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Kinetics of nitrous oxide (N_2O) formation and reduction by *Paracoccus pantotrophus*

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Abstract

Nitrous oxide (N_2O) is a powerful greenhouse gas emitted from wastewater treatment, as well as natural systems, as a result of biological nitrification and denitrification. While denitrifying bacteria can be a significant source of N_2O, they can also reduce N_2O to N_2. More information on the kinetics of N_2O formation and reduction by denitrifying bacteria is needed to predict and quantify their impact on N_2O emissions. In this study, kinetic parameters were determined for *Paracoccus pantotrophus*, a common denitrifying bacterium. Parameters included the maximum specific reduction rates, $\hat{q}$, growth rates, $\hat{\mu}$, and yields, Y, for reduction of NO_3^- (nitrate) to nitrite (NO_2^-), NO_2^- to N_2O, and N_2O to N_2, with acetate as the electron donor. The $\hat{q}$ values were 2.9 gN gCOD^{-1} d^{-1} for NO_3^- to NO_2^-, 1.4 gN gCOD^{-1} d^{-1} for NO_2^- to N_2O, and 5.3 gN gCOD^{-1} d^{-1} for N_2O to N_2. The $\hat{\mu}$ values were 2.7, 0.93, and 1.5 d^{-1}, respectively. When N_2O and NO_3^- were added concurrently, the apparent (extant) kinetics, $\hat{q}_{app}$, assuming reduction to N_2, were 6.3 gCOD gCOD^{-1} d^{-1}, compared to 5.4 gCOD gCOD^{-1} d^{-1} for NO_3^- as the sole added acceptor. The $\hat{\mu}_{app}$ was 1.6 d^{-1}, compared to 2.5 d^{-1} for NO_3^- alone. These results suggest that NO_3^- and N_2O were reduced concurrently. Based on this research, denitrifying bacteria like *P. pantotrophus* may serve as a significant sink for N_2O. With careful design and operation, treatment plants can use denitrifying bacteria to minimize N_2O emissions.

Keywords: *Paracoccus pantotrophus*, Nitrous oxide, Denitrification, Maximum specific reduction rates, Kinetics

Introduction

Nitrous oxide (N_2O) is a potent greenhouse gas with a global warming potential 300-fold greater than CO_2 (IPCC 2006). It also is a major concern for ozone depletion in the stratosphere (Ravishankara et al. 2009). In recent years, wastewater treatment processes, especially those employing biological nutrient removal (BNR), have been found to be significant sources of N_2O (Ni and Yuan 2015). The most common sources of N_2O in BNR processes are ammonium-oxidizing bacteria (AOB) and heterotrophic denitrifying bacteria (DNB) (Law et al. 2012). AOB can form significant amounts of N_2O, especially when the dissolved oxygen (DO) concentrations are low, or during transitions from anoxic to aerobic conditions (Chandran et al. 2011; Sabba et al. 2015). During denitrification, N_2O can form when insufficient electron donor is available, when the pH is excessively high, when sufficient copper is lacking, or when inhibitors of the N_2O reductase, such as DO, hydrogen sulfide, high nitrite (NO_2^-) or ammonia (NH_3) concentrations, are present (Tallec et al. 2008; Bergaust et al. 2010; Lu and Chandran 2010; Pan et al. 2012, 2013a).

While DNB can be a source of N_2O emissions, they also can scavenge N_2O and reduce it to N_2 (Zumft and Kroneck 2007). For example, N_2O produced by nitrifying bacteria can be reduced by DNB in the anoxic zone of a suspended-growth process or in the deeper portions of a biofilm (Ikeda-Ohtsubo et al. 2013).

A better understanding, and quantification, of the kinetics of N_2O reduction by DNB is critical to predicting N_2O emissions from wastewater treatment processes and developing strategies for N_2O mitigation. Since N_2O reduction may take place in the presence of NO_3^-, it also is important to explore the kinetics when both acceptors are present (Schreiber et al. 2012). These parameters are needed for more recent mathematical models that explicitly include N_2O as a state variable, such as those developed by (Ni and

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Yu 2008; Hiatt and Grady 2008; Ni et al. 2011; Pan et al. 2013b).

In this research, we determined denitrification kinetics of a pure culture of *Paracoccus pantotrophus* (formerly *Thiosphaera pantotropha*), a versatile denitrifying bacterium isolated from denitrifying wastewater treatment processes (Robertson and Kuenen 1983). We used a multistep model including the reduction of NO_3^- to NO_2^-, NO_2^- to N_2O, and N_2O to N_2, and determined the biomass yield (Y), $\hat{q}$, and maximum growth rate ($\hat{\mu}$) for each step. We also determined the apparent $\hat{q}$ and $\hat{\mu}$, based solely on donor oxidation and biomass formation, for the reduction of NO_3^- to N_2 and concurrent reduction of NO_3^- and N_2O. Our objective was to gain a better understanding of the mechanisms of N_2O formation and reduction by DNB.

Materials and methods

Bacterial strain and growth medium

We used a pure culture of *P. pantotrophus* (ATCC 35512) in this study. A minimal growth medium was used, consisting of 1.386 g Na_2HPO_4, 0.849 g KH_2PO_4, 0.02 g $MgSO_4{\cdot}7H_2O$, and 0.1 g $(NH_4)_2SO_4$, 0.1 mL Ca–Fe solution, and 0.1 mL trace mineral solution (Nerenberg et al. 2002). The medium also included a trace amount of Luria–Bertani (LB) broth, at 1 % of the usual concentration, to minimize microbial aggregation during growth. All chemicals were analytical grade. Nitrogen gas was UHP grade and NO_3^- was added as needed to obtain the desired initial concentrations. N_2O gas was 99.5 % purity and was added into the headspace.

Batch studies

Batch tests were carried out in 1-L glass bottles with 200 mL of minimal medium. Bottles were capped with a cored rubber stopper containing a sectioned Balch tube with a butyl rubber stopper and aluminum crimp seal, allowing for sample collection. Bottles were successively vacuum-degassed to −1.7 atm and pressurized with either N_2 or N_2O at 1.3 atm, three times. The final headspace contained either N_2 or N_2O at 1.3 atm. Batch tests were carried out at least in triplicate.

Bottles were inoculated with 100 µL of *P. pantotrophus* culture with an optical density at 600 nm (OD_{600}) of 0.6. Bottles were shaken on their sides at 150 rpm at room temperature (22 °C). The medium was amended with acetate as an electron donor and carbon source, with an initial concentration of 650 mgCOD L^{-1} (600 mg/L as acetate). When NO_3^- was used, its initial concentration was 50 mgN L^{-1}.

Analytical methods

Acetate, NO_3^-, and NO_2^- were analyzed using a Dionex ICS2500 ion chromatograph (IC, Dionex Corporation, Sunnyvale, CA) with a 4-mm Dionex AS-11 column, an AG-11 guard column, and a conductivity detector. The program consisted of a 5-min equilibration with 4 mM sodium hydroxide eluent, injection of the sample, a 9-min isocratic run at 4 mM, and a linear gradient from 4 to 50 mM sodium hydroxide over 2 min. A Dionex ASRS suppressor was used in internal recycle mode. Injection was performed with a Dionex AS40 automated sampler. The injection volume was 200 µL. The detection limit for acetate, NO_3^-, and NO_2^- was approximately 0.1 mgN L^{-1}. The biomass concentration was assessed with a spectrophotometer via the OD_{600} (UV10, Thermo, Rochester, NY) and converted to dry weight (DW) using a conversion factor. A conversion factor of 385 mgDW L^{-1} per OD unit was determined following (Nerenberg et al. 2006).

Determination of parameters

The maximum specific growth rates, $\hat{\mu}$ (d^{-1}), maximum specific substrate utilization rates, $\hat{q}$ (gCOD $gCOD^{-1}$ d^{-1} or gN $gCOD^{-1}$ d^{-1}), and yields, Y (gCOD $gCOD^{-1}$ or gCOD gN^{-1}), were determined by parameter fitting (Reichert et al. 1995; Wild et al. 1995). A three-step model was used, including (1) NO_3^- reduction to NO_2^-, (2) NO_2^- reduction to N_2O, and (3) N_2O reduction to N_2. The model lumped NO reduction together with NO_2^- reduction, as NO reduction to N_2O is very fast and NO accumulation during denitrification is minimal (Schreiber et al. 2012).

The process matrix is shown in Table 1 while the model components and the kinetic and stoichiometric parameters are shown in Additional file 1: Tables S1 and S2. Since the NO_3^-, N_2O, and acetate concentrations were well above their expected half-saturation constants for essentially the entire duration of the tests, the half saturation constants K_s for NO_3^-, NO_2^-, N_2O, and acetate were not determined experimentally. Values were taken from (Ni et al. 2011). The specific rate of decay coefficient, b, also was considered insignificant compared to the maximum growth rates and therefore not independently determined. The value for b was taken as 0.15 d^{-1} (Rittmann and McCarty 2001).

The experimental strategy consisted of (1) determining the $\hat{q}$, Y, and $\hat{\mu}$ for N_2O using batch tests with N_2O as the sole added acceptor; (2) after incorporating the parameters for N_2O into the denitrification model (Table 1), determining the $\hat{q}$, Y, and $\hat{\mu}$ for reduction of NO_3^- to NO_2^-, as well as the $\hat{q}$ for reduction of NO_2^- to N_2O, from batch tests with NO_3^- as the sole added acceptor. When NO_3^- was added, accumulation of NO_2^- occurred at values greatly exceeded the reported K_s for NO_2^-, which typically are below 1 mgN L^{-1}. This accumulation allowed the $\hat{q}$ value for NO_2^- reduction to be determined from the

Table 1 Process matrix for denitrification model

Components reactions	$S_{NO3\text{-}N}$ mgN L^{-1}	$S_{NO2\text{-}N}$ mgN L^{-1}	$S_{N2O\text{-}N}$ mgN L^{-1}	S mgCOD L^{-1}	X mgCOD L^{-1}	Rate expression
Nitrate reduction (NAR, NAP)	$-\frac{1-Y_{NO_3^-}}{1.14Y_{NO_3^-}}$	$\frac{1-Y_{NO_3^-}}{1.14Y_{NO_3^-}}$		$\frac{-1}{Y_{NO_3^-}}$	1	$\hat{q}_{NO_3^-} \times Y_{NO_3^-} \times \frac{S_{NO_3^-}}{K_{NO_3^-}+S_{NO_3^-}} \times \frac{S_S}{K_S+S_S} \times X_H$
Nitrite reduction (NIR)		$-\frac{1-Y_{NO_2^-}}{1.14Y_{NO_2^-}}$	$\frac{1-Y_{NO_2^-}}{1.14Y_{NO_2^-}}$	$\frac{-1}{Y_{NO_2^-}}$	1	$\hat{q}_{NO_2^-} \times Y_{NO_2^-} \times \frac{S_{NO_2^-}}{K_{NO_2^-}+S_{NO_2^-}} \times \frac{S_S}{K_S+S_S} \times X_H$
Nitrous oxide reduction (N_2OR)			$-\frac{1-Y_{NO_2^-}}{0.57Y_{NO_2^-}}$	$\frac{-1}{Y_{NO_2^-}}$	1	$\hat{q}_{NO_2^-} \times Y_{NO_2^-} \times \frac{S_{NO_2^-}}{K_{NO_2^-}+S_{NO_2^-}} \times \frac{S_S}{K_S+S_S} \times X_H$
Cell decay					−1	$-b_H \times X_H$

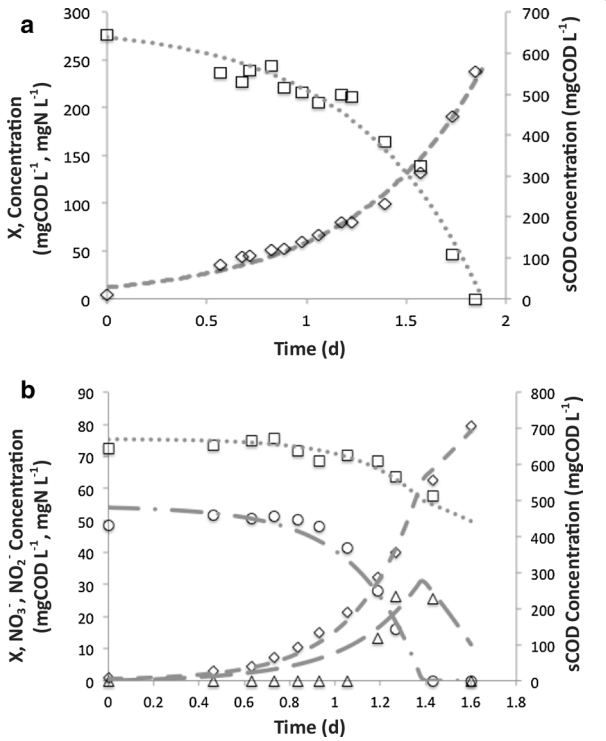

Fig. 1 Typical batch and modeling (data fitting) results for **a** N_2O as sole electron acceptor, **b** NO_3^- as sole added electron acceptor; model sCOD (*dotted line*), model biomass (— -), model NO_3^- (— ·), model NO_2^- (— —), experimental sCOD (*square*), experimental biomass (*diamond*), experimental NO_3^- (*circle*), experimental NO_2^- (*triangle*)

NO_3^- reduction test. The Y for reduction of NO_2^- to N_2O, in gCOD/gCOD, was assumed to be the same as the Y for reduction of N_2O to N_2 (Hiatt and Grady 2008; Ni et al. 2011).

Tests were also carried out with NO_3^- plus N_2O as concurrently added acceptors. For these tests, as well as for the previous tests with NO_3^- as the sole added acceptor, we determined apparent (extant) parameters $\hat{q}_{app}$, Y_{app} and $\hat{\mu}_{app}$. These were determined solely from acetate oxidation and biomass growth data, without considering acceptor utilization. Thus, these parameters reflect the concurrent use of multiple acceptors. The model was adapted from Ni et al. (2011) implemented using AQUASIM (Reichert et al. 1995; Wild et al. 1995). Parameters were determined using AQUASIM's parameter estimation function. Each batch test was carried out at least in triplicate. The reported values are the average and standard deviation.

Results

Parameters for partial reduction steps

Typical plots for the batch tests are shown in Fig. 1. The tests with N_2O as the sole electron acceptor showed vigorous growth. Since one atmosphere of pure N_2O gas was supplied in the headspace, and the bottles were vigorously shaken, the theoretical value of N_2O in the aqueous phase was 905 mg L^{-1} and therefore non-rate-limiting. This was confirmed by the exponential growth observed throughout the tests with N_2O as the sole acceptor. Because N_2O was in excess, acetate was fully consumed during the experiment. In contrast, the tests with NO_3^- as the sole added electron acceptor had an initial NO_3^- concentration of only 50 mgN L^{-1}. In these tests, acetate was only partially consumed and the final biomass concentration was much lower.

Data fitting was used to determine kinetic parameters from the experimental data. Parameters included the $\hat{\mu}$, $\hat{q}$, and Y for reduction of NO_3^- to NO_2^-, NO_2^- to N_2O, and N_2O to N_2. Results are summarized in Table 2. The $\hat{\mu}$ for NO_3^- reduction to NO_3^- was highest (2.7 d^{-1}), and that for NO_2^- reduction to N_2O was the lowest (0.93 d^{-1}). The $\hat{\mu}$ for N_2O reduction (1.7 d^{-1}) was lower than for NO_3^-, but around double that for NO_3^-. Note that these rates are for individual denitrification steps. The observed growth rates on NO_3^- or NO_3^-, where the reduction products are utilized concurrently, would probably be higher.

The $\hat{q}$ can be expressed in terms of the acceptor (gN gCOD d^{-1}) or in terms of the donor (gCOD $gCOD^{-1}$ d^{-1}). The first is useful for identifying kinetic bottlenecks during sequential reduction of nitrogen oxides, as

Table 2 Summary of kinetic and stoichiometric parameters

Reactions	$\hat{\mu}$		$\hat{q}$		Y
	d^{-1}	gCOD $gCOD^{-1}$ d^{-1}	gN $gCOD^{-1}$ d^{-1}	gCOD $gCOD^{-1}d^{-1}$	gCOD gN^{-1}
$NO_3^- \rightarrow NO_2^-$	2.7	6.0 ± 1.5	2.9 ± 0.72	0.45 ± 1.5	0.93 ± 0.72
$NO_2^- \rightarrow N_2O$	0.93	2.6 ± 0.44	1.4 ± 0.25	0.36[a]	0.65
$N_2O \rightarrow N_2$	1.7	4.8 ± 0.48	5.3 ± 0.27	0.36 ± 0.02	0.32 ± 0.27

[a] NO_2^- yields were assumed to be the same as N_2O

the downstream rate must be equal or higher than the upstream to avoid significant intermediate accumulation. The second is useful when assessing donor demand resulting from different combinations of acceptors. The two forms are related by stoichiometry.

In terms of N, the $\hat{q}$ for reduction of NO_3^- to NO_3^- was 2.9 gN gCOD d^{-1}, and for reduction of NO_3^- to N_2O was 1.4 gN g $CODd^{-1}$ (Table 2). The $\hat{q}$ for reduction of N_2O was highest at 5.3 gN gCOD d^{-1}. When examining the COD oxidation results, the highest $\hat{q}$ was obtained for NO_3^- reduction to NO_3^-, at 6.0 gCOD $gCOD^{-1}$ d^{-1}, consistent with its high growth rate. The $\hat{q}$ for NO_3^- reduction to N_2O was only 2.6 gCOD $gCOD^{-1}$ d^{-1}, while N_2O was 4.8 gCOD $gCOD^{-1}$ d^{-1}.

Batch tests with concurrent addition of NO_3^- and N_2O

Batch tests were used to compare the reduction rates of NO_3^-, as the sole added acceptor, with rates of concurrently added NO_3^- and N_2O. In order to explore the aggregate specific rates of growth and donor oxidation, the batch tests were fitted to determine the "apparent" or extant specific growth rates and donor utilization rates. Figure 2 shows the resulting plots and Table 3 summarizes the parameters. The combined addition of N_2O and NO_3^- slowed the apparent $\hat{\mu}$ from 2.5 to 1.6 d^{-1}. However, the apparent $\hat{q}$ increased from 5.4 to 6.3 gCOD $gCOD^{-1}$ d^{-1}.

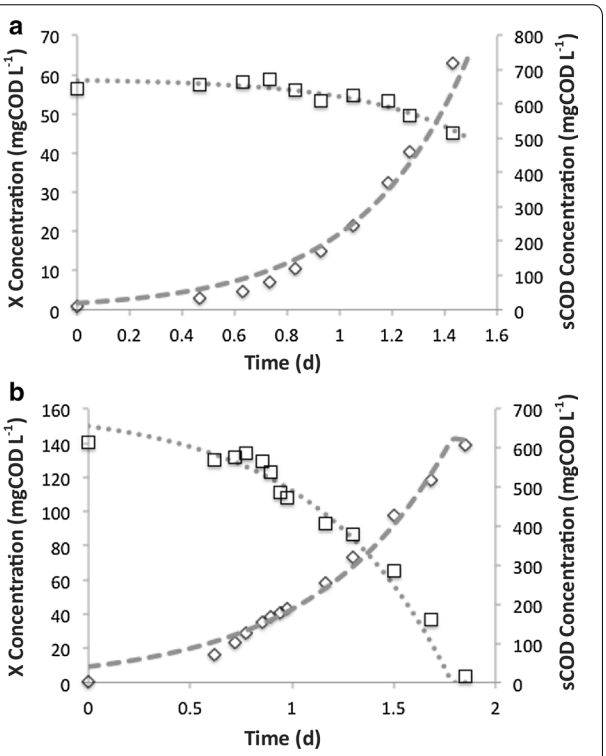

Fig. 2 Typical batch tests for the determination of apparent rates for **a** NO_3^- and **b** NO_3^- plus N_2O. Model sCOD (*dotted line*), model biomass (*dashed line*), experimental sCOD (*square*), experimental biomass (*diamond*)

Discussion

Kinetic parameters for the denitrification pathway for *P. pantotrophus* were determined. The growth rates on N_2O are high, suggesting that DNB can thrive when N_2O is the sole electron acceptor. When NO_3^- and N_2O are supplied together, the growth rates are higher than with N_2O alone, but lower than with NO_3^- alone.

The lower $\hat{q}$ value for NO_2^- indicates a bottleneck on the denitrification pathway, i.e., when NO_3^- is present at non-rate-limiting concentrations, NO_2^- necessarily accumulates, and the observed rate of N_2O reduction is limited to the maximum rate of N_2O formation from NO_2^-. Since the $\hat{q}$ for N_2O, expressed as N, is around triple that of NO_2^- and almost double that of NO_3^-, there appears to be significant capacity for N_2O reduction concurrently with NO_3^- or NO_2^-. In fact, our research shows that *P. pantotrophus* can concurrently utilize NO_3^- and N_2O. Thus, DNB should be able to reduce externally supplied N_2O concurrently with NO_3^- or NO_2^-.

Few sets of kinetic data for the individual reduction steps have been previously reported. While some values have been reported for mixed culture (Additional file 1: Tables S3–S5), very few studies have assessed pure culture kinetics values. While environmental systems typically are based on mixed cultures, such mixed cultures are not reproducible and may give false indications of the mechanisms and regulation of denitrification. For example, for a given inoculum, a reduction test for N_2O typically will be different from the community for a

Table 3 Summary of apparent parameters

Reactions	$\hat{\mu}_{app}$		$\hat{q}_{app}$		Y_{app}
	d^{-1}	gCOD gCOD^{-1} d^{-1}	gN gCOD^{-1} d^{-1}	gCOD gCOD^{-1} d^{-1}	gN gCOD^{-1} d^{-1}
$NO_3^- \rightarrow N_2$	2.5 ± 0.96	5.4 ± 0.48	0.99 ± 0.09[a]	0.48 ± 0.09	2.6 ± 0.09[a]
$NO_3^- + N_2O \rightarrow N_2$	1.6 ± 0.11	6.3 ± 1.3	1.7 ± 0.34[a]	0.25 ± 0.03	0.95 ± 0.03[a]

[a] Calculated from donor utilization data, considering NO_3^- reduction to N_2

NO_3^- reduction test (Shade et al. 2013). The latter could select for bacteria that reduce NO_3^- to NO_2^- over denitrifiers, so NO_2^- accumulation would be due to microbial selection, not the intrinsic kinetics of a denitrifying system.

Values for $\hat{q}$ were reported by several researchers (von Schulthess et al. 1994; Wild et al. 1994; von Schulthess et al. 1995; Wild et al. 1995; Wicht 1996) (Additional file 1: Tables S3–S5). However, these values vary widely from 0.88 to 11.1 gN gCOD d^{-1} for a mixed culture grown on N_2O (Additional file 1: Table S5). In other studies, $\hat{\mu}$ values were reported for growth on pure cultures of denitrifying bacteria using N_2O as an acceptor, but not for NO_3^- to NO_2^- or NO_2^- to N_2O (Strohm et al. 2007). The $\hat{\mu}$ for N_2O in this study was 1.7 d^{-1}, falling in the range that was previously reported for *P. denitrificans* (Koike and Hattori 1975), 1.37–2.57 d^{-1}. The $\hat{q}$ values fall within the range of values previously reported for mixed cultures of denitrifying bacteria when N_2O is reduced to N_2. The yields on N_2O presented in this paper are consistent with previous studies on the closely related DNB species *P. denitrificans* and *Pseudomonas stutzeri,* using acetate as an electron donor.

When examining the batch tests where N_2O an NO_3^- were both supplied as electron acceptors, the results suggest that N_2O was being reduced concurrently with NO_3^-, leading to higher specific rates of donor utilization. The addition of N_2O may have diverted electron equivalents from NO_3^- to N_2O, which has a lower specific growth rate. This could lead to the lower overall apparent specific growth rate. Competition for electron carriers in DNB has been proposed by some researchers, who incorporated it in a metabolic model (Pan et al. 2013b, 2015). This approach has much greater complexity than conventional models, but may be warranted in cases where the donor oxidation rate is limiting (Pocquet et al. 2016).

The results from this study provide important insights into the mechanisms of N_2O formation and consumption by denitrifying microorganisms. In particular, the parameters may be important for assessing the role of DNB in scavenging N_2O produced by nitrifiers or due to incomplete denitrification (Sabba et al. 2015). N_2O may be produced at a given time or location within a process, but could potentially be consumed at a different time or location by N_2O-reducing microorganisms such as *P. pantotrophus*.

The role of DNB in producing and consuming N_2O is illustrated schematically in Fig. 3. In Fig. 3a, a biofilm is supplied with ammonium, DO, and COD. N_2O is formed by AOB, especially as the DO decreases, and some also is produced by the DNB. However, DNB provide a sink for N_2O in the anoxic zone, so only a fraction of the produced N_2O escapes to the bulk liquid (Sabba et al., submitted). If COD does not reach the base of the biofilm, little or no N_2O will be reduced. Thus, all formed N_2O will be released to the bulk (Fig. 3b). Another example is a denitrifying filter (Fig. 3c). If an influent containing COD and NO_3^- enters the top, NO_3^- is reduced first, with some

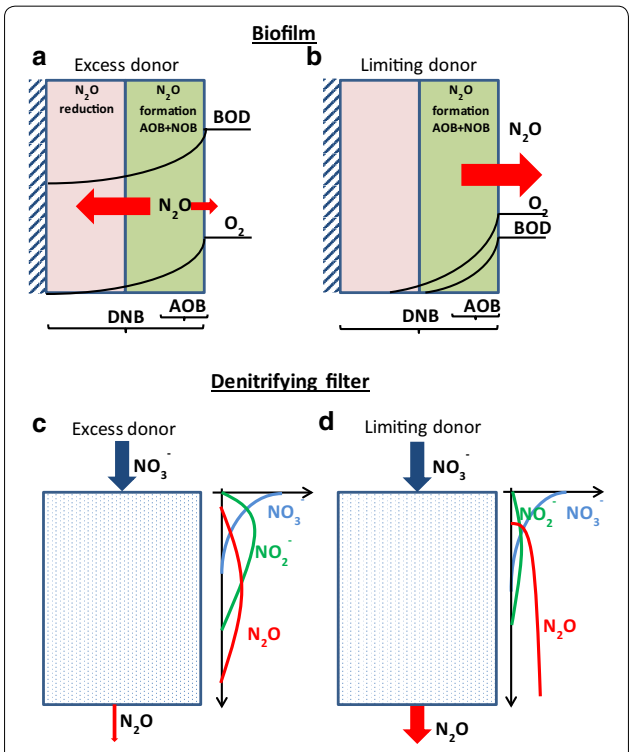

Fig. 3 *Top panels* theoretical behavior of denitrifying bacteria in biofilms under (**a**) excess or (**b**) limiting electron donor conditions. *Lower panels* theoretical nitrogen profiles in a denitrifying filter in presence of (**c**) excess or (**d**) limiting electron donor

NO_2^- and N_2O accumulation. Then NO_2^- is reduced, and finally N_2O is fully reduced towards the bottom. Again, if COD is limiting (Fig. 3d), N_2O can break through the filter and be emitted to the environment. This breakthrough of N_2O was recently demonstrated in a full-scale denitrifying filter (Bollon et al. 2016).

Our research suggests that, while DNB be a source of N_2O, proper management of treatment conditions can allow DNB to scavenge N_2O previously produced by AOB or DNB. This is especially true for biofilm systems or denitrifying filters, where zones of N_2O formation may be adjacent to, or precede, zones where DNB can scavenge N_2O. Providing anoxic conditions and sufficient electron donor is a key for effective N_2O scavenging.

Additional file

Additional file 1. Additional tables.

Abbreviations

AOB: ammonium oxidizing bacteria; BNR: biological nitrogen removal; CO_2: carbon dioxide; COD: chemical oxygen demand; DNB: denitrifying bacteria; DW: dry weight; DO: dissolved oxygen; IC: ion chromatography; H_2O: water; LB: Luria–Bertani; N_2O: nitrous oxide; NH_3: ammonia; NO: nitric oxide; NO_2^-: nitrite; OD: optical density; O_2: oxygen.

Authors' contributions

BLRD conducted the batch study experiments, determined the kinetic parameters using the model, and analyzed the experimental data. FS, JPP, and RN helped interpret data. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

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