy (Droege and Hill, 2008). With 454-pyrosequencing, thousands of operational taxonomic units (OTUs) can be identified in a reasonable period of time. Therefore, this technique can provide wider and more complete information about microbial community structures than previous conventional molecular biology techniques. For these reasons, 454-pyrosequencing has

gained interest in the study of complex microbial communities such as those of wastewater treatment environments (Hu et al., 2012; Xie et al., 2013). Despite the potential of 454-pyrosequencing, the use of this novel technique with anammox reactors is still scarce (Costa et al., 2014; Pereira et al., 2014).

Hence, the performance of an anammox reactor before and after the occurrence of a high and long term temperature shock (46 ºC for 8 days) is presented. The research was focused on exploring the microbial community structure changes during the N-removal recovery process of the anammox reactor. With that purpose, 454-pyrosequencing technique was used.

2. Materials and methods

2.1 Experimental set-up description

The anammox process was carried out in a sequencing batch reactor (SBR) with a working volume of 10 L, a diameter of 20 cm and a height of 61 cm. The SBR was operated in cycles of 6 h, divided in four phases: mixed filling (300 min), mixing (30 min), settling (20 min) and effluent withdrawal (10 min). The volumetric exchange ratio was of 25%. Biomass was mixed using a mechanical stirrer operated at rotating speed of 72 rpm. Nitrogen gas was flushed into the headspace at an average flow rate of 300 mL min^{-1}. The pH was not controlled but ranged between 7.5 – 8.5 during one cycle. The temperature before and after the temperature shock was maintained constant at 35 ºC. The reactor was fed with a mineral medium containing (mg L^{-1}): $KHCO_3$ (100); H_2PO_4 (50); $CaCl_2 \cdot 2H_2O$ (100); $MgSO_4 \cdot 7H_2O$ (200); $FeSO_4$ (6.3); EDTA (6.3) and 1.25 mL L^{-1} of a trace elements solution (van de Graaf et al., 1996). The required amounts of nitrite

and ammonium in the form of $NaNO_2$ and $(NH_4)_2SO_4$ were added to this mineral medium (as specified in the Results and Discussion section, Figure 1).

The SBR was operated for more than 700 days previously to the temperature shock with nitrogen loading rate (NLR) ranging from 0.20 – 0.55 g N L^{-1} d^{-1}. The biomass had an average Specific Anammox Activity (SAA) of 0.40±0.05 g N g^{-1} VSS d^{-1}.

The temperatures shock lasted 8 days (from days -8 to 0 in Figure 1) and consisted in a step change of the temperature in the anammox SBR from 35 to 46ºC.

2.2 Analytical methods

Ammonium was measured by means of an ammonium analyzer (AMTAX, Hach Lange, Germany). Nitrate and nitrite concentrations were analyzed with ionic chromatography using an ICS-2000 Integrated Reagent-Free IC system (DIONEX Corporation, USA) which performs ion analyses using suppressed conductivity detection. Mixed liquor total suspended solids (TSS), mixed liquor volatile suspended solids (VSS) were measured according to Standard Methods (APHA, 2008).

SAA was determined in batch assays, measuring the overpressure produced by di-nitrogen gas production of anammox bacteria (adapted from Dapena-Mora et al. 2007). Nitrifying activity was determined in an aerobic batch assay, measuring the ammonium, nitrite and nitrate concentration over time. Dissolved Oxygen (DO) concentration, pH and temperature were maintained in this assay at 0.5-1.0 mg O_2 L^{-1}, 7.5 ± 0.1 and 34 ± 1 ºC, respectively.

2.3 Granular sludge morphology

The Anammox granules for scanning electron microscope (SEM) observation were washed three times with water to remove impurities. For fixation, cells were immersed

in 2.5% (vol/vol) glutaraldehyde (pH 7.4) for 2 h. The samples were then processed according to conventional electron microscopy methods as described in Julián et al., (2010). Anammox granules were observed with an S-570 scanning electron microscope (Hitachi Ltd., Japan) at an accelerating voltage of 30 kV.

2.4 Microbial diversity analysis

2.4.1 DNA Isolation

DNA was extracted from samples using the MoBio PowerBiofilm™ DNA Isolation Kit (MoBio Laboratories, USA), following the manufacturer protocol, with the exception of solution BF3, that was added to 200 mL instead of the 100 mL recommended by the manufacturer. The quality and quantity of extracted DNA were measured by using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). All DNA samples were adjusted to 25 ng mL^{-1} for tagged pyrosequencing.

2.4.2 Bacteria and anammox specific pyrosequencing for 16S rRNA

Purified community DNA samples were sent to the Research and Testing Laboratory (Lubbock, TX, USA) for Bacterial Tag-Encoded FLX-Titanium Amplicon Pyrosequencing (bTEFAP) and data processing. Samples were amplified for pyrosequencing using a forward and reverse fusion primer. The forward primer was constructed with (5'-3') the Roche A linker (CCATCTCATCCCTGCGTGTCTCCGACTCAG), an 8bp barcode and the appropriated forward primer selected for bacteria diversity assay (530F: GTGCCAGCMGCNGCGG) or for anammox specific diversity assay (Amx368F: TTCGCAATGCCCGAAAGG; (Cornish Shartau et al., 2010). The reverse fusion

primer was constructed with (5'-3') a biotin molecule, the Roche B linker (CCTATCCCCTGTGTGCCTTGGCAGTCTCAG), and the proper reverse primer for bacteria diversity assay (1100R: GGGTTNCGNTCGTTR) or for anammox specific diversity assay (Amx820R: AAAACCCCTCTACTTAGTGCCC; Cornish Shartau et al. (2010)). Amplifications were performed in 25 µl reactions with Qiagen HotStar Taq master mix (Qiagen Inc, Valencia, California), 1µl of each 5µM primer, and 1µl of template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosytems, Carlsbad, California) under the following thermal profile: 95ºC for 5 min, then 35 cycles of 94ºC for 30 sec, 54ºC for 40 sec, 72ºC for 1 min, followed by one cycle of 72ºC for 10 min and 4ºC hold.

Amplification products were visualized with eGels (Life Technologies, Grand Island, New York). Products were then pooled equimolar and each pool was cleaned with Diffinity RapidTip (Diffinity Genomics, West Henrietta, New York), and size selected using Agencourt AMPure XP (BeckmanCoulter, Indianapolis, Indiana) following Roche 454 protocols (454 Life Sciences, Branford, Connecticut). Size selected pools were then quantified and diluted for use in emPCR reactions, which were performed and subsequently enriched. Sequencing followed established manufacture protocols (454 Life Sciences, Branford, Connecticut).

2.4.3 Biodiversity analysis and phylogenetic classification

Quality of data obtained from sequencing was evaluated and denoised and chimeras were removed from the data set at Research and Testing Laboratory. USEARCH was used for dereplication, clustering (4% divergence) and consensus sequence generation and poor reads were then eliminated (Edgar, 2010). For general bacteria diversity analysis, reads shorter than 250 bps and larger than 673 bps were trimmed. The same

was done for Anammox specific diversity analysis but for reads shorter than 274 bps and larger than 675 bps. Chimera detection and removal was performed by executing UCHIME (Edgar et al., 2011) in *de novo* mode on the clustered data. In order to determine the identity of each remaining sequence, the sequences must first be quality checked and then clustered into Operational Taxonomic Units (OTU) using the UPARSE algorithm (Edgar, 2013). Each OTU is then identified using the USEARCH global alignment algorithm (Edgar, 2010) and a database of high quality sequences derived from NCBI maintained by Research and Testing Laboratory.

For each OTU, the top six matches from the high quality database were kept and confidence values were assigned to each taxonomic level by taking the number of taxonomic matches that agree with the best match at that level and dividing that by the number of high quality sequence matches that were found. Each OTU was then assigned taxonomic information using the lowest common taxonomic level whose confidence value was above 51%. OTUs that received no matches against the high quality sequences are identified as “no hit”. After resolving the number of sequences per OTU, the percentage of each organism was individually calculated for each sample. Data obtained provided relative abundance information within and among individual samples. Relative abundances of reads were calculated by taxonomic level for each library. Values represent the percentage of reads of sequences obtained at each taxonomic identity (according to the degree that of similarity described above) within the total set of readings from the library.

Indices of biological diversity were calculated for all libraries (Table 1 and Figures S1-S3 in Supplementary Materials) indicating all libraries were comparable in terms of abundance percentages and that good coverage of diversity was reached.

3. Results and discussion

3.1 Nitrogen removal recovery after the temperature shock

During the temperature shock (days -8 to 0 in Figure 1), a progressive accumulation of ammonium and nitrite was observed in the reactor. Simultaneously, the Nitrogen Removal Rate (NRR) decreased from 0.250 to 0.023 g N L^{-1} d^{-1}. The colour of the supernatant at the end of the temperature shock (day 0) presented a slight orange colour (data not shown), suggesting the lysis of anammox cells. On day 7 after the temperature shock, a SAA test was performed, but not measurable activity was obtained. Similar results were found by Dosta et al. (2008) when, after performing two consecutive SAA tests at 45 ºC to an anammox culture, almost no activity was measured and the supernatant presented an slight orange color. These authors suggested that this orange color could be due to the release of cytochrome c after the lysis of anammox cells due to the temperature shock.

To enhance the anammox activity recovery, the ammonium and nitrite influent concentrations were decreased to minimize any substrate inhibition of the anammox bacteria (Jin et al., 2012). From day 7 to day 105, a progressive increase of the NRR was observed in the reactor (Figure 1). As depicted in Figure 2, from days 0 to 22 the ratio of nitrate production to ammonium consumption showed higher values than the 0.26 g $N\text{-}NO_3^-$ g^{-1} $N\text{-}NH_4^+$ expected according to anammox stoichiometry (van de Graaf et al., 1996). However, from day 22 onwards the nitrate to ammonium stoichiometric ratio stabilized to 0.25±0.05 g $N\text{-}NO_3^-$ g^{-1} $N\text{-}NH_4^+$. The average nitrite to ammonium ratio was stable at 1.3±0.1 g $N\text{-}NO_2^-$ g^{-1} $N\text{-}NH_4^+$ during all the experimental period (Figure 2).

On day 40, nitrite effluent concentration was lower than 3 mg $N\text{-}NO_2^-$ L^{-1}, and NRR did not vary from days 32 to 40 (Figure 1), which suggested that anammox growth rate was limited by low substrate concentration. Accordingly, the ammonium and nitrite influent concentrations were progressively increased, which allowed the NRR increase to 0.25 g N L^{-1} d^{-1} on day 70. This NRR was similar to that obtained just before the temperature shock. Also on day 70, the SAA of the anammox granules was of 0.150±0.005 g N g^{-1} VSS d^{-1}. This SAA was lower than the average 0.40±0.05 g N g^{-1} VSS d^{-1} measured before the temperature shock. Similar SAA values to that obtained previous to the temperature shock (i.e. 0.381±0.040 g N g^{-1} VSS d^{-1}) could not be measured until day 166.

3.2 SEM images

The morphological differences between the anammox granules on days 13 and 42 after the temperature shock were inspected with SEM. Granules on day 13 presented a moderately rough surface (See Figure S4-A in Supplementary Materials). When granules were inspected at a higher magnification, a consortium of rod, coccus and filamentous bacteria seemed to be growing over an apparently inert substratum (See Figure S4-C in Supplementary Materials) without any colony structure.

For granules taken on day 42, a much rougher surface could be observed compared with granules from day 13 (See Figure S4-B in Supplementary Materials). At a higher magnification, the bacteria in the image seemed to grow in colonies and the inert substratum was not observed (See Figure S4-D in Supplementary Materials). It seems like the temperature shock killed most cells from the granule and then, new cells were growing in colonies over the structure of the old granule.

The small round cells could presumably be anammox cells, since they are typically visualized under the microscope as small coccoid cells with a diameter of about 0.8 µm (Kartal et al., 2013). If this is the case, the number of anammox cells in the picture taken on day 42 (Figure S4-D) seemed to be greater than that in picture from day 13 (Figure S4-C).

3.3 Effect of temperature shock over microbial community diversity

3.3.1 Microbial diversity in anammox granules using 530F-1100R primers

The microbial community structure in the anammox reactor after the temperature shock was analyzed through the bTEFAP approach using the primers 530F-1100R. Three amplicon libraries, namely AMX_13, AMX_45 and AMX_166, were constructed with biomass samples obtained 13, 45 and 166 days after the temperature shock, respectively. A total of 17770 (AMX_13), 12568 (AMX_ 45) and 20940 (AMX_166) sequence reads were obtained. After quality analysis and removal of low quality sequences, 43964 sequences were annotated, corresponding to 14725 high-quality V4-V6 tags of the 16S rRNA-gene in library AMX_13, to 11200 in library AMX_45 and to 18039 in AMX_166, with average lengths of 419, 411 and 504 bp per sequence, respectively. Later, unique OTUs were clustered in taxa and finally, 66 (AMX_13), 51 (AMX_45) and 24 (AMX_166) genera were estimated. Species similarities were found for a total of 1793 (AMX_13), 1297 (AMX_45) and 548 (AMX_166) clusters.

Chao1, Shannon and E indices estimated at 97% of similarity for the three biomass samples (Table 1) showed a progressive decrease of microbial diversity as the reactor progressively recovered the anammox activity after the temperatures shock, since the values of these indices in AMX_13 were higher than that of AMX_45, and much higher

than that in AMX_166. The increase of diversity of bacteria just after the temperature shock was reasonable, since many opportunistic bacteria could grow on lysis products from died cells during the temperature shock.

In AMX_13 library, corresponding to the reactor just after the temperature shock, β-Proteobacteria was the most abundant group at class level with 14% of total sequences while Planctomycetia was only 7%, indicating low anammox abundance (Figure 3). Other classes, such as Actinobacteria (7%), δ-Proteobacteria (6%) or Clostridia (4%), presented significant abundances confirming a high microbial diversity after the temperature shock. It is widely known that Planctomycetia class contains all known anammox bacteria, while β-proteobacteria class contains several groups of nitrifying, denitrifying and other N-cycle related microorganisms, among many others.

At genus level, any predominant group was observed, and a very diverse number of genera presented relative abundances between 1-7% (Figure 4). Many of these genera, (i.e. *Pelobacter, Zooglea, Eubacterium, Chloroflexus* or *Clostridium*, among others), contained bacteria capable of performing anaerobic metabolism, which could presumably degrade organic products from cell lysis that took place during the temperature shock. Anammox bacteria genera *Brocadia* and *Candidatus Kuenenia* only had a 3% of abundance each (Figure 4). Two strictly aerobic genera, *Nitrospira* and *Derxia* had significant abundances, 4% and 7% respectively (Figure 4) in spite of the anoxic conditions of the reactor.

In AMX_45, a similar community structure than that in AMX_13 was observed at both class and genus levels, with two main differences (Figures 3 and 4). On one hand, the Planctomycetacia class increased its abundance from 7% on AMX_13 to 35% in AMX_45 (Figure 3). In fact, *Brocadia* genus represented 33% overall reads, indicating a clear anammox bacteria recovery from days 13 to 45 (Figure 4). On the other hand, at

both class and genus level, most of all the other groups presented lower abundances in AMX_45 than those observed in AMX_13 (Figures 3 and 4). The only non-anammox genus whose abundance increased from AMX_13 to AMX_45 was *Anaeromyxobacter* (2% overall reads in AMX_45), a group of anaerobic bacteria (Figure 4).

In AMX_166 library, corresponding to the fully recovered reactor, Planctomycetia was clearly the most abundant class, accounting for 46% of total reads, followed by β-proteobacteria with 9% of total reads (Figure 3). The clear the predominance of Planctomycetia class in AMX_166 suggested that the reactor was enriched in anammox bacteria (Figure 3).

As shown in Figure 4, results at genus level confirmed the enrichment of the reactor in anammox biomass, since 99% of reads within Planctomycetia class corresponded to *Brocadia*, while the other 1% corresponded to *Candidatus Kuenenia*, both, two known anammox genera. Accordingly, 46% of total reads were identified as anammox bacteria. Apart from *Brocadia*, only genera *Nitrosospira* (4%) and *Derxia* (3%) and *Petrimonas* (2%), presented abundance above 1% of total sequences (Figure 4). *Nitrosospira* genus are ammonium-oxidising bacteria (AOB), while *Derxia* genus contains N-fixing microorganisms. Both, *Nitrosospira* and *Derxia* are strictly aerobic bacteria. *Petrimonas* genus are anaerobic fermentative bacteria.

Total AOB in AMX_166 accounted 6% of total sequences (as the sum of *Nitrosospira*, *Nitrosomonas* and *Nitrococcus* genera). However, the ammonium to nitrite stoichiometric ratios measured in the reactor (Figure 2) were quite similar to those typically reported in the literatures for anammox reactors (van der Star et al., 2007), suggesting that AOB in the anammox granules were not very active. The AOB activity of the granular biomass from the anammox reactor was checked with a batch assay performed on day 160. During first 24h, a nitrite formation rate of 0.9 ± 0.4 mg N L^{-1} d^{-1}

was measured while nitrate formation was not detected. The batch assay was left 24 additional hours and the AOB activity increased in the range of one order of magnitude, to 10 ± 2 mg N L^{-1} d^{-1} while nitrite-oxidizing bacteria (NOB) activity remained undetectable. These results confirmed, on one hand, that AOB were present in the reactor with a very low activity whereas NOB were not present. But on the other hand, the AOB activity test suggested that AOB could be rapidly reactivated if enough oxygen is supplied. The DO sources that allowed the growth of aerobic bacteria in the anammox reactor were the DO in the feeding (ca. 3-4 mg O_2 L^{-1}) and air entering the reactor during discharge phases. Probably, the N_2 flushed into the anammox reactor (ca. 0.3 L min^{-1}) was not enough to totally displace the oxygen entering the head-space volume of the reactor (10 L), preventing to maintain fully anaerobic conditions. In fact, an average and relatively constant DO concentration of 0.4 mg O_2 L^{-1} was measured during two different SBR cycles on days 150 and 160 using a DO probe. Despite oxygen presence, AOB activity did not probably go further in the reactor, unlike in the batch assay, due to high external boundary layer resistance caused by the low stirring speed (72 rpm) in the anammox reactor. The presence of AOB in an anoxic anammox reactor has been reported previously (Kindaichi et al., 2007). These authors suggested that AOB could remain due to the ability of some AOB of reducing nitrite to N_2O or NO gases under oxygen-limiting conditions.

3.3.2 Anammox diversity in biomass samples using Amx368F-Amx820R

An anammox-specific primer (Amx368F-Amx820R) was used to more precisely determine the anammox community in the biomass samples. Relative abundances at species level are shown in Figure 5. Sequence reads from the three biomass samples could be classified into 5 groups: *Brocadia anammoxidans*, *Brocadia fulgida*, *Brocadia*

sp., *Candidatus Kuenenia sp.* and "No Hit". As shown in Figure 5, *Brocadia anammoxidans* was clearly identified as the most abundant anammox species in the fully recovered reactor sample with 95% of total reads, as well as in AMX_45 library, with 93% of total reads (Figure 5, AMX_45). In contrast, in AMX_13 library, just after the temperature shock, *Candidatus Kuenania sp*. was the most abundant specie (61%), while *Brocadia anammoxidans* accounted for 32% of total sequences (Figure 5, AMX_13). Other 6% of sequences were classified as *Brocadia fulgida* in AMX_13. The niche differentiation between *Brocadia* and *Candidatus Kuenenia* genera is still not clear. According to several studies, the genus *Brocadia* would presumably be an r-strategist (i.e., better growth rate but lower substrate affinity) (Oshiki et al., 2011; Puyol et al., 2013), while the genus *Candidatus Kuenenia* could be a K-strategist (i.e., lower growth rate but better substrate affinity) (van der Star et al., 2008). In this study, ammonium was clearly in excess during all the reactor operation (Figure 1). Nitrite was also not completely consumed during all the reactor operation (around 4.0±1.9 mg N-NO_2 L^{-1} from days 40 to 140, and higher than 4.0 mg N-NO_2 L^{-1} from day 140 to 166 (Figure 1). This continuous excess of substrate would favour the proliferation of a r-strategist population, i.e., a *Brocadia*-like anammox genus, as experimentally obtained. Also, the slight excess of nitrite at the end of the cycle could be an indication that the anammox population had a high nitrite half-saturation coefficient, which would be in accordance with the hypothesis that *Brocadia*-like populations are r-strategist. For example, Puyol et al., (2013) reported a nitrite half-saturation coefficient of 5 mg N-NO_2 L^{-1} for a *Brocadia spp*.-dominated anammox culture.

4. Conclusions

- The microbial community structure changed during the N-removal recovery process, from a very diverse microbial community just after the temperature shock, to an anammox enriched community in the fully recovered reactor.
- Anammox abundance results were in agreement with the N-removal performance results and SAA measured in the reactor during the recovery process.
- Anammox population shifted from *Candidatus Kuenenia sp.* just after the temperature shock to *Brocadia anammoxidans*. This population shift was in agreement with the hypothesis of different kinetic strategies (i.e. either r or K-strategist) of *Brocadia* and *Candidatus Kuenenia* genera, as shown in the literature.

5. Acknowledgements

Authors want to thanks to Dr. Eric Rees and Dr. John Delton Hanson (Research and Testing Laboratory) for their invaluable help. T. Bezerra wants to thanks her funding to the programs of the Conselho Nacional de Desenvolvimento Científico e Tecnológico and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CNPq/CAPES) from the Brazil government.

6. References

1. Abma, W.R., Driessen, W., Haarhuis, R., van Loosdrecht, M.C.M., 2010. Upgrading of sewage treatment plant by sustainable and cost-effective separate treatment of industrial wastewater. Water Sci. Technol. 61, 1715–1722.
2. APHA, 2008. Standard methods for the examination of water and wastewater. American Water Association (Ed.), Washington, DC.

3. Byrne, N., Strous, M., Crépeau, V., Kartal, B., Birrien, J.-L., Schmid, M., Lesongeur, F., Schouten, S., Jaeschke, A., Jetten, M., Prieur, D., Godfroy, A., 2009. Presence and activity of anaerobic ammonium-oxidizing bacteria at deep-sea hydrothermal vents. ISME J. 3, 117–123.
4. Cornish Shartau, S.L., Yurkiw, M., Lin, S., Grigoryan, A. a, Lambo, A., Park, H.-S., Lomans, B.P., van der Biezen, E., Jetten, M.S.M., Voordouw, G., 2010. Ammonium concentrations in produced waters from a mesothermic oil field subjected to nitrate injection decrease through formation of denitrifying biomass and anammox activity. Appl. Environ. Microbiol. 76, 4977–4987.
5. Costa, M.C.M.S., Carvalho, L., Leal, C.D., Dias, M.F., Martins, K.L., Garcia, G.B., Mancuelo, I.D., Hipólito, T., Mac Conell, E.F.A., Okada, D., Etchebehere, C., Chernicharo, C.A.L., Araujo, J.C., 2014. Impact of inocula and operating conditions on the microbial community structure of two anammox reactors. Environ. Technol. 35, 1811-1822.
6. Dapena-Mora, A., Fernández, 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.
7. Dosta, J., Fernández, I., Vázquez-Padín, J.R., Mosquera-Corral, A., Campos, J.L., Mata-Alvarez, J., Méndez, R., 2008. Short- and long-term effects of temperature on the Anammox process. J. Hazard. Mater. 154, 688–693.
8. Droege, M., Hill, B., 2008. The Genome Sequencer FLX System--longer reads, more applications, straight forward bioinformatics and more complete data sets. J. Biotechnol. 136, 3–10.

9. Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460–2461.
10. Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194–2200.
11. Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods 10, 996–998.
12. Hu, B., Zheng, P., Tang, C., Chen, J., van der Biezen, E., Zhang, L., Ni, B., Jetten, M.S.M., Yan, J., Yu, H.-Q., Kartal, B., 2010. Identification and quantification of anammox bacteria in eight nitrogen removal reactors. Water Res. 44, 5014–5020.
13. Hu, M., Wang, X., Wen, X., Xia, Y., 2012. Microbial community structures in different wastewater treatment plants as revealed by 454-pyrosequencing analysis. Bioresour. Technol. 117, 72–79.
14. Hu, Z., Lotti, T., de Kreuk, M., Kleerebezem, R., van Loosdrecht, M.C.M., Kruit, J., Jetten, M.S.M., Kartal, B., 2013. Nitrogen removal by a nitritation-anammox bioreactor at low temperature. Appl. Environ. Microbiol. 79, 2807–2812.
15. Jin, R.-C., Yang, G.-F., Yu, J.-J., Zheng, P., 2012. The inhibition of the Anammox process: A review. Chem. Eng. J. 197, 67–79.
16. Julián, E., Roldán, M., Sánchez-Chardi, A., Astola, O., Agustí, G., Luquin, M., 2010. Microscopic cords, a virulence-related characteristic of Mycobacterium tuberculosis, are also present in nonpathogenic mycobacteria. J. Bacteriol. 192, 1751–1760.
17. Kartal, B., de Almeida, N.M., Maalcke, W.J., Op den Camp, H.J.M., Jetten, M.S.M., Keltjens, J.T., 2013. How to make a living from anaerobic ammonium oxidation. FEMS Microbiol. Rev. 37, 428–461.

18. Kartal, B., Kuenen, J.G., van Loosdrecht, M.C.M., 2010. Sewage treatment with anammox. Science 328, 702–703.
19. Kindaichi, T., Tsushima, I., Ogasawara, Y., Shimokawa, M., Ozaki, N., Satoh, H., Okabe, S., 2007. In situ activity and spatial organization of anaerobic ammonium-oxidizing (anammox) bacteria in biofilms. Appl. Environ. Microbiol. 73, 4931–4939.
20. Lackner, S., Gilbert, E.M., Vlaeminck, S.E., Joss, A., Horn, H., van Loosdrecht, M.C.M., 2014. Full-scale partial nitritation/anammox experiences--an application survey. Water Res. 55, 292–303.
21. Liu, S., Zhang, Z., Ni, J., 2015. Behavior detection and activity recovery of damaged anammox bacteria culture after accidental overheating. Chem. Eng. J. 259, 70-78.
22. Lopez-Vazquez, C.M., Kubare, M., Saroj, D.P., Chikamba, C., Schwarz, J., Daims, H., Brdjanovic, D., 2014. Thermophilic biological nitrogen removal in industrial wastewater treatment. Appl. Microbiol. Biotechnol. 98, 945–956.
23. Lotti, T., Kleerebezem, R., van Erp Taalman Kip, C., Hendrickx, T., Kruit, J., van Loosdrecht, M.C.M., 2014. Anammox growth on pretreated municipal wastewater. Environ. Sci. Technol. 48, 7874-7880.
24. Oshiki, M., Shimokawa, M., Fujii, N., Satoh, H., Okabe, S., 2011. Physiological characteristics of the anaerobic ammonium-oxidizing bacterium “Candidatus Brocadia sinica”. Microbiology 157, 1706–1713.
25. Park, H., Rosenthal, A., Jezek, R., Ramalingam, K., Fillos, J., Chandran, K., 2010. Impact of inocula and growth mode on the molecular microbial ecology of anaerobic ammonia oxidation (anammox) bioreactor communities. Water Res. 44, 5005–5013.

26. Pereira, A.D., Leal, C.D., Dias, M.F., Etchebehere, C., Chernicharo, C.A.L., de Araújo, J.C., 2014. Effect of phenol on the nitrogen removal performance and microbial community structure and composition of an anammox reactor. Bioresour. Technol. 166, 103–111.
27. Puyol, D., Carvajal-Arroyo, J.M., Garcia, B., Sierra-Alvarez, R., Field, J. a, 2013. Kinetic characterization of Brocadia spp.-dominated anammox cultures. Bioresour. Technol. 139, 94–100.
28. Rysgaard, S., Glud, R.N., 2004. Anaerobic N2 production in Arctic sea ice. Limnol. Oceanogr. 49, 86–94.
29. Strous, M., Kuenen, J.G., Jetten, M.S., 1999. Key physiology of anaerobic ammonium oxidation. Appl. Environ. Microbiol. 65, 3248–3250.
30. Toh, S.K., Webb, R.I., Ashbolt, N.J., 2002. Enrichment of autotrophic anaerobic ammonium-oxidizing consortia from various wastewaters. Microb. Ecol. 43, 154–167.
31. Van de Graaf, A.A., de 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.
32. Van der Star, W.R.L., Abma, W.R., Blommers, D., Mulder, J.-W., Tokutomi, T., Strous, M., Picioreanu, C., van Loosdrecht, M.C.M., 2007. Startup of reactors for anoxic ammonium oxidation: experiences from the first full-scale anammox reactor in Rotterdam. Water Res. 41, 4149–4163.
33. Van der Star, W.R.L., Miclea, A.I., van Dongen, U.G.J.M., Muyzer, G., Picioreanu, C., van Loosdrecht, M.C.M., 2008. The membrane bioreactor: a novel tool to grow anammox bacteria as free cells. Biotechnol. Bioeng. 101, 286–294.

34. Wett, B., Omari, A., Podmirseg, S.M., Han, M., Akintayo, O., Gómez Brandón, M., Murthy, S., Bott, C., Hell, M., Takács, I., Nyhuis, G., O'Shaughnessy, M., 2013. Going for mainstream deammonification from bench to full scale for maximized resource efficiency. Water Sci. Technol. 68, 283–289.
35. Xie, B., Lv, Z., Hu, C., Yang, X., Li, X., 2013. Nitrogen removal through different pathways in an aged refuse bioreactor treating mature landfill leachate. Appl. Microbiol. Biotechnol. 97, 9225–9234.

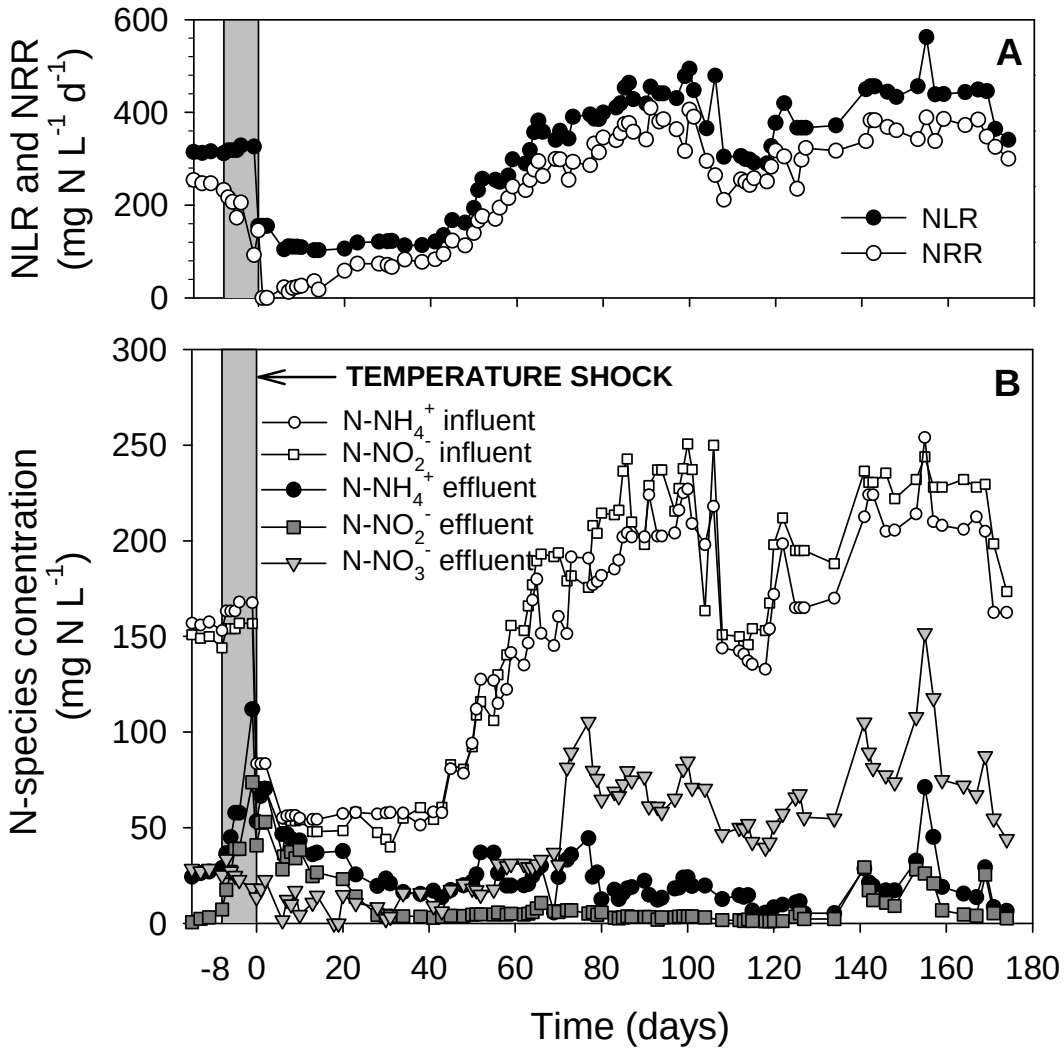

Figure 1. Time course of nitrogen removal performance measured in the anammox reactor before and after the temperature shock event (highlighted with a gray band). (A) nitrogen loading and removal rate; (B) influent and effluent ammonium, nitrite and nitrate concentration.

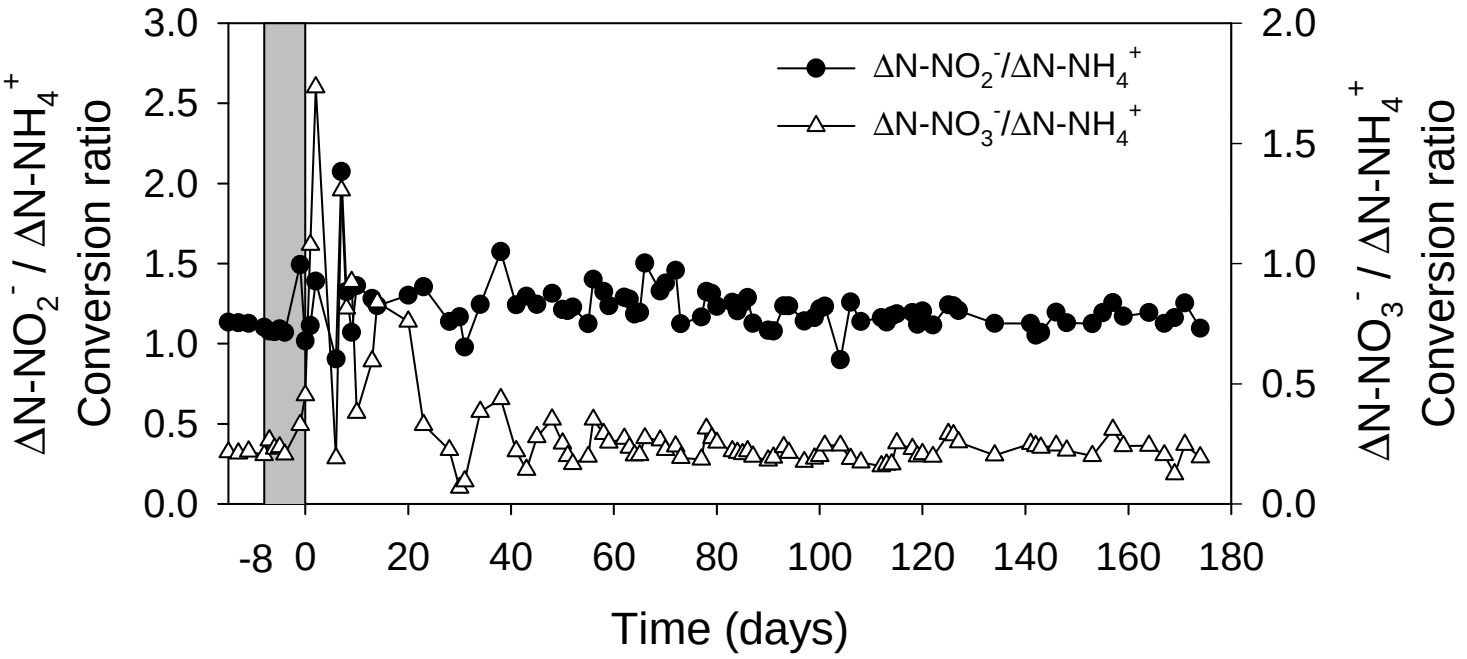

Figure 2. Time course of nitrite to ammonium and nitrate to ammonium conversion ratios in the anammox reactor calculated from effluent N-species concentration in the effluent. The temperature shock event is highlighted with a gray band.

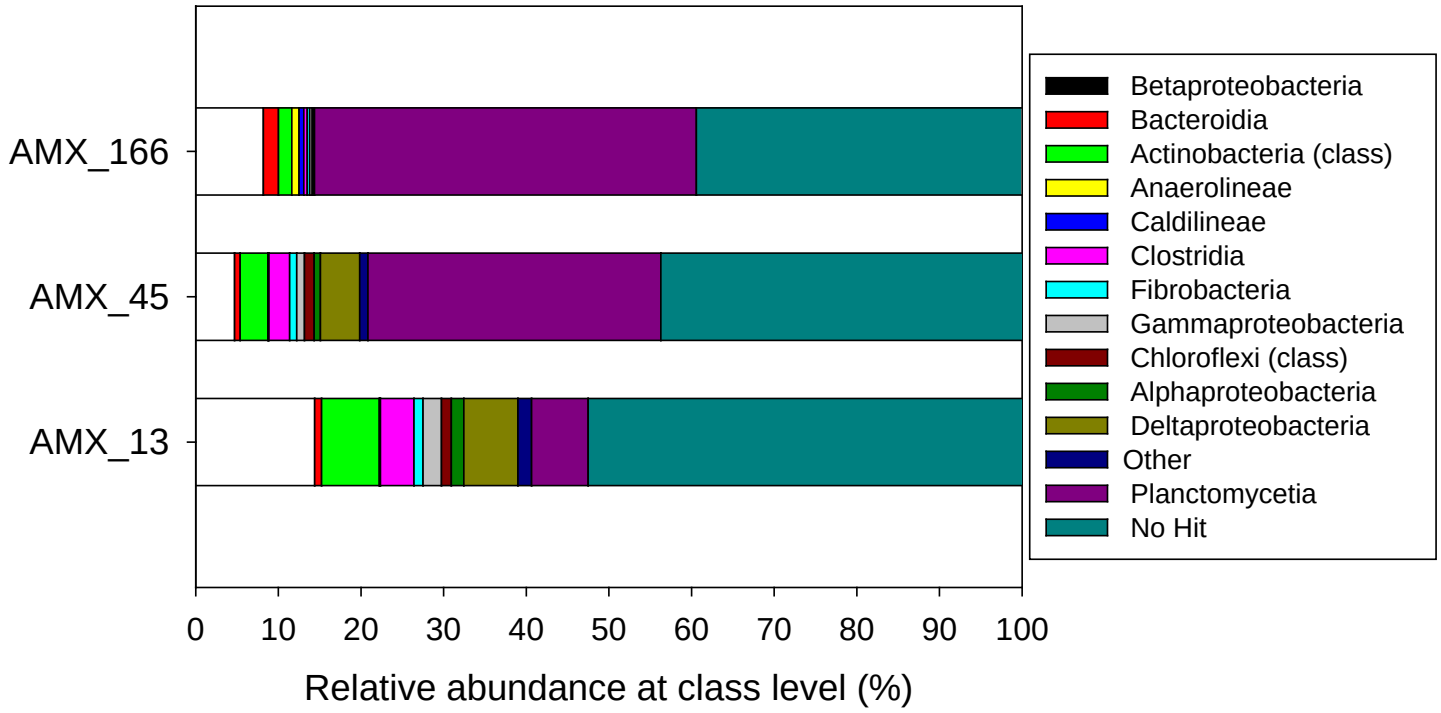

Figure 3. Microbial diversity at class level of libraries AMX_13, AMX_45 and AMX_166 obtained using primers 530F-1100F. Relative abundance was defined as the number of sequences affiliated with that taxon divided by the total number of sequences per sample (%). Classes making up less than 1% were defined as “Others”.

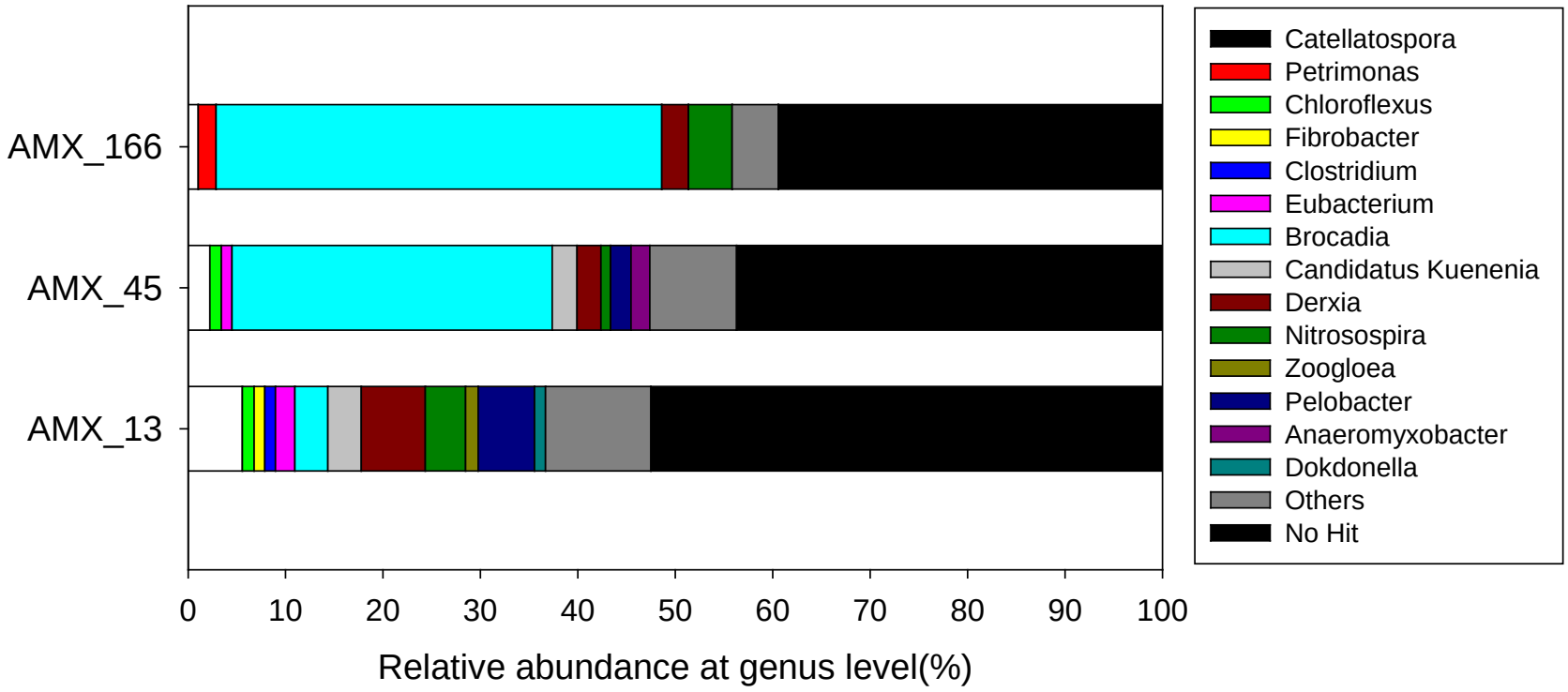

Figure 4. Microbial diversity at genus level of libraries AMX_13, AMX_45 and AMX_166 obtained using primers 530F-1100F. Relative abundance was defined as the number of sequences affiliated with that taxon divided by the total number of sequences per sample (%). Genera making up less than 1% were defined as “Others”.

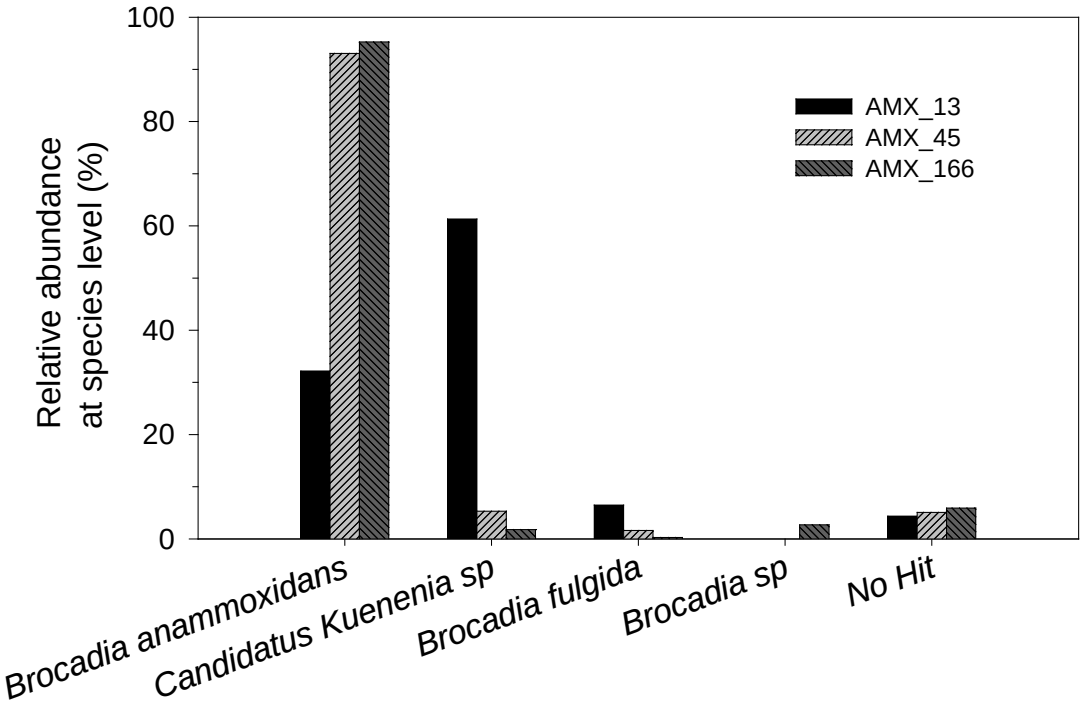

Figure 5. Relative abundance of anammox species for libraries AMX_13, AMX_45 and AMX_166 obtained using Amx368F-Amx820R specific primer. Relative abundance was defined as the number of sequences affiliated with that taxon divided by the total number of sequences per sample (%).

Table 1. Indices of richness Chao1, diversity Shannon (H’) and E of Eubacteria at 97% of similitude for libraries AMX_13, AMX_45 and AMX_166 and day at which biomass samples were obtained after the temperature shock.

Library	Day	Number of reads	Chao1	Shannon (H')	E
AMX_13	13	14725	2388	6.38	0.85
AMX_45	45	11200	1807	5.84	0.82
AMX_166	166	18039	630	3.56	0.56

Figure captions

Figure 1. Time course of nitrogen removal performance measured in the anammox reactor before and after the temperature shock event (highlighted with a gray band). (A) nitrogen loading and removal rate; (B) influent and effluent ammonium, nitrite and nitrate concentration.

Figure 2. Time course of nitrite to ammonium and nitrate to ammonium conversion ratios in the anammox reactor calculated from effluent N-species concentration in the effluent. The temperature shock event is highlighted with a gray band.

Figure 3. Microbial diversity at class level of libraries AMX_13, AMX_45 and AMX_166 obtained using primers 530F-1100F. Relative abundance was defined as the number of sequences affiliated with that taxon divided by the total number of sequences per sample (%). Classes making up less than 1% were defined as “Others”.

Figure 4. Microbial diversity at genus level of libraries AMX_13, AMX_45 and AMX_166 obtained using primers 530F-1100F. Relative abundance was defined as the number of sequences affiliated with that taxon divided by the total number of sequences per sample (%). Genera making up less than 1% were defined as “Others”.

Figure 5. Relative abundance of anammox species for libraries AMX_13, AMX_45 and AMX_166 obtained using Amx368F-Amx820R specific primer. Relative abundance was defined as the number of sequences affiliated with that taxon divided by the total number of sequences per sample (%).