Journal of Environmental Chemical Engineering 9 (2021) 106622

Contents lists available at ScienceDirect

Journal of Environmental Chemical Engineering

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

Microbial communities in an anammox reactor treating municipal wastewater at mainstream conditions: Practical implications of different molecular approaches

Pía Oyarzúa [a], Patricia Bovio-Winkler [b], Claudia Etchebehere [b], María Eugenia Suárez-Ojeda [a,*]

[a] GENOCOV Research Group, Department of Chemical, Biological and Environmental Engineering, School of Engineering-Edifici Q, Universitat Autònoma de Barcelona, Campus UAB, 08193 Bellaterra, Barcelona, Spain
[b] Microbial Ecology Laboratory, Department of Microbial Biochemistry and Genomic, Biological Research Institute "Clemente Estable", Avenida Italia 3318, CP 11600 Montevideo, Uruguay

ARTICLE INFO

Editor: Yang Liu

Keywords:
Mainstream anammox
Microbial community
Amplicon sequencing
Metagenomics
qPCR

ABSTRACT

Biases on molecular techniques used to study microbial communities in the anammox process are not always considered. This work aimed to evaluate different molecular approaches to study bacterial communities in an anammox reactor treating real urban wastewater at mainstream conditions. 16S rRNA amplicon sequencing with different primer sets, metagenomics, and qPCR were evaluated. An enriched anammox reactor feed with synthetic wastewater was used as a positive control. We reported that, despite its *in silico* coverage, the universal primers 515F-909R had, in fact, very low coverage (1%) of the Planctomycetota phylum. The functional genes approach showed a taxonomic resolution at the species level, although genomic database updates are a bias to be considered. From the phylogenetic analysis, coherence among the approaches was obtained. Primers targeting anammox 16S rRNA and hydrazine synthase genes in qPCR showed consistency in the quantification, making them useful for comparing similar investigations. According to the results, recommendations on the biomolecular approach to be used were outlined.

1. Introduction

The anammox process is carried out by anammox bacteria, described as a monophyletic order ("*Candidatus*" Brocadiales) in the Planctomycetes phylum that have the metabolic ability to catalyze the one-to-one conversion of ammonium and nitrite under anoxic conditions to form dinitrogen gas [32,48].

Recent research on autotrophic nitrogen removal has been focused on partial nitrification (PN) or partial denitrification (PD) coupled to anammox for its implementation in the mainstream line of urban WWTP, encouraged by the intention of achieving energy-neutral plants [12,36,46,54]. Despite the increasing experimental studies, there are still many challenges to improve the nitrogen removal efficiency at mainstream conditions, including: low NH_4^+ concentrations, variable nitrogen loads, low temperatures during winter season, proliferation of nitrite oxidizing bacteria (NOB), nitrate buildup, competition between different classes of bacteria (autotrophic, heterotrophic), long-term process stability and stringent-effluent-quality requirements (Du et al., 2019), [22,28,38,46].

There is evidence that under the same operational conditions, the nitrogen removal efficiency of various anammox systems may differ owing to the difference in the dominant anammox species, [9,62]. To date, six anammox genera have been described, including "*Candidatus*" *(Ca.)*. Brocadia, *Ca.* Kuenenia, *Ca.* Scalindua, *Ca.* Anammoxoglobus, *Ca.* Jettenia and *Ca.* Anammoximicrobium, all of them detected in different WWTPs [20,62]. These lineages all have "*Candidatus*" status as it is not yet possible to culture them in pure culture and must be grown in laboratory enrichments [30]. According to Yarza et al. (2014), [65] the candidate taxonomic unit is a "biological entity that is delineated by a monophyletic set of sequences with a sequence identity that stays within, or very close to, the taxonomic threshold that is proposed for a given rank".

Giving the metabolic differences among the anammox community, better identification of the existing anammox bacteria in a bioreactor is of utmost importance to understand the process and improve its operation [9]. But also, other microbial communities, whose identity and

* Corresponding author.
E-mail address: mariaeugenia.suarez@uab.cat (M.E. Suárez-Ojeda).

https://doi.org/10.1016/j.jece.2021.106622
Received 19 July 2021; Received in revised form 21 September 2021; Accepted 17 October 2021
Available online 20 October 2021

relationship with the anammox bacteria are still under investigation [15], are important. In this sense, Lawson et al. [30] proposed different metabolic networks between anammox and heterotrophic bacteria. Until now, research about anammox systems working at mainstream conditions in real environments have provided insights into the taxonomic composition and, about the key nitrogen cycle microorganisms [22,30,33,7], but the main focus of these investigations was on operating conditions from an engineering perspective [10]. As a result, the actual knowledge is insufficient to reveal the complexity of the anammox community composition and their metabolic network.

In this sense, DNA-based molecular techniques are the most widely used for studying the microbial ecology in this kind of bioreactors [42, 47]. These approaches include PCR-based methods, as amplicon sequencing and qPCR, which utilize 16S rRNA gene as a molecular marker for detection, identification, and/or quantification of bacterial communities. Since the 16S rRNA gene is not necessarily related to the physiology of anammox bacteria, the use of functional gene markers as hydrazine synthase (hzsA), nitrite reductase (nirS), among others, may provide alternatives for assessing these communities in anammox reactors [18,23,53]. The most recent developed molecular approach is metagenomic sequencing, which allows a functional and taxonomic analysis, besides obtaining a higher resolution of microbial and anammox diversity compared to amplicon sequencing [2,30].

Despite the wide variety of molecular approaches, biases in each of them are not always considered. As an example, reliability of PCR-based methods predominantly depends on the specificity and efficiency of the PCR primers [51]. Since the PCR primers are designed *in silico*, their characteristics depend on the type of biomass, the type of sample, and many other factors inherent to the PCR reaction itself. Therefore, their practical applications do not always offer an acceptable performance [53]. Thus, when trying to compare similar investigations, several problems arise, like the inconsistency of the data obtained with the different methods, as shown by Orschler et al. [42] that compared amplicon sequencing of different hypervariable regions of 16S rRNA gene in samples from partial nitrification/anammox (PN/A) reactors.

Therefore, this work aimed to evaluate different molecular approaches to determine the whole bacterial and the anammox communities' structure in a granular-sludge anammox reactor treating real wastewater at mainstream conditions. Also, a comparison with an enriched anammox reactor treating synthetic medium was used to validate the techniques and the scope and biases of each one of the approaches used.

2. Materials and methods

Different molecular approaches to determine the whole bacterial and the anammox communities' structure were evaluated in a granular-sludge anammox reactor treating real wastewater at mainstream conditions. For a better understanding of the strategies used in every step of this work, we define six DNA-based approaches (Table 1), coming from three techniques: amplicon sequencing, metagenomics, and qPCR. The amplicon sequencing technique was performed using three primer sets, where each primer was considered an individual approach: a) universal primers 515 b) universal primers 515F-909R and c) specific anammox primers Amx368F-Amx820R. The shot-gun sequencing metagenomic was the second technique evaluated, including two different approaches: d) 16S rRNA sequences extracted from the metagenomic data, and e) functional taxonomy assigned based on functional genes. These five approaches were sequencing-related and their respective relative abundances were obtained. The final technique performed was qPCR (approach f), where primers targeting 16S rRNA gene for anammox populations, and two primer sets targeting functional genes were used. This last technique allowed an absolute quantification. Sections 2.1 and 2.2 contains the details about sampling and DNA extraction, respectively while in the rest of the sections each approach is explained in detail.

Table 1
DNA-based approaches used in this work to determine the whole bacterial and the anammox communities' structure in a granular-sludge anammox reactor treating real wastewater at mainstream conditions.

Molecular technique	Details	Approach
Amplicon sequencing	Set of primers used: 515F-806R	a
	Set of primers used: 515F-909R	b
	Set of primers used: Amx368F-Amx820R	c
Shot-gun sequencing metagenomic	16S rRNA sequences from metagenomic data	d
	Functional taxonomy from functional genes	e
qPCR	Functional genes (hydrazine synthase gen and nitrite reductase gen) + Bacteria 16S rRNA gen + Anammox bacteria 16S rRNA gen	f

2.1. Anammox reactors description

Samples were obtained from two different reactors. The first reactor corresponds to an anammox sludge sequential-batch-reactor (SBR) working at 35 ºC under stable conditions for more than six years [21]. This SBR was fed with a synthetic medium as influent (180 ± 30 mg $N\text{-}NH_4^+$ L^{-1}, 190 ± 30 mg $N\text{-}NO_2^-$ L^{-1}) and operated with a nitrogen loading rate (NLR) of 0.45 ± 0.09 g N L^{-1} d^{-1}. The nitrogen removal rate (NRR) was 0.36 ± 0.09 g N L^{-1} d^{-1}. Further details about operation and performance of this reactor have been reported elsewhere [21]. The second reactor was an up-flow anammox-sludge-bed (UAnSB) reactor working at 20 ºC at the time of sampling. This UAnSB was fed with a wastewater from a partial nitritation reactor treating the real effluent exiting the primary settler of an urban WWTP (150.000 inhabitants equivalent) located in an industrial area of Catalonia, NE Spain. The composition of this wastewater was: 26 ± 8 mg $N\text{-}NH_4^+$ L^{-1}, 36 ± 9 mg $N\text{-}NO_2^-$ L^{-1}, 2 ± 2 mg $N\text{-}NO_3^-$ L^{-1}, 60 ± 14 mg COD L^{-1}, among other compounds).

Samples of the bottom of this reactor were taken during a stable operation period of 81 days, when it was operated with NLR of 0.10 ± 0.02 g N L^{-1} d^{-1}; NRR 0.10 ± 0.02 g N L^{-1} d^{-1}, nitrogen removal efficiency (NRE) was 87 ± 3% (for more details, see the work of Juan-Díaz et al. [22]. Samples taken from both reactors, were washed 3 times (5000*g*, 5 min) with 1 mL of 1X Phosphate Saline Buffer (PBS) and the pellet was stored at −80 ºC for DNA extraction. Sample from SBR reactor was labelled as "AM" and considered as the enriched anammox reactor treating synthetic influent. The other sample, coming from UAnSB reactor was labelled as "S1″.

2.2. DNA extraction

For total genomic DNA obtention, frozen samples were thawed, centrifuged (5000*g*, 5 min) and 0.35 g of wet pellet were used for the Soil DNA Isolation Plus Kit (Norgen Biotek, Thorold, ON, Canada) according to the manufacturer's instructions. DNA quantity and integrity were checked with a ND-1000 Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and stored at −80 ºC until further use for amplicon and metagenomic sequencing. A final quantification of DNA was determined with the Qubit fluorometric quantitative analyzer (Life Technologies, Carlsbad,CA, USA) for qPCR assays.

2.3. Bacterial 16S rRNA gene analysis by amplicon sequencing

Amplicon sequencing paired-end (PE) service of 16S rRNA genes on the Illumina MiSeq platform (2 × 300 bp) was performed by RTL Genomics (Lubbock, TX, USA) for detecting bacteria and anammox bacteria using primers shown in Table 2. To avoid differences in the results due to the target of different hypervariable region (HVR) of the 16S rRNA gene, two universal primer set targeting the HVR of the 16S rRNA gene (V4-V5) were used as suggested by several researchers [51,53,6]. The Silva database Test Prime tool [26] was used to evaluate primer

Table 2
Primers used in this study for 16S rRNA amplicon sequencing and qPCR.

Application	Probe/Primer set	Sequence (5′−3′)	Specificity	Annealing Temperature (°C)		Reference
16S rRNA gene amplicon	515F	GTGCCAGCMGCCGCGGTAA	Bacteria and Archaea	–	[55]	
	806R	GGACTACHVGGGTWTCTAAT				
	909R	CCCCGYCAATTCMTTTRAGT	Bacteria and Archaea		[64]	
	AMX368F	TTCGCAATGCCCGAAAGG	16S rRNA gen specific for anammox bacteria	–	[57]	
	AMX820R	AAAACCCCTCTACTTAGTGCCC				
qPCR	519F	CAGCMGCCGCGGTAANWC	Bacteria 16S rRNA gen	52	[61]	
	907R	CCGTCAATTCMTTTRAGTT				
	AMX809F	GCCGTAAACGATGGGCACT	16S rRNA gen specific for anammox bacteria	56	[63]	
	AMX1066R	AACGTCTCACGACACGAGCTG				
	AnnirS379F	TCTATCGTTGCATCGCATTT	Nitrite reductase gen	54	[58]	
	AnnirS821R	GGATGGGTCTTGATAAACA				
	AnhzsA1597F	WTYGGKTATCARTATGTAG	hydrazine synthase gen	51	[18]	
	AnhzsA1857R	AAABGGYGAATCATARTGGC				

coverage with settings recommended by Zhou et al. [53]: two maximum number mismatches and one site of 0-mismatch zone at 3′ end.

2.4. Data processing of amplicon sequencing

FastQC v.0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to assess raw sequences quality control and adaptors contamination. Demultiplexed sequences were imported into QIIME2™ v.2019.10 (https://qiime2.org). DADA2 denoise-paired plug-in [4] was used to quality filtering, trimming of poor-quality bases, de-replication, chimera filtering, merging paired-end reads, and the identification of amplicon sequence variants (ASV). The resulting feature table was used for taxonomic assignment based on the Silva SSU 138 rRNA reference database pre-clustered at 99% sequence identity with the QIIME2 q2-feature-classifier plugin [16]. The Bray-Curtis metric was used for calculation of beta diversity and a non-metric multidimensional scaling (NMDS) plot was constructed with metaMDS function (vegan package) and ggplot function (ggplot2 package) using R software version 3.5.1 (R Core Team 2013) in R Studio environment Version 1.0.153. The raw data was deposited at National Center for Biotechnology Information (NCBI) under accessing number: PRJNA728853.

2.5. Metagenomic analysis

DNA samples were analyzed using shotgun sequencing performed in Illumina Novaseq6000 sequencer (Macrogen, Seoul, South Korea) (2 × 100 bp, PE). The metagenomic data was processed and analysed with the following tools: FastQC (v0.11.9) for assessing sequence quality, Trimmomatic (v0.36) for removal of ambiguous reads, adapters, and low-quality sequences to obtain high quality reads [3] with the settings SLIDINGWINDOW:4:25 and MINLEN:90, MEGAHIT v1.2.3-beta [31] for metagenome assembly. Contigs shorter than 1000 bp were filtered. After this, metagenomic annotation and functional classification were predicted against the KEGG GENES database using MetaErg [11]. On the other side, Ribosomal Small Subnubit (SSU) from 16S rRNA genes were reconstructed from metagenomic reads using Metaxa2 v.2.1 [1] together with the SILVA SSU 138 rRNA database. Assembly of shotgun reads assigned to the 16S rRNA gene was performed using EMIRGE [39]. The raw data was deposited at NCBI under accessing number: PRJNA728853.

2.6. Phylogenetic analysis

Three phylogenetic trees were constructed to determine the position of anammox bacteria retrieved by the different strategies. As all primer sets used for amplicon sequencing produced different length sizes of the PCR products, it was decided to construct one tree for each primer set. In this way, we avoided losing information by trimming sequences. The trees included: representative sequences of ASVs classified as Brocadiaceae family from amplicon sequencing for each primer set, full-length 16S rRNA gene sequences reconstructed from metagenome, sequences belonging to the *Ca.* Brocadiales order from an anammox study [53] and related environmental sequences found in GenBank using BLASTN algorithm. All sequences were aligned using SILVA v1.2.11 [45] and phylogenetic trees were performed using the maximum likelihood method in MEGA7 software [27]. 16S rRNA sequence from *Pirellula staleyi* (AJ231183) as an outgroup for rooting trees [53]. Bootstrap values were obtained from data resampling of 500 replicates.

2.7. Quantification of different anammox genes and total bacteria by qPCR

Two functional genes (hzsA and nirS) and one specific 16S rRNA method were used to quantify the proportion of anammox in both reactors. Moreover, the total bacterial population was quantify using 16S rRNA gene bacterial-target primers. Table 2 shows the primers and its annealing temperatures. The calibration curves were constructed with positive controls. For these controls, plasmids pTOP_Blunt_V2 containing as insert specific anammox bacterial 16S rRNA and for the functional genes encoding hzsA and nirS were manufactured by Macrogen Inc (Seoul, South Korea). Accession number of reference genes used for synthesis were: AB277765.1, JN703716.1 and CT573071.1. Genomic DNA previously extracted from a strain of *Pseudomonas fluorescens* was used as standard for the 16S rRNA gene bacterial quantification. The standard samples were diluted to yield 10-fold dilutions in the range of 10^4-10^8 DNA copies μl^{-1} and subsequently used to generate q-PCR standard curves. The R^2 value for each standard curve exceeded 0.99. All PCR reactions were performed in a Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia). Fluorescence was detected at the end of the extension step. The qPCR protocol was as follow: 95 °C for 5 min followed by 40 cycles at 95 °C for 10 s, 20 s at the annealing temperature and 72 °C for 20 s. Each PCR mixture (20 μl) contained 2 μl of diluted DNA template, 10 μM of each primer, and 10 μl of 2X KAPA SYBR® FAST qPCR Master Mix Universal (Kapa Biosystems, Wilmington, MA, USA). The specificity of the amplified PCR product was assessed by performing a melting curve analysis; fluorescence readings were consecutively collected during the melting process from 60 to 95 °C at a heating rate of 5 °C s^{-1}. DNA concentrations were determined using the Qubit fluorometric quantitative analyzer (Life Technologies, CarlsbadCA, USA) and gene copy numbers obtained were normalized by DNA concentrations in corresponding individual samples. All quantitative PCR reactions were carried out in triplicates and statistical analysis was performed with Infostat software. Log transformed gene copy numbers were subjected to ANOVA analysis. Tukey's test was used to determine which genes copies were statistically distinct (5% of significance was used). The efficiencies of the quantitative PCR assays were over 80%, and the R^2 value always exceeded 0.98.

3. Results and discussion

3.1. Microbial community composition

The microbial community composition of the samples taken from two anammox reactors: reactor S1 (anammox reactor treating urban wastewater at mainstream conditions) and reactor AM (enriched anammox reactor treating synthetic influent as a positive control) was determined using: amplicon sequencing (approaches a and b) and shotgun sequencing metagenomic (approaches d and e).

Relative abundances of the four datasets were compared at the class level (Fig. 1A) showing that Anaerolineae, Brocadiae, Gammaproteobacteria and Ignavibacteria were the most abundant classes in both reactors, although the relative abundances vary among techniques and the reactor samples. These classes correspond to the phyla Chloroflexi, Planctomycetota, Proteobacteria and Bacteroidota, respectively. This so-called "core microbiome", have been found in the microbial communities of anammox bioreactors, being similar in several types of reactors, despite the differences in the operation of the reactor and the composition of the influent wastewater, and the molecular approach used to determine the community composition [2,30,37].

A very low coverage of the Brocadiae class was observed using the universal primers 515F-909R. In the AM reactor, the results showed that 16S rRNA amplicon with universal primers 515F-909R detected 1% while the universal primers 515F-806R presented a relative abundance of 75% (Fig. 1A). In this sense, we have selected two universal primer set targeting the same hypervariable region of the 16S rRNA gene (V4-V5), that showed similar coverage efficiencies and specificity for anammox bacterial detection on *in silico* evaluation with SILVA test prime, as recommended by various investigations (Table 3) [34,51,53]. Nevertheless, our results showed that primer set 515F-909R did not cover most of the organisms from the Brocadiae class present in our reactors, underrepresenting the anammox community despite the estimated *in silico* coverage (96%). In a similar work developed by Chen et al. [8] studying the community structure of a partial nitritation system, amplicon sequencing was performed with the primer set 515F-909R, but Planctomycetes or anammox bacteria were not detected, although it was not reported as an issue. This absence of Planctomycetes disagree with Zhao et al. [52] whose pointed out that anammox reaction occurred in the partial nitritation system. Differences between *in silico* and in practical results have been previously reported by Yang et al. [51]. This may be due to the fact that *in silico* predictions do not consider chemical reactions and limitations that can occur in the PCR tube and therefore cannot truly approximate to the practical applications [19], another possibility is that the microorganism predominant were not covered by this primer set and the sequences from these microorganisms were not deposited in the databases. In contrast, the primer set 515F-806R gave us a better representation of the anammox community, but it could lead to an underestimation of the other phyla on the enriched anammox reactor treating synthetic influent. This is due to the amplification bias among taxa with low estimated abundance in amplicons, as was demonstrated by Campanaro et al. [5]. These results agree with those of Ferrera and Sánchez [14], showing that there are fundamental biases related to the PCR amplification and the primer set used that impact on the quantitative information that was obtained from these approaches.

The S1 reactor, instead, was dominated by the heterotroph's classes Ignavibacteria, Anaerolineae and Gammaproteobacteria, not anammox. The class Brocadiae was found in very low abundance (3%) with primer set 515F-806R and it was not detected with the universal primer set 515F-909R. The difference in abundance of the Brocadiales class between both reactors was expected, given the characteristics of the influent. The synthetic medium provided to the AM reactor and operation conditions were the ideal ones for enhancing the anammox enrichment, while the presence of organic matter and other compounds in real wastewater that feeds the S1 reactor enhance the growth of heterotrophic bacteria to the detriment of anammox bacteria. Function of Ignavibacteria and Anaerolinea classes are not yet completely understood, but based on transcriptional insights on interactions with anammox, they seem to be polymer-degrading, fermentative anaerobes, and participates in the nitrogen removal supporting complete denitrification [15,30]. Also, they could supply short-chain volatile fatty acids and alcohols to support other communities as Proteobacteria, due to their ability to hydrolysate extracellular compounds of anammox bacteria [2,22].

In this reactor, as primers 515F-909R did not detect Brocadiae class, Ignavibacteria represented the main class (90%). For the other two datasets based on 16S rRNA gene (approaches a and d), no major differences were observed between the relative abundances of the different

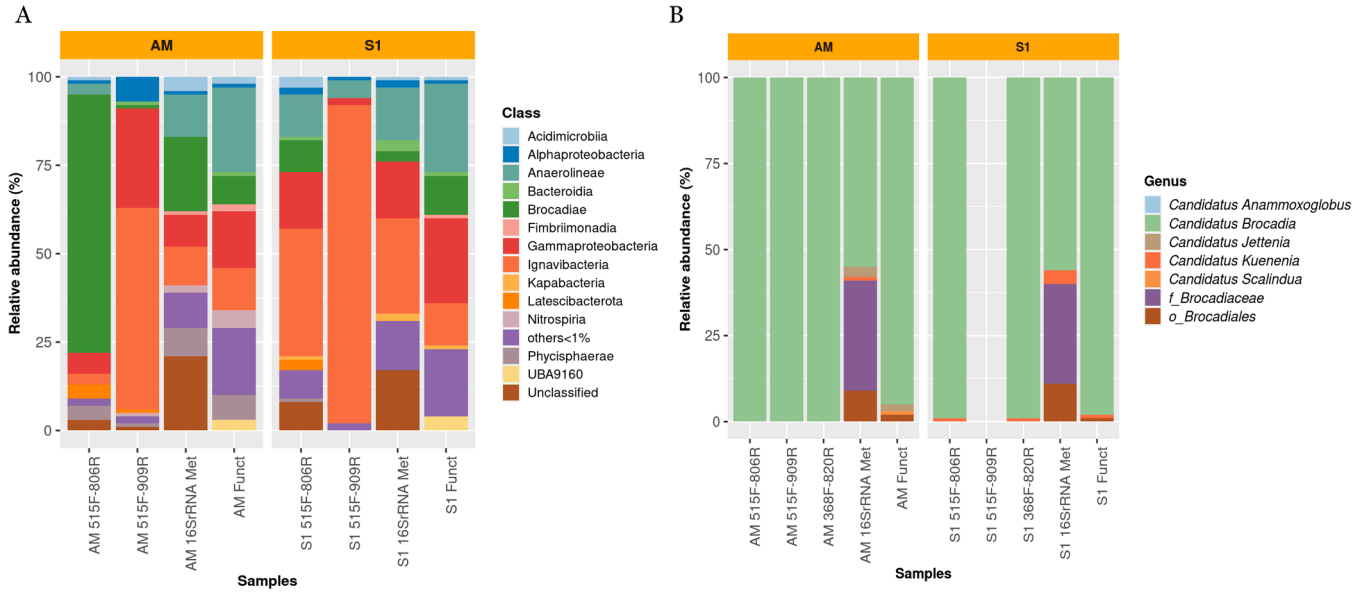

Fig. 1. A) Relative abundance of identified classes of the microbial community in anammox reactors using four different molecular approaches. Classes whose relative abundance among all the techniques were less than 1% were grouped as "others". B) Relative abundance of identified anammox bacteria at the genus level using five different molecular approaches. When classification at the genus level was not obtained, the previous taxonomic level was indicated as f (family) and o (order).

Table 3
In silico primer coverage prediction for the primers used in this study restricted to de phylum Planctomycetes in SILVA Test Prime analysis (SSU Ref 138 NR). The numbers represent the percentage of sequences deposited in the database targeted with each primer sets. A heat map color code was used, red color means lower values while green color means higher values.

	Target gene	Universal 16S rRNA			Anammox 16S rRNA	
	Primers set	515F-806R[a]	515F-909R[b]	519F-907R[c]	Amx368F-Amx820R[d]	Amx809F-Amx1066R[e]
Planctomycetota		94	96	96	0.9	43
Brocadiae		90	90	90	52	92
Brocadiales		91	90	91	54	92
Brocadiaceae		85	85	85	93	92
"Candidatus" Anammoxoglobus		67	67	67	67	100
"Candidatus" Brocadia		91	91	91	97	97
"Candidatus" Jettenia		94	94	94	88	88
"Candidatus" Kuenenia		87	87	87	93	80
scalinduaceae		98	94	96	13	98
"Candidatus" Scalindua		98	94	96	13	98

[a]Caporaso et al. (2012)

[b]Tuan et al. (2014)

[c]Stubner (2002)

[d]Gu et al. (2007)

[e]Tsushima et al. (2007)

classes belonging to the core microbiome mentioned above.

Important differences in the relative abundance at Brocadiae class were observed among techniques in the AM reactor, and nevertheless, in the S1 reactor were in a similar range (3–11%). Differences between amplicon sequencing and metagenomic and even between the two metagenomics approaches were obtained. This could be explained because a common bias for all the techniques studied is the databases limitations, although there are several high quality, comprehensive and curated 16S rRNA gene databases compared to genomic databases [44]. The main differences in terms of relative abundance were obtained with functional taxonomy when all approaches were compared. Metagenomics approaches, on the contrary, avoids PCR biases [35] and have resulted in a turning point in studying phylogenetic and functional diversity of wastewater treatment systems [14] since it reach a much higher resolution of taxonomic annotation [34,44]. But, on the other hand, its limitations include a higher cost and bionformatics complexity

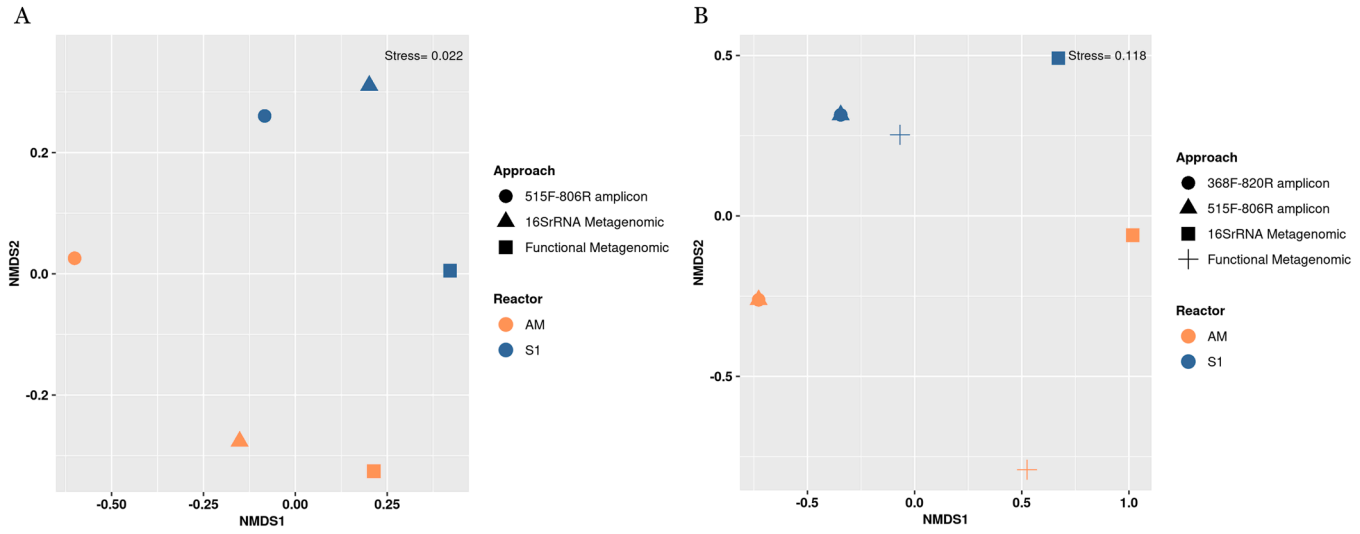

Fig. 2. NMDS ordination using A) the class level relative abundance data of the whole microbial community; B) the genus level relative abundance data of detected anammox bacteria. Communities' similarities are based on the Bray–Curtis index which includes the relative abundances on each approach.

than PCR based methods [34].

To determine how the samples analyzed with different approaches were grouped, an NMDS analysis was performed. The samples from amplicon sequencing using the primers 515F-909R were excluded from the analysis as this primer set did not detect Brocadiae class in the S1 reactor samples. The results obtained using the relative abundance of the different classes showed that the communities were grouped by reactor (S1 and AM) (Fig. 2A). The impact of the bias on the results was different for the two reactors treating different influents. Thus, major difference between techniques were observed in AM reactor, where Anaerolineae class was underrepresented in the amplicon sequencing compared to the metagenome in the AM reactor, but, on the contrary, Brocadiae class was overrepresented comparing with the metagenomic approach (Table 4). These differences would be explained because shotgun sequencing (metagenomics) has more power to identify less abundant taxa than amplicon sequencing of the16S rRNA gene [13], causing a shift in the relative abundance estimation of higher abundant taxa determined by amplicon sequencing. In our study, the concordance between the functional and amplicon taxonomic assignment in the S1 reactor indicates that the last is a good method to visualize the functional abilities of this reactor.

3.2. Anammox bacteria community analysis

To specifically study the anammox community, a third set of primers was also used for amplicon sequencing (approach c). The primers Amx368F-Amx820R were designed to target anammox bacteria and have 93% of coverage for the Brocadiaceae family when checked with SILVA test prime SSU138 RefNR database (Table 3). Anammox sequences from each approach (a, b, c, d and e) classified at the Brocadiae class were separately analyzed and relative abundances of anammox community at deeper taxonomic levels were determined.

Leaving aside amplicon sequencing data obtained with primers 515F-909R which did not detect Planctomycetota in the S1 reactor, 100% of Brocadiales order was detected. At family level, all approaches detected mainly Brocadiaceae in the two samples. 1% of Scalinduaceae was found in the AM reactor with functional taxonomy (Table 4). All other methods studied were consistent in the composition of the anammox community down to the family taxonomic level (phylum, class, order, and family). Approximately 10% of the sequences from both reactors' samples were not classified at the family level with the16S rRNA metagenomic approach.

The classification at the genus level reveals that the anammox community in both reactor samples correspond mainly to *Ca.* Brocadia genus (Fig. 1B). All the approaches showed similar detection abilities in both reactors. The communities obtained by amplicon sequencing with the three primer sets showed no other genus than *Ca.* Brocadia in the AM reactor, while a low abundance of *Ca.* Kuenenia was detected in the S1 reactor. As we previously mentioned, the primers 515F-909R did not detect anammox at any taxonomic level in the S1 reactor. The 16S rRNA metagenomic approach detected mainly *Ca.* Brocadia and small abundances of *Ca.* Jettenia and/or *Ca.* Kuenenia in both reactors, nevertheless, showed a low resolution at the genus level. A high proportion of the sequences were classified at previous taxonomic levels as order and family (41% and 40%, for AM and S1 reactors, respectively) (Table 4). *Ca.* Brocadia was also the genus of anammox with the highest abundance detected by functional taxonomy in both reactors. The NMDS analysis (Fig. 2B) showed that S1 samples grouped together while for AM samples the communities retrieved by the two metagenomics approach were closer positioned and the communities obtained using the 16 S rRNA approaches were identical for the two set of primers.

About the anammox bacteria community on S1 reactor, the dominant genus was *Ca.* Brocadia. This has been reported in several mainstream studies [28,36,38]. Some studies on anammox WW treatment technologies have reported that there would be a dominant genus of anammox bacteria under specific growth conditions [24], so this will depend on the capacity to adapt to the mainstream wastewater [40].

Functional taxonomy from metagenomes allowed obtaining a classification at species level (Fig. 3). The predominant species of anammox bacteria in the reference reactor, AM, were *Ca.* Brocadia sinica and *Ca.* Brocadia fulgida (45% each). In the S1 reactor, *Ca.* Brocadia fulgida (66%) and *Ca.* Brocadia caroliniensis (18%) had higher relative abundance. These differences could be associated with the influent characteristics, including the concentration of nitrogen compounds and the presence of organic matter. According to Narita et al. (2017), [59] the differences in physiological characteristics among anammox bacteria are one of the essential key factors to understand niche differentiation. As an example, *Ca.* Brocadia fulgida has been reported to be dominant on different deammonification systems [29,33,50]. Interestingly, Winkler et al. [50], reported an accumulation of this specie in granular sludge once nitrite was omitted from the feed. Also, this species was reported to consume some organic carbon forms such as propionate, formate, and dimethylamine [56]. Nevertheless, until now there is not consensus about the anammox species that predominated in mainstream nitrogen removal processes [33].

Down genus level, all techniques cover the same/similar anammox bacteria, but the 16S rRNA sequences extracted from the metagenome approach lost resolution in the taxonomic assignment and on the contrary, functional taxonomy of the metagenome had a better resolution on assignment at the specie level. Nevertheless, considering that functional taxonomy from metagenomes is a new approach, the obtained results would depend on database updates and completeness, and one shall take into account that genome annotations pipelines are nowadays on constant changes [11].

Table 4
Comparative table of the relative abundance obtained for the anammox microorganisms with the different approaches.

		AM					S1				
Approach		515F-806R[a]	515F-909R[b]	Amx368F-Amx820R[c]	16S rRNA metagenome[d]	Functional metagenome[e]	a	b	c	d	e
Taxonomic classification											
Phylum	Plancomycetota	79	1	100	40	18	11	0	100	5	11
Class	Brocadiae	75	1	100	27	8	9	0	100	3	11
Order	Brocadiales	100	100	100	100	100	100	0	100	100	100
Family	Brocadiaceae	100	100	100	90	99	100	0	100	89	99
Genus	*Ca. Brocadia*	99.8	100	100	55	95.4	99	0	99	56	98
	Ca. Jettenia	0	0	0	3	2	0	0	0	0	0.4
	Ca. Kuenenia	0.2	0	0	1	0.5	1	0	1	4	0.9

[a] amplicon sequencing with universal primers 515F-806R,
[b] amplicon sequencing with universal primers 515F-909R and
[c] amplicon sequencing with specific anammox primers Amx368F-Amx820R,
[d] 16S rRNA sequences extracted from the metagenomic data, and
[e] functional taxonomy assigned based on functional gene

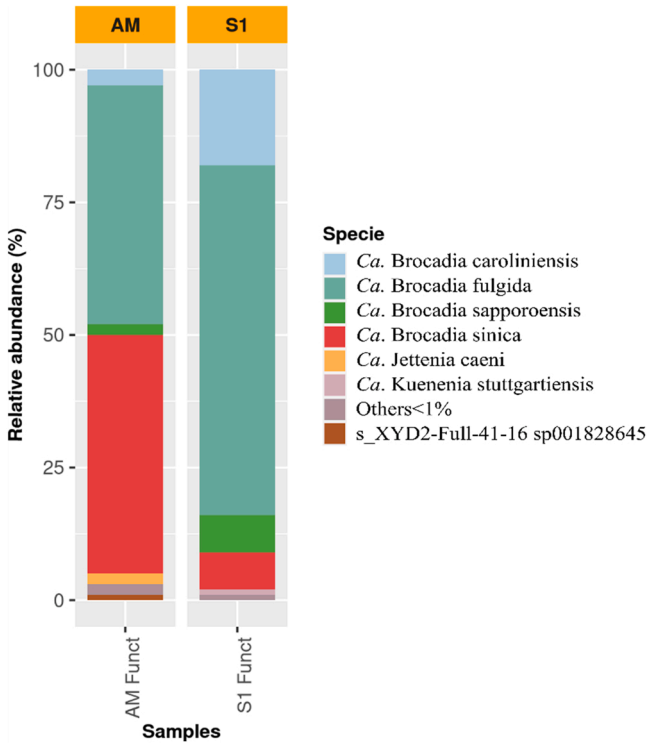

Fig. 3. Relative abundance of identified anammox bacteria at the specie level using functional genes approach from metagenomes. Contigs with abundance below 1% were grouped as "Others". Results presented as "s" (specie) are not associated to a known "*Candidatus*" specie.

3.3. Phylogeny of anammox microorganisms predominant in the samples

A phylogenetic analysis was performed to elucidate the relationship between the anammox sequences obtained by the different 16S rRNA-based approaches and the complete 16S rRNA genes retrieved from the metagenome without any primers bias. For the amplicon sequence analysis, the sequences were grouped in ASVs (sequences with 100% similarity). As different regions were targeted by the different primer sets, three phylogenetic trees were constructed, one for each primer set (Fig. 4). Comparing the phylogenetic trees, coherence between all approaches in the phylogenetic analysis was observed. Most abundant ASVs retrieved with the different amplicon sequencing approaches with the specific set of primers (AMX368F-AMX820R, approach c) and with the universal primers (515F-806R, approach b), always cluster in the same branch with a sequence retrieved by the metagenomics approach.

Some ASVs sequences retrieved with the specific primers did not cluster with any of the sequences retrieved from the metagenomic approach. Due to the low coverage of the set of primers (515F-909R) only one ASV was retrieved which cluster with a sequence from the metagenomic approach.

Most of the ASV sequences were positioned in two clusters (A and B) within the *Ca.* Brocadia genus. On one side, Cluster A grouped the most abundant ASVs of the S1 sample with *Ca.* Brocadia fulgida reference sequences (around 70% of relative abundance of ASVs retrieved with approaches b and c) and with two sequences retrieved from the metagenome (Full length AM metagenome 1 and Full length S1 metagenome 1). The dominant anammox in the S1 reactor was *Ca.* Brocadia fulgida. Anammox communities dominated by this specie have been reported in a variety of studies on mainstream effluent treated by anammox and PN/A processes, though, it was mainly detected by other methods [28,30,36]. So, to the authors' knowledge, there is a lack of DNA sequencing data available about the anammox "*Candidatus*" species present in mainstream anammox which allows a comparison of our results. The next ASVs in decreasing abundance order (12% in Fig. 4A and 17% in Fig. 4B) were bunched in the same clade as the environmental clone Pla PO55–3 sequence (GQ356105), and the sequence from the recently described "*Candidatus*" Brocadia pituitae [41]. On the other side, Cluster B involved the dominant ASVs of the AM sample (100%) which were also present in the S1 sample (relative abundance of 8% in Figs. 4A and 4B). These ASVs and two sequences retrieved from the metagenome (Full length AM metagenome 2 and 3) were bunched in a clade, close but in a different branch to the environmental clone ZMP-3 sequence (GQ175287) and distant to the reference's sequences of *Ca.* Brocadia sinica, *Ca.* Brocadia anammoxidans and *Ca.* Brocadia brasiliensis. Clusters C, D, E and F grouped sequences from other anammox genera: *Ca.* Kuenenia, *Ca.* Anammoxoglobus, *Ca.* Jettenia and *Ca.* Scalindua respectively. From these clusters, only in cluster C, sequences from ASVs (1% of relative abundance) and from the genome were positioned. As expected by the low coverage of this primer set, the sequences obtained with the primers 515F-909R detected only one ASV positioned in cluster B for the AM sample (Fig. 4C).

From this phylogenetic analysis, we were struck by the fact that the sequences from the entire anammox population of our reference reactor (AM) and, also detected in the S1 reactor (8% of relative abundance), were positioned into a clade that is not directly associated with any previously described species. According to Whitman (2016), sequence of genomic DNA may also serve as the type of material that unambiguously identifies the species. Herein, we propose a new anaerobic ammonium oxidation (anammox) bacterium, namely "*Candidatus*" Brocadia barcinensis. Representative sequences were submitted to GeneBank with accession numbers: MZ242076 and MZ242077. This result was preliminarily confirmed using the genomic centric approach from metagenome [43].

3.4. 16S rRNA and functional genes quantification by qPCR

To specifically quantify our target population, qPCR was performed targeting two functional genes: hszA and nirS. Moreover, a specific 16S rRNA primer set targeting all the anammox 16S rRNA genes was included in the analysis and first checked on a good performance *in silico* (see primer coverage in Table 3) and in practical test according to different authors [51,53]. In order to compare the values from the different reactors a boxplot was constructed, and statistical analysis was performed (Fig. 5). The results showed that 16S rRNA gene copies were significantly different for both reactors with higher values for the S1 reactor. In the enriched anammox reactor treating synthetic influent, values of 1.04×10^6- 1.17×10^6 gene copies ng^{-1} DNA of the 16S rRNA-amx gen were obtained. In the anammox reactor treating urban wastewater at mainstream conditions, values ranging from 2.32×10^5 to 2.58×10^5 gene copies ng^{-1} DNA of the 16S rRNA-amx gen were obtained. There were no statistically significant differences between the anammox marker genes (16S rRNA-amx and hzsA) in both reactors. Comparing both reactors, a lower abundance of 16S rRNA-amx was obtained in S1. Consistency in the anammox community quantification of either 16S-amx gene and hzsA gene on both reactors showed that these primers are a good choice for estimating our target and serve as a guide to further works that use anammox biomass for treating mainstream MWW. Regarding nirS gene, a low quantification was obtained in both reactors compared to the other studied genes. Also, a statistically different quantification between both reactors was obtained, being lower in the AM reactor. Han et al. [17] showed that for a sample of WWTP, nirS primer set presented 100% efficiency, though, it was able to detect a large portion of denitrifiers. Other works evaluating this gene, showed that it was absent in some genomes of *Ca.* Brocadia [60], therefore, it is not available for the detection and quantitative analysis of this genus [51], which is the main genus detected in our reactors.

Beyond the target gene, it is important to understand that the primers influence the PCR based methods for relative quantification (as we have shown on amplicon sequence analysis) and for absolute quantification

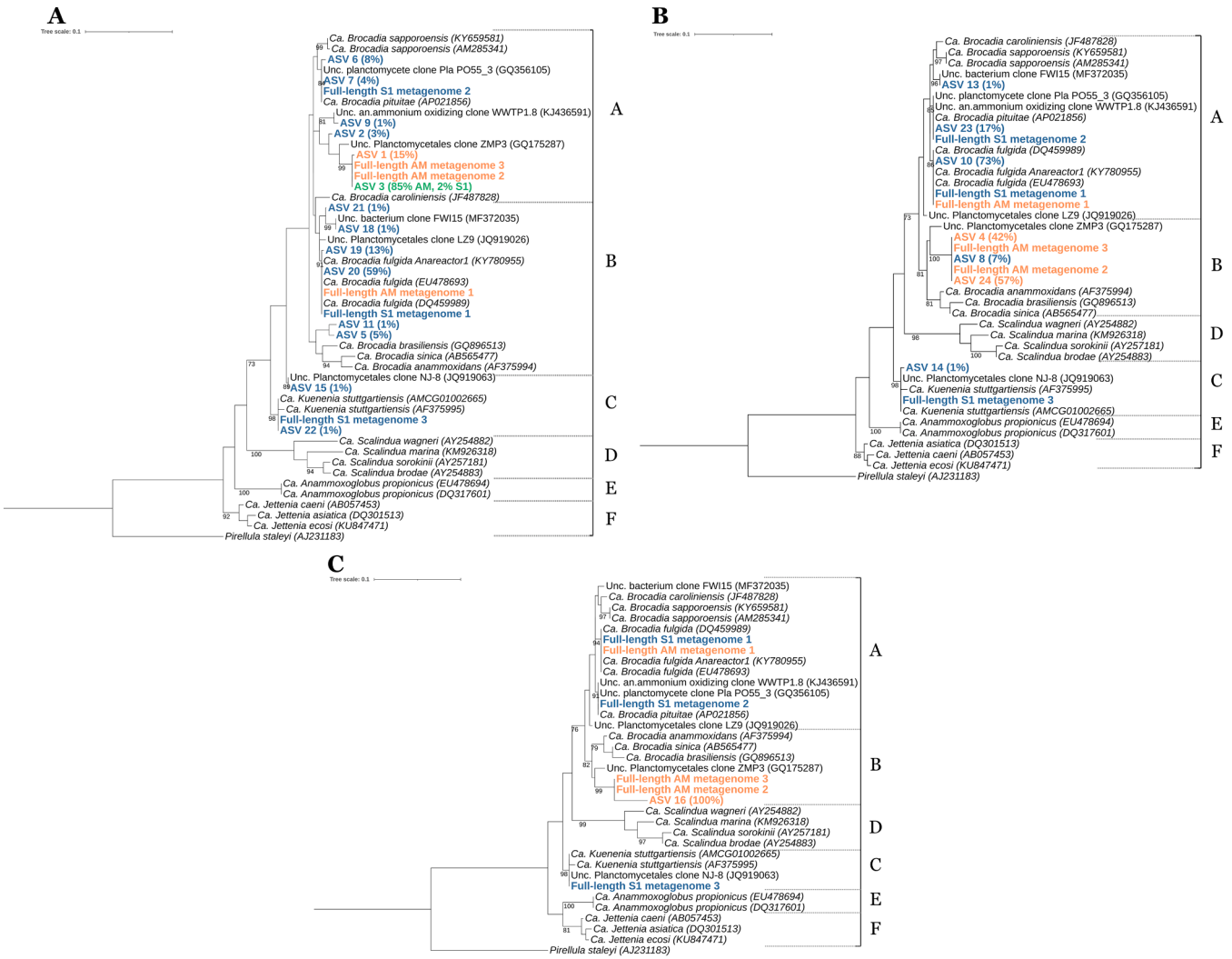

Fig. 4. Phylogenetic Maximum Likelihood trees based on 16S rRNA gene sequences from amplicon sequencing with primer set Amx368F-Amx820R (A), 515F-806R (B) and 515F-909R (C). ASVs of this study and full-length sequences of 16S rRNA gene reconstructed from metagenomes are shown in colors for each reactor. The relative abundance of each ASV is shown in parentheses. Accession numbers of the reference sequences and environmental clones of anammox bacteria are shown in parentheses. Bootstrap values (greater than or equal to 70%) based on 500 replications are shown at branch nodes. *Pirellula staleyi* was used as an outgroup for rooting trees. Bar indicates 0.01 substitutions per site.

[42,51]. Besides, the differences between *in silico* and practical application have been proved, which is why we pointed out that standardization is required in this field, before conducting more in-depth analyzes, such as changes in communities over time.

In addition to the primer bias, it is difficult to compare our results with similar studies. This, because in general, the results are reported as the relative abundance of certain microbial groups (i. e., AOB, NOB, others) normalized to the abundance of total bacteria. Considering the variability of the copy numbers of 16S rRNA gen per genome in bacteria [25], the estimated population from the 16S rRNA gene qPCR results have a tendency to differ from the true number of target populations. As an example, normalizing our data with the gene's copies of total bacteria a 13% and 0.3% of anammox bacteria abundance was obtained to the AM and S1 reactor, respectively. Thus, clearly underestimate the relatives' abundances of anammox bacteria in the anammox enriched culture (AM reactor). Also, the results should not be compared with other works, unless the same primers are used [42]. Furthermore, when the results have been reported as absolute quantification, since there is no consensus on the normalization of the data, they have been expressed as a function of the sample volume [42,49], ng of DNA [32]; this study), g of dry weight [17], among others.

4. Conclusions

This work serves as a basis for studying the microbial ecology and relationships in anammox reactors working at mainstream conditions. Thus, based on our previous analysis we are able to recommend amplicon sequencing with primer set 515F-806R if the research wants to determine anammox microorganisms. However, if the focus is on the anammox community relationships, a combination of amplicon sequencing with specific primers and qPCR, targeting 16S rRNA or hzsA genes, would be more reliable. Both approaches allowed a comparison to determine the community dynamics. Metagenomics approaches have also solved this goal but are more expensive to use for that purpose, but, with the drastic decrease in sequencing costs this approach will be used in a near future.

CRediT authorship contribution statement

P. Oyarzúa: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft. **P. Bovio-Winkler**: Methodology, Validation, Formal analysis, Writing – review & editing. **C. Etchebehere**: Conceptualization, Resources, Writing – review &

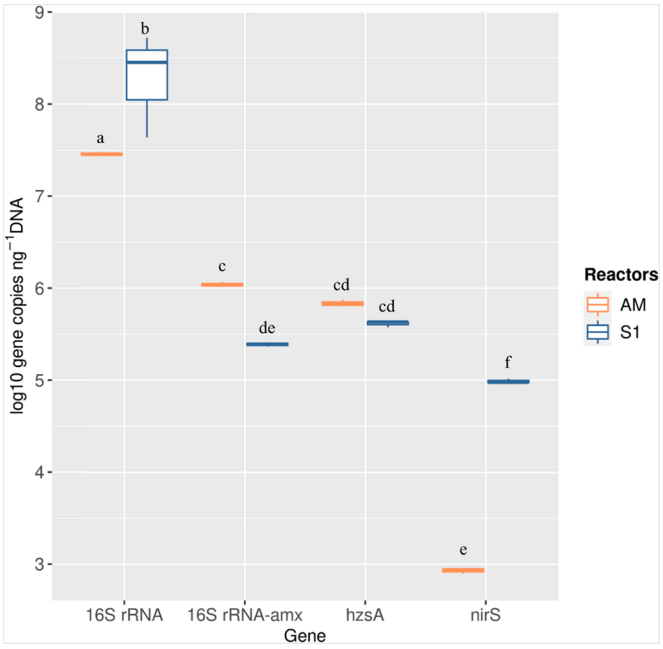

Fig. 5. Boxplot of absolute quantification of bacterial genes in anammox reactors by qPCR. 16S rRNA gene for total bacteria, 16S rRNA-amx for anammox bacteria, and functional genes hzsA and nirS were quantified. A logarithmic scale was chosen for the y-axis. The X on each box represents the mean value from triplicate qPCR reactions. The error bars stand for the standard deviations. Means with one letter in common are not significantly different ($p > 0.05$).

editing, Supervision. **M.E. Suárez-Ojeda**: Conceptualization, Resources, Writing – review & editing, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was funded by Spanish Ministry of Economy and Competitiveness: "HIPATIA: Towards the implementation of the concept of biorefinery and energy self-sustainability in an urban wastewater treatment plant" (CTQ2017-82404-R). P. Oyarzúa is funded by the National Agency for Research and Development (ANID) / Scholarship Program / DOCTORADO BECAS CHILE/2017- 72180362. C. Etchebehere and P. Bovio-Winkler are funded by SNI-ANII. P. Bovio-Winkler is also funded by CAP-CSIC.

References

[1] J. Bengtsson-Palme, M. Hartmann, K.M. Eriksson, C. Pal, K. Thorell, D.G.J. Larsson, R.H. Nilsson, metaxa2: improved identification and taxonomic classification of small and large subunit rRNA in metagenomic data, Mol. Ecol. Resour. 15 (2015) 1403–1414, https://doi.org/10.1111/1755-0998.12399.

[2] A.S. Bhattacharjee, S. Wu, C.E. Lawson, M.S.M. Jetten, V. Kapoor, J.W.S. Domingo, K.D. McMahon, D.R. Noguera, R. Goel, Whole-community metagenomics in two different anammox configurations: process performance and community structure, Environ. Sci. Technol. 51 (2017) 4317–4327, https://doi.org/10.1021/acs.est.6b05855.

[3] A.M. Bolger, M. Lohse, B. Usadel, Trimmomatic: a flexible trimmer for Illumina sequence data, Bioinformatics 30 (2014) 2114–2120, https://doi.org/10.1093/bioinformatics/btu170.

[4] B.J. Callahan, P.J. Mcmurdie, M.J. Rosen, A.W. Han, A.J.A. Johnson, S.P. Holmes, DADA2: high-resolution sample inference from Illumina amplicon data, Nat. Methods (2016) 13, https://doi.org/10.1038/nmeth.3869.

[5] S. Campanaro, L. Treu, P.G. Kougias, X. Zhu, Taxonomy of anaerobic digestion microbiome reveals biases associated with the applied high throughput sequencing strategies, Sci. Rep. (2018) 1–12, https://doi.org/10.1038/s41598-018-20414-0.

[6] A.W.Y. Chan, J. Naphtali, H.E. Schellhorn, High-throughput DNA sequencing technologies for water and wastewater analysis, Sci. Prog. 102 (2019) 351–376, https://doi.org/10.1177/0036850419881855.

[7] H. Chen, T. Liu, J. Li, L. Mao, J. Ye, X. Han, M.S.M. Jetten, J. Guo, Larger anammox granules not only harbor higher species diversity but also support more functional diversity, Environ. Sci. Technol. 54 (2020) 14664–14673, https://doi.org/10.1021/acs.est.0c02609.

[8] J. Chen, Y. Lu, Z. Ouyang, J. Zhang, Regulation of partial nitritation in constructed rapid infiltration system and analysis of microbial community structure, Pol. J. Environ. Stud. 29 (2019) 33–43, https://doi.org/10.15244/pjoes/99908.

[9] S. Cho, C. Kambey, V.K. Nguyen, Performance of anammox processes for wastewater treatment: a critical review on effects of operational conditions and environmental stresses, Water (2020) 12, https://doi.org/10.3390/w12010020.

[10] Z. rui Chu, K. Wang, X. kun Li, M. ting Zhu, L. Yang, J. Zhang, Microbial characterization of aggregates within a one-stage nitritation-anammox system using high-throughput amplicon sequencing, Chem. Eng. J. 262 (2015) 41–48, https://doi.org/10.1016/j.cej.2014.09.067.

[11] X. Dong, M. Strous, An integrated pipeline for annotation and visualization of metagenomic contigs, Front. Genet. 10 (2019) 1–10, https://doi.org/10.3389/fgene.2019.00999.

[12] R. Du, S. Cao, H. Zhang, X. Li, Y. Peng, Flexible nitrite supply alternative for mainstream anammox: advances in enhancing process stability, Environ. Sci. Technol. 54 (2020) 6353–6364, https://doi.org/10.1021/acs.est.9b06265.

[13] F. Durazzi, C. Sala, G. Castellani, G. Manfreda, D. Remondini, A.De Cesare, Comparison between 16S rRNA and shotgun sequencing data for the taxonomic characterization of the gut microbiota, Sci. Rep. (2021) 1–10, https://doi.org/10.1038/s41598-021-82726-y.

[14] I. Ferrera, O. Sánchez, Insights into microbial diversity in wastewater treatment systems: how far have we come? Biotechnol. Adv. 34 (2016) 790–802, https://doi.org/10.1016/j.biotechadv.2016.04.003.

[15] G. Gonzalez-Gil, R. Sougrat, A.R. Behzad, P.N.L. Lens, P.E. Saikaly, Microbial community composition and ultrastructure of granules from a full-scale anammox reactor, Microb. Ecol. 70 (2015) 118–131, https://doi.org/10.1007/s00248-014-0546-7.

[16] M. Hall, R.G. Beiko, 2018. Chapter 8 1849, 113–129.

[17] P. Han, U. Klümper, A. Wong, M. Li, J.G. Lin, Z. Quan, M. Denecke, J.D. Gu, Assessment of molecular detection of anaerobic ammonium-oxidizing (anammox) bacteria in different environmental samples using PCR primers based on 16S rRNA and functional genes, Appl. Microbiol. Biotechnol. 101 (2017) 7689–7702, https://doi.org/10.1007/s00253-017-8502-3.

[18] H.R. Harhangi, M. Le Roy, T. van Alen, B. lan Hu, J. Groen, B. Kartal, S.G. Tringe, Z.X. Quan, M.S.M. Jetten, H.J.M. Op den Camp, Hydrazine synthase, a unique phylomarker with which to study the presence and biodiversity of anammox bacteria, Appl. Environ. Microbiol. 78 (2012) 752–758, https://doi.org/10.1128/AEM.07113-11.

[19] A. Henriques, T. Cereija, A. MacHado, N. Cerca, In silico vs in vitro analysis of primer specificity for the detection of Gardnerella vaginalis, Atopobium vaginae and Lactobacillus spp, BMC Res. Notes 5 (2012) 2–5, https://doi.org/10.1186/1756-0500-5-637.

[20] B. lan Hu, P. Zheng, C. jian Tang, J. wei Chen, E. van der Biezen, L. Zhang, B. jie Ni, M.S.M. Jetten, J. Yan, H.Q. Yu, B. Kartal, Identification and quantification of anammox bacteria in eight nitrogen removal reactors, Water Res. 44 (2010) 5014–5020, https://doi.org/10.1016/j.watres.2010.07.021.

[21] E. Isanta, T. Bezerra, I. Fernández, M.E. Suárez-Ojeda, J. Pérez, J. Carrera, Microbial community shifts on an anammox reactor after a temperature shock using 454-pyrosequencing analysis, Bioresour. Technol. 181 (2015) 207–213, https://doi.org/10.1016/j.biortech.2015.01.064.

[22] X. Juan-Díaz, P. Julio, J. Carrera, Effective dampening of temperature effects in an anammox reactor treating real mainstream wastewater, J. Water Process Eng. (2020), https://doi.org/10.1016/j.jwpe.2020.101853.

[23] P. Junier, V. Molina, C. Dorador, O. Hadas, O.S. Kim, T. Junier, K.P. Witzel, J. F. Imhoff, Phylogenetic and functional marker genes to study ammonia-oxidizing microorganisms (AOM) in the environment, Appl. Microbiol. Biotechnol. 85 (2010) 425–440, https://doi.org/10.1007/s00253-009-2228-9.

[24] B. Kartal, L. Van Niftrik, J. Rattray, J.L.C.M.C.M. Van De Vossenberg, M.C. Schmid, J. Sinninghe Damsté, M.S.M.M. Jetten, M. Strous, Candidatus "Brocadia fulgida": an autofluorescent anaerobic ammonium oxidizing bacterium, FEMS Microbiol. Ecol. 63 (2008) 46–55, https://doi.org/10.1111/j.1574-6941.2007.00408.x.

[25] J.A. Klappenbach, P.R. Saxman, J.R. Cole, T.M. Schmidt, Rrndb: the ribosomal RNA operon copy number database, Nucleic Acids Res 29 (2001) 181–184, https://doi.org/10.1093/nar/29.1.181.

[26] A. Klindworth, E. Pruesse, T. Schweer, J. Peplies, C. Quast, M. Horn, F.O. Glöckner, Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies, Nucleic Acids Res. 41 (2013) 1–11, https://doi.org/10.1093/nar/gks808.

[27] S. Kumar, G. Stecher, K. Tamura, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol. 33 (2016) 1870–1874, https://doi.org/10.1093/molbev/msw054.

[28] M. Laureni, P. Falås, O. Robin, A. Wick, D.G. Weissbrodt, J.L. Nielsen, T.A. Ternes, E. Morgenroth, A. Joss, Mainstream partial nitritation and anammox: long-term process stability and effluent quality at low temperatures, Water Res. 101 (2016) 628–639, https://doi.org/10.1016/j.watres.2016.05.005.

[29] M. Laureni, D.G. Weissbrodt, I. Szivák, O. Robin, J.L. Nielsen, E. Morgenroth, A. Joss, I. Sziv, O. Robin, J. Lund, E. Morgenroth, A. Joss, ScienceDirect activity and growth of anammox biomass on aerobically pre-treated municipal wastewater, Water Res. 0 (2015) 325–336, https://doi.org/10.1016/j.watres.2015.04.026.
[30] C.E. Lawson, S. Wu, A.S. Bhattacharjee, J.J. Hamilton, K.D. McMahon, R. Goel, D. R. Noguera, Metabolic network analysis reveals microbial community interactions in anammox granules, Nat. Commun. 8 (2017) 1–12, https://doi.org/10.1038/ncomms15416.
[31] D. Li, C.M. Liu, R. Luo, K. Sadakane, T.W. Lam, MEGAHIT: An ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph, Bioinformatics 31 (2015) 1674–1676, https://doi.org/10.1093/bioinformatics/btv033.
[32] X.R. Li, B. Du, H.X. Fu, R.F. Wang, J.H. Shi, Y. Wang, M.S.M. Jetten, Z.X. Quan, The bacterial diversity in an anaerobic ammonium-oxidizing (anammox) reactor community, Syst. Appl. Microbiol. 32 (2009) 278–289, https://doi.org/10.1016/j.syapm.2009.03.002.
[33] W. Liu, X. Ji, J. Wang, D. Yang, Y. Shen, C. Chen, F. Qian, P. Wu, Microbial community response to influent shift and lowering temperature in a two-stage mainstream deammonification process, Bioresour. Technol. 262 (2018) 132–140, https://doi.org/10.1016/j.biortech.2018.04.082.
[34] Y.X. Liu, Y. Qin, T. Chen, M. Lu, X. Qian, X. Guo, Y. Bai, A practical guide to amplicon and metagenomic analysis of microbiome data, Protein Cell (2020), https://doi.org/10.1007/s13238-020-00724-8.
[35] R. Logares, S. Sunagawa, G. Salazar, F.M. Cornejo-Castillo, I. Ferrera, H. Sarmento, P. Hingamp, H. Ogata, C. de Vargas, G. Lima-Mendez, J. Raes, J. Poulain, O. Jaillon, P. Wincker, S. Kandels-Lewis, E. Karsenti, P. Bork, S.G. Acinas, Metagenomic 16S rDNA Illumina tags are a powerful alternative to amplicon sequencing to explore diversity and structure of microbial communities, Environ. Microbiol. 16 (2014) 2659–2671, https://doi.org/10.1111/1462-2920.12250.
[36] T. Lotti, R. Kleerebezem, Z. Hu, B. Kartal, M.K. De Kreuk, C. Van Erp Taalman Kip, J. Kruit, T.L.G. Hendrickx, M.C.M. Van Loosdrecht, Pilot-scale evaluation of anammox-based mainstream nitrogen removal from municipal wastewater, Environ. Technol. 36 (2015) 1167–1177, https://doi.org/10.1080/09593330.2014.982722.
[37] A.V. Mardanov, A.V. Beletsky, N.V. Ravin, E.A. Botchkova, Y.V. Litti, A. N. Nozhevnikova, Genome of a novel bacterium "candidatus jettenia ecosi" reconstructed from the metagenome of an anammox bioreactor, Front. Microbiol. 10 (2019) 1–12, https://doi.org/10.3389/fmicb.2019.02442.
[38] Y. Miao, J. Zhang, Y. Peng, S. Wang, An improved start-up strategy for mainstream anammox process through inoculating ordinary nitrification sludge and a small amount of anammox sludge, J. Hazard. Mater. 384 (2020), 121325, https://doi.org/10.1016/j.jhazmat.2019.121325.
[39] C.S. Miller, B.J. Baker, B.C. Thomas, S.W. Singer, J.F. Banfield, EMIRGE: reconstruction of full-length ribosomal genes from microbial community short read sequencing data, Genome Biol. (2011) 12, https://doi.org/10.1186/gb-2011-12-5-r44.
[40] A. Nejidat, D. Diaz-Reck, N. Massalha, A. Arbiv, A. Dawas, C. Dosoretz, I. Sabbah, Abundance and diversity of anammox bacteria in a mainstream municipal wastewater treatment plant, Appl. Microbiol. Biotechnol. 102 (2018) 6713–6723, https://doi.org/10.1007/s00253-018-9126-y.
[41] T. Okubo, A. Toyoda, K. Fukuhara, I. Uchiyama, Y. Harigaya, M. Kuroiwa, T. Suzuki, Y. Murakami, Y. Suwa, H. Takami, The physiological potential of anammox bacteria as revealed by their core genome structure, DNA Res 28 (2021) 1–12, https://doi.org/10.1093/dnares/dsaa028.
[42] L. Orschler, S. Agrawal, S. Lackner, On resolving ambiguities in microbial community analysis of partial nitritation anammox reactors, Sci. Rep. 9 (2019) 1–10, https://doi.org/10.1038/s41598-019-42882-8.
[43] P. Oyarzúa, P. Bovio-Winkler, C. Etchebehere, M.E. Suárez-Ojeda, 2021. Metabolic potential of metagenome-assembled genomes (MAGs) in two anammox reactors working at different operating conditions. In preparation.
[44] R. Poretsky, L.M. Rodriguez-R, C. Luo, D. Tsementzi, K.T. Konstantinidis, Strengths and limitations of 16S rRNA gene amplicon sequencing in revealing temporal microbial community dynamics, PLoS One (2014) 9, https://doi.org/10.1371/journal.pone.0093827.
[45] E. Pruesse, J. Peplies, F.O. Glöckner, SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes, Bioinformatics 28 (2012) 1823–1829, https://doi.org/10.1093/bioinformatics/bts252.
[46] C. Reino, M.E. Suárez-Ojeda, J. Pérez, J. Carrera, Stable long-term operation of an upflow anammox sludge bed reactor at mainstream conditions, Water Res. 128 (2018) 331–340, https://doi.org/10.1016/j.watres.2017.10.058.
[47] M. Schmid, S. Schmitz-Esser, M. Jetten, M. Wagner, ^{16}S–^{23}S rDNA intergenic spacer and 23S rDNA of anaerobic ammonium-oxidizing bacteria: Implications for phylogeny and in situ detection, Environ. Microbiol. 3 (2001) 450–459, https://doi.org/10.1046/j.1462-2920.2001.00211.x.
[48] M. Strous, J.J. Heijnen, J.G. Kuenen, M.S.M. Jetten, The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms, Appl. Environ. Microbiol. 50 (1998) 589–596, https://doi.org/10.1007/s002530051340.
[49] Z. Wang, S.Q. bin, Ni, J. Zhang, T. Zhu, Y. guang Ma, X. lin Liu, Q. Kong, M. sheng Miao, Gene expression and biomarker discovery of anammox bacteria in different reactors, Biochem. Eng. J. 115 (2016) 108–114, https://doi.org/10.1016/j.bej.2016.09.002.
[50] M.K.H. Winkler, R. Kleerebezem, M.C.M. Van Loosdrecht, Integration of anammox into the aerobic granular sludge process for main stream wastewater treatment at ambient temperatures, Water Res. 46 (2012) 136–144, https://doi.org/10.1016/j.watres.2011.10.034.
[51] Y. Yang, M. Li, H. Li, X.Y. Li, J.G. Lin, M. Denecke, J.D. Gu, Specific and effective detection of anammox bacteria using PCR primers targeting the 16S rRNA gene and functional genes, Sci. Total Environ. (2020), https://doi.org/10.1016/j.scitotenv.2020.139387.
[52] Z. Zhao, J. Luo, B. Jin, J. Zhang, B. Li, B. Ma, X. An, S. Zhang, B. Shan, Analysis of bacterial communities in partial nitritation and conventional nitrification systems for nitrogen removal, Sci. Rep. 8 (2018) 1–9, https://doi.org/10.1038/s41598-018-30532-4.
[53] Z. Zhou, Q. Wei, Y. Yang, M. Li, J.D. Gu, Practical applications of PCR primers in detection of anammox bacteria effectively from different types of samples, Appl. Microbiol. Biotechnol. 102 (2018) 5859–5871, https://doi.org/10.1007/s00253-018-9078-2.
[54] S. Cao, A. Oehmen, Y. Zhou, Denitrifiers in Mainstream Anammox Processes: Competitors or Supporters? Environ. Sci. Technol. 53 (2019) 11063–11065, https://doi.org/10.1021/acs.est.9b05013.
[55] J.G. Caporaso, C.L. Lauber, W.A. Walters, D. Berg-Lyons, J. Huntley, N. Fierer, S. M. Owens, J. Betley, L. Fraser, M. Bauer, N. Gormley, J.A. Gilbert, G. Smith, R. Knight, Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms, ISME J. 6 (2012) 1621–1624, https://doi.org/10.1038/ismej.2012.8.
[56] S. Cho, C. Kambey, V.K. Nguyen, Performance of anammox processes for wastewater treatment: Acritical review on effects of operational conditions and environmental stresses, Water (Switzerland) 12 (2020), https://doi.org/10.3390/w12010020.
[57] A.Z. Gu, P.B. Pedros, A. Kristiansen, A. Onnis-Hayden, A. Schramm, Nitrifying Community Analysis in a Single Submerged Attached-Growth Bioreactor for Treatment of High-Ammonia Waste Stream, Water Environ. Res. 79 (2007) 2510–2518, https://doi.org/10.2175/106143007x254566.
[58] M. Li, T. Ford, X. Li, J.D. Gu, Cytochrome cd1-containing nitrite reductase encoding gene nirS as a new functional biomarker for detection of anaerobic ammonium oxidizing (anammox) bacteria, Environ. Sci. Technol. 45 (2011) 3547–3553, https://doi.org/10.1021/es103826w.
[59] Y. Narita, L. Zhang, Z. Kimura, M. Ali, T. Fujii, S. Okabe, Enrichment and physiological characterization of an anaerobic ammonium-oxidizing bacterium 'Candidatus Brocadia sapporoensis', Syst. Appl. Microbiol. 40 (2017) 448–457, https://doi.org/10.1016/j.syapm.2017.07.004.
[60] M. Oshiki, M. Ali, K. Shinyako-Hata, H. Satoh, S. Okabe, Hydroxylamine-dependent anaerobic ammonium oxidation (anammox) by "Candidatus Brocadia sinica", Environ. Microbiol. 18 (2016) 3133–3143, https://doi.org/10.1111/1462-2920.13355.
[61] S. Stubner, Enumeration of 16S rDNA of Desulfotomaculum lineage 1 in rice field soil by real-time PCR with SybrGreenTM detection, J. Microbiol. Methods 50 (2002) 155–164, https://doi.org/10.1016/S0167-7012(02)00024-6.
[62] H.P. Trinh, S.-H. Lee, G. Jeong, H. Yoon, H.-D. Park, Recent developments of the mainstream anammox processes: Challenges and opportunities, J. Environ. Chem. Eng. 9 (2021), 105583, https://doi.org/10.1016/j.jece.2021.105583.
[63] I. Tsushima, Y. Ogasawara, T. Kindaichi, H. Satoh, S. Okabe, Development of high-rate anaerobic ammonium-oxidizing (anammox) biofilm reactors, Water Res. 41 (2007) 1623–1634, https://doi.org/10.1016/j.watres.2007.01.050.
[64] N.N. Tuan, Y.C. Chang, C.P. Yu, S.L. Huang, Multiple approaches to characterize the microbial community in a thermophilic anaerobic digester running on swine manure: A case study, Microbiol. Res. 169 (2014) 717–724, https://doi.org/10.1016/j.micres.2014.02.003.
[65] P. Yarza, P. Yilmaz, E. Pruesse, F.O. Glöckner, W. Ludwig, K.-H. Schleifer, W. B. Whitman, J. Euzéby, R. Amann, R. Rosselló-Móra, Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences, Nat. Rev. Microbiol. 12 (2014) 635–645, https://doi.org/10.1038/nrmicro3330.