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PN major contributor
Hydroxylamine Pathway

**Comparison of nitrous oxide emission between a partial and full nitrification
enriched ammonia-oxidising culture**

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Abstract

Nitrification systems are known to be a source of nitrous oxide (N₂O) emission, however, the contribution from partial and full nitrification systems remains controversial. In this study, N₂O emission from a partial and full nitrification culture was investigated. In all tests, nitrite, dissolved oxygen concentration and pH levels were controlled within a similar range limiting ammonium concentration to be the only variable. The results reveal with the same amount of ammonium removed, the full nitrification culture produced far greater N₂O than the partial nitrification culture for both pulse (25 – 36 times) and continuous feeding modes (2 - 110 times). The relative gene expression data indicate that under pulse feeding there is a decreasing trend of *nirK* and *norB* genes for the partial and full nitrification culture respectively while under continuous feeding, decreasing *norB* trends were observed for both. This possibly indicated the hydroxylamine pathway was favoured for the partial nitrification culture while the hybrid N-nitrosation pathway maybe the major contributor for the full nitrification culture. These findings improve our understanding on N₂O production pathways and enable researchers to propose better mitigation strategies.

Keywords: Nitrous oxide; Ammonia-oxidising bacteria; Partial Nitrification; Full Nitrification.

1. Introduction

A rising regulatory demand to achieve greater nitrogen removal from wastewater treatment plants (WWTPs), while reducing infrastructure expenditure and operation costs, has resulted in the advancement of a few novel biological nitrogen removal (BNR) processes (Su et al., 2019; Sun et al., 2016). Partial nitrification (PN) based processes such as the completely autotrophic nitrogen removal over nitrite (CANON) and its alternatives are appealing for treating waste due to less consumption of energy (for aeration) and organic carbon (for denitrification) (Abbas et al., 2015).

The carbon and energy savings of PN processes result from restricting ammonia oxidation to nitrite rather than to nitrate. On the other hand, nitrite is known to promote nitrous oxide (N_2O) production particularly during nitrification and denitrification (Foley et al., 2010; Kampschreur et al., 2008b; Wang et al., 2016). N_2O is a very powerful greenhouse gas with a warming effect about 265 times stronger than carbon dioxide (Stocker et al., 2013) and its presence would also lead to the destruction of the stratospheric ozone layer (Ravishankara et al., 2009). A N_2O emission factor of 1% in a WWTP would elevate the carbon footprint by about 30% (De Haas and Hartley, 2004), hence even low amounts of N_2O emission is unwanted.

Research shows that during biological wastewater treatment, N_2O is emitted mainly from autotrophic and heterotrophic denitrification. Ammonia oxidising bacteria (AOB) have been proven to be a major contributor to N_2O emissions in WWTPs (Chandran et al., 2011; Kampschreur et al., 2009; Tallec et al., 2006). Based on current knowledge, AOB produce N_2O mainly via three pathways (i) the reduction of NO_2^- also called nitrifier denitrification (Stein, 2011), (ii) as a byproduct of the incomplete oxidation of hydroxylamine (NH_2OH) to NO_2^- (Caranto et al., 2016; Stein, 2011) and (iii) hybrid N-

nitrosation ($\text{NH}_2\text{OH} + \text{NO}_2^- + \text{H}^+ \rightarrow \text{N}_2\text{O} + 2\text{H}_2\text{O}$) via biotic and abiotic pathways (Frame et al., 2016; Spott et al., 2011; Terada et al., 2017).

Up to date, only a handful of studies have done a direct comparison between N_2O emission from PN and full nitrification (FN) cultures. It is mainly reported that PN cultures produce more N_2O than FN cultures (Ahn et al., 2011; Gong et al., 2014; Rodriguez-Caballero et al., 2013; Schneider et al., 2014; Wei et al., 2014). However, certain factors like dissolved oxygen (DO) (Ahn et al., 2011; Gong et al., 2014; Rodriguez-Caballero et al., 2013) and substrate loading rate differed during the comparisons. DO has been reported to play an important role in N_2O emission and affects the different pathways undertaken (Kampschreur et al., 2008a; Law et al., 2012; Peng et al., 2014; Tallec et al., 2006). The hydroxylamine pathway is favoured at higher DO while the nitrifier denitrification pathway is favoured under oxygen limited conditions. The influence of heterotrophs also played a part in these comparison studies (Gong et al., 2014; Wei et al., 2014). In one contrary study, it was reported that a FN culture produced more N_2O than a PN culture (Brotto et al., 2015). Thus, it needs to be determined whether nitrogen removal processes based on nitrite accumulation are indeed greater contributors of N_2O than FN based processes under comparable operating conditions.

In WWTPs, there are varying configurations such as Sequential Batch Reactor (SBR), Plug-Flow Reactor (PFR), Step-Feed, Completely Stirred Tank Reactor (CSTR) which result in different substrate loading methods and rates can have an effect on N_2O production (Frison et al., 2015; Quan et al., 2012; Zheng et al., 2015). In one study, a SBR (pulse feeding) produced far less N_2O (0.02 – 0.54%) compared to a CSTR (continuous feeding) (0.25 – 22.7%) (Terada et al., 2013). This warrants further

investigation into different feeding methods to increase our understanding of N_2O production pathways across different cultures.

Thus, the aims of this study were to (i) verify under similar operating conditions if an enriched PN culture does indeed produce more N_2O than an enriched FN culture, (ii) investigate if the type of feeding method would have an impact on the N_2O emission factor, (iii) and use relative gene expression data to understand N_2O production pathways under the different conditions.

2. Materials and Methods

2.1 Reactor operation for PN and FN culture

The PN culture was enriched in a lab-scale SBR with a working volume of 5 L. The SBR was operated in identical 8 h cycles. The cycle consisted of 2×5 min feeding, 2×200 min aeration, 20 s sludge discharge, 55 min settling, and 15 min discharge. The mixed liquor volatile suspended solid (MLVSS) concentration was maintained at approximately 2 ± 0.1 g/L with a sludge retention time (SRT) of 20 d. 2 L of synthetic wastewater was fed every cycle giving a hydraulic retention time (HRT) of 20 h. The SBR was fed with synthetic wastewater mimicking characteristics of anaerobic digestion liquor. pH and DO were continuously monitored online using Mettler Toledo meters and controlled between 0.3 and 0.4 mg O_2/L by a programmed logic controller (PLC). The composition of wastewater was 3.38 g/L NH_4HCO_3 (600 mg $\text{NH}_4\text{-N/L}$), 6.9 g/L NaHCO_3 , 0.064 g/L each of KH_2PO_4 and K_2HPO_4 and 2 mL of trace element stock solution. The trace element stock solution contained: 1.25 g/L EDTA, 0.55 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.40 g/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 1.275 g/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.40 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$,

0.05 g/L $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 1.375 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.25 g/L $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 44.4 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$.

The FN culture was enriched in a lab-scale SBR with a working volume of 5 L. The SBR was operated in identical 8 h cycles. The cycle consisted of 5 sub-cycles of 10 min feeding (aeration on) and 70 min aeration. This was followed by 5 min sludge discharge, 45 min settling, 25 min discharge and 5 min idle. The MLVSS concentration was maintained at approximately 1.9 ± 0.1 g/L with a SRT of 5 d. 1.5 L of synthetic wastewater was fed every cycle giving a HRT of 1.1 d. DO was not controlled but in the range of 0 to 7.18 mg O_2 /L. The composition of wastewater was 1000 mg-N/L as $(\text{NH}_4)_2\text{SO}_4$, 0.064 g/L each of KH_2PO_4 and K_2HPO_4 and 2 mL of trace element stock solution. The trace element stock solution contained the same chemical composition as PN stock. pH was controlled by external addition of NaHCO_3 .

Both SBRs had been operated for more than 12 months and displayed stable performance before the batch experiments were carried out. NH_4^+ removal efficiency for the PN reactor was approximately 96% with < 20 mg $\text{NO}_3\text{-N/L}$ at all times in the effluent. NH_4^+ removal efficiency for the FN culture was almost 100% with complete conversion to NO_3^- . Characterisation of the microbial community was carried out by Fluorescence in situ hybridisation (FISH) according to the procedure described in Fuchs et al. (2007). The probes used for both cultures were EUBmix (Daims et al., 1999) which covers all bacteria, NSO1225 for all AOB, Ntspa662 for *nitrospira* (Mobarry et al., 1996) and NIT3 for *nitrobacter* (Wagner et al., 1996). 20 FISH images were taken for each reactor using a laser scanning microscope (Carl Zeiss LSM 880 Airyscan) and analysed using Imaris software to determine the percentage of the bacteria of interest. Reported values are mean percentages with standard deviations.

2.2 Batch experiment design

All batch tests were carried out in a sealed 2 L batch cylindrical reactor. In each batch experiment, 300 mL or 375 mL culture was withdrawn from the PN or FN SBR respectively. The sludge was then washed with $1 \times$ PBS to remove remaining nitrogen source. The washed sludge was placed in mineral salt medium similar to that of the respective reactors and the final working volume was 500 mL. MLVSS concentration was 1.0 ± 0.2 g/L. pH was monitored by a pH meter (Melter Toledo, Switzerland) and controlled between 7.5 and 8.0 with a PLC by addition of NaHCO_3 . Temperature and stirring was controlled at 30 ± 1 °C and 250 rpm respectively by a heating and stirring plate. DO of the mixed liquor was maintained at 1.0 ± 0.1 mg O_2 /L by manually adjusting the flow rates of compressed air and purified N_2 gas. DO, pH, temperature and N_2O readings were allowed to stabilize before commencement of the batch test.

For pulse feed batch tests (Table 1), low NH_4^+ concentration was used to prevent high NO_2^- accumulation (> 50 mg-N/L) which may reduce N_2O emissions (Law et al., 2013). For continuous feed batch tests, experiments were performed at various increasing flow rates as means to increase the NH_4^+ loading rate and hence total NH_4^+ removed. Feed solution contained 30 mg NH_4^+ -N/L, 30 mg NO_2^- -N/L and mineral salt medium similar to that of reactor operation. The reason for the addition of NO_2^- in continuous feed experiments was to keep NO_2^- concentrations relatively constant in a certain range (30 – 40 mg-N/L) throughout the experiment and hence isolate NH_4^+ addition as the only variable.

2.3 Data collection and analysis

The total gas flow rate into the reactor was constantly controlled at 0.6 L/min during experiment to facilitate the calculation of N₂O emission (see below). Total gas flow rate and cumulative gas volume into the reactor was measured and logged by a digital gas flow meter (Aalborg, USA). Off-gas exited the reactor from a single port at the top of the reactor. A T-joint at the off-gas port split gas flow into a gas phase N₂O analyser (Model 46i Nitrous oxide analyser, Thermo Scientific, USA) and a gas relief valve. During experiment, the N₂O analyser drew off-gas at a flow rate of 0.5 L/min for N₂O analysis and data was logged every minute. Excess off-gas exited the reactor through the gas relief valve.

Mixed liquor samples were collected every 15 min for NH₄⁺, NO₂⁻ and NO₃⁻ analysis. Collected samples were immediately filtered with 0.45 µm disposable syringe filters and stored at 4 °C till analysis. NH₄⁺ analysis was done with the Nessler ammonia - nitrogen reagent set (HACH, USA). Analysis for NO₂⁻, NO₃⁻, mixed liquor suspended solid (MLSS) and MLVSS were done according to standard methods (APHA, 2012).

2.4 Calculation

Total amount of NH₄⁺ removed was taken to be the maximum total nitrogen concentration (mg-N/L) which was very similar to the initial measured NH₄⁺ concentration. N₂O emission (mg) was calculated by multiplying the measured gas phase N₂O concentration and the known gas flow rate. The N₂O emission factor was calculated by dividing the total N₂O produced over total NH₄⁺ removed and expressing the results in terms of percentage. All values above were normalized with the MLVSS concentration.

2.5 RNA extraction and quantification

Total RNA was isolated from biomass samples stored at -80 °C. For pulse feed batch tests, samples were withdrawn during peak N₂O emission while for continuous feed batch tests, samples were withdrawn at 45 min during stable N₂O emission period. Due to unfortunate circumstances, batch test #6 pulse feeding samples were lost. RNA content was extracted using a commercially available kit (Qiagen mini RNA prep) following the instructions from the manufacturer. Additional DNA removal was carried out before cDNA synthesis (RNase-free DNase set, Qiagen). cDNA was reverse transcribed from 2 µg of total RNA using the SuperScript III first-strand synthesis system (Invitrogen) according to the manufacturer's protocols. The cDNA was then used as the template for qPCR reactions on ABI StepOnePlus system (Life Technologies, Foster City, CA) using SYBR green with the Rox detection system (Kapa Biosystems). Primers (efficiency >90% for all primers) were obtained from Integrated DNA Technologies and their sequences are listed in Table S1.

PCR was carried out with 2 µL of first-strand cDNA in a total volume of 20 µL containing 0.2 mM of each primer, 10 µL of PCR KAPA SYBR FAST qPCR Master Mix ABI (2X) Prism™, 0.4 µL ROX high. Amplification parameters used were: initial activation 3 min at 95 °C, then 40 cycles of 3 s at 95 °C and 30 s at respective appropriate annealing temperature. Experiments were performed in triplicates for all the genes. The amount of each target gene was normalized to a reference gene 16S and compared to control samples. Data analysis was performed using the 2- $\Delta\Delta$ CT method, where $\Delta\Delta$ CT = Δ CT (sample) – Δ CT (baseline), Δ CT = CT (target gene) – CT (16S), and CT is the threshold cycle value for the amplified gene. Primer specificity and the absence of primer-dimers were confirmed via melt curve analysis, performed for each and every q-RT-PCR assay conducted (data not shown).

3. Results

3.1 Microbial Community

For the PN culture, $85.91 \pm 8.75\%$ of the EUBMix probe targeted cells bound to the NSO1225 probe (Figure S1a) while NIT 3 and NTSP 662 probes did not give any signals, indicating the absence of *Nitrobacter* and *Nitrospira* genera respectively. For the FN culture, $83.07 \pm 11.38\%$ of the EUBMix probe targeted cells bound to the NSO1225 probe (Figure S1b) while $3.84 \pm 5.22\%$ of the EUBMix probe targeted cells bound to the NIT3 probe (Figure S1c), specific for the *Nitrobacter* genera. All the NTSP 662 probes did not give any signals, indicating the absence of the *Nitrospira* genera. Generally, FN cultures are expected to have a lower abundance of AOB. However in this study, due to short SRT of the FN culture, the abundance of NOB could be restricted giving rise to a higher AOB population.

3.2 N₂O Emission under Pulse Feeding

Figure 1a shows the N₂O emission profiles for the PN culture batch tests. Upon addition of NH₄⁺, N₂O rapidly built up in the first few minutes of all experiments and peaked around the 7th to 8th min regardless of initial NH₄⁺ concentration or peak NO₂⁻ concentration. N₂O emission started to decline after at a slower rate. N₂O profile along with NH₄⁺, NO₂⁻ and NO₃⁻ profiles are shown in Figure 1b for test #6. The profiles of all other tests displayed very similar trends (data not shown). The results of total NH₄⁺ removed, N₂O emission and N₂O emission factor for the PN culture are summarized in Table 2. In general, increasing the initial NH₄⁺ concentration corresponded to an increase in N₂O emitted and N₂O emission factor. The highest achieved N₂O emission

factor was 0.44% for 28.9 mg NH_4^+ -N removed/g MLVSS. A linear positive correlation ($R^2 = 0.89$) between NH_4^+ removed and N_2O emission was observed (Figure S2a).

Figure 1c shows the N_2O emission profiles for the FN culture batch tests. N_2O emission was detected in all tests and the peak N_2O emission time varied. It occurred when NH_4^+ was almost fully consumed or when NO_2^- was near to its highest concentration. Hence a higher initial NH_4^+ concentration would result in a later peak N_2O emission time. N_2O in headspace declined at a faster rate than the built up. A higher initial pulse feed NH_4^+ concentration lead to a higher peak N_2O emission. N_2O profiles along with NH_4^+ , NO_2^- and NO_3^- profiles are shown in Figure 1d for test #6. The profiles of all other tests displayed very similar trends (data not shown). In general, increasing the initial NH_4^+ concentration corresponded to an increase in N_2O emitted and N_2O emission factor (Table 2). The highest achieved N_2O emission factor was 11.6% for 27.0 mg NH_4^+ -N removed/g MLVSS. A linear positive correlation ($R^2 = 0.96$) between NH_4^+ removed and N_2O emitted was observed (Figure S2b).

3.3 N_2O Emission under Continuous Feeding

For the PN culture, N_2O emission was detected in all batch tests immediately after NH_4^+ was introduced. Generally, N_2O emission rapidly built up in the initial 20 min of NH_4^+ loading and remained relatively stable between the 40th and 60th min regardless of background NH_4^+ , NO_2^- , NO_3^- concentrations. For the highest NH_4^+ loading rate batch test, N_2O emission appeared to be on the rise throughout the duration of the experiment (Figure 2a). NH_4^+ accumulation occurred in all batch tests. Slight accumulation (<1 mg NH_4^+ -N/L) occurred at the lowest NH_4^+ loading rate. Gradual increase in NH_4^+ accumulation was observed as the NH_4^+ loading rate increased. Profile of NH_4^+ , NO_2^- ,

NO₃⁻ concentrations and N₂O emission profile for continuous feed partial nitrification (CFPN) batch test #8 is shown Figure 2b. NO₂⁻ concentrations were stable in the range of approximately 29 – 40 mg-N/L while NO₃⁻ concentrations were less than 1 mg-N/L for all batch tests. Increasing the NH₄⁺ loading rate corresponded to higher NH₄⁺ removed and hence a higher N₂O emission rate and factor (Table 3). The highest N₂O emission factor of 0.077% was achieved for 20.2 mg NH₄⁺-N removed/g MLVSS. A linear positive correlation ($R^2 = 0.98$) between NH₄⁺ removed and N₂O emitted was observed (Figure S2c).

For the FN culture, similar N₂O emission trend was detected (Figure 2c). No NH₄⁺ accumulation was observed for batch #1, the lowest NH₄⁺ loading rate for this set of experiments. Slight accumulation was observed in all other batch tests. Increasing the flow increased the amount of accumulation of NH₄⁺. A typical profile of NH₄⁺, NO₂⁻, NO₃⁻ concentrations and N₂O emission profile for continuous feed full nitrification (CFFN) batch test #8 is shown Figure 2d. NO₂⁻ concentrations were in the range of 24 – 40 mg-N/L for all batch tests. In general, increasing the NH₄⁺ loading rate increased N₂O emission factor (Table 3). The highest N₂O emission factor of 8.15% was achieved for 18.3 mg NH₄⁺-N removed/g MLVSS. An exponential positive correlation ($R^2 = 0.88$) between NH₄⁺ removed and N₂O emitted was observed (Figure S2d).

3.4 Gene Expression

Batch test #1, where the lowest NH₄⁺ loading rate was applied, was used as the reference for all other batch tests. For pulse feed partial nitrification (PFPN), *amoA* relative expression levels decreased despite increasing background NH₄⁺ concentration (Figure 3a). No clear trends were detected for *hao*, *nirK* and *norB* (Figure S3a, S3b,

S3c). For the pulse feed full nitrification (PFFN), *nirK* relative expression levels decreased with increasing NH_4^+ loading and increasing NO_2^- concentrations (Figure 3b). *norB* expression levels also displayed a decreasing trend (Figure 3c). No clear trends were detected for *amoA*, and *hao* (Figure S3d, S3e). For CFPN batch tests, *hao* relative gene expression levels showed a steady decline for lower NH_4^+ loading and remained similar at higher NH_4^+ loading (Figure 3d). For *norB* expression levels, there was an increasing trend for increasing NH_4^+ loading (Figure 3e). No clear trends were detected for *amoA*, and *nirK* (Figure S4a and S4b). For CFFN batch tests, *hao* and *norB* gene expression levels displayed an increasing trend (Figure 3f and 3g). No clear trends were detected for *amoA* and *nirK* (Figure S4c and S4d).

4. Discussion

4.1 Comparison of N_2O emission factors between PN and FN Culture

NO_2^- concentration for all pulse feed batch tests at any time point was in the range of 0 – 50 mg-N/L which was reported to stimulate N_2O production (Law et al., 2013; Peng et al., 2015). The PN batch tests had a slightly higher amount of NO_2^- than the FN batch tests due to limited NOB activity which should translate into a higher amount of N_2O produced. However, total N_2O emitted and N_2O emission factors from the FN culture were much higher than that of the PN culture which is contrary to what most studies reported (Ahn et al., 2010; Gong et al., 2014; Rodriguez-Caballero et al., 2013; Schneider et al., 2014; Wei et al., 2014). Across all pulse feed batch tests, the FN culture had a N_2O emission factor approximately 25– 36 times more than the PN culture for similar amounts of total NH_4^+ -N removed. By keeping DO, pH, NH_4^+ and NO_2^-

relatively similar in each respective comparison batch tests, the only factor that could have led to such a significant difference would be related to the culture.

Previous studies on N_2O emission from enriched PN culture showed that hybrid N-nitrosation (Terada et al., 2017) and NH_2OH via $HNO\cdot$ (Law et al., 2012) were the respective dominant N_2O producing pathways. However, DO levels differed between these studies and were 1 – 4 mg O_2/L and <1 mg O_2/L respectively. High levels of NO_2^- have been shown to inhibit N_2O production. Increasing NO_2^- beyond 50 mg-N/L to 1000 mg-N/L has shown to decrease N_2O production of a PN culture (Law et al., 2013). NO_2^- or free nitrous acid (FNA) has displayed the ability to hamper NO_2^- reducing potential in *Paracoccus denitrificans*, either by inactivating NO_2^- reductase (Baumann et al., 1997) or preventing the translation of *nirK* gene. Furthermore, FNA was also observed to inhibit up to 40% denitrification action of an enriched denitrifying polyphosphate accumulating culture at low concentrations (Zhou et al., 2007).

Hence it is postulated that the high NO_2^- (approximately 550 mg-N/L) experienced in the parent reactor caused a short term irreversible inhibition on the *nirK* gene limiting the nitrifier denitrification pathway. This resulted in the NH_2OH production pathway to be the dominant N_2O production pathway for this PN culture. This can further substantiate that the peak N_2O emission for all PFPN batch tests occurred around the 7th min, indicating that an increase in NO_2^- concentrations after the 7th min did not have a stimulating effect on N_2O production. For the FN batch tests, no NO_2^- inhibition was expected and thus the high N_2O emission factor could be because the hydroxylamine, nitrifier denitrification and hybrid N-nitrosation pathways were all contributing.

In continuous feeding, the FN culture had a N_2O emission factor 2 – 110 times more than the PN culture across all batch tests. At higher NH_4^+ loading rates (batch test #7 and #8), the difference was almost consistent at around 100 to 110 times. It should be noted that unlike previous studies, DO, pH, NH_4^+ and NO_2^- concentrations were kept relatively consistent in this study to provide a fair comparison. Some studies reported higher N_2O emission factors from their PN culture compared to FN culture during the continuous feeding (Gong et al., 2014; Schneider et al., 2014; Wei et al., 2014). However, the influence of heterotrophs cannot be neglected as the influent in their studies had high COD concentration. Hence it was likely that AOB would not dominate the bacteria population in their studies.

Heterotrophic bacteria growing on dead cells could potentially contribute to total N_2O emitted. However, in the continuous feeding experiment prior to addition of NH_4^+ , NO_2^- was present but a negligible amount of N_2O was detected (data not shown). Nitrogen mass balance indicates minimal presence of heterotrophic denitrifying activity. Furthermore, due to the washing process, no external carbon sources were available to drive heterotrophic denitrification. The enzyme activity of heterotrophic denitrification is also known to be strongly inhibited by the availability of DO (Von Schulthess et al. 1994).

Generally, the level of mRNA should reflect the abundance of relevant protein and thus relate with the biomass activity. However, for the PFPN batch tests, *amoA* expression decreased despite increasing ammonia oxidation rates. Similar discrepancies have been reported (Cua and Stein, 2011; Yu and Chandran, 2010), which showed mRNA levels did not necessarily correlate with biomass activities. Other factors such as post-transcriptional and post-translational regulation, or discrepancy in the ability to acquire

electrons, energy and reductant, may also affect the biological activities (Kuo et al., 2010). In this study, even though both PN and FN culture had similar abundance and genome of AOB, relative gene expression differs among them when subjected to similar operating conditions. Previous studies have shown genome structure (Stein et al., 2007) and gene regulation of AOB in response to NO_2^- vary even between similar strains of *N. eutropha* and *N. europaea* (Cua and Stein, 2011). The control and regulation of the different N_2O production pathways also vary in different AOB isolates and strains (Stein, 2011), hence the result in here is not unexpected.

The *norB* and *nirK* relative gene expression levels for the PFFN batch tests showed a decreasing trend despite increasing levels of NO_2^- . A possible explanation would be that as NH_4^+ loading increased and NO_2^- accumulation increased and there was a shift in N_2O producing pathway towards the hybrid N-nitrosation resulting in a much higher N_2O emission factor compared to the PN culture. It has been noted that the hybrid N-nitrosation pathway can be the dominant N_2O production pathway (Terada et al., 2017). The N_2O emission factor is comparable to other FN studies of 7.47% and 8.50% respectively (Gong et al., 2014; Wei et al., 2014).

4.2 Comparison of N_2O emission factors between feeding methods

For a similar total amount of NH_4^+ -N removal, pulse feeding had a higher N_2O emission factor compared to continuous feeding (Table 4). Studies that reported pulse feeding or shock loading producing more N_2O was due to NO_2^- build up (Burgess et al., 2002; Kampschreur et al., 2008a). However, in this study both PN and FN culture had a similar amount of NO_2^- present, hence the difference in N_2O emissions was unlikely to be due to NO_2^- influence. One contrary study which reported that continuous feeding

produced more N₂O than pulse feeding attributed it to the microbial community difference with the SBR (0.02 – 0.54%) containing more halophilic and halotolerant AOB while the CSTR (0.25 – 22.7%) contained more *Nitrosospira* spp. NO₂⁻ conditions were also different in the SBR and CSTR (Terada et al., 2013).

For batch #1, CFPN test had a higher amount of NO₂⁻ than the PFPN test throughout the duration of the experiment. However, its N₂O emission factor was nearly twice as little. NO₂⁻ within the range of 0 – 50 mg-N/L has been reported to stimulate N₂O emission through the nitrifier denitrification pathway (Law et al., 2013), however it was not reflected in this study. This further points to the NH₂OH pathway being the dominant N₂O producing pathway for the PN culture. For the CFPN tests, *hao* relative expression levels decreased which could have translated into less N₂O produced via the hydroxylamine pathway resulting in a lower N₂O emission factor for the continuous feed compared to the pulse feed. The hydroxylamine pathway is hypothesised to be the dominant pathway for the PN culture due to high nitrite experienced in the parent reactor limiting the nitrifier denitrification pathway (Law et al., 2013).

For the FN culture, generally pulse feeding had a higher N₂O emission factor than continuous feeding (Table 4). As the total amount of NH₄⁺-N removed increased, the difference in N₂O emission factors diminished which is contradictory to the PN results which are maintained around 2 – 4 times difference. It was reported that NH₄⁺ shock loading can result in an increase in N₂O emission due to an increase in NO₂⁻ concentration (Burgess et al., 2002). However, in this study, the CFFN batch tests had NO₂⁻ concentrations in the range of 24 – 40 mg-N/L which were higher than some of the PFFN batch tests but still produced much less N₂O. It is suggested that pulse feeding creates a bigger overloading stress on AOB and can cause a greater NH₂OH build up

due to AOB having higher NH_2OH accumulation rates (Schmidt et al., 2004). The results above together with decreasing trends in *norB* and *nirK* relative gene expression (Figure 3b and 3c), indicating less dependency on the nitrifier denitrification pathway, could mean the overall production of N_2O during PFFN batch tests is mainly driven by the hybrid N-nitrosation and the hydroxylamine pathway.

Although studies have identified nitrifier denitrification to be the dominant N_2O producing pathway under low DO and high NO_2^- conditions (Kampschreur et al., 2008b; Shaw et al., 2006; Tallec et al., 2006), a recent study using isotopic techniques indicated that the hybrid N-nitrosation and hydroxylamine pathway produced more N_2O than the nitrifier denitrification pathway (Terada et al., 2017). Hence, it possible that for this study the N_2O production by the hydroxylamine pathway is greater than the nitrifier denitrification pathway. For the CFFN batch tests, the increase in *hao* and *norB* relative gene expression levels (Figure 3f and 3g) indicate a shift towards the hydroxylamine pathway which is different from the PFFN batch tests. The hybrid N-nitrosation pathway can produce more N_2O (Terada et al., 2017) than the hydroxylamine pathway which could be why the PFFN batch tests produced more N_2O than the CFFN batch test.

Although it is recognised that WWTPs in reality experience far more complex interactions, this study provides evidence that PN cultures do not always necessarily produce more N_2O than FN cultures as previous reported (Ahn et al., 2011; Gong et al., 2014; Rodriguez-Caballero et al., 2013; Schneider et al., 2014; Wei et al., 2014) and that AOB cultivation method also plays an important role in mitigating N_2O emission. Controlling the NH_4^+ loading can help reduce N_2O emissions and WWTPs should try to minimise shock loadings. The major contributing pathway for N_2O production differs

across cultures and hence further research should be done to distinguish them and propose better mitigation strategies.

5. Conclusions

The PN culture produced far less N₂O under both pulse and continuous feeding compared to the FN culture. The difference in N₂O emission factors were attributed to the different N₂O production pathways. Feeding modes also affected the N₂O production pathways.

- For the PN culture, the hydroxylamine pathway was dominant under both pulse and continuous feeding due to inhibition of the *nirK* gene.
- For the FN culture, under pulse feeding, the dominant pathway was shifted towards the hybrid N-nitrosation. Under continuous feeding, the hydroxylamine pathway increased contribution resulting in a lower N₂O emission factor.

Declaration of Interest

None.

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Figure Captions

Figure 1 – Profiles of (a) N₂O emission for PFFN (b) corresponding nitrogen profiles for batch test #6 (c) N₂O emission for PFFN and (d) corresponding nitrogen profiles for batch test #6

Figure 2 – Profiles of (a) N₂O emission for CFFN batch tests (b) corresponding nitrogen profiles for a batch test #8 (c) N₂O emission for CFFN batch tests (d) corresponding nitrogen profiles for batch test #8

Figure 3 – Relative gene expression for (a) *amoA* and corresponding NH₄⁺ concentration in PFFN batch test (b) *nirK* and corresponding NO₂⁻ concentration in PFFN batch test (c) *norB* in PFFN batch test (d) *hao* (e) *norB* for CFFN batch test (f) *hao* (g) *norB* for CFFN batch test

Figure 1

Figure 2

Figure 3

Table 1

Experiment conditions for pulse and continuous feed batch tests

Pulse Feeding			Continuous Feeding		
Batch test #	Initial NH_4^+ concentration (mg-N/L)	Initial NO_2^- (mg-N/L)	Batch test #	NH_4^+ Loading rate (mg-N/min)	Initial NO_2^- (mg-N/L)
1	20	0	1	0.068	30
2	25	0	2	0.096	30
3	30	0	3	0.172	30
4	35	0	4	0.201	30
5	40	0	5	0.238	30
6	50	0	6	0.292	30
7	60	0	7	0.321	30
			8	0.410	30

Table 2

Results for PFPN and PFFN batch test

Batch test #	PFPN			PFFN		
	Total NH_4^+ -	Total N_2O	N_2O	Total NH_4^+ -N	Total N_2O	N_2O
	N removed	emitted	Emission	removed	emitted	Emission
	(mg/g MLVSS)	(mg/g MLVSS)	factor (%)	(mg/g MLVSS)	(mg/g MLVSS)	factor (%)
1	10.9	0.012	0.11	8.4	0.286	3.4
2	14.0	0.021	0.15	11.8	0.436	3.7
3	17.8	0.033	0.19	11.4	0.706	6.2
4	16.3	0.046	0.28	12.8	0.954	7.4
5	23.7	0.061	0.26	19.6	1.45	7.4
6	21.4	0.064	0.30	23.7	2.51	10.6
7	28.9	0.128	0.44	27.0	3.12	11.6

Table 3

Results for CFPN and CFFN batch test

Batch test #	Partial Nitrification			Full Nitrification		
	Total NH_4^+ - N removed (mg/g MLVSS)	Total N_2O emitted (mg/g MLVSS)	N_2O emission factor (%)	Total NH_4^+ -N removed (mg/g VSS)	Total N_2O emitted (mg/g VSS)	N_2O emission factor (%)
1	2.31	0.0004	0.017	3.29	0.024	0.73
2	3.96	0.0026	0.065	4.55	0.0081	0.18
3	8.61	0.0055	0.064	8.19	0.095	1.16
4	10.5	0.0062	0.059	10.3	0.118	1.14
5	12.4	0.0077	0.062	12.9	0.271	2.11
6	14.6	0.0097	0.066	13.1	0.495	3.77
7	14.1	0.0088	0.062	12.5	0.855	6.86
8	20.2	0.0154	0.077	18.3	1.49	8.15

Table 4

Comparison of N₂O emission between pulse and continuous feeding for PN and FN cultures

Partial Nitrification					Full Nitrification				
Pulse Feeding		Continuous Feeding		Ratio*	Pulse Feeding		Continuous Feeding		Ratio*
Total		Total			Total		Total		
NH ₄ ⁺ -N	N ₂ O	NH ₄ ⁺ -N	N ₂ O		NH ₄ ⁺ -N	N ₂ O	NH ₄ ⁺ -N	N ₂ O	
removed	Emission	removed	Emission		removed	Emission	removed	Emission	
(mg/g MLVSS)	Factor (%)	(mg/g MLVSS)	Factor (%)		(mg/g MLVSS)	Factor (%)	(mg/g MLVSS)	Factor (%)	
10.9	0.113	10.5	0.059	1.9	8.43	3.4	8.19	1.16	2.93
		12.4	0.062	2.4	11.8	3.7	10.3	1.14	3.25
14.0	0.148	14.6	0.066	2.2	11.4	6.2	12.9	2.11	3.51
		14.1	0.062	2.4	12.8	7.4	13.1	3.77	1.96
21.4	0.297	20.2	0.077	3.9			12.5	6.86	1.08
23.7	0.257	23.8	0.104	2.5	19.6	7.4	18.3	8.15	0.91

*Ratio of pulse feeding N₂O emission factor to continuous feeding N₂O emission factor

Highlights

- Full nitrification culture produced more N_2O than partial nitrification culture
- Relative gene expression was used to determine the dominant pathway
- Hydroxylamine pathway was favoured for the partial nitrification culture
- For the full nitrification culture, N_2O pathways depended on the feeding method