Effect of COD/N ratio on N_2O production during nitrogen removal by aerobic granular sludge

V. F. Velho, B. S. Magnus, G. C. Daudt, J. A. Xavier, L. B. Guimarães and R. H. R. Costa

ABSTRACT

N_2O-production was investigated during nitrogen removal using aerobic granular sludge (AGS) technology. A pilot sequencing batch reactor (SBR) with AGS achieved an effluent in accordance with national discharge limits, although presented a nitrite accumulation rate of 95.79% with no simultaneous nitrification–denitrification. N_2O production was 2.06 mg L^{-1} during the anoxic phase, with N_2O emission during air pulses and the aeration phase of 1.6% of the nitrogen loading rate. Batch tests with AGS from the pilot reactor verified that at the greatest COD/N ratio (1.55), the N_2O production (1.08 mgN_2O-N L^{-1}) and consumption (up to 0.05 mgN_2O-N L^{-1}), resulted in the lowest remaining dissolved N_2O (0.03 mgN_2O-N L^{-1}), stripping the minimum N_2O gas (0.018 mgN_2O-N L^{-1}). Conversely, the carbon supply shortage, under low C/N ratios, increased N_2O emission (0.040 mgN_2O-N L^{-1}), due to incomplete denitrification. High abundance of ammonia-oxidizing and low abundance of nitrite-oxidizing bacteria were found, corroborating the fact of partial nitrification. A denitrifying heterotrophic community, represented mainly by *Pseudoxanthomonas*, was predominant in the AGS. Overall, the AGS showed stable partial nitrification ability representing capital and operating cost savings. The SBR operation flexibility could be advantageous for controlling N_2O emissions, and extending the anoxic phase would benefit complete denitrification in cases of low C/N influents.

Key words | aerobic granular sludge, COD/N ratio, nitrogen removal, N_2O production

V. F. Velho (corresponding author)
B. S. Magnus
G. C. Daudt
J. A. Xavier
L. B. Guimarães
R. H. R. Costa
Federal University of Santa Catarina,
Florianópolis, SC,
Brazil
E-mail: *vivianevelho@yahoo.com.br*

V. F. Velho
Catarinense Federal Institute of Education, Science and Technology,
Campus Camboriú, CEP: 88340 055 Camboriú, SC,
Brazil

NOMENCLATURE

AGS	aerobic granular sludge
AND	alternating nitrification and denitrification
AOB	ammonium-oxidizing bacteria
BOD	biochemical oxygen demand
COD	chemical oxygen demand
DO	dissolved oxygen
FA	free ammonia
FISH	fluorescence *in situ* hybridization
GHG	greenhouse gas
HRT	hydraulic retention time
N_2O	nitrous oxide
NLR	nitrogen loading rate
NO	nitric oxide
NOB	nitrite-oxidizing bacteria
SBR	sequencing batch reactor
SND	simultaneous nitrification and denitrification
SRT	sludge retention time
SVI	sludge volume index
TSS	total suspended solids
VSS	volatile suspended solids

doi: 10.2166/wst.2017.502

INTRODUCTION

Sequencing batch reactors (SBR) with granular biomass have been successfully used to treat municipal and industrial wastewaters (Beun *et al.* 1999; Arrojo *et al.* 2004). Aerobic granular sludge (AGS) has a compact structure with excellent settling properties, presenting diverse microbial species and high biomass retention (Liu & Tay 2004). The existence of substrate profiles across the granule depth enables simultaneous aerobic and anoxic processes in the same bioparticle, resulting in a very good performance of

Downloaded from https://iwaponline.com/wst/article-pdf/241589/wst076123452.pdf
by guest

organic matter and nitrogen removal (De Kreuk *et al.* 2005). Aerobic and anoxic zones coexist within the granules even when dissolved oxygen (DO) concentration is high in the bulk liquid, which favours simultaneous nitrification and denitrification (SND) in this system. According to Ju *et al.* (2007), there are three different mechanisms that make SND possible in aerobic granular SBR. (i) Aerobic and anoxic zones within the granules. (ii) Aerobic and anoxic zones inside the reactor. (iii) The presence of microorganisms able to perform nitrifier denitrification and heterotrophic nitrification.

A specific microbial distribution and mass transfer gradient in AGS provides a potential alternative for partial nitrification (Shi *et al.* 2011). Some studies applying AGS process showed the establishment of partial nitrification in low-strength real wastewater conditions (Coma *et al.* 2012; Wagner *et al.* 2015; Guimarães *et al.* 2017). Partial nitrification has gained interest in biological nitrogen removal from wastewater, since it reduces carbon limitation concerns and acts as a shortcut nitrogen removal system combined with anaerobic ammonium oxidation (anammox) process (Ge *et al.* 2015). An effluent composition close to a molar ammonium to nitrite ratio of 1:1 is best for the anammox process. Nevertheless, the conditions for partial nitrification can increase nitrous oxide (N_2O) production, which is a greenhouse gas (GHG) with 300-fold stronger effect than carbon dioxide in terms of global warming potential.

In the biological nitrogen removal process, nitrite and/or nitrate produced from nitrification are reduced to nitrogen gas by denitrifiers, which is directly associated with N_2O generation. The amount of N_2O released is relatively small compared to the quantity of total nitrogen removed during the processes (Gao *et al.* 2016). Wastewater treatment processes are extremely important in microbial N_2O emission (Kong *et al.* 2013). N_2O generation is associated with three different metabolic pathways: (i) incomplete hydroxylamine oxidation, (ii) nitrifier denitrification, (iii) heterotrophic denitrification (Kampschreur *et al.* 2009). Therefore, the microbial composition of AGS has an important influence on the behaviours and pathways of N_2O emissions (Gao *et al.* 2016). Furthermore, an increase in N_2O production and emission can result from some operational conditions such as COD/N ratio, DO concentration, nitrite concentration and pH (Kampschreur *et al.* 2009).

Although information about nitrogen conversion in aerobic granules is abundant, very few studies have focused on N_2O production and emission by this system. Since N_2O is considered the third most important contributor to climate change (IPCC 2014), it is important to verify its emission by this technology, which has been widely applied, especially via studies using real wastewater. Additional knowledge of the microbial processes and the factors controlling N_2O production in nitrifying/denitrifying AGS systems is required to develop strategies to reduce emissions of this GHG. Therefore, this study aimed (i) to monitor the N_2O production and emissions from an SBR with AGS treating domestic wastewater and (ii) to verify the maximum N_2O emissions for different COD/N ratios. The microbial composition was also investigated in order to clarify the nitrogen biological processes.

MATERIALS AND METHODS

Set-up of a pilot SBR with AGS

A pilot SBR (0.25 m in diameter and 3.0 m in height) with working volume of 98 L and volumetric exchange ratio of 56% was used to provide the formation of granular sludge. Natural biomass accumulation and granulation occurred without inoculation. The selection conditions such as the settling time, superficial upflow air velocity and the volume exchange ratio were set to stimulate the growth of the granular biomass from wastewater. The SBR was operated at room temperature ($23 \pm 2^{\circ}C$) in anoxic/oxic (A/O) cycles of 6 hours, consisting of 3 min feeding, 90 min anoxic, 248 min aeration, 15 min settling, 3 min effluent withdrawal and 1 min idle. During the 90 min anoxic phase, air pulses (turned on every 15 minutes for 10 seconds) were applied in order to keep the sludge mixed through the liquid column. The hydraulic retention time (HRT) was 7.14 hours and the sludge retention time (SRT) was 8 ± 2 days. The pH in the system was recorded but not controlled, and fluctuated between 6.5 and 7.5. Aeration was provided by airflow at 32 L min^{-1}, using a membrane diffuser (B&FDIAS, Brazil) placed at the bottom of the reactor in a superficial upflow air velocity of 1.2 cm s^{-1}, which resulted in a DO at saturation level (7.2–8.5 mg L^{-1}).

Real domestic wastewater from a sewage network of Florianópolis, Santa Catarina, Brazil (27°35′49″S/48°32′56″W), fed the SBR at the beginning of each cycle. The influent concentrations of total chemical oxygen demand (COD_{tot}), soluble chemical oxygen demand (COD_s), and ammonium-nitrogen (NH_4^+-N) were 380 ± 101 mgCOD_{tot} L^{-1}, 179 ± 29 mgCOD_s L^{-1}, and 71 ± 13 mgNH_4^+–N L^{-1}, respectively. The reactor was operated for 120 days, with a biomass concentration of 1.3 gTSS L^{-1} and 1.1 gVSS L^{-1}, respectively. The AGS granule size was between 0.2 and 0.4 mm and the

Downloaded from https://iwaponline.com/wst/article-pdf/241589/wst076123452.pdf
by guest

sludge volume index (SVI) was 70 mL g^{-1}. The performance of system was monitored through the cycles, with samples taken regularly for COD_s, NH_4^+-N, and NO_x-N analysis, while N_2O was measured in its dissolved and gaseous states. The detailed study over the operational cycle in the pilot SBR with AGS was conducted to verify the nitrogen removal processes and the production and emission of N_2O associated with these processes.

Setup of batch experiments using biomass from the pilot SBR

Batch experiments were performed according to Kim & Kim (2011), using AGS taken from the end of the aeration phase of the pilot SBR. The sludge (2.1 L) was stirred and aerated for 20 hours in order to deplete COD and ammonium sources. Subsequently, the sludge was divided into three equal volumes (0.7 L) to be used in the three different assays varying the type of wastewater. The experimental bench system is represented in Figure 1, and comprised: (1) wastewater storage flask, (2) peristaltic pump, (3) compressed air, (4) bench reactor, (5) stirring table, (6) dissolved N_2O sensor, (7) N_2O gas sensor.

For each batch assay, the bench reactor was filled with AGS (0.7 L) and the specific wastewater (0.1 L), which had different characteristics and gave distinct COD/NH_4^+-N ratios (Table 1). The domestic raw sewage was taken from the same municipal wastewater network that was used in the pilot reactor. The denitrified domestic sewage is the effluent sampled at the end of the anoxic phase of the pilot SBR. The synthetic sewage was a solution of NH_4Cl prepared without a carbon source. The operational cycle conditions were

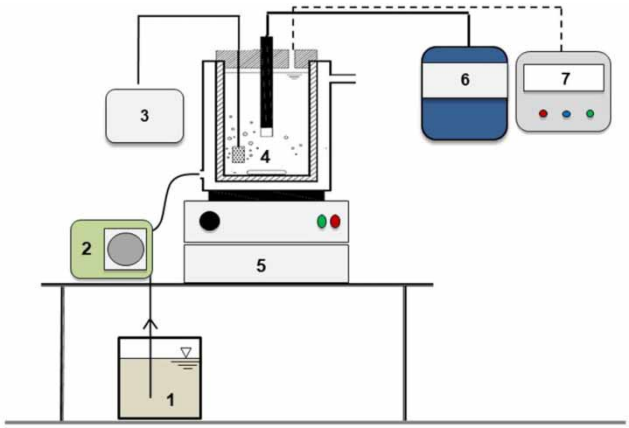

Figure 1 | Bench experimental design. (1) Wastewater storage flask, (2) peristaltic pump, (3) compressed air, (4) bench reactor, (5) stirring table, (6) dissolved N_2O sensor, (7) N_2O gas sensor.

Table 1 | Experimental conditions in the three batch tests, varying C/N ratios, using AGS from the pilot SBR

Parameter	Domestic raw sewage	Denitrified domestic sewage	Synthetic sewage
TSS (g TSS L^{-1})	1.1	1.0	1.0
COD_s (mg COD_s L^{-1})	103	52	No addition of carbon
NH_4^+-N (mg NH_4^+ L^{-1})	67	45	45
COD/NH_4^+-N ratio	1.55	1.17	0

the same as for the pilot SBR and the conditions at the beginning of each assay are shown in Table 1. The batch experiments were performed simulating the operational cycle of the pilot reactor, consisting of 90 min of anoxic stirring, 240 min of aeration, 15 min of settling, totalling 345 min. Compressed air was provided through an air porous stone diffuser at the bottom of the reactor; DO was kept at 2 mg L^{-1}.

Analytical methods

The cycle of the pilot SBR and each batch experiment were monitored for following: NH_4^+-N, NO_2^--N, NO_3^--N, COD_s and solids every 45 min (during the anoxic phase) and every hour (during the aerobic phase). The parameters were analyzed in accordance with *Standard Methods* (APHA 2012). The nitrite accumulation rate was calculated based on Wei *et al.* (2014) and free ammonia (FA) according to Anthonisen *et al.* (1976).

The N_2O gas flow was continuously analysed (every minute) by an infra-red analyser (Guardian NG, Edinburgh, UK) with a range between 0 and 3,000 ppm. In addition, dissolved N_2O concentration was measured with an N_2O micro-sensor (N_2O Wastewater System, Unisense A/S, Denmark) with a working range between 0–1.5 mgN_2O-N L^{-1} and a detection limit of 0.005 mgN_2O-N L^{-1}. The N_2O production rate was calculated according to the method described by Hu *et al.* (2011).

Microbiological methods

The identification of active bacterial populations in the sludge used for the assays was carried out by fluorescence *in situ* hybridization (FISH). Biomass samples were fixed with 4% paraformaldehyde solution and hybridized using the following probes: EUB338 I, II and III to stain all bacteria; NSO190 for ammonium-oxidizing bacteria (AOB) population; Ntspa662 for *Nitrospira*; GAO431 and 989 for

Downloaded from https://iwaponline.com/wst/article-pdf/241589/wst076123452.pdf
by guest

member of '*Candidatus* Competibacter'; PAO462, 651 and 841 to stain '*Candidatus* Accumulibacter phosphatis'; PAE997 for *Pseudomonas* spp. (Amann *et al.* 1995). The detailed sequences of FISH probes can be checked in Probebase (Greuter *et al.* 2016).

DNA sequencing was performed using MiSeq® Illumina technology for sequencing by synthesis (SBS) (Neoprospecta, Brazil). The DNA was extracted from biomass by applying the protocol of MoBio PowerBiofilm™ DNA extraction kit (MoBio Laboratories, USA). The rRNA 16S gene, V3/V4 region, was been amplified using the 341F (CCTACGGGRS GCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) primers, with Illumina adapters, required for sequencing. The amplification was performed in 35 cycles at 50°C annealing temperature, where each sample was amplified in triplicate. The sequencing was performed in Illumina MiSeq, using a V2 kit, with a single-end 300 cycle run. The system guaranteed the reading of 100,000 sequences with sampling taxonomic identification and quantification of the number of sequences obtained from each taxon. Operational taxonomic unit (OTU) picking was performed using BLASTN 2.2.28 against GreenGenes 13.8 database. To attribute taxonomy, only sequences with hits of 99% of identity in alignment covered over 99% were considered.

RESULTS AND DISCUSSION

Pilot SBR with AGS treating domestic wastewater

Treatment performance

Table 2 presents the performance of the pilot SBR with AGS during long-term operation (120 days). The BOD_5 and ammonium removal were greater than 80%, attending the Brazilian discharge limits (120 mg L^{-1} or 60% of removal efficiency for BOD_5 – Brasil 2011) with effluent concentration lower than 35 mg BOD_5 L^{-1}. Final phosphorous effluent concentrations reached effluent quality criteria of Santa Catarina state law ($\leq$4 mg L^{-1} of total phosphorus – Santa Catarina 2009). The high standard deviation is attributed to natural fluctuation in the municipal sewage network and also to the influence of rainwater inputs.

Figure 2 shows a typical cycle profile of nitrogen compounds, production and emission of N_2O, and concentrations of COD_s and DO during pilot SBR operation. The reactor was fed with a COD/N ratio of 2.53, with loading rates of 0.41 kgCOD_s m^{-3} d^{-1} and 0.16 kgNH_4^+-N m^{-3} d^{-1}. Ammonium was oxidized mainly to nitrite during the aeration phase and negligible nitrate formation was verified (effluent concentrations of 20 mgNO_2^--N L^{-1} and 0.95 mgNO_3^--N L^{-1}), indicating partial nitrification (Figure 2(a)). The nitrite accumulation rate was 95.79%, implying the activity of nitrite-oxidizing bacteria (NOB) was fully limited

Table 2 | Influent and effluent concentrations and removal efficiencies in the pilot SBR with AGS for the legally required parameters

Parameter	Influent (mg L^{-1}) (n = 14)	Effluent (mg L^{-1}) (n = 14)	Removal (%)
COD_{tot}	400 ± 101	136 ± 23	64
COD_s	174 ± 29	55 ± 9	68
BOD_5	221 ± 36	31 ± 7	86
NH_4^+-N	54 ± 13	8 ± 5	84
Total phosphorus	5.1 ± 1.0	4.0 ± 1.0	16
TSS	145 ± 48	61 ± 12	54

Average ± standard deviation. n = number of samples.

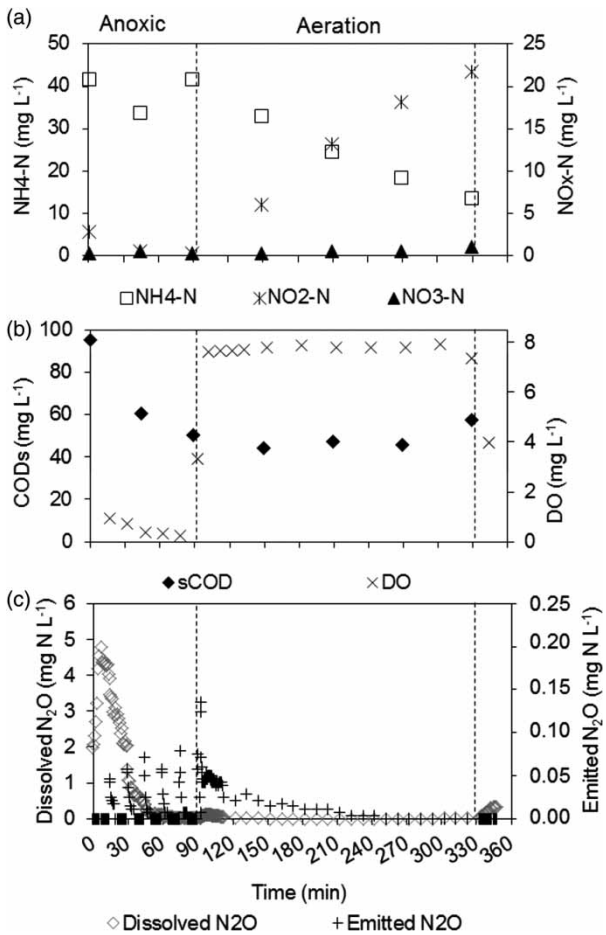

Figure 2 | Pilot SBR operational cycle with AGS treating domestic wastewater. During the 360 min cycle, the concentration profile of NH_4^+-N, NO_2^--N and NO_3^--N (a); COD and DO (b); and N_2O production and emission (c) were measured over time.

Downloaded from https://iwaponline.com/wst/article-pdf/241589/wst076123452.pdf
by guest

in the partial nitrification in the pilot reactor. This phenomenon likely results from the uncoupled activities of AOB and NOB (Wei *et al.* 2014), and it commonly occurs in reactors with aerobic granules (Isanta *et al.* 2012; Wagner *et al.* 2015; Guimarães *et al.* 2017).

Nitrite and COD_s (Figure 2(a) and 2(b)) decreased during the anoxic phase, indicating the process of heterotrophic denitrification in this period. Previous studies have shown that SND could occur in AGS as a result of DO diffusion limitations, which creates anoxic zones inside the granule (Shi *et al.* 2011; Wei *et al.* 2014). However, SND was not observed in the pilot SBR of this study. The granular sludge size obtained was in a range of 0.2–0.4 mm, which is probably not large enough to create the anoxic core. Overall, nitrogen removal was performed by partial nitrification and denitrification under alternating anoxic and aerobic conditions inside the reactor. According to Guo *et al.* (2010) and Wei *et al.* (2014), the requirements for oxygen consumption and carbon source are reduced during partial nitrification and subsequent nitrite denitrification. Therefore, the method is considered cost-effective for the treatment of wastewater with low COD/N ratios. However, the accumulation of nitrite might lead to negative effects, such as inducing and/or increasing the N_2O emission via denitrification (Kampschreur *et al.* 2009).

N_2O production and emission

The fraction of influent nitrogen converted to N_2O was 1.6%, while N_2O emission from the oxidized ammonium during the cycle was 2.26%. This value represents a N_2O emission of 3.15 $mgN_2O\text{-}N\ (gVSS)^{-1}$ and 2.06 $mgN_2O\ L^{-1}$, in terms of nitrogen emitted as N_2O per litre of treated sewage. According to Kong *et al.* (2013) and Castro-Barros *et al.* (2016), the N_2O emissions from biological nutrient removal processes vary substantially among the studies (0.01–25% of N removed) due to different operational conditions, wastewater and reactor types, and N_2O measurement methods. N_2O emissions from municipal wastewater treatment plants are estimated as approximately 0.5% of the nitrogen loading rate (NLR) (IPCC 2006). A previous partial nitrification study with AGS reactor reported N_2O emissions of 3.8% of NLR (Shi *et al.* 2011).

Although the N_2O emissions occurred during the air pulses in the anoxic phase and during the aeration phase by stripping (Figure 2(c)), N_2O is likely produced mainly during the anoxic period as a result of the denitrification process. In order to verify that, N_2O was measured in the liquid phase (Figure 2(c)), showing that the greatest dissolved fraction was during the first 15 min of the anoxic period. Many factors have been reported to increases N_2O production during denitrification, such as low COD/N ratio (<3.5), low pH (<6.5) and short solids retention time (<1 day) (Kishida *et al.* 2004; Kampschreur *et al.* 2009; Quan *et al.* 2012). The SBR studied here was operated at a low COD/N ratio (2.5), pH of 6.5–7.5 and SRT of 8 days, indicating C/N ratio as a relevant factor that could induce N_2O production. Similar results were obtained by Quan *et al.* (2012) in an AGS reactor treating synthetic wastewater, which verified that increasing COD/N ratio and aeration rate would reduce N_2O emissions.

Batch experiments with AGS from the pilot SBR

The effect of different COD/N ratios on N_2O production and emission

In order to verify the maximum emission of N_2O from the AGS of the pilot reactor, batch experiments were carried out with different and low COD/N ratios. Figure 3(a)–3(c) show the NH_4^+-N, NO_3^--N and NO_2^--N concentrations and Figure 3(d)–3(f) N_2O production and emission over one cycle under different COD/N ratios. NH_4^+-N concentration remained unchanged during the anoxic phase for the three batch experiments. When the aeration started, NH_4^+-N concentration decreased linearly with increasing NO_2^--N concentration, following the partial nitrification phenomena already observed in the pilot reactor. The effluent ammonium concentration decreased to 21, 21 and 15 $mg\ L^{-1}$, while nitrite increased to 14.8, 10.7 and 12 mg/L at COD/N ratios of 1.55, 1.17 and 0, respectively. Nitrate was negligible during the three batch experiments (Figure 3(a)–3(c)) and no significant SND was observed in aerobic conditions. Total nitrogen removal was higher at increased COD/N ratios (32% at ratios 0; 44% at ratio 1.55).

The main factors associated with nitrite accumulation in this study can be FA inhibition and DO limitation (Ge *et al.* 2015). The FA concentration fluctuated in the batch cycle tests. At the COD/N ratio of 1.55, it varied from 0.31 to 0.16 $mg\ FA\ L^{-1}$, from the beginning to the end of the cycle, while at lower ratios (1.17 and 0) it varied from 0.24 to 0.14 $mg\ FA\ L^{-1}$. These FA values are in the range of NOB inhibition, which is from 0.1 to 1.0 $mg\ FA\ L^{-1}$ (Anthonisen *et al.* 1976). DO limitation inside the granules should be considered even if a sufficient DO concentration is maintained in the bulk liquid (greater than 2 $mg\ L^{-1}$) for complete nitrification. The oxygen penetration depth provided different DO levels in the granular sludge affecting

Downloaded from https://iwaponline.com/wst/article-pdf/241589/wst076123452.pdf
by guest

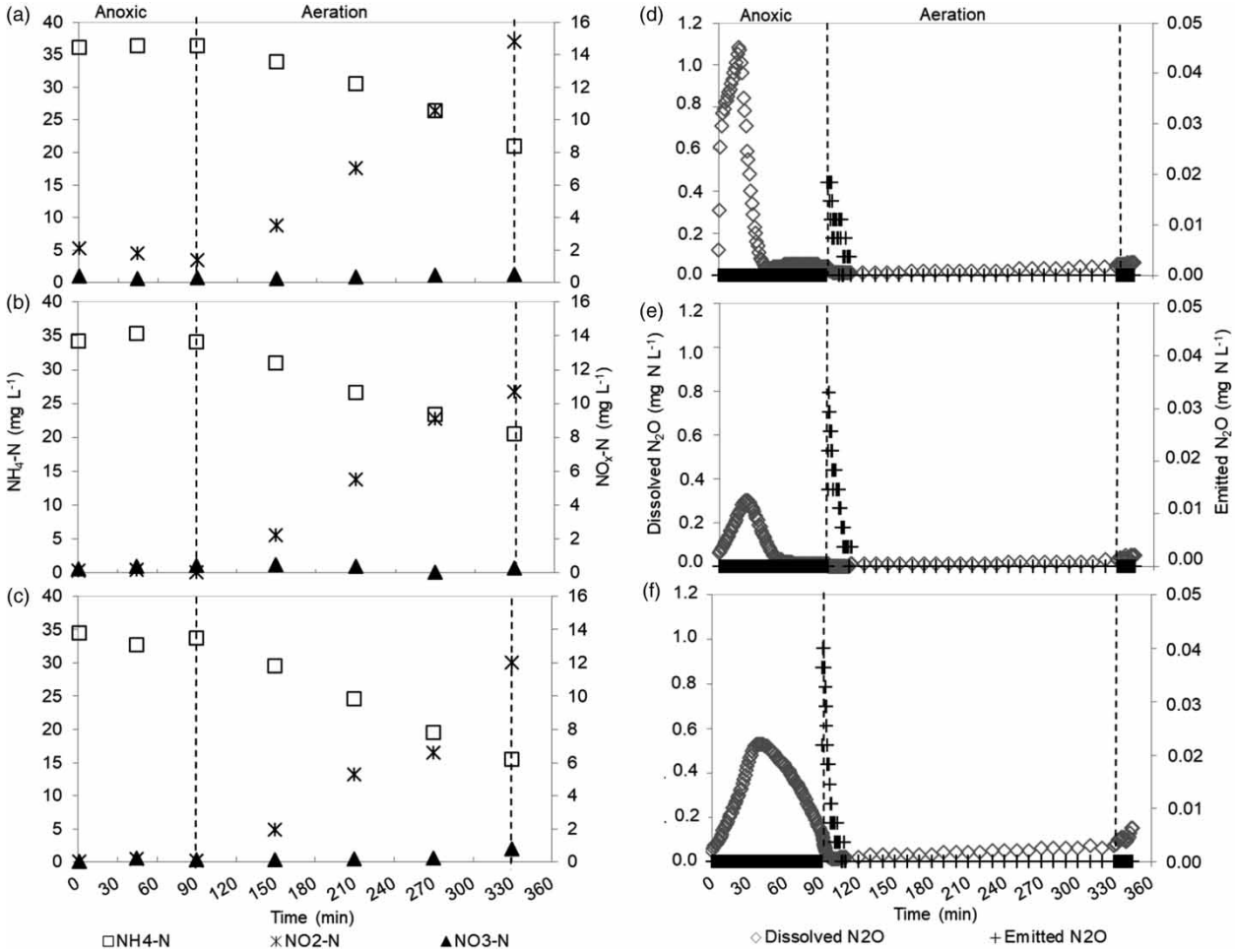

Figure 3 | Bench-scale SBR operational cycle with AGS from the pilot system. During the 360 min cycle, the concentration profile of NH_4^+-N, NO_2^--N and NO_3^--N and the effect of different COD/N ratios on N_2O production/emission were monitored. (a) and (d) COD/N = 1.55; (b) and (e) COD/N = 1.17; (c) and (f) COD/N = 0.

the activity and distribution of microorganisms. DO limitation inside the granules limited the growth of NOB and thus helped to maintain partial nitrification (Vázquez-Padín *et al.* 2010). Furthermore, Liang *et al.* (2015) stated that the presence of organic matter in the influent could promote the suppression of NOB, and then ensured the stable operation of partial nitrification. According to the authors NOB could not outcompete for DO with the heterotrophic bacteria and AOB.

N_2O was mainly produced in the anoxic phase and briefly in the aerobic phase in all experimental batch assays, as shown by N_2O dissolved concentrations in Figure 3(d)–3(f). During anoxic phase, the dissolved N_2O was formed and also consumed, which is likely a result of nitrite reduction followed by N_2 production, in a heterotrophic denitrification process. The N_2O emitted was maximal at the beginning of the aerobic period and stabilized at a low concentration after this initial spike. At COD/N ratio of 1.55, a maximum concentration of 1.08 mg N_2O-N L^{-1} was produced in 17 min followed by a rapid decrease in the next 21 min up to 0.05 mg N_2O-N L^{-1}. The remaining N_2O in the liquid (0.03 mg N_2O-N L^{-1}) was stripped to the gas phase as soon as the aeration started, represented by the peak in 0.018 mgN_2O-N L^{-1} of emitted gas (Figure 3(d)). Conversely, at the lowest COD/N ratio, N_2O was produced at half the speed (36 min) at a maximum concentration of 0.53 mgN_2O-N L^{-1}, and not fully consumed, leaving 0.13 mgN_2O-N L^{-1}, which was subsequently stripped in the aeration phase up to 0.040 mgN_2O-N L^{-1} of emitted gas (Figure 3(f)). The increased N_2O emission during low C/N ratios can be ascribed to incomplete denitrification, induced by the carbon supply shortage (Lemaire *et al.* 2006), which in the case of a ratio equal to 0, might be replaced by the use of internal carbon sources. Since N_2O reduction to N_2 is the last step of denitrification, after nitrate or nitrite reduction to N_2O, if no carbon source is available, N_2O gas will possibly accumulate (Yang *et al.* 2009). Additionally, NO_2^--N

Downloaded from https://iwaponline.com/wst/article-pdf/241589/wst076123452.pdf
by guest

accumulation resulting from incomplete nitrification and denitrification may inactivate N_2O reductase, thus increasing N_2O production and emissions (Kampschreur *et al.* 2009).

Partial nitrification for saving capital and operational SBR strategy to reduce N_2O emission

Nitrogen removal via nitrite decreases the energy consumption, by decreasing aeration, and reduces carbon limitation concerns (Yang *et al.* 2009; Ge *et al.* 2015). During the pilot SBR operation and batch experiments of the present study, partial nitrification with 95.79% and 96.98% of NO_2^--N accumulation were observed, respectively, showing that AGS had excellent and stable partial nitrification ability. Therefore, the stable partial nitrification process achieved by the AGS system in the present study could benefit other processes. This phenomenon is suitable as pre-treatment of the anammox process, which does not require a carbon source and reduces nitrite directly to N_2 without N_2O production.

However, special attention should be given to the nitrite accumulation process, since its conditions usually lead to N_2O production. N_2O yield based on the removed total nitrogen was estimated in order to quantify and analyse the nitrogen flux during nitrogen removal in one operational cycle. Thus, 2.79%, 9.43% and 7.99% of the removed total nitrogen was converted to N_2O-N at COD/N ratios of 1.55, 1.17 and 0, respectively. The N_2O levels emitted in each cycle assay were 1.28, 1.77 and 1.76 mgN_2O-N L^{-1}, meaning 1.22%, 2.50% and 2.55% of the NLR at COD/N ratio of 1.55, 1.17 and 0, respectively. These N_2O fractions emitted are greater than expected N_2O emissions in wastewater treatment plants (0.5% by IPCC 2006), but lower than studies with partial nitrification in AGS systems (3.8% by Shi *et al.* 2011). This relatively low N_2O emission could be compensated with the combination of AGS and anammox systems, since this last shows no N_2O production.

Additionally, the present batch experiments showed that at higher C/N ratios, less N_2O was emitted, as a result of complete denitrification. However, the use of external carbon or controlling the organic loading rate in full-scale systems can be costly and unfeasible. Therefore, the batch tests showed that in very low C/N ratio conditions, which could be associated with rain events or a specific effluent, an extension of the anoxic phase would lead to enough N_2O reduction, decreasing its emission. In this way, the SBR operation flexibility could be beneficial for controlling N_2O emission, diminishing the greenhouse effect. Yang *et al.* (2009) have suggested the application of step-feed SBR to reduce N_2O production, which provides an external carbon source at the end of aeration to reduce nitrite and then N_2O. Lochmatter *et al.* (2013) have tested different aeration rates to promote alternating nitrification and denitrification (AND) conditions. The AND strategies were designed alternating aerobic/anoxic phases during the famine phase, and N_2O emissions significantly decreased with COD loads of 2.1–2.4 mg L^{-1} d^{-1}.

Microbial community characterization

Microbial characterization using FISH of the sludge from the pilot SBR used in the bench assays revealed a diversified community. AOB belonging to the bacterial genus *Nitrosomonas* were detected in high abundance in the aerobic granules. The genus *Nitrospira* was the identified NOB, but at low abundance, corroborating with the partial nitrification observed at pilot and bench SBR. Accumulation of nitrite could induce nitrifier denitrification activity by AOB, which uses nitrite as the terminal electron acceptor to produce N_2O (Kampschreur *et al.* 2009; Gao *et al.* 2016). Heterotrophic denitrifiers from genus *Pseudomonas* were detected in high abundance, while glycogen- and polyphosphate-accumulating organisms (GAO and PAO) were in low abundance in the AGS. Besides the use of external carbon for heterotrophic denitrification, PAO and GAO organisms can perform denitrification using intracellular carbon (Lemaire *et al.* 2006), which could also have contributed to N_2O production.

The bacterial communities were also verified using the advanced method of high-throughput sequencing. Samples of the pilot SBR were characterized on days 21 and 119, the latter coinciding with the batch experiments' accomplishment (Figure 4). Both samples were globally composed of the same predominant populations present in relative abundances above 5% of the total bacterial community and members of family Xanthomonadaceae, Comamonadaceae, Microbacteriaceae and Rhodobacteraceae. These families are commonly detected on wastewater treatment systems (Weissbrodt *et al.* 2014). Xanthomonadaceae showed the greatest relative abundance (6–9-fold higher than other organisms) throughout the experiments. Additionally, Flavobacteriaceae and Bradyrhizobiaceae were identified in relative abundances above 5% on day 119.

Conditions applied in the pilot SBR favoured genus *Pseudoxanthomonas* sp. from the Xanthomonadaceae family, with relative abundances increasing from 30% (day 21) to 45% (day 119). These organisms belong to the heterotrophic denitrifying community and can produce exopolysaccharides, which in AGS systems is essential for supporting the granule structure (Adav *et al.* 2010; Weissbrodt *et al.* 2014). *Acidovorax* sp. (family Comamonadaceae) was detected on the samples

Downloaded from https://iwaponline.com/wst/article-pdf/241589/wst076123452.pdf
by guest

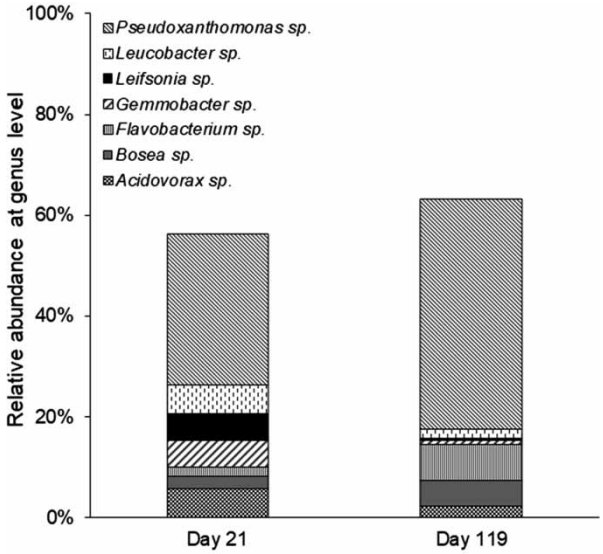

Figure 4 | Microbial diversity at genus level in the AGS sampled from pilot SRB on days 21 and 119. Only relative abundance greater than 5% of the total sequences was considered.

(day 21: 6%; day 119: 2%), which are aerobic organisms that are able to grow anaerobically using nitrate or nitrite as terminal electron acceptors. In activated sludge, *Acidovorax* have been demonstrated to denitrify (McIlroy *et al.* 2015). The Microbacteriaceae family, represented by the genera *Leucobacter* and *Leifsonia*, was previously found as the predominant population on the surface of mature granules, and shows a high growth rate (Kim & Lee 2011). *Flavobacterium* sp. (day 21: 2%; day 119: 7%), commonly seen in activated sludge, has an aerobic metabolism, but anaerobic growth is also possible for some species. Interestingly, various polysaccharides are hydrolyzed by several species in the genus (McIlroy *et al.* 2015). Overall, these predominant populations on the AGS of the present study are facultative ordinary heterotrophic organisms, which are aerobic and able to denitrify. Therefore, the microbial characterization supports the heterotrophic denitrification as the main N_2O production and consumption process, during the anoxic phases of the pilot SBR and bench assays, which agrees with physical–chemical results previously discussed.

CONCLUSIONS

N_2O emission was verified in the SBR with AGS as a result of partial nitrification and heterotrophic denitrification processes. High concentrations of FA and DO limitation inside the granules might be related to high nitrite accumulation playing an important role in N_2O production. N_2O was mainly produced during the anoxic phase and its highest emissions were measured under low COD/N ratio conditions, due to incomplete denitrification induced by the carbon supply shortage. AOB was at high abundance, while NOB was found at low abundance in granules. Denitrifying heterotrophics represented the majority community in the sludge, with the genus *Pseudoxanthomonas* predominating (6–9-fold more abundant than others organisms). Overall, the AGS had shown excellent and stable partial nitrification ability representing capital and operating cost savings. The SBR operation flexibility could be advantageous for controlling N_2O emissions, and the extension of anoxic phase in cases of low C/N influents would benefit complete denitrification.

ACKNOWLEDGEMENTS

The authors would like to thank FAPESC, FINEP, CAPES and CNPq for financial support in terms of costs and providing scholarships.

REFERENCES

Adav, S. S., Lee, D. J. & Lai, J. Y. 2010 Microbial community of acetate utilizing denitrifiers in aerobic granules. *Appl. Microbiol. Biotechnol.* **85**, 753–762.

Amann, R. I., Ludwig, W. & Schleifer, K. 1995 Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews* **59** (1), 143–169.

American Public Health Association (APHA) 2012 *Standard Methods for the Examination of Water and Wastewater*, 22nd edn. APHA, AWWA (American Water Works Association), and WEF (Water Environment Federation), Washington, DC.

Anthonisen, A. C., Loehr, R. C., Prakasam, T. B. S. & Srinath, E. G. 1976 Inhibition of nitrification and nitrous acid compounds. *Journal of the Water Pollution Control Federation* **48** (5), 835–852.

Arrojo, B., Mosquera-Corral, A., Garrido, J. M. & Méndez, R. 2004 Aerobic granulation with industrial wastewater in sequencing batch reactors. *Water Research* **38**, 3389–3399.

Beun, J. J., Hendriks, A., van Loosdrecht, M. C. M., Morgenroth, M., Wilderer, P. A. & Heijnen, J. J. 1999 Aerobic granulation in a sequencing batch reactor. *Water Research* **33**, 2283–2290.

Brasil2011 *Resolução Conama n.430. Dispõe sobre as condições e padrões de lançamento de efluentes (Conama Resolution n.430. Provides the Conditions and Standards for the Effluents Discharge)*. National Environment Council, Brasília, Brazil, 89p.

Castro-Barros, C. M., Rodríguez-Caballero, A., Volcke, E. I. P. & Pijuan, M. 2016 Effect of nitrite on the N_2O and NO production on the nitrification of low-strength ammonium wastewater. *Chem. Eng. J.* **287**, 269–276.

Coma, M., Verawaty, M., Pijuan, M., Yuan, Z. & Bond, P. L. 2012 Enhancing aerobic granulation for biological nutrient

Downloaded from https://iwaponline.com/wst/article-pdf/241589/wst076123452.pdf
by guest

removal from domestic wastewater. *Bioresour. Technol.* **103** (1), 101–108.

De Kreuk, M. K., Heijnen, J. J. & van Loosdrecht, M. C. M. 2005 Simultaneous COD, nitrogen, and phosphate removal by aerobic granular sludge. *Biotechnol. Bioeng.* **2**, 761–769.

Gao, M., Yang, S., Wang, M. & Wang, X. 2016 Nitrous oxide emissions from an aerobic granular sludge system treating low-strength ammonium wastewater. *Journal of Bioscience and Bioengineering* **122** (5), 601–605.

Ge, S., Wang, S., Yang, X., Qiu, S., Li, B. & Peng, Y. 2015 Detection of nitrifiers and evaluation of partial nitrification for wastewater treatment: a review. *Chemosphere* **140**, 85–98.

Greuter, D., Loy, A., Horn, M. & Rattei, T. 2016 ProbeBase-an online resource for rRNA-targeted oligonucleotide probes and primers: new features 2016. *Nucleic Acids Research* **44** (1), 586–589.

Guimarães, L. B., Mezzari, M. P., Daudt, G. C. & da Costa, R. H. R. 2017 Microbial pathways of nitrogen removal in aerobic granular sludge treating domestic wastewater. *Journal of Chemical Technology & Biotechnology* **92** (7), 1756–1765.

Guo, J., Peng, Y., Huang, H., Wang, S., Ge, S., Zhang, J. & Wang, Z. 2010 Short- and long-term effects of temperature on partial nitrification in a sequencing batch reactor treating domestic wastewater. *Journal of Hazardous Materials* **179**, 471–479.

Hu, Z., Zhang, J., Xie, H., Li, S., Wang, J. & Zhang, T. 2011 Effect of anoxic/aerobic phase fraction on N_2O emission in a sequencing batch reactor under low temperature. *Bioresour. Technol.* **102**, 5486–5491.

IPCC 2006 *Guidelines for National Greenhouse Gas Inventories.* IGES, Japan.

IPCC 2014 *Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Core Writing Team, R. K. Pachauri & L. A. Meyer (eds)]. IPCC, Geneva, Switzerland, 151p.

Isanta, E., Suárez-Ojeda, M. E., Val del Río, Á., Morales, N., Pérez, J. & Carrera, J. 2012 Long term operation of a granular sequencing batch reactor at pilot scale treating a low-strength wastewater. *Chemical Engineering Journal* **198–199**, 163–170.

Ju, L. K., Huang, L. & Trivedi, H. 2007 Simultaneous nitrification, denitrification, and phosphorus removal in single-tank low-dissolved oxygen systems under cyclic aeration. *Water Environment Research* **79** (8), 912–920.

Kampschreur, M. J., Temmink, H., Kleerebezem, R., Jetten, M. S. M. & van Loosdrecht, M. C. M. 2009 Nitrous oxide emission during wastewater treatment. *Water Research* **43**, 4093–4103.

Kim, D. & Kim, Y. 2011 Effect of ammonium concentration on the emission of N_2O under oxygen-limited autotrophic wastewater nitrification. *J. Microbiol. Biotechnol.* **21** (9), 988–994.

Kim, H. J. & Lee, S. S. 2011 *Leucobacter kyeonggiensis* sp. nov., a new species isolated from dye wastewater. *The Journal of Microbiology* **49** (6): 1044–1049.

Kishida, N., Kim, J. H., Kimochi, Y., Nishimura, O., Sasaki, H. & Sudo, R. 2004 Effect of C/N ratio on nitrous oxide emission from swine wastewater treatment process. *Water Sci. Technol.* **49** (5–6), 359–365.

Kong, Q., Zhang, J., Miao, M., Tian, L., Guo, N. & Liang, S. 2013 Partial nitrification and nitrous oxide emission in an intermittently aerated sequencing batch biofilm reactor. *Chem. Eng. J.* **217**, 435–441.

Lemaire, R., Meyer, R., Taske, A., Crocetti, G. R., Keller, J. & Yuan, Z. 2006 Identifying causes for N_2O accumulation in a lab-scale sequencing batch reactor performing simultaneous nitrification, denitrification and phosphorus removal. *Journal of Biotechnology* **122**, 62–72.

Liang, Y., Li, D., Zeng, H., Zhang, C. & Zhang, J. 2015 Rapid start-up and microbial characteristics of partial nitrification granular sludge treating domestic sewage at room temperature. *Bioresour. Technol.* **196**, 741–745.

Liu, Y. Q. & Tay, J. H. 2004 State of the art of biogranulation technology for wastewater treatment. *Biotechnology Advances* **22**, 533–563.

Lochmatter, S., Gonzalez-Gil, G. & Holliger, C. 2013 Optimized aeration strategies for nitrogen and phosphorus removal with aerobic granular sludge. *Water Research* **47**, 6187–6197.

McIlroy, S. J., Saunders, A. M., Albertsen, M., Nierychlo, M., McIlroy, B., Hansen, A. A., Karst, S. M., Nielsen, J. L. & Nielsen, P. H. 2015 MiDAS: the field guide to the microbes of activated sludge. *Database* **2015** (2), 1–8.

Quan, X., Zhang, M., Lawlor, P. G., Yang, Z. & Zhan, X. 2012 Nitrous oxide emission and nutrient removal in aerobic granular sludge sequencing batch reactors. *Water Research* **46**, 4981–4990.

Santa Catarina 2009 *Lei n.14.675. Institui o Código Estadual do Meio Ambiente e estabelece outras providências (State Law n.14.675. Establishes the Environment State Code).* Florianópolis, Brazil, 64p.

Shi, Y., Wang, X., Yu, H., Xie, H., Teng, S., Sun, X., Tian, B. & Wang, S. 2011 Aerobic granulation for nitrogen removal via nitrite in a sequencing batch reactor and the emission of nitrous oxide. *Bioresour. Technol.* **102**, 2536–2541.

Vázquez-Padín, J. R., Figueroa, M., Campos, J. L., Mosquera-Corral, A. & Méndez, R. 2010 Nitrifying granular systems: a suitable technology to obtain stable partial nitrification at room temperature. *Separation and Purification Technology* **74**, 178–186.

Wagner, J., Guimarães, L. B., Akaboci, T. R. V. & Costa, R. H. R. 2015 Aerobic granular sludge technology and nitrogen removal for domestic wastewater treatment. *Water Sci. Technol.* **71** (7), 1040–1046.

Wei, D., Shi, L., Zhang, G., Wanga, Y., Shi, S., Wei, Q. & Du, B. 2014 Comparison of nitrous oxide emissions in partial nitrifying and full nitrifying granular sludge reactors treating ammonium-rich wastewater. *Bioresour. Technol.* **171**, 487–490.

Weissbrodt, D. G., Shani, N. & Holliger, C. 2014 Linking bacterial population dynamics and nutrient removal in the granular sludge biofilm ecosystem engineered for wastewater treatment. *FEMS Microbiol. Ecol.* **88**, 579–595.

Yang, Q., Liu, X., Peng, C., Wang, S., Sun, H. & Peng, Y. 2009 N_2O production during nitrogen removal via nitrite from domestic wastewater: main sources and control method. *Environ. Sci. Technol.* **43**, 9400–9406.

First received 8 June 2017; accepted in revised form 12 September 2017. Available online 27 September 2017

Downloaded from https://iwaponline.com/wst/article-pdf/241589/wst076123452.pdf
by guest