Anammox start-up strategies: the use of local mixed activated sludge seed versus Anammox seed

Bilge Alpaslan Kocamemi, Duygu Dityapak, Neslihan Semerci, Esra Keklik, Alper Akarsubası, Mert Kumru and Halil Kurt

ABSTRACT

The start-up period of Anammox systems is still a big challenge due to the unavailability of large volumes of slowly growing Anammox seed locally in most countries. This study aims to evaluate the effects of seeding strategy on the start-up and enrichment period of Anammox systems by monitoring both process performance and microbial population dynamics. Two different seeding strategies, the use of mixed activated sludge culture from a local STP and the use of enriched Anammox culture transported from abroad, were comparatively studied in SBR systems operated for 410 days. The enriched Anammox seed from abroad inhibited seriously during transportation. Anammox activity re-started after 195 days' recovery period. An active Anammox culture was successfully enriched within 95 days from a local activated sludge source without seeding any Anammox. The Anammox population reached levels of 10^{11} copies/ng at the end of 410 days' enrichment period. Based on FISH, *Ca.* Brocadia anammoxidans and *Ca.* Scalindua species were dominant in the enriched culture. The maximum TNRR was observed as 430 mg N/day. DGGE analyses revealed a drastic change in the microbial community (56%) with Anammox enrichment. Phylogenetic analysis demonstrated a significant decrease in phylotype Proteobacteria and increase in phylotypes Planctomycetes, Chloroflexi and Acidobacteria with enrichment.

Key words | anaerobic ammonium oxidation (Anammox), enrichment, molecular techniques, nitrogen removal, seeding strategy, start-up

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INTRODUCTION

In the past few decades, conventional biological treatment technologies, which are quite successful and satisfactorily achieve the required discharge limits, have been replaced with novel technologies that are more difficult but much cheaper to operate. The anaerobic ammonium oxidation (Anammox) process, which is an anaerobic chemolithoautotrophic nitrogen removal process discovered by Mulder *et al.* (1995), has received great interest as a very cost effective alternative to conventional nitrification and denitrification processes. The Anammox process is mediated by a group of Planctomycetes bacteria associated with at least five genera (Gonzalez-Martinez *et al.* 2018) and converts ammonium directly into dinitrogen gas under anaerobic conditions, with nitrite as the electron acceptor in the absence of any organic carbon source. Hence, it allows saving of oxygen and organic matter, minimizes sludge production and prevents greenhouse gases emissions. Many studies have demonstrated that the Anammox process is quite capable of replacing the conventional biological nitrogen removal technologies especially for the treatment of ammonium-rich and low organic carbon containing wastewaters, such as centrate from anaerobic digesters (Lackner *et al.* 2015; Rikmann *et al.* 2018). However, the long start-up period of the process due to the slow growth rate of Anammox bacteria seriously limits its full-scale applications. The first full-scale Anammox reactor was taken into operation in 2002 at the sludge treatment plant Sluisjesdijk in Rotterdam, The Netherlands, in order to treat anaerobic digesters' centrate (Van der Star *et al.* 2007). The start-up period was about 3 years. Since then, several full-scale centrate Anammox reactors have been started up, mostly in Europe (Lackner *et al.* 2014). The main obstacle for worldwide full-scale Anammox applications is the availability of large volumes of enriched Anammox seed. There are studies (Table 1) searching for

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Table 1 | Summary of the studies evaluating Anammox start-up period in SBR systems using different inocula

Type of seed sludge	Source of seed sludge	Location of treatment plant	Feed wastewater	Start-up period (days)	Observed specific Total Nitrogen removal rates (SNR) (gN/g VSS·d)	ΔNH_4-N: ΔNO_2-N: ΔNO_3	Anammox bacterial community composition after enrichment	Quantity of Anammox bacteria after enrichment	Reference
Enriched Anammox sludge	Full-scale Anammox plant	Strass, Austria	Partially nitrified anaerobic digester centrate	150	NR[a]	1:1.21:0.13	Brocadia	10^8–10^9 copies/ml	Park *et al.* (2010)
Enriched granular Anammox sludge	Laboratory-scale fluidized bed reactor	–	Synthetic wastewater	NR[a]	0.45[b]	1:1.32:0.26	–	–	Strous *et al.* (1998)
Mixed activated sludge biomass	Urban landfill leachate treatment plant, urban WWTP	Spain	Synthetic wastewater	60	0.94[c]	1:1.32:0.23	Brocadia	–	Lopez *et al.* (2008)
Activated sludge	Aerobic SBR unit at a domestic WWTP	Korea	Synthetic wastewater	35	0.18[c]	1:0.91:0.45	Jettenia, Brocadia, Kuenenia	6.4×10^8 copies/ml	Bae *et al.* (2010)
Activated sludge	Anaerobic unit of an A_2/O system at a domestic WWTP	Korea	Synthetic wastewater	35	0.23[c]	1:0.77:0.31	Jettenia, Brocadia, Kuenenia	3×10^8 copies/ml	Bae *et al.* (2010)
Activated sludge	Anoxic unit of an A_2/O system at a domestic WWTP	Korea	Synthetic wastewater	35	0.23[c]	1:0.80:0.30	Jettenia, Brocadia, Kuenenia	1.5×10^9 copies/ml	Bae *et al.* (2010)
Activated sludge	Oxic unit of an A_2O system at a domestic WWTP	Korea	Synthetic wastewater	50	0.18[c]	1:1.04:0.13	Jettenia, Brocadia, Kuenenia	5×10^6 copies/ml	Bae *et al.* (2010)
Activated sludge	Return activated sludge line of a municipal WWTP	Arizona, USA	Synthetic wastewater	50	NR[a]	1:1.32:0.20	Brocadia, Kuenenia	–	Sun *et al.* (2011)
Activated sludge	Oxic unit of a municipal WWTP	Arizona, USA	Synthetic wastewater	51	NR[a]	NR[a]	Brocadia, Kuenenia	–	Sun *et al.* (2011)
Activated sludge	Oxidation ditch unit of a municipal WWTP	Arizona, USA	Synthetic wastewater	134	NR[a]	1:1.41:0.21	Brocadia, Kuenenia	–	Sun *et al.* (2011)
Activated sludge	Membrane biological reactor at a municipal WWTP	Lancaster, USA	Synthetic wastewater	134	NR[a]	1:1.37:0.23	Brocadia, Kuenenia	–	Sun *et al.* (2011)

Activated sludge	Return activated sludge line of a municipal WWTP	San Jose, USA	Synthetic wastewater	204	NR[a]	1:1.31:0.20	Brocadia, Kuenenia	–	Sun *et al.* (2011)
Activated sludge	Anoxic tank of a WWTP	Bangkok, Thailand	Synthetic wastewater	90	2.59	1:1.50: NR[a]	Brocadia, Kuenenia	–	Saricheewin *et al.* (2010)
Activated sludge	NR[a]	NR[a]	Synthetic wastewater	90	0.08[d]	1:1.50:0.04	Brocadia, Kuenenia	–	Chamchoi & Nitisoravut (2007)
Activated sludge	Anoxic tank at a centralized WWTP	Bangkok, Thailand	Synthetic wastewater	100	0.13	1:1.38: NR[a]	Brocadia, Kuenenia	–	Noophan *et al.* (2009)
Anaerobic activated sludge	Upflow anaerobic sludge blanket (UASB) reactor	–	Synthetic wastewater	90	0.08[d]	1:1.50:0.04	Brocadia, Kuenenia	–	Chamchoi & Nitisoravut (2007)
Methanogenic granular activated sludge	Industrial WWTP treating alcohol distillery wastewater	The Netherlands	Synthetic wastewater	ND[e]	–	–	–	–	Sun *et al.* (2011)
Methanogenic granular activated sludge	Industrial WWTP treating brewery wastewater	Spain	Synthetic wastewater	ND[e]	–	–	–	–	Sun *et al.* (2011)
Anaerobic digestion sludge	NR[a]	NR[a]	Synthetic wastewater	90	0.08[d]	1:1.50:0.04	Brocadia, Kuenenia	–	Chamchoi & Nitisoravut (2007)
Anaerobically digested sewage sludge	Municipal WWTP	Arizona, USA	Synthetic wastewater	185	NR[a]	NR[a]	Brocadia, Kuenenia	–	Sun *et al.* (2011)
Enriched granular Anammox sludge	The first full-scale UASB type Anammox reactor in the world	Rotterdam, The Netherlands	Synthetic wastewater	195	0.12	1:1.45:0.21	Brocadia, Scalindua	1.6×10^{10} copies/ng	This study (SBR1)
Activated sludge	Return activated sludge line of a municipal WWTP	Istanbul Turkey	Synthetic wastewater	75	0.03	1:1.41:0.20	Brocadia, Scalindua	3.1×10^{11} copies/ng	This study (SBR2)

[a]NR: not reported.
[b]Calculated by assuming content of biomass 0.5 g protein/g biomass.
[c]Not reported directly in the paper. Calculated based on the reported NH_4-N, NO_2.N removal rates and VSS values.
[d]Calculated by assuming MLVSS/MLSS ratio as 0.8.
[e]ND: not detected.

the presence of Anammox bacteria in local treatment plants and evaluating the enrichment of Anammox bacteria from various types of sludges (e.g. activated sludge, anaerobic digester sludge, methanogenic granules). However, there are still many countries that are unaware that their local mixed activated sludge in treatment plants contains minor quantities of Anammox species and can be enriched for Anammox species. The worldwide widespread use of the Anammox process can only be possible if newly started Anammox reactors can initially be seeded with locally available mixed activated sludge and later enriched for Anammox species. Hence, research studies should focus on the feasibility of using mixed local sludges containing low quantities of Anammox bacteria in comparison with using enriched Anammox seed to be exported from other countries. In these studies, molecular techniques evaluating microbial diversity and population dynamics through the enrichment period have to be coupled with the process performance since they are quite valuable in identifying the most suitable inocula for quick start-up of Anammox reactors.

This study aims to evaluate the effects of seeding strategy on the start-up and enrichment period of Anammox systems by monitoring both process performance and microbial population dynamics. Two different seeding strategies, the use of a mixed activated sludge sample from a local sewage treatment plant in Turkey and the use of an enriched Anammox sludge sample transported from the first worldwide full-scale Anammox reactor in the Netherlands, were comparatively studied in sequencing batch type reactor (SBR) systems operated for 410 days. The nitrogen removal performance of the systems was monitored in terms of ammonium removal, nitrite removal and nitrate production efficiencies. To study the microbial population dynamics, the diversity and abundance of Anammox bacteria were monitored via denaturating gradient gel electrophoresis (DGGE) and quantitative real-time polymerase chain reaction (Q-RT-PCR) measurements, respectively. Cloning, sequencing and phylogenetic analyses were also performed to characterize the microbial population in the reactors, especially after the enrichment period.

MATERIALS AND METHODS

Seed sludges

An enriched Anammox sludge sample was obtained from the first full-scale Anammox reactor in the world, which started to operate in 2002 at the Sluisjesdijk sludge treatment plant in Rotterdam, The Netherlands (Van der Star *et al.* 2007). The local mixed activated sludge sample was taken from the return activated sludge line of the Pasakoy BNR sewage treatment plant, which has been operating since 2000 in Istanbul, Turkey.

Start-up and operation of sequencing batch reactors (SBRs)

Two SBRs, with a working volume of 1 L and 4.5 L, were inoculated with the enriched Anammox sludge seed (SBR-1) and the local mixed activated sludge seed (SBR-2), respectively. The working volume of the SBR-1 was increased from 1 L to 4.5 L on operation day 286. The initial biomass concentration in the reactors was around 3,000 mg VSS/L. The reactors were operated continuously for 410 days by feeding with the stock synthetic wastewater, as described previously (Alpaslan Kocamemi & Dityapak 2014). The ammonium and nitrite loadings to the reactors were increased stepwise depending on the treatment efficiencies. Nitrate was fed continuously to the reactors for the consumption of organic matter possibly generated from endogenous decay activity during the early stages of start-up and possible sulfate reduction to sulfide, which may inhibit Anammox bacteria.

The operational cycles of the reactors, including the fill, react, settle and decant phases, were kept at around 24 hours using PLC systems. For the SBR-1, HRT was around 8 days until day 118 and then decreased to 3.6 days between days 118 to 286, and later kept at a constant value of 4.5 days. For SBR-2, HRT was 4.5 days throughout the study. The reactors' water and headspace were continuously flushed with 95% Ar-5% CO_2 gas mixture to maintain anaerobic conditions and to supply an inorganic carbon source. They were operated at a fixed temperature of around 34°C using thermostated water jackets. Complete mixing conditions were provided by means of magnetic stirrers. pH, temperature, dissolved oxygen (DO), oxidation reduction potential (ORP) and conductivity values were continuously monitored and recorded using Hach Lange probes. The recorded values and their correlations with the treatment efficiencies have been discussed in the previous studies (Alpaslan Kocamemi & Dityapak 2014). Throughout the 410 day operational period, water temperature, pH and DO values were in the ranges of 34.0 ± 1.1, 7.4 ± 0.3 and 0 ± 0.04 in SBR-1 and 34.4 ± 1.3, 7.7 ± 0.3, and 0 ± 0.07 in SBR-2.

FISH analyses

Biomass samples for fluorescence in situ hybridization (FISH) analyses were collected with 50% ethanol (v/v) and stored at −20°C. They were fixed with 4% (v/v) paraformaldehyde. Hybridization with fluorescently labelled Eub 338 I, II, III, Planc 046, Amx368, Amx820, BS820, Kst1275 oligonucleotides was performed as described by Daims *et al.* (2005). Images were taken with a Leica TCS SP2 confocal laser scanning microscope.

DNA extraction

Biomass (2 ml) was harvested by centrifugation (10 min, 13,000 rpm) and the Fast DNA Spin Kit (MP Bio, USA) was used for extraction as described in the manufacturer's instructions.

Eubacterial PCR

Eubacterial specific, 16S rRNA gene fragments were amplified using universal bacterial primers (VfGC-Vr) (Muyzer *et al.* 1993). All PCR reactions were performed in a C1000™ Thermal Cycler (BIORAD, USA). The PCR program was set as 5 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 55 °C, 40 s at 72 °C; and extension for 10 min at 72 °C for bacterial samples. Another primer set named pA-AGA GTT TGA TCC TGG CTC AG and pH-AGG GAG GTG ATC CAG CCG CA (Edwards *et al.* 1989) was also used for cloning.

Quantitative-real time PCR (Q-RT PCR) for Anammox bacteria

A combination of forward Pla46f-GGA TTA GGC ATG CAA GTC and reverse Amx667r-ACC AGA AGT TCC ACT CTC (van der Star *et al.* 2007) primers were used for quantifying Anammox bacteria. Q-RT PCR was performed with an iCycler iQ5 thermocycler and real-time detection system (Bio-Rad, Berkeley, CA). The program of the thermal cycler was initiated with 5 min at 95 °C denaturing step, followed by 45 cycles of 20 s at 95 °C denaturation, 20 s at 55 °C annealing, and 60 s at 72 °C of extension steps.

Denaturating gradient gel electrophoresis (DGGE)

DGGE was performed by using the DCode universal mutation detection system (Bio-Rad). 1 mm 10% (w/v) polyacrylamide (37:5:1 acrylamide:bisacrylamide) gels containing denaturant gradients of 35 to 65% were used to separate eubacterial PCR products. Gels were run at 60 °C and 180 V for 330 minutes. The gels were stained with 0.5 µg/mL ethidium bromide in 1× TAE buffer for 20 min. Images were recorded using the GelDoc imaging system (Bio Rad, CA) after a 15 min destaining process in 1× TAE buffer. DGGE band pattern was analysed using Bionumerics Software Version 5.1 (Applied Maths, Belgium). Calculation of similarities of the DGGE profiles was based on the Pearson product moment correlation coefficient.

Cloning, sequencing and phylogenetic analysis

A clone library of universal bacterial 16S rRNA gene was constructed using the PCR products and a TOPO TA Cloning Kit for Sequencing (Invitrogen, USA) as described in the manufacturer's instructions. Primers named pA and pH targeting a large fragment of the 16S rRNA gene were used for PCR prior to clone library analysis. The sequencing of the cloned nucleotides was performed with an automatic sequencer (ABI, USA). Affiliated phylotypes for the sequences of 16S rRNA gene were checked at the Ribosomal Database Project. Phylogenetic trees were constructed using the neighbour-joining method under the total gap remotion and Kimura's two-parameter substitution model and visualized using the MEGA 5 software package. The bootstrap analysis was conducted on 1,000 resamplings.

Nucleotide sequence accession numbers

The sequences determined in this study have been deposited in the GenBank database (accession number JN590920-JN590971).

Scanning electron microscope (SEM) observations

The morphology of biomass in both SBR-1 and SBR-2 were observed by environmental scanning electron microscope (Philips XL30 ESEM-FEG/EDAX).

Analytical methods

NH_4^+-N, NO_2^--N and NO_3^--N concentrations were determined by a dual injection ion chromatograph (Shimadzu Prominence HIC-SP) equipped with Shimpack IC-SC1 (150 × 4.6 mm) and Shimpack IC-SA2 (250 × 4 mm) columns. Mixed liquor volatile suspended solids (MLVSS) concentrations were determined in accordance with the Method 2540 E in *Standard Methods* (APHA/AWWA/WEF 1999).

RESULTS AND DISCUSSION

Process performance

The influent and effluent NH_4^+, NO_2^- and NO_3^- profiles of the SBR-1 (inoculated with enriched Anammox seed) and the SBR-2 (inoculated with local mixed activated sludge seed) are shown in Figures 1 and 2, respectively. The loading and removal rates calculated based on these data are summarized in Table 2 and shown graphically in a previous study (Alpaslan Kocamemi & Dityapak 2014). For both reactors, in spite of using different seed sludges, the 410-day operation period could be divided into three (3) main phases (Alpaslan Kocamemi & Dityapak 2014) : (I) early start-up, (II) start-up, (III) enrichment. Based on the sequence of processes observed in the reactors, each main phase was subdivided and re-named as follows: *Phase I – Early Start-Up (*I-A: intense endogenous denitrification, I-B: endogenous denitrification), *Phase II – Simultaneous Endogenous Denitrification and Anammox Processes*, *Phase III – Anammox Process* (III-A: unsteady-state, III-B: steady-state).

Both SBR-1 and SBR-2 were started up by feeding with a low level of total nitrogen. NH_4^+-N and NO_2^--N were around 5 ± 2 mg/L and 8 ± 2 mg/l for SBR-1, 35 ± 6 mg/L and 21 ± 6 mg/l for SBR-2. In the early stages of start-up, *Phase I-A* (Figures 1 and 2 and Table 2), a slight increase in NH_4^+-N and significant decrease in NO_2^--N and NO_3^--N levels were observed concurrently with decreasing MLVSS. The start-up MLVSS values of around 3,000 mg/l dropped to the levels of 1,200 mg/l for SBR-1 and 2,300 mg/l for SBR-2 at the end of *Phase I-A*. These observations strongly indicated that endogenous denitrification due to decay of seed sludges predominated in both systems and a significant amount of sludge wash-out occurred. pH, ORP and conductivity profiles of both systems (Alpaslan Kocamemi & Dityapak 2014) were well consistent with the dominance of endogenous denitrification. The NO_2^--N: NH_4^+-N removal ratios ($\Delta_{NO2\text{-}N}$: $\Delta_{NH4\text{-}N}$) and the NO_3^--N production : NH_4^+-N removal ratios ($\Delta_{NO3\text{-}N}$:$\Delta_{NH4\text{-}N}$) (Supplementary Figure 1, available with the online version of this paper) exhibited negative values due to denitrification activity in the systems. In the earlier studies (e.g. Chamchoi & Nitisoravut 2007), similar observations were commonly reported with varying durations depending on type of seed sludge, reactor configuration, and environmental conditions. Endogenous denitrification activity was more severe and longer in the reactor inoculated with enriched Anammox seed (SBR-1). This continued intensely for about one month (*Phase I-A*) and then with decreasing rate (*Phase I-B*) until day 110. In SBR-2, however, intense endogenous denitrification activity was observed only during the first week of operation (*Phase I-A*) and ended completely in a month (*Phase I-B*). Anammox bacteria in the enriched seed of SBR-1, which was brought from Rotterdam to Istanbul after a long transportation time, might temporarily cease their activity as they are very sensitive to changes in environmental conditions and this might cause severe decay of the seed biomass. In the case of SBR-2, cell lysis of aerobic bacteria in mixed seed sludge was inevitable under the anaerobic conditions prevailing in the Anammox system. However, the high fraction of facultative heterotrophs in the mixed consortium of SBR-2 probably provided quick consumption of organics released from the decay of biomass and hence helped to shorten the endogenous denitrification phase significantly. Moreover, a few operational changes in SBR-2 (e.g. longer settling time, shorter hydraulic retention time, enhanced CO_2 solubilization and strict anaerobic conditions) might favor this situation. Starting from day 111 for SBR-1 and day 61 for SBR-2, a decreasing rate of endogenous denitrification activity was followed by observation of the first signs of Anammox activity (*Phase II*), slight NH_4^+-N losses coincidentally with decreasing NO_2^--N levels. In both systems, NO_3^--N decreases were still continuing but with slower rate compared to *Phase I*, indicating minimized endogenous denitrification activity. Influent concentrations fed to the reactors were 26 ± 13 mg NH_4^+-N/l and 25 ± 10 mg NO_2^--N/l for SBR-1, and 28 ± 15 mg NH_4^+-N/l and 44 ± 22 mg NO_2^--N/l for SBR-2. The $\Delta_{NO2\text{-}N}$: $\Delta_{NH4\text{-}N}$ (Supplementary Figure 1) were significantly higher than that stoichiometrically expected for the Anammox process due to still ongoing denitrification activity in the systems. At day 195 for SBR-1 and day 75 for SBR-2, NH_4^+-N and NO_2^--N concentrations in the reactors started to fall, consistent with the Anammox stoichiometry (Supplementary Figure 1) although there was still no NO_3^--N decrease in the reactors. NO_3^--N production, which is the main indicator of the dominance of the Anammox process over denitrification was first observed on days 226 for SBR-1 and 92 for SBR-2.

With the start of NO_3-N formation in *Phase III*, NH_4^+-N and NO_2^--N loadings to the reactors were gradually increased to enrich the Anammox culture (Figures 1 and 2). Influent concentrations were 105 ± 35 mg NH_4^+-N/l and 146 ± 56 mg NO_2^--N/l for SBR-1, 109 ± 38 mg NH_4^+-N/l and 158 ± 56 mg NO_2^--N/l for SBR-2. In *Phase III-A*, prior to steady-state being reached, the loadings reached the levels of 170 mg/d NH_4^+-N and 237 mg/d NO_2^--N for SBR-1 and 171 mg/d NH_4^+-N and 284 mg/d NO_2^--N for

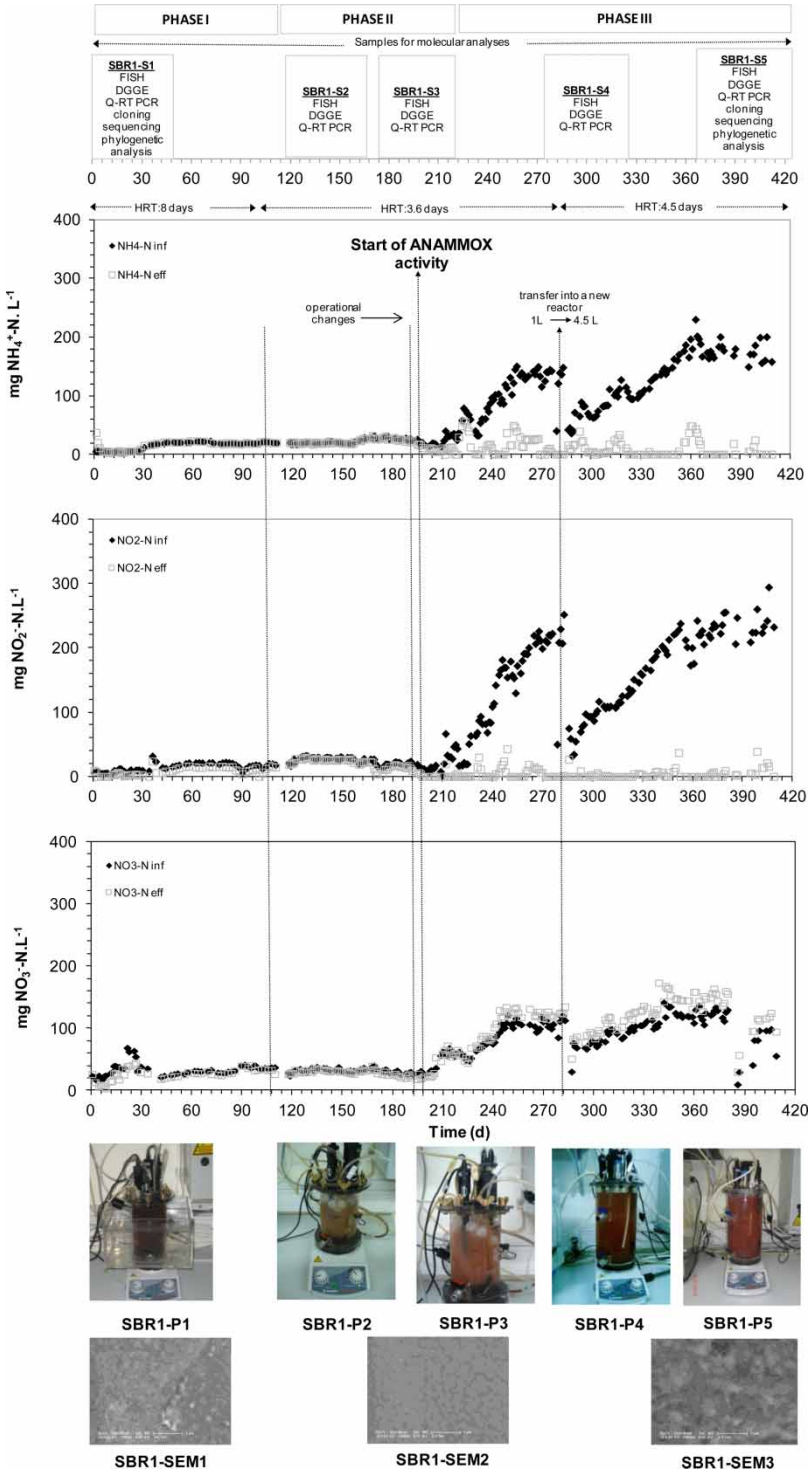

Figure 1 | Chronological illustration of influent and effluent ammonium, nitrite and nitrate concentrations of SBR-1 during 410 days of operation.

SBR-2. A sharp nitrogen loading decrease in SBR-1 at day 286 (Figure 1) was due to the transfer of the 1 L reactor's contents into a larger reactor (4.5 L). In a short time, loadings could be gradually increased depending on the treatment efficiency. NH_4^+-N and NO_2^--N removal efficiencies were around 90–100% for both systems. Total nitrogen removal rate (TNRR) reached the maximum levels of 402 mg N/d for SBR-1 and 430 mg N/d for SBR-2. The corresponding

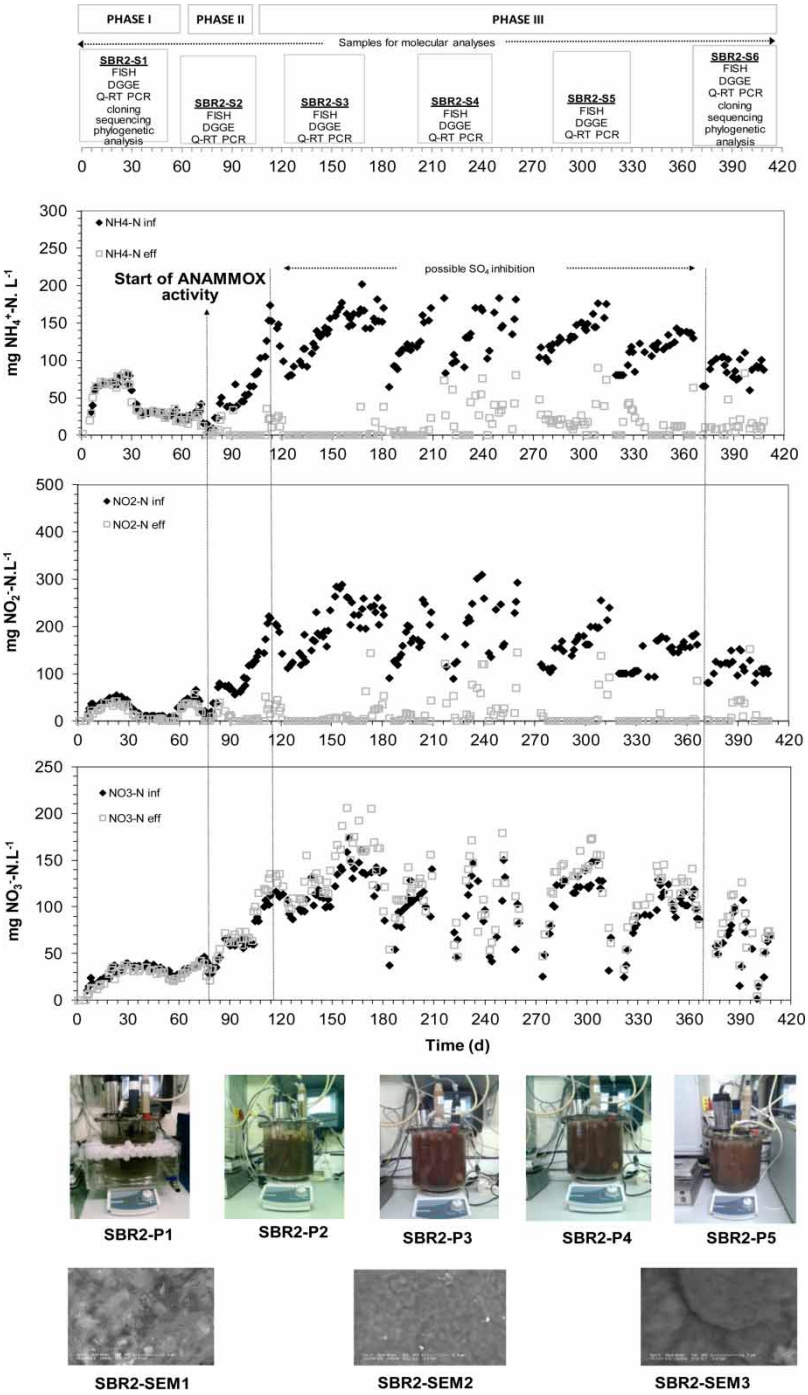

Figure 2 | Chronological illustration of influent and effluent ammonium, nitrite and nitrate concentrations of SBR-2 during 410 days of operation.

maximum specific total nitrogen removal rates (sTNRR) were 0.1 g N/g VSS.d for SBR-1 and 0.07 g N/g VSS.d for SBR-2. The $\Delta_{NO2\text{-}N} : \Delta_{NH4\text{-}N}$ and $\Delta_{NO3\text{-}N} : \Delta_{NH4\text{-}N}$ ratios (Supplementary Figure 1) were observed to be 1.48 and 0.22 for SBR-1 and 1.44 and 0.17 for SBR-2, respectively. During steady-state operation (*Phase III-B*), SBR-1 and SBR-2 were continuously fed with 185 ± 25 mg NH_4^+-N/l, 242 ± 50 mg NO_2^--N/l and 128 ± 34 mg NH_4^+-N/l, 172 ± 59 mg NO_2^--N/l, respectively. TNRR was around 404 mg N/d for SBR-1 with a removal efficiency of over 95%. The corresponding average sNRR was around 0.08 gN/gVSS.d. However, for SBR-2, the loadings were decreased to the

Table 2 | Comparison of Anammox start-up and enrichment periods in SBR-1 and SBR-2 systems

	Phase I				Phase II		Phase III			
	I-A intense endogenous denitrification		I-B endogenous denitrification		Endogenous denitrification and Anammox		III-A Anammox (unsteady-state)		III-B Anammox (steady-state)	
	SBR-1	SBR-2	SBR-1	SBR-2	SBR-1	SBR-2	SBR-1	SBR-2	SBR-1	SBR-2
Duration (operation day)	0.30	0–7	31–110	8–60	111–226	61–92	227–353	93–155	354–410	156–410
NH_4^+-N loading rate, mg/d	4.2 ± 1	34.8 ± 6	2.4 ± 0.2	45.6 ± 22	6.5 ± 3	28 ± 15	8→170	38→171	179 ± 17	121 ± 35
NH_4^+-N release(−)/removal (+) rate[a], mg/d	−0.2 ± 1	−7.5 ± 9	0.003 ± 0.1	1.3 ± 6	1.4 ± 2.2	13 ± 17	8→165	38→150	167 ± 17	107 ± 32
NO_2^+-N loading rate, mg/d	7.4 ± 3	21.3 ± 6	2.2 ± 0.6	25 ± 17	6.1 ± 2.4	42 ± 24	15→237	56→284	225 ± 24	165.8 ± 55
NO_2^+-N removal rate, mg/d	5.3 ± 2	13 ± 9	0.8 ± 0.4	9 ± 5	2.5 ± 2.9	24 ± 22	12→237	56→280	220 ± 21	148 ± 53
NO_3^+-N loading rate, mg/d	36 ± 17	12.4 ± 3	4 ± 0.6	31 ± 6	9.5 ± 2.8	41 ± 12	13→141	58→142	103 ± 32	97.7 ± 3.5
NO_3^+-N removal (–)/production(+) rate[b], mg/d	−14 ± 7	−6.5 ± 2	−0.4 ± 0.4	−0.4 ± 0.4	−0.65 ± 1.2	−0.5 ± 6	2→28	2→47	26 ± 11	18 ± 23
Start of Anammox activity (operation day)	–	–	–	–	195	75	–	–	–	–
Start of NO_3-N production (operation day)	–	–	–	–	–	–	226	92	–	–
ΔNO_2^--N/ΔNH_4-N	–	–	–	–	–	–	1.48 ± 0.18	1.44 ± 0.14	1.34 ± 0.07	1.39 ± 0.22
ΔNO_3^--N/ΔNH_4-N	–	–	–	–	–	–	0.22 ± 0.12	0.17 ± 0.10	0.17 ± 0.07	0.22 ± 0.16
Sulfate loading mg/d	31 ± 23	224 ± 72	22 ± 4	315 ± 80	90 ± 37	400 ± 189	1,127 ± 814	1,793 ± 559	2,245 ± 837	2,308 ± 792
Similarity to seed sludge observed by DGGE analysis (%)	–	–	–	–	67–77	76	72	64	71	46 → 38
Quantity of Anammox bacteria, copies/ng DNA	1.4×10^9	2.1×10^6	–	–	1.4×10^9	1×10^7	1.4×10^9–2.7×10^{10}	5.8×10^7	1.6×10^{10}	5.3×10^{10}–3×10^{11}
MLVSS, mg/l	3067→1289	3000→2320	988 ± 152	1480 ± 170	634 ± 157	987 ± 85	617 ± 210	1376 ± 257	1099 ± 162	1868 ± 327

[a]Increase in effluent NH_4^+-N concentration with respect to influent is mentioned with (−) sign as release rate.
[b]Decrease in effluent NO_3^+-N concentration with respect to influent is mentioned with (−) sign as removal rate.

levels of 121 mg/d NH_4^+-N and 166 mg/d NO_2^--N due to sudden NH_4^+-N and NO_2^--N accumulations observed on a few operational days (e.g. days 184, 260, 308 and 360 (Figure 2). On these days, SO_4^{2-} entering the system with $(NH_4)_2SO_4$ loading was around 3,000 mg/L. As a mixed activated sludge, the sulfate-reducing bacteria content of the SBR-2 seed sludge was presumably non-ignorable. Hence, the sudden accumulations might be related to the inhibition of sensitive Anammox organisms due to reduction of SO_4^{2-} to S^{2-} by sulfate-reducing bacteria. The TNRR was kept at around 287 mg N/d for SBR-2 with a removal efficiency of around 90%. The corresponding average sNRR was around 0.03 gN/gVSS.d. In *Phase III-B*, $\Delta_{NO2\text{-}N}$: $\Delta_{NH4\text{-}N}$ and $\Delta_{NO3\text{-}N}$: $\Delta_{NH4\text{-}N}$ ratios were observed to be 1.34 and 0.17 for SBR-1 and 1.39 and 0.22 for SBR-2 (Supplementary Figure 1).

The overall results indicated that the use of local sludge has a significant positive effect on the start-up of Anammox reactors in comparison to the seed transported from abroad. The first attempt at Anammox enrichment from local activated sludge in Turkey became successful. An active Anammox culture could be enriched from a local source of activated sludge in around 3 months. In contrast, the use of highly enriched Anammox seed containing three orders of magnitude greater Anammox population with respect to SBR-2 (Table 2) could cause an extension of the start-up period of up to 6 months due to serious inhibition of the seed culture during the travelling period. The mixed consortium in the local activated sludge helped to maintain the necessary environmental conditions for Anammox bacteria. The operational conditions and influent characteristics were identified as the main factors having a significant impact on the Anammox start-up period. The absence of molecular oxygen, keeping pH around 7.52, providing efficient settlement of sludge, introduction and good solubilization of CO_2 were defined as the major operational factors directly accelerating the start-up period. Influent characteristics, especially the nitrite loading level, were also found to be quite important.

The maximum TNRR increased up to 404 mg/d for SBR-1 and 430 mg/d for SBR-2. The corresponding maximum sTNRR were 0.1 gN/gVSS.d for SBR-1 and 0.07 for SBR-2. Although the sTNRR of SBR-2 appeared lower than SBR-1, nitrogen removal rates expressed per MLVSS were not directly comparable. First, the granular characteristics of Anammox sludge did not allow the taking of a homogenous sample from the systems for MLVSS measurements. Second, the Anammox species fractions in total MLVSS were quite different in SBR-1 and SBR-2. SBR-2 was started up using mixed activated sludge, and hence it contained various bacterial species other than Anammox species even after it was enriched for Anammox. However, qPCR results (Table 2) demonstrated that the Anammox species in SBR-2 appeared more active than those in SBR-1. The sTNRR values observed in SBR systems were reported to be in the range of 0.0012–0.45 g N/gVSS.d in various past studies (Table 1).

Regardless of inocula source, after the cessation of endogenous denitrification activity in the reactors, stoichiometric molar ratios based on nitrite and ammonium consumption and nitrate formation were consistent with the previously reported stoichiometry of the Anammox process (Chamchoi & Nitisoravut 2007).

Morphological analysis of microbial communities

ESEM micrographs of the SBR-1 revealed that the microorganisms in the seed sludge (Figure 1, SBR-1-SEM1) were mostly spherical in shape with a heterogenous surface and high degree of compactness. About 1 month after the start of Anammox activity (Figure 1, SBR-1-SEM2), the spherical granules were regular in shape with a homogenous smooth surface and loose distribution, which might allow better access to nutrients. Similarly to the seed sludge, the 215-day enriched culture re-exhibited a heterogenous surface and high degree of compactness (Figure 1, SBR-1-SEM3). However, as opposed to the seed, the enriched biomass had a cauliflower-like appearance, which is a typical characteristic of Anammox enrichment cultures. The clusters were partly embedded in EPS. The color changes of the SBR-1 biomass were consistent with the ESEM analyses and the treatment performance. The red granular appearance of the seed sludge from the Rotterdam Anammox plant disappeared in a few days and was replaced with big brown flocs. The dark brown color (Figure 1, SBR-1-P1) turned into light yellow (Figure 1, SBR-1-P2) with the decrease in endogenous denitrification activity. After the start of Anammox activity, the color of the sludge gradually turned to red, which is the distinct feature of Anammox bacteria, accompanied by an increase in cytochrome content.

The ESEM micrograph of the SBR-2 seed (Figure 2, SBR-2-P1) was quite different to the SBR-1 micrograph. The shape of the bacterial clusters could not be identified properly since the bacteria in the clusters were heavily encapsulated with EPS (Figure 2, SBR-2-SEM2). Protozoa like organisms were apparent. As the culture enriched for Anammox, the morphology of the biomass (Figure 2, SBR-2-SEM2) resembled the morphology of the enriched biomass in SBR-1 (Figure 1,

SBR-1-SEM3). The spherical shape microorganisms with a compact heterogenous surface were still covered with a thin EPS layer. Following the 335-day enrichment period, the compact granular structure exhibited a typical cauliflower-like appearance. Similarly to SBR-1, in the course of the enrichment period the color changes of the SBR-2 biomass were consistent with the ESEM analyses results and the treatment performance. The initial brown color of the seed sludge (Figure 2, SBR-2-P1) initially turned to light yellow (Figure 2, SBR-2-P2) and gradually to the red color (Figure 2, SBR-2-P3-P5).

Microbial community structure analysis

The microbial population dynamics in both reactors were followed via FISH, DGGE and qPCR measurements through the enrichment period.

FISH analyses

FISH analyses were performed regularly during the 410-day operational period for both SBR-1 and SBR-2 (Supplementary Figures 2 and 3, available with the online version of this paper). At the start-up of SBR-1, in *Phase I-A*, seed sludge from an enriched Anammox containing reactor in Rotterdam STP exhibited only a few microcolonies belonged to Planctomycetales and no signals were detected in *Phase I-B*, indicating a change in microbial community due to inhibition. Hybridization signals of SBR-1 re-started following the start of Anammox activity in *Phase II* with probes specific to *Ca.* Brocadia anammoxidans and *Ca.* Scalindua. The enrichment of both genera was apparent in FISH images in *Phase III.* No signal was observed for *Ca.* Kuenenia stuttgartiensis throughout the 410-day operation period. At start-up, SBR-2 seeded with local mixed activated sludge showed no signal with a probe specific to Planctomycetales, indicating the initial absence of a significant population of Anammox. With the start of Anammox activity, in *Phase II*, *Ca.* Brocadia anammoxidans and *Ca.* Scalindua became apparent. In *Phase III*, hybridization signals with *Ca.* Brocadia anammoxidans and *Ca.* Scalindua increased significantly, indicating enrichment of the Anammox population in SBR-2. Hybridization signals specific to *Ca.* Kuenenia stuttgartiensis were not observed. Similarly to the present study, *Ca.* Brocadia anammoxidans was the most commonly observed dominant Anammox population in the reactors seeded with municipal activated sludge (Table 1). However, finding a *Ca.* Scalindua population in the enriched culture was unexpected, since *Ca.* Scalindua has been observed mostly in a marine environment. In spite of starting up with different seed sludges, in both SBR-1 and SBR-2, the enriched Anammox population contained the same genera, Brocadia and Scalindua, as a result of 410 days' continuous feeding with wastewater of the same origin.

DGGE analyses

DGGE analyses revealed that SBR-1 (Figure 3(a)) consisted of fewer taxa with respect to SBR-2 (Figure 3(b)). SBR-2, which was inoculated with mixed activated sludge, exhibited a diverse microbial profile. The microbial community in the SBR-1 seed shifted to a different composition in mid Phase II (SBR-1-S2) compared to the start-up (SBR-1-S1). Dissimilarity was observed to be around 33%. This explains why no Anammox activity was observed till day 195 in Phase II. After the start of Anammox activity, the DGGE profile remained relatively stable.

The microbial community profiles at the end of *Phase-II* and during *Phase-III* (SBR-1-S3, SBR-1-S4 and SBR-1-S5) consisted of similar taxa with 90–95% similarity values.

As opposed to SBR-1, in SBR-2 (Figure 3(b)), during *Phase I*, *Phase II* and *Phase III-A* the microbial community gradually changed (SBR-2-S2) with respect to the seed culture (SBR-2-S1). Dissimilarity with the seed sludge reached about 36% at day 121 (SBR-2-S3). One hundred days later than the start of Anammox activity (day 95) in *Phase III-A*, the microbial community drastically changed and the dissimilarity to the seed sludge increased to 56%. During the steady enrichment period of *Phase III-B*, the DGGE profile remained stable. The microbial community profiles (SBR-2-S4, SBR-2-S5 and SBR-2-S6) consisted of similar taxa with 90–95% similarity values.

Increase in band intensities through S1-S6 sludge samples of SBR-1 and SBR-2 suggested that enrichment promoted the dominance of these populations in the process.

The comparison of the normalized DGGE banding profiles of SBR-1 and SBR-2 (Figure 3(c)) indicated that the enriched cultures in SBR-1 and SBR-2 are 80% similar to each other. The observed similarities among the communities of different seed enrichments suggested that using different seed sludge samples in the start-up period could not have led to selection of a different microbial population at the end of enrichment.

Q-RT PCR

The enrichment of Anammox bacteria was quantified with qPCR analyses through the 410-day operational period at

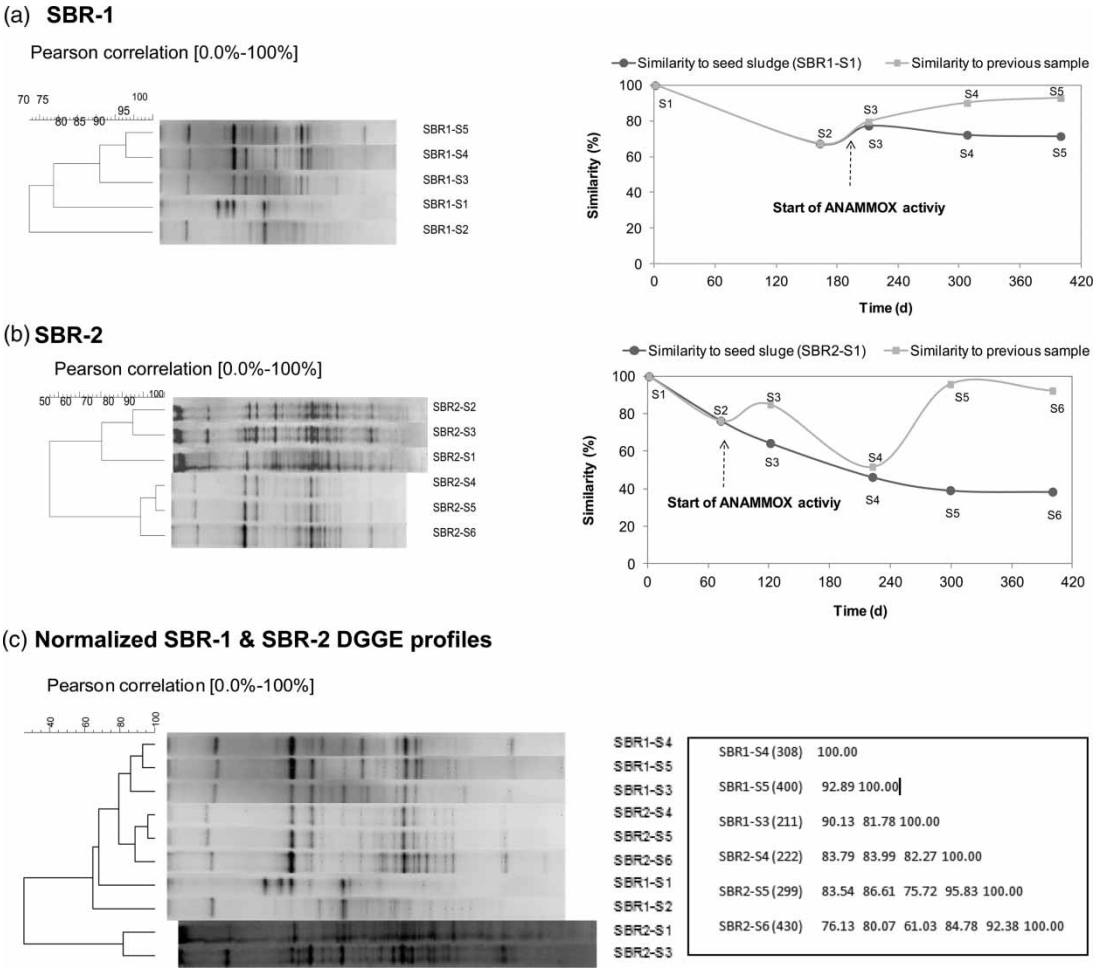

Figure 3 | DGGE profiles and similarity percentages calculated based on the Pearson product moment correlation coefficient for (a) SBR-1, (b) SBR-2, (c) normalized SBR-1 and SBR-2.

regular time intervals (Table 2). The systems were started up with seed sludges containing Anammox bacteria in the order of 10^9 for SBR-1 and 10^6 for SBR-2. As it was taken from an active Anammox reactor, the SBR-1 seed had initially three orders of magnitude greater than the SBR-2 seed, which was mixed activated sludge. However, the process efficiency data demonstrated that the highly abundant Anammox cells in SBR-1 were inactive. The low Anammox population in the SBR-2 seed explains not observing any Anammox activity initially. In *Phase II*, no change was observed in Anammox copy numbers in SBR-1, just before and after the start of Anammox activity. The Anammox population increased only one order of magnitude from 10^9 to 10^{10} in *Phase III*. This indicated that the increase in Anammox process efficiency observed for SBR-1 in *Phase III* was mainly due to activation and adaptation of the initially inactive Anammox population, not significant enrichment. In contrast, the abundance of the Anammox population in SBR-2 mixed activated sludge seed increased one log in *Phase-II* just before the start of observing Anammox activity. In *Phase III*, increasing loading conditions resulted in a three log jump in the Anammox population, from 10^7 to 10^{10}. This sudden jump explains the drastic change in microbial population observed via DGGE analysis in the same period. Increasing loadings resulted in one log further increase in the Anammox population and the abundance of Anammox cells reached the level of 10^{11}. A significant correlation was found between the increase in copy numbers of the 16S rRNA gene of Anammox bacteria and the increase in nitrogen removal rates.

Cloning, sequencing and phylogenetic analysis

Cloning, sequencing and phylogenetic analyses were also performed to characterize the microbial population in the reactors, before and after the enrichment period.

Table 3 | Sequenced clones of SBR-1 and SBR-2 matching with the closest RDP database affiliates from four different libraries

Clone name	Closest affiliate in RDP database	Phylum	Isolation source	Similarity
SBR1_S1_Clone – 1 (2)	Uncultured bacterium; C35_D63_H_B_H09; EF559129	Bacteroidetes	Mesophilic anaerobic digester	0.922
SBR1_S1_Clone – 2 (3)	*Pseudomonas* sp. Y2 – 1 – 1.AY515308	Proteobacteria	Sewage aerobic denitrification bacteria	1.000
SBR1_S1_Clone – 3 (7)	*Pseudomonas* sp. C23.; GQ241352	Proteobacteria	Expanded granular bed reactor	0.939
SBR1_S1_Clone – 5	Uncultured bacterium; KIST-JJY012; EF584532	Planctomycetes	Mixed granules from Anammox reactor	0.993
SBR1_S1_Clone – 9	Uncultured *Chryseobacterium* sp. Clone EF033490	Bacteroidetes	Denitrifying dispersed-growth reactors	0.99
SBR1_S1_Clone – 11	*Serratia proteamaculans*; TRS1-WB.; FN997641	Proteobacteria	Estuarine sediment	0.965
SBR1_S1_Clone – 16	*Lysobacter concretionis* (T); Ko07; AB161359	Proteobacteria	Granular sludge	0.982
SBR1_S5_Clone – 1 (2)	Uncultured bacterium; KIST-JJY023; EF594055	Chloroflexi	Mixed granules from Anammox reactor	0.945
SBR1_S5_Clone – 2 (6)	Uncultured bacterium; B124; HQ640593	Chloroflexi	Partial nitrifying-Anammox municipal wastewater reactor	0.936
SBR1_S5_Clone – 3 (4)	Uncultured bacterium; Dok07; FJ710726	Planctomycetes	Anaerobic ammonium oxidation reactor	0.987
SBR1_S5_Clone – 4	Uncultured *Pseudomonas* sp.; Filt.60; HM152648	Proteobacteria	Biological degreasing system	0.897
SBR1_S5_Clone – 5	Uncultured bacterium; ctg1_TOPO2 – 1; EU708509	Proteobacteria	Anoxic arsenite oxidizing denitrifying enrichment culture	0.953
SBR1_S5_Clone – 7 (2)	Uncultured *Chloroflexibacterium*; AKYH652; AY921687	Chloroflexi	Farm soil adjacent to a silage storage bunker	0.921
SBR1_S5_Clone – 10	Uncultured bacterium; Dok29; FJ710748	Acidobacteria	Anaerobic ammonium oxidation reactor	0.833
SBR1_S5_Clone – 12	Uncultured bacterium; TX5A_95; FJ152803	Proteobacteria	Alkaline saline soils of the former lake Texcoco	0.904
SBR1_S5_Clone – 18 (2)	Uncultured bacterium; SBR2037; X84576	Chloroflexi	Activated sludge	0.814
SBR2_S1_Clone – 1	Uncultured bacterium; 125ds10; AY212576	Proteobacteria	Water 10 m downstream of manure	0.851
SBR2_S1_Clone – 2 (6 clones)	Uncultured *Pseudomonas* sp.; Filt.60; HM152648	Proteobacteria	Biological degreasing systems	0.947
SBR2_S1_Clone – 4	Uncultured bacterium; A4 – 191; GQ898063	Firmicutes	Feces from human	0.959
SBR2_S1_Clone – 6 (2)	Uncultured bacterium; Dok07; FJ710726	Planctomycetes	Anaerobic ammonium oxidation reactor	0.934
SBR2_S1_Clone – 9	Uncultured bacterium; A4_435; EU763269	Firmicutes	Fecal sample	0.911
SBR2_S1_Clone – 12	Uncultured bacterium; ncd1006e09c1; HM341090	Bacteroidetes	Skin, popliteal fossa	0.738
SBR2_S6_Clone – 1	Uncultured eubacterium; 92; AJ412676	Proteobacteria	Denitrifying reactor	0.879
SBR2_S6_Clone – 2 (2)	Uncultured bacterium; Dok07; FJ710726	Planctomycetes	Anaerobic ammonium oxidation reactor	0.960

(*continued*)

Table 3 | continued

Clone name	Closest affiliate in RDP database	Phylum	Isolation source	Similarity
SBR2_S6_Clone – 3 (2)	Uncultured bacterium; KIST-JJY024; EF594056	Chloroflexi	Mixed granules from Anammox reactor	0.860
SBR2_S6_Clone – 4 (2)	*Pseudomonas* sp. Y2 – 1 – 1; AY515308	Proteobacteria	Sewage	0.955
SBR2_S6_Clone – 5	Uncultured bacterium; B15; HQ640522	Planctomycetes	Partial nitrifying-Anammox municipal wastewater reactor	0.947
SBR2_S6_Clone – 7 (2)	Uncultured bacterium; AMIA4; AM935488	Chloroflexi	Pilot-scale bioremediation process of a hydrocarbon-contaminated soil	0.714
SBR2_S6_Clone – 8	Uncultured bacterium; LR A2-26; DQ988307	Proteobacteria	Coking wastewater treatment reactor	0.659

Similarity (A seqmatch score (S_ab) values indicate the number of (unique) 7-base oligomers shared between your sequence and a given RDP sequence divided by the lowest number of unique oligos in either of the two sequences).

Phylogenetic trees of 16S rRNA clone library sequences of sludge samples at start-up (SBR-1-S1 and SBR-2–S1) and after the 410-day enrichment period (SBR-2–S1) and SBR-2–S6) were formed (Supplementary Figures 4 and 5, available with the online version of this paper). The sequenced clones of SBR-1 and SBR-2 were matched with the closest RDP database affiliates from four different libraries. The closest affiliates in the RDP databases are summarized in Table 3. Sixteen clones from the SBR-1 seed (SBR-1-S1) and 12 clones from the SBR-2 seed (SBR-2-S1) were sequenced. The dominant phylum detected in the SBR-1 seed from an active Anammox reactor were Proteobacteria (75%) and Bacteroidetes (19%). A clone belonging to Planctomycetes (6%) was also found. In the SBR-2 seed, which was taken from a local sewage treatment plant return activated sludge line, Proteobacteria (58%) was the most abundant phyla. Clones belonging to Firmicutes (17%), Planctomycetes (17%) and Bacteroidetes (8%) were also found. Members of the Bacteroidetes and Firmicutes phyla were closely related to filamentous bacteria detected in several other types of sludge (Li *et al.* 2009). At the end of the 410-day enrichment period, the relative abundance of the dominant bacterial communities in the seed sludges of SBR-1 and SBR-2 changed. The phylum Proteobacteria reduced significantly in both SBR-1 (15%) and SBR-2 (36%). The phylum Bacteroidetes in the SBR-1 and SBR-2 seed sludges and the phylum Firmicutes in the SBR-2 seed completely disappeared. As the enrichment proceeded and Anammox activity was maintained, agreeably clone library analysis demonstrated that the phylotypes Planctomycetes, Chloroflexi and Acidobacteria that are usually found in Anammox reactors became dominant in both SBR-1 (85%) and SBR-2 (64%). Especially, the abundance of phylotypes highly similar to the Chloroflexi phylum in both reactors agrees with previous studies where more than half of the clones detected in an Anammox reactor were found to be Chloroflexi-like bacteria (Li *et al.* 2009). These bacteria are thought to be important role players in the granulation process in the reactor.

CONCLUSION

The widespread use of the Anammox process world-wide is still limited by the availability of large volumes of local Anammox seed in most countries. The evaluation of the start–up period of Anammox systems seeded with local activated sludge compared to enriched Anammox bacteria is quite valuable since the purchase of enriched Anammox seed from other countries will significantly increase the capital cost of newly constructed Anammox plants. This study allowed the evaluation of the start-up of an Anammox system seeded with local mixed activated sludge in comparison to a system seeded with enriched Anammox sludge transported from abroad. The local mixed activated sludge was successfully enriched for an active Anammox population in 3 months. The Anammox population reached the level of 10^{11} Anammox copies/ng at the end of 410 days. The enriched Anammox seed transported from abroad was inhibited seriously during transportation and recovered over a very long period of time (195 days), most probably due to the absence or low population of different microbial species that could minimize the constituents causing inhibition of the Anammox species. Despite the different seeding strategies applied, the 410-day enriched Anammox populations contained the same genera, Brocadia and

Scalindua. The relative abundances of the dominant bacterial communities changed significantly with Anammox enrichment. The initially dominant phylum Proteobacteria in both seeds decreased with Anammox enrichment while the phylum Bacteroidetes and phylum Firmicutes disappeared completely. The phylotypes Planctomycetes, Chloroflexi and Acidobacteria become dominant at the end of the enrichment period.

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