**O₂ versus N₂O respiration in a continuous microbial enrichment**

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### Abstract

Despite its ecological importance, essential aspects of microbial N₂O reduction—such as the effect of O₂ availability on the N₂O sink capacity of a community—remain unclear. We studied N₂O vs. aerobic respiration in a chemostat culture to explore (i) the extent to which simultaneous respiration of N₂O and O₂ can occur, (ii) the mechanism governing the competition for N₂O and O₂, and (iii) how the N₂O-reducing capacity of a community is affected by dynamic oxic/anoxic shifts such as those that may occur during nitrogen removal in wastewater treatment systems. Despite its prolonged growth and enrichment with N₂O as the sole electron acceptor, the culture readily switched to aerobic respiration upon exposure to O₂. When supplied simultaneously, N₂O reduction to N₂ was only detected when the O₂ concentration was limiting the respiration rate. The biomass yields per electron accepted during growth on N₂O are in agreement with our current knowledge of electron transport chain biochemistry in model denitrifiers like *Paracoccus denitrificans*. The culture’s affinity constant (Kₛ) for O₂ was found to be two orders of magnitude lower than the value for N₂O, explaining the preferential use of O₂ over N₂O under most environmentally relevant conditions.

### Keywords

Nitrous oxide · Mixotrophy · Enrichment · Chemostat

### Introduction

Coping with rising levels of the potent greenhouse gas nitrous oxide (N₂O) in the atmosphere calls for the development of mitigation strategies to reduce N₂O accumulation and emission in soil management and wastewater treatment (WWT). The presence and activity of N₂O-reducing organisms in fertilized soils and WWT plants, such as bacteria and archaea harboring nosZ-type genes, may be key in such mitigating strategies (Thomson et al. 2012). Nitrous oxide reductase (N₂OR), the enzyme encoded by the nosZ gene, is a terminal reductase present in some microbial respiratory electron transport chains (ETC) that catalyzes the only microbial reaction known to consume N₂O, converting it to innocuous N₂ (which constitutes 79% of the Earth’s atmosphere). Although N₂O reduction is generally associated to denitrifying organisms, many N₂O reducers lack reductases other than N₂OR (i.e., nitrate-, nitrite-, or nitric oxide-reductase; Hallin et al. 2018). However, most, if not all, denitrifiers—and presumably N₂O reducers—are facultative aerobes, having the terminal oxidases necessary for O₂ respiration (van Spanning and Richardson 2007).

Based on what is known on the biochemistry of model organisms like *Paracoccus denitrificans*, N₂O and O₂ respiration presumably share the core of the ETC (Chen and Strous 2013), with electrons branching out to O₂ (via cytochrome oxidases), N₂O (via N₂OR), or other NOₓ (in denitrifying N₂O reducers) depending on electron acceptor availability. It is a common notion that, when both N₂O and O₂ are available, N₂O reducers will consume O₂ preferentially over N₂O (and other NOₓ; Shapleigh 2013). Even though N₂O is a stronger electron acceptor than O₂ in terms of thermodynamics, a number of authors have shown that N₂O respiration is energetically less efficient than aerobic respiration, resulting in lower biomass growth yields per substrate (Koike and Hattori 1975; Stouthamer et al. 1982; Beun et al. 2000). We cannot rule out the existence of a more energy-efficient N₂O reduction process (Conthe et al. 2018a), considering the broad phylogenetic diversity of N₂O reducers and our limited knowledge regarding non-denitrifying N₂O reducers in particular. However, given the growth yields reported in literature, it

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would make evolutionary sense for microorganisms to favor aerobic respiration over the respiration of N compounds to optimize energy conservation in the cell. Intriguingly, the physical mechanism directing electrons to O₂ preferentially over other N compounds, when both electron acceptors are available, remains unclear.

Regulatory systems on a transcriptional or post-transcriptional level have been shown to shut down denitrification in the presence of oxygen in a variety of organisms (Zumft 1997). For instance, the NosZ protein of Paracoccus denitrificans and Pseudomonas stutzeri is inhibited by O₂ in vitro (Coyle et al. 1985; Alef and Ferguson 1982), which could be a form of allosteric regulation in vivo. It has also been proposed that N₂OR is— for reasons unknown— less competent than the cytochrome oxidases involved in respiration of O₂ in the “competition” for electrons in the ETC (Qu et al. 2015). Nevertheless, diverse studies have reported the occurrence of denitrification in the presence of O₂ (termed aerobic denitrification; Chen and Strous 2013 and references therein). Regarding N₂O reduction more specifically, a significant degree of N₂OR transcription and activity has been found under aerated conditions (Körner and Zumft 1989; Qu et al. 2015).

From a greenhouse gas mitigation point of view, it is interesting to study O₂ and N₂O mixotrophy—or the capability of microorganisms to simultaneously respire O₂ and N₂O—in order to understand how frequentoxic-anoxic shifts during nitrogen removal from wastewater, in space or time, may affect the N₂O-reducing capacity of activated sludge. WWTP design and operation vary greatly, but universal questions to address are, e.g., (a) if N₂OR activity can persist in aerated zones consuming nitrification-derived N₂O potentially minimizing greenhouse gas emissions or (b) if, on the contrary, N₂OR is relatively less active than the other N oxidases in the presence of O₂, leading to N₂O accumulation in the aerobic-anoxic transition zones.

We explored O₂ versus N₂O respiration in a continuous enrichment culture selected and grown with N₂O as the sole electron acceptor and fully characterized—in terms of stoichiometry and community composition—in a previous study (Conte et al. 2018b). The culture had been found to be composed of a relatively simple microbial community dominated by Dechlorobacter-like Betaproteobacteria. In this study, operation of the chemostat was continued and the N₂O-limited steady-state conditions were intermittently interrupted to perform short-term batch experiments in situ, with varying concentrations of N₂O, O₂, or both N₂O and O₂ simultaneously, to determine (i) whether O₂ is, in fact, preferentially consumed over N₂O when both electron acceptors are available, (ii) under which O₂ concentrations (if any) N₂O consumption can take place, and (iii) to begin to unravel the mechanism governing the electron flow in the ETC to O₂ or N₂O.

**Materials and methods**

**Chemostat operation**

Following the work presented in Conte et al. (2018b), a microbial enrichment using acetate as a carbon and energy source and exogenous N₂O as the sole electron acceptor was maintained under N₂O-limiting conditions in a continuous culture at 20 °C, pH 7, and a dilution rate of 0.026 ± 0.001 h⁻¹. The reactor set-up, operation, sampling, and medium composition are described in detail in Conte et al. (2018b, c). One hundred percent pure N₂O gas diluted in Argon gas was fed to the chemostat at a total flow rate of 200 ml/min and the offgas from the reactor was recirculated at a rate of 700 ml/min, resulting in an incoming N₂O concentration of roughly 0.30%. The stability of the culture in terms of conversion rates and microbial community composition was monitored by regular sampling of the broth and biomass and via online monitoring of the acid (1 M HCl) dosing (a proxy for acetate consumption in the system) and offgas composition.

**Batch experiments**

The steady-state conditions of the culture were briefly interrupted on different operation days in order to perform batch experiments in situ and determine the maximum conversion rates of the enrichment under non-limiting conditions (Figure S1). The medium and effluent pumps were switched off and the gas supply rates of O₂ (from a bottle of pure O₂) and/or N₂O were modified to achieve different electron acceptor concentrations within the system in random steps. Two main types of batches were performed: (1) supplying a single electron acceptor—either N₂O or O₂—at different concentrations or (2) supplying N₂O and O₂ simultaneously, keeping the N₂O gas supply rate constant and varying that of O₂. Additionally, we performed a batch test in which a constant O₂ gas supply rate was maintained while varying that of N₂O as well as short batch tests with either NO₃⁻ or NO₂⁻ to assess the denitrifying capacity of the culture. Note that gas recirculation was maintained during the experiments, causing an apparent delay between the conversions in the chemostat and the offgas concentration values measured. To avoid acetate depletion, a concentrated solution of sodium acetate was added to the broth at the start of the experiments and the 1 M HCl solution used for pH control during continuous operation was replaced by 1 M acetic acid for the duration of the experiment. For the batch tests with NO₃⁻ and NO₂⁻, these compounds were supplied as 1 M KNO₃ or 1 M KNO₂.

**Analytical procedures**

Samples from the reactor for analysis of acetate and NH₄⁺ were immediately filtered after sampling (0.45-μm pore size
poly-vinylidene difluoride membrane, Merck Millipore, Carrigtohill, Ireland). Acetate was measured with a Chrompack CP 9001 gas chromatograph (Chrompack, Middelburg, The Netherlands) equipped with an HP Innowax column (Agilent Technologies, Santa Clara, CA, USA) and a flame ionization detector. Ammonium, NO$_3^−$, and NO$_2^−$ concentrations were determined spectrophotometrically using cuvette test kits (Hach Lange, Düsseldorf, Germany). For the estimation of biomass concentration, the volatile suspended solids (VSS) concentration was determined by centrifuging 0.2 L of the enrichment, drying the pellet overnight at 105 °C, and then burning the pellet at 550 °C for 2 h to determine the ash content. Additionally, the optical density of the culture (at a wavelength of 660; OD$_{660}$) was monitored. Concentrations of N$_2$O, N$_2$, and CO$_2$, Argon, and O$_2$ in the headspace of the reactor were measured online via mass spectrometry (Prima BT, Thermo Scientific). The dissolved O$_2$ concentration in the broth during the batch tests with O$_2$ was measured with two types of oxygen sensors: a Clark electrode calibrated in the range of 0–20.8% and an optical oxygen probe calibrated in range 0–2% (Presens, Regensburg, Germany).

Calculations

Elemental and electron balances during steady state were set up as described in Conthe et al. (2018a, b, c). During the batch tests, the conversion rates ($r$, in mol h$^{-1}$) for O$_2$ and N$_2$O were calculated from the measured ingoing and outgoing gas composition and the argon supply rate (see Figures S2–S6 and Tables S2–S6 for details). The average biomass concentration value for each experimental step was derived from the ammonium uptake rates (see for example Figure S4b) and used to calculate the corresponding biomass specific rates ($q$, in mol CmolX$^{-1}$ h$^{-1}$). A standard and constant biomass composition of CH$_{1.8}$O$_{0.5}$N$_{0.2}$ (Roels 1980). The $q_{N2O-N}$ and $q_{NO3-N}$ or $q_{NO2-N}$ obtained for each step were plotted against the corresponding concentration of dissolved O$_2$ or N$_2$O in the broth in order to determine the $q_{\text{max}}$ and $K_s$ of the enrichment for O$_2$ and N$_2$O.

The concentration of dissolved O$_2$ was obtained experimentally with the DO probes while the concentration of dissolved N$_2$O was estimated given a $k_{LaN2O}$ of 180 h$^{-1}$—obtained by scaling the experimentally derived $k_{LaO2}$ (Janssen and Warmoeskerken 1987) and deriving the corresponding $K_{La\text{broth}}$ and $K_{La\text{headspace}}$ assuming a broth of 6 s (1800 and 50 h$^{-1}$, respectively). A Monod model fitting the results was obtained by minimizing the sum of squared errors using the Microsoft Excel software.

The thermodynamic efficiency of metabolic growth using acetate as an electron donor and O$_2$, N$_2$O, or NO$_3^−$ as an electron acceptor can be interpreted by the Gibbs free energy ($\Delta G_0$) dissipated per C mole of biomass growth or per electron-equivalent used for respiration. These values were calculated based on Kleerebezem and van Loosdrecht (2010) and using the thermodynamic values found in Thauer et al. (1977)—please refer to Table S7 for more details.

DNA extraction and 454 amplicon sequencing of 16S rRNA gene

The taxa-based community composition of the enriched culture during the period of operation presented in this study was determined by 454 amplicon sequencing of the 16S rRNA gene following the procedure described in Conthe et al. (2018a, b, c) and the sequences are available at NCBI under BioProject accession number PRJNA413885.

Results

Continuous operation and microbial community composition of the N$_2$O-reducing enrichment

A culture enriched from activated sludge using acetate as a carbon source and electron donor and exogenous N$_2$O as the sole electron acceptor was studied for a total period of 155 days (>100 volume changes) in a chemostat under electron acceptor (N$_2$O) limiting conditions (Figure S1). The start-

| Compound | q$_{N2O-N}$ | q$_{NO3-N}$ or q$_{NO2-N}$ | q$_{N2-N}$ | q$_{Acetate-C}$ |
|----------|-------------|---------------------------|------------|---------------|
| Steady state | −0.033 ± 0.001$^b$ | 0.034 ± 0.001$^b$ | −0.017 ± 0.001$^b$ | |
| N$_2$O batch | −0.131 ± 0.004$^b$ | 0.126 ± 0.008$^b$ | −0.067 ± 0.009$^c$ | |
| NO$_3^−$ batch | −0.007 ± 0.000$^c$ | 0.004 ± 0.000$^c$ | −0.003 ± 0.000$^c$ | |
| N$_2$O + NO$_3^−$ batch | −0.033 ± 0.000$^c$ | 0.042 ± 0.000$^c$ | |

$^a$ N$_2$O gas supply was kept on during addition of 1 mM KNO$_3$.

$^b$ Standard deviation calculated from at least three independent measurements.

$^c$ Standard deviation calculated by LINEST least squares method.
up and characterization of the enrichment during the first 70 days of operation, in terms of conversion rates, stoichiometry, and microbial community composition, are described in Conthe et al. (2018b). During the subsequent period reported here, the conversion rates and corresponding biomass yields remained consistent with the previous period, characterized by steady-state growth on acetate oxidation coupled to \( \text{N}_2 \text{O} \) reduction to \( \text{N}_2 \) (Tables 1 and 2). Furthermore, 454 amplicon sequencing of the 16S rRNA gene of the microbial community confirmed the continued prevalence of a \( \text{Dechlorobacter} \)-like OTU (Figure S1), transiently co-occurring (around day 100) with two other closely related OTUs classified as \text{Azonexus} and uncultured \text{Rhodocyclaceae}.

### O\( _2 \) vs. \( \text{N}_2\text{O} \) batch tests: affinity and yields

Batch experiments with varying supply rates of either \( \text{N}_2\text{O} \) or \( \text{O}_2 \) were performed on days 106 and 132, respectively (Fig. 1). The maximum biomass specific conversion rates of \( \text{N}_2\text{O} \) \( (q_{\text{max}}^{\text{N}_2\text{O}}) \) and acetate were identified by increasing the \( \text{N}_2\text{O} \) supply rate to non-limiting conditions. The \( q_{\text{max}}^{\text{N}_2\text{O}} \) values

### Table 2

| Parameter | Units | \( \text{N}_2\text{O} \)<sup>a</sup> | \( \text{NO}_3^- \)<sup>b</sup> | \( \text{O}_2 \)<sup>c</sup> |
|-----------|-------|-------|-------|-------|
| \( Y_{\text{XS}} \) | Biomass yield on acetate | Cmol<sub>x</sub>/Cmol<sub>Ac</sub> | 0.36 ± 0.03 | 0.38 | 0.45 |
| \( Y_{\text{Xe}} \) | Biomass yield on e<sup>-</sup> transported in catabolic process | Cmol<sub>x</sub>/mol<sub>e</sub> | 0.16 ± 0.01 | 0.15 | 0.19 |
| \( \Delta G_{\text{MET}}^{01} \) | Metabolic energy change per mole donor<sup>d</sup> | kJ/Cmol<sub>x</sub> | −1078 | −620 | −479 |
| \( \Delta G_{\text{eCAT}}^{01} \) | Metabolic energy change per electron transferred in catabolism | kJ/mol e<sup>-</sup> | −159 | −96 | −101 |

<sup>a</sup> Steady state data, this study
<sup>b</sup> Steady state data—no significant accumulation of intermediates (Conthe et al.; data unpublished)
<sup>c</sup> Batch experiment data in \( \text{N}_2\text{O} \) reducing enrichment, this study

Fig. 1 Offgas data from the batch experiments with varying concentrations of a \( \text{N}_2\text{O} \); day 106, b and \( \text{O}_2 \); day 132. For the experiment with \( \text{O}_2 \), the dissolved oxygen concentration (DO) was measured both with a Clark electrode (DO<sub>_1</sub>) and an optical sensor (DO<sub>_2</sub>). The affinity of the culture for \( \text{N}_2\text{O} \) and \( \text{O}_2 \) was determined from these experiments (see Fig. 5). The asterisk mark time points at which acetate had been depleted and was added to the culture.
identified were roughly fourfold higher than the actual biomass specific conversion rates during steady state (Table 1). When exposed to varying concentrations of O₂, the culture was able to switch to aerobic respiration in the order of seconds. The maximum O₂ reducing capacity (\(q_{\text{O}_2}^{\text{max}}\)) was comparable to N₂O respiration when expressed per mole electron accepted. NO₃⁻ and NO₂⁻ reducing capacities were much lower compared to N₂O or O₂ (<15% of the maximum N₂O or O₂ reduction rate; Table 1).

Plotting the biomass-specific electron transfer rate (\(q_e\)) at different dissolved O₂ (DO) or N₂O concentrations, we could determine the apparent \(K_e\) for O₂ or N₂O by fitting a Monod model to the data (Fig. 2). Given the confidence intervals, the absolute value for this parameter could not be identified accurately, but the results demonstrate clearly that the \(K_e\) value for O₂ is 1 or 2 orders of magnitude smaller compared to \(K_e\)-N₂O. The maximum biomass-specific conversion rate of O₂ (\(q_{\text{O}_2}^{\text{max}}\)) was roughly two times lower than that of N₂O (\(q_{\text{N}_2\text{O}}^{\text{max}}\)) per mole of electron acceptor but the conversion rates expressed as electron equivalents (\(q_e^{\text{max}}\)) were comparable for both processes, since double the electrons are taken up during the reduction of O₂ to H₂O compared to N₂O to N₂.

The biomass yields per mole of electron donor (determined from the steady-state growth on N₂O in the chemostat, and from the batch experiments with O₂ as the sole electron acceptor) are presented in Table 2.

### Simultaneous O₂ and N₂O batch tests

Batch experiments with excess N₂O and varying concentrations of O₂, supplied simultaneously, were performed on days 110 and 155 (Figs. 3 and 4). The maximum electron transfer rate (\(q_e^{\text{max}}\))—combining the electron transfer capacities of N₂O and O₂—summed up to a value comparable with the...
$q_e^\text{max}$ found during the N$_2$O- or O$_2$-only experiments. N$_2$O reduction to N$_2$ co-occurred with aerobic respiration only at relatively low concentrations of O$_2$ (Fig. 3d). The experiments performed on days 110 and 155 differed regarding the O$_2$ concentration range at which N$_2$O reduction could co-occur (roughly < 4 and < 1.5 μM O$_2$ on days 110 and 155, respectively) but, nevertheless, N$_2$O reduction in the presence of O$_2$ contributed to no more than a small fraction of the total electron acceptor capacity (generally < 20% of $q_e$-tot; Fig. 4). An additional batch experiment on day 113, with a constant supply of O$_2$ and a varying supply of N$_2$O, also showed that N$_2$O reduction was undetectable in the presence of relatively high concentrations of O$_2$ (<5 μM; Fig. 3c).

**Discussion**

Aerobic respiration was distinctly favored over N$_2$O respiration in the enrichment despite the fact that the culture had been operated for an extensive number of generations with N$_2$O as only electron acceptor. Upon a sudden change in supply from N$_2$O to O$_2$, the culture readily switched to O$_2$ respiration and, when both electron acceptors were available, N$_2$O reduction was only observed at relatively low concentrations of O$_2$ (<4 μM = 0.13 mg O$_2$/L). Under conditions of electron acceptor excess (N$_2$O and/or O$_2$), growth in the system was likely limited by the electron supply rate to the electron transport chain (see Fig. 5) and not by the capacity of N$_2$OR or O$_2$ reductases.
This was inferred from the fact that the maximum electron acceptor capacity of the culture was comparable for N$_2$O and O$_2$ respiration (i.e., $q_{e\text{-N}_2O}^{\text{max}} \approx q_{e\text{-O}_2}^{\text{max}}$), and could be due to kinetic limitations in acetate uptake, acetate oxidation in the citric acid cycle, or in some shared component of the ETC itself.

The overall electron transfer capacity during the simultaneous respiration of N$_2$O and O$_2$ (i.e., $q_{e\text{-TOT}}^{\text{max}}$) was comparable to $q_{e\text{-N}_2O}^{\text{max}}$ or $q_{e\text{-O}_2}^{\text{max}}$. This suggests that "aerobic N$_2$O respiration" (by analogy to aerobic denitrification) generally occurs if the electron supply rate to the ETC exceeds the electron accepting capacity of the O$_2$ reductases. In other words, N$_2$O respiration complements aerobic respiration primarily when O$_2$ is limiting. Nonetheless, our results indicate that, under O$_2$-limiting conditions, N$_2$O reducers can use O$_2$ and N$_2$O mixotrophically as proposed by Chen and Strous 2013 (Fig. 5). We cannot exclude heterogeneity in electron acceptor use within the population in our bioreactor leading for example to most of the culture respiring O$_2$ and a side population reducing N$_2$O. Under the microscope, we did not observe formation of aggregates or biofilms which could create anoxic niches in spite of the O$_2$ supply (data not shown), yet oxygen gradients and anoxic microzones could still form around suspended cells if O$_2$ diffusion rate is slower than the respiration rate. Nevertheless, with the strong sparging and mixing conditions imposed on the culture, we would expect that most cells would be exposed to comparable environmental conditions.

The $K_s$ values of the enrichment culture were in the same range as the $K_{oe}$ values reported for purified N$_2$O OR and different O$_2$ reductases in literature, i.e., in the μM range for N$_2$O and mM range for O$_2$ (Pouvreau et al. 2008 and references therein, Yoon et al. 2016). The relatively high $K_{S\text{-N}_2O}$ (two orders of magnitude higher than for O$_2$) is noteworthy in a culture presumably well-adapted to N$_2$O-limiting conditions. Also the observation that, even after a prolonged absence of O$_2$ in the environment, the cellular machinery specific for aerobic respiration (i.e., cytochrome oxidases) was constitutively present (in contrast to NO$_3^-$ and NO$_2^-$ reductases). According to these results, the preferential use of O$_2$ over N$_2$O in natural systems could be attributed to a difference in affinity ($\mu\text{max}/K_s$) for O$_2$ and N$_2$O.

With regard to efficiency of N$_2$O respiration versus O$_2$ respiration, our chemostat enrichment cultures corroborate studies in literature (Koike and Hattori 1975; Stoutamer et al. 1982; Beun et al. 2000) and predictions based on our knowledge of the ETC in model denitrifiers (Chen and Strous 2013): with biomass yields per mole of acetate during growth with N$_2$O (or NO$_2^-$) roughly 1/3 lower than yields during O$_2$ respiration (Table 2). The relatively low growth yields on N$_2$O imply that N$_2$O reduction to N$_2$ is, thermodynamically, a very inefficient process with high energy dissipation. Thus, ensuring the maximization of energy conservation during microbial growth may be the evolutionary driver behind the preferential flow of electrons to O$_2$ over N$_2$O.

We cannot provide a conclusive answer regarding which cellular mechanism governs the preferential use of O$_2$ in the presence of excess N$_2$O observed. However, the instantaneous switch from N$_2$O to O$_2$ respiration suggests that the preference for O$_2$ over N$_2$O is regulated at the metabolome level and is independent from transcriptional regulation, e.g., by control of enzyme activity, like allosteric inhibition of N$_2$OR, or simply a higher affinity of O$_2$ reductases for the electrons coming from a common quinone pool.

Translated to the environmental conditions in a WWT plant, the results from this study suggest that oxic-anoxic transitions are unlikely to result in N$_2$O emissions associated to denitrification as a result of N$_2$OR inhibition by O$_2$ since the enrichment culture readily switched back and forth between O$_2$ and N$_2$O respiration. This implies that (a) either N$_2$OR is not directly inhibited by O$_2$ in vivo or (b) inhibition is readily reversible once O$_2$ is depleted.

On the other hand, the fact that aerobic respiration is so strongly favored over N$_2$O respiration would make it a challenge to exploit the N$_2$O sink capacity of activated sludge in the aerated/nitrification zones of WWT plants. The range in which significant N$_2$O consumption co-occurred with O$_2$ consumption in our experiments was narrow: roughly up to 1.5–4 μM O$_2$, i.e., 0.05–0.13 mg O$_2$/L, presumably below common DO values in the aerated tanks of WWTP (Tchobanoglous and Burton 2002).

The very high affinity for oxygen minimizes the range of dissolved oxygen concentrations in which O$_2$ and N$_2$O respiration could occur in parallel. However, a beneficial difference in full-scale systems compared to our enrichment, in terms of avoiding N$_2$O accumulation, may be that mass transfer limitation induced oxygen limitation within the activated sludge flocs provide anoxic zones, prone to N$_2$O reduction, even when O$_2$ is present in the bulk liquid (Picioreanu et al. 2016). This, together with the fact that N$_2$O is much more soluble than O$_2$, could perhaps be exploited to enhance the N$_2$O sink capacity of activated sludge.
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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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

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