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Nanoarchitected structure and population dynamics of anaerobic ammonium oxidizing (anammox) bacteria in a wastewater treatment plant

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ABSTRACT

Studies on microbial community and population dynamics are essential for the successful development, monitoring and operation of biological wastewater treatment systems. Especially for novel or sustainable systems such as the anaerobic ammonium oxidizing (anammox) process that are not yet well explored. Here we collected granular microbial sludge samples and investigated a community of anammox bacteria over a period of four years, divided into eight stages in a full scale simultaneous partial nitrification, anammox and denitrification (SNAD) process for treating landfill leachate. Specific qPCR primers were designed to target and quantify the two most abundant anammox species, *Candidatus Kuenenia stuttgartiensis* (KS) and *Candidatus Brocadia anammoxidans* (BA). The two species were monitored and could explain the dynamic shift of the anammox community corresponding to the operating conditions. Using the newly designed KS-specific primer (KSqF3/KSqR3) and BA-specific primer (BAqF/BAqR), we estimated the amounts of KS and BA to be in the range of 6.2×10^6 to 5.9×10^8 and 1.1×10^5 to 4.1×10^7 copies μg^{-1} DNA, respectively. KS was found to be the dominant species in all anammox granules studied and played an important role in the formation of granules. The KS/BA ratio was positively correlated to the size of granules in the reactor and ammonia nitrogen removal efficiency of the treatment plant.

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Nomenclature

AAOB	aerobic ammonium oxidizing bacteria
Anammox	anaerobic ammonium oxidizing
AOA	ammonia-oxidizing archaea
AOB	ammonia-oxidizing bacteria
BA	<i>Candidatus Brocadia anammoxidans</i>
EB	eubacteria
HRT	hydraulic retention time
KS	<i>Candidatus Kuenenia stuttgartiensis</i>
MLSS	mixed liquor suspended solids
MLVSS	mixed liquor volatile suspended solids

NOB	nitrate oxidizing bacteria
PCR	polymerase chain reaction
qPCR	quantitative PCR
RE	removal efficiency
SNAD	simultaneous partial nitrification, anammox and denitrification
SRT	sludge retention time
SS	suspended solids
TA	total species of anammox bacteria
VLR	volumetric loading rate
VSS	volatile suspended solids

1. Introduction

Anaerobic ammonium-oxidizing (anammox) bacteria participate in the microbial nitrogen cycle that connects nitrite reduction and ammonium oxidation under anoxic conditions (Strous et al., 1998). The anammox process is a more eco-friendly and cost-effective technology compared to the traditional nitrification and denitrification process for nitrogen removal (Jetten et al., 1997; Kartal et al., 2010; Kartal et al., 2011). The draft genome and biochemistry of the anammox pathway has been studied by metagenomics and metaproteomics analyses of enriched anammox bacteria (Kartal et al., 2011; Jetten et al., 2009; Lawson et al., 2017; Bagchi et al., 2016; Speth et al., 2016; Speth et al., 2017; Speth et al., 2015; Gori et al., 2013; Russ et al., 2012; Strous et al., 2006). For wastewater treatment systems, the anammox process needs to be combined with partial nitrification either in a two reactor system such as SHARON-Anammox or a one reactor system such as the CANON (completely autotrophic nitrogen removal over nitrite) process (Jetten et al., 1997; Sliekers et al., 2002; Guo et al., 2008; Park et al., 2010; Vazquez-Padin et al., 2010). However, these nitrogen removal systems must be functionally stable over a long period of time, which depends on the activity of the microbial communities involved (Gentile et al., 2007). Therefore, studies on the dynamics of microbial communities are necessary for designing a functionally successful and stable wastewater treatment plant.

Molecular biology techniques such as real-time quantitative polymerase chain reaction (qPCR), and fluorescence in situ hybridization (FISH) have been used successfully to detect and quantify anammox bacteria (Jaeschke et al., 2009; Kuenen, 2008; Lam et al., 2009; Li et al., 2009; Ni et al., 2010; Schmid et al., 2008; Winkler et al., 2012; Zhu et al., 2011). Several studies have been reported on the biodiversity and abundance of aerobic and anaerobic ammonium-oxidizing bacteria in different samples using the qPCR technique (Lam et al., 2009; Ni et al., 2010; Zhu et al., 2011). Some studies have indicated that qPCR analyses of samples from anammox reactors show that anammox bacteria may comprise approximately 50 % of the total bacterial 16S rRNA genes, but that aerobic ammonium-oxidizing bacteria (AOB) still may make 3% of these anaerobic community (Quan et al., 2008). Other qPCR based studies have found about 16 % of anammox bacteria (*Candidatus Kuenenia stuttgartiensis* and *Candidatus Jettenia* were detected) and less than 1% of AAOB in an anammox reactor. By now qPCR is a widely accepted tool to study and quantify the anammox and nitrifying bacteria in various ecosystems such as soil, marine, and high temperature habitats (Lam et al., 2009; Dang et al., 2010; Humbert et al., 2010; Lam et al., 2007; Li et al., 2010; Li et al., 2011a; Zhu et al., 2013).

Recently, some studies have shown that the growth rate of anammox bacteria can be improved with doubling times as low as 3 days (Zhang et al., 2017; Liu and Ni, 2015). However, so far not much information is available on the dynamic shifts and interactions with other N-cycle bacteria present in the bioreactors. In order to understand the microbial community compositions of nitrifiers, denitrifiers, and anammox bacteria in more detail, we monitored the microbial N-cycle

communities for four years in a full-scale simultaneous partial nitrification, anammox, and denitrification (SNAD) process for treating landfill leachate. In our earlier work, anammox bacteria were observed by FISH and PCR in the leachate treatment reactor (Wang et al., 2010; Huang et al., 2013). In this study, we report the dynamic shift of the anammox community corresponding to the operating conditions of the leachate treatment reactor, using specifically designed primers for targeting the two most dominant anammox species, *Kuenenia stuttgartiensis* (KS) and *Brocadia anammoxidans* (BA), in relation to other community members. The correlations between the granule size and the ammonium nitrogen removal efficiency and KS/BA ratio were also evaluated in this study, and the implications for full-scale practice were discussed.

2. Materials and methods**2.1. Leachate treatment plant**

The present study was conducted based on a full-scale landfill-leachate treatment plant located in Taiwan. This treatment plant has been in operation since 1992 with an average flow of 304 m³ d⁻¹. The leachate treatment plant is an integrated fixed-film activated sludge plant with an aeration tank followed by a clarifier. The plant simultaneous partial nitrification, anammox and denitrification processes do occur, which involves a complex nitrogen-related microbial community (Wang et al., 2010). The working volume, hydraulic retention time (HRT) and suspended solids retention time (SRT) of the aeration tank were 384 m³, 1.3 d and 12–18 d, respectively. The average concentrations of mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were 2110 mg L⁻¹ and 1560 mg L⁻¹, respectively, in the aeration tank. A blower into the aeration tank delivered the air by fine bubble tubular diffusers. The fine bubble diffusing system in the aeration tank provided both mixing and oxygen transfer. The DO concentration of the aeration tank was maintained at approximately 0.3 mg L⁻¹. The temperature was fluctuated under the influence of ambient temperature within 22–30 °C during sampling periods.

2.2. Sampling and DNA extraction

The anammox bacteria grew in the forms of granules and suspended solids. The granules and suspended solid growth samples were taken from a full-scale treatment aeration tank and preserved in completely sealed tubes. The samples were stored in a refrigerator at 4 °C until the DNA extraction step. The DNA extraction procedure was performed within 48 h after sampling.

Samples were collected during the four-year period divided into eight different stages to study granule sizes and microbial community. Granular sludge changed in color and size with time, and both red granules and black granules were collected in different time periods. In stage II, granular sludge was disintegrated and only suspended sludge was collected. The total genomic DNA of the sample was extracted using the PowerSoil® DNA isolation kit (MO BIO Laboratories, USA) and

UltraClean Microbial DNA isolation kit (MO BIO Laboratories, USA). The DNA concentration was determined by a Gene Quant pro (Amersham Biosciences, Pittsburgh, PA, USA).

2.3. PCR and qPCR procedures

To design the primer set, we collected the 16S rRNA sequences of anammox bacteria through GenBank database. Sequences were aligned by CLUSTALW (<http://www.genome.jp/tools/clustalw/>). Candidate primers were chosen from a highly conserved region, with the following characteristics: average length (20 base pairs), GC content (50–60 %), melting temperature (55–70 °C), and less secondary structure (confirmed through the Oligo Analyzer program offered by Integrated DNA Technologies). The specificity of the primers was checked by cloning and *in silico* analysis (Primer-BLAST). The primer sets KSqF3/KSqR3 and BAqF/BAqR were used to detect KS and BA, respectively.

PCR was performed in a 48 well MJ Mini™ Gradient Thermal-Cycler (BIO-RAD, USA). Primer sets are listed in Table 1. Each reaction was performed in a 50 µL volume containing 10 µL of GoTaq® Flexi Buffer, 5 µL of MgCl₂ (25 mM), 1 µL of PCR Nucleotide Mix (10 mM each dNTP), 1 µL of DNA template (<0.5 µg), 1 µL of each primer (10 µM), 0.25 µL of GoTaq® Hot start polymerase (5 u/µL), and 30.75 µL of sterilized water (Promega, USA). The cycling parameters were 5 min at 95 °C and 35 cycles of 30 s at 95 °C, 30 s at 57 °C for 11f/1512r and CTO189fA/B + CTO189fC (2:1 ratio)/CTO654r, 56 °C for BAqF/BAqR, 58 °C for Nitro-1198f/Nitro-1423r, 60 °C for NSR-1113f/NSR-1264r, 52 °C for KSqF3/KSqR3, 53 °C for amoA-1F/amoA-2R, 51 °C for nirS-1F/nirS-6R, cnorB-2F/cnorB-6R, 16S-5'/16S-3', and AnnirS379F/AnnirS821R, and 1 min at 72 °C, and finally 5 min at 72 °C. The PCR results were confirmed by agarose gel electrophoresis and DNA sequencing.

The most commonly used DNA-binding dye for qPCR is SYBR Green-based dye. The fluorescence increased up to 1,000-fold when binding with double strand DNA compared to free dye. In this study, quantification was based on the fluorescent dye SsoFast™EvaGreen® Supermix (BIO-RAD, USA), performed in 48-well optical reaction plates placed in a 48 well MJ Mini™ Gradient Thermal-Cycler (BIO-RAD, USA). Primer

sets are listed in Table 1. Each reaction was performed in a 10 µL volume containing 1 µL of DNA template (5 ng), 0.5 µL (<0.5 µM) of each primer set, 3 µL of sterilized water and 5 µL of SsoFast™EvaGreen® Supermix (BIO-RAD, USA). The cycling parameters were 30 s at 95 °C and 40 cycles of 5 s at 95 °C and 5 s at 52 °C for KSqF3/KSqR3, 56 °C for BAqF/BAqR, 54 °C for BACT1369F/PROK1492R, 58 °C for Amx809F/Amx1066R, followed by a dissociation stage (95 °C for 15 s, 65 °C for 15 s, followed by a slow ramp to 95 °C). There were no detectable peaks associated with primer-dimer artifacts and no other nonspecific PCR amplification products were observed during the melting process. Specificity of the qPCR product was confirmed by agarose gel electrophoresis and only the expected product size was obtained. The qPCR amplification efficiencies were 95–105 % for BAqF/BAqR and KSqF3/KSqR3. The R² value was greater than 0.99 for all of the standard curves, as shown in Supplementary Figure S1. The standard curve was constructed using a cloned 16S rRNA fragment of anammox bacteria in the pGEM-T easy vector (Promega, USA).

2.4. Chemical analyses

The pH and ORP in the landfill-leachate treatment plant were monitored online using the digital pH and ORP meters (Suntex PC320, Taiwan), respectively. All chemical analyses including ammonium, nitrite, nitrate, COD, and BOD were performed according to the Standard Methods (Rice et al., 2012). Ammonium and nitrite concentrations were determined by colorimetric methods, whereas nitrate was measured spectrophotometrically. The organic matter content of the sample was expressed as COD and BOD. Moreover, the solids in the aeration tank including suspended solids (SS), volatile suspended solids (VSS), MLSS and MLVSS were determined according to the Standard Methods.

3. Results and discussion

3.1. Detection of bacterial community with two primer sets by using PCR

The results of PCR reactions showed that the bacterial community in

Table 1
List of primers used in this study.

Target	Primer	Sequence (5'→3')	Annealing temp (°C)	Reference
Most bacteria	11F	GTTTGATCCTGGCTCAG	57	(Amann et al., 1995)
	1512R	GGYTACCTTGTTACGACTT		
	BACT1369F	CGGTGAATACGTTTCYCGG	54	(Suzuki et al., 2000)
AOB	PROK1492R	GGWTACCTTGTTACGACTT		
	CTO189fA/B	GGAGRAAAGCAGGGGATCG	57	(Kowalchuk et al., 1997)
	CTO189fC	GGAGGAAAGTAGGGGATCG		
	CTO654r	CTAGCYTTGTAGTTTCAAACGC		
	amoA-1F	GGGGTTTCTACTGGTGGT	53	(Rotthauwe et al., 1997)
NOB	amoA-2R	CCCCTCKGSAAAGCCTTCTTC		
	Nitro-1198f	ACCCCTAGCAAATCTCAAAAAACCG	58	(Knapp and Graham, 2007)
	Nitro-1423r	CTTCACCCAGTCGCTGACC		
	NSR-1113f	CCTGCTTTCAGTTGCTACCG	60	(Dionisi et al., 2002)
Denitrifier	NSR-1264 r	GTTTGCAGCGCTTTGTACCG		
	nirS-1F	CCTAYTGGCCGCCRCART	51	(Braker et al., 1998)
	nirS-6R	CGT TGAACITRCCGGT		
	cnorB-2F	GACAAGNNNTACTGGTGGT	51	(Braker and Tiedje, 2003)
Most Anammox	cnorB-6R	GAANCCCCANACNCCNGC		
	16S-5'	TGGCGGCGTGGTTTAGGC	51	(Fujii et al., 2002)
	16S-3'	GGTTACCTTGTTACGACT		
	Amx809F	GCCGTAACGATGGGCACT	58	(Tsushima et al., 2007a)
	Amx1066R	AACGCTCACGACAGAGCTG		
KS	AnnirS379F	TCTATCGTTGCATCGCATTT	51	(Li et al., 2011b)
	AnnirS821R	GGATGGGTCTTGATAAACA		
	KSqF3(82–103)	GCCTTTGAGATGGGAATAACTG	52	This study
	KSqR3(202–182)	CAAGGAACAGAATTCCTAGCA		
BA	BAqF (620–641)	AATGGCGGCCGAAACTGCATGG	56	This study
	BAqR (896–878)	AGCCTTGGCGCCGTACTCC		

K = G or T; R = A or G; S = G or C; Y = C or T; N = A, C, G or T.

AOB: ammonia-oxidizing bacteria; NOB: nitrate oxidizing bacteria; KS: *Candidatus Kuenenia stuttgartiensis*; BA: *Candidatus Brocadia anammoxidans*.

the investigated full-scale landfill-leachate treatment reactor comprised of AOB (lane: CTO, amoA), NOB (lane: NSR, nitro), denitrifying bacteria (lane: nirS), and anammox bacteria (lane: 16s-3'-16s-5', AnnirS, KS, BA) (Fig. 1), suggesting that all functional bacterial populations necessary for a successful SNAD system were present. Primer set AnnirS379F-AnnirS821R, designed to target anammox bacteria of *Kueenia* and *Scalindua* genera, showed a positive band on the lane of AnnirS, while primer set KSqF3-KSqR3, specifically targeting KS showed a clear positive result on the lane of KS. Therefore, the KS specific primers designed in this study were assumed to be suitable to detect KS via PCR. Although the band on the lane of BA was relatively weak, the band was detected at the expected position. The relatively weak signal of BA compared to KS may be due to the lower concentration of BA in this system (see also next section).

3.2. The variation in community structure of anammox bacteria with time

A series of quantitative PCR were done to quantify the anammox bacteria over time. The primers were based on the sequences of cloned 16S rRNA of KS and BA. The quantification range of KSqF3-KSqR3 spanned seven orders of magnitude, from 1.5×10^4 to 1.5×10^{10} copies, and eight orders of magnitude, from 1.4×10^3 to 1.4×10^{10} copies were obtained for BAqF-BAqR by setting the lowest concentration of template according to the specification of the supplier. The detection limit for KS specific primer and BA specific primer were 1.5×10^8 copies μg^{-1} and 1.4×10^7 copies μg^{-1} DNA, respectively. The quantification based on 16S rRNA of bacteria (EB) and total species of anammox bacteria (TA) was done according to Suzuki et al. (2000) and Tsushima et al. (2007a, 2007b) (Suzuki et al., 2000; Tsushima et al., 2007a; Tsushima et al., 2007b). The average copy numbers of 16S rRNA in bacterial and an anammox cell have been assumed to be 3.6 copies and 1 copy, respectively (Strous et al., 2006; Harms et al., 2003).

Table 2 shows the copy number of each sample per μg of extracted DNA. For granules collected in stage I, 2.6×10^8 or 1.8×10^8 copies of anammox bacteria per μg DNA were detected in red granules and black granules, respectively. Although the concentration of anammox cells had dropped in the sample collected in stage V, still 1.6×10^7 copies per μg DNA could be detected. Concentration of anammox bacteria was relatively higher in this full-scale landfill-leachate treatment plant during the sampling period compared to well controlled anammox reactors described by Quan et al. (2008) and Li et al. (2009) (Li et al., 2009; Quan et al., 2008) or in other habitats such as black sea suboxic zone, oilfield production water and mangrove sediments (Lam et al., 2007; Li et al., 2010; Li et al., 2011a) (Table 3).

After this initial screen, we focused our monitoring on two anammox species, KS and BA. By applying the newly designed KS-specific primer to qPCR, the estimated amount of KS was in the range of 6.7×10^5 to 5.9×10^8 copies μg^{-1} DNA (Table 2). The amount of KS was relatively high in stage I, stage III and stage VI. Interestingly, the ratio of TA to EB was relatively higher in stage I (51.1 %), stage III (90.1 %) and stage VI (93.1 %), while relatively lower during stages IV and VIII (8.9–15.1%) (Table 4). In addition, the dynamic changes of KS to TA ratio were highly correlated with TA to EB ratio. From Table 4, relatively high ratio of KS to TA occurs in stage I (92.0 %), stage III (90.0 %), while the lowest in stage VIII (0.66 %). On the other hand, the quantitative analysis of BA was performed with the newly designed BA-specific primer. The estimated copy number of BA per μg genomic DNA was in the range of 1.1×10^5 to 1.9×10^8 with the highest amount occurring in stage VIII and lowest amount occurring in stage V (Table 2). The ratio of BA to TA is always under 10.7 % during stage I to stage VII, and even less than 0.2 % in stage I and stage VI, showing that BA is the less dominant anammox species in the community at initial stages (Table 4). These results indicate that anammox bacteria comprise a substantial part of the total bacteria in this system, and that both KS and BA are detectable with the newly designed primer sets with high efficiency and specificity as previous work (Han et al., 2013). The dominant genus of anammox population shifted from *Candidatus Kueenia* to *Candidatus Brocadia*, consisting with the lower ammonium strength at stage VII. In mature granules the ratios of TA to EB were higher than 50 %, and KS was found to be dominant species.

3.3. The characteristics of water

Water samples were collected from the aeration tank on a regular basis, and the influent/effluent characteristics of the aeration tank were analyzed (Table S1 for detail characteristics). The pH values in the influent and effluent were 7.8 and 7.0, respectively. The decline in pH was observed due to a substantial partial nitrification reaction, which produced acidity and consumed significant alkalinity. The influent concentrations of nitrogen and organics (COD) varied during the studied period as real landfill leachate is being treated in the plant. These variations in influent might be the reason for the dynamic changes of KS to TA ratio, and TA to EB ratio. The average influent COD was varied between ~ 800 to 1000 mg L^{-1} in stage I to V, and was quite low ~ 600 , ~ 300 and $\sim 500 \text{ mg L}^{-1}$ during stage VI, stage VII and stage VIII, respectively. The average influent $\text{NH}_4^+\text{-N}$ concentration was $\sim 500 \text{ mg L}^{-1}$ till stage VI and after this it decreased to $\sim 300 \text{ mg L}^{-1}$ in stage VIII. The total COD was consumed mostly by denitrification.

Fig. 1. Results of PCR by performing agarose gel electrophoreses. Lane 1 to lane 12 represents the DNA marker, and primer set to target the following genes: 16S rRNA of most bacteria, 16S rRNA of AOB, amoA of AOB, 16S rRNA of *Nitrospira* sp. (NOB), 16S rRNA of *Nitrobacter* sp. (NOB), nirS of denitrifier, cnorB of denitrifier, 16S rRNA of most anammox, nirS of most anammox, 16S rRNA of KS and 16S rRNA of BA, respectively. The arrowhead indicated the expected size of PCR product.

Table 2
Bacterial and Anammox quantification analyses of the 16S rRNA gene.

Stage	Sample	EB ^a	TA ^a	KS ^a	BA ^a
I	Red granule	$1.86 \pm 0.62 \times 10^9$	$2.64 \pm 0.85 \times 10^8$	$2.43 \pm 0.65 \times 10^8$	$4.61 \pm 1.71 \times 10^5$
I	Black granule	$8.82 \pm 0.42 \times 10^8$	$1.78 \pm 0.01 \times 10^8$	$1.46 \pm 0.01 \times 10^8$	$1.41 \pm 0.31 \times 10^5$
III	Red granule	$2.63 \pm 0.01 \times 10^9$	$6.58 \pm 0.01 \times 10^8$	$5.92 \pm 0.01 \times 10^8$	$4.41 \pm 0.01 \times 10^7$
IV	Red granule	$7.75 \pm 0.01 \times 10^9$	$1.91 \pm 0.01 \times 10^8$	$4.76 \pm 0.01 \times 10^7$	$3.83 \pm 0.01 \times 10^5$
V	Red granule	$2.04 \pm 0.01 \times 10^8$	$1.59 \pm 0.01 \times 10^7$	$6.17 \pm 0.11 \times 10^6$	$1.13 \pm 0.11 \times 10^5$
VI	Red granule	$2.08 \pm 0.39 \times 10^9$	$5.38 \pm 0.69 \times 10^8$	$1.83 \pm 0.33 \times 10^8$	$3.72 \pm 0.71 \times 10^5$
VII	Red granule	$2.14 \pm 0.68 \times 10^9$	$1.19 \pm 0.26 \times 10^8$	$4.29 \pm 0.52 \times 10^7$	$1.27 \pm 0.13 \times 10^7$
VIII	Red granule	$2.83 \pm 0.16 \times 10^9$	$2.19 \pm 0.48 \times 10^8$	$1.45 \pm 0.42 \times 10^6$	$1.91 \pm 0.38 \times 10^8$
VIII	Red granule	$4.28 \pm 0.37 \times 10^9$	$2.20 \pm 0.47 \times 10^8$	$2.12 \pm 0.73 \times 10^6$	$1.44 \pm 0.00 \times 10^7$
VIII	Red granule	$2.15 \pm 0.20 \times 10^9$	$9.01 \pm 2.14 \times 10^7$	$6.74 \pm 2.01 \times 10^5$	$1.16 \pm 0.23 \times 10^7$

TA: total species of anammox bacteria; EB: eubacteria; KS: *Candidatus Kuenenia stuttgartiensis*; BA: *Candidatus Brocadia anammoxidans*.

a: copy numbers per μg of extracted total genomic DNA, and values calculated from triplicate.

Table 3
The abundance of anammox bacteria from various ecosystems.

Detected anammox bacteria	Source of samples	Reference
2.6×10^3 cells mL^{-1}	black sea suboxic	(Lam et al., 2007)
4.6×10^8 cells mL^{-1}	reactor	(Ni et al., 2010)
2.4×10^{10} copies of P8-type AnAOB μg^{-1} DNA	reactor	(Quan et al., 2008)
3.7×10^7 - 8.6×10^9 cells mL^{-1}	reactor	(Hu et al., 2010)
6.6×10^2 - 4.9×10^4 copies mL^{-1}	oilfield production water	(Li et al., 2010)
7.5 – 36.5×10^4 copies g^{-1}	sediments	(Li et al., 2011a)
6.6×10^8 copies μg^{-1} DNA	landfill-leachate treatment plant	This study

Table 4
The size and microbial community (Bacterial and Anammox) of the collected granules.

Stage	Condition	TA/EB %	KS/TA %	BA/TA %	KS:BA
I	red granule, 4.11 ± 1.71 mm ^a	51.1	92.0	0.17	527
III	red granule, 0.80 ± 0.24 mm ^a	90.1	90.0	6.70	13
IV	red granule, 0.66 ± 0.23 mm ^a	8.9	24.9	0.20	124
V	red granule, 1.10 ± 0.32 mm ^a	28.1	38.8	0.71	55
VI	red granule, 1.65 ± 1.16 mm ^a	93.1	34.0	0.07	492
VII	red granule, 0.47 ± 0.28 mm ^a	20.0	36.1	10.7	3.4
VIII	red granule, less than 0.1 mm ^a	27.9	0.66	87.2	0.008
VIII	red granule, less than 0.1 mm ^a	18.5	0.96	6.55	0.15
VIII	red granule, less than 0.1 mm ^a	15.1	0.75	12.9	0.058

TA: total anammox bacteria; KS: *Candidatus Kuenenia stuttgartiensis*; BA: *Candidatus Brocadia anammoxidans*.

a: average diameter of granules.

Ammonium was completely converted into nitrogen gas ($\sim 90\%$) and nitrate ($\sim 10\%$) by simultaneous nitrification and anammox reaction in the reactor. The nitrite was utilized immediately by both anammox and denitrifying bacteria. Therefore, the nitrite concentration in effluent was found to be low and varied between 0.9 and 15.7 mg N L^{-1} . Nitrate, which was absent in influent, was found in the effluent probably as by product of anammox reaction¹. The average monthly NH_4^+ -N removal efficiency (RE) was always greater than $\sim 50\%$ during the studied period and it was higher in stage II ($69 \pm 9\%$), stage III ($67 \pm 8\%$), stage VI ($66 \pm 13\%$) and stage VIII ($66 \pm 27\%$). The maximum average COD removals ($60 \pm 5\%$) were also found in stage VI.

3.4. The relationship between anammox community, granule size and plant performance

Anammox bacteria were reported to preferentially aggregate and form biofilm, flocs and granules (Chen et al., 2010; Trigo et al., 2006;

Daverey et al., 2015; Liang et al., 2016; Chen et al., 2017; Wang et al., 2018). Therefore, the degree of aggregation may reflect the activity of anammox process that influences the performance of wastewater treatment. In this study, nitrogen removal efficiency could be directly correlated to size of granules. The correlation coefficient is high (0.87) between NH_4^+ -N RE and size of granule (Table S2). The size and microbial community of the collected granules and samples, and the ammonia nitrogen volumetric loading rate (VLR), NH_4^+ -N RE and removed amount of ammonia nitrogen (kg NH_4^+ -N $\text{m}^{-3} \text{d}^{-1}$) for each sampling stage are listed in Tables 4 and 5, respectively. VLR was varied between 0.188 to 0.504 kg N $\text{m}^{-3} \text{d}^{-1}$ as influent NH_4^+ -N concentration fluctuated during the studied period. The molecular analysis of suspended sludge samples revealed very low % of TA/EB and KS/TA (Table S3) compared to the granules. This further confirmed that anammox bacteria grow preferentially in granules. Granular sludge collected in this study varied in size and color (Table 4, Figure S2). In the beginning of sampling (stage I), the average diameter of granules was the largest with red granules as big as 4.11 mm and black granules as 3.41 mm. During stage IV, the size of granules decreased to 0.66 and 0.80 mm, and then recovered to 1.65 mm in the stage VI. The smallest average diameter of granules occurred in stage VIII. The RE was varied with respect to granule size and it was the highest (80 %) in stage I and the lowest in stage VII (44 %) (Table 5). The NH_4^+ -N RE reached near 80 % in early stage, while lower down to less than 50 % gradually. The amount of removed ammonia nitrogen was relatively high in stage I and V, while relatively low since stage VI. The change in granule size could be correlated with the specific anammox species. The results of this study suggested that high numbers of KS over BA (KS:BA ratio > 492) gave a better granule formation and RE in the treatment plant (Table 4). On the other hand, high BA population compared to KS (KS:BA ratio < 124) negatively affected the granule formation and RE. This may be due to lower conversion rates, different substrate affinities, different cell morphologies and granule formation capacities. Moreover, the correlation coefficients are high between RE and KS/BA (0.88), total anammox bacteria and KS (0.88), size of granule and KS/BA (0.84) (Table S2), indicating the dominant anammox species play important role both in NH_4^+ -N RE and formation of granules. Results of this study can

Table 5
Ammonia nitrogen removed in the various stages.

Stage	VLR ^a (kg N $\text{m}^{-3} \text{d}^{-1}$)	RE ^b (%)	NH_4^+ -N removed (kg N $\text{m}^{-3} \text{d}^{-1}$)
I	0.397	80	0.318
III	0.390	45	0.228
IV	0.356	39	0.139
V	0.504	59	0.297
VI	0.188	72	0.135
VII	0.260	44	0.114

^a: volumetric loading rate.

^b: removal efficiency.

contribute to the further research of nanoarchitected structure and population dynamics of anammox system, and may facilitate the start-up of anammox bioreactor.

4. Conclusions

Overall, this study can be concluded as follows:

- (1) A four-year study on bacterial community of a SNAD landfill leachate treatment plant was carried out. The bacterial community in a SNAD landfill leachate treatment plant comprised of AOB, NOB, denitrifying bacteria, and anammox bacteria. Compared to well-controlled laboratory reactors, the anammox bacteria in this full scale plant were relatively high (1.6×10^7 to 6.6×10^8 copies μg^{-1} DNA).
- (2) The newly designed KS-specific primer (KSqF3/KSqR3) and BA-specific primer (BAqF/BAqR) were very suitable for qPCR quantification and yielded estimates of maximally 5.9×10^8 KS and 4.4×10^7 BA copies μg^{-1} DNA, respectively.
- (3) The ratio of all anammox bacteria to bacteria peaked in at 90–93 % with KS being the dominant anammox species.
- (4) The correlation coefficients are high between ammonium nitrogen removal efficiency and KS/BA (0.88), TA and KS (0.88), size of granule and KS/BA (0.84), ammonium nitrogen removal efficiency and size of granule (0.87).

CRedit authorship contribution statement

Yu-Tzu Huang: Conceptualization, Methodology, Software, Validation, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Shiou-Shiou Chen:** Formal analysis, Investigation, Data curation, Visualization. **Mike S.M. Jetten:** Writing - review & editing, Supervision. **Jih-Gaw Lin:** Writing - original draft, Writing - review & editing, Supervision.

Declaration of Competing Interest

There are no conflicts to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jhazmat.2020.122714>.

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