Water Research 151 (2019) 381–387

Contents lists available at ScienceDirect

Water Research

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

Denitrification as an N_2O sink

Monica Conthe [a], Pawel Lycus [b], Magnus Ø. Arntzen [b], Aline Ramos da Silva [c], Åsa Frostegård [b], Lars R. Bakken [b], Robbert Kleerebezem [a], Mark C.M. van Loosdrecht [a, *]

[a] Department of Biotechnology, Delft University of Technology, Delft, the Netherlands
[b] Faculty of Chemistry, Biotechnology and Food Sciences, Norwegian University of Life Sciences, Ås, Norway
[c] Bioclear Earth, Rozenburglaan 13, 9727 DL, Groningen, Netherlands

ARTICLE INFO

Article history:
Received 16 September 2018
Received in revised form
29 November 2018
Accepted 30 November 2018
Available online 24 December 2018

Keywords:
Nitrous oxide
Denitrification
Activated sludge
Wastewater treatment

ABSTRACT

The strong greenhouse gas nitrous oxide (N_2O) can be emitted from wastewater treatment systems as a byproduct of ammonium oxidation and as the last intermediate in the stepwise reduction of nitrate to N_2 by denitrifying organisms. A potential strategy to reduce N_2O emissions would be to enhance the activity of N_2O reductase (NOS) in the denitrifying microbial community. A survey of existing literature on denitrification in wastewater treatment systems showed that the N_2O reducing capacity ($V_{maxN2O \rightarrow N2}$) exceeded the capacity to produce N_2O ($V_{maxNO3 \rightarrow N2O}$) by a factor of 2–10. This suggests that denitrification can be an effective sink for N_2O, potentially scavenging a fraction of the N_2O produced by ammonium oxidation or abiotic reactions. We conducted a series of incubation experiments with freshly sampled activated sludge from a wastewater treatment system in Oslo and found that the ratio $\alpha = V_{maxN2O \rightarrow N2}/V_{maxNO3 \rightarrow N2O}$ fluctuated between 2 and 5 in samples taken at intervals over a period of 5 weeks. Adding a cocktail of carbon substrates resulted in increasing rates, but had no significant effect on α. Based on these results – complemented with qPCR and metaproteomic data – we discuss whether the overcapacity to reduce N_2O can be ascribed to gene/protein abundance ratios (nosZ/nir), or whether in-cell competition between the reductases for electrons could be of greater importance.

1. Introduction

With a global warming potential roughly 300 times greater than CO_2, N_2O can be a major contributor to the greenhouse gas footprint of a wastewater treatment plant (WWTP; Daelman et al., 2013). N_2O accumulates during biological nitrogen removal from wastewater as a byproduct of nitrification by ammonia oxidizing bacteria and/or as a result of incomplete denitrification by heterotrophic denitrifying bacteria in the activated sludge (Kampschreur et al., 2009; Schreiber et al., 2012). The fact that most of the emission of N_2O occurs in aerated nitrification zones in the full-scale could be taken to suggest that nitrification is the primary source of N_2O, but this is far from clear since the N_2O stripped off by aeration could a) originate from non-aerated anoxic zones or b) be produced by denitrification in anoxic microsites within the aerated nitrification zones. Attempts to discriminate N_2O produced via nitrification or denitrification by isotopomer analyses (Wunderlin et al., 2013) or by correlating a wide range of process variables to emissions in a long term N_2O-monitoring campaign in a full-scale WWTP (Daelman et al., 2015) have not been conclusive. Furthermore, N_2O can be produced via abiotic reactions between intermediates of nitrification and denitrification, e.g. between NO_2^- and hydroxylamine (Soler-Jofra et al., 2016) or reduced iron species (Kampschreur et al., 2011). The relative contribution of all these different processes to N_2O accumulation remains unresolved and makes it a challenge to develop greenhouse gas mitigation strategies in full-scale systems.

A number of studies have focused on reducing the production of N_2O during nitrogen removal (Lu and Chandran, 2010; Perez-Garcia et al., 2017; Ribera-Guardia et al., 2014; Wunderlin et al., 2012) but far fewer have focused on *increasing* the *consumption* of N_2O as an equally valid - and arguably more simple - strategy to reduce emissions. While ammonia oxidizing bacteria (AOB) are invariably net sources of N_2O, denitrifying organisms are either net sources or net sinks, both producing and consuming this gas (as shown in Fig. 1a). The propensity of a wastewater treatment system, be it of the activated sludge-type or other, to emit N_2O will be strongly dependent on the intrinsic capacity of its heterotrophic denitrifying

---

[*] Corresponding author. Van der Maasweg 9, 2629 HZ, Delft.
E-mail address: m.c.m.vanloosdrecht@tudelft.nl (M.C.M. van Loosdrecht).

https://doi.org/10.1016/j.watres.2018.11.087

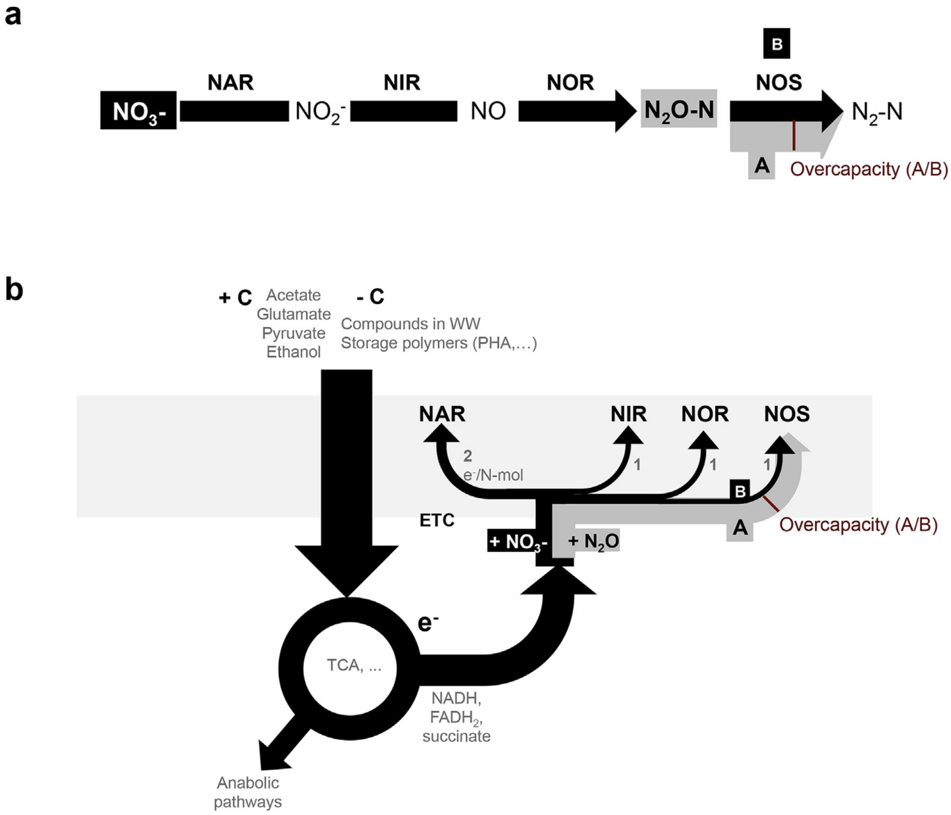

Fig. 1. The denitrification pathway visualized in terms of (a) NO$_x$ substrate or (b) electron flow distribution in the ETC. The thickness of black and gray arrows represents the hypothetical proportional flux of N or e$^-$-equivalents during incubation with NO$_3^-$ (assuming no accumulation of intermediates) or N$_2$O, respectively and the difference in width in N or e$^-$ flux through NOS represents a cell or community's overcapacity for N$_2$O reduction. In (b) we assume that all 4 denitrifying enzymes share a common electron pool. A more complex mixed culture might be partly (or fully) composed of truncated denitrifiers, meaning that the arrows would be segregated in different cells, and different reductases could have access to electron pools of different sizes depending on the cell's metabolic capacity - or preference - to use some electron donor compounds over others.

community to reduce N$_2$O. A community with low N$_2$O reductase (NOS) activity relative to the other reductases (i.e. nitrate reductases, NAR, nitrite reductases, NIR, and nitric oxide reductases, NOR) will be a strong N$_2$O-source, while one with high relative NOS activity will emit less N$_2$O and may even be able to function as a net sink for N$_2$O produced during nitrification, as observed in microcosm experiments with Leca-particle biofilms in Mao et al. (2008).

The degree of NOS activity - and the resulting N$_2$O sink/source strength - of an ecosystem will ultimately depend on a) the genetic potential of the denitrifying community within and/or b) on the overall physiology of said community (including regulation phenomena, enzyme kinetics, electron affinity of the different reductases, etc). Microorganisms can harbor different combinations of denitrification genes in their genome (Graf et al., 2014; Lycus et al., 2017; Roco et al., 2017; Shapleigh, 2013): e.g. denitrifiers lacking the *nosZ* gene encoding NOS are widespread, as are organisms solely equipped with *nosZ* (coined non-denitrifying N$_2$O reducers in Sanford et al., 2012; Hallin et al., 2018, and referred to as such from here on). Thus, microbial community structure can play a role in the N$_2$O sink/source potential of a system. But even in denitrifying organisms harboring all the reductases necessary to complete the denitrification pathway (i.e. NAR/NAP, NIR, NOR, and NOS), transcriptional regulation and post transcriptional phenomena may cause an imbalance in the activity of these enzymes, leading to the release of N$_2$O and/or other intermediate products (i.e. NO$_2^-$ and NO; Liu et al., 2013; Lycus et al., 2017). Such imbalances have been associated with e.g. the presence of O$_2$, significant NO$_2^-$ accumulation, low C/N ratios, storage polymer metabolism and, not the least, rapid fluctuations in these parameters (Foley et al., 2010; Kampschreur et al., 2008; Law et al., 2012; Lu and Chandran, 2010; Otte et al., 1996; Wunderlin et al., 2012).

In order to assess the intrinsic N$_2$O reduction capacity of activated sludge and its potential use in full-scale N$_2$O emission mitigation strategies, an inventory was made of literature studies reporting maximum conversion rates for NO$_3^-$, NO$_2^-$, and N$_2$O in a variety of heterotrophic denitrifying systems. Below we compiled the ratios of maximum rates of N$_2$O production (from NO$_3^-$) to N$_2$O-reduction, which in general were not explicitly reported, as a proxy for the N$_2$O sink capacity of these systems and calculated the steady state concentrations of N$_2$O ([N$_2$O]$_{ss}$), an estimation of the N$_2$O-concentrations at which denitrification changes from being a net source of N$_2$O ([N$_2$O]<[N$_2$O]$_{ss}$) to become a net sink for N$_2$O ([N$_2$O]>[N$_2$O]$_{ss}$). Most studies involved lab-scale sequencing batch reactors (SBRs) run for prolonged periods of time, and the resulting microbial population likely had little similarities to that of the activated sludge used as inoculum. An exception is Wicht (1996), who determined N$_2$O vs. NO$_3^-$ consumption rates for activated sludge. However, acetate was used as a sole carbon and energy source, neglecting the contribution of microorganisms unable to use acetate in the NO$_3^-$ and N$_2$O rates reported. In the present study we complement the existing literature by comparing the N$_2$O and NO$_3^-$ conversion rates of fresh activated sludge from a full-scale WWTP, with and without the addition of a mix of organic electron donors, and at 12 °C, a value within the temperature range of the wastewater during most part of the sampling. Furthermore, we address the potential role of (i) the microbial gene and protein abundance in the N$_2$O sink capacity of the sludge - by quantifying the ratio of *nir* vs. *nosZ* genes and NIR *vs.* NOS proteins - and (ii) of differences in electron affinity amongst denitrifying reductases by means of batch tests with the simultaneous addition of NO_3^- and N_2O. Based on the results obtained, we discuss the reasons why denitrification is potentially a source of N_2O in full-scale systems, and the possibility of exploiting the N_2O sink potential as a mitigation strategy to reduce emissions of this potent greenhouse gas.

2. Materials and methods

2.1. NO_3^- and N_2O batch tests with activated sludge

Batch tests were performed in 120 ml serum flasks filled with 50 ml of untreated, undiluted, fresh activated sludge from one of the pre-denitrification tanks of the Bekkelaget WWTP, which is a modified Ludzack-Ettinger (MLE)-type plant in Oslo, Norway (see Figure S1 and for a scheme of the process units, also described inVenkatesh and Elmi, 2013). Samples were taken over a period of 5 weeks in April and May 2015, and later in October 2015 and May 2017. Immediately after sampling, the activated sludge was transported to the lab on ice, dispensed in serum flasks while stirring for sample heterogeneity, and used for batch tests within 4 h after sampling. Preliminary tests showed that conversion rates were not affected by which process unit of the WWTP the activated sludge was obtained from (data not shown).

The flasks, once filled with the 50 ml of activated sludge sample and 3.5 cm long Teflon covered magnets, were sealed with rubber septa and metallic crimps, helium-washed with 6 cycles of vacuum and refilling of the headspace, and placed in the robotized incubation system described in Molstad et al. (2007). After a period of 15 min with stirring at 600 rpm for the temperature of the samples to equilibrate with the surrounding water bath at 12 °C, the flasks were injected with either 1 ml of pure N_2O gas (using a gas tight syringe, aiming for a final headspace concentration of 1% N_2O or 0.9 mM N_2O–N) or 1 mM NO_3^- (from a 0.5M stock solution of $NaNO_3$) or both. These batch tests were conducted both with and without the addition of an external electron donor –a mixture of acetate, pyruvate, ethanol and glutamic acid– which was injected into the serum flasks to a final concentration of 0.5 mM for each electron donor, immediately before the injection of N_2O or NO_3^-. The transport coefficient for the transfer of gas between the headspace and the liquid was calculated to be $10^{-3}\,L\,s^{-1}$ at the stirring speed used - 600 rpm -, meaning that roughly 5–6 min were necessary for the gas-liquid concentrations to reach an equilibrium, as demonstrated in Figure S2. Therefore, to avoid confounding transport and N_2O reduction kinetics a period of 6.3 min was kept between the injection of N_2O and the first sampling of the headspace. Thereafter, the concentration of NO, N_2O, N_2, CO_2, He and O_2 in the headspace was regularly analyzed by the robotized system and the corresponding concentration of NO, N_2O, and N_2 in the liquid calculated as described in Molstad et al. (2007). When relevant, 100 μL of slurry sample was collected manually for the immediate determination of NO_3^- and NO_2^- concentrations (see below). After verifying that results were reproducible (see Figure S3), replicate runs were sacrificed in exchange for a higher time resolution of the conversion rates (the sampling frequency of the robotized incubation system being limited by the length of the GC run and the number of flasks). For our purposes, we only considered the initial consumption rates (i.e. approximately during the first hour of incubation) to avoid the potential effect of changes in enzyme pools or depletion/accumulation of storage polymers (e.g. PHB) on N_2O reduction rates. The buffering capacity of the activated sludge itself was sufficient to maintain the pH in the range of 6.5–7.5 during the batch tests (the initial pH being 6.5 ± 0.2; data not shown).

Control experiments with either 15% of acetylene in the headspace or with autoclaved activated sludge (15 min at 121 °C; both treatments effectively inhibiting NOS activity) were performed.

2.2. Analytical procedures

NO_3^- and NO_2^- concentrations were determined by measuring the amount of nitric oxide (NO) produced by the reaction with vanadium (III) chloride in HCl at 95 °C ($NO_2^- + NO_3^-$) and the reaction with sodium iodide in acetic acid at room temperature (NO_2^- only) using the purger system coupled to the Sievers Nitric oxide analyser NOA280i (Braman and Hendrix, 1989; Cox, 1980).

2.3. qPCR and metaproteomics

Activated sludge samples were fixed in 100% ethanol (1 ethanol: 1 sample) and DNA was extracted using FastDNA® SPIN Kit for Soil (MP Biomedicals). The primers and PCR conditions used are found in Table S1. Given the potential PCR biases, and the fact that genes are not always expressed, as evidenced by the lacking correlation between gene numbers and related functions in microbial communities (Rocca et al., 2014; Lycus et al., 2017), we also performed an Orbitrap-based mass spectrometry analysis of the proteins. For this, we used a curated database where all the bacterial genera reported to be abundant in activated sludge, anaerobic digesters and influent wastewater (based on MiDAS survey of 24 Danish wastewater treatment plants Mielczarek et al., 2013) were included. The protein extraction procedure aimed at the periplasmic fraction of proteins adapting the protocol for spheroplasts generation (Kučera, 2003). 50 ml of activated sludge was centrifuged at 10 000 g for 20 min and the pellet was used for protein extraction. The pellet was resuspended in 20 ml of 0.1 M Tris-HCl, pH 8.0, 20% sucrose, 1 mM EDTA, 60 mg lysozyme (Fluka) and incubated for 30 min at 37 °C, followed by addition of 25 ml of ice-cold H_2O and gentle mixing by inverting the tube. The sample was then incubated on ice for another 10 min and centrifuged at 10000 g for 20 min. The supernatant containing water soluble proteins was then concentrated on VivaSpin centrifugal concentrator (Sartorius) with the 30 kDa cutoff. Concentrated preparate was used for proteomic analysis. More details can be found in Supplementary Materials.

2.4. Analysis of literature data

We selected studies that reported rates of nitrate reduction in the presence of nitrate excess (R_{NO3}), and rates of N_2O-reduction under conditions of N_2O excess and absence of other nitrogen oxyanions (R_{N2O}), which were taken as estimates of the maximum rates of N_2O production ($V_{\mathrm{maxNO3} \rightarrow \mathrm{N2O}}$) assuming no significant accumulation of intermediates, and the maximum rates of N_2O reduction ($V_{maxN2O \rightarrow \mathrm{N2}}$), respectively. We calculated the ratio $\alpha = V_{maxN2O \rightarrow N2}/V_{\mathrm{max}NO3 \rightarrow N2O}$ with the data from these studies and we used this data to estimate steady state N_2O concentration during denitrification (at high nitrate concentrations, $>>K_S$, no extra N_2O added). Assuming the gross production of N_2O to be as measured ($= V_{\mathrm{max}NO3 \rightarrow N2O}$), and the N_2O reduction rate a simple Michaelis Menten function of the N_2O concentration the following differential equation can be set up:

$$d[N_2O]/dt = V_{\mathrm{max}NO3 \rightarrow N2O} - V_{\mathrm{maxN2O \rightarrow N2}} * [N_2O]/([N_2O] + k_{\mathrm{mN2OR}}) \quad (1)$$

Where $[N_2O]$ is the concentration in mol L^{-1} of N_2O in the liquid and k_{mN2OR} is the half saturation constant in mol L^{-1} for N_2O reductase. Solving for $[N_2O]$ when $d[N_2O]/dt = 0$ the steady state N_2O concentration ($[N_2O]_{\mathrm{ss}}$) can be obtained:

$$[N_2O]_{ss} = k_{mN2OR}/(\alpha-1), \text{ where } \alpha = V_{maxN2O\rightarrow N2} / V_{maxNO3\rightarrow N2O} \quad (2)$$

3. Results and discussion

3.1. Overcapacity of N_2O reduction in activated sludge and other denitrifying systems

A number of studies in literature report the maximum rates, as measured in batch tests in the absence of substrate limitation, for the different steps of denitrification in activated sludge (Wicht, 1996) and denitrifying SBRs (Itokawa et al., 2001; Pan et al., 2012, 2013; Ribera-Guardia et al., 2014; Wang et al., 2014). We calculated the ratio $\alpha = V_{maxN2O\rightarrow N2}/V_{maxNO3\rightarrow N2O}$, which was not explicitly reported in these studies, as an indication of the N_2O sink (or source) potential of the denitrifiying community in these systems. Interestingly the α values obtained showed that N_2O reduction rates were consistently higher than the corresponding NO_3^- reduction rates, by a factor between 2 and 10 (Table 1). We consider α values > 1 to represent the overcapacity of the N_2O reduction step relative to the rest of the denitrification pathway (as illustrated in Fig. 1**)** and a measure of the potential N_2O sink capacity of the denitrifying community in these systems.

We carried out additional batch experiments to determine the $V_{maxN2O\rightarrow N2}$ and $V_{maxNO3\rightarrow N2O}$ in freshly sampled activated sludge taken during a 5-week sampling campaign at the Bekkelaget WWTP, and on two subsequent occasions (Fig. 2). The α values obtained from these tests ranged from 2 to 5, reflecting a persistent N_2O reduction overcapacity of the activated sludge over time (Fig. 3). The overcapacity was apparent in the batch tests both with and without the addition of a mixture of acetate, pyruvate, glutamic acid, and ethanol carbon substrate (rates increased by a factor of roughly 3–5 in the presence of the carbon substrate – Fig. S4). In the batch tests provided with external N_2O, the measured rate of N_2O depletion sometimes exceeded the measured rates of N_2 production by 5–10% (data not shown) and we considered that this could be due to strong sorption of N_2O to the activated sludge or conversion via an abiotic pathway other than reduction to N_2. However tests with acetylene in the headspace or with autoclaved sludge did not provide any evidence for loss of N_2O and the difference was therefore attributed to error propagation in the calculation of gas-liquid mass transfer of N_2O from the headspace to the sludge which do not affect the N_2 production rates (Figure S2).

3.2. N_2O overcapacity and NOS/NIR ratio of the microbial community

The *nosZ* and *nir* gene abundance in the activated sludge, determined by qPCR, showed that copy numbers of the genes encoding for NOS (*nosZI* + *nosZII*) were higher but in the same order of magnitude as NIR (*nirK* + *nirS*), with a *nosZ/(nirS* + *nirK)* abundance ratio of ~2 (Table S2). The abundance of NIR and NOS proteins measured by means of a metaproteomic assay, showed that protein numbers were, on the contrary, greater for NIR than for NOS ($1.19*10^9$ NIR *vs.* $6.4*10^8$ NOS), but nevertheless close to the same order of magnitude. Taken together, the gene and protein abundance data suggests that the efficient N_2O reduction in activated sludge is likely not a result (i) of a numerical dominance of NOS over NIR or (ii) of a relatively abundant population of non-denitrifying N_2O reducers in the sludge.

N_2O overcapacity in the context of electron competition in the electron transport chain.

Electron competition amongst the different denitrifying reductases could create a bias in the N_2O sink potential reflected in α (note that the total electron flux for an equivalent amount of N_2O–N reduction to N_2 is 5 times greater during the batch tests with NO_3^- than in those provided with only N_2O). Denitrification is a sequential process in terms of substrates, but a branched process in terms of electron flow within the electron transport chain (ETC; see Fig. 1, **a** *vs.* **b**) and there is evidence that, even under conditions of electron acceptor excess, the electron supply rate to the ETC may not match the combined electron accepting capacity of the denitrifying reductases (Pan et al., 2013). To assess whether a lower affinity of NOS for electrons relative to the other reductases, would

Table 1
Ratio of the maximum N_2O consumption and production rates (from NO_3^-) reported in literature and in this study (expressed as α) and steady state concentrations of N_2O ($[N_2O]_{ss}$) during denitrification in these systems, expressed as a fraction of the culture's Ks for N_2O.

Reference	System	C source	Conditions	$\alpha = V_{maxN2O\rightarrow N2}/V_{maxNO3\rightarrow N2O}$[a]	$[N_2O]_{ss}$ Fraction of K_s[b]
This study	Activated sludge	Mix[c] + WW		2–5	0,5-1
Ribera-Guardia et al. (2014)	Denitrifying SBR[d]	Acetate		3,0	0,5
		Ethanol		3,6	0,38
		Methanol		7,5	0,15
		Mix		3,4	0,41
Pan et al. (2013)	Denitrifying SBR[d]	Methanol	pH 7	8,4	0,14
Pan et al. (2012)	Denitrifying SBR[d]	Methanol	pH 6	3,3	0,43
			pH 7	6,4	0,19
			pH 8	8,6	0,13
			pH 9	10,5	0,11
Wang et al. (2014)	Denitrifying SBR[d]	Acetate	4 °C	3,3	0,43
			20 °C	1,9	1,11
			34 °C	1,9	1,11
Itokawa et al. (2001)	Nitrifying-denitrifying SBR[c]	Acetate	COD/N 3.5	2,2	0,83
			COD/N 5.0	3,5	0,4
Wicht (1996)	Activated sludge	Acetate		4,0	0,33
Hassan et al. (2016)	Soil			0,5-5	0,33-∞[e]
Hassan et al. (2016)	*Paracoccus denitrificans*	Succinate	NO_2^-	2[f]	0,14

[a] In the literature studies, $V_{maxNO3\rightarrow N2O}$ was estimated from RNO_3^- (see text for explanation).
[b] Steady state N_2O-concentration expressed as fractions of k_{mN2O} (see text for explanation).
[c] C source mixture included acetate, ethanol, glutamate and pyruvate.
[d] SBR inoculated with activated sludge.
[e] No steady state concentration is reached if $RN_2ON/RNO_3 < 1$.
[f] The value is for cultures grown by denitrification through many generations. Much higher α-values are measured for a period after transition to anoxia because all cells express NOS, while only a fraction express NIR (Hassan et al., 2016).

affect the NOS overcapacity highlighted above (electron competition being absent in our determination of $V_{maxN2O\rightarrow N2}$) we performed additional batch tests providing N_2O and NO_3^- to the sludge simultaneously. In the presence of both N_2O and NO_3^- the total flux going through NOS decreased compared to the N_2O-only experiments (indicating at least some degree of electron competition) but N_2O overcapacity persisted, providing evidence that NOS can effectively compete with the other denitrifying reductases (Fig. 3c). Similar conclusions can be reached from the results of batch experiments with denitrifying SBR cultures in Ribera-Guardia et al. (2014) and Pan et al. (2013), though it remains to be seen if the competitiveness of NOS would persist under, for example, more extreme conditions of C limitation, pH, microaerophilic conditions, etc.

3.3. Implications for full-scale WWT systems

Given the literature survey and our results, it would seem that (1) a varying degree of N_2O reduction overcapacity is universal in denitrifying (heterotrophic) communities – true for a broad range of pH and temperature values, COD/N ratios, organic electron donors, and irrespective of whether microbial cultures are exposed to fully anoxic or alternatingly oxic-anoxic conditions or electron competition phenomena, and (2) that this NOS overcapacity is a physiological characteristic of denitrifying microorganisms rather than a result of the genetic potential of the microbial community. Indeed, NOS overcapacity has also been (non-explicitly) reported for pure cultures of the full-fledged denitrifier *Paracoccus denitrificans:* with conversion rates of $N_2O{-}N$ 2 to 6 times higher than those of NO_2^- depending on whether the culture had been exposed to oxic conditions shortly before a switch to anoxia or had been growing for a number of generations under anoxic conditions (Bergaust et al., 2012; Hassan et al., 2016).

We are not aware of a conserved regulatory or post-regulatory mechanism hardwiring denitrifying cells to overexpress the N_2O reduction step relative to the other denitrification steps. The existence of such a mechanism would be a surprising explanation given the diversity of denitrifying regulatory phenotypes found even within a same genus (Liu et al., 2013). Furthermore, given that protein numbers of NOS were lower than NIR, NOS overcapacity is more likely to be a result of enzyme activity or electron affinity than of gene overexpression. Whatever the mechanism behind it, a hardwired NOS overcapacity could be a competitive strategy

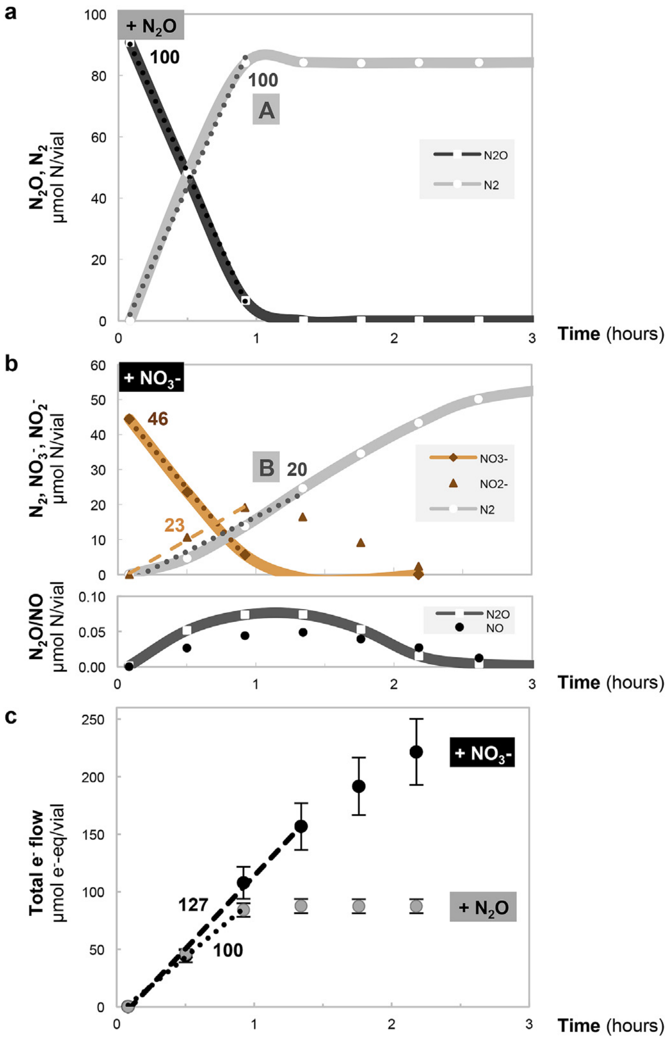

Fig. 2. Example of parallel N_2O (a) and NO_3^- (b) batch incubation tests with the activated sludge collected on one of the sampling days. The maximum N_2O reduction and N_2O production rates of the sludge ($V_{maxN2O\rightarrow N2}$; labelled A and $V_{maxNO3\rightarrow N2O}$; labelled B - in µmol N $vial^{-1}$ h^{-1}) were obtained from the linear regression of the data points during the first hour of the experiments (see Fig. 3b). (c) Cumulative electron flux to denitrification in the two treatments.

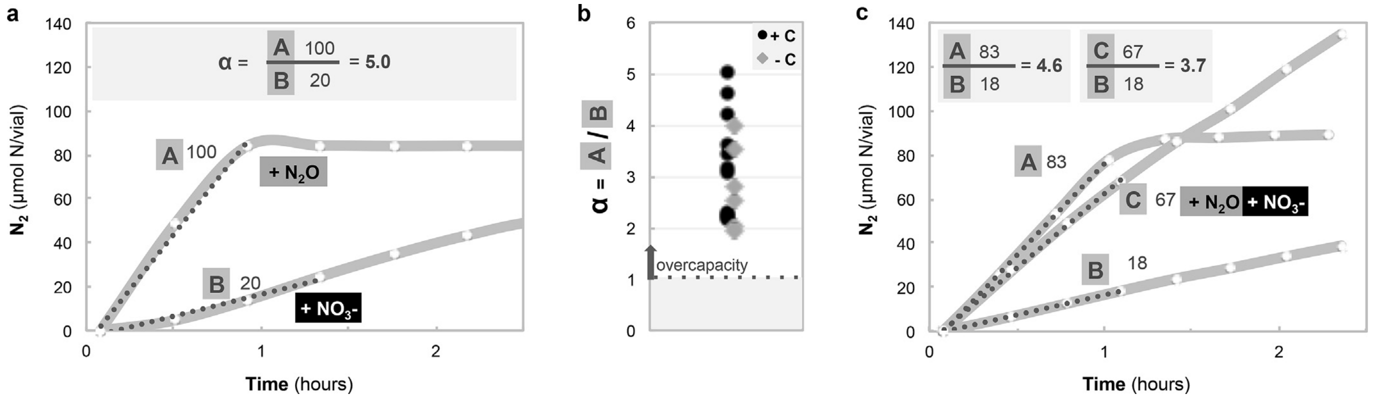

Fig. 3. Overcapacity on N_2O reductase activity in the activated sludge samples. (**a**) Example of how the data from the batch experiments in Fig. 2 was used to calculate α. For simplicity – we derived $V_{maxNO3\rightarrow N2O}$ from the production rate of N_2 during the batch tests with NO_3^-, given that N_2O-N accounted for less than 1% of $N_2{-}N$ produced during the first hour. The N_2 production rate is a proxy for the N or e^- - equivalent flux through NOS. (**b**) α values determined from the N_2 production rates shown in Figure S4 on different sampling days with (+C) and without (−C) the addition of the cocktail of carbon substrates. (**c**) Example of N_2 production rates during a batch experiment provided with N_2O (A) or NO_3^- (B) or both N_2O and NO_3^- simultaneously (C).

evolved to maximize the effective electron accepting capacity of denitrifying cells, which could be particularly advantageous in systems like WWTP with frequently fluctuating availability of electron donor and electron acceptor limitations (e.g. we estimated that any given denitrifying species in the Bekkelaget activated sludge would be exposed to oxic/anoxic transitions in the range of 12–104 times per generation - see Figure S1).

Unfortunately, an overcapacity of N_2O reduction (which reflects maximum conversion rates under substrate excess) is not a guarantee that N_2O will not accumulate and be emitted to the atmosphere in a wastewater system. The affinity constant (K_s) of the culture for the N_2O determines the steady state N_2O concentration ($[N_2O]_{ss}$) at which the denitrifying community changes from being a net source of N_2O to become a net sink, and relatively high steady state N_2O concentrations during denitrification imply a greater likelihood of N_2O stripping into the gas phase (the degree of which will depend on the gas-liquid mass transfer of the system). Using the data obtained in literature and in this study, we estimate the steady state N_2O concentrations to be in the range of $0.1–1.1{*}K_s$ (Table 1), and assuming K_s values for N_2O in the range of 0.6–3.4 μM (based on K_m values determined by Hassan et al., 2016 and Pouvreau et al., 2008), this would mean concentrations of 0.07–3.74 μM, equivalent to a partial pressure range $2–100{*}10^{-6}$ atm at 10 °C (given a solubility of N_2O of 0.039 mol $L^{-1}atm^{-1}$) or a concentration range of 2–100 ppmv of N_2O in the gas phase (if in equilibrium with the liquid). This relatively low concentration range suggests that denitrification is likely to be a net sink for N_2O in activated sludge systems, able to consume part of the N_2O produced by nitrification or abiotic reactions.

The observation that N_2O reduction overcapacity in denitrifying communities is widespread should be considered in modeling efforts and in the development of N_2O mitigation strategies during nitrogen removal from wastewater. For example, carrousel-type systems, or MLE systems with increased recirculation rates, could be less prone to emissions than e.g. MLE systems with a low recirculation rate since, microbial communities are subjected to more frequent oxic-anoxic shifts. Under such conditions nitrification derived N_2O would be more rapidly transferred to the anoxic zones and readily consumed by N_2O reducing microorganisms, instead of being stripped to the atmosphere.

4. Conclusions

- The N_2O reducing capacity of denitrifying microbial communities generally exceeds their capacity to produce N_2O by a factor of 2–10, making denitrification a potential N_2O sink in wastewater treatment systems, scavenging N_2O derived not only from denitrification but also from ammonium oxidation and abiotic reactions of NO_2^-.
- Numbers in the same order of magnitude of NIR and NOS, both in terms of genes and proteins, suggest that the overcapacity observed in denitrifying systems is a characteristic of denitrifier physiology, rather than a consequence of the genetic composition of the microbial community.

Declaration of interests

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

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Acknowledgements

The authors would like to warmly thank Bekkelaget (Morten Rostad Haugen, Tommy Angeltvedt, Jessica Gunnarsson) and VEAS workers (Anne-Kari Marsteng and Ida Skaar).

This work was funded by the European Commission (Marie Curie ITN NORA, FP7- 316472).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2018.11.087.

References

Bergaust, L., van Spanning, R.J.M., Frostegård, A., Bakken, L.R., 2012. Expression of nitrous oxide reductase in *Paracoccus denitrificans* is regulated by oxygen and nitric oxide through FnrP and NNR. Microbiology 158, 826–834.

Braman, R.S., Hendrix, S.A., 1989. Nanogram nitrite and nitrate determination in environmental and biological materials by vanadium(III) reduction with chemiluminescence detection. Anal. Chem. 61, 2715–2718.

Cox, R.D., 1980. Determination of nitrate and nitrite at the parts per billion level by chemiluminescence. Anal. Chem. 52, 332–335.

Daelman, M.R.J., van Voorthuizen, E.M., van Dongen, L.G.J.M., Volcke, E.I.P., van Loosdrecht, M.C.M., 2013. Methane and nitrous oxide emissions from municipal wastewater treatment - results from a long-term study. Water Sci. Technol. 67, 2350–2355.

Daelman, M.R.J., van Voorthuizen, E.M., van Dongen, U.G.J.M., Volcke, E.I.P., van Loosdrecht, M.C.M., 2015. Seasonal and diurnal variability of N_2O emissions from a full-scale municipal wastewater treatment plant. Sci. Total Environ. 536, 1–11.

Foley, J., de Haas, D., Yuan, Z., Lant, P., 2010. Nitrous oxide generation in full-scale biological nutrient removal wastewater treatment plants. Water Res. 44, 831–844.

Graf, D.R.H., Jones, C.M., Hallin, S., 2014. Intergenomic comparisons highlight modularity of the denitrification pathway and underpin the importance of community structure for N_2O emissions. PLoS One 9 e114118.

Hallin, S., Philippot, L., Löffler, F.E., Sanford, R.A., Jones, C.M., 2018. Genomics and ecology of novel N_2O-reducing microorganisms. Trends Microbiol. 26, 43–55.

Hassan, J., Qu, Z., Bergaust, L.L., Bakken, L.R., 2016. Transient accumulation of NO_2^- and N_2O during denitrification explained by assuming cell diversification by stochastic transcription of denitrification genes. PLOS Comput. Biol. PLoS Comput Biol 12.

Itokawa, H., Hanaki, K., Matsuo, T., 2001. Nitrous oxide production in high-loading biological nitrogen removal process under low cod/n ratio condition. Water Res. 35, 657–664.

Kampschreur, M.J., Kleerebezem, R., de Vet, W.W.J.M., van Loosdrecht, M.C.M., 2011. Reduced iron induced nitric oxide and nitrous oxide emission. Water Res. 45, 5945–5952.

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

Kampschreur, M.J., van der Star, W.R.L., Wielders, H. a, Mulder, J.W., Jetten, M.S.M., van Loosdrecht, M.C.M., 2008. Dynamics of nitric oxide and nitrous oxide emission during full-scale reject water treatment. Water Res. 42, 812–826.

Kučera, I., 2003. Passive penetration of nitrate through the plasma membrane of Paracoccus denitrificans and its potentiation by the lipophilic tetraphenylphosphonium cation. Biochim. Biophys. Acta Bioenerg. 1557, 119–124.

Law, Y., Ye, L., Pan, Y., Yuan, Z., 2012. Nitrous oxide emissions from wastewater treatment processes. Philos. Trans. R. Soc. Lond. B Biol. Sci. 367, 1265–1277.

Liu, B., Mao, Y., Bergaust, L., Bakken, L.R., Frostegård, Å., 2013. Strains in the genus *Thauera* exhibit remarkably different denitrification regulatory phenotypes. Environ. Microbiol. 15, 2816–2828.

Lu, H., Chandran, K., 2010. Factors promoting emissions of nitrous oxide and nitric oxide from denitrifying sequencing batch reactors operated with methanol and ethanol as electron donors. Biotechnol. Bioeng. 106, 390–398.

Lycus, P., Lovise Bøthun, K., Bergaust, L., Shapleigh, J.P., Bakken, L.R., Frostegård, Å., 2017. Phenotypic and genotypic richness of denitrifiers revealed by a novel isolation strategy. Nat. Publ. Gr. 11, 2219–2232.

Mao, Y., Bakken, L.R., Zhao, L., Frostegård, Å., 2008. Functional robustness and gene pools of a wastewater nitrification reactor: comparison of dispersed and intact biofilms when stressed by low oxygen and low pH. In: FEMS Microbiology Ecology, pp. 167–180.

Mielczarek, A.T., Saunders, A.M., Larsen, P., Albertsen, M., Stevenson, M., Nielsen, J.L., Nielsen, P.H., 2013. The Microbial Database for Danish wastewater treatment plants with nutrient removal (MiDas-DK) - a tool for understanding activated sludge population dynamics and community stability. Water Sci. Technol. 67, 2519–2526.

Molstad, L., Dörsch, P., Bakken, L.R., 2007. Robotized incubation system for monitoring gases (O_2, NO, N_2O N_2) in denitrifying cultures. J. Microbiol. Methods 71, 202–211.

Otte, S., Grobben, N.G., Robertson, L.A., Jetten, M.S., Kuenen, J.G., 1996. Nitrous oxide production by *Alcaligenes faecalis* under transient and dynamic aerobic and anaerobic conditions. Appl. Environ. Microbiol. 62, 2421–2426.

Pan, Y., Ni, B.-J., Bond, P.L., Ye, L., Yuan, Z., 2013. Electron competition among nitrogen oxides reduction during methanol-utilizing denitrification in wastewater treatment. Water Res. 47, 3273–3281.

Pan, Y., Ye, L., Ni, B.-J., Yuan, Z., 2012. Effect of pH on N_2O reduction and accumulation during denitrification by methanol utilizing denitrifiers. Water Res. 46, 4832–4840.

Perez-Garcia, O., Mankelow, C., Chandran, K., Villas-Boas, S.G., Singhal, N., 2017. Modulation of nitrous oxide (N_2O) accumulation by primary metabolites in denitrifying cultures adapting to changes in environmental C and N. Environ. Sci. Technol. 51 (23), 13678–13688 acs.est.7b03345.

Pouvreau, L.A.M., Strampraad, M.J.F., Van Berloo, S., Kattenberg, J.H., de Vries, S., 2008. NO, N_2O, and O_2 reaction kinetics: scope and limitations of the Clark electrode. Methods Enzymol. 436, 97–112.

Ribera-Guardia, A., Kassotaki, E., Gutierrez, O., Pijuan, M., 2014. Effect of carbon source and competition for electrons on nitrous oxide reduction in a mixed denitrifying microbial community. Process Biochem. 49, 2228–2234.

Rocca, J.D., Hall, E.K., Lennon, J.T., Evans, S.E., Waldrop, M.P., Cotner, J.B., Nemergut, D.R., Graham, E.B., Wallenstein, M.D., 2014. Relationships between protein-encoding gene abundance and corresponding process are commonly assumed yet rarely observed. ISME J. 9, 1693–1699.

Roco, C.A., Bergaust, L.L., Bakken, L.R., Yavitt, J.B., Shapleigh, J.P., 2017. Modularity of nitrogen-oxide reducing soil bacteria: linking phenotype to genotype. Environ. Microbiol. 19, 2507–2519.

Sanford, R.A., Wagner, D.D., Wu, Q., Chee-Sanford, J.C., Thomas, S.H., Cruz-García, C., Rodríguez, G., Massol-Deyá, A., Krishnani, K.K., Ritalahti, K.M., Nissen, S., Konstantinidis, K.T., Löffler, F.E., 2012. Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. Proc. Natl. Acad. Sci. U. S. A 109, 19709–19714.

Schreiber, F., Wunderlin, P., Udert, K.M., Wells, G.F., 2012. Nitric oxide and nitrous oxide turnover in natural and engineered microbial communities: biological pathways, chemical reactions, and novel technologies. Front. Microbiol. 3, 372.

Shapleigh, J.P., 2013. Denitrifying prokaryotes. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.), The Prokaryotes: Prokaryotic Physiology and Biochemistry. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 405–425.

Soler-Jofra, A., Stevens, B., Hoekstra, M., Picioreanu, C., Sorokin, D., van Loosdrecht, M.C.M., Pérez, J., 2016. Importance of abiotic hydroxylamine conversion on nitrous oxide emissions during nitritation of reject water. Chem. Eng. J. 287, 720–726.

Venkatesh, G., Elmi, R.A., 2013. Economic-environmental analysis of handling biogas from sewage sludge digesters in WWTPs (wastewater treatment plants) for energy recovery: case study of Bekkelaget WWTP in Oslo (Norway). Energy 58, 220–235.

Wang, X., Yang, X., Zhang, Z., Ye, X., Kao, C.M., Chen, S., 2014. Long-term effect of temperature on N_2O emission from the denitrifying activated sludge. J. Biosci. Bioeng. 117, 298–304.

Wicht, H., 1996. A model for predicting nitrous oxide production during denitrification in activated sludge. Water Sci. Technol. 34, 99–106.

Wunderlin, P., Mohn, J., Joss, A., Emmenegger, L., Siegrist, H., 2012. Mechanisms of N_2O production in biological wastewater treatment under nitrifying and denitrifying conditions. Water Res. 46, 1027–1037.

Wunderlin, P., Lehmann, M.F., Siegrist, H., Tuzson, B., Joss, A., Emmenegger, L., Mohn, J., 2013. Isotope signatures of N_2O in a mixed microbial population system: constraints on N_2O producing pathways in wastewater treatment. Environ. Sci. Technol. 47, 1339–1348.