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MECHANISMS OF N_2O PRODUCTION IN BIOLOGICAL WASTEWATER TREATMENT: FROM PATHWAY IDENTIFICATION TO PROCESS CONTROL

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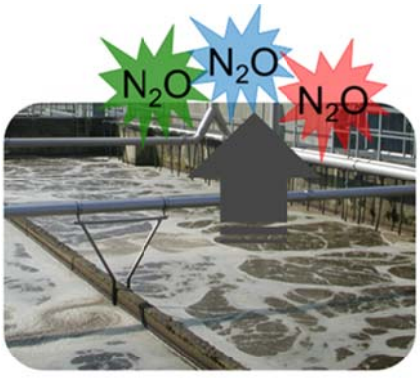

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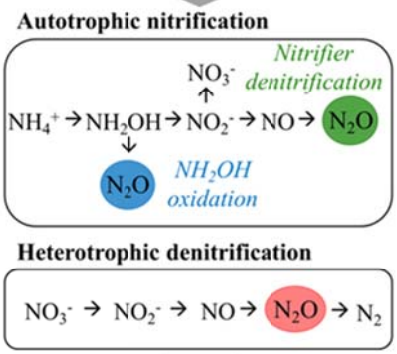

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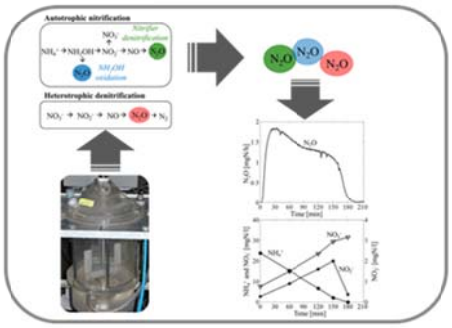

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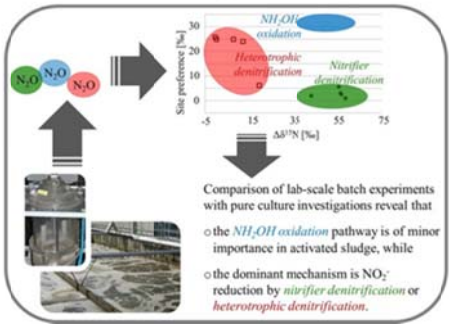

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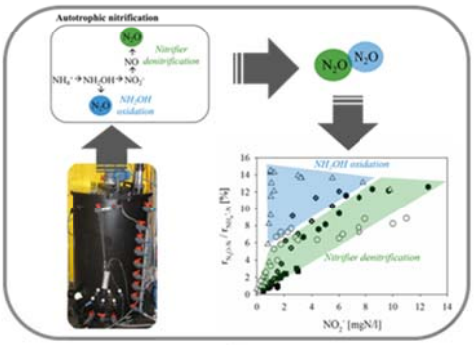

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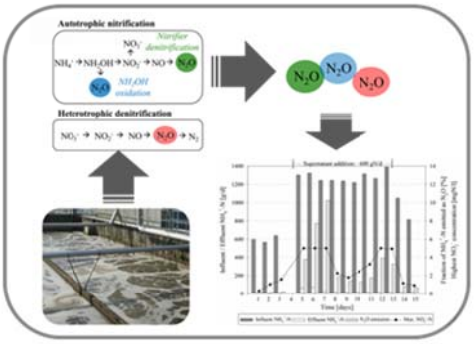

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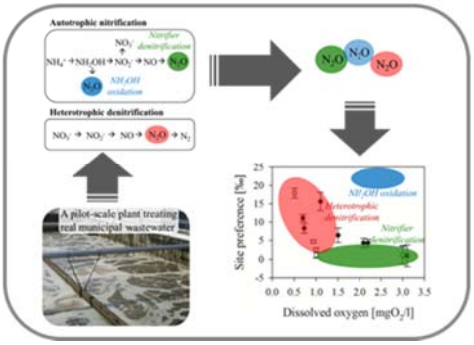

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LIST OF FREQUENTLY USED ABBREVIATIONS

AOB	Ammonia oxidizing bacteria
NOB	Nitrite oxidizing bacteria
AMO	Ammonia monooxygenase, an important enzyme of AOB
CHF	Swiss currency
COD	Chemical oxygen demand, a surrogate measure for organic pollutant concentration in the sector of wastewater mostly proportional to the total organic content
$\delta^{15}N^{\alpha,\beta}$	Relative differences of isotope ratios for the inner (α) and the outer (β) nitrogen atom in the asymmetric N_2O molecule
GHG	Greenhouse gas
HAO	Hydroxylamine oxidoreductase, an important enzyme of AOB
HET	Heterotrophic microorganisms
Isotopomer	Molecules containing the same number of isotopic atoms but with differing positions
N	Nitrogen
NH_4^+	Ammonium
NH_2OH	Hydroxylamine
NO_2^-	Nitrite
NO_3^-	Nitrate
N_2O	Nitrous oxide
NO	Nitric oxide
PE	Person equivalents
SP	Site preference, the difference between the $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$
WWTP	Wastewater treatment plant

ZUSAMMENFASSUNG

Lachgas (N_2O) ist ein starkes Treibhausgas und bedeutend an der Zerstörung der stratosphärischen Ozonschicht beteiligt. Seine Bildung und Freisetzung in die Atmosphäre ist deshalb von grosser Umweltrelevanz. In der biologischen Abwasserreinigung kann N_2O sowohl während der Nitrifikation (Oxidation von Ammonium zu Nitrat), wie auch durch die heterotrophe Denitrifikation (Reduktion von Nitrat zu Luftstickstoff) gebildet werden. Die simultane Aktivität mehrerer Bildungswege erschwert eine eindeutige Identifizierung der dominanten biochemischen Bildungsmechanismen. Auf Grund der grossen Umweltrelevanz von N_2O sowie des ungenügenden Prozessverständnisses der N_2O Bildung in der Abwasserreinigung, werden in dieser Arbeit die wichtigsten Bildungsprozesse sowie die relevanten Prozessparameter untersucht. Dazu wurde eine Methode angewandt, die es erlaubt die positionsabhängige Verteilung von ^{15}N (genannt Site Preference, SP), wie auch den Anteil von ^{15}N im N_2O Molekül zu bestimmen. Darauf aufbauend wurden Betriebsstrategien formuliert und getestet, welche die N_2O Emissionen aus der Abwasserreinigung minimieren.

Es konnte gezeigt werden, dass die N_2O Bildung unter aeroben Bedingungen durch die Nitrifikanten dominiert wird, wobei hohe Ammonium- und Nitrit- Konzentrationen die N_2O Produktion begünstigen. Ein Beispiel stellt die Dosierung von Faulwasser dar, in deren Folge die Ammonium- und Nitrit-Konzentrationen mit den N_2O Emissionen deutlich korreliert haben. In diesem Sinne wurde getestet, ob NO_2^- indirekt über die N_2O Messung detektiert werden kann. Dies wurde für ein Nitritations-Anammox Prozess getestet, bei welchem NO_2^- ein wichtiges Zwischenprodukt darstellt, da seine Konzentration ein Indikator für eine mangelnde Prozessstabilität ist. Der Zusammenhang zwischen gelöstem NO_2^- und erhöhter N_2O Emission konnte bestätigt werden, wobei weitere Bildungswege (z.B. die NH_2OH Oxidation) berücksichtigt werden müssen. Insgesamt sind die Resultate jedoch vielversprechend.

Die Mechanismen der N_2O Bildung sind jedoch noch nicht vollständig verstanden, was eine Abschätzung der Emissionsfaktoren schwierig macht. Zudem sind die Emissionen räumlich und zeitlich sehr variabel und werden durch eine Vielzahl von Faktoren beeinflusst. Aus diesem Grund sind aktuelle Emissionsabschätzungen noch mit einer grossen Unsicherheit verbunden. Daraus wird gefolgert, dass für die gezielte Optimierung einer Anlage eine fix installierte kontinuierliche N_2O-Abluftmessung vorteilhaft ist. Damit kann neben der N_2O Emissionsüberwachung auch die Prozessstabilität überwacht werden. Abschliessend kann gesagt werden, dass auf Grund des N_2O Emissionspotentials energetische Optimierungen von Abwasserreinigungsverfahren, ohne Mitberücksichtigung der N_2O Emissionen, nicht sinnvoll erscheinen.

SUMMARY

Nitrous oxide (N_2O) is a strong greenhouse gas, and involved in the destruction of the stratospheric ozone layer. Emissions to the atmosphere are therefore harmful for the environment. In biological wastewater treatment, N_2O can be produced in different process steps: during nitrification (the oxidation of ammonia to nitrate) and during heterotrophic denitrification (the reduction of nitrate to dinitrogen gas). However, identifying the most important N_2O production pathways is a complex issue, since all of them might be active simultaneously. The aim of this work was therefore (i) to identify the most important N_2O production pathway in biological wastewater treatment based on isotopomeric analysis in combination with emission pattern, (ii) to evaluate the impact of relevant operating parameters as well as (iii) to test operating strategies reducing these emissions.

Results indicate that NO_2^- reduction (presumably by ammonia oxidizing bacteria) is the dominant N_2O production pathway under aerobic conditions. The contribution from NH_2OH oxidation in wastewater treatment, however, cannot be completely excluded, but is deemed only of minor importance in this investigation. The addition of digester liquid, equivalent to a temporary increase of the nitrogen load, to a pilot-scale activated sludge plant showed that high nitrogen loads accelerated N_2O emission significantly, correlating positively with the NO_2^- build-up in the nitrification activated sludge tanks. As such, operating strategies reducing NO_2^- accumulation are considered to emit only low amounts of N_2O. Given the correlation of soluble NO_2^- with N_2O emission, the application of N_2O analysis as a potential indirect measure for dissolved NO_2^- was tested for a nitritation-anammox process. Results clearly confirmed this correlation but showed that also other pathways are relevant for N_2O emission in this process (e.g. NH_2OH oxidation or yet undefined toxic components). Thus this is a promising approach and needs to be further investigated.

N_2O production is a complex issue, since strongly depending on the individual plant operating conditions. This makes it difficult to extrapolate from one treatment plant to another. Given the N_2O emission potential, plant optimization from an energetic point of view does not make sense without considering N_2O emission. Further, a continuous N_2O off-gas online monitoring concept for full-scale plants is considered favorable in order to minimize overall climate impact of wastewater treatment. A financial greenhouse gas crediting system could be a potent incentive to promote widespread adoption of the here proposed approach.

Chapter 1

General Introduction

Introduction

In this doctoral thesis, the production and emission of nitrous oxide (N_2O) from biological wastewater treatment is investigated, with a specific focus on pathway identification, plant operating reduction strategies and the potential use of N_2O off-gas real-time data for process control.

In the following introduction, the emphasis lies on (i) the environmental relevance of N_2O, (ii) some facts about the historical context, (iii) the current state of knowledge concerning N_2O production mechanisms and important pathways, and (iv) its relevance in the field of biological nutrient removal.

Managing the nitrogen cycle and environmental relevance of N_2O

The nitrogen (N) cycle (Figure 1) is essential for life, as N (present in the redox states from -3 to +5) is a nutrient of primary importance for all organisms, since being an important component of proteins, enzymes and genetic material (e.g. DNA). The N cycle is driven by microbial activities, involving a high diversity of organisms (Barton and Atwater 2002; Jetten 2008). Until recent decades, the biological N availability was basically driven by naturally occurring N_2 fixation by several types of bacteria and algae (Barton and Atwater 2002). However, human-derived N_2 fixation, e.g. by the Haber-Bosch process or by cultivation of legumes, significantly increased global N fixation rate throughout the last century. This resulted in significant quantitative changes of the N cycle and led to world-wide environmental problems, such as eutrophication of coastal waters, acid rain, stratospheric ozone depletion as well as to an increase in the atmospheric N_2O concentration (Figure 2; Kampschreur 2010).

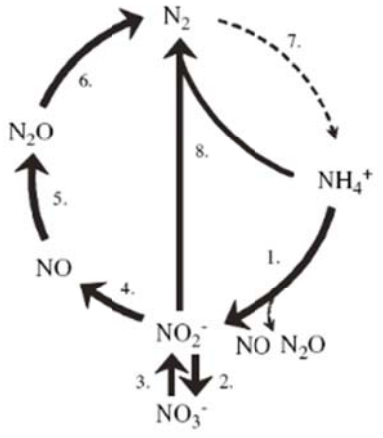

Figure 1. *Microbial N cycle: (1) aerobic ammonia (NH_4^+) oxidation, (2) aerobic nitrite (NO_2^-) oxidation, (3) nitrate (NO_3^-) reduction to NO_2^-, (4) to (6) heterotrophic denitrification, (7) N_2 fixation, (8) anaerobic ammonium oxidation (Anammox; Kampschreur et al., 2009).*

N_2O is an unwanted gas since of great environmental relevance: (i) it is a greenhouse gas (GHG) with a global warming potential of about 300 times higher than that of carbon dioxide (CO_2) (IPCC 2007), and (ii) it is involved in the destruction of the stratospheric ozone layer. Since the ban on chlorofluorocarbons (CFCs) in the 1980-ies,

N_2O is estimated to be the most important threat to the ozone layer of the 21st century (Kramlich and Linak 1994; IPCC 2007; Ravishankara et al., 2009). Compared to the pre-industrial level, the atmospheric N_2O concentration has increased by about 18 % (from about 270 ppb to up to 324.2 ppb in the year 2011; WMO GHG Bulletin (2012); Figure 2), primarily due to human activities, and due to an atmospheric residence time of about 114 years (IPCC 2001; IPCC 2007). The major sink of N_2O is the stratospheric reaction with molecular oxygen to nitric oxide (NO). Latter induces the destruction of the stratospheric ozone layer (Kramlich and Linak 1994; Ravishankara et al., 2009; Wuebbles, 2009).

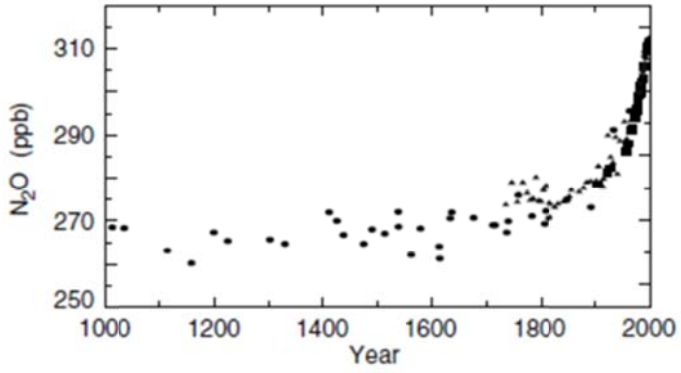

Figure 2. *Atmospheric N_2O concentrations over the last 1000 years (IPCC 2001).*

About two thirds of overall N_2O emission is estimated to originate from agricultural activities, such as application of fertilizer, animal manure management and so forth, while fuel combustion, industrial production of adipic acid as well as of nitric acid are other important source (USEPA 2009). The contribution of N_2O emission from wastewater treatment to total GHG emission is estimated to be approximately 0.25 % (Kampschreur et al., 2009) (please see also 'Importance of N_2O emission in the field of wastewater treatment' in this chapter). In Switzerland, total N_2O emission is estimated to cause about 6 % of the total GHG emission (FOEN 2008). As such, given global climate change as well as the current political GHG debate, emission reduction is considered of increasing importance, and is highly expected to appear on the political agendas in the near future. Switzerland, for example, has committed, based on the new CO_2 act (in force since 1.1.2013), to reduce domestic GHG emission, until 2020, by at least 20 % with respect to the emission level in 1990 (http://www.bafu.admin.ch/klima/12325/index.html?lang=de; September 2013).

N_2O from a historical point of view: Medical application vs. rocket science

N_2O is color-less, nonflammable, and commonly known as 'laughing gas', due to its euphoric effect when inhaled. Therefore, the popularity nowadays among young people is not surprising. The gas was first synthesized by Joseph Priestly in the 18th century. However, it took almost a century, till Horace Wells, a dentist, successfully applied N_2O as an anesthetic drug (Figure 3). Still, his colleagues remained skeptical since the public demonstration by Horace Wells had been only partly successful. In 1863, however, N_2O started to be applied more frequently for medical purposes, and is

nowadays still used in dentistry (www.general-anaesthesia.com; www.wikipedia.com; Mai 2013).

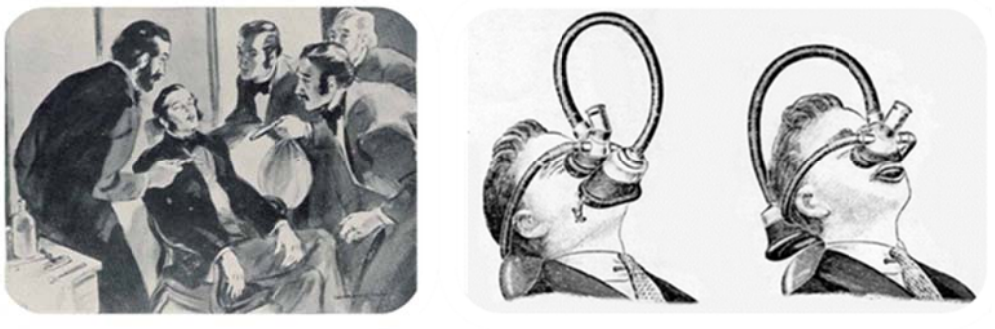

Figure 3. *Horace Wells uses N_2O as an anesthetic for tooth extraction in a self-experiment (left; http://lifeboat.com/ex/utopian.surgery; September 2013). Inhalant systems used for N_2O application (right; www. http://general-anaesthesia.com/images/nitrous-oxide.htm; September 2013).*

Moreover, N_2O is also used as an oxidizer (at elevated temperatures similarly effective as oxygen) in rocket engines or in vehicles, since N_2O delivers extra oxygen which enables the engine to burn more fuel and thus to increase its power (www.wikipedia.com, Mai 2013). At Stanford university, for example, there are engineering attempts to use N_2O as a fuel for rocket thrusters (http://woods.stanford.edu/environmental-venture-projects/high-rate-microbial-production-nitrous-oxide-energy-generation; September 2013). Or a recent patent application by the Robert Bosch GMBH suggests to increase block heating work power by adding N_2O (presumably originating from biological wastewater treatment) to CH_4 produced during anaerobic sludge digestion (Patent number: PCT/EP2011/057571). This illustrates that N_2O has a wide technical application. In the environment (and in biochemical processes within engineered reactors), however, N_2O is an unwanted microbial by-product, due to its negative impacts on the global climate and the stratospheric ozone layer. Process understanding and optimization, leading to minimal microbial N_2O production, is therefore subject to increasing research activities, which is also the focus of this thesis. As such, the next section will provide a brief overview over the current understanding of the microbial N cycle and how it is linked to N_2O production (additional information is provided in chapter 2 of this thesis).

Microorganisms involved in the nitrogen cycle and in N_2O production

The biological N cycle (Figure 1) involves a complex interaction of many microorganisms and enzymes, depending on the environmental conditions. Nitrification and heterotrophic denitrification, both relevant for the N cycle, are also used in wastewater treatment plants to achieve nutrient removal (Figure 4). Basically, nitrification is the stepwise autotrophic oxidation of ammonia (NH_4^+) via nitrite (NO_2^-) to nitrate (NO_3^-) by the use of oxygen (O_2). Ammonia-oxidizing bacteria (AOB) and nitrite oxidizers (NOB) are two groups of microorganism involved in this process (Colliver and Stephenson 2000; Ward et al., 2011).

Denitrification is the heterotrophic reduction of NO_3^- to atmospheric nitrogen (N_2), with NO_2^-, nitric oxide (NO) and N_2O as obligatory intermediates (detailed overview given in Zumft 1997).

Ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB)

During the autotrophic oxidation of NH_4^+ to NO_3^-, O_2 is used as the terminal electron acceptor and CO_2 as the carbon source (Colliver and Stephenson 2000). In activated sludge, the oxidation from NH_4^+ via NH_2OH to NO_2^- is performed by AOB (such as *Nitrosomonas oligotropha*, *Nitrosomonas europaea*, and *Nitrosospira*, a β-subclass of proteobacteria). NOB perform the oxidation of NO_2^- to NO_3^- (e.g. *Nitrobacter*, an α-subclass of proteobacteria and *Nitrospira* an independent line within the domain of bacteria; Bock and Wagner 2006). Under regular operating conditions, *Nitrosomonas* were reported to be the dominant AOB species and *Nitrospira* the dominant NOB species at low NH_4^+ and NO_2^- concentrations (Purkhold et al., 2000; Freitag et al., 2005; Manser et al., 2005).

The enzymes required for the oxidation of NH_4^+ to NO_2^- are ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO). The latter being the energy-generating step: four electrons are released, two of them are required for the AMO reaction while the other two are used for energy generation. The enzyme for the oxidation of NO_2^- to NO_3^- is called nitrite oxidoreductase (NO_2-OR) (Colliver and Stephenson 2000; Bock and Wagner 2006).

Heterotrophic denitrifying microorganisms (HET)

Heterotrophic denitrification is the reduction of NO_3^- to N_2 by use of organic substrate as the electron donor and carbon source. It is carried out by prokaryotes (bacteria as well as archaea), such as *Paracoccus denitrificans* and *Alcaligenes faecalis* (Zumft 1997). The N_2O release is expected to be linked to the activity of N_2O-reductase enzymes relative to the activity of N_2O forming enzymes (Alinsafi et al., 2008; Knowles 1982; von Schulthess et al., 1994). For example, low dissolved oxygen concentrations may result in incomplete denitrification (Stouthamer 1991), and strong N_2O-reductase enzyme inhibition in the presence of O_2 (von Schulthess et al., 1994).

Denitrification requires four reductases: NO_3^-- (NaR / Nap), NO_2^-- (NiR), NO (NoR) and N_2O (N_2OR) reductase (Bergaust, 2008; Zumft 1997). The genes encoding these proteins are activated by several signals: both, (i) no (or low) O_2 concentrations as well as (ii) the presence of denitrification intermediates (e.g. NO_2^-, NO) are needed for their expression (Bergaust et al., 2008). In general, regulation of these enzymes helps to avoid toxic NO_2^- and NO concentrations, as e.g. reported for *Nitrosomonas europaea* expressing NO_2^- reductase (NirK) at toxic NO_2^- levels (Beaumont et al., 2004).

N_2O production pathways in wastewater treatment

N_2O production in biological wastewater treatment is generally attributed to nitrification and heterotrophic denitrification. According to Kampschreur et al. (2009),

there are three main routes for N_2O production (Figure 4), which are called hydroxylamine oxidation, nitrifier denitrification and heterotrophic denitrification, respectively (adapted from Wunderlin et al., 2012):

- Hydroxylamine oxidation refers to the production of N_2O from intermediates of biological hydroxylamine oxidation (e.g. HNO, $N_2O_2H_2$; Poughon et al., 2001; Law et al., 2012), being probably related to a highly imbalanced metabolic activity of AOB (Yu et al., 2010), or to chemical decomposition of NH_2OH as well as to chemical oxidation with NO_2^- as an electron acceptor (chemo-denitrification; Ritchie and Nicholas 1972; Stuven et al., 1992).
- Nitrifier denitrification is attributed to reduction of NO_2^- by AOB in combination with NH_4^+, hydrogen or pyruvate as electron donors, e.g. at O_2-limiting conditions or elevated NO_2^- concentrations (Stuven et al., 1992; Colliver and Stephenson 2000; Wrage et al., 2001).
- During heterotrophic denitrification, however, N_2O is an obligate intermediate and its production therefore assumed to be due to an imbalanced activity of N-reducing enzymes, e.g. due to O_2 inhibition (Baumann et al., 1997; Lu and Chandran 2010), NO_2^- accumulation (von Schulthess et al., 1994), or a limited availability of biodegradable organic compounds (Itokawa et al., 2001).

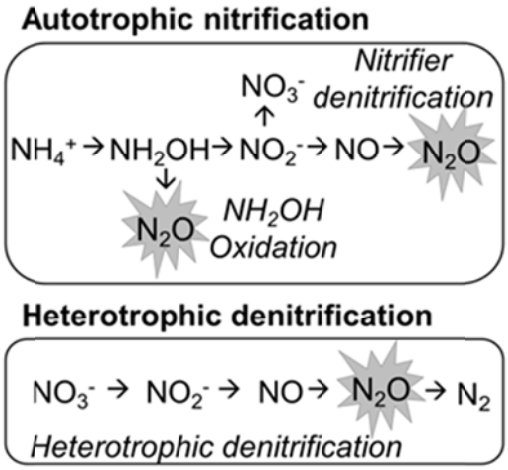

Figure 4. *Overview of N_2O production pathways during autotrophic nitrification and heterotrophic denitrification. During nitrification, N_2O can be produced via nitrifier denitrification or NH_2OH oxidation. During heterotrophic denitrification, N_2O is an obligate intermediate (adapted from Wunderlin et al., 2013).*

Even though, the dominant N_2O production pathways in biological nutrient removal have been identified (Figure 4), their relative contribution under defined process conditions is not yet clear to avoid unnecessary emissions. As such, no consensus has been achieved so far about the most important parameter favoring N_2O production. The scientific discussion, however, has been focused on some factors that have been recognized to be strongly linked to N_2O production. These are (i) low dissolved O_2 concentration during nitrification as well as heterotrophic denitrification, (ii) the accumulation of NO_2^- and / or NH_4^+ during nitrification due to high nitrogen loads in

combination with limited aeration, and (iii) a low ratio of readily biodegradable organic compounds to NO_3^- during heterotrophic denitrification (Kampschreur et al., 2009; Ahn et al., 2010; Desloover et al., 2012; Wunderlin et al., 2012).

A continuously and fast growing understanding of the N_2O production mechanisms and the relevant impacting parameters within the near future is highly expected, since in addition to the present thesis, an increasing number of research groups worldwide are working on this topic.

Importance of N_2O emission in the field of wastewater treatment

In conventional biological wastewater treatment, N removal occurs via microbial nitrification and heterotrophic denitrification. Most of the wastewater treatment plants in Switzerland are designed for nutrient removal. Basically, aerobic conditions are needed for autotrophic nitrification, while anoxic conditions (absence of O_2) and a sufficient amount of organic carbon is required to support heterotrophic denitrification (detailed overview given in Law et al., 2012).

In the last decade, significant efforts have been made to reduce the energy consumption of wastewater treatment plants, mainly by lowering the aeration of the nitrification stage to the required minimum (Kampschreur et al., 2009), because aeration is responsible for about 50 % of the total energy consumption of a wastewater treatment plant (VSA, 2008). This leads to low dissolved O_2 concentrations in the bioreactors used for nitrification, which might in combination with high N loads be a trigger for N_2O production (please see also 'N_2O production pathways wastewater treatment' in this chapter).

The literature is currently inconsistent about the quantities of N_2O emitted during wastewater treatment: reported values are ranging from 0 to 25 % (Kampschreur et al., 2009; Law et al., 2012). Recently, a measuring campaign across the United States showed that 0.01 to 3.3 % of the removed N is emitted as N_2O (Ahn et al., 2010). And in another investigation, where N_2O was measured continuously over one year on a full-scale treatment plant in the Netherlands, an emission of 3 % was reported (Daelman et al., 2012). These wide ranges clearly indicate that N_2O emission is dynamic, plant specific, and not yet sufficiently understood.

Figure 5 shows a rough estimation of GHG emission (CO_2, CH_4, N_2O) from wastewater treatment. Currently, an average N_2O emission of 0.5 % with respect to influent N, is proposed as an acceptable emission level, since in this case, the respective global warming potential is somewhat smaller compared to the one of aeration energy consumption. Moreover, in a recent investigation it is discussed that in addition to biological nutrient removal, N_2O production can also be relevant during sludge incineration (please see Appendix A). Data from two Swiss sludge mono-incineration plants indicate that around 0.2 to 1 % of the N influent load was emitted as N_2O (equivalent to about 10 to 47 $gCO_{2,equiv}$/PE/d), which is comparable to estimated N_2O emission from the main water line. Moreover, N_2O emissions from sludge incineration were negatively correlated with incineration temperatures, being higher at low

temperature and vice-versa. Accordingly, the here presented comparison (Figure 5), clearly illustrates that wastewater treatment plant optimization requires a broad view, focusing on more than just one aspect like aeration energy, and that all relevant GHG emissions need to be taken into account.

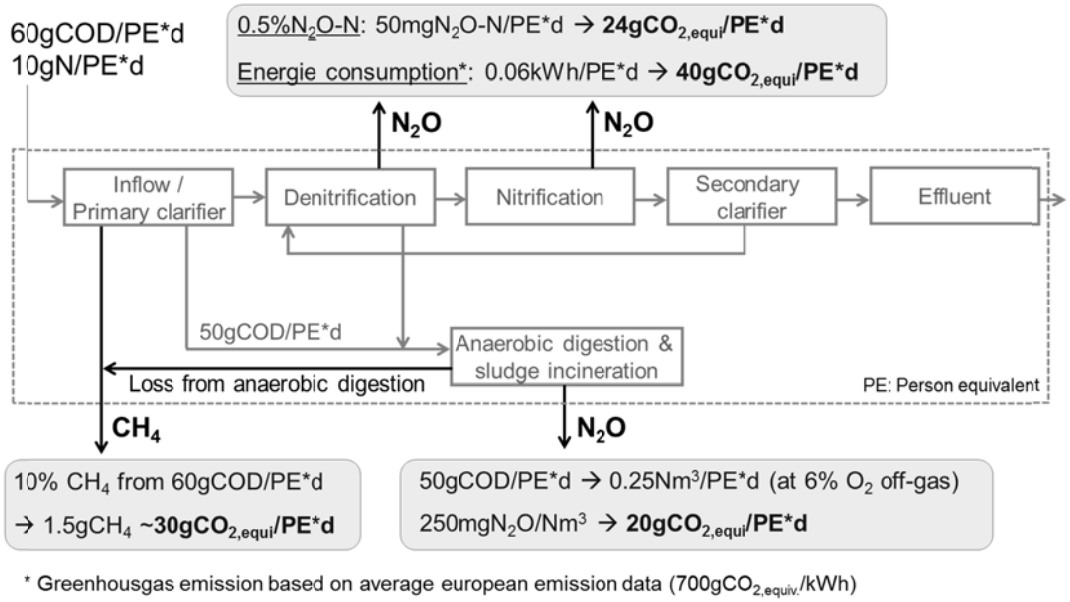

Figure 5. *Estimation of GHG emission in wastewater treatment (adapted from Wunderlin et al., 2013): N_2O is produced during biological nutrient removal as well as during sludge incineration; CH_4 originates most probably from the sewer system, primary clarifier and sludge digestion and storage (e.g. contribution of past-digester if not connected to the biogas system).*

General objectives of this thesis

The overall objective of this thesis is to identify mechanisms of N_2O production and how the resulting emissions can be reduced in biological wastewater treatment. Therefore, the focus will be on mixed microbial cultures used for state-of-the-art municipal wastewater treatment. To address these objectives, the work will be structured around the following milestones (please refer also to the graphical thesis overview on page iv and v):

(1) Providing a sound literature overview, and discussing suitable methods available to elucidate N_2O production mechanisms (**Chapter 2**).

(2) Investigating the N_2O production pathways based on N_2O emission pattern in a lab-scale bioreactor (**Chapter 3**).

(3) Identifying the N_2O site-specific isotopic signature of individual microbial processes in a lab-scale reactor, based on quantum cascade laser absorption spectrometry (QCLAS; **Chapter 4**).

(4) Validating the isotope approach during regular operation of a pilot-scale wastewater treatment plant (**Chapter 5**).

(5) Evaluating the importance of the different operating strategies, and the impact of key factors linked to N_2O production (**Chapter 6**).

(6) Discussing and exemplifying the potential future role of N_2O off-gas measurement in wastewater treatment and process control (**Chapter 7 and 8**).

Significance of the work

This thesis contributes to a better understanding of the relevant N_2O production pathways, including the discussion of N_2O impacting process parameters. However, the wide range of emissions observed, as well as the multiple factors correlating with them, result in complex and dynamic N_2O emission patterns, which makes it challenging to formulate general plant operating strategies for keeping N_2O emissions low. Therefore, it is suggested to implement a continuous on-line N_2O off-gas measurement at full-scale plants. Investment costs are estimated to be higher, but still in the same order of magnitude, compared to conventional commercially available ion selective electrodes, as usually applied for online NH_4^+ or NO_3^- measurement (please see also Chapter 8). Consequently, every plant can be optimized individually with respect to its overall carbon footprint. Moreover, the implementation of a financial GHG crediting system, as suggest by Wang et al. (2011), could be an additional incentive to promote widespread adoption of a continuous N_2O off-gas monitoring concept.

The site-specific isotopic signatures of N_2O produced during biological nutrient removal, and determined in this study, is a novel approach in the field of wastewater treatment, and will very likely be increasingly applied in future work. Accordingly, this method has the potential to substantially improve the understanding of N_2O emission dynamics in biological nutrient removal, especially when combined with other tools, such as molecular approaches (e.g. Yu et al., 2010) and mathematical modeling (e.g. Ni et al., 2011; Ni et al., 2012; please see also Chapter 2).

Our improved understanding of the involved mechanisms is deemed to be relevant well beyond wastewater treatment, since the biochemical processes also occur in aquatic environments (e.g. surface waters, sediments), in agricultural soils (the dominant global N_2O source) as well as other ecosystems and technical processes, and play a crucial role in the global N cycle.

Outline

In the first part of this thesis (**chapters 3 to 5**), the mechanisms of N_2O production are investigated, based on concentration and emission data combined with the nitrogen isotopic signature of N_2O. With the latter being a novel approach in the field of biological nutrient removal. In the second part (**chapter 6**), the dynamics and levels of

N_2O emissions are studied in pilot-scale. Finally, in **chapter 7** and **8**, N_2O off-gas measurement as a process control parameter was investigated for a nitritation-anammox process, which is judged to be a promising approach for future applications and a necessary stepping stone toward implementation in conventional full-scale plants.

In the first part of **Chapter 2** an overview is given over the current state of knowledge concerning N_2O production pathways and mechanisms, while the second part focuses on novel methods for future investigations of N_2O emissions from natural as well as engineered systems. One of the presented methods is about the nitrogen isotopic signature of N_2O (site preference, $\delta^{15}N$), which is a novel tool in biological wastewater treatment and applied in this thesis (see chapter 3 and 4).

In **Chapter 3** the mechanisms of N_2O production are investigated in a lab-scale bioreactor. Based on N_2O emission data in combination with NH_4^+, NO_2^- and NO_3^- concentration profiles, production pathways and mechanisms are discussed. The main conclusion is that there was a small contribution of NH_2OH oxidation at the beginning of the aeration phase when NH_4^+ concentration is high but NO_2^- still low, while in the course of nitrification a shift to nitrifier denitrification driven N_2O production is observed.

Chapter 4 introduces the nitrogen isotopic signature of N_2O as a novel method in biological wastewater treatment (see Chapter 2). The isotopic signature of the different N_2O production pathways was investigated based on lab-scale experiments where substrate availability was controlled in order to 'promote' the different production routes. The data confirm that under aerobic conditions, NO_2^- reduction, presumably by AOB, is the dominant N_2O production pathway. The contribution from NH_2OH oxidation is only of minor importance. This is an important aspect concerning the actual debate about the N_2O production mechanisms in biological wastewater treatment.

In **Chapter 5** the nitrogen isotopic signature of N_2O was applied to a pilot-scale treatment plant operated at different dissolved oxygen concentrations. The data confirm the findings of Chapter 3 and 4, where NO_2^- reduction was postulated as the dominant N_2O production mechanism under aerobic conditions. Moreover, it was confirmed that analyzing N_2O nitrogen isotopic signature is a promising tool for pathway identification in biological wastewater treatment.

The effect of digester liquid addition to a pilot-scale activated sludge plant was studied in **Chapter 6**. Results show that an increase in influent N load resulted in elevated N_2O emission, correlating positively with the NO_2^- build-up in the nitrification reactor. This underscores the fact that an operating strategy at low dissolved NO_2^- (<2 mgN/l) and with equalized N loads prevents substantial N_2O emission.

In **Chapter 7** and **8** the potential of N_2O as an indirect measure for dissolved NO_2^- will be discussed in detail. This application is based on a positive correlation between dissolved NO_2^- and N_2O off-gas concentration (as reported for conventional treatment schemes; see e.g. chapter 6). A nitritation-anammox reactor was operated at different aeration

rates in order to control NO_2^- accumulation, which is feasible due to the absence of NOBs, and due to anammox inhibition if molecular oxygen is not promptly depleted by AOB activity. It is shown that N_2O emission can be controlled by adjusting the airflow rate, with high emissions at high aeration rates, and vice-versa. Moreover, NO_2^- reduction and NH_2OH oxidation seemed to contribute to N_2O production as independent mechanisms, and thus did not always result in a clear linear NO_2^-/N_2O correlation. However, reactor operation at minimal N_2O emission avoided situations of NO_2^- accumulation, which is expected to improve overall process stability of nitritation-anammox reactors, and thus suggests incorporating continuous N_2O off-gas measurement in the process control.

Appendix A shows an article about N_2O emission of sludge incineration (in German).

Appendix B shows an overview article about N_2O emission of biological wastewater treatment (in German).

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Chapter 2

Nitric oxide and nitrous oxide turnover in natural and engineered microbial communities: biological pathways, chemical reactions and novel technologies

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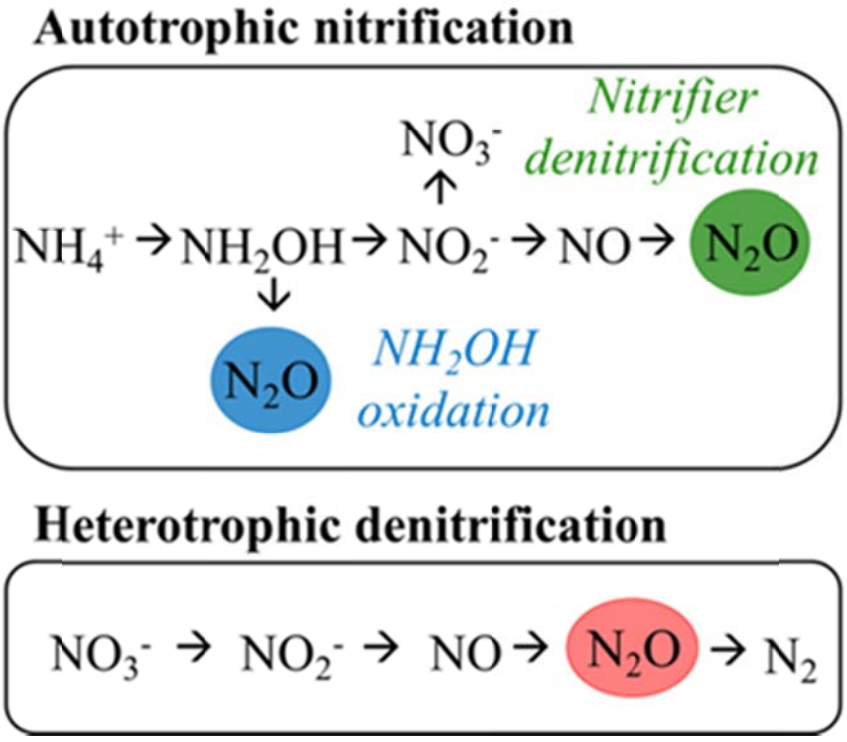
Graphical abstract



Abstract

Nitrous oxide (N_2O) is an environmentally important atmospheric trace gas because it is an effective greenhouse gas and it leads to ozone depletion through photo-chemical nitric oxide (NO) production in the stratosphere. Mitigating its steady increase in atmospheric concentration requires an understanding of the mechanisms that lead to its formation in natural and engineered microbial communities. N_2O is formed biologically from the oxidation of hydroxylamine (NH_2OH) or the reduction of nitrite (NO_2^-) to NO and further to N_2O. Our review of the biological pathways for N_2O production shows that apparently all organisms and pathways known to be involved in the catabolic branch of microbial N-cycle have the potential to catalyze the reduction of NO_2^- to NO and the further reduction of NO to N_2O, while N_2O formation from NH_2OH is only performed by ammonia oxidizing bacteria. In addition to biological pathways, we review important chemical reactions that can lead to NO and N_2O formation due to the reactivity of NO_2^-, NH_2OH and nitroxyl (HNO). Moreover, biological N_2O formation is highly dynamic in response to N-imbalance imposed on a system. Thus, understanding NO formation and capturing the dynamics of NO and N_2O build-up are key to understand mechanisms of N_2O release. Here, we discuss novel technologies that allow experiments on NO and N_2O formation at high temporal resolution, namely NO and N_2O microelectrodes and the dynamic analysis of the isotopic signature of N_2O with quantum cascade laser based absorption spectroscopy. In addition, we introduce other techniques that use the isotopic composition of N_2O to distinguish production pathways and findings that were made with emerging molecular techniques in complex environments. Finally, we discuss how a combination of the presented tools might help to address important open questions on pathways and controls of nitrogen flow through complex microbial communities that eventually lead to N_2O build-up.

Key words

Isotopic signature; micro-sensors; molecular tools; dinitrogen oxide; nitrogen monoxide; pathway identification; quantum cascade laser based absorption spectrometry, site preference

Introduction

Nitric oxide (NO) and nitrous oxide (N_2O) are atmospheric trace gases that influence atmospheric chemistry and the greenhouse effect. Biological and chemical processes produce N_2O on the earth surface (Crutzen, 1979). Entering the stratosphere, N_2O is converted to NO by photo-oxidation. NO together with nitrogen dioxide (NO_2) participate in a set of reactions that transfer ozone (O_3) to molecular oxygen (O_2), thereby leading to O_3 layer depletion. In fact, N_2O is and will remain the dominant O_3-depleting substance in the 21st century (Ravishankara et al., 2009), since the use of chlorofluorocarbons has been restricted by the Montreal Protocol. In addition, N_2O is a potent greenhouse gas. The infrared radiative forcing of one N_2O molecule is 206 times that of one carbon dioxide (CO_2) molecule (Stein and Yung, 2003). Together with the long atmospheric lifetime of N_2O (~ 120 years) this results in a ~300 times higher global warming potential of N_2O than that of CO_2 on a per molecule basis. Overall, N_2O contributes 6 to 8 % to the anthropogenic greenhouse effect, despite its relatively low atmospheric concentration (~322 ppbv) (Montzka et al., 2011).

Over the last 100 years atmospheric N_2O concentrations have been steadily increasing due to the massive introduction of fixed nitrogen into the environment by humans (IPCC, 2001). Counteracting the further increase of N_2O in the atmosphere will rely on (i) decreasing the introduction of fixed nitrogen into the environment by humans, (ii) exactly quantifying the important environmental sources of N_2O, and (iii) implementing effective strategies to mitigate its formation in nitrogen-transforming, man-made ecosystems such as agriculture and wastewater treatment. Thus, there is an urgent need to understand the mechanisms that underpin the formation of N_2O in natural and engineered microbial communities.

In this review, we will outline the current state-of-the-art on biological and chemical processes that can produce and consume N_2O and NO - an important precursor of N_2O in many biological pathways. We will discuss pathways that produce NO and N_2O in natural and engineered microbial communities and experimental approaches that can be used to distinguish between different pathways in these systems. Importantly, NO and N_2O formation can be highly dynamic and occur at small spatial scales. Thus, we will further introduce two novel technologies that provide such data and how they can lead to mechanistic insight: (i) NO and N_2O microelectrodes and (ii) the analysis of the site preference in N_2O measured with quantum cascade laser based absorption spectrometry. In addition, we discuss the challenges of incorporating molecular biological techniques in this scheme.

Biological pathways for NO and N_2O production

The study of laboratory cultures for pathways and controls of NO and N_2O production in different organisms has generated considerable knowledge, which was partly reviewed recently (Stein, 2011; Chandran et al., 2011). Figure 1 shows that the sequential reduction of nitrite (NO_2^-) to NO and further to N_2O can be performed by all organisms involved in the catabolic branch of the N-cycle. While all N-cycle organisms can perform

these reactions it is currently believed that denitrifiers and ammonia oxidizing bacteria (AOB) and archaea (AOA) are the most important environmental sources of N_2O. However, in the following section we additionally review the evidence for NO and N_2O production by nitrite oxidizing bacteria (NOB), anaerobic methane (N-AOM) and ammonia oxidizing bacteria (anammox), and bacteria that perform dissimilatory nitrate reduction to ammonia (DNRA). Even though it is clear that these bacteria can produce NO and N_2O there is only few information on the controls, conditions and magnitude for NO and N_2O production by these bacteria in the laboratory and in the environment. This should be an important aspect of future research as e.g. DNRA and anammox are the major N-conversion pathways in some important environments.

Denitrification. The key enzyme for NO formation during denitrification is nitrite reductase (Nir). Purification and characterization of Nir from several bacteria revealed two entirely different periplasmic enzymes: a heme-containing cytochrome cd1 Nir (NirS) and a copper-containing Nir (NirK) as reviewed by Cutruzzolà (1999). Reduction of NO to N_2O is mediated by respiratory nitric oxide reductases (Nor). Respiratory Nor proteins are integral membrane proteins that fall into two groups: one is a cytochrome bc complex that can use c-type cytochromes as electron donors (cNor), whereas the other one lacks a cytochrome c component and accepts electrons from quinols (qNor; sometimes termed NorZ) (Hendriks et al., 2000; Zumft, 2005). Few bacteria use qNor for classical denitrification. Rather, qNor is mainly encoded by pathogenic bacteria that use it for NO detoxification and the survival of anoxic periods when expressed in concert with Nir, as shown for *Neisseria spp.* (Anjum et al., 2002; Rock et al., 2007). The final step in denitrification is mediated by nitrous oxide reductase (Nos), a multi-copper enzyme that reduces N_2O to dinitrogen (N_2) (Zumft and Kroneck, 2007).

N_2O reduction by Nos is the only known N_2O consuming process that can counteract release of N_2O from ecosystems (Richardson et al., 2009). Accumulation of N_2O is often observed in pure cultures (Otte et al., 1996; Baumann et al., 1996; Kester et al., 1997; Bergaust et al., 2010) and mixed microbial communities (Firestone and Tiedje, 1979; Firestone et al., 1980; Morley et al., 2008; Kampschreur et al., 2008b; Schreiber et al., 2009; Elberling et al., 2010; Pellicer-Nàcher et al., 2010; Liengaard et al., 2011) during transitions from anoxic to oxic conditions or vice versa (Table 1). Even in pure cultures the physiological basis for this is not well understood because it probably has multiple, strain-specific reasons. It has been hypothesized that Nos is - unlike Nir and Nor - inhibited by O_2 (Morley et al., 2008), but in pure cultures evidence for O_2-insensitive (Berks et al., 1993) and O_2-sensitive (Otte et al., 1996) Nos have been reported. Likewise, it has been argued that expression of Nos is slower than that of the preceding denitrification enzymes (Stief et al., 2009; Firestone et al., 1980), but in *Paracoccus denitrificans* Nos synthesis is faster (Baumann et al., 1996; Bergaust et al., 2010) and in *Pseudomonas stutzeri* Nos is even constitutively expressed at low levels (Körner and Zumft, 1989). More studies on Nos expression in relation to N_2O production pathways and on Nos inhibition by O_2 are needed with environmentally relevant isolates and mixed microbial communities. Additional factors that lead N_2O accumulation are the slower turnover of Nos at low pH as compared to nitrate reductase (Nar), Nir and Nor (Richardson et al., 2009; Bergaust et al., 2010), low pH during Nos assembly (Bergaust et

al., 2010), inhibition of Nos by nitrous acid formed from NO_2^- at low pH (Zhou et al., 2008), inhibition of Nos by exogenously produced NO (Frunzke and Zumft, 1986; Schreiber, in preparation) or hydrogen sulfide (H_2S) (Sørensen et al., 1980) and copper limitation (Granger and Ward, 2012).

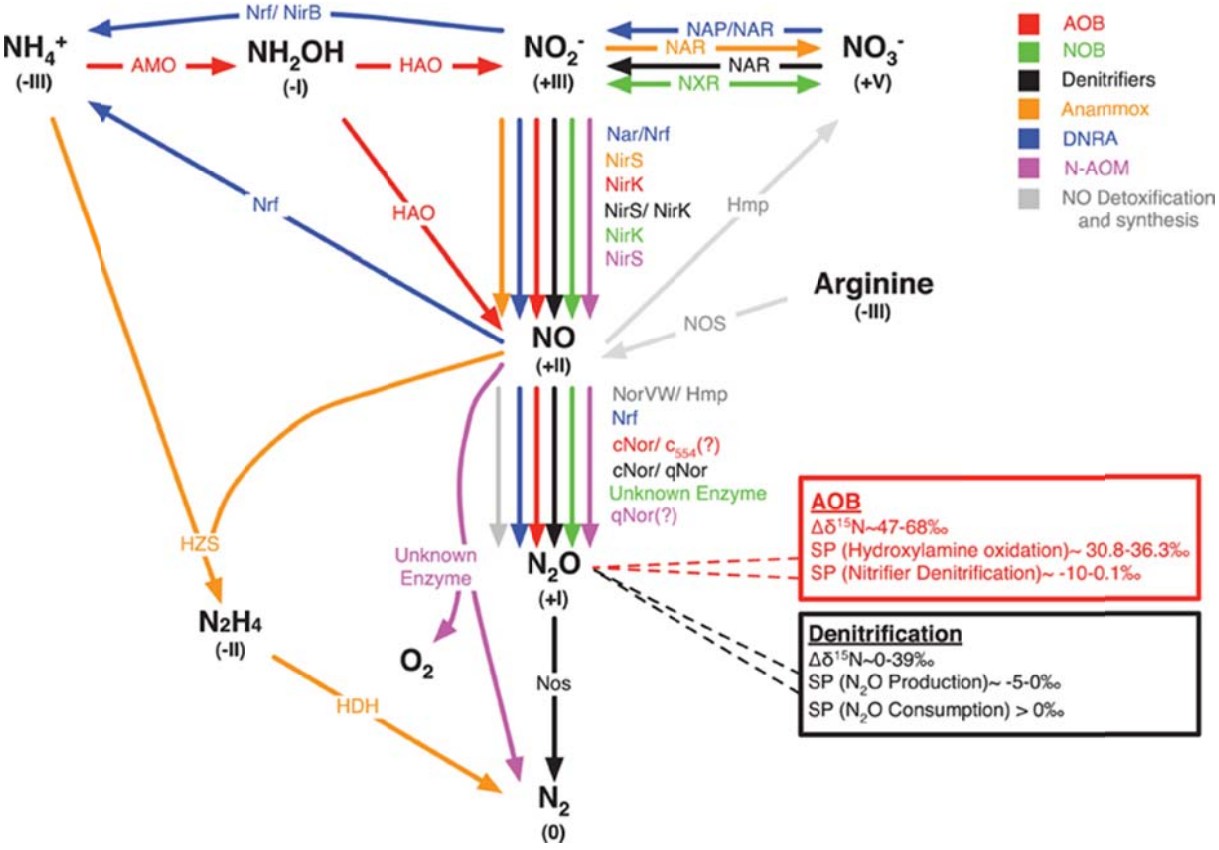


Figure 1. *Biological pathways for NO and N_2O turnover in the catabolic branch of the N-cycle plus NO synthesis and detoxification. Different colors are allocated to different microbial guilds or turnover pathways: AOB (red) – ammonia oxidizing bacteria; NOB (green) – nitrite oxidizing bacteria; anammox (orange) – anaerobic oxidation of ammonia; DNRA (blue) – dissimilatory nitrate/nitrite reduction to ammonia; N-AOM (purple) – oxygenic nitrite-dependent anaerobic oxidation of methane. Key enzymes of each microbial guild are depicted that are known to mediate the conversion from one chemical N-species into another: AMO – ammonia monooxygenase; HAO - hydroxylamine oxidoreductase; NXR - nitrite oxidoreductase; Nar – membrane-bound nitrate reductase; Nap – periplasmic nitrate reductase; NirK – copper-containing nitrite reductase; NirS - cytochrome cd1 nitrite reductase; Nrf - cytochrome c nitrite reductase; NirB - cytoplasmic nitrite reductase; cNor – nitric oxide reductase that accepts electrons from c-type cytochromes; qNor - nitric oxide reductase that accepts electrons from quinols; c_{554} - cytochrome c_{554}; NorVW – flavorubredoxin, Hmp – flavohemoglobins; HZS – hydrazine synthase; HDH – hydrazine dehydrogenase; Nos – nitrous oxide reductase; NOS – nitric oxide synthase; unknown enzymes – nitric oxide dismutation to N_2 and O_2 during N-AOM and nitrous oxide producing enzyme in NOB. Roman numbers in brackets denote the oxidation state of the chemical N-species. The red and the black box denote the*

isotopic composition ($\delta^{15}N$) and the site preference (SP) in isotopomers of N_2O produced by AOB and denitrifiers, respectively.

Ammonia oxidizing bacteria (AOB). High levels of NO and N_2O can be produced by pure cultures of aerobic AOB (Lipschultz et al., 1981; Kester et al., 1997; Shaw et al., 2006), but the mechanism is not completely understood. Generally, two different pathways are inferred. First, the activity of nitrifier-encoded NirK and cNor reduces NO_2^- to NO and N_2O in a pathway termed nitrifier denitrification (Poth and Focht, 1985; Wrage et al., 2001; Schmidt et al., 2004b). A few reports exist on N_2 formation by AOB during nitrifier denitrification, but a nosZ gene or functional Nos in AOB was not demonstrated (Poth, 1986; Schmidt et al., 2004b; Schmidt, 2009). The term nitrifier denitrification is somewhat misleading as it has until now not been shown that it is a true dissimilatory process for energy conservation and growth, but rather may be a detoxification mechanism to counteract the accumulation of NO_2^- to toxic concentrations (Beaumont et al., 2002, 2004a, 2004b).

In the second pathway, N_2O is formed by hydroxylamine (NH_2OH) oxidation. The current model is that hydroxylamine oxidoreductase (HAO) oxidizes NH_2OH to NO (Hooper, 1968; Hooper and Terry, 1979). NO is then reduced to N_2O by a yet unidentified Nor; a potential candidate is cytochrome c_{554} (Upadhyay et al., 2006). However, the catalytic cycle of HAO, including its intermediates and its catalytic potential are a subject of ongoing debate (Hendrich et al., 2002; Cabail and Pacheco, 2003; Cabail et al., 2005; Fernández et al., 2008; Kostera et al., 2008) and as of yet direct formation of N_2O from HAO or other reactions cannot be excluded. Indeed, the difference in the site preference (SP) of N_2O produced by NH_2OH oxidation and nitrifier denitrification indicates that N_2O might be produced by HAO by a mechanism that (i) either does not involve NO reduction by canonical Nor used for nitrifier denitrification or (ii) does proceed via a completely different mechanism without free NO as intermediate (discussed in section 'site preference' and 'HNO as intermediate of enzymatic hydroxylamine oxidation'). Both nitrifier denitrification and NH_2OH oxidation require O_2 to activate ammonia (NH_3) with ammonia monooxygenase (AMO) to NH_2OH, which serves as a substrate for HAO or as electron donor to nitrifier denitrification. A pathway in which AOB perform denitrification with organic substrates instead of NH_3 as electron donor (Schmidt, 2009) should be considered heterotrophic denitrification performed by AOB. Ammonia oxidizing archaea (AOA) have also been demonstrated to produce N_2O probably by pathways akin to AOB (Santoro et al., 2011).

The relative importance of NH_2OH oxidation and nitrifier denitrification for NO and N_2O production is still debated. Based on pure culture investigations Yu et al. (2010) hypothesized that a high NH_3 oxidation activity favors N_2O production via NH_2OH oxidation. Similarly, Wunderlin et al. (2012) found that NH_2OH oxidation is favored by high NH_3 and low NO_2^- concentrations, and a high nitrification rate in a mixed culture for treating municipal wastewater. Moreover, stable nitrogen isotopes work with AOB pure cultures showed that NH_2OH oxidation contributes to N_2O production mainly at high O_2 whereas nitrifier denitrification is more active at low O_2 concentrations (Sutka et al. 2006).

Nitrite oxidizing bacteria (NOB). NOB form NO and N_2O during denitrification of nitrate (NO_3^-) or NO_2^- with pyruvate or glycerol as electron donor under anoxic conditions (Freitag et al., 1987; Ahlers et al., 1990), but a known NO reductase could not be identified in the genomes of different *Nitrobacter* species and *'Candidatus Nitrospira defluvii'* (Starkenburg et al., 2006, 2008b; Lücker et al., 2010). Under anoxic conditions nitrite oxidoreductase (NXR) mediates NO_3^- reduction to NO_2^-, while it mediates the reverse reaction under oxic conditions (Freitag et al., 1987). NOB actively express NirK, which co-purifies with NXR, in the presence of NO_2^- and if O_2 concentrations are low (Ahlers et al., 1990; Starkenburg et al., 2008a). NO generated by NOB-NirK is thought to direct cellular electron flux either toward O_2 respiration at high O_2 concentrations or toward NADH synthesis by reversibly inhibiting cytochrome oxidase at low O_2 concentrations. An interesting question to explore in natural communities would be whether NO produced by AOB or denitrifying bacteria can influence the activity of NOB.

Dissimilatory nitrate reduction to ammonia (DNRA). NO and N_2O turnover by bacteria that perform DNRA has been mainly investigated in *Escherichia coli* and *Salmonella typhimurium*. In *E. coli*, NO formation is mediated by cytochrome c nitrite reductase (Nrf) under anoxic conditions in the presence of NO_3^- and NO_2^- (Corker and Poole, 2003). NO detoxifying enzymes, such as flavorubredoxin, may further reduce NO to N_2O. On the other hand, *E. coli* Nrf reduces NO to N_2O or NH_3 if electrons are donated to the enzyme at high or low potential, respectively (Costa et al., 1990), contributing to detoxification of exogenously generated NO (van Wonderen et al., 2008). Aerobic and anaerobic NO formation from NO_2^- in *S. typhimurium* is mediated by membrane-bound nitrate reductase (Nar). Under aerobic conditions, activity of NO detoxifying Hmp (see below) oxidizes NO to NO_3^- resulting in non-detectable NO concentrations in culture suspensions (Gilberthorpe and Poole, 2008).

Anaerobic methane and ammonia oxidizing bacteria. Bacteria that mediate the oxygenic nitrite-dependent oxidation of methane (N-AOM) and anaerobic ammonia oxidation (anammox) have been shown to use NO as an intracellular intermediate produced by NO_2^- reduction via NirS while they consume exogenous NO without concurrent N_2O formation (Ettwig et al., 2010; Kartal et al., 2010, 2011). Rather, N-AOM dismutates NO to form N_2 and O_2, while anammox couples the reduction of NO to a condensation with NH_3 to produce hydrazine (N_2H_4). Both have the genetic potential to reduce NO to N_2O; anammox bacteria encode for flavorubredoxin (Strous et al., 2006) and N-AOM encodes for qNor (Ettwig et al., 2010). However, physiological data for both indicates that they withstand rather high NO levels (N-AOM 20 µmol L-1, anammox 7 µmol L-1) without activating anaerobic NO detoxification mechanisms.

$NO_2^- \rightarrow NO \rightarrow N_2O$. central steps in the N-cycle. Generally, the reduction of NO_2^- to NO is a central step in the catabolic branch of the N-cycle, because it can be carried out by all involved organisms (Figure 1). The reduction of NO_2^- to NO is central for energy conservation in denitrification, anammox and N-AOM. In contrast, during NO_2^- oxidation and nitrifier denitrification the reduction of NO_2^- to NO is involved in regulating metabolic homeostasis or the removal of toxic NO_2^- (Beaumont et al., 2002, 2004a; Starkenburg et al., 2008).

The reduction of NO to N_2O is, besides a potential direct formation of N_2O from NH_2OH in AOB, the only known biochemical reaction that produces N_2O. NO reduction to N_2O is central for energy conservation only in denitrification (Zumft, 1997). The function of cNor in AOB is unclear. cNor is expressed and metabolically active during aerobic growth (Beaumont et al., 2004b). Knock-out mutants of cNor have lower growth rate and yield in chemostats (Schmidt et al., 2004b) but not in batch culture (Beaumont et al., 2004b). In chemostats, cNor regulates the free NO concentration to an optimal, non-toxic level and contributes to recovery of AOB from anaerobic conditions (Schmidt et al., 2004b). On the other hand, stripping NO from AOB cultures leads to the inhibition of growth, arguing for NO being an obligate intermediate of AOB (Zart et al., 2000).

NO detoxification and NO synthesis. Most bacteria encode for enzymes involved in NO detoxification. This is true for bacteria inside and outside the catabolic N-cycle. Flavohemoglobins (Hmp) mediate the O_2-dependent detoxification of NO to NO_3^- with NO dioxygenase activity (Gardner et al., 1998). In contrast, the anaerobic detoxification of NO is mediated by Flavodiiron NO reductase (flavorubredoxin [NorVW]) and Hmp by reducing NO to N_2O (Kim et al., 1999; Gardner et al., 2002; Gomes et al., 2002).

An alternative, less explored route to N_2O formation is via the synthesis of NO from arginine by NO synthases (NOS) and subsequent reduction of NO to N_2O by cNor, qNor, Hmp or NorVW. Because NOS was discovered in the medical field it shares a similar abbreviation with N_2O reductases (Nos). Until now, NOS has only been detected in a few bacterial –mostly gram-positive – species (Sudhamsu and Crane, 2009) and synthesized NO seems to remain intracellular (Shatalin et al., 2008; Schreiber et al., 2011). However, NOS activity has also been reported in blooming, pelagic diatoms (Vardi et al., 2006). More research is needed to elucidate if NOS-derived NO is a significant source for N_2O emitted from phytoplankton blooms in oceans and freshwater.

Chemical reactions in NO and N_2O turnover

Chemical production of NO and N_2O from inorganic nitrogen compounds at ambient temperatures are well known phenomena in soil science (van Cleemput and Samater, 1996) and atmospheric chemistry (Lammel and Cape, 1996). In soil science, the chemical processes leading to NO and N_2O are often summarized as chemo-denitrification (Chalk and Smith, 1983). NH_2OH and NO_2^- (or its acid HNO_2) are the main precursors for chemical production of NO and N_2O in wastewater or natural waters. In the following, we discuss chemical reactions involving HNO, NH_2OH and NO_2^- that can be responsible for the release of NO and N_2O. We will also discuss the possible significance of chemical N_2O production during biological NH_2OH oxidation.

Significance of HNO. In many studies on chemical N_2O production, HNO is postulated as the direct precursor of N_2O (see below): HNO dimerizes via hyponitrous acid ($H_2N_2O_2$), to N_2O and H_2O (Bonner and Hughes, 1988).

$$2\ HNO \rightarrow H_2N_2O_2 \rightarrow N_2O + H_2O \qquad \text{Equation 1}$$

It can be assumed that formation of HNO in natural and wastewater follows the same mechanisms that are used to synthesize HNO (DuMond and King, 2011) in the laboratory: (i) disproportionation of NH_2OH derivatives containing good leaving groups attached to the nitrogen atom and (ii) decomposition of nitroso compounds (X-N=O, where X represents a good leaving group). Chemical HNO production is a likely process for wastewater treatment, since nitrification can produce considerable amounts of both, HNO_2, which is a precursor for nitrosation agents (e.g. dinitrogen trioxide N_2O_3, Bonner and Stedman, 1996), and NH_2OH.

Recently, medical researchers have started to reevaluate the relevance of HNO for physiologically and biologically systems (Fehling and Friedrichs, 2011). The increased interest in HNO is due to the fact that HNO lifetime in aqueous solutions is much longer than previously assumed: the HNO dimerization rate constant has been reassessed to be on the order of $8{\cdot}10^5$ $M^{-1}{\cdot}s^{-1}$ instead of the previously reported value of $2{\cdot}10^9$ $M^{-1}{\cdot}s^{-1}$, and the pKa value of HNO has been redetermined to be 11.4 instead of the old value of 4.2 (Shafirovich and Lymar, 2002). It is likely that the importance of HNO has also been underestimated in the research on N_2O emissions. Analytical determination of HNO is very challenging (Miranda, 2005), because HNO is short-lived. However, computer simulations could be a helpful tool to assess the importance of HNO in N_2O formation (Law et al., 2012).

HNO_2 disproportionation. A well understood process for NO production is the disproportionation of HNO_2 (Udert et al., 2005). Since the pKa value of the NO_2^-/HNO_2 couple (pKa = 3.29; Schwartz and White, 1981) is far below 7, this process releases relevant amounts of NO only under acidic conditions. The disproportionation of HNO_2 can be described with Equation 2. The products - NO and NO_2 - are in equilibrium with N_2O_3 (Eq. 5) which is an important agent for nitrosation (Bonner and Stedman, 1996). Under aerobic conditions, NO will be further oxidized to NO_2. Since NO_2 reacts with H_2O to form HNO_2 and NO_3^-, the reaction scheme (Eq. 2 to 4) is ultimately a chemical pathway for the oxidation of NO_2^- to NO_3^-.

$$2\ HNO_2 \leftrightarrow NO + NO_2 + H_2O \qquad \text{Equation 2}$$

$$NO + 0.5\ O_2 \rightarrow NO_2 \qquad \text{Equation 3}$$

$$2\ NO_2 + H_2O \leftrightarrow HNO_2 + NO_3^- + H^+ \qquad \text{Equation 4}$$

$$NO + NO_2 \leftrightarrow N_2O_3 \qquad \text{Equation 5}$$

Since the kinetic and equilibrium constants for Equations 2 to 5 are known, the production of NO can be calculated (Udert et al., 2005). Depending on the aeration intensity, substantial losses of nitrogen oxides can occur during chemical HNO_2 oxidation. The stripped nitrogen oxides are mainly HNO_2, but also NO is lost.

Iron-mediated reduction of NO_2^-. Ferrous iron (Fe(II)) can reduce NO_2^- to NO and, in the second reaction step, NO to N_2O (Kampschreur et al., 2011)

$NO_2^- + Fe^{2+} + 2H^+ \rightarrow Fe^{3+} + NO + H_2O$ $\Delta G^\circ = 35.8 \cdot kJ\ reaction^{-1}$
Equation 6

$NO + Fe^{2+} + H^+ \rightarrow Fe^{3+} + 0.5\ N_2O + 0.5\ H_2O$ $\Delta G^\circ = -38.9\ kJ\ reaction^{-1}$
Equation 7

The first reaction is thermodynamically not possible under standard conditions, but in natural waters ferric iron (Fe(III)) will precipitate and thereby draw the Gibbs free energy to negative values. Iron-mediated reduction of NO_2^- was described as one of the sources of N_2O in soils (Van Cleemput, 1998). Recently, Kampschreur et al. (2011) postulated that this process can contribute significantly to N_2O production in wastewater treatment, if NO_2^- and Fe(II) are present concomitantly. One example for such a system is nitrogen removal from anaerobic digester effluents via nitritation/denitrification or nitritation/anammox. Digester supernatants can contain high amounts of Fe(II), because iron salts are used to precipitate phosphate and Fe(II) will be released in the anaerobic digester due to the reducing conditions. Hu et al. (2001) reported an additional reaction of NO_2^- with iron: under acidic conditions NO_2^- is reduced in the presence of metallic iron to N_2 and NH_3. They propose a mechanism, in which metallic iron is oxidized at low pH releasing Fe^{2+} ions and molecular hydrogen (H_2). NO_2^- is then reduced by H_2 to N_2 and NH_3.

Oxidation of NH_2OH by Fe(III). Iron not only mediates NO and N_2O production from NO_2^-. As Fe(III), it also oxidizes NH_2OH to N_2O. This process can be used for the analytical determination of trace amounts of NH_2OH (Butler and Gordon, 1986a). The general equation for the reaction is

$4\ Fe(III) + 2\ NH_2OH \rightarrow 4\ Fe(II) + N_2O + H_2O + 4\ H^+$ Equation 8

In this reaction, N_2O formation strongly depends on the pH value. In experiments with distilled water and natural seawater, Butler and Gordon (1986b) found that at pH 3, N_2O recovery was 80 %, while at a pH value of 9.5, N_2O production was negligibly low. The authors hypothesized that at high pH values, HNO, reacts with O_2 to produce NO_2^- and H_2O. However, it is also known that HNO can react with NH_2OH to N_2 (Bonner et al. 1978, Eq. 10). Chemical production of N_2O via NH_2OH oxidation by Fe(III) is a likely process during nitrification, because Fe(III) compounds are ubiquitous in natural waters and wastewater treatment systems.

Reaction of NH_2OH with HNO_2 and HNO. Döring and Gehlen (1961) investigated the reaction of NH_2OH and HNO_2. They described the process as nitrosation of NH_2OH. The overall reaction can be written as

$NH_2OH + HNO_2 \rightarrow N_2O + 2\ H_2O$ Equation 9

In their reaction scheme, Döring and Gehlen (1961) included $H_2N_2O_2$ (the dimer of HNO) as a direct precursor for N_2O. At neutral pH values, N_2O_3 is the relevant nitrosation agent. There are several reaction pathways for N_2O_3 formation from HNO_2. Formation of N_2O_3 from HNO_2 is given by Equation 2 and 5. A kinetic constant for nitrosation of

NH_2OH is given by Döring and Gehlen (1961) and together with the kinetic constants for Equation 1 and 4 (Udert et al., 2005) the N_2O production from NH_2OH and HNO_2 can be estimated. Some of the NH_2OH can also react with the intermediate HNO to form N_2 (Bonner et al., 1978)

$$HNO + NH_2OH \rightarrow N_2 + 2\ H_2O \qquad \text{Equation 10}$$

Disproportionation of NH_2OH. The disproportionation of NH_2OH can be described with the following equation (Bonner et al., 1978)

$$4\ NH_2OH \rightarrow 2\ NH_3 + N_2O + H_2O \qquad \text{Equation 11}$$

In pure water, this process is very slow with slightly higher degradation rates at elevated pH values. At pH 3 and 25±3 °C, Bonner et al. (1978) observed no NH_2OH disproportionation over 2 months, while 12 to 18% of the NH_2OH was degraded over two months at pH 13.5. Complexes of transition metals can accelerate NH_2OH disproportionation considerably (Alluisetti et al., 2004). Jenni et al. (2012) also observed N_2O formation within minutes, although the experiment was conducted in a phosphate buffer solution without transition metals. The disproportionation might have been catalyzed by the steel surface of an electrode immersed in the reactor, but this hypothesis still has to be proven.

Autoxidation of NH_2OH. Oxidation of NH_2OH with O_2 (autoxidation, Eq. 12) is a slow process, although faster than NH_2OH disproportionation.

$$2\ NH_2OH + O_2 \rightarrow N_2O + 3\ H_2O \qquad \text{Equation 12}$$

Again, trace concentrations of metals can strongly accelerate the process. Anderson (1964) reported that in an aerated solution with 1 $mmol \cdot L^{-1}$ NH_2OH and 1 $\mu mol \cdot L^{-1}$ cupric sulfate 30% of the NH_2OH was oxidized within 1 hour, while only 2.5 % were degraded without cupric sulfate addition (pH between 7.8 and 7.9, 30°C). Cu is by far the most potent catalyzer for the autooxidation of NH_2OH followed by Co(II), Fe(II), Mn(II) and Zn(II) (Moews Jr and Audrieth, 1959). Since most wastewaters and natural waters contain some traces of metals, autoxidation of hydroxylamine cannot a priori be excluded as a source of N_2O.

HNO as intermediate of enzymatic NH_2OH oxidation. Several authors postulated that HNO was a likely intermediate of HAO due to the observed N_2O production (Ritchie and Nicholas, 1972; Anderson 1964). Igarashi et al. (1997) could show that the crystal structure of HAO in *Nitrosomonas europaea* is in agreement with the following two step reaction

$$NH_2OH \rightarrow (HNO) + 2\ H^+ + 2\ e^- \qquad \text{Equation 13}$$

$$(HNO) + H_2O \rightarrow HNO_2 + 2\ H^+ + 2\ e^- \qquad \text{Equation 14}$$

Based on this scheme, an imbalance of the two reaction steps could lead to an accumulation of HNO and subsequently to chemical N_2O production (Eq. 1). Law et al. (2012) developed four different metabolic computer models to elucidate the

mechanisms of aerobic N_2O production in a nitritation reactor. The best fit of the measurement data was achieved with a model based on chemical HNO production. The other models, which represented three different metabolic pathways for the enzymatic reduction of nitrite and NO to N_2O, could not reproduce the measurement data satisfactorily. Indeed, we think that the positive site preference (SP) of N_2O produced during NH_2OH oxidation can be explained by a kinetic isotope effect acting during the chemical cleavage of a symmetric intermediate such as $H_2N_2O_2$ formed by dimerization of two HNO molecules (Eq. 1; Toyoda et al., 2005). In addition, the studies of Law et al. (2012) and of Udert et al. (2005) exemplify that computer models are powerful tools to elucidate the mechanisms of N_2O and NO production, especially when the processes contain microbial as well as chemical reaction steps.

Relevant environments for chemical reactions. In the last years, nitrogen treatment of high-strength wastewaters such as digester supernatant, manure and urine have received considerable attention. Based on our literature review, these systems are particularly prone to chemical production of NO and N_2O because of high NH_3 oxidation rates and high concentrations of the intermediate NH_2OH. Furthermore, some treatment schemes include NO_2^- accumulation as a process step, for example SHARON®. Ubiquitous iron compounds, e.g. from phosphate precipitation or as sensors and reactor walls, are another factor that can support the production of NO and N_2O. At the current stage of knowledge, it is hard to estimate the contribution of chemical processes to the overall NO and N_2O production. Many chemical processes have been described, but with the exception of HNO_2 disproportionation and the reaction of HNO_2 with NH_2OH, the kinetic data are insufficient for a reliable prediction of the production rates. Chemical production of NO and N_2O can also occur in natural environments, where high ammonia inputs meet low pH values such as strongly fertilized soils (van Cleemput and Samater, 1996) or poorly buffered lakes (Schuurkes and Mosello, 1988). Furthermore, chemical oxidation of NO and N_2O is an important process in the atmosphere (Lammel and Cape, 1996).

NO and N_2O formation in natural environments

Nitric oxide. NO production and consumption has been studied in soils. The studies used inhibition of nitrification with low concentrations of acetylene (~10 Pa) to distinguish between NO turnover by nitrification and denitrification, assuming that acetylene does not inhibit N_2O reductase at these concentrations. O_2 availability, as regulated by soil moisture content, is the main factor controlling the mechanisms of NO release (Bollmann and Conrad, 1998). While denitrification is the only process that releases NO under anoxic conditions, nitrification dominates NO release under oxic conditions with highest rates at low O_2 concentrations. In addition, soil pH, NH_4^+, NO_3^-, NO_2^- and respiration are important soil variables that affect NO turnover (Gödde and Conrad, 2000).

Measurements of NO in seawater are rare, because concentrations are low and turnover is fast due to its reactivity. However, Zafiriou et al. (1980) found that surface water of the central equatorial Pacific is a NO source to the atmosphere. Here, NO is

formed by photolysis of NO_2^- during daytime and reaches concentrations in the picomolar range (Zafiriou and True, 1979). Moreover, NO is formed by microbial processes in the O_2 minimum zone of the eastern tropical North Pacific (Ward and Zafiriou, 1988). Here, maximum NO turnover and concentration coincide with low O_2 concentrations (10 – 100 µmol L-1) and some nitrification activity overlying the O_2 minimum zone. In contrast, NO turnover and concentrations are low in the core of the O_2 minimum zone. The exact source of NO remained unidentified, but it was hypothesized that nitrifiers produce NO under reduced O_2 concentrations and that denitrifiers establish rather low NO concentrations in the core of the O_2 minimum zone. NO formation has been measured in marine sediments (Schreiber et al., 2008) and a more detailed study of NO turnover has been performed in freshwater sediments (Schreiber in preparation). Both studies will be discussed in the section focusing on microelectrodes.

Nitrous oxide. Generally, N_2O formation has been investigated to greater detail and in a wider variety of habitats as compared to NO, because its environmental impact is considered to be stronger than that of NO and its turnover is easier to measure due to its chemical stability. At present anthropogenic N_2O emissions account for ~40 % of the global N_2O emissions (Montzka et al., 2011). Current estimates state that ~50 % of the anthropogenic N_2O is emitted from soils (Stein and Yung, 2003), 10 % from estuaries and freshwater habitats (Beaulieu et al., 2011) and 3.2 % are emitted from wastewater treatment plants (WWTP) (Kampschreur et al., 2009). We caution that future adjustments to these estimates are likely, and that these averages do not capture the high variability in emissions from selected environments. Recent work has suggested that emissions from WWTPs in particular are highly variable and may in some cases be up to an order of magnitude greater than previous estimates (Lotito et al, 2012; Ahn et al., 2010). Soils and aquatic habitats exposed to intense agricultural activities are the largest sources due to high N-input through fertilization. Since mixed microbial communities in soils are the largest anthropogenic source for N_2O, its formation has been intensively studied and was recently reviewed (Baggs, 2011). N_2O formation in WWTP has been reviewed by Kampschreur et al. (2009).

The ocean is an important source of N_2O accounting for ~30 % of the natural N_2O emission (Stein and Yung, 2003). Large areas of the ocean are thought to be in equilibrium with the atmosphere, but regions of O_2 depletion are significant sources of N_2O (Elkins et al., 1978). In O_2 minimum zones, N_2O is generally produced to concentrations in the nanomolar range as O_2 reaches low concentrations (Yoshida et al., 1989; Naqvi et al., 2000; Farias et al., 2007; Nicholls et al., 2007). High N_2O accumulation was observed in surface water of the Arabian Sea and explained with frequent, turbulence-induced aeration of suboxic surface water (Naqvi et al., 2000). Likewise, O_2 fluctuations, induced by the El Nino-Southern oscillation, have been proposed to affect N_2O emission from the O_2 minimum zone of the eastern South Pacific (Farias et al., 2007). Furthermore, marine and freshwater sediments emit N_2O (Meyer et al., 2008; Nielsen et al., 2009). NO and N_2O formation in sediments will be discussed in more detail in the section focusing on microelectrodes.

The occurrence of animals such as earthworms (Horn et al., 2003) in soils and macrofauna in fresh -or seawater habitats (Stief et al., 2009; Heisterkamp et al., 2010) enhances the emission of N_2O in response to anthropogenic N-input. These animals ingest denitrifying bacteria and stimulate their activity probably with delayed expression of N_2O reduction leading to enhanced N_2O emissions.

Experimental approaches

In most investigated habitats NO and N_2O formation has been attributed to the NH_2OH pathway by AOB, nitrifier denitrification and heterotrophic denitrification. There are three approaches to determine the contribution of the different pathways:

(1) Indirect inference of pathways by excluding the activity of all other possible pathways, which can be achieved by using inhibitors or by removing the substrate (Kampschreur et al., 2008b; Stief et al., 2009; Schreiber et al., 2009; Wunderlin et al., 2012)

(2) Measuring the isotopic signature of N_2O (^{15}N natural abundance or site preference) and comparing the data to values of pure cultures (Yoshida, 1988; Yoshida et al., 1989; Sutka et al., 2006; Well et al., 2006; Charpentier et al., 2007; Wunderlin et al., under review).

(3) Application of ^{15}N isotopically-enriched substrates and mass spectrometric measurements of N_2O (Bateman and Baggs, 2005; Baggs, 2008).

In complex systems all of these approaches suffer from the coupled nature of nitrification and denitrification. This especially applies to studies where bulk measurements have been done even though micro-environmental heterogeneities are expected; e.g. in aggregates in wastewater treatment systems or in soil particles. In addition, it has become clear that NO and N_2O are dynamically produced in response to changing environmental conditions (Kampschreur et al., 2008b; Schreiber et al., 2009). Transient NO and N_2O concentrations can be orders of magnitude higher than under steady state. Conventional mass spectrometric measurements do not allow measurements with high temporal and spatial resolution, making approach 2 and 3 inaccessible to microscale and dynamic analysis of NO and N_2O.

Table 1. *Transient formation of NO and N_2O in different habitats.*

Habitat	Pertubation	NO [µM]				N_2O [µM]				Possible pathway	Reference
		baseline	peak	build-up[a]	recovery[b]	baseline	peak	build-up[a]	recovery[b]		
tropical soil (slurries)	oxic-anoxic					0	200-400	13-20 h	6-10 h	Denitrification	Liengaard et al. 2011
agricultural soil (cores)	oxic-anoxic by liquid-manure injection					< 1	200	27 h	48 h	Denitrification	Markfoged et al. 2011
agricultural soil (aggregates)	oxic-anoxic by tryptone addition					< 1	400	19.5 h	n.d.	Denitrification	Hojberg et al. 1994
permafrost soil (cores)	oxic-anoxic by thawing					< 1	2.5	36 h	n.d.	Denitrification	Elberling et al. 2010
nitrifying and denitrifying biofilm	oxic-anoxic	< 0.03	1.1	5-7 min	15 min	0.5	5	5 min	15 min	AOB	Schreiber et al. 2009
	oxic-anoxic	< 0.03	0.3	30 min	n.d.	< 0.1	3	30 min	n.d.	Denitrification	
	NO_2^- addition	< 0.03	1.3	0.5 min	20 min					AOB	
	NO_2^- addition	0.05	0.4	1 min	n.d.					Denitrification	
full scale nitritation reactor	influent shut-down	15 ppm[c]	80 ppm[c]	~10 min	1	10	110	4.5 h	n.d.	AOB/Denitrification and reduced gas stripping	Kampschreur et al. 2008a
complex nitrifying culture	oxic-anoxic	0.3 ppm[c]	2.5 ppm[c]	~ 8 min	n.d.	2	11	10 min	n.d.	AOB	Kampschreur et al. 2008b
	NO_2^- addition	0.2 ppm[c]	0.45 ppm[c]	15 min	45 min	2.4	3.4	15 min	30 min	AOB	
membrane-aerated biofilm	oxic-anoxic					< 1	70	25 min	60 min	AOB	Pellicer-Nàcher et al., 2010
	anoxic-oxic					20	45	20 min	25 min	Denitrification	
freshwater sediment	salinity increase					0	4	9 h	22 h	Denitrification	Nielsen et al., 2009
marine sediment	salinity decrease NO_3^- increase					0	2.5	2	7 h	Denitrification	
Arabian sea water	oxic-anoxic					0.05	1.5	72 h	48 h	Denitrification/AOB	Naqvi et al., 2000

[a]time to reach peak concentrations; [b]time to recover to a new steady-state concentration (not necessarily to baseline concentration); cconcentration in ppm instead of µM because it was measured in the gas phase

Novel analytical methods

In the following sections, we will discuss different analytical methods (microelectrodes, mass spectrometry and quantum cascade laser based absorption spectroscopy) that can be used to allocate NO and N_2O production to certain pathways by using one of the three approaches outlined above. Combining these methods and thus the different approaches will lead to a more firm pathway allocation. Microelectrodes can measure with high temporal and spatial resolution and in combination with other microelectrodes (NH_4^+, NO_3^-, NO_2^-, O_2) approach 1 can be used to allocate source pathways. Further, quantum cascade laser based absorption spectroscopy can measure the site preference in N_2O dynamically and can be used to allocate N_2O production pathways with approach 2. In addition, we will discuss the potential for other techniques that measure the isotopic composition of N_2O and molecular methods to aid the understanding of NO and N_2O formation in complex environments.

Microelectrodes to capture micro-environmental distribution and temporal dynamics of NO and N_2O

NO and N_2O microelectrodes

Microelectrodes belong to the tool box of microbial ecologists since Revsbech et al. introduced an O_2 microelectrode in the early 1980's (Revsbech et al., 1980). The first N_2O microelectrode for microbial ecology (Revsbech et al., 1988) was a combined O_2/N_2O sensor where an O_2-reducing gold cathode was placed in front of an N_2O-reducing silver cathode (both polarized at -800 mV) to avoid the interference of O_2 with N_2O detection. These sensors where difficult to manufacture and had a short life-time. Thus, Andersen et al. (2001) introduced an improved O_2-insensitive N_2O microelectrode. Insensitivity to O_2 is achieved by placing a reservoir filled with alkaline ascorbate solution for the chemical reduction of O_2 in front of the N_2O-reducing cathode, which is separated from the ascorbate reservoir with a gas permeable silicone membrane. These N_2O microelectrodes have a sensitivity of ~0.5 µmol L^{-1} and a spatial resolution of ~60 µm.

Electrochemical NO sensors for the detection of NO in biological systems are available since the early 1990's (Shibuki, 1990). Amperometric sensing of NO is commonly achieved by the oxidation of NO at a working electrode polarized with 0.7 - 0.9 V vs. a reference electrode (Ag/AgCl or Calomel) leading to the following anodic reaction:

$$NO + 2\,H_2O + 3\,e^- \rightarrow NO_3^- + 4\,H^+ \qquad \text{Equation 15}$$

The resulting current is proportional to the NO concentration and can be detected as the analytical signal. Electrodes are reported as single anode-type electrodes or as combined sensors (Figure 2). In combined sensors, the reference electrode and the sensing electrode are placed together in an internal electrolyte compartment that is separated from the sample by a gas permeable, non-conductive membrane (Clark-type,

Figure 2B), whereas single anode-type electrodes use the aqueous sample as an electrolyte and complete the measuring circuit by submerging an external reference electrode into it (Figure 2A). Charged interferences like NO_2^- and ascorbate are typically repelled by constructing combined sensors with hydrophobic membranes like chloroprene (Shibuki, 1990), PTFE (Teflon™) (Lee et al., 2004), sol-gels (Shin et al., 2005), polystyrene (Kitamura et al., 2000) or silicone (Schreiber et al., 2008), or by depositing conductive Nafion™ on single anode-type electrodes (Malinski and Taha, 1992; Friedemann et al., 1996; Bedioui and Villeneuve, 2003).

Most of the previously described NO electrodes have been optimized to detect NO at low nanomolar or even picomolar concentration. This has been achieved by increasing the sensing surface with a subsequent loss of spatial resolution. Single-anode type sensors commonly rely on carbon-fibers that have a length of up to several millimeters and combined sensors have openings in the high micrometer to millimeter range. Microelectrodes with long, exposed sensing surfaces are not applicable for profiling in stratified microbial systems because the concentration of the analyte might change along the sensing surface. The obtained signal is then an integrated measure of the concentrations along the electrode. Similarly, combined electrodes with wide openings are also problematic for profiling applications, since the step size of different measurement points in a depth profile should not be smaller than 2 times the outer diameter of the electrode (Gieseke and de Beer, 2004). In addition, single-anode sensors are not robust enough to be inserted in a sturdy sediment or soil sample since the particles will damage the Nafion membrane that confers selectivity against NO_2^-. Consequently, applications of NO electrodes – commercially supplied by World Precision Instruments (Sarasota, Florida, USA) – in microbiology were restricted to detection of NO in pure culture suspensions (e.g. Corker and Poole, 2003).

Recently, an NO microelectrode was introduced that is applicable to study complex, stratified microbial communities in sediments and biofilms (Schreiber et al., 2008). The NO microelectrode is a combined (Clark-type) sensor with a carbon-fiber anode (+ 750 mV) placed behind a gas permeable silicon membrane (Figure 2B). The sensor has a detection limit of 0.030 µmol L^{-1} and a spatial resolution of ~60 µm. Thus, the sensor is optimized to provide sufficient sensitivity for NO concentrations produced in complex, N-cycling microbial communities and sufficient spatial resolution to measure in microbial biofilms, sediments and soils. The robust Clark-type design allows measurements in sturdy soil and sediment samples. It has been made commercially available through Unisense A/S (Arhus, Denmark), who also supplies N_2O microelectrodes.

Interferences. H_2S interferes with NO measurement as it passes the silicone membrane and is readily oxidized at the sensing anode. A sensitive H_2S microsensor (Jeroschewski et al., 1996) should thus be used to rule out any interference of H_2S in the measurements or –if possible- experiments must be designed to avoid active sulfate reduction in the sample by excluding sulfate from the medium. Jenni et al. (2012) investigated the interferences of CO_2 , O_2 and various nitrogen compounds commonly found in wastewater treatment on NO and N_2O sensors. They found that NO interfered

with the N_2O measurement, while the NO sensors were sensitive on NH_3, NH_2OH, HNO_2 and N_2H_4. If high concentrations of these compounds are expected, it is recommended to check the concentrations of interfering compounds. No significant interferences were found by CO_2 and O_2. The cross-sensitivities can be corrected with calibration curves that are determined before the experiments. Jenni et al. (2012) also reported a significant temperature dependency. The NO signal increased by about 3.5 % per 1 °C and the N_2O signal by 3.9 % per 1 °C. The temperature dependencies can be corrected with exponential functions.

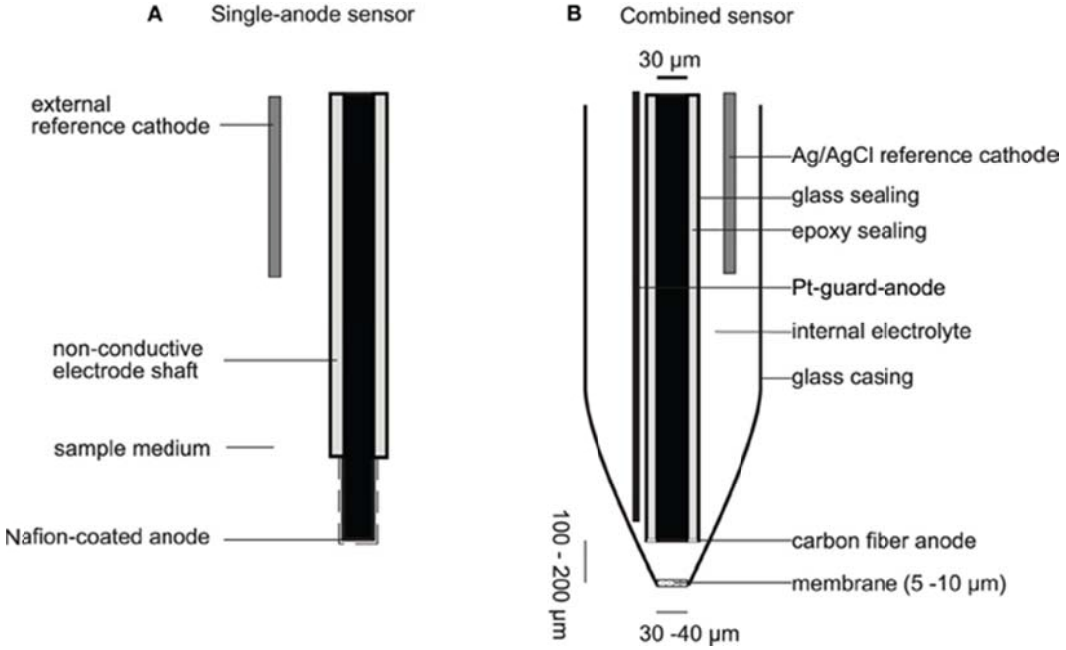


Figure 2. *NO microelectrodes. (A) depicts a typical single-anode type NO sensor with a long sensing anode, which is coated with Nafion to confer selectivity against charged interferences. The anode and reference cathode are directly emerged into the sample medium. Some sensor designs integrate the cathode into the electrode shaft. (B) depicts the NO microelectrode for measurements in biofilms and sediments as reported by Schreiber et al. (2008). This sensor is also an example for a combined NO sensor (Clark-type) where sensing anode and reference cathode are separated from the sample medium by a gas permeable membrane. Drawing is not to scale.*

Application of NO microelectrodes

The novel NO microelectrode has been applied to study NO formation in permeable marine (Schreiber et al., 2008) and river (Schreiber et al., in preparation) sediments. The results showed that in steady-state NO is produced in oxic/micro-oxic sediment strata reaching concentrations of 0.13 µmol L^{-1} in river and 0.5 µmol L^{-1} in marine sediments. In both sediments NO produced in the oxic zone was consumed in the anoxic zone. It was hypothesized that NO was produced by AOB in the oxic zone. Labeling experiments with a ^{15}N-labeled NO donor in the river sediment suggested that denitrification actively consumes exogenously produced NO.

Furthermore, the NO microelectrodes have been applied together with N_2O microelectrodes in two N-cycling microbial biofilms; namely a complex NH_4^+-fed biofilm with nitrifying and denitrifying activity (Schreiber et al., 2009) and human dental plaque that was naturally exposed to high NO_3^- and NO_2^- in saliva (Schreiber et al., 2010). The study in dental plaque showed that plaque denitrified under aerobic conditions, that NO and N_2O was produced by denitrification and that NO and N_2O concentrations increased with decreasing pH. Aerobic denitrification has also been reported from permeable marine sediments (Gao et al., 2010) and from isolated (Patureau et al., 2000) or extracted soil bacteria (Morley et al., 2008). Until now, it is not known in which environments aerobic denitrification plays an important role and if it is an environmentally significant NO and N_2O emission pathway. NO, N_2O, NO_2^-, NO_3^- and O_2 microelectrodes will be crucial to determine the importance of aerobic denitrification for NO and N_2O release for complex ecosystems, because these sensors allow the simultaneous detection of NO, N_2O, NO_2^-, NO_3^-, O_2 concentrations at high spatial resolution and their relation to denitrification activity.

Studying a complex N-cycling biofilm revealed the dynamics of NO and N_2O formation upon perturbations in a system where nitrification and denitrification co-exist (Schreiber et al., 2009). The concomitant use of an O_2 microelectrode and a set of control experiments enabled assignment of NO and N_2O formation under oxic conditions to AOB and under anoxic conditions to denitrifiers. It also showed that AOB produce NO and N_2O under fully oxic conditions if NO_2^- concentrations are high. This is in agreement with other observations (Beaumont et al., 2004a, 2004b; Shaw et al., 2006) and contradicts the assumption that AOB require low O_2 to release NO and N_2O (Lipschultz et al., 1981; Poth and Focht, 1985; Kester et al., 1997; Beaumont et al., 2004a; Kampschreur et al., 2008b). The high temporal resolution of the microelectrodes allow to detect transient bursts (seconds to minutes) of NO and N_2O. The bursts occurred by AOB upon O_2 removal and upon NO_2^- addition by both AOB and denitrifiers. The bursts only occurred if the perturbations were exerted upon metabolically active AOB and denitrifiers. In both scenarios NO and N_2O are formed in parallel confirming that NO is the preceding intermediate of N_2O in the N_2O production pathways in this biofilm. An important contribution by Yu et al. (2010) showed that an AOB pure culture accumulated only NO, not N_2O, upon transition from oxic to anoxic conditions. In mixed microbial communities were AOB and heterotrophic denitrifiers co-exist this could lead to NO release by AOB and immediate reduction to N_2O by heterotrophic denitrifiers or anaerobic detoxification via NorVW and Hmp. This mixed source of N_2O during transient oxic to anoxic conditions has to be taken into account when determining the pathways with isotopic techniques. It has been argued that N_2O transiently accumulates during transition from anoxic to oxic conditions because O_2 inhibits Nos while denitrification still proceeds, but direct evidence for this hypothesis is weak. Using both NO and N_2O microelectrodes would allow to test this because N_2O accumulation should not be accompanied by NO accumulation if the denitrification sequence is inhibited at the level of Nos.

Application of N_2O microelectrodes

In many habitats steady-state N_2O concentrations are below or at the detection limit of the N_2O microelectrode. Thus, the N_2O microelectrode has commonly been used to estimate the denitrification potentials in stratified microbial communities such as sediments, biofilms and aggregates in combination with the acetylene inhibition technique (Revsbech et al., 1988). Acetylene (~10 kPa) inhibits N_2O reductase and leads to the accumulation of high amounts of N_2O.

More recently, N_2O microelectrodes have been used to study N_2O production without acetylene inhibition in natural samples. These studies revealed that N_2O concentrations in the micromolar range are expected when the system is exposed to a perturbation (Table 1). Transient accumulation of high N_2O concentrations were achieved by any perturbation that affects the ambient O_2 concentration: flooding of soils with water (Liengaard et al., 2011; Markfoged et al., 2011), creating an organic hotspot around a soil aggregate (Hojberg et al., 1994), thawing of permafrost soils (Elberling et al., 2010), and decreasing the O_2 supply to wastewater-grown biofilms (Kampschreur et al., 2008a, 2008b; Schreiber et al., 2009; Pellicer-Nàcher et al., 2010). In addition, increased input of NO_3^-, NO_2^- or NH_4^+ to sediments, soils and biofilms (Hojberg et al., 1994; Meyer et al., 2008; Schreiber et al., 2009; Nielsen et al., 2009), organic inputs, salinity fluctuations in sediments (Nielsen et al., 2009) and changes of pH due to microbial activity in a denitrifying, dental biofilm (Schreiber et al., 2010) lead to increased micro-environmental N_2O levels. Importantly, in many of these studies N_2O accumulated in a transient manner making time-course measurements necessary to capture the N_2O peak and the accumulation time span. The high spatial resolution of the N_2O microelectrode allowed allocating processes that mitigate the emission of N_2O to the atmosphere in soils, sediments and wastewater treatment biofilms. N_2O that is produced by denitrification in deeper layers is consumed during its diffusion toward the sediment-water interface in nutrient-enriched mangrove sediments (Meyer et al., 2008), toward the soil-atmosphere interface in a thawed permafrost soil (Elberling et al., 2010) or in a soil aggregate exposed to an organic hotspot (Hojberg et al., 1994). Likewise, N_2O release from a membrane-aerated biofilm reactor was minimized by N_2O-reducing microbes placed above AOB that produced N_2O due to perturbations induced by an intermittent aeration regime (Pellicer-Nàcher et al., 2010).

Outlook

From the investigations of transient NO and N_2O accumulation it emerges that two scenarios with distinct dynamics are important. First, N_2O accumulates over hours to days, because it mirrors the onset of denitrification activity. Depending on the system it decreases because N_2O reduction pathways are turned on with a delay or denitrification activity decreases due to substrate limitation. Ahn et al. (2011) even observed that peak NO and N_2O emissions after a shift to O_2-limitation in a nitrifying reactor were lasting for several month before adaptation on the metabolic or community level decreased the emissions. Second, perturbation of active AOB or denitrifiers leads to burst-like (within seconds to minutes) release of NO and N_2O. The

exact biochemical mechanisms for this require further research directly on the involved enzymes. Moreover, future research must show the contributions of the two types of transitions to the N_2O budget and could use this as a framework to mitigate peak N_2O releases to the atmosphere. Mitigation strategies could aid at avoiding perturbations or confining the N_2O-releasing processes into a diffusion-limited environment that is overlaid with N_2O-consuming microbial communities.

N_2O source partitioning based on the nitrogen and oxygen isotopic signature

In recent years, the isotopic signature of N_2O has been used as a powerful tool to assign N_2O production pathways to nitrification and heterotrophic denitrification in different ecosystems such as soils, rivers, sea, wastewater treatment (Yoshida et al., 1989; Yamagishi et al., 2007; Baggs, 2008; Koba et al., 2009; Baulch et al., 2011; Park et al., 2011; Toyoda et al., 2011). N_2O is a linear molecule (N^{β}-N^{α}-O) with one nitrogen atom at the center position (N^{α}) bound to oxygen, and one at the end position (N^{β}) bound to N^{α}. The three most abundant N_2O isotopic species in the atmosphere are $^{14}N^{15}N^{16}O$ (0.37 %), $^{15}N^{14}N^{16}O$ (0.37 %) and $^{14}N^{14}N^{16}O$ (> 99 %). Isotope abundances are usually reported in the δ-notation (in per-mil; ‰), $\delta\,^{15}N = [(R_{sample}/R_{reference}) - 1] \times 1000$, where R is the ratio of $^{15}N/^{14}N$ of a sample (R_{sample}) with respect to atmospheric N_2 as the reference ($R_{reference}$) (Mariotti et al., 1981).

The intramolecular distribution of the nitrogen isotopes ($^{14}N^{15}NO$ versus $^{15}N^{14}NO$) is termed site preference (SP) and is expressed as the relative difference in $\delta\,^{15}N$ between the α and the β position ($SP = \delta\,^{15}N^{\alpha} - \delta\,^{15}N^{\beta}$) (Toyoda and Yoshida, 1999). In analogy to the δ-notation, the isotopomer analysis denotes the relative difference of the $^{15}N/^{14}N$ isotope ratio for a given position ($\delta\,^{15}N^{\alpha}$, $\delta\,^{15}N^{\beta}$) with respect to the standard (e.g. $\delta\,^{15}N^{\alpha} = [(^{15}R^{\alpha}/^{15}R^{\alpha}{}_{reference}) - 1] \times 1000$, whereas $^{15}R^{\alpha} = [^{14}N^{15}N^{16}O]/[^{14}N^{14}N^{16}O]$ and $^{15}R^{\alpha}{}_{reference}$ is the isotope ratio of the standard material (N_2O) (see below)) (Toyoda and Yoshida, 1999). The SP has the advantage of being independent of the isotopic signature of the respective substrates (e.g. NH_4^+ or NO_3^-) and of being specific for pathways (enzymes) involved in N_2O formation (Toyoda et al., 2005; Sutka et al., 2006).

Microbial (enzymatic) processes usually lead to an isotopic fractionation due to different transformation rates of ^{14}N and ^{15}N, resulting in isotopically lighter end-products than molecules in prior steps (Stein and Yung, 2003). Thus, the average $^{15}N/^{14}N$ ratio of N_2O, termed as $\delta\,^{15}N^{bulk}{}_{N2O}$, can be used to distinguish different production pathways in complex samples if the isotopic signature of the pure bacterial culture is known. However, the meaning of $\delta\,^{15}N^{bulk}{}_{N2O}$ can be limited since it is strongly dependent on the isotopic signature of the substrate, which usually is unknown, as well as on the physiological activity (Mariotti et al., 1981). Additionally, the isotopic composition of an intermediate (e.g. N_2O during heterotrophic denitrification) is

affected by production (NO_3^- reduction) as well as consumption (N_2O reduction) processes.

In addition to nitrogen isotopes, oxygen isotope ratios are also increasingly used in order to better distinguish between the N_2O formation pathways (Yoshinari and Wahlen, 1985; Kool et al., 2007; Baggs, 2008; Frame and Casciotti, 2010). In this case $\delta^{18}O$ denotes the relative difference in the $^{18}O/^{16}O$ ratio of N_2O (R_{sample}) with respect to the reference ($R_{reference}$), in per-mil (‰), usually being the Vienna Standard Mean Ocean Water (VSMOW) ($\delta^{18}O = [(R_{sample}/R_{reference}) - 1] \times 1000$) (Wahlen and Yoshinari, 1985).

Table 2. *Advantages and disadvantages of isotope-ratio mass spectrometry (IRMS), quantum cascade laser based absorption spectroscopy (QCLAS) and membrane-inlet mass spectrometry (MIMS).*

	Advantages	**Disadvantages**
IRMS	• Well known, widely applied method	• Lab-based method
	• Measurement of $\delta^{15}N^{\alpha}$, $\delta^{15}N^{\beta}$ and $\delta^{18}O$	• Low temporal resolution (flask-sampling)
		• Requirement of standard gases (not commercially available)
QCLAS	• Portable, enabling field measurement campaigns	• Requirement of standard gases (not commercially available)
	• Continuous measurement (high temporal resolution) of $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$	
MIMS	• High sample throughput	• Application limited to isotope labeling / tracer experiments
	• Low sample volume required	
	• Long-term measurement possible	
	• online measurements with high temporal resolution possible	

Analysis of the isotopic signature of N_2O

There are basically two different analytical techniques available to analyze N_2O nitrogen isotopic signatures at natural abundance levels (Table 2): (i) the isotope-ratio mass spectrometry based technique (IRMS) (Brenninkmeijer and Röckmann, 1999; Toyoda and Yoshida, 1999), and (ii) the recently developed quantum cascade laser based absorption spectrometry (QCLAS) (Waechter et al., 2008).

IRMS-based method is widely applied with an excellent precision and accuracy (Mohn et al., 2010). Nevertheless, the calibration procedure of the intramolecular nitrogen isotope distribution in N_2O is still under debate. Originally, two alternative approaches have been proposed, one by Toyoda and Yoshida (1999) and one by Brenninkmeijer and Röckmann (1999), which resulted in a difference in SP of about 30 ‰ for tropospheric

N_2O. The analysis of the SP by IRMS techniques relies on the N_2O^+ and NO^+ fragment ions at the mass-to-charge ratio (m/z) 44, 45, 46 (for N_2O) and m/z 30, 31 (for NO). However, both calibration approaches do not take into account the isotope effects associated with the formation of NO^+ in the ion source of the mass spectrometer. Recently, Westley et al. (2007) investigated these discrepancies in more detail and found that these isotope effects have much smaller impact on the calibration procedure proposed by Toyoda and Yoshida (1999) (see below), and supported therefore this procedure as the most accurate basis for a community standard.

Furthermore, IRMS is a lab-based technique. Thus, the time resolution of N_2O isotopic analysis during field measurement campaigns is therefore limited (Waechter et al., 2008). Nevertheless, in addition to nitrogen isotopes, the oxygen isotopic signature can also be analyzed routinely by IRMS.

QCLAS is a novel approach for site-specific analysis of nitrogen isotopes, with the advantage of a high sensitivity, time resolution, and portability, the latter of which enables field measurement campaigns (Waechter et al., 2008). This was demonstrated by Mohn et al. (2012), who recently presented first data of a high precision real-time analysis of site-specific isotopic signatures of atmospheric N_2O above a grassland plot. The measurement campaign was run over three weeks with almost 550 analyzed gas samples. It was demonstrated that a continuous measurement of the N_2O isotopic signature allowed improved detection of the dynamics of N_2O production (before and after fertilizer application to the grassland plot), and thus opens a completely new field of applications. In another study, isotopic signature of N_2O, produced during batch-scale experiments with activated sludge, were analyzed in real time, which permitted to trace short-term fluctuations in SP and $\delta^{15}N^{bulk}_{N2O}$, allowing to identify N_2O production pathways in biological wastewater treatment (Wunderlin et al., under review).

The QCLAS is based on direct absorption laser spectroscopy in the mid-infrared range for simultaneous measurement of the most abundant N_2O isotopic species, such as $^{14}N^{15}N^{16}O$, $^{15}N^{14}N^{16}O$ and $^{14}N^{14}N^{16}O$ (Waechter et al., 2008; Mohn et al., 2010). In order to enable high precision analysis (e.g. a precision of < 0.1 ‰ for $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$) (Waechter et al., 2008) a combination with a pre-concentration unit is essential at ambient or sub-ambient mixing ratios (Mohn et al., 2010, 2012). For example, with the liquid nitrogen-free, fully-automated pre-concentration unit built by Mohn et al. (2010), N_2O can be concentrated by a factor of 200 (e.g. from ambient concentrations to around 60 ppm) from 10 L gas samples within 20 min.

Calibration. For both techniques, IRMS as well as QCLAS, an adequate calibration procedure needs to be applied, since instrumental nonlinearity and drifts impact the accuracy of the isotope ratio measurement (e.g. $\delta^{15}N^{bulk}_{N2O}$ values depend on the N_2O gas concentration) (Waechter et al., 2008). However, international standards are not commercially available so far. Therefore, they need to be prepared and analyzed from other laboratories (intercalibration) for $\delta^{15}N^{bulk}_{N2O}$, $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$, to ensure that measurements are performed on a common scale and that results are comparable

between laboratories (Westley et al., 2007). So far, the calibration procedure proposed by Toyoda and Yoshida (1999), as mentioned above, is accepted as the provisional basis for a community standard: N_2O is synthesized via thermal decomposition of isotopically characterized NH_4NO_3, since it is known that the nitrogen atom at the center (α) position of N_2O originates from NO_3^-, while the end (β) nitrogen comes from NH_4^+. Using this calibration procedure a SP of tropospheric N_2O of 18.7 ± 2.2 ‰ is measured (Westley et al., 2007).

Membrane-inlet mass spectrometry (MIMS) was proposed as another promising tool to study the dynamics of N_2O production in ^{15}N labeling experiments. MIMS has a high sample throughput (within minutes), allows direct analysis of liquid or gas samples and requires only low sample amounts (Bauer, 1995; Baggs, 2008) (Table 2). Recently, it was coupled with an automated sampling and calibration unit (ASCU), and was tested in a long-term ^{15}N-NO_3^- tracer experiment over 7 days. It was confirmed that ^{15}N measurements of N_2 and N_2O, detected as N_2 at m/z 28, 29 and 30 (N_2O was reduced to N_2 in an elemental copper furnace prior to analysis), are in good agreement with IRMS-based analysis (Eschenbach and Well, 2011).

The membrane-inlet part can also be combined with a quadrupole mass spectrometer for simultaneous online measurement of different m/z ratios (e.g. $^{15,15}N_2O$ at m/z = 46, $^{14,15}N_2O$ at m/z = 45, $^{15,15}N_2$ at m/z = 30, $^{14,15}N_2$ at m/z = 29) with a time resolution of 1 to 2 minutes (Ettwig et al., 2010; Gao et al., 2010). Nevertheless, the interpretation of spectra corresponding to a certain gas mixture might be difficult since one peak can correspond to different atomic compositions (e.g. $^{14,14}N_2^+$ and CO^+ at m/z = 28). This problem is reduced by applying ^{15}N labeled substrates, where the only important remaining correction needed is for m/z = 30, which consist of the signal from the $^{15,15}N_2^+$ fragment of $^{15,15}N_2O$, the $^{14}NO^+$ fragment of $^{14,14}N_2O$ and $^{15,15}N_2$) (Thomsen et al., 1994).

Isotopic signature of N_2O: site preference, $\delta^{15}N$ and $\delta^{18}O$

Site preference. The SP is a promising tool for N_2O source partitioning since it is specific to pathways involved and independent of the respective substrates (Sutka et al., 2006) (Table 3). For N_2O production via NH_2OH oxidation by typical AOB pure cultures values in the range of 30.8 ± 5.9 to 35.6 ± 1.4 ‰ were measured (Sutka et al., 2003, 2004, 2006), which is in agreement with recently reported SP values of marine AOA (30.8 ± 4.4 ‰) (Santoro et al., 2011). In contrast, Frame and Casciotti (2010) estimated 36.3 ± 2.4 ‰ for a marine AOB. For nitrifier denitrification by AOB, the following SP values were reported: 0.1 ± 1.7 ‰ (Sutka et al., 2006), -0.8 ± 5.8 ‰ (Sutka et al., 2003, 2004) and -10.7 ± 2.9 ‰ (Frame and Casciotti, 2010). For N_2O production via heterotrophic denitrification SP values in the range of -5.1 to 0 ‰ were reported (Toyoda et al., 2005; Sutka et al., 2006). Nitric oxide reductases (Nor) likely determine the SP of N_2O during nitrifier denitrification as well as heterotrophic denitrification. The SP for both pathways is in the same range indicating that the involved Nor's in AOB (cNor) and heterotrophic denitrifiers (cNor or qNor) (Stein and Yung, 2003; Stein, 2011) share a similar enzymatic mechanism. In case free NO is formed during NH_2OH oxidation, any NO molecule that is funneled into nitrifier or heterotrophic denitrification (either directly or via initial

oxidation to NO_2^-) would result in N_2O with an SP of ~0 ‰ masking its initial NH_2OH source.

The most probable explanation for a positive SP during NH_2OH oxidation is a preferable ^{14}N-^{16}O bond cleavage of a symmetric intermediate such as hyponitrite (-$^{16}O^{14}N^{15}N^{16}O$-), leading to an enrichment of ^{14}N-^{15}N-^{16}O (Toyoda et al., 2005; Schmidt et al., 2004a). In the current model of N_2O formation from NH_2OH oxidation, NH_2OH is reduced to NO, which is further reduced to N_2O by an unidentified Nor. However, the positive SP of N_2O formed from NH_2OH oxidation can only be explained (i) if the involved Nor has a different mechanism than Nor's mediating nitrifier and heterotrophic denitrification or (ii) if N_2O is formed by a different mechanism, which does not involve free NO. We suggest mechanisms involving HNO: either by formation of free $H_2N_2O_2$ with further chemical decomposition to N_2O (discussed in section 'HNO as intermediate of enzymatic NH_2OH oxidation') or a site specific enzymatic cleavage of -ONNO- as discussed above (Toyoda et al., 2005; Schmidt et al., 2004a). Further insights in the enzymatic mechanism of HAO and potentially HAO-associated Nor with careful chemical control experiments are needed to elucidate the biochemical mechanism of N_2O formation during NH_2OH oxidation.

Furthermore, a positive SP is, in addition to NH_2OH oxidation, also an indicator for increasing importance of the heterotrophic N_2O reductase activity relative to N_2O production (substantially greater activity than 10 % compared to production) (Yamagishi et al., 2007; Jinuntuya-Nortman et al., 2008; Koba et al., 2009). As a consequence, N_2O reduction to N_2 might lead to an overestimation of N_2O production by NH_2OH oxidation, or vice versa. Nevertheless, further investigations are necessary in order to determine the individual signatures under conditions more representative for ecosystems with mixed culture populations (Wunderlin et al., under review).

Under nitrifying conditions, N_2O can theoretically be produced simultaneously via NH_2OH oxidation as well as nitrifier denitrification. Thus, based on SP literature data, the individual contribution (F_{NN}: NH_2OH oxidation; F_{ND}: nitrifier denitrification) can be calculated from the following isotopomer mixing model:

$$F_{ND} = (1 - F_{NN}) = \frac{(SP_{tot} - SP_{NN})}{(SP_{ND} - SP_{NN})} \qquad \text{Equation 16}$$

where SP_{ND} and SP_{NN} are the end-member SP signatures of the NH_2OH oxidation and nitrifier denitrification pathway, respectively, as reviewed above, and SP_{tot} the measured signature of the individual produced N_2O (Frame and Casciotti, 2010).

$\delta^{15}N$. Wide ranges for $\delta^{15}N^{bulk}_{N2O}$ were reported so far, mainly due to limited information about the isotopic signature of the substrates or to both a huge complexity determined by multiple transformation processes involving different enzymes, as well as variable reaction rates or mechanisms affecting isotopic fractionation (Perez et al., 2006) (Table 3). For example, it was shown that isotopic fractionation during NH_3 oxidation is variable, depending mainly on the amino acid sequences for the α-subunit of AMO of the different investigated pure culture AOB

(Casciotti et al., 2003). However, N_2O produced by AOB during nitrifier denitrification or NH_2OH oxidation is basically more strongly depleted in ^{15}N ($\Delta\ \delta\ ^{15}N = \delta\ ^{15}N_{substrate} - \delta\ ^{15}N^{bulk}_{N2O}$; in the range of between 40 to 68 ‰) compared to heterotrophic denitrification, where N_2O is an obligate intermediate and the fractionation therefore depends on both production and consumption processes ($\Delta\ \delta\ ^{15}N$ of 0 to 39 ‰) (Yoshida, 1988; Yoshida et al., 1989; Stein and Yung, 2003; Perez et al., 2006; Koba et al., 2009; Park et al., 2011).

$\delta\ ^{18}O$. The oxygen isotopic signature of N_2O ($\delta\ ^{18}O$) is also used as a tool for N_2O source partitioning, even though this approach faces a couple of difficulties: for example, N_2O production via NH_2OH oxidation as well heterotrophic N_2O reduction result in a positive correlation between the $\delta\ ^{18}O$ in N_2O and SP (Frame and Casciotti, 2010) (Table 3). Furthermore, $\delta\ ^{18}O$ enrichment factors are scarce and highly variable (Park et al., 2011), and are reported to be strongly influenced by oxygen exchange or incorporation, such as (i) oxygen incorporation (from dissolved O_2) into NH_2OH during the oxidation of NH_4^+ to NH_2OH, (ii) oxygen incorporation (from H_2O) into NO_2^- during the oxidation of NH_2OH to NO_2^-, and (iii) oxygen exchange between NO_2^-/NO_3^- and H_2O (Kool et al., 2007). For example, it was shown that 64 to 94 % of the oxygen atoms in the precursors of N_2O were exchanged with oxygen atoms in H_2O (Snider et al., 2009; Park et al., 2011), which underscores the fact that the understanding and quantification of the effect of oxygen exchange between H_2O and dissolved nitrogen species is and will remain challenging. Isotopic labeling is a promising approach to overcome such difficulties (see below), but up to now the natural abundance oxygen isotopic signature should be used with caution in N_2O source partitioning studies (Kool et al., 2007, 2010).

N and O labeling. Beside natural abundances, nitrogen and oxygen isotope labeling techniques have been applied to study and quantify N_2O production pathways (Table 3). For example, Poth and Focht (1985) investigated the relative importance of the NH_2OH oxidation and nitrifier denitrification pathway in *Nitrosomonas europaea* pure culture by applying $^{14}N\text{-}NH_4^+$ in combination with $^{15}N\text{-}NO_2^-$. Based on the large amounts of double-labeled $^{15,15}N_2O$ (m/z = 46), it was concluded that nitrifier denitrification is the dominant pathway. Baggs and Blum (2004) determined the relative contribution of nitrification and denitrification to $^{15}N\text{-}N_2O$ production by the application of $^{14}NH_4{}^{15}NO_3$ and $^{15}NH_4{}^{15}NO_3$. However, such conventional ^{15}N labeling techniques do not allow to distinguish between NH_2OH oxidation and nitrifier denitrification in mixed population systems (Kool et al., 2010). As a consequence, a dual isotope approach was applied, based on ^{18}O-labeling of H_2O as well as ^{15}N-labeling of NH_4^+ or NO_3^- (Wrage et al., 2005). The basic concept behind is, that AOB use oxygen from O_2 for the oxidation of NH_4^+ to NH_2OH, but oxygen from H_2O for the oxidation of NH_2OH to NO_2^- (see above). As such, the ^{18}O signature of N_2O produced via nitrifier denitrification reflect to 50 % the signature of O_2 and to the other 50 % the signature of H_2O, which is in this study artificially enriched in ^{18}O (Kool et al., 2007), under the assumption that no further oxygen is exchanged between NO_2^- and H_2O. In contrast, the ^{18}O signature of N_2O derived from NH_2OH oxidation reflects to 100 % the signature of O_2 (Wrage et al., 2005;

Kool et al., 2010). Nevertheless, the effect of oxygen exchange has to be taken into account.

Table 3. *Advantages and disadvantages of SP, $\delta^{15}N^{bulk}$ and $\delta^{18}O$, on a natural abundance or labeled level (adapted from Baggs (2008)).*

	Advantages	Disadvantages
Site preference (SP)	• Independent of isotopic signature of substrates	• Unknown pathways might affect SP
	• Noninvasive method	• SP from pure culture bacteria have to be known
	• Specific for pathways involved	
$\delta^{15}N^{bulk}$	• Characteristic fractionation of different pathways (depending on the rate limiting step)	• Depending on the isotopic signature of the substrate, as well as the physiological activity
	• Noninvasive method	• Multiple reaction steps (branching effects) cause uncertainty
$\delta^{18}O$	• Noninvasive method	• Oxygen exchange between N species and O_2 or H_2O difficult to quantify
	• Additional information to nitrogen isotopic signature	
Isotope labeling of N and O	• Isotopically enriched substrates are not significantly impacted by kinetic isotope fractionation	• The use of ^{18}O labeled H_2O is not suitable under field conditions
	• Quantification of individual pathways	• Isotopically labeled substances might impact microbial activity

Natural samples

The analysis of the natural abundance isotopic signature of N_2O emitted from ecosystems such as soils, rivers or biological wastewater treatment indicate that N_2O from terrestrial and aquatic sources is depleted in ^{15}N compared to tropospheric N_2O ($\delta^{15}N$ = 7 ‰ and $\delta^{18}O$ = 20.7 ‰) (Stein and Yung, 2003), but also show a huge variability and complexity, making process identification ambiguous at large scale. For example, in biological wastewater treatment an average $\delta^{15}N^{bulk}_{N2O}$ of -9.6 ‰, SP of 16 ‰ and $\delta^{18}O$ of 22 to 44.3 ‰ were estimated (Yoshinari and Wahlen, 1985; Toyoda et al., 2011), indicating that nitrification as well as denitrification contributed to N_2O production. N_2O emitted from agricultural soils is reported to be strongly depleted in $\delta^{15}N^{bulk}_{N2O}$ (e.g. -34 ‰) (Park et al., 2011), referring to nitrification dominated N_2O production. Isotopic signatures of N_2O emitted from rivers and streams are in the range of -18 to 2.4 ‰ ($\delta^{15}N^{bulk}$), -6 to 31 ‰ (SP) and 17 to 53 ‰ ($\delta^{18}O$) being in line with values reported above, which indicates to be highly influenced by sources such as agriculture or municipal wastewater treatment (Toyoda et al., 2009; Baulch et al., 2011). This is underscored by a recent study that investigates the oxygen and intramolecular nitrogen isotopic composition of N_2O, confirming that nitrogen-based fertilizer application was largely

responsible for the rise in N_2O atmospheric concentration during the last 65 years (Park et al., 2012).

Outlook

In this section, the isotopic signature of N_2O, especially the SP, is discussed to be a powerful tool to distinguish N_2O production pathways. Recent technological advances, e.g. the development and application of the quantum cascade laser absorption spectroscopy, now allow a high temporal resolution in the analysis of the isotopic changes of N_2O. Nevertheless, an adequate calibration procedure still needs to be applied, since instrumental nonlinearity and drifts impact the accuracy of the isotope ratio measurement, and calibration standards are not commercially available so far. It is a pressing issue to further investigate the characteristic isotopic signatures of the individual N_2O production pathways in mixed microbial communities under controlled conditions, in order to more accurately interpret isotopic signatures from complex environmental systems. Further, it is important to study N_2O isotopic signatures with respect to involved microbial communities, enzymatic reaction mechanisms and enzymatic transformation rates. The use of the oxygen isotopic signature of N_2O as a reliable tool for pathway identification requires the elucidation of mechanisms and rates of oxygen exchange in the future.

Molecular approaches to understanding microbial NO and N_2O formation

While abiotic variables such as dissolved O_2, pH, NO_2^-, and other nitrogen compounds have long been recognized to exert a strong influence on rates of microbial NO and N_2O emissions, the importance of microbial community composition and dynamics to such emissions is still little understood (Wallenstein et al., 2006). As such, researchers have recently begun supplementing process-level NO and N_2O emission measurements in a variety of environments with molecular techniques aimed at characterizing abundance, diversity, community structure, and activity of microbial guilds involved in nitrogen cycling. Here, we briefly introduce emerging molecular approaches to the delineation of key pathways, communities, and controls of NO and N_2O production, and we summarize recent applications of these tools.

Quantifying the genetic potential for N_2O consumption

An appealing focus for application of molecular tools in environmental samples is direct quantification via the quantitative polymerase chain reaction (qPCR) of relevant functional genes (Smith and Osborn, 2008). Such an approach most commonly targets DNA, not RNA, and is thus a measure of genetic potential in the environment and not the activity.

Owing to the relative independence of each catabolic step, denitrification has been described as having a modular organization (Zumft, 1997). Indeed, Jones et al. (2008)

concluded based on an analysis of 68 sequenced genomes of heterotrophic denitrifiers that approximately 1/3 lacked the nosZ gene encoding for N_2O reductase and thus lack the genetic capacity for N_2O reduction. Based on this assessment, researchers have hypothesized that the ratio of nosZ to the sum of nirK and nirS encoding for copper and cytochrome cd1-type nitrite reductases, respectively, is representative of the fraction of denitrifiers in a given environment that generate N_2O as a catabolic end product. Environments with high nosZ/(nirK+nirS) ratios are likely associated with a high capacity for N_2O consumption, and thus for low N_2O emissions. Commonly used primers and qPCR conditions for genes relevant for NO and N_2O turnover during N-cycling are available in the literature and are listed in Table 4, and thus the measurement of such ratios are feasible with little method development. Application of such tools has commonly shown a lower abundance of nosZ compared to other denitrifying reductases, particularly in soil environments (Henry et al., 2006; Hallin et al., 2009; Bru et al., 2011).

First assessments of this hypothesis are somewhat conflicting. In favor for the hypothesis, Philippot et al. (2009) demonstrated a negative correlation between nosZ proportional abundance and $N_2O/(N_2 + N_2O)$ ratio in grassland pasture soil. In a follow-up study, Philippot et al. (2011) dosed three soils with several dilutions of a denitrifying bacterial isolate known to lack the nosZ gene, and measured the response at the DNA level of nirK, nirS, and nosZ genes via qPCR. N_2O emissions increased in all soils upon dosing of the nosZ-deficient isolate. However, in two of the three soils, the increase in denitrification potential (relative to non-inoculated controls) was higher than the measured increase in N_2O emissions, suggesting that the original denitrifier community was capable of acting as a sink for N_2O production. Moreover, ratios of N_2O emissions to total denitrifying end products ($N_2O + N_2$) in non-inoculated soils were not correlated to nosZ/(nirK+nirS). While the authors acknowledge that abundance of nosZ deficient denitrifiers may not be as important in soils with a high N_2O uptake capacity, their results clearly demonstrate that abundance of denitrifiers incapable of N_2O reduction can influence denitrification end products in natural environments. Similarly, Morales et al. (2010) document a strong positive correlation between the difference in nirS and nosZ gene abundance (nirS-nosZ; nirK was not quantified) and N_2O emissions in 10 soils. Garcia-Lledo et al. (2011) suggested that a significant decrease in nosZ gene abundance during periods of high NO_3^- content in a constructed wetland might be indicative of increased genetic capacity for (unmeasured) N_2O emissions.

In contrast, Čuhel et al. (2010) detail a significant but, puzzlingly, positive correlation in grassland soil between nosZ/(nirS+nirK) ratios and $N_2O/(N_2 + N_2O)$, but caution that the relative importance of denitrifier community composition and enzyme regulation relative to proportion of nosZ deficient community members remains uncertain. In line with this result, Braker and Conrad (2011) found similar ratios of nosZ/(nirS+nirK) via Most Probable Number (MPN-) PCR in three soils with profoundly different $N_2O/(N_2 + N_2O)$ ratios, and concluded that the hypothesis that a higher abundance of denitrifiers lacking nosZ is linked to increased N_2O emissions may be an oversimplification.

The genetic potential for N_2O production via nitrifier denitrification in AOB (and possibly AOA) could theoretically be measured via qPCR of the nirK and norB genes. Design of such analyses is hampered due to the fact that AOB nirK and norB genes are not phylogenetically distinct from that of heterotrophic denitrifying organisms (Cantera and Stein, 2007; Garbeva et al., 2007). In addition, NorB is not the only NO reductase in AOB (Stein, 2011).

Table 4. *Reported primers and literature references relevant for NO and N_2O turnover during N-cycling.*

Target gene[1]	Primer name	Nucleotide sequence (5'-3')	References
b-AOB (amoA)	amoA-1F	GGG GTT TCT ACT GGT GGT	(Rotthauwe et al., 1997)
	amoA-2R	CCC CTC KGS AAA GCC TTC TTC	
AOA (amoA)	Arch-amoAF	STA ATG GTC TGG CTT AGA CG	(Francis et al., 2005)
	Arch-amoAR	GCG GCC ATC CAT CTG TAT GT	
narG	narG-F	TCG CCS ATY CCG GCS ATG TC	(Bru et al., 2007)
	narG-R	GAG TTG TAC CAG TCR GCS GAY TCS G	
napA	V17m	TGG ACV ATG GGY TTY AAY C	(Bru et al., 2007)
	napA4r	ACY TCR CGH GCV GTR CCR CA	
nirK	nirK1F	GGM ATG GTK CCS TGG CA	(Braker et al., 1998, 2012)
	nirK5R	GCC TCG ATC AGR TTR TGG	
	nirK876	ATY GGC GGV AYG GCG A	(Henry et al., 2004)
	nirK1040	GCC TCG ATC AGR TTR TGG TT	
nirS	nirS1F	CCT AYT GGC CGC CRC ART	Braker et al. 1998, 2012
	nirS6R	CGT TGA ACT TRC CGG T	
	cd3aF	GTS AAC GTS AAG GAR ACS GG	(Throbäck et al., 2004)
	R3cd	GAS TTC GGR TGS GTC TTG A	(Michotey et al., 2000)
norB	cnorB-2F	GAC AAG NNN TAC TGG TGG T	(Braker and Tiedje, 2003)
	cnorB-6R	GAA NCC CCA NAC NCC NGC	(Geets et al., 2007)
nosZ	nosZ2F	CGC RAC GGC AAS AAG GTS MSS GT	Henry et al. 2006
	nosZ2R	CAK RTG CAK SGC RTG GCA GAA	
	nosZF	CGC TGT TCI TCG ACA GYC AG	(Rich et al., 2003)
	nosZR	ATG TGC AKI GCR TGG CAG AA	(Kloos et al., 2001)

[1]amoA – subunit A of ammonia monooxygenase, b-AOB - ammonia oxidizing bacteria, narG – subunit G of membrane bound nitrate reductase; napA – subunit A of periplasmic nitrate reductase; nirK - copper-type nitrite reductase; nirS - cytochrome cd1 nitrite reductase; norB – subunit B of nitric oxide reductase; nosZ – subunit Z of nitrous oxide reductase

Community structure and diversity impacts on NO and N_2O production

In addition to monitoring abundance of nosZ deficient denitrifiers, PCR-based tools are now being applied to the investigation of links between community structure and N_2O emissions for both nitrifiers and denitrifiers. For this purpose, community structure is commonly profiled via cultivation-independent molecular fingerprinting methods, such as terminal restriction fragment length polymorphism (T-RFLP) or denaturing gradient gel electrophoresis (DGGE), targeting either 16S rRNA fragments specific to the

functional guild of interest or functional genes (for example, nirK or amoA) directly. In addition, traditional cloning and Sanger sequencing and, increasingly, barcoded amplicon-based pyrosequencing of functional genes are often employed for robust phylogenetic comparisons. Readers are referred to Prosser et al. (2010) for a detailed methodological description of these and other nucleic-acid based methods. Multivariate statistical analyses such as canonical correspondence analysis (CCA), redundancy analysis (RDA) (Ramette, 2007; Wells et al., 2009), or path analysis (Avrahami and Bohannan, 2009) can then be used to explore the interplay between abiotic variables, community composition, and extant process rates.

It should be emphasized that the molecular and statistical tools highlighted above are most commonly used in microbial ecology to explore correlations, rather than causal associations, between community structure and function in complex microbial communities. As discussed in detail by Reed and Martiny (2007) directly testing causal relationships between microbial community composition or diversity and ecosystem processes is significantly more difficult, but experimental approaches often drawn from classical ecology are now being adapted to this challenge. We anticipate that future studies testing the functional significance of microbial community structure to NO or N_2O production will benefit greatly from these approaches.

Studies targeting the relationship between nitrifier community composition and greenhouse gas production are sparse at present, despite the fact that ample molecular tools are available for this purpose. Avrahami and Bohannan (2009) employed a combination of qPCR and T-RFLP to explore the response of N_2O emission rates and betaproteobacterial AOB abundance and composition in a California meadow to manipulations in temperature, soil moisture, and fertilizer concentration. While a complex interaction between factors was determined to directly and indirectly contribute to N_2O emission rates, path analysis suggested that the major path by which NH_4^+ influenced emission rates in the high N fertilization treatment was indirectly via two specific AOB clusters. This observation suggested a significant relationship between AOB community structure and N_2O emission rates. It is important to note that this study did not attempt to discriminate between the nitrifier denitrification and hydroxylamine oxidation pathways for AOB-linked N_2O production, nor was the relative importance of heterotrophic denitrification versus nitrification for overall N_2O emissions directly compared.

Assessment of the importance of DNRA as a process, and diversity therein, to NO and N_2O production is in its infancy. It has been suggested that our understanding of this little understood phenomena would benefit from the future investigations employing molecular techniques to quantify abundance and diversity of the nrf gene in conjunction with either modeling or stable isotope-based methods (Baggs, 2011). To our knowledge, such an assessment has yet to be conducted.

The relationship between denitrifier community composition and N_2O emissions, while still ambiguous, has been studied in more detail. Palmer et al. (2010) investigated narG (encoding for membrane-bound nitrate reductase, Nar) and nosZ phylogenetic diversity

in a low-pH fen via gene clone libraries and T-RFLP. They documented novel narG and nosZ genotypes and a phylogenetically diverse low-pH adapted denitrifier community, and suggested that the novel community structure may be responsible for complete denitrification and low N_2O emissions under in situ conditions. In a more recent study, Palmer et al. (2011) investigated denitrifier gene diversity in peat circles in the arctic tundra via barcoded amplicon pyrosequencing of narG, nirK/nirS, and nosZ, and found evidence that high and low N_2O emission patterns were associated with contrasting denitrifier community composition. Braker et al. (2012) found that, of three soils profiled, the soil with the most robust denitrification (lowest N_2O/N_2 ratio) harbored the most diverse denitrifier community, as measured via nosZ and nirK sequence diversity, suggesting that differences in community composition (higher diversity) are associated with ecosystem-level functional differences. In denitrifying bioreactors, population dynamics tracked via 16S rRNA-based T-RFLP were strongly correlated to NO_2^- appearance and emissions of N_2O (Gentile et al., 2007). In contrast, Rich and Myrold (2004) found little relationship between nosZ phylogenetic diversity as measured via T-RFLP in wet soils and creek sediments in an agrosystem, and suggested that activity and community composition were uncoupled in this ecosystem.

Taken together, the body of literature reviewed here suggests that, in at least some cases, community structure and diversity can play a functionally significant role in microbial N_2O emissions. The importance of community composition relative to environmental parameters and metabolic adaptation in response to transient conditions (for example, shifts in patterns of gene expression or regulation) in determining N_2O production, however, remains poorly understood. A worthwhile, but challenging future research direction would be to tease apart the influence of whole community metabolic adaptation versus community shifts on NO/N_2O emissions in mixed microbial communities.

A role for variation in regulatory response

Differences in transcriptional and translational regulation as well as enzyme activity have also been highlighted as potentially critical modulators of microbial NO or N_2O production (Richardson et al., 2009; Bergaust et al., 2011; Braker and Conrad, 2011). Such differences likely contribute to observed associations between community structure and greenhouse gas production discussed above. Strong regulation at the transcriptional, translational, and enzyme level is likely occurring in both nitrifier and denitrifier communities, and such regulation complicates attempts to directly relate abundance or diversity of functional guilds to process rates (Braker and Conrad, 2011). Similarly, transient near-instantaneous NO and N_2O accumulation in active nitrifying and denitrifying biofilms in response to O_2 or NO_2^- perturbations, as measured with high temporal resolution via microelectrodes, strongly suggests that dynamics are controlled in some cases at the enzyme level (Schreiber et al., 2009). Indeed, culture-based assays targeting denitrifier isolates from two soils demonstrated substantial diversity in sensitivity of Nos enzymes to O_2 and provided a physiological underpinning for a previously observed link between denitrifier community composition and rate of N_2O production (Cavigelli and Robertson, 2000).

Gene expression can be readily quantified with reverse transcriptase quantitative PCR (RT-qPCR), and researchers are now beginning to explore the relationship between gene expression patterns for critical functional genes (amoA, hao, nirK, nirS, norB, and nosZ) and NO/N_2O emissions. Yu et al. (2010) used such an approach to quantify expression of amoA, hao, nirK, and norB in chemostats of *Nitrosomonas europaea* during initiation and recovery from transient anoxic conditions. Surprisingly, expression profiles of nirK and norB were not strongly linked; strong overexpression of nirK concomitant with NO accumulation was observed upon initiation of anoxia, and at the same time norB, amoA, and hao gene transcripts declined in abundance. N_2O emissions peaked during recovery to aerated conditions, but did not correlate strongly to gene expression. The methods of Yu et al. (2010) provide a robust road map for examining relationships between nitrifier gene expression and NO/N_2O emissions in mixed communities in environmental settings, though it should be noted that such an analysis is complicated by the polyphyletic nature of the AOB nirK and norB genes.

RT-qPCR has also been used to assess the relationship between gene expression and NO/N_2O production in systems dominated by denitrifiers. Liu et al. (2010) quantified the relationship between nirS, nirK, and nosZ gene pools, their transcription products, and gas kinetics (NO, N_2O, and N_2) as a function of pH in soils. Interestingly, neither gene pool abundance, nor transcription rates could explain a profound increase in N_2O emissions at low pH. The authors attribute the observed N_2O:N_2 product ratio to post-transcriptional phenomenon, although it is also plausible that enhanced chemo-denitrification may play a role.

A worthy future contribution could be made via direct environmental metatranscriptomic assessment of patterns in microbial gene expression in environments with different or varying rates of NO or N_2O production. Metatranscriptomics is the direct sequencing of cDNA generated via reverse transcription of environmental RNA transcripts, and therefore provides a picture of currently transcribed genes in a given environment (Morales and Holben, 2010). In line with the results of Liu et al. (2010), it is important to recognize that measurement of the size or diversity of the gene transcript pool neglects post-transcriptional regulation governing, for example, the assembly of N_2O reductase and enzyme activity (Braker and Conrad, 2011). As of yet, variations in post-transcriptional regulation at the community level and its effect on NO/N_2O production has been little explored in nitrifying and denitrifying pure cultures and communities. Critical insights in this regard may be possible in the future from an approach coupling metatranscriptomics and metaproteomics—that is, direct measurement of the composition of the proteome in an environment.

A need for an integrated approach to NO and N_2O turnover in complex microbial communities

NO and N_2O can be produced by many different biological and chemical reactions. Considerable progress has been made to allocate NO and N_2O production to certain

biological pathways, but commonly some uncertainty remains, because many processes share the same reaction sequence for N_2O production via NO and NO_2^-. We delineated basically 3 independent approaches to allocate pathways (indirect inference; isotopic signature of N_2O, and isotopic labeling). Parallel use of these approaches will increase confidence in the interpretation. The possibility for various chemical reaction that produce and consume NO and N_2O additionally complicate the picture. Chemical reactions can be important in engineered systems that employ waters with concentrated N-contents and in natural systems, where low pH values coincide with high ammonia inputs. However, in most natural systems and in municipal wastewater treatment, chemical reactions will probably not be the main contributors of NO and N_2O emissions. Nevertheless, the possibility of chemical NO and N_2O production has to be considered when interpreting measurements. Experiments with inactivated biomass could help to give a first estimation of the chemical production rates. However, care has to be taken since the chemical conditions that facilitate chemical NO and N_2O production such as pH and trace metal availability are in turn shaped by microbial activity.

Molecular methods have largely been applied independently from the stable isotope and microelectrode approaches. Ample opportunities exist for integration of these techniques. Indeed, it is clear that such an integrated approach is critical to assessing the importance of microscale heterogeneity in environmental parameters, microbial community structure and stability, and genetic regulation to observed process-level N_2O emission rates.

Joint use of stable isotope methods in conjunction with molecular techniques appears particularly important, given reported difference in isotope effects depending on the community structure of nitrifiers (Casciotti et al., 2003) or denitrifiers (Toyoda et al., 2005) present. In addition, linking source-partioned N_2O as measured via stable isotope techniques to the underlying microbial communities via molecular approaches may allow a more significant measure of the strength of coupling between microbial diversity and measured emissions (Baggs, 2008, 2011). One promising way forward is to assess environmental conditions that favor a shift of dominant N_2O production pathway (for example, from denitrification to nitrification, or vice versa) as measured via stable isotope methods, and to simultaneously link such a shift to diversity and abundance of functional gene pools and transcripts via PCR-based molecular approaches. Such an approach has the potential to yield insights into the relative importance of dominant functional guilds, community composition, and activity in determining microbial NO/N_2O production rates. A fruitful first application would be to combine stable isotope-based methods with the molecular approach pioneered by Yu et al. (2010) for delineating the relationship between transcriptional response of the model AOB *Nitrosomonas europaea* and NO/N_2O production. This coupled approach would allow conclusive verification of conditions proposed by Chandran et al. (2011) to favor a switch between nitrifier denitrification and NH_2OH oxidation as dominant sources NO and N_2O production.

Similarly, it is clear that molecular tools and microelectrodes are complementary to study NO and N_2O turnover. An excellent example of such integration is provided by Okabe et al. (2011), who profiled microscale gradients in N_2O emissions in anammox granules and compared these profiles to spatial location of AOB, as measured via fluorescence in-situ hybridization (FISH). Based on their results, the authors concluded that putative heterotrophic denitrifiers in the inner part of the granule, not AOB, were likely responsible for the majority of the extant N_2O process emissions. A similar approach is likely applicable in a wide variety of environments, including flocs, sediments, soils, and microbial mats. In addition, use of either FISH probes with higher phylogenetic resolution or depth stratified DNA/RNA extraction coupled to PCR-based measurements may allow a direct microscale assessment of links between microbial diversity and activity and NO/N_2O production profiles. Such a microscale assessment is important because stratified environments likely contain both regions of N_2O production and consumption that are masked during bulk NO/N_2O concentration measurements or DNA/RNA extractions. In addition, microelectrode measurements with high temporal resolution should be combined with qPCR to better understand the regulation of NO and N_2O peak emissions from different environments.

The conditions for NO and N_2O formation in pure cultures and by chemical reactions begin to be better understood. Furthermore, several recent technological advancements allow researcher to investigate the regulation of NO and N_2O formation in complex environments at high spatial and temporal resolution. These advancements provide a cornerstone to understand and mitigate the release of NO and N_2O from natural and engineered environments.

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Chapter 3

Mechanisms of N_2O production in biological wastewater treatment under nitrifying and denitrifying conditions

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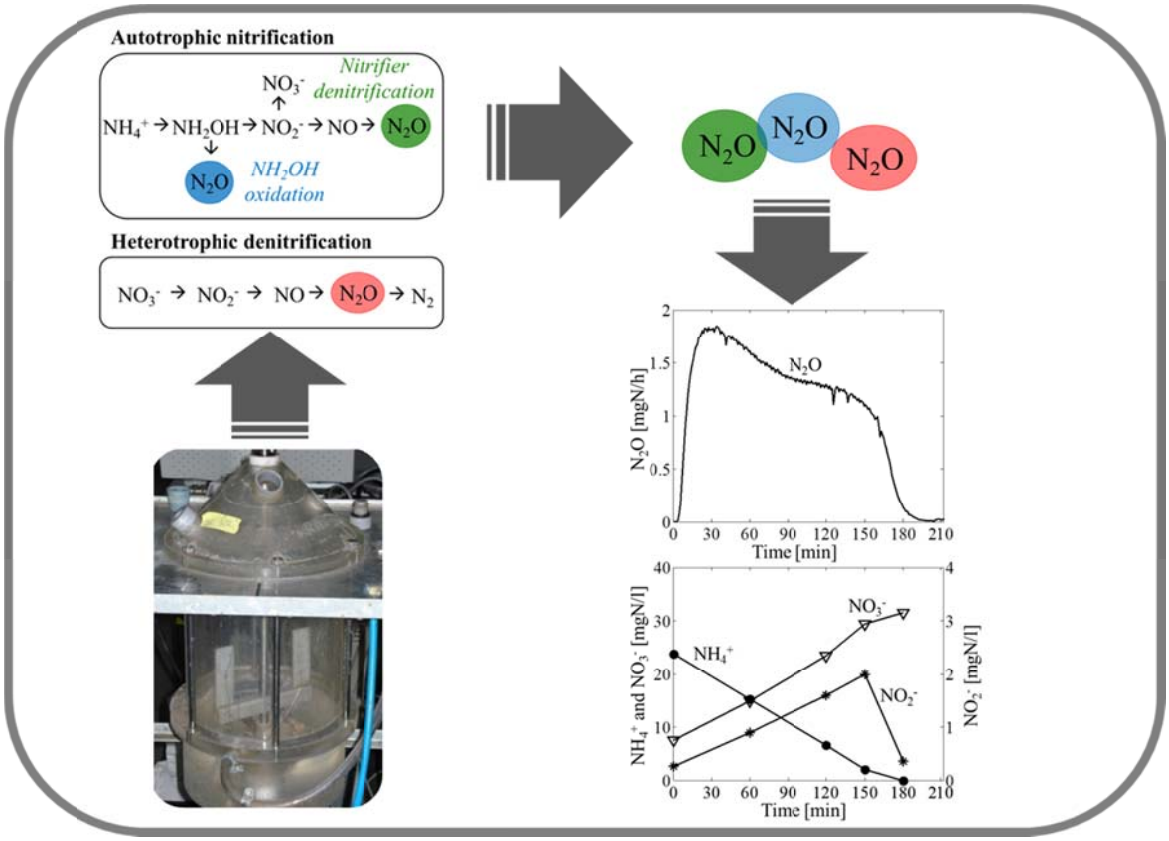
Graphical abstract



Abstract

Nitrous oxide (N_2O) is an important greenhouse gas and a major sink for stratospheric ozone. In biological wastewater treatment, microbial processes such as autotrophic nitrification and heterotrophic denitrification have been identified as major sources; however, the underlying pathways remain unclear. In this study, the mechanisms of N_2O production were investigated in a laboratory batch scale system with activated sludge for treating municipal wastewater. This relatively complex mixed population system is well representative for full-scale activated sludge treatment under nitrifying and denitrifying conditions.

Under aerobic conditions, nitrite oxidation experiments have confirmed nitrifier denitrification to be the dominant, strongly nitrite-dependent N_2O production pathway. Furthermore, N_2O is produced via hydroxylamine oxidation, as has been shown by the addition of hydroxylamine. In both sets of experiments, N_2O production was highest at the beginning of the experiment, then decreased continuously and ceased when the substrate (nitrite, hydroxylamine) had been completely consumed. In ammonia oxidation experiments, N_2O peaked at the beginning of the experiment when the nitrite concentration was lowest. This indicates that N_2O production via hydroxylamine oxidation is favored at high ammonia and low nitrite concentrations, and in combination with a high metabolic activity of ammonia-oxidizing bacteria (at 2 to 3 mgO_2/l); the contribution of nitrifier denitrification increases at higher nitrite and lower ammonia concentrations towards the end of the experiment.

Under anaerobic conditions, nitrate reducing experiments confirmed that N_2O emission is low under optimal growth conditions for heterotrophic denitrifiers. However, N_2O and nitric oxide (NO) production rates increased significantly under unfavorable conditions, for example in the presence of oxygen or nitrite.

Keywords

Biological wastewater treatment; denitrifying condition; hydroxylamine oxidation; nitrous oxide; nitric oxide; nitrifying condition

Introduction

Nitrous oxide (N_2O) is an important greenhouse gas, about 310 times more effective than carbon dioxide (CO_2), and a major sink for stratospheric ozone (S.A. Montzka and S. Reimann et al., 2011; Ravishankara et al., 2009; IPCC 2007). Limiting anthropogenic N_2O emission is thus an urgent requirement. It is estimated that about two thirds of the overall N_2O is emitted by microbial processes occurring mainly in agriculture, but also in biological wastewater treatment (USEPA 2009). In fact, N_2O emissions have been shown to dominate total greenhouse gas emissions from biological wastewater treatment (Wunderlin et al., 2010). In addition to N_2O, nitric oxide (NO) could also be emitted, which is toxic for microorganisms (Zumft 1993) and contributes to the destruction of the stratospheric ozone layer (Crutzen 1979).

N_2O production in biological wastewater treatment is associated with autotrophic nitrification and heterotrophic denitrification. Nitrification is the stepwise autotrophic oxidation of ammonia (NH_4^+) to nitrite (NO_2^-) by ammonia-oxidizing bacteria (AOB) and further to nitrate (NO_3^-) by nitrite-oxidizing bacteria (NOB). Denitrification is the reduction of nitrate (NO_3^-) to atmospheric nitrogen (N_2) by heterotrophic denitrifiers (HET), with nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O) as obligatory intermediates. According to Kampschreur et al. (2009), there are three main routes for N_2O production (Figure 1):

- Hydroxylamine oxidation: production of N_2O from intermediates of biological hydroxylamine oxidation (HNO, $N_2O_2H_2$; Poughon et al., 2001), probably related to a highly imbalanced metabolic activity of AOB (Yu et al., 2010), or by chemical decomposition of hydroxylamine as well as by chemical oxidation with NO_2^- as an electron acceptor (chemodenitrification; Stüven et al., 1992; Ritchie and Nicholas 1972).
- Nitrifier denitrification: reduction of NO_2^- by AOB in combination with ammonia, hydrogen or pyruvate as electron donors, e.g. at oxygen-limiting conditions or elevated nitrite concentrations (Wrage et al., 2001; Colliver and Stephenson 2000; Stüven et al., 1992).
- Heterotrophic denitrification: production of N_2O by heterotrophic denitrifiers due to an imbalanced activity of nitrogen-reducing enzymes, e.g. due to oxygen inhibition (Lu and Chandran 2010; Baumann et al., 1997), nitrite accumulation (von Schulthess et al., 1994), or a limited availability of biodegradable organic compounds (Itokawa et al., 2001).

In the last decade, significant efforts have been made to better understand the mechanisms of N_2O production (Rassamee et al., 2011; Yu et al., 2010; Tallec et al., 2006; Burgess et al., 2002; Itokawa et al., 2001; von Schulthess et al., 1994). As a result, several parameters favoring N_2O production were identified: low dissolved oxygen concentration, accumulation of nitrite, rapidly changing (dynamic) conditions or a low ratio of COD to N-compounds during heterotrophic denitrification (Kampschreur et al., 2009).

It is generally believed that nitrifier denitrification is the main N_2O production pathway in biological wastewater treatment under aerobic conditions (Colliver and Stephenson 2000), but its importance with respect to hydroxylamine oxidation and heterotrophic denitrification remains unclear. Therefore, a better understanding of these mechanisms is essential to formulate operating strategies that minimize N_2O emissions.

In this study, we analyzed N_2O and NO production (emission patterns) from activated sludge under nitrifying and denitrifying conditions in a laboratory-scale batch reactor. A homogeneously mixed system was chosen to allow the selection of specific microbial activities (AOB, NOB, HET) without the use of (i) chemical inhibitors potentially interfering with activated sludge in a complex way, or (ii) pure cultures that may behave differently than mixed culture systems, such as activated sludge. This is the first study investigating the combination of nitrifier denitrification, heterotrophic denitrification and hydroxylamine oxidation as relevant N_2O (and NO) production pathways in conventional activated sludge used to treat real municipal wastewater.

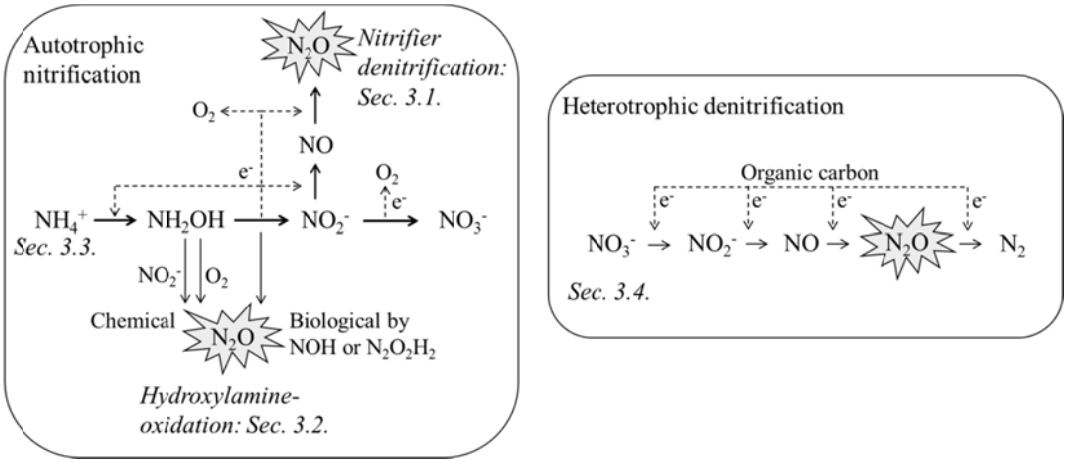


Figure 1: *Scheme of relevant N_2O production pathways in biological wastewater treatment. During autotrophic nitrification (left figure), N_2O can be produced either via nitrifier denitrification or hydroxylamine oxidation (chemically as well as biologically). During heterotrophic denitrification (right figure), N_2O is an obligate intermediate in the nitrogen reduction chain.*

Materials and methods

Maintenance of sludge and experimental setup

Batch experiments were carried out with activated sludge taken from our pilot scale wastewater facility at Eawag, which was operated continuously for several weeks at the same sludge age before the experimental phase. It treats around 60 population equivalents and consists of primary clarification followed by activated sludge treatment with nitrification, pre-denitrification and secondary clarification. It was operated with a solid retention time of around 22 days (11 days aerobic) and total suspended solid (TSS) concentrations between 4 and 4.4 g/l. N_2O emissions from the

aerobic tank were low during normal operation (below 0.1% of the nitrogen load entering the biological compartment), but correlated positively with the incoming nitrogen peak in the morning. Nitrification was complete (average NH_4^+ and NO_2^- concentrations below 0.7 mgN/l in the effluent), with average nitrogen removal above 60%.

Experiments were carried out in a laboratory- scale batch reactor (Figure 2) with a working volume of 6.9 liters and a headspace of 1.2 liters. The reactor was controlled by a programmable logical controller (PLC) linked to a SCADA interface for data visualization and storage. The water temperature was held at 20.1±1.2°C. A continuous gas flow of 1 l/min was supplied by a mass flow controller (red-y smart series, Vögtlin Instruments AG) during nitrification as well as during denitrification experiments in order to strip N_2O and NO from the liquid phase and analyze it in the off-gas (see section 'Analytical procedures'). Under aerobic conditions, on / off regulation (switching between high-purity nitrogen gas and synthetic air) was applied to adjust the oxygen concentration. Under anaerobic conditions, nitrogen gas was continuously fed into the reactor. The pH was measured with a pH electrode (Orbisint CPS11, Endress+Hauser; calibration at pH 7 and 9), and was held at 7.1±0.2 by a pH controller through the addition of CO_2 (>99.9% CO_2, Carbagas). The dissolved oxygen concentration was measured with an oxygen sensor (Oxymax H, Endress+Hauser; operational range from 0.01 to 20 mgO_2/l).

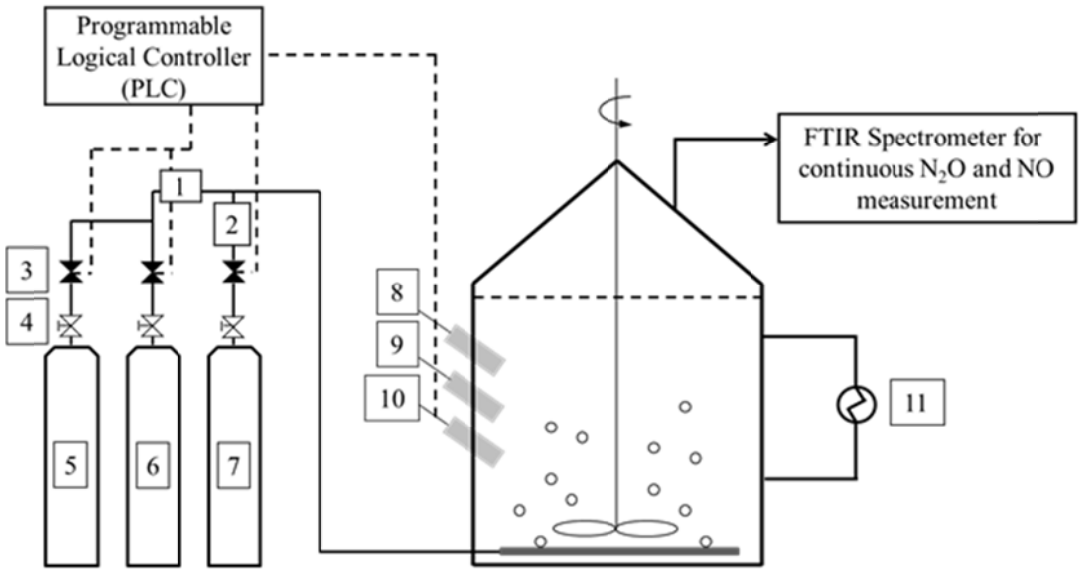


Figure 2: *Schematic diagram of the laboratory-scale batch reactor. (1) and (2) mass flow controller; (3) solenoid valve (controlled); (4) pressure regulator; (5) synthetic air; (6) high purity N_2 gas; (7) pure CO_2; (8) oxygen sensor; (9) pH sensor; (10) temperature sensor; (11) temperature controller.*

Nitrifying and denitrifying batch experiments

For nitrifying experiments, activated sludge was taken from the nitrification reactor of our pilot-scale wastewater facility before 8 a.m. (before morning urea and COD peak) and filled into the lab-scale batch reactor. Each nitrifying experiment was carried out at

a constant oxygen concentration (set point): 0.5, 1, 2 or 3 mgO_2/l. The experiments were started by adding the substrate (25 mgNH_4^+-N/l, NH_4HCO_4 from Merck; 15 mgNO_2^--N/l, $NaNO_2$ from Merck; 10 mgNH_2OH-N/l, NH_2OH-Cl from Fluka; Table 1). Except for simultaneous nitrifying and denitrifying experiments, no external organic carbon source was provided in order to prevent heterotrophic activity, which was confirmed by nitrogen mass balances and a constant NO_3^- concentration after the experiment. The substrate addition initialized N_2O production, but also NO production in some nitrite oxidation experiments. Neither N_2O nor NO was emitted before the substrate addition.

For the denitrifying experiments, sludge was taken from the nitrification reactor of our pilot-scale wastewater facility in the afternoon and was stored overnight in the batch-scale reactor under anaerobic conditions in order to remove the remaining dissolved nitrogen compounds (mainly NO_3^-). The experiments were launched by adding organic carbon (Na acetate from Merck; always >100 mgCOD/l) and nitrate (20 mgNO_3^--N/l, $NaNO_3$ from Fluka; Table 1). The substrate addition initiated N_2O production, as well as NO production during suboptimal growth conditions, while neither N_2O nor NO was produced before the substrate addition. For experiments at low dissolved oxygen concentrations, an N_2 / O_2 gas mixture with only about 5% oxygen was applied in order to better adjust the dissolved oxygen concentration in the reactor.

The nitrifying and denitrifying experiments were both stopped when N_2O (and NO) production ceased and the dissolved nitrogen species were completely oxidized (NH_4^+, NH_2OH or NO_2^- in the nitrifying experiment) or reduced (NO_3^- in the denitrifying experiment). During all experiments, N_2O as well as NO were continuously analyzed in the off-gas and liquid samples were periodically taken (see section 'Analytical procedures'). The disappearance and appearance rates of NH_4^+, NH_2OH, NO_2^- and NO_3^- were calculated using regression analysis. Average N_2O and NO emission rates were calculated based on time integration of emission loads.

Analytical procedures

The N_2O and NO concentrations in the off-gas were continuously (1 minute temporal resolution) analyzed by FTIR spectroscopy (GASMET CX-4000, Temet Instruments, Helsinki), equipped with a heated (40°C) flow-through gas cell with a 9.8 m path length. The quantification limits for N_2O and NO are 0.25 and 5 ppm respectively, and the expanded standard uncertainty for both components is around 10% (95% confidence level; Mohn et al., 2008). Liquid grab samples were taken regularly (every 15 to 60 minutes) and immediately filtered through a 0.7µm filter (GF/F Whatman) and a 0.45 μ m syringe filter (Chromafil Membranfilter from Macherey), then stored at 4°C. NH_4^+-N was analyzed photometrically using a Foss FIAstar flow injection 5000 analyzer (detection limit 0.2 mgN/l; uncertainty around 5% at 95% confidence level). NO_3^--N and NO_2^--N were determined by anion chromatography (761 compact IC, Metrohm; detection limit for both components 0.2 mgN/l; uncertainty around 5% at 95% confidence level). Commercial photochemical test kits (Hach Lange GmbH, Düsseldorf, Germany) were used to measure low NO_2^- concentrations (LCK 341; detection limit 0.015mgN/l) and COD (LCK 314, LCK 414, or LCK 114).

Experiment	Added substrate (initial concentration)	O_2 concentration	Nitrogen conversion rate	N_2O-N (NO-N) production rate[#]	Y N_2O[##] (Y NO[##])
		[mg/l]	[mgN/gTSS*h]	[mgN/gTSS*h[	[%]
NH_4^+ oxidation	NH_4^+ (25 mgN/l)	0.6±0.2	1.1[a]	0.040 (<LOD)	3.8
	NH_4^+ (25 mgN/l)	1.0±0.2	1.4[a]	0.037 (<LOD)	2.7
	NH_4^+ (25 mgN/l)	1.9±0.2	1.9[a]	0.039 (<LOD)	2.0
	NH_4^+ (25 mgN/l)	3.1±0.2	2.6[a]	0.033 (<LOD)	1.3
NO_2^- oxidation	NO_2^- (15 mgN/l)	0.6±0.2	1.8[a]	0.028 (<LOD)	1.5
	NO_2^- (15 mgN/l)	1.1±0.2	1.8[a]	0.15 (0.008)	8.4 (0.5)
	NO_2^- (15 mgN/l)	2.1±0.3	3.0[a]	0.031 (<LOD)	1.0
	NO_2^- (15 mgN/l)	3.1±0.2	2.2[a]	0.18 (0.018)	8.9 (0.8)
NH_2OH oxidation	NH_2OH (10 mgN/l)	1.1±0.2	0.6[c]	0.043 (<LOD)	6.9
	NH_2OH (10 mgN/l)	2.2±0.2	0.7[c]	0.062 (<LOD)	8.5
NO_3^- reduction (+Na acetate)	NO_3^- (20 mgN/l)	0	4.8[b]	0.009 (<LOD)	0.2
	NO_3^- (20 mgN/l)	0.01±0.01	3.7[b]	0.029 (<LOD)	0.8
	NO_3^- (20 mgN/l)	0.04±0.03	2.1[b]	0.196 (0.008)	9.5 (0.4)
	NO_3^- (20 mgN/l)	0.13±0.15	4.4[b]	0.450 (0.12)	10.3 (2.6)
	NO_3^- (20 mgN/l)	0.4±0.2	3.0[b]	0.565 (0.017)	18.9 (0.6)
Continuous addition of NH_4^+	NH_4^+ (10 mgN/l)	1.1±0.2	1.4[c]	0.032 (<LOD)	2.3
	NH_4^+ (10 mgN/l)	2.1±0.2	2.1[c]	0.023 (<LOD)	1.1
	NH_4^+ (10 mgN/l)	3.1±0.2	2.1[c]	0.018 (<LOD)	0.9
Simultaneous nitrification / denitrification	NH_4^+ (25 mgN/l), acetate (>100 mgCOD/l)	2.0±0.3	1.8[a]	0.270 (<LOD)	15.0

a) Nitrogen (NH_4^+ or NO_2^-) oxidation rate

b) Nitrogen (NO_3^-) reduction rate

c) Nitrogen (NO_3^-) production rate

[#] The N_2O and NO production rates were calculated on the basis of the N_2O and NO concentrations in the off-gas and the gas flowrate. The N_2O and NO concentrations were measured in ppm (every 60 seconds) and transformed into μgN_2O-N/h (1ppmN_2O=1.149 μgN_2O-N/l_{air} at 20°C and 1bar) and µgNO-N/h (1ppmNO=0.58 µgNO-N/l_{air} at 20°C and 1bar) respectively by multiplying the concentration with the gas flowrate (Q_{gas}=60 l/h).

[##] Y N_2O (and Y NO) was calculated by dividing the average N_2O-N (NO-N) emission rate by the nitrogen conversion rate and multiplied by 100%.

<LOD: below limit of detection

Table 1: *Summary of experiments and relevant parameters: added substrates and initial concentrations; oxygen concentrations; nitrogen conversion rates (nitrogen oxidation under nitrifying conditions and reduction under denitrifying conditions); N_2O emission rates in relation to the nitrogen conversion rates.*

Results and discussion

N_2O production during nitrite (NO_2^-) oxidation under aerobic conditions

Figure 3 shows two typical profiles of the N_2O emission rate and the concentrations of NO_2^- and NO_3^- (the initial nitrite concentration was around 15 mgN/l), at 2.1 and 3.1 mgO_2/l respectively. The nitrite degradation rate was in the range from 1.8 to 3 mgN/gTSS*h, depending on the oxygen concentration (Table 1). Most of the nitrite was oxidized to nitrate, which indicates that there was no significant heterotrophic activity. NO was only produced under conditions with high N_2O production rates, where NO peaked before N_2O. In most experiments, the NO concentration was close or below the FTIR detection limit and therefore not shown in in Figure 3 A. The production of N_2O started as soon as nitrite was added and ceased when all the nitrite was consumed. The N_2O production was dynamic and variable, but not oxygen-dependent: there was a high N_2O emission rate compared to the nitrogen oxidation rate (8.4 and 8.9%) at 1.1 and 3.1 mgO_2/l (Figure 3, A and B), but only a low emission rate (1.0 to 1.5%) at 0.6 and 2.1 mgO_2/l (Figure 3, C and D; Table 1). The different N_2O emissions seem to be due to variable maximum NO_2^- oxidation rates: the NO_2^- oxidation rate was low at high N_2O production and vice-versa (Table 1).

The NO_2^- oxidation experiments always showed the highest N_2O production rate after NO_2^- addition (when the NO_2^- concentration was highest), and decreased in parallel with the NO_2^- concentration (Figure 3). This is consistent with Tallec et al. (2006), who also showed a positive correlation between the nitrite concentration and N_2O production. NO_2^- is often considered to be one of the key parameters responsible for N_2O production (see Kampschreur et al., 2009; Bock et al., 1995; Remde and Conrad, 1990). It is generally assumed that an accumulation of nitrite leads to increased nitrifier denitrification activity by AOB with nitrite instead of oxygen as the terminal electron acceptor; ammonia is thought to be the corresponding electron donor (Kampschreur et al., 2009; Colliver and Stephenson 2000): as reported by Kim et al. (2010), N_2O emissions were significantly enhanced when ammonia was added together with nitrite to activated sludge. In our experiments, ammonia is unlikely to act as an electron donor since no external ammonia was added, and it was not detectable in the bulk media; the inhibition of the ammonia oxidation (by allylthiourea) confirmed that there was no significant ammonia production from biomass decay (data not shown). Therefore, hydrogen, pyruvate or other electron donors have to be considered.

At high N_2O production rates, the production of NO, a precursor of N_2O in the nitrogen reduction chain, underscores nitrifier denitrification to be the dominant N_2O production pathway (Figure 3, C and D). In our setup, heterotrophs cannot be assumed to contribute significantly to N_2O production, because (i) no external organic carbon was added, (ii) most of the nitrite was converted into nitrate, and (iii) the enzyme activity of heterotrophic denitrifiers is known to be strongly affected by the availability of dissolved oxygen because this process operates mainly under anaerobic conditions (von Schulthess et al., 1994).

As shown in this section, nitrifier denitrification by AOB is the dominant N_2O production pathway under aerobic conditions, low COD loads and the presence of nitrite.

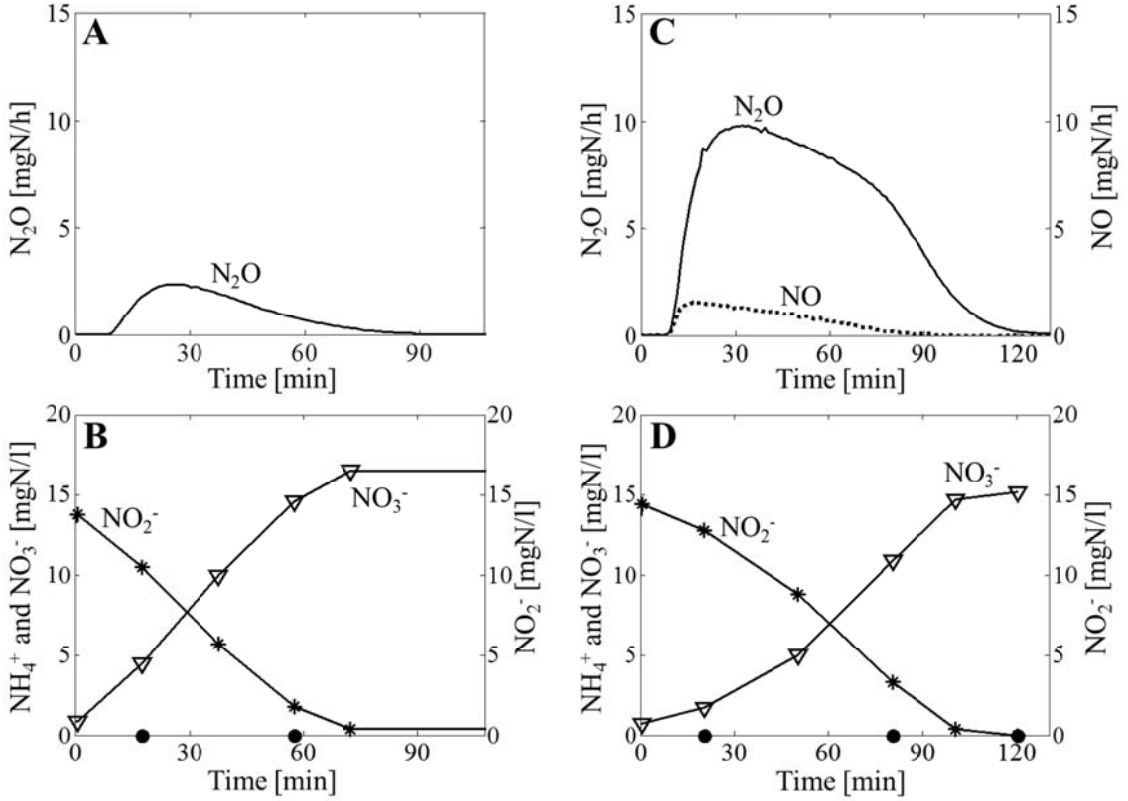


Figure 3: *Effect of NO_2^- addition (15 mgN/l, at time zero) to activated sludge, at 2.1 mgO_2/l (A, B) and at 3.1 mgO_2/l (C, D) respectively. N_2O (A, C) and NO (C) production started after substrate (NO_2^-) addition. Symbols: N_2O emission rate (solid line), NO emission rate (dotted line), NH_4^+ concentration (closed circles), NO_3^- concentration (open triangles), and NO_2^- concentration (stars).*

N_2O production during hydroxylamine (NH_2OH) oxidation under aerobic conditions

A representative profile of the N_2O emission rate and the concentrations of NH_4^+, NO_2^- and NO_3^- (the initial hydroxylamine concentration was around 10 mgN/l; Table 1) is given in Figure 4 (A and B). NH_4^+ production occurred at a constant rate of around 0.1 mgN/gTSS*h (at 1 and 2 mgO_2/l) up to around 0.6 mgN/gTSS*h (at 0 mgO_2/l), which could be due to (enzymatic) NH_2OH decay (this was not observed in experiments without activated sludge). Nitrite accumulated up to 0.4 mgN/l, and the NO_3^- production rate was in the range from 0.6 to 0.7 mgN/gTSS*h (Table 1). The NO_2^- concentration as well as the NO_3^- production rate were significantly lower than during the ammonia oxidation experiments (see next section). Nevertheless, the N_2O production rate (43 to 62 µgN/gTSS*h) was comparable to the ammonia oxidation experiment, and therefore significantly higher in relation to the nitrogen oxidation rate (6.9 to 8.5% of the oxidized NH_2OH (calculated from NO_3^- accumulation) was emitted as

N_2O, compared to 1.3 to 3.8% for ammonia oxidation experiments; Table 1). N_2O production started as soon as hydroxylamine was added and reached its maximum concentration around 30 minutes after the substrate addition. The N_2O concentration then decreased even though the nitrite level remained at around 0.4 mgN/l. The nitrogen mass balance shows that most of the NH_2OH is converted into NO_3^-, indicating that the N_2O emission rate decreases in parallel with the NH_2OH concentration. This emission pattern points to biological hydroxylamine oxidation as the most important N_2O production pathway. Nitrifier denitrification, which is strongly correlated with the nitrite concentration (see the section before) may additionally account for some of the emitted N_2O. It was already observed that N_2O is produced during biological NH_2OH oxidation (Sutka et al., 2006), and it is assumed that intermediates such as HNO and $N_2O_2H_2$ are directly involved in its formation (Poughon et al., 2001; Ritchie and Nicholas, 1972).

In our oxidation experiments, the N_2O production rate was in the same range or, at elevated oxygen concentrations (2 mgO_2/l), even higher than during the NH_4^+ oxidation experiments. This is in agreement with Kim et al. (2010), who explained this phenomenon by a higher availability of electrons during hydroxylamine oxidation (compared to ammonia oxidation) because of a lack of the electron-consuming ammonia oxidizing step (see Figure 1). As a consequence, more electrons are available for nitrite reduction and N_2O production (since nitrite reduction by AOB stops at N_2O). A further distinction between the contribution of hydroxylamine oxidation and nitrifier denitrification would require alternative techniques, such as the analysis of the N_2O site-specific isotopic signature (Wunderlin et al., 2010; Koba et al., 2009; Sutka et al., 2006). However, N_2O production from NH_2OH is not expected to be dominant in biological wastewater treatment, as the NH_2OH concentration in activated sludge is assumed to be much lower than in our batch experiment; NH_2OH oxidation to NO_2^- is the energy-generating step in AOB, an accumulation is therefore unfavorable from an energetic point of view (Casciotti et al., 2003).

The above results may also be due to chemical N_2O production. This was tested by adding NH_2OH to tap water (without activated sludge; Figure 4 C and D). Under aerobic conditions, low amounts of N_2O were produced, but were slightly increased by further addition of NO_2^-. On the basis of these experiments, chemical decomposition of NH_2OH as well as a chemical reaction between NH_2OH and NO_2^- could be responsible for the N_2O production; the following mechanisms are hypothesized for this process (Stüven et al., 1992; Ritchie and Nicholas, 1972):

$$NH_2OH + 0.5O_2 \rightarrow 0.5N_2O + 1.5H_2O \qquad \text{Equation 1}$$

$$NH_2OH + NO_2^- + H^+ \rightarrow N_2O + 2H_2O \qquad \text{Equation 2}$$

However, as N_2O production from NH_2OH in tap water was low as compared to activated sludge, chemical N_2O production is not expected to be dominant in biological wastewater treatment (Figure 4, A and C).

Based on the above arguments we conclude that, in addition to nitrifier denitrification, biological NH_2OH oxidation must be considered as a potentially relevant pathway for N_2O production.

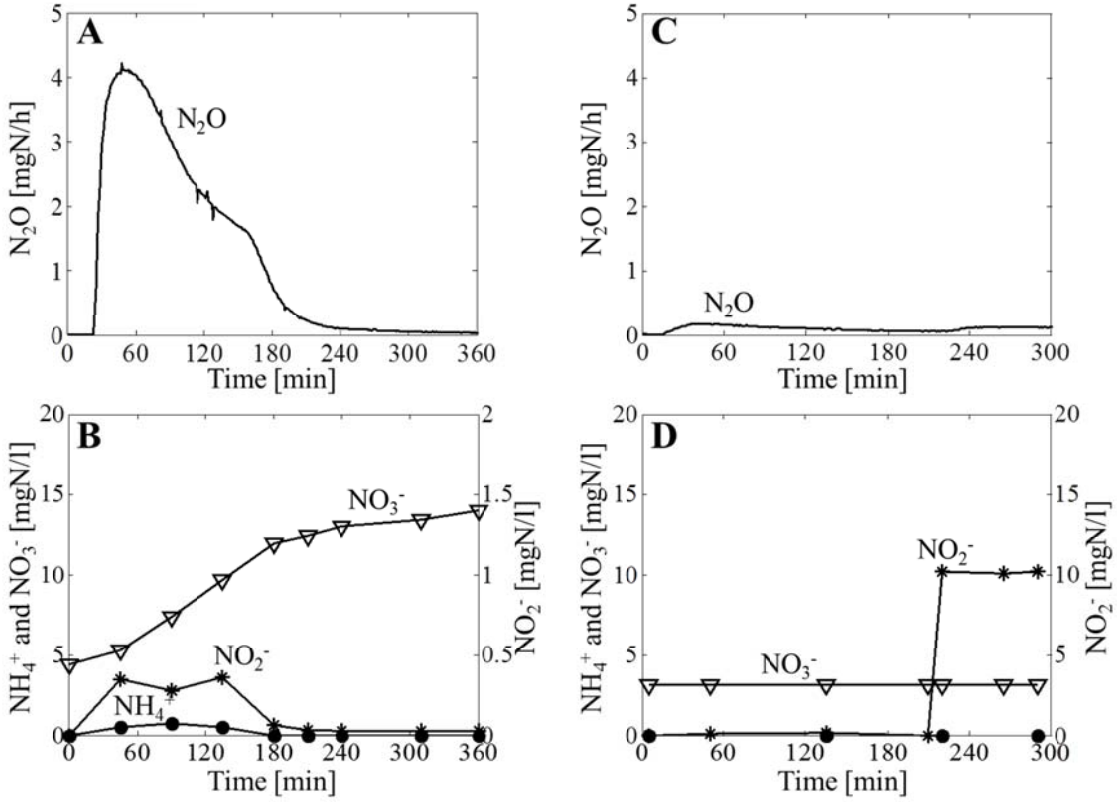


Figure 4: *Effect of NH_2OH addition (10 mgN/l, at time zero) to activated sludge, at 1.1 mgO_2/l (A, B) and to tap water, at 3.1 mgO_2/l (C, D; including NO_2^- addition after 210 minutes) respectively. N_2O (A, C) production started after substrate (NH_2OH) addition. Symbols: N_2O emission rate (solid line), NH_4^+ concentration (closed circles), NO_3^- concentration (open triangles), and NO_2^- concentration (stars; please note the different scale for NO_2^-).*

N_2O production during ammonia (NH_4^+) oxidation under aerobic conditions

During NH_4^+ oxidation experiments, NO_2^- accumulated up to 2 mgN/l (Figure 5). The ammonia degradation rates were in the range from 1.1 to 2.6 mgN/gTSS*h (Table 1), depending on the dissolved oxygen concentration. Most of the ammonia was converted into nitrate, which confirms the presence of AOB and NOB, and indicates no significant heterotrophic activity. At 2 mgO_2/l (Figure 5, A and B), N_2O production started as soon as ammonia was added and reached its maximum concentration around 20 to 30 minutes after substrate addition, when NH_4^+ was still high and NO_2^- very low. At 0.6 mgO_2/l (Figure 5, C and D), the N_2O production rate reached a plateau of rather constant emissions while NH_4^+ oxidation was ongoing. In all experiments, N_2O emissions ceased when ammonia and nitrite were completely oxidized. The N_2O production rates were in the range from 33 to 40 µgN/gTSS*h, which corresponds to 1.3 to 3.8% of the ammonia oxidation rate (Table 1). During continuous ammonia addition,

the N_2O emission rate was in the range from 18 to 32 µgN/gTSS*h, which corresponds to 0.88 to 2.3% of the ammonia oxidation rate (Table 1) and thus comparable to the NH_4^+ (peak) addition experiments. It is also similar to the values reported in the literature: Tallec et al. (2006) measured N_2O emissions from nitrifying activated sludge in the range from 0.1 to 0.4%, compared to ammonia oxidation; N_2O emissions in the range from 0.05 to 3.3% were observed for pure-culture AOB (Hynes and Knowles, 1984; Yoshida, 1988). Recently, Kim et al. (2010) reported a value of 2.9% from nitrifying activated sludge. Our experiments showed quite comparable N_2O emission rates at different oxygen concentrations. In contrast, the NH_4^+ fraction emitted as N_2O was higher at low oxygen concentrations. This is in agreement with Tallec et al. (2006), who reported a maximum yield (ratio of emitted N_2O to oxidized nitrogen) at an oxygen concentration of 1 mgO_2/l. It is hypothesized that oxygen-limiting conditions during nitrification lead to an accumulation of nitrite, since NOB have a lower affinity to oxygen than AOB, possibly due to a different half-saturation constant (Blackburne et al., 2008) and therefore a lower activity. Given that AOB can use nitrite instead of oxygen as the electron acceptor, nitrifier denitrification is held responsible for the increased N_2O emissions (Kampschreur et al., 2009; Wrage et al., 2001). Burgess et al. (2002) observed a strong correlation between the build-up of nitrite and N_2O emissions in combination with ammonia shock loads, which is consistent with our data. At high concentrations of dissolved oxygen, N_2O emissions peaked at the beginning of the experiment, when NH_4^+ was highest and NO_2^- lowest. This indicates that N_2O might partly be produced via NH_2OH oxidation, when ammonia is in excess, nitrite at low concentrations and the nitrogen oxidation rate is high. This is consistent with the findings of Sutka et al. (2006), who showed that an increasing oxygen concentration in *N. europaea* cultures decreased the relative importance of NO_2^- reduction relative to NH_2OH oxidation in N_2O production. Moreover, they suggested that an increase in NO_2^- concentration could have contributed to N_2O production via nitrite reduction. Similarly, Yu et al. (2010) reported a positive correlation between N_2O production and NH_4^+ concentration in *N. europaea* cultures and concluded that N_2O production via NH_2OH oxidation contributed even more when ammonia oxidation is shifted from low towards its maximum specific activity.

Based on the results presented in the two sections before, we conclude that in ammonia oxidation experiments (this section) NH_2OH oxidation and nitrifier denitrification contribute to the N_2O production: nitrifier denitrification is dominant but hydroxylamine oxidation becomes increasingly relevant at high ammonia and low nitrite concentrations. Therefore, a systematic experimental approach, as chosen in this study, proves to be appropriate to study the mechanisms of N_2O production in a complex, mixed population system.

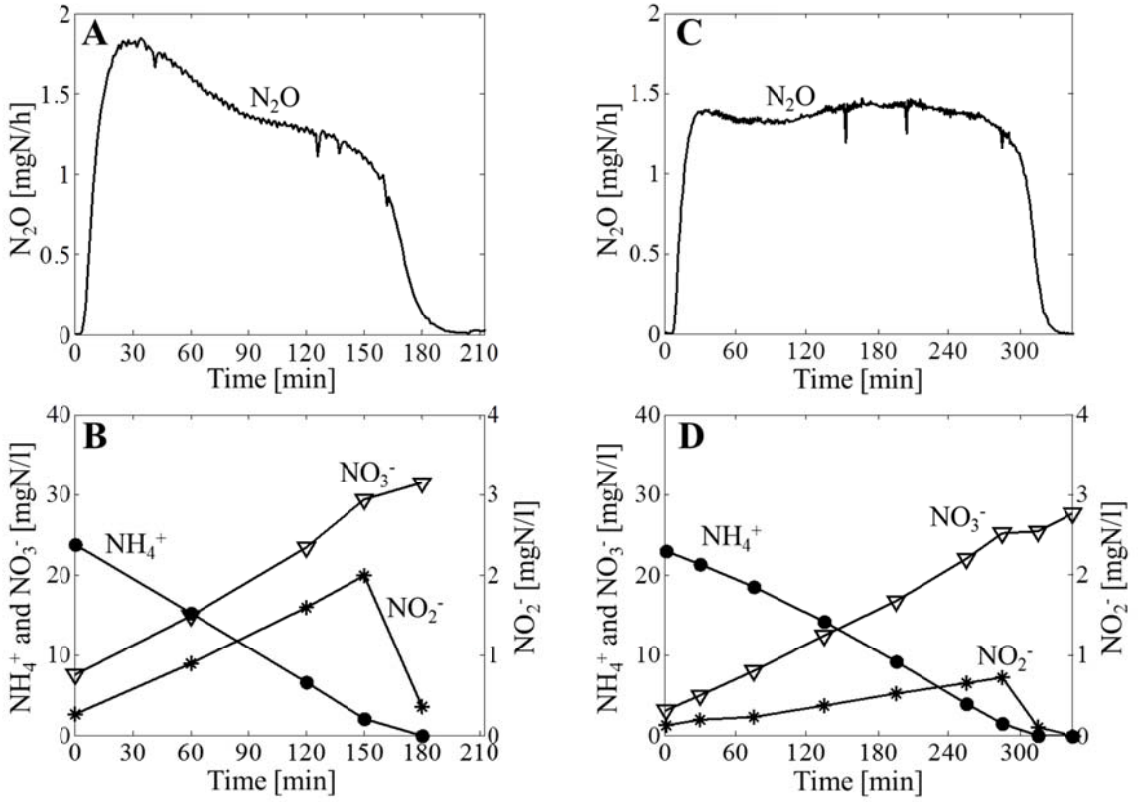


Figure 5: *Effect of NH_4^+ addition (25 mgN/l, at time zero) to activated sludge, at 2 mgO2/l (A, B) and 0.6 mgO_2/l (C, D) respectively. N_2O production (A, C) started right after NH_4^+ addition. Symbols: N_2O emission rate (solid line), NH_4^+ concentration (closed circles), NO_3^- concentration (open triangles), and NO_2^- concentration (stars).*

N_2O production during nitrate (NO_3^-) reduction under anaerobic and low aerobic conditions

Under optimal anaerobic conditions (Figure 6, A and B), the NO_3^- reduction rate was around 4.8 mgN/gTSS*h, and NO_2^- reached 4 to 5 mgN/l towards the end of the experiment. The N_2O emission rate was around 9 µgN/gTSS*h (0.2% of the NO_3^- reduction rate), which is a factor of 3.1 to 20 lower compared to the oxidation experiments (NH_4^+ oxidation, NH_2OH oxidation or NO_2^- oxidation; Table 1). N_2O production started immediately after substrate addition and peaked towards the end of the experiment when nitrite accumulated to maximum concentrations. Accordingly, von Schulthess et al. (1994) reported a positive correlation between N_2O and NO_2^- concentrations for anaerobic growth conditions.

In the presence of oxygen (Figure 6, C and D), the NO_3^- reduction rate was in the range from 2.1 to 4.4 mgN/gTSS*h, which is lower than under completely anaerobic conditions. Additionally, nitrite accumulated up to around 9 mgN/l, and the N_2O production rate was significantly higher than under optimal conditions, with values of 29 to 565 µgN/gTSS*h, which is equivalent to between 0.79 and 18.9% of the NO_3^- reduction rate. NO was also produced, at a rate of 8 to 120 mgN/gTSS*h, e.g. 0.37 to 2.6% of the NO_3^- reduction rate (Table 1; Figure 6, C). The reason for the variable profile

of N_2O and NO emissions in Figure 6 C remains unclear, but it may be due to (i) inhibition of the NO-reducing enzyme by an accumulation of NO_2^- or (ii) an imprecise O_2 regulation of the batch reactor because high concentrations of dissolved N_2O and NO seem to interfere with the dissolved oxygen concentration measurement. Our findings are consistent with the studies of Kampschreur et al. (2009) and von Schulthess et al. (1994), which reported that dissolved oxygen availability, especially in combination with other factors such as nitrite accumulation, is expected to lead to increasing N_2O production. An unbalanced activity of the nitrogen-reducing enzymes is considered to be the dominant mechanism for heterotrophic N_2O production, e.g. N_2O reductase is reported to be particularly inhibited by oxygen (Bonin et al., 2002; Baumann et al., 1997). The ratio of COD to N-compounds is not assumed to be a relevant factor in impacting N_2O emissions in our denitrifying experiments, since it was always higher than 5 (COD was always >100 mgCOD/l; the highest initial NO_3^- concentration was 20 mgN/l), and Itokawa et al. (2001) reported almost no N_2O emissions at COD / N ratios above 5, but more than 20% under conditions of limiting organic carbon availability (COD / N ratios below 3.5).

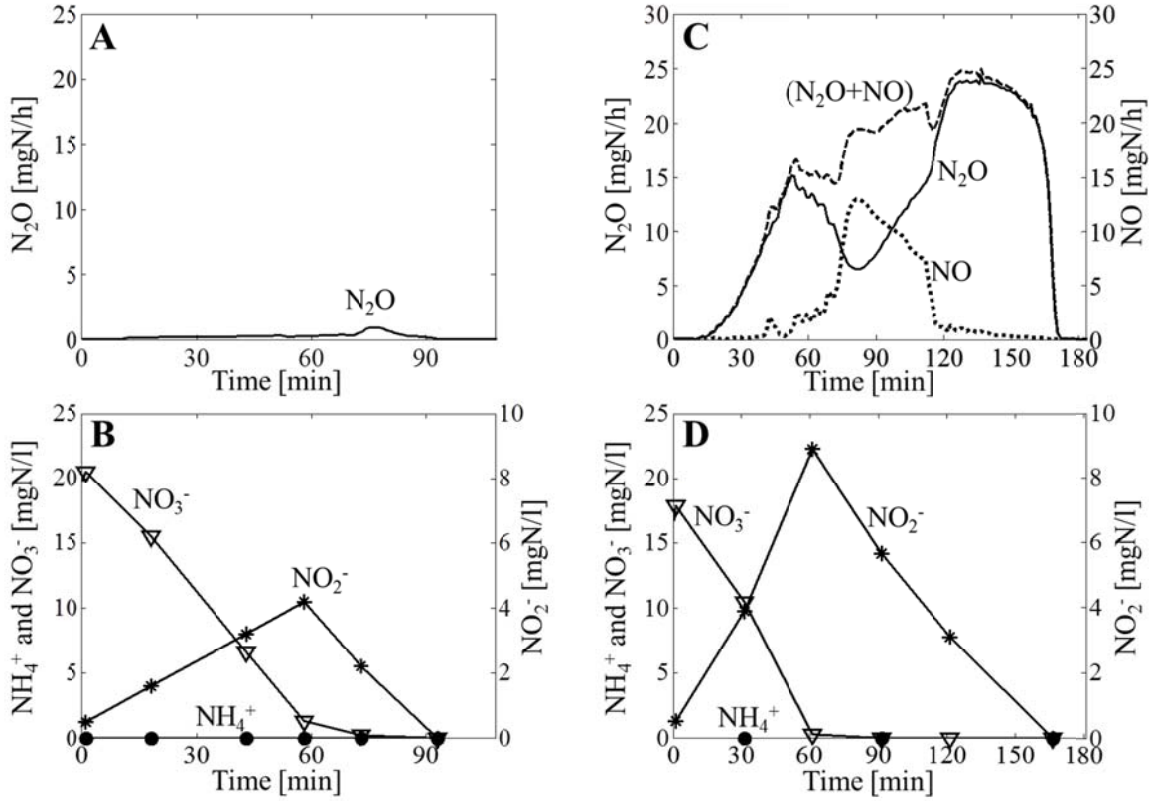


Figure 6: *Effect of NO_3^- addition (20 mgN/l, at time zero) to activated sludge, at 0 mgO_2/l (only high purity N_2 flow; A, B) and controlled at 0.13 mgO_2/l (synthetic air with low oxygen partial pressure; C, D) respectively. Symbols: N_2O emission rate (solid line; please note the different scale for N_2O), NO emission rate (dotted line), and total N_2O and NO emission rate (dashed line); NH_4^+ concentration (closed circles), NO_3^- concentration (open triangles), NO_2^- concentration (stars; COD concentration was always >100 mgCOD/l).*

As demonstrated in this section, the production of N_2O by HET was low under optimal growth conditions, but increased significantly in the presence of oxygen or nitrite accumulation.

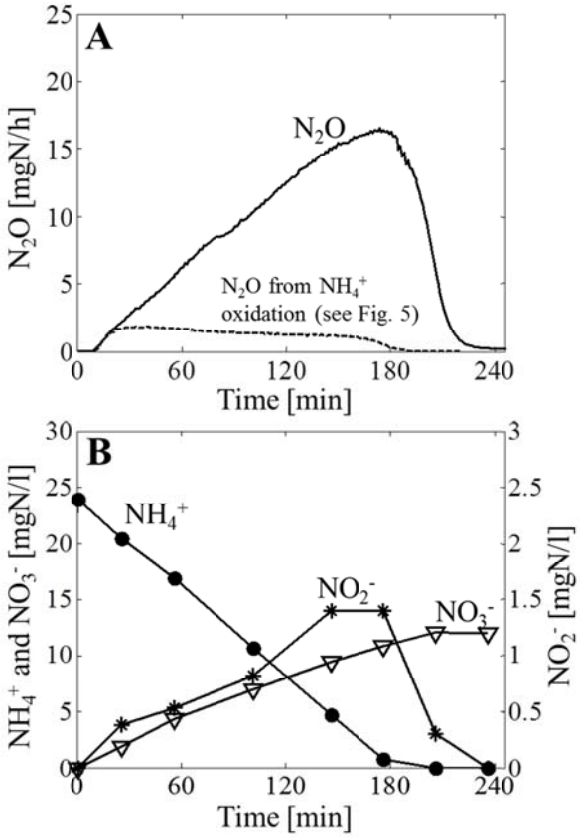


Figure 7: *Effect of NH_4^+ (25 mgN/l, at time zero) and acetate addition (COD concentration was always >100 mgCOD/l) to activated sludge, at 2 mgO2/l (A, B). N_2O production (A) started after NH_4^+ addition. Symbols: N_2O emission rate (solid line), NH_4^+ concentration (closed circles), NO_3^- concentration (open triangles), and NO_2^- concentration (stars). In contrast, negligible N_2O emissions (dashed line) occurred in the NH_4^+ oxidation experiment at 2 mgO_2/l and no additional organic carbon (data taken from Figure 5, A).*

N_2O production during simultaneous nitrifying and denitrifying conditions under aerobic conditions

Figure 7 shows a profile of the N_2O emission rate and the concentrations of NH_4^+, NO_2^- and NO_3^-. Organic substrate was always available in excess (>100 mgCOD/l). Nitrite accumulated up to 1.4 mgN/l during the experiment, and the ammonia degradation rate was 1.8 mgN/gTSS*h at 2 mgO_2/l, which is comparable to the values obtained in NH_4^+ oxidation experiments without any additional carbon source (see Table 1). Around 50% of the ammonia was converted into NO_3^- which indicates simultaneous heterotrophic activity. N_2O production started as soon as the substrate was added and reached a maximum concentration at the end of the experiment when nitrite accumulated to its highest concentrations. N_2O emissions almost ceased entirely when all the ammonia (and nitrite) was oxidized, even though heterotrophic reduction of

NO_3^- was still going on. The N_2O production rate was in the range of 270 µgN/gTSS*h, which is around 15% of the ammonia oxidation rate or around 34% of the denitrification rate (calculated from the NH_4^+ oxidation rate minus the NO_3^- production and NO_2^- net production rate), and therefore much higher than in NH_4^+ oxidation batch experiments. The N_2O emission and NO_2^- concentration patterns were different to those in the NH_4^+ oxidation experiments, but resembled those in the NO_3^- reduction experiment under anaerobic conditions. Nitrite reduction by HET thus dominates the N_2O production. Given that both the NH_4^+ and the organic substrate are present at non-limiting concentrations, it is to be expected that O_2 limitation will develop in the inner core of bigger sludge flocs (Hamersley and Howes 2002). Thus, heterotrophic denitrification is to be expected inside the flocs, even though some enzymes, like the N_2O reductase, might still be inhibited due to the presence of oxygen.

This section clearly indicates that under aerobic conditions (2 mgO_2/l) and high COD loads, heterotrophs dominate the N_2O production. Additional methods, such as the analysis of the N_2O site-specific isotopic signature, are greatly needed to obtain a more accurate allocation of these emissions to heterotrophic denitrification, nitrifier denitrification and biological hydroxylamine oxidation.

Strategies for reducing N_2O emissions

Our data clearly support nitrifier denitrification by AOB under aerobic conditions as the dominant N_2O production pathway, where NO_2^- positively correlates with N_2O emissions. Biological NH_2OH oxidation is hypothesized to contribute to N_2O production mainly at high NH_4^+ and low NO_2^- concentrations in combination with a high nitrogen oxidation rate.

The production of N_2O by heterotrophic denitrification is likely to be of minor importance when operated without significant NO_2^- accumulation (<2 mgN/l; von Schulthess et al., 1994) and under completely anaerobic conditions.

Therefore, to avoid N_2O emissions, biological wastewater treatment plants should be operated at low NH_4^+ and NO_2^- concentrations, which means a high solid retention time (large population with effective protection against N-peaks and extended denitrification), equalization of load variation (e.g. with digester liquid) and optimal control of the sludge recycling depending on the COD and NO_3^- loads in the anoxic zone.

Under aerobic conditions (2 mgO_2/l) and high COD loads, NO_2^- reduction by HET is the dominant N_2O producer. Low oxygen concentrations (<1 mgO_2/l; Tallec et al., 2006) are therefore expected to reduce the N_2O production due to a higher heterotrophic N_2O reductase activity which is particularly sensitive to oxygen (and inhibited at elevated oxygen concentrations such as at 2 mgO_2/l). Simultaneous nitrification / denitrification at high oxygen concentrations and high COD loads should consequently be avoided.

Conclusions

Batch experiments with activated sludge treating municipal wastewater have shown that N_2O is produced under both nitrifying (aerobic) and denitrifying (anaerobic) conditions:

- AOB are the main N_2O producers under aerobic conditions and low COD loads; the N_2O production pathway is dominated by nitrifier denitrification.
- At high ammonia and low nitrite concentrations in combination with a high nitrogen oxidation rate, biological N_2O production via hydroxylamine oxidation is favored. However, an alternative technique such as the analysis of the N_2O site-specific isotopic signature has to be applied to confirm the activity of this pathway and to further distinguish between the contribution of hydroxylamine oxidation and nitrifier denitrification (Wunderlin et al., 2010; Koba et al., 2009; Sutka et al., 2006).
- The production of N_2O by HET is of minor importance when the plant is operated under optimal growth conditions. Suboptimal conditions, such as the presence of oxygen or the accumulation of nitrite, significantly increased both N_2O and NO production rates. Oxygen input into anoxic zones should therefore be strongly prevented.
- At simultaneous nitrification and denitrification, N_2O production is dominated by heterotrophic NO_2^- reduction, depending strongly on the nitrite concentration, the COD load and the dissolved oxygen concentration. Simultaneous nitrification / denitrification at high oxygen concentrations (2 to 3 mgO2/l) and high COD loads should consequently be avoided.
- This study provides direct evidence that strategies to study and avoid N_2O emissions must consider nitrifier denitrification, hydroxylamine oxidation as well as heterotrophic denitrification.

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Chapter 4

Isotope signatures of N_2O in a mixed microbial population system: Constraints on N_2O producing pathways in wastewater treatment

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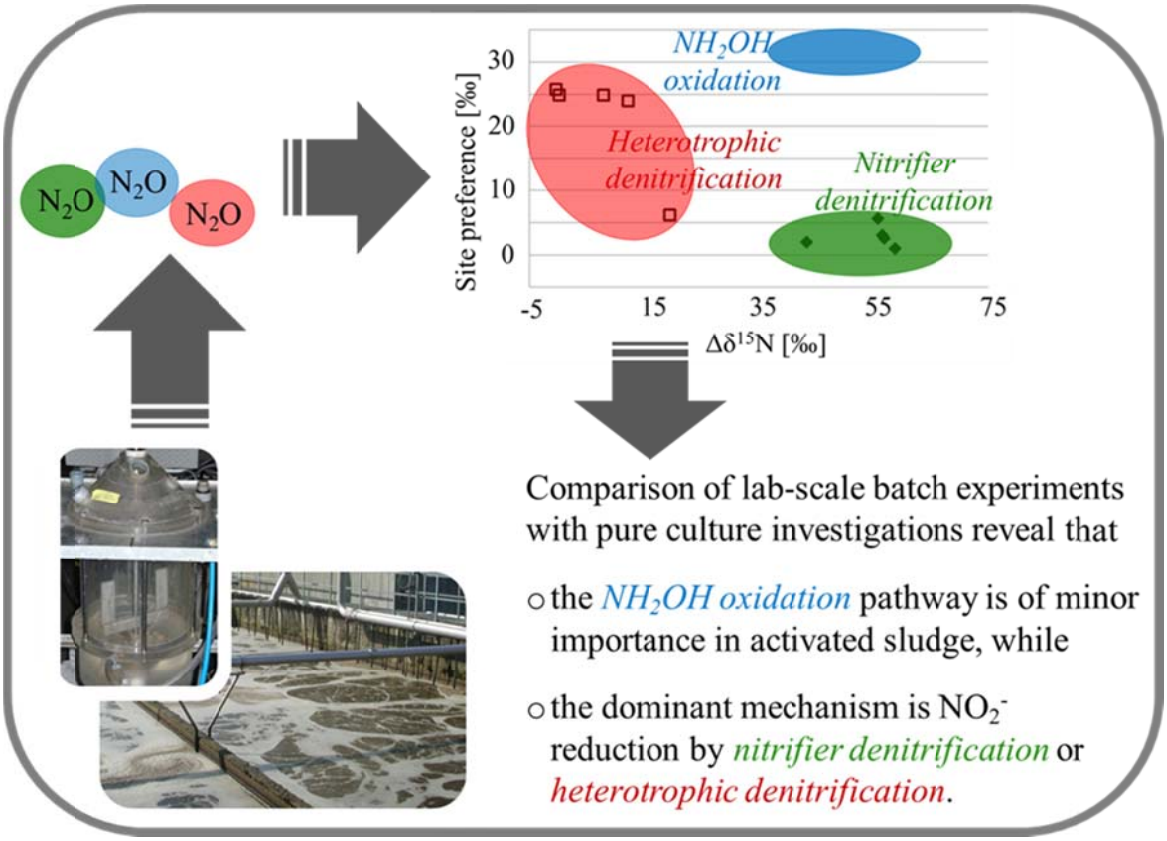
Graphical abstract



Abstract

We present measurements of site preferences (SP) and bulk $^{15}N/^{14}N$ ratios ($\delta\,^{15}N^{bulk}_{N2O}$) of nitrous oxide ($N_2O$) by quantum cascade laser absorption spectroscopy (QCLAS) as a powerful tool to investigate N_2O production pathways in biological wastewater treatment. QCLAS enables high-precision N_2O isotopomer analysis in real time. This allowed us to trace short-term fluctuations in SP and $\delta\,^{15}N^{bulk}_{N2O}$ and, hence, microbial transformation pathways during individual batch experiments with activated sludge from a pilot-scale facility treating municipal wastewater. On the basis of previous work with microbial pure cultures, we demonstrate that N_2O emitted during ammonia (NH_4^+) oxidation with a SP of -5.8 to 5.6 ‰ derives mostly from nitrite (NO_2^-) reduction (e.g. nitrifier denitrification), with a minor contribution from hydroxylamine (NH_2OH) oxidation at the beginning of the experiments. SP of N_2O produced under anoxic conditions was always positive (1.2 to 26.1 ‰), and SP values at the high end of this spectrum (24.9 to 26.1 ‰) are indicative of N_2O reductase activity. The measured $\delta\,^{15}N^{bulk}_{N2O}$ at the initiation of the NH_4^+ oxidation experiments ranged between -42.3 and -57.6 ‰ (corresponding to a nitrogen isotope effect $\Delta\,\delta\,^{15}N = \delta\,^{15}N_{substrate} - \delta\,^{15}N^{bulk}_{N2O}$ of 43.5 to 58.8 ‰), which is considerably higher than under denitrifying conditions ($\delta\,^{15}N^{bulk}_{N2O}$ 2.4 to -17 ‰; $\Delta\,\delta\,^{15}N$ = 0.1 to 19.5 ‰). During the course of all NH_4^+ oxidation and nitrate (NO_3^-) reduction experiments, $\delta\,^{15}N^{bulk}_{N2O}$ increased significantly, indicating net ^{15}N enrichment in the dissolved inorganic nitrogen substrates (NH_4^+, NO_3^-) and transfer into the N_2O pool. The decrease in ^{15}N during NO_2^- and NH_2OH oxidation experiments is best explained by inverse fractionation during the oxidation of NO_2^- to NO_3^-.

Keywords

N_2O production; nitrification; denitrification; pathway identification; stable nitrogen isotopes; isotopomer; laser spectroscopy

Introduction

Nitrous oxide (N_2O) is an important greenhouse gas and a major sink for stratospheric ozone (Ravishankara et al., 2009; S.A. Montzka and S. Reimann et al., 2011). It is estimated that about two thirds of anthropogenic N_2O emissions can be attributed to microbial processes occurring mainly in agricultural soils, or managed lawns but also in biological wastewater treatment (USEPA 2009; Townsend-Small et al., 2011).

Microbial nitrogen (N) transforming processes such as autotrophic nitrification and heterotrophic denitrification have been identified as major N_2O sources. However, the partitioning between these processes with respect to global N_2O emissions and the respective mechanisms are still unclear. There are three main microbial pathways involved in N_2O formation (Figure 1; Kampschreur et al., 2009): (i) N_2O production as a side product during hydroxylamine (NH_2OH) oxidation to nitrite (NO_2^-), probably related to high metabolic activity (Yu et al., 2010), (ii) the reduction of NO_2^- by ammonia-oxidizing bacteria (AOB), known as nitrifier denitrification (Colliver and Stephenson 2000), and (iii) the production of N_2O by heterotrophic denitrifiers (HET), resulting from an unbalanced activity (e.g. due to inhibition) of nitrogen-reducing enzymes (or in some cases from a lack of N_2O reductase; Baumann et al., 1997). The reduction of N_2O to N_2 by HET is currently considered to be the dominant microbial sink for N_2O (Schreiber et al., 2012).

A promising approach to trace N_2O source and sink processes is to analyze the nitrogen (and oxygen) isotope composition and intramolecular distribution of ^{15}N on the central (α) and terminal (β) positions of the asymmetric N_2O molecules (Toyoda and Yoshida 1999; Sutka et al., 2006; Baggs 2008). Bulk nitrogen isotope ratios of N_2O are reported in the conventional δ-notation, in per-mil (‰), $\delta^{15}N = [(R_{N2O}/R_{reference}) - 1] \times 1000$, where R is the nitrogen isotope ratio ($^{15}N/^{14}N$), and atmospheric N_2 (AIR) serves as the reference (Mariotti et al., 1981; Coplen 2011). Analogously, $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ denote the relative enrichment of ^{15}N in the central (N^{α}) position ($^{14}N^{15}N^{16}O$) or in the terminal (N^{β}) position ($^{15}N^{14}N^{16}O$) with respect to the reference. The site preference (SP) is defined as $SP = \delta^{15}N^{\alpha} - \delta^{15}N^{\beta}$ (Brenninkmeijer and Röckmann 1999; Toyoda and Yoshida 1999).

Most of the studies that have used N_2O isotopic measurements so far were field studies, in which the responsible N_2O source processes, for instance, were not well constrained (Westley et al., 2006; Yamagishi et al., 2007; Koba et al., 2009; Toyoda et al., 2009; Ostrom et al., 2010). In this regard, it is helpful that microbial N transformation processes are typically associated with kinetic isotope fractionation, e.g. the discrimination of the ^{15}N-containing molecules during most enzymatic reactions. During nitrification, N_2O is a side-product. Therefore, the nitrogen isotope fractionation is generally higher than during heterotrophic denitrification, where N_2O is assumed to be an obligate intermediate and the isotope fractionation is the net result of production and consumption processes (Figure 1; SI, Table S1; Yamagishi et al., 2007; Koba et al., 2009).

In methodological investigations with pure cultures designed to identify the isotopic signature produced by specific enzymatic pathways, the following SP values were reported: (i) -10.7 ± 2.9 to 0.1 ± 1.7 ‰ for nitrifier denitrification by AOB (Sutka et al., 2003; Sutka et al., 2004; Sutka et al., 2006; Frame and Casciotti 2010), (ii) 30.8 ± 5.9 to 36.3 ± 2.4 ‰ for NH_2OH oxidation (Sutka et al., 2003; Sutka et al., 2004; Sutka et al., 2006; Frame and Casciotti 2010; Santoro et al., 2011), and (iii) -5 to 0 ‰ for N_2O production by HET only (e.g. at inhibited N_2O reductase; Figure 1; SI, Table S1 for a detailed overview; Toyoda et al., 2005; Sutka et al., 2006). A strongly positive SP might also be the result of the subsequent heterotrophic N_2O reduction to N_2, as the ^{14}N-O bond is preferentially broken during this enzymatic transformation step, leading to an enrichment in ^{14}N-^{15}N-O in the residual N_2O (Figure 1; Ostrom et al., 2007; Yamagishi et al., 2007). For N_2O production by the nitrification process, a SP of about 33 ‰ was assumed, mainly on the basis of the findings of a study with *N. europaea* in a pure culture (Sutka et al., 2006; suggesting the importance of NH_2OH oxidation in overall N_2O production by this nitrifier). In contrast, Toyoda et al. (2011) estimated a SP of 4.5 ‰ for N_2O production in an aerated (oxic) tank of a biological wastewater treatment plant and suggested that the contribution of NH_2OH oxidation (with a presumably much higher SP) during the nitrification pathway is less important in mixed cultures.

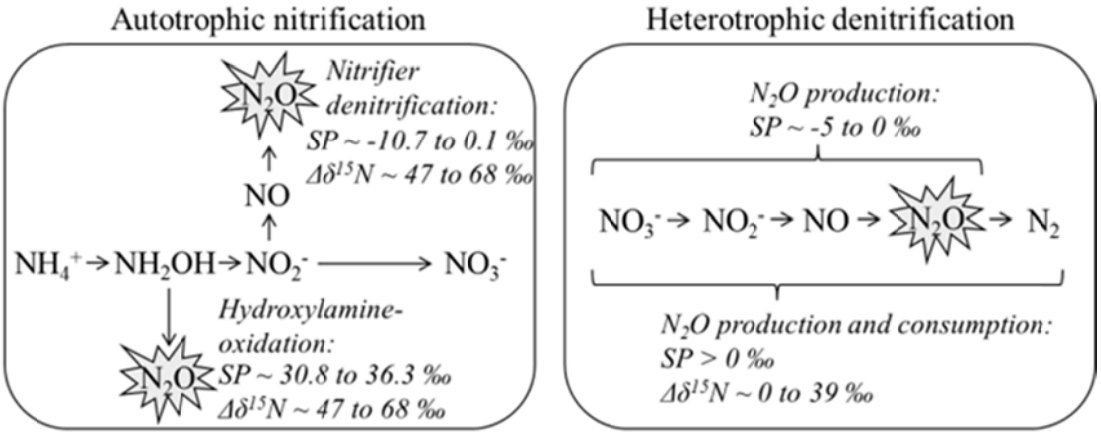


Figure 1. *Scheme of relevant N_2O production pathways in biological wastewater treatment, adapted from Wunderlin et al. (2012), including SP and $\Delta\ \delta^{15}N$ (= $\delta^{15}N_{substrate} - \delta^{15}N^{bulk}_{N2O}$) values reported from cultivation studies (Yoshida 1988; Sutka et al., 2003; Sutka et al., 2004; Toyoda et al., 2005; Sutka et al., 2006; Ostrom et al., 2007; Yamagishi et al., 2007; Koba et al., 2009; Frame and Casciotti 2010; Santoro et al., 2011). Additional information is given in the SI, Table S1.*

The goal of the present study is to identify N_2O production pathways of an activated sludge system in a lab-scale batch reactor operated under specific process conditions. We applied quantum cascade laser absorption spectroscopy (QCLAS) as a novel technique to analyze the site-specific isotope composition of N_2O in real time. The net N_2O nitrogen isotopic signatures are compared to published pure-culture investigations where the active pathways are known. A mixed population system was chosen to more adequately mimic the situation in a full-scale plant, as compared to pure culture investigations. Our lab-scale experiments were performed to test the

following hypotheses: (i) nitrifier denitrification is the dominant N_2O production pathway during nitrification (NH_4^+ oxidation), (ii) NH_2OH addition is conductive to N_2O production by NH_2OH oxidation, while (iii) the addition of NO_2^- to activated sludge fosters N_2O production by nitrifier denitrification, and (iv) HET is assumed to be the main pathway under anoxic conditions with N_2O reductase activity being regulated by the dissolved oxygen concentration (Table 1).

Materials and methods

Experimental setup for batch scale experiments

In the present investigation, batch experiments were carried out with activated sludge sampled from the aerobic (nitrifying) reactor of a pilot scale municipal wastewater treatment plant adapted to NH_4^+ nitrification before the daily NH_4^+ peak load (before 8 a.m.; for more details see SI Text S1). A laboratory batch-scale reactor with a working volume of 6.9 L and a headspace of 1.2 L was used (for more details see Wunderlin et al., 2012). The wastewater temperature was held at 20 ± 1.2 °C. Continuous gas flow was maintained at 1 standard liter per minute using a mass flow controller (Red-y Smart series, Vögtlin Instruments, Switzerland) during both nitrification and denitrification experiments in order to strip N_2O (and NO) from the liquid phase for subsequent analysis. The dissolved oxygen concentration was adjusted by automated oxygen-controlled (by Oxymax H, Endress + Hauser) purging either with high purity nitrogen gas or synthetic air (20.5% O_2 in N_2). Under anoxic conditions, nitrogen gas was continuously purged through the reactor. The pH was measured with a pH electrode (Orbisint CPS11, Endress + Hauser, calibration at pH 7 and 9), and was held constant at 7.1 ± 0.2 using a pH controller via the addition of CO_2 (> 99.9% CO_2, Carbagas).

Nitrification experiments were carried out at oxygen concentrations typical for full-scale plants: 0.5, 1, 2 or 3 mgO_2/l. The experiments were started by adding either NH_4^+, NO_2^- or NH_2OH (25 mg NH_4^+-N/l, NH_4HCO_3 from Merck, Switzerland; 15 mgNO_2^--N/l, $NaNO_2$ from Merck; 2, 5, 10 or 15 mgNH_2OH-N/l, NH_2OH-HCl from Fluka, Switzerland). Denitrification experiments were performed at zero or very low dissolved oxygen concentrations (<0.2 mgO_2/l), and were launched by adding organic carbon (sodium acetate from Merck; always > 100 mg COD/l; COD: chemical oxygen demand) and NO_3^- (10 or 20 mgNO_3^--N/l, $NaNO_3$ from Fluka). Samples were preconditioned to remove ambient NO_3^- present in the activated sludge through denitrification, prior to the start of the actual denitrification experiment with controlled substrate amendments. Preconditioning of the nitrification experiments included continuous aeration of the batch-scale reactor to remove ambient NH_4^+ by nitrification. An overview of the experiments is given in Table 1. The nitrogen isotopic signature of the substrates used in the experiments was analyzed by elemental analyzer (EA) - isotope-ratio mass-spectrometry (IRMS; for more details about the analytical aspects, see SI, Text S2).

Experiments under nitrifying and denitrifying conditions were stopped when N_2O (and NO) production ceased and the dissolved N substrate was completely oxidized (NH_4^+,

NH_2OH or NO_2^- in the nitrification experiments) or reduced (NO_3^- in the denitrification experiments). More detailed descriptions of the experimental setup, the batch experiments and the respective emission data are given in Wunderlin et al. (2012).

Liquid analysis procedures

Grab samples were taken regularly (every 15 to 60 minutes) and were immediately filtered through a 0.7 µm filter (GF/F Whatman) and a 0.45 µm syringe filter (Chromafil Membranfilter from Macherey), before being stored at 4 °C until their analysis within 24 hours. NH_4^+-N was analyzed photometrically using a Foss FIAstar flow injection 5000 analyzer (detection limit 0.2 mgN/l; relative uncertainty 5%). NO_3^--N and NO_2^--N were determined by ion chromatography (761 compact IC, Metrohm; detection limit for both components 0.2 mgN/l; relative uncertainty 5%). Commercial photochemical test kits (Hach Lange GmbH, Düsseldorf, Germany) were used to measure low NO_2^- concentrations (LCK 341; detection limit 0.015 mgN/l) and COD (LCK 314, LCK 414, or LCK 114).

Analysis of N_2O concentration and isotopic composition

The N_2O and NO concentrations in the outflowing gas (off-gas) were continuously (1 minute temporal resolution) analyzed by FTIR spectroscopy (GASMET CX-4000, Temet Instruments, Finland), equipped with a heated (40°C) flow-through gas cell of 9.8 m path length (see SI, Figure S1). The quantification limits for N_2O and NO are 0.25 and 5 ppm respectively, and the expanded standard uncertainty for both components is around 10% (2 σ confidence level; Mohn et al., 2008).

The exhaust gas was dynamically diluted with synthetic air (Messer, Switzerland) to constant N_2O concentrations (around 300 ppb), using two mass flow controllers (Red-y Smart series, Vögtlin Instruments, Switzerland), in order to ensure a high precision of the isotope measurement (see SI, Text S3, Figure S1). At the pump outlet, the pressure was adjusted to 4 bar using a pressure relief valve. Humidity as well as CO_2 were removed from the gas flow to ppm or even ppb levels, respectively, applying a permeation drier (MD-050-72S-1, PermaPure Inc., USA) and a chemical CO_2 trap filled with Ascarite (4 g, 10 - 35 mesh, Fluka, Switzerland) and $Mg(ClO_4)_2$ (2 x 2 g, Fluka, Switzerland). Finally, the sample gas was passed through a sintered metal filter (SS-6F-MM-2, Swagelok, USA) and directed to a preconcentration unit (Mohn et al., 2010). This fully automated liquid-nitrogen free preconcentration device was used to increase the N_2O concentrations by a factor of 200, from ambient level to around 70 ppm N_2O (Mohn et al., 2010; Mohn et al., 2012). The N_2O content from 10 liters of diluted off-gas was adsorbed onto a porous polymer adsorption trap (HayeSep D 100-120 mesh, Hayes Separations Inc., USA) at a flowrate of 500 standard cubic centimeters per minute, then desorbed by resistive heating combined with synthetic air purging, and finally introduced into the evacuated multipass cell of the QCLAS (Waechter et al., 2008). Successive preconcentration cycles resulted in a temporal resolution of 30 minutes for N_2O isotopomer analysis. The accuracy of this procedure was ensured by preconcentration of N_2O with a known isotopic composition (standard I) and

subsequent QCLAS analysis at least once daily. The high precision analysis capacity and equivalence to IRMS has recently been demonstrated in an inter-comparison study (Köster et al., 2013).

The QCLAS employed in this study allowed simultaneous quantification of the three most abundant N_2O isotopic species ($^{14}N^{14}N^{16}O$, $^{15}N^{14}N^{16}O$, $^{14}N^{15}N^{16}O$). For both $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$, a precision better than 0.1 ‰ was obtained with six minutes of spectral averaging at mixing ratios of 70 ppm N_2O. Before and after each experiment, standard gases were analyzed to calibrate the $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ measurements, and to correct for drifts. The standard gas itself was calibrated against primary standards by QLAS. Standard I: $\delta^{15}N^{\alpha}$ = 2.1 ± 0.1‰, $\delta^{15}N^{\beta}$ = 2.0 ± 0.2 ‰, 246.9 ± 0.1 ppm N_2O; standard II: $\delta^{15}N^{\alpha}$ = 25.0 ± 0.1 ‰, $\delta^{15}N^{\beta}$ = 24.8 ± 0.2 ‰, 249.1 ± 0.1 ppm N_2O (the precision indicated is the standard error of the mean). Primary standard gases were analyzed ($\delta^{15}N^{\alpha}$, $\delta^{15}N^{\beta}$ and $\delta^{15}N^{bulk}_{N2O}$) by IRMS at the Tokyo Institute of Technology, Japan (Toyoda and Yoshida 1999).

Table 1. *Summary of batch experiments: added substrates (initial concentrations and their isotopic composition) to activated sludge taken from the nitrification reactor of our pilot plant, and dissolved oxygen concentrations; SP, $\delta^{15}N^{bulk}$ of N_2O (both at the beginning and end of the experiments), $\Delta\ \delta^{15}N$ of produced N_2O and the quantitative interpretation of SP data. NN refers to NH_2OH oxidation, ND to AOB nitrifier denitrification, HET to heterotrophic denitrification and N_2O red. to N_2O reductase activity. Additional information is given in Wunderlin et al (2012).*

Experiment	Added substrate	O_2	$\delta^{15}N^{bulk}_{N2O}$ *		Net N isotope effect ($\Delta\delta^{15}N$)#	Site preference (SP)*		Interpretation of microbial processes based on SP##	
			start	end		start	end	start	end
		[mg/l]	[‰]	[‰]	[‰]	[‰]	[‰]		
NO_2^- oxidation ($\delta^{15}N$ = -30.5‰) Hypothesis: Nitrifier denitrification (ND)	NO_2^- 15 mgN/l	0.6	-64.4		-	0.2		100%ND	
	15 mgN/l	0.6	-56.3	-72.9	25.8	-1.1	-2.1	100%ND	100%ND
	15 mgN/l	1.1	-54.9	-67.1	24.4	-0.2	-1.1	100%ND	100%ND
	15 mgN/l	2.1	-59.7	-72.4	29.2	-3.0	-3.4	100%ND	100%ND
	15 mgN/l	3.1	-57.5	-69.2	27.0	-2.1	-3.9	100%ND	100%ND
NH_2OH oxidation ($\delta^{15}N$ = -71.3‰) Hypothesis: NH_2OH oxidation (NN) and ND	NH_2OH 2 mgN/l	2.1	-62.3		-	25.2		89%NN 11%ND	
	5 mgN/l	2.1	-76.0	-86.3	4.7	26.4	19.9	93%NN 7%ND	72%NN 28%ND
	10 mgN/l	1.1	-63.9	-50.7	-7.4	30.7	3.7	100%NN	19%NN 81%ND
	10 mgN/l	2.2	-68.2	-83.1	-3.1	29.0	20.7	100%NN	74%NN 26%ND
	15 mgN/l	2.3	-70.1	-79.2	-1.2	27.5	25.8	97%NN 3%ND	91%NN 9%ND
	10 mgN/l§	2.1	-65.7	-68.4	-5.6	30.5	30.2	100% NN§§	100% NN§§

	NH_4^+								
NH_4^+ peak addition ($\delta^{15}N$ = 1.15‰) Hypothesis: ND and NN	25 mgN/l	0.6	-57.6	-34.7	58.8	0.9	-2.0	10%NN 90%ND	100%ND
	25 mgN/l	1.0	-55.5	-25.8	56.7	3.0	-1.0	16%NN 84%ND	3%NN 97%ND
	25 mgN/l	1.9	-54.7	-29.0	55.9	5.6	0.4	25%NN 75%ND	8%NN 92%ND
	25 mgN/l	2.0	-55.8	-25.3	57.0	2.5	-2.3	15%NN 85%ND	100%ND
	25 mgN/l	3.1	-42.3	-39.6	43.5	1.9	-1.8	13%NN 87%ND	100%ND
Continuous NH_4^+ addition ($\delta^{15}N$ = 1.15‰) Hypothesis: ND and NN	9 mgN/l	1.1	-54.8	-35.7	56.0	-0.5	-4.5	5%NN 95%ND	100%ND
	9 mgN/l	2.1	-50.2	-31.7	51.4	-1.2	-5.8	3%NN 97%ND	100%ND
	9 mgN/l	3.1	-48	-33.6	49.2	-1.2	-4.9	3%NN 97%ND	100%ND
	15 mgN/l	2.9	-51.4	-30.9	52.6	1.1	-2.3	10%NN 90%ND	100%ND
NO_3^- reduction ($\delta^{15}N$ = 2.5‰) Hypothesis: hetero-trophic denitrification (HET) and N_2O reductase	NO_3^-								
	20 mgN/l	0	2.4	50.1	0.1	24.9	25.4	HET and strong N_2O red.	
	20 mgN/l	0	-5.3	13.2	7.8	24.9	26.1	HET and strong N_2O red.	
	10 mgN/l	<0.1	3.0		-	25.8		HET and strong N_2O red.	
	10 mgN/l	<0.1	-9.6	9.3	12.1	24.0	14.7	HET and low N_2O red.	
	20 mgN/l	0.1-0.2	-17.0	31.5	19.5	6.2	1.2	HET and low N_2O red.	

* SP and $\delta^{15}N^{bulk}_{N2O}$ values at the beginning and the end of experiments, respectively. In experiments with the same start and end value only one sample was analyzed.
[#] Net nitrogen (N) isotope effect calculated from the difference between $\delta^{15}N$substrate and $\delta^{15}N^{bulk}_{N2O}$, by using the first data point, leading to a slight bias since some of the substrate was at that time already oxidized.
[##] Calculated based on the isotopomer mixing model, presented in equation 1.
[§] Control experiment in tap water.
[§§] Assumption that 100 % of N_2O is produced by chemical NH_2OH oxidation.

RESULTS AND DISCUSSION

Isotopic signature of N_2O produced during NO_2^- oxidation experiments

The SP of N_2O produced during NO_2^- oxidation experiments, where nitrifier denitrification is hypothesized to be the dominant pathway, ranged between 0.2 ‰ and -3.9 ‰ (-1.7 ± 1.3 ‰ on average; Figure 2 A; Table 1), which is consistent with previous work. For example, Sutka and co-workers (Sutka et al., 2003; Sutka et al., 2004; Sutka et al., 2006) and Frame and Casciotti (2010) reported average SP values of 0.1 ± 1.7 and -10.7 ± 2.9 ‰ for nitrifier denitrification by *N. multiformis*, *N. europaea* and *N. marina* in batch cultures, respectively (Figure 1). However, our data show a systematic decrease in SP from the beginning (-0.2 to -3 ‰) towards the end of the experiments (-1.1 to -3.9 ‰), when NO_2^- was almost depleted (Table 1). On the basis of their SP data, Toyoda et al. (2005) proposed that during N_2O production by enzymatic NO reduction, one NO molecule is bound to the active center of the NO reductase (e.g. cNOR or qNOR; Hendriks et al., 2000; Stein 2010), followed by the binding of the second NO molecule. Assuming that ^{15}N-O binds preferentially to the active center of the enzyme compared to ^{14}N-O, and that oxygen is abstracted from the NO molecule bound to the active

center, ^{15}N would be enriched at the terminal (β) position (^{15}N-^{14}N-O; resulting in a negative SP). In our experiments, the N_2O production rate was highest at the beginning (highest off-gas concentration of about 140 ppm at a constant air flow rate of 1 standard liter per minute) and steadily decreased in parallel with the NO_2^- concentration (SI, Text S4, Figure S2 A and B, and see also Wunderlin et al., 2012). While we lack an obvious explanation for the observed SP decrease, we speculate that SP is somehow affected by (i) the N_2O production rate, or (ii) by a (slow) switch in N_2O production process in the course of an experiment, for example from a partial contribution of NH_2OH oxidation with a positive end-member SP to 'sole' nitrifier denitrification activity.

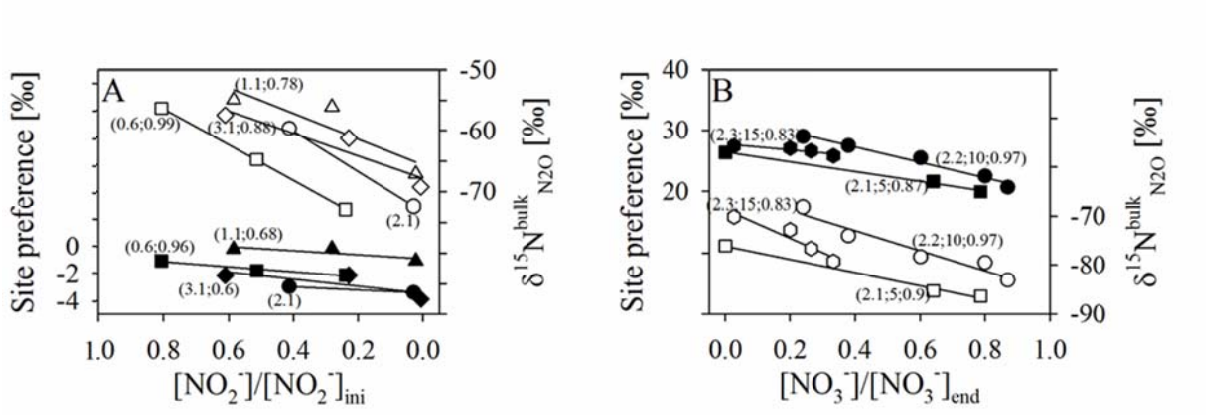


Figure 2. *$\delta^{15}N^{bulk}$ (open symbols) and SP (closed symbols) of N_2O emitted during (A) NO_2^- ($\delta^{15}N$ = -30.5 ‰) oxidation, and (B) NH_2OH ($\delta^{15}N$ = -71.3 ‰) oxidation experiments. The reaction progress is either reported as the ratio of the actual versus the initial NO_2^- concentration (experiment starts at 1.0 in A), or by the ratio of the actual versus the final NO_3^- concentration (experiment starts at 0.0 in B). In parentheses: set-points of dissolved oxygen concentration (in mg/l; left), NH_2OH concentration (in mgN/l; middle in B), and R^2 (right). The uncertainty (2 σ confidence level) for SP and $\delta^{15}N^{bulk}_{N2O}$ is ± 0.3 ‰ and for $[NO_2^-]/[NO_2^-]_{ini}$ and $[NO_3^-]/[NO_3^-]_{ini}$ better than ± 0.1.*

The first measurement of $\delta^{15}N^{bulk}_{N2O}$ was in the range of -54.9 to -59.7 ‰ (Figure 2 A; Table 1). The apparent net N isotope effect ($\Delta\,\delta^{15}N = \delta^{15}N_{NO2-} - \delta^{15}N^{bulk}_{N2O}$; $\delta^{15}N$ of $NaNO_2$ = -30.5 ‰; Table 1), calculated on the basis of the initial N_2O isotopic composition thus lies between 24.4 and 29.2 ‰. However, the actual value for $\Delta\,\delta^{15}N$ may be a few ‰ lower (extrapolation of data shown in Figure 2 A), since about 20 to 40 % of the initial NO_2^- was already oxidized to NO_3^- when the first N_2O sample was analyzed (SI, Text S4, Figure S2 A and B). Nevertheless, our estimated range is consistent with the literature data, where, for example $\Delta\,\delta^{15}N$ of 24.4 and 35.1 ‰ are reported for nitrifier denitrification of *N. multiformis* and *N. europaea*, respectively, in pure culture (Sutka et al., 2003; Sutka et al., 2004; Sutka et al., 2006). In the course of our experiments, $\delta^{15}N^{bulk}_{N2O}$ systematically decreased to values between -67.1 and -72.9 ‰ (Figure 2 A; Table 1), a trend which seems to contradict 'normal fractionation' during progressive substrate depletion, in this case NO_2^- reduction to N_2O (e.g. where the product $\delta^{15}N^{bulk}_{N2O}$ is lower than the substrate $\delta^{15}N$, e.g. $\delta^{15}N$-NO_2^-, and $\delta^{15}N$ of both

substrate and product increase with ongoing reaction). The decreasing trend in $\delta^{15}N$ indicates that the $\delta^{15}N$ of the NO_2^- itself represents a 'moving baseline' which is affected not only by N isotope fractionation during the reduction to N_2O, but also by the oxidation to NO_3^- (preventing the use of a Rayleigh model to calculate N isotope effects for N_2O production). Oxidation of NO_2^- to NO_3^- in the ocean has been reported to be associated with an inverse N isotope effect (e.g. a negative $\Delta\ \delta^{15}N$; Casciotti 2009), and it appears that this effect seems to dominate the isotopic changes of the NO_2^- (and hence of N_2O).

Isotopic signature of N_2O produced during NH_2OH oxidation experiments

During NH_2OH oxidation experiments, where NH_2OH oxidation is hypothesized to be the dominant N_2O production pathway, the SP of N_2O ranged between 26.4 and 30.7 ‰ at the beginning of the experiments (28.4 ± 1.9 ‰ on average; Figure 2 B; Table 1). This was significantly higher than the SP observed during NO_2^- oxidation experiments (SP in the range of -0.2 to -3.0 ‰; Figure 2 A), where nitrifier denitrification by AOB was the dominant N_2O producing process. But in agreement with previous work, where average SP values of 32.5 ± 0.6 ‰, 33.5 ± 1.2 ‰ and 30.8 ± 5.9 ‰ were measured for the N_2O production via NH_2OH oxidation by *N. multiformis*, *N. europaea* and *Methylococcus capsulatus*, respectively (Sutka et al., 2003; Sutka et al., 2004; Sutka et al., 2006). SP values of 36.3 ± 2.4 ‰ were determined for the marine β-proteobacterium *Nitrosomonas marina C-113a* (Figure 1; Frame and Casciotti 2010). The systematic decrease in SP in the course of our experiments (Figure 2 B), in addition to the decreasing trend observed for $\delta^{15}N^{bulk}{}_{N2O}$ (see below), underscores that NH_2OH oxidation is dominant at the beginning of the experiments, while nitrifier denitrification becomes important with ongoing depletion of NH_2OH substrate. By assuming an average SP of 28.5 ‰ for pure N_2O production via NH_2OH oxidation (SP_{NN}; this section) and an average SP of -2 ‰ for AOB nitrifier denitrification (SP_{ND}; see section before), the partitioning of both processes with regards to total N_2O production can be estimated (F_{ND} and F_{NN}, respectively), even though a marginal contribution from additional (not considered) pathways cannot be completely excluded (equation 1; Frame and Casciotti 2010):

$$F_{ND} = (1 - F_{NN}) = \frac{(SP_{tot} - SP_{NN})}{(SP_{ND} - SP_{NN})} \qquad \text{Equation 1}$$

Accordingly, a site preference (SP_{tot}) generally in between of 20 and 25 ‰ (as observed towards the end of several of the experiments shown in Figure 2 B) suggests that ~12 to ~28 % of the total N_2O production in the nitrifying batch experiments can eventually be attributed to nitrifier denitrification (F_{ND}). At the extreme, in one of the experiments at 1.1 mgO_2/l (Table 1; SI, Figure S2 C), we measured a SP of 3.7 ‰ at the end of the experiment when NH_2OH is low suggesting that nitrifier denitrification by AOB can almost completely take over N_2O production (see also Figure 3 A).

The exact mechanism of N_2O formation during NH_2OH oxidation is unknown, but can be hypothesized that it is produced either via chemical breakdown of nitroxyl radicals (NOH; Law et al., 2012), or via NO production by hydroxylamine oxidoreductase (HAO), which is subsequently reduced to N_2O by an NO reductase such as cytochrome c_{554} (Upadhyay et al., 2006; Chandran et al., 2011). For both scenarios, a positive SP can be interpreted as an indicator of a reaction via a symmetric intermediate, where the cleavage of the ^{14}N-O bond is preferred over that of the ^{15}N-O bond, resulting in a ^{15}N enrichment at the central (α) position (^{14}N-^{15}N-O; Schmidt et al., 2004).

The $\delta^{15}N^{bulk}$ values of N_2O produced at the beginning of the NH_2OH oxidation experiments were in the range of -63.9 to -76 ‰ (Figure 2 B). On the basis of these values, $\Delta\ \delta^{15}N$ between -7.4 and 4.7 ‰ were estimated ($\delta^{15}N$ of NH_2OH-HCl = -71.3 ‰; Table 1). Yet, the observed N isotope effect range is consistent with reports from the literature, with $\Delta\ \delta^{15}N$ values for N_2O production via NH_2OH oxidation of -2 ‰ for *N. europaea* and *N. multiformis*, and -5.7 ‰ for *M. trichosporium* respectively (Sutka et al., 2006). A similar value was measured for *N. capsulatus* (-3.1 ‰; Sutka et al., 2003; Sutka et al., 2004) and for the marine β-proteobacterium *Nitrosomonas marina C-113a* (6.7 ‰; Frame and Casciotti 2010). As in the NO_2^- oxidation experiments, we observed systematically decreasing $\delta^{15}N^{bulk}_{N2O}$ values to -79.2 to -86.3 ‰ with progressive reaction (Figure 2 B). This trend is best explained by the above mentioned increasing contribution of nitrifier denitrification activity by AOB to the total N_2O production. Indeed, NO_2^-, the precursor substrate of N_2O in nitrifier denitrification, accumulated by up to 0.4 mgN/l during the experiment (in parallel with N_2O), yielding maximum off-gas concentrations of about 60 ppm (SI, Text S4, Figure S2 C and D). The inverse fractionation during partial NO_2^- oxidation to NO_3^- can explain the negative trends as described for the NO_2^- oxidation experiments (see above and Casciotti 2009). In the experiment at 1.1 mgO_2/l (shown in SI, Figure S2 C and D), we monitored the N_2O isotopic signature until NH_2OH was almost completely oxidized (in contrast to the other NH_2OH oxidation experiments shown in Figure 2 B): at very low N_2O production rates during the second half of the incubation, $\delta^{15}N^{bulk}_{N2O}$ increased again from a minimum of -81.1 to -50.7 ‰. We consider this increase in $\delta^{15}N^{bulk}_{N2O}$ to be the result of a paralleling increase of the $\delta^{15}N$ of the precursor N (NH_2OH and NO_2^-) and N_2O pools according to Rayleigh distillation kinetics, dominating the overall N isotope dynamics towards substrate depletion.

In a control experiment, where we added NH_2OH to tap water (without activated sludge), N_2O was produced with an average $\delta^{15}N^{bulk}$ value of -67.2 ± 1.8 ‰ ($\Delta\ \delta^{15}N$ of around -4.1 ± 1.8 ‰) and an average SP of 30.3 ± 0.2 ‰ (Table 1). SP remained essentially invariant and only a minor decrease in $\delta^{15}N^{bulk}_{N2O}$ (from -65.7 to -68.4 ‰) was observed. As NO_2^- production was marginal in the absence of activated sludge (data not shown), we conclude that the observed signatures are characteristic for purely inorganic NH_2OH oxidation. Indeed, Toyoda et al. (2005) reported an average SP value of 30.1 ‰ for inorganic NO_2^- reduction and 29.5 ‰ for inorganic NH_2OH oxidation respectively, and proposed an N_2O formation mechanism via hyponitrite ($N_2O_2^{2-}$), a symmetric

intermediate. Also here, the positive SP can be explained by the preferential cleavage of the ^{14}N-O bond of the -O-^{14}N-^{15}N-O- molecule (similar to the mechanism discussed above).

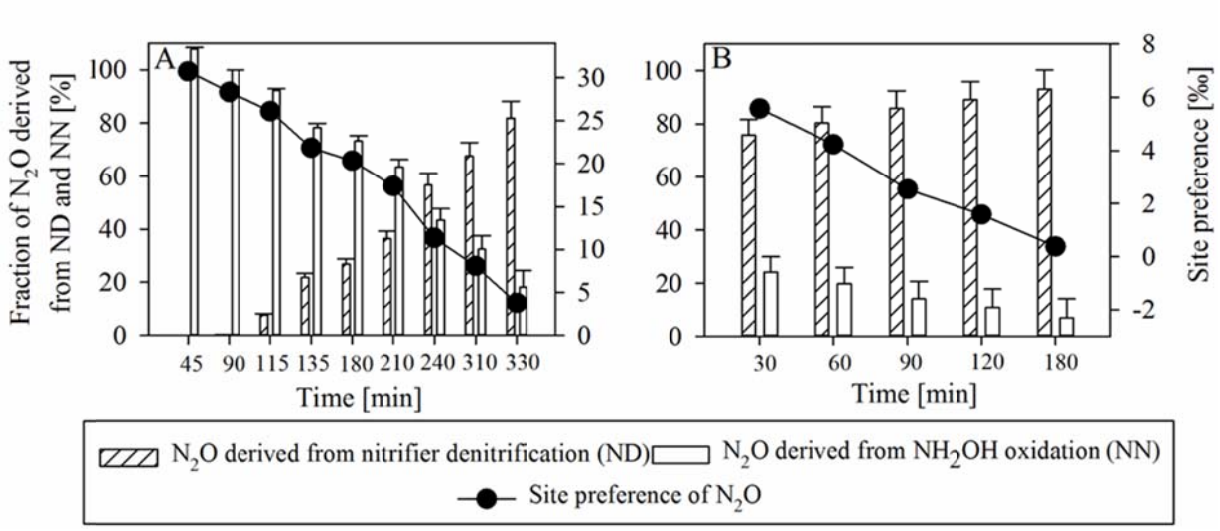


Figure 3. *Quantitative apportioning of AOB nitrifier denitrification (ND) and NH_2OH oxidation (NN) according to the observed SP and the isotopomer mixing model (equation 1) during (A) NH_2OH oxidation at 1.1 mgO_2/l and (B) NH_4^+ oxidation (peak addition) at 1.9 mgO_2/l. Substrate was added at time zero. The uncertainty (2 σ confidence level) for SP is ± 0.3 ‰*

Isotopic signature of N_2O produced during NH_4^+ oxidation experiments

During 'conventional' nitrification (NH_4^+ oxidation) where nitrifier denitrification was hypothesized to be the dominant N_2O production pathway, the SP of the N_2O (determined at the onset of the experiments) ranging from 0.9 to 5.6 ‰ and from -5.8 to 1.1 ‰ during peak and continuous NH_4^+ addition, respectively (Figure 4 A; Figure 5; Table 1). In the peak addition experiments, SP decreased to values of 0.4 to -2.3 ‰ with ongoing reaction, similar to the values observed in the NO_2^- oxidation experiments and reported for nitrifier denitrification in pure culture (see section 'Isotopic signature of N_2O produced during NO_2^- oxidation experiments'). O_2 and NH_4^+ concentrations seemed to influence the SP, with higher SP values at elevated dissolved O_2 and high NH_4^+ concentrations at the beginning of the experiment (Figure 4 B). For example, the SP ranged between 2.5 and 5.6 ‰ at ~2 mgO_2/l, and was significantly lower (0.9 ‰) at 0.6 mgO_2/l (at the same initial NH_4^+ concentration). SP was minimal towards the end of the experiment when NO_2^- concentrations peaked and almost all the NH_4^+ was consumed (Figure 4 B and C, Figure 5 A and B). This trend further underscores the growing importance of nitrifier denitrification by AOB with ongoing reaction and the progressive depletion of NH_4^+ and the accumulation of NO_2^- (see also Wunderlin et al., 2012). In contrast, during continuous NH_4^+ addition (Figure 5 C and D), SP was quite constant and only decreased after the cessation of NH_4^+ addition (and the putative shift to nitrifier denitrification dominated N_2O production as described above). Our SP data

further indicate that an increase in NH_4^+ concentration from 9 to 15 mgN/l fosters N_2O production by NH_2OH oxidation (Table 1).

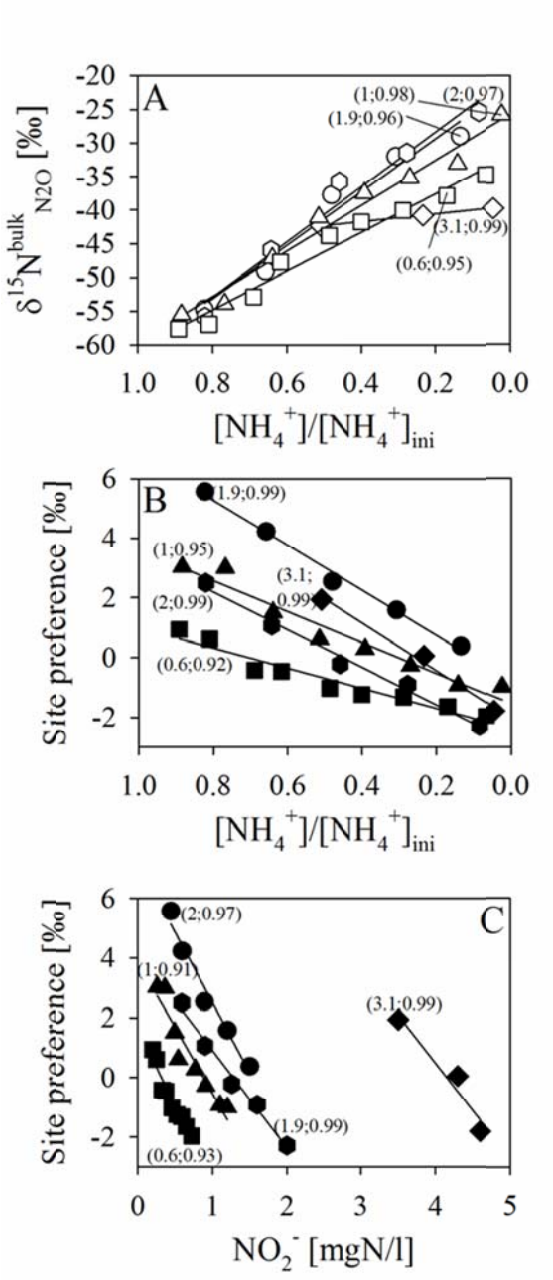


Figure 4. *(A) $\delta^{15}N^{bulk}$, and (B) SP of N_2O produced during NH_4^+ ($\delta^{15}N$ = 1.15 ‰) oxidation experiments. The concentration of NH_4^+ is expressed as the ratio of the instantaneous NH_4^+ concentration to the initial NH_4^+ concentration (experiment starts at 1.0). (C) SP of N_2O emitted as a function of the NO_2^- concentration (experiment starts at 0 mgN/l). Note that in the experiment at 3 mgO_2/l, a rapid NO_2^- built-up occurred, and therefore no SP value was obtained at lower NO_2^- concentrations. In parentheses: set-point of dissolved oxygen concentrations (in mg/l; left) and R^2 (right). The uncertainty (2 σ confidence level) for SP and $\delta^{15}N^{bulk}_{N2O}$ is ± 0.3 ‰, and for $[NH_4^+]/[NH_4^+]_{ini}$ better than ± 0.05.*

Similar to the approach described above (equation 1), and assuming average SPs of 28.5 ‰ for pure N_2O production via NH_2OH oxidation (derived from NH_2OH oxidation

experiments) and of -2 ‰ for NO_2^- reduction (derived from NO_2^- oxidation experiments) respectively, the contribution from both pathways can be assessed, even though the marginal contribution from other pathways cannot be completely excluded at this point. Accordingly, a site preference (SP_{tot}) of 0.9 to 5.6 ‰ at the onset of the peak addition experiments indicates a maximum contribution of NH_2OH oxidation to the total N_2O production of about 25 %. This contribution systematically decreases to less than ~7 % towards the end of the experiments (Figure 3 B). This observation appears to be qualitatively consistent with a recent study by Yu et al. (2010), who speculated that during initial N_2O production in a pure *N. europaea* culture NH_2OH oxidation is more important because NH_2OH accumulates transiently at high NH_4^+ oxidation rates, e.g. under oxic conditions in an ammonia-fed chemostat or at high NH_4^+ loads (Chandran et al., 2011). Furthermore, in a nitritation system, the NH_4^+ oxidation rate correlated exponentially with the specific N_2O production rate, which can be explained by the chemical breakdown of NOH (presumably formed during NH_2OH oxidation; Law et al., 2012), and in a pure culture of *N. europaea* it was shown that increased dissolved oxygen concentrations decreased the relative importance of nitrifier denitrification to NH_2OH oxidation (Sutka et al., 2006; Frame and Casciotti 2010). In other mixed culture nitrification experiments, the observed SP values suggest a dominance of nitrifier denitrification (over NH_2OH oxidation) with regard to N_2O production (Toyoda et al., 2011).

The $\delta\,^{15}N^{bulk}$ of N_2O emitted at the onset of NH_4^+ oxidation experiments ranged between -42.3 and -57.6 ‰ (Figure 4 A), which translates into $\Delta\,\delta\,^{15}N$ values of 43.5 to 58.8 ‰ ($\delta\,^{15}N$ of NH_4HCO_3 = 1.15 ‰; Table 1) (again being slightly biased by a potential ^{15}N enrichment prior to the first measurement). Nevertheless, these $\Delta\,\delta\,^{15}N$ values are similar to data reported for N_2O production via nitrification (50 ‰ by Toyoda et al., 2011; 47 to 68 ‰ by Koba et al., 2009; 56.9 ‰ by Frame and Casciotti 2010; Figure 1). In the course of our incubation experiments, $\delta\,^{15}N^{bulk}{}_{N2O}$ values systematically increased to -25.3 to -39.6 ‰ due to ^{15}N enrichment in the substrate N pool (NH_4^+ and transformation products) as result of N isotopic fractionation according to the Rayleigh distillation kinetics during the stepwise oxidation to NO_2^- and NO_3^- (Figure 4 A; SI, Text S2 and S5, Figure S4 A). In the experiment with 3.1 mgO_2/l, the initial $\delta\,^{15}N^{bulk}{}_{N2O}$ was significantly higher (-42.3 ‰) than in the other NH_4^+ oxidation experiments (-54.7 to -57.6 ‰), and thus the net $\Delta\,\delta\,^{15}N$ was significantly lower (43.5 ‰). In addition, NO_2^- accumulation was highest in the high-O_2 experiment (up to almost 5 mgN/l; Figure 4 C), indicating that the NO_2^- oxidation to NO_3^- was the limiting step. In a mixed population system, the $\Delta\,\delta\,^{15}N$ naturally depends on the importance of each single transformation process. However, there are ambiguities with respect to the relationship between biogeochemical process reaction rate and N isotope fractionation (e.g. Kritee et al., 2012), and the reduction in N isotope fractionation is often linked to low substrate concentrations (e.g. Lehmann et al., 2007; Granger et al., 2008). In the high-O_2 experiment (at 3.1 mgO_2/l), NO_2^- was clearly not limiting N_2O production, and hence we assume that it is the high NH_4^+ oxidation rate in combination with a comparatively sluggish NO_2^- oxidation, which leads to NO_2^- accumulation and in turn to the partial

expression of an inverse fractionation, that ultimately results in an overall lowered net N isotope effect for N_2O production.

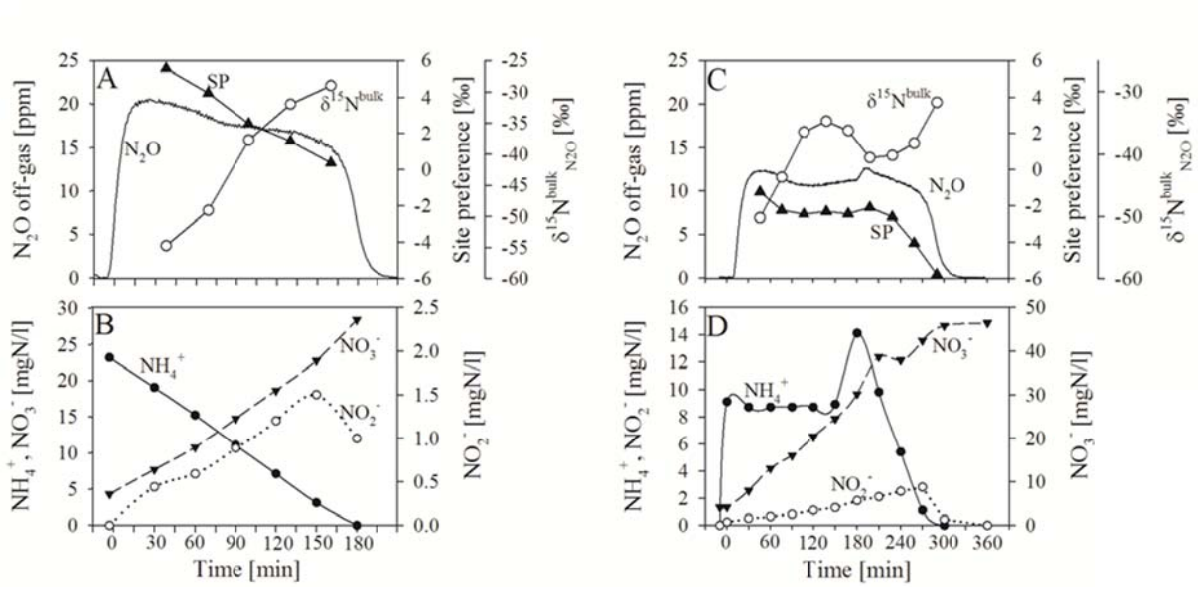


Figure 5. *N_2O data time series of NH_4^+ ($\delta^{15}N$ = 1.15 ‰) peak addition (at 1.9 mgO_2/l; A and B) and NH_4^+ continuous addition (at 1.9 mgO_2/l; C and D) experiments; dissolved nitrogen species (NH_4^+, NO_2^-, NO_3^-), N_2O concentrations, as well as isotopic composition (adapted from Wunderlin et al., 2012, where additional information about nitrogen conversion rates, N_2O production rate, yield and a detailed discussion of concentration / emission patterns is provided).*

Isotopic signature of N_2O produced during NO_3^- reduction experiments

Under anoxic conditions, N_2O SP values of around 25 ‰ seem to be consistent with previous work (Ostrom et al., 2007; Yamagishi et al., 2007; Koba et al., 2009), where an increasing SP was explained by the partial reduction of N_2O to N_2 (Figure 6). However, Toyoda et al. (2005) determined SP values of ~24 ‰ for *Pseudomonas fluorescens*, even though N_2O reduction was inhibited by acetylene . At low levels of dissolved oxygen (< 0.1 - 0.2 mgO_2/l) and in the presence of accumulated NO_2^- (> 4 mgN/l), SP values lower than 25 ‰ indicate the production of N_2O by heterotrophic denitrification, with reduced impact from N_2O reduction, since N_2O production by heterotrophic denitrification in absence of N_2O reduction results in SP values of between 0 and -5 ‰ (Figure 1 and SI, Table S1; Toyoda et al., 2005; Sutka et al., 2006). Our interpretation of the N_2O isotopic signatures is in accordance with the N_2O emission profiles and emission rates under varying dissolved oxygen concentrations presented in Wunderlin et al. (2012), assuming (partial) inhibition of the N_2O reductase at low dissolved oxygen concentrations, or the presence of NO_2^- (> 4 mgN/l).

Under anoxic conditions (0 mgO_2/l), initial $\delta^{15}N^{bulk}$ values were in the range from -17 to 2.4 ‰, and increased systematically to values between 9.3 and 50.1 ‰ at the end of the experiments (Figure 6; Table 1). That is, the N_2O generated had a significantly higher $\delta^{15}N^{bulk}$ than was observed during NH_4^+ oxidation conditions (-42.3 to -57.6 ‰), albeit

similar $\delta^{15}N$ substrates ($\delta^{15}N$ of $NaNO_3$ = 2.5 ‰; $\delta^{15}N$ of NH_4HCO_3 = 1.15 ‰). The isotopic signature of dissolved NO_2^- and NO_3^- (SI, Text S2 and S5, Figure S4 B) indicates that during heterotrophic denitrification the change of the $\delta^{15}N^{bulk}_{N2O}$ (-17 to 50.1 ‰) paralleled that of the precursor NO_2^- (-23.5 to 43 ‰) within the respective experiments, with a consistent $\delta^{15}N$ offset between the two N pools due to the N isotope fractionation during NO_3^- reduction to N_2O. The $\Delta\ \delta^{15}N$ (= $\delta^{15}N_{NO3-}$ - $\delta^{15}N^{bulk}_{N2O}$) of N_2O produced during heterotrophic denitrification ranged between 0.1 and 19.5 ‰, a range that includes values reported from pure culture investigations (~13 ‰ for NO_2^- reduction; Sutka et al., 2006). However, our values are at the lower end of data reported for heterotrophic denitrification in the natural environment (0 to 39 ‰; Koba et al., 2009; Figure 1). This might partly be due to an underestimation of our $\Delta\ \delta^{15}N$ values, since a significant fraction of the initial NO_3^- was already reduced before the first N_2O sample was collected.

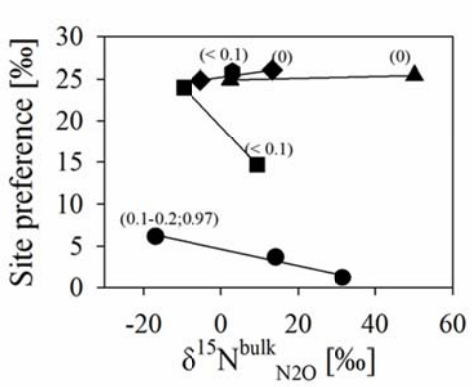


Figure 6. *SP is plotted against $\delta^{15}N^{bulk}_{N2O}$ under NO_3^- ($\delta^{15}N$ = 2.5 ‰) reducing conditions (experiments start at low $\delta^{15}N^{bulk}_{N2O}$). In parentheses: set-point of dissolved oxygen concentration (in mg/l). The uncertainty (2 σ confidence level) for SP and $\delta^{15}N^{bulk}_{N2O}$ is ± 0.3 ‰.*

The isotopic signature of N_2O as a tool to differentiate between the contribution of nitrification and heterotrophic denitrification

During the multi-step process of NH_4^+ oxidation, the SP of N_2O indicates that NO_2^- reduction, presumably by AOB nitrifier denitrification, is the dominant N_2O production pathway in the investigated activated sludge system. However, NH_2OH oxidation contributes up to about 25 % to the total N_2O production, preferentially at dissolved O_2 concentrations higher than 1 mgO_2/l and NH_4^+ concentrations higher than 10 mgN/l. On the other hand, the isotopic signature of the N_2O produced during heterotrophic denitrification is significantly different from nitrification-derived N_2O. Still, N_2O source partitioning remains a difficult task under low dissolved oxygen concentrations where, in addition to NH_2OH oxidation and nitrifier denitrification by AOB, heterotrophic denitrification may also contribute significantly to N_2O production. Concomitant N_2O reduction by HET further complicates the interpretation of net N_2O isotope effects.

Our results underscore the value, but also the limitations, of N_2O isotopomer analysis in investigating the pathways of, and controls on, N_2O production and co-occurring N transformations in biological (municipal) wastewater treatment. Especially in combination with parameters commonly reported in wastewater treatment, such as the concentrations of dissolved O_2, N species, COD, but also N_2O and NO, the isotopic signature of N_2O provides additional insight into the pathways directly involved in N_2O production. Our data elucidating the N isotope dynamics associated with N_2O production in wastewater treatment may be integrated in future numerical models in order to establish a more quantitative framework for predicting and understanding N_2O production in biological wastewater treatment plants.

Nomenclature

Isotopomer:	Molecules containing the same isotopes but with differing isotope positions; e.g. N_2O with ^{15}N in the central (^{14}N-^{15}N-O) or the end (^{15}N-^{14}N-O) position (Müller 1994)
R:	Nitrogen isotope ratio, $^{15}N/^{14}N$
$\delta\,^{15}N_{compound}$:	$(R_{compound} - R_{ref}) / R_{ref}$ x 1000, with atmospheric nitrogen as the reference material (ref)
$\delta\,^{15}N^{\alpha}$ and $\delta\,^{15}N^{\beta}$:	Relative differences of isotope ratios for the inner (α) and the outer (β) nitrogen atom in the asymmetric N_2O molecule
SP:	Site preference, the difference between the $\delta\,^{15}N^{\alpha}$ and $\delta\,^{15}N^{\beta}$
$\delta\,^{15}N^{bulk}{}_{N2O}$:	The average between $\delta\,^{15}N^{\alpha}$ and $\delta\,^{15}N^{\beta}$ in N_2O
$\Delta\,\delta\,^{15}N$:	The net nitrogen (N) isotope effect, which approximates the apparent fractionation of a multiple step reaction, expressed as the difference between $\delta\,^{15}N$substrate and $\delta\,^{15}N^{bulk}{}_{N2O}$; according to Koba et al. (2009) and Sutka et al. (2006).

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Supporting information available

Additional information about the analysis of the nitrogen isotopic composition of dissolved nitrogen species, the experimental setup for the analysis of N_2O isotopic composition, and the time series of NO_2^- oxidation and NH_2OH oxidation experiments is given in the supporting information. This information is available free of charge via the Internet at http://pubs.acs.org/.

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Supporting Information for Chapter 4

Isotope signatures of N_2O in a mixed microbial population system: Constraints on N_2O producing pathways in wastewater treatment

This chapter has been published as:

Wunderlin, P.; Lehmann, M. F.; Siegrist, H.; Tuzson, B.; Joss, A.; Emmenegger, L.; Mohn, J. Isotope signatures of N_2O in a mixed microbial population system: Constraints on N_2O producing pathways in wastewater treatment. *Environmental Science and Technology,* **2013**, *47* (3), 1339-1348.

Table S1. *Reported values of characteristic nitrogen isotopic signatures (SP, $\Delta\ \delta^{15}N$) for NH_2OH oxidation, nitrifier denitrification, and heterotrophic denitrification.*

SP [‰]	Isotope fractionation [‰]	Species	Reference
NH_2OH oxidation			
30.8 ± 5.9	-3.1	*Methylococcus capsulatus*	Sutka et al. (2003); Sutka et al. (2004)
33.5 ± 1.2	-2	*Nitrosomonas europaea*	Sutka et al. (2006)
32.5 ± 0.6	-2	*Nitrosomonas mulitiformis*	Sutka et al. (2006)
35.6 ± 1.4	-5.7	*Methylosinus trichosporium*	Sutka et al. (2006)
36.3 ± 2.4[a]	6.7[a]	*Nitrosomonas marina C-113a*	Frame and Casciotti (2010)
29.5 ± 1.1	n.d.	*Inorganic NH_2OH oxidation*	Toyoda et al. (2005)
Nitrification: NO_2^- reduction by nitrifier denitrification			
-0.8 ± 5.8	35.1	*Nitrosomonas europaea*	Sutka et al. (2003); Sutka et al. (2004)
0.1 ± 1.7	24.4	*Nitrosomonas multiformis*	Sutka et al. (2006)
-10.7 ± 2.9[b]	56.9[b]	*Nitrosomonas marina C-113a*	Frame and Casciotti (2010)
Nitrification: Combination of NO_2^- reduction by nitrifier denitrification and/or NH_2OH oxidation			
31.4 ± 4.2	46.9	*Nitrosomonas europaea*	Sutka et al. (2006)
30.8 ± 4.4	n.d.	*Marine ammonia oxidizing archaea*	Santoro et al. (2011)
n.d.	68	*Nitrosomonas europaea*	Yoshida (1988)
4.5[c]	48.4 ± 0.3[c]	*Mixed population system; WWTP*	Toyoda et al. (2011)
Heterotrophic denitrification: NO_3^- and/or NO_2^- reduction			
-0.6 ± 1.9[d]	12.7[d]	*Pseudomonas chlororaphis*	Sutka et al. (2006)
-0.5 ± 1.9[d]	n.d.[d]	*Pseudomonas aureofaciens*	Sutka et al. (2006)
-0.5 ± 1.9[e]	n.d.[e]	*Pseudomonas chlororaphis*	Sutka et al. (2006)
-0.5 ± 0.6[e]	36.7[e]	*Pseudomonas aureofaciens*	Sutka et al. (2006)
23.3 ± 4.2[f]	17 to 39[f]	*Pseudomonas fluorescens*	Toyoda et al. (2005)
-5.1 ± 1.8[f]	10 to 22[f]	*Paracoccus denitrificans*	Toyoda et al. (2005)

[a] NH_4^+ oxidiation: Dominant contribution from NH_2OH oxidation (experiment at high O_2 concentrations).

[b] Dominant contribution from nitrifier denitrifcation (experiment at low O_2 concentrations).

[c] Nitrifier denitrification as dominant N_2O production pathway in the oxic tank.

[d] NO_2^- eduction

[e] NO_3^- eduction

[f] From NO_3^- to N_2O

Text S1. Characterization of the activated sludge and the pilot-scale treatment plant

Batch experiments were carried out with activated sludge taken from the nitrification reactor before the daily NH_4^+ peak load (before 8 a.m.) of the pilot scale facility at Eawag treating municipal wastewater. The plant consists of a primary clarification unit followed by activated sludge treatment (pre-denitrification, nitrification) and secondary clarification (sludge recirculation was twice the influent flow). It was operated with a solids retention time of ~20 - 22 days (10 - 11 days aerobic) and total suspended solid (TSS) concentrations between 4 and 4.4 g/l. Nitrification was complete, with average total nitrogen removal above 60%. The nitrification reactor is operated at dissolved oxygen concentrations in the range from 0.5 to 2 mgO_2/l. Therefore, batch-scale experiments were carried out at dissolved oxygen concentrations in a similar range (0.5 to 3 mgO_2/l). The dissolved nitrogen species in the activated sludge tanks were usually below 10 mgN/l.

Text S2. Analytical details of the nitrogen isotopic composition of substrates and dissolved nitrogen species

For measuring $\delta\,^{15}N$, aliquots of the samples (NH_4HCO_3, $NaNO_2$, NH_2OH-HCl and $NaNO_3$) were weighed into tin capsules (Säntis). Samples were combusted at 1020 °C with excess oxygen in an elemental analyzer (Thermo quest, CE instruments) and the resulting combustion gases passed through a reduction furnace at 650 °C. After removal of water with magnesium perchlorate and purification in a gas chromatographic column, N_2 was measured on-line with an isotope-ratio mass-spectrometer (IRMS; Micromass). Nitrogen isotope ratios are reported in the conventional delta notation with respect to atmospheric N_2 (AIR). The analytical reproducibility is ± 0.2 ‰ for $\delta\,^{15}N$.

In selected experiments, the isotope signature of dissolved NO_x (= NO_3^- + NO_2^-) and NO_3^- was measured: (i) in the ammonia oxidation experiment at 2.1 mgO_2/l with an initial ammonia concentration of ~25 mgN/l, and (ii) in the denitrification experiment under anoxic conditions with an initial nitrate concentration of ~20 mgN/l (Figure S3 and S4).

Nitrogen (N) isotope ratio measurements of naturally occurring NO_x (= NO_2^- + NO_3^-) were performed using the denitrifier method (Sigman et al., 2001; Casciotti et al., 2002). Briefly, sample NO_x or NO_3^- (after nitrite removal from the sample) is quantitatively converted to N_2O by cultured denitrifying bacteria. The N_2O was automatically extracted, purified and analyzed on-line using a gas-bench preparation system coupled to a continuous flow isotope-ratio mass-spectrometer (CF-IRMS; Thermo Finnigan DeltaPlus XP). The general target sample size was 50 nmol *N. Pseudomonas chlororaphis* (ATCC #13985; formerly *Pseudomonas aureofaciens*) were used for the NO_x conversion. Data were corrected for the procedural blank contribution, which was always less than 1% of the sample size. N isotope ratios are reported in the conventional

delta (δ) notation in per-mil (‰): $\delta^{15}N = (R_{sample}/R_{reference}) - 1] \times 1000$, where R represents the ratio of ^{15}N to ^{14}N in the sample and in atmospheric N_2 (AIR) respectively. Isotope values were calibrated using international NO_3^- reference materials (IAEA-N3, USGS-34) with assigned $\delta^{15}N$ values of 4.7 and -1.8 ‰ (versus atmospheric N2) respectively. The analytical precision of the method is generally better than ± 0.2 ‰. For separate $\delta^{15}N$-NO_3^- measurement, NO_x samples were amended with 20 µL per 1 ml sample of 1% sulfamic acid (dissolved in 10 % vol / vol HCl) solution to remove any nitrite in the sample prior to the bacterial conversion of the remaining NO_3^- to N_2O (Granger and Sigman 2009). $\delta^{15}N$-NO_2^- was then derived on the basis of the following isotope mass balance equation (1):

$$\delta^{15}N_{NO_2^-} = \left\{\delta^{15}N_{NO_x} * [NO_x] - \delta^{15}N_{NO_3^-} * [NO_3^-]\right\} / [NO_2^-] \qquad [1]$$

It is important to note that this indirect approach can lead to a significant error for $\delta^{15}N$ of NO_2^- in the low-nitrite / high-nitrate samples. Samples with nitrite concentrations lower than 0.2 mgN/l were not considered.

Text S3. Details of experimental setup and analysis of N_2O isotopic composition

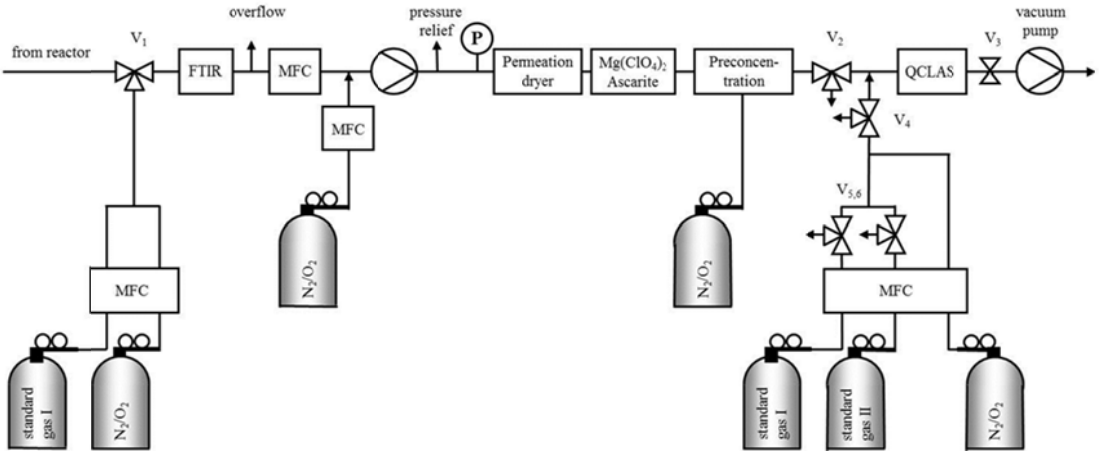

Figure S1. *Experimental setup for analysis of N_2O mixing ratios and its site-specific isotopic composition. The reactor setup is identical to the study in Wunderlin et al.Wunderlin et al., 2012. MFC: mass flow controller; FTIR: fourier transform infrared spectroscopy; P: pressure sensor; v: valve; QCLAS: quantum cascade laser absorption spectroscopy.*

The laser spectrometer (QCLAS) was operated in batch mode, alternating between preconcentrated sample gas from the batch-scale reactor and a calibration gas (standard I diluted to 70 ppm), to correct for drift effects. Prior to the analysis, the gas cell was evacuated by a scroll pump (TriScroll 300, Varian), then flushed for 4 minutes with 10 standard cubic centimeters per minute of purge gas at reduced pressure (1 kPa), before the downstream on-off valve (V3 in Figure S1) was closed (Mohn et al., 2012). As purge gas we used either synthetic air (prior to analysis of preconcentrated off-gas) or

reference gas (prior to analysis of standard I). The multipass cell was subsequently filled with pre-concentrated air or reference gas respectively until a cell pressure of 8 kPa was reached. Finally, the multipass cell was closed by switching the three-way valve V2 or V4, and the gas sample was analyzed for 6 minutes (Figure S1).

Text S4. Time series of NO_2^- oxidation and NH_2OH oxidation experiments

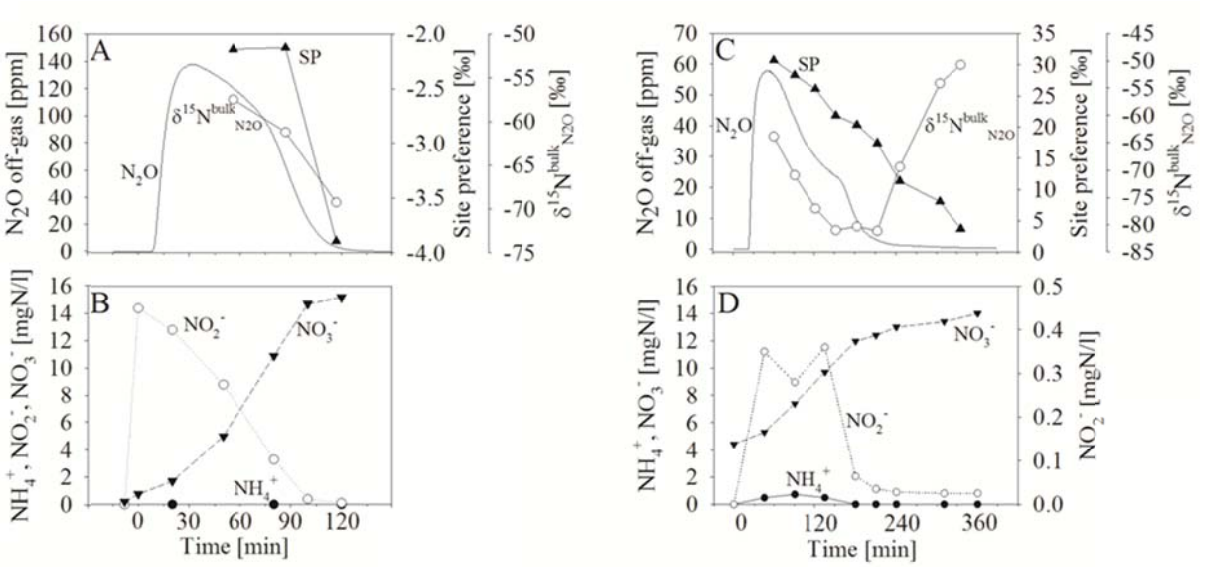

Figure S2. *N_2O data time series of NO_2^- (at 3.1 mgO_2/l; A and B) and NH_2OH (at 1.1 mgO_2/l; C and D) oxidation experiments: dissolved nitrogen species (NH_4^+, NO_2^-, NO_3^-), N_2O mixing ratios, as well as isotopic composition. Additional information about nitrogen conversion rates, N_2O production rate, yield and a detailed discussion of concentration / emission patterns is given in Wunderlin et al. (2012).*

Figure S2 displays time series data of dissolved nitrogen species (NH_4^+, NO_2^-, NO_3^-), N_2O off-gas concentrations, and N_2O isotopic signatures (SP, $\delta^{15}N^{bulk}_{N2O}$) during an NO_2^- oxidation experiment (Figure S2 A and B), as well as an NH_2OH oxidation experiment (Figure S2 C and D). Data indicate that during both NO_2^- oxidation and NH_2OH oxidation experiments, most of the nitrogen is oxidized to NO_3^-.

SP data of the two experiments are significantly different: during NO_2^- oxidation the SP is slightly negative (-0.2 to -3.9 ‰), while SP is positive during NH_2OH oxidation experiments (26.4 to 30.5 ‰ at the beginning; Table 1). The continuous decrease in SP during NH_2OH oxidation is likely to be due to a shift from NH_2OH oxidation to nitrifier denitrification dominated N_2O production in the course of the experiment (for a more detailed discussion see section 'Isotopic signature of N_2O production during NH_2OH oxidation' in the manuscript).

During progressive NO_2^- oxidation, decreasing $\delta^{15}N^{bulk}_{N2O}$ is best explained by the inverse kinetic isotopic fractionation during the oxidation of NO_2^- to NO_3^-. In the NH_2OH

oxidation experiment, a decreasing $\delta^{15}N^{bulk}_{N2O}$ during the first part of the incubation is followed by an increase after 200 minutes. We suspect that this changing trend is due to a shift of NH_2OH oxidation dominated N_2O production to nitrifier denitrification, as also concluded from the SP data (see above).

Text S5. Nitrogen isotopic composition of dissolved nitrogen species

Figure S3 A shows the dissolved nitrogen species (NH_4^+, NO_2^-, NO_3^-) in the course of an ammonia oxidation experiment. The NH_4^+ oxidation rate was approximately 1.9 mgN/gTSS*h (TSS: total suspended solids). Towards the end of the experiment, NO_2^- accumulates by up to 1.1 mgN/l.

Dissolved nitrogen species (NO_2^-, NO_3^-) under denitrifying conditions (at 0 mgO_2/l) are shown in Figure S3 B. The NO_3^- reduction rate was about 4.2 mgN/gTSS*h. NO_2^- accumulated by up to 4 mgN/l towards the end of the experiment when NO_3^- was almost depleted.

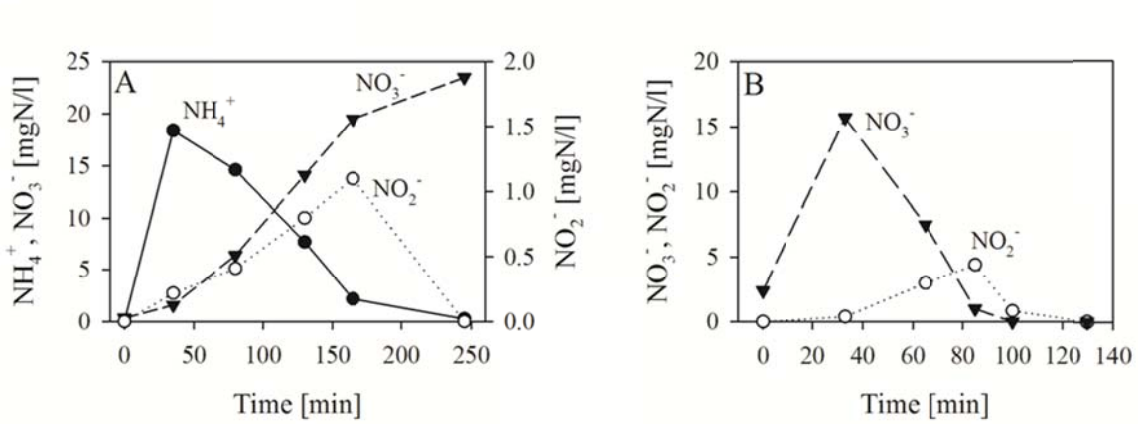

Figure S3. *Concentrations of dissolved nitrogen species (NH_4^+, NO_2^-, NO_3^-) of (A) in the ammonia oxidation experiment at 2.1 mgO_2/l, and (B) in the denitrification experiment under anoxic conditions. Note that total nitrogen was 15 % lower at the beginning compared to the end of the experiment (after 250 minutes in Figure A), which can probably be explained by ammonia adsorption to the surface of activated sludge flocs.*

The N isotope data of dissolved NO_3^- and NO_2^- of both experiments are depicted in Figure S4 (see also Text S2). Under nitrifying conditions, the $\delta^{15}N$ of ammonia was approximated as follows:

$$\delta^{15}N_{NH_4^+} = \frac{\left[NH_4^+\right]_{ini}}{\left[NH_4^+\right]_t} * \left\{ \delta^{15}N_{NH_4^+\,Substrate} - \left(1 - \frac{\left[NH_4^+\right]_t}{\left[NH_4^+\right]_{ini}} \right) * \delta^{15}N_{NO_x} \right\} \qquad [2]$$

where $[NH_4^+]_{ini}$ and $[NH_4^+]_t$ are the initial and instantaneous NH_4^+ concentrations respectively and the $\delta^{15}N$-NH_4^+ substrate is the $\delta^{15}N$ of the added NH_4^+ salt (Mariotti et

al., 1981). A strong ammonium $\delta^{15}N$ increase was observed over the course of the experiment, corresponding to an N isotope effect (change of isotope ratio) of ~19 ‰, in agreement with reports from the literature (Casciotti 2009; Toyoda et al., 2011). The calculated N isotopic signature of NO_2^- ranges between -31.6 ‰ at the beginning and -7.4 ‰ towards the end of the experiment, paralleling the $\delta^{15}N$ trend of NH_4^+ (Rayleigh distillation; Figure S4 A). Nitrite is depleted more strongly in ^{15}N compared to NO_3^-, which is explained by inverse kinetic fractionation during the oxidation of NO_2^- to NO_3^- (Casciotti 2009). The $\delta^{15}N$ of NO_3^- decreases during the initial phase of the batch experiment, to -26 ‰. At the end of the experiment, when most of the ammonia is converted into NO_3^-, its $\delta^{15}N$ value is similar to the (initial) NH_4^+ educt, as expected in a closed system where the oxidation of ammonia to nitrate is the dominant process. The slightly positive initial $\delta^{15}N\text{-}NO_3^-$ at time zero is hypothesized to be due to the mixing of nitrified 'new' nitrate with some residual nitrate (0.34 mgN/l; Figure S3 A), slightly enriched in ^{15}N.

Under denitrifying conditions (Figure S4 B), the $\delta^{15}N\text{-}NO_3^-$ increases from ~0.5 ‰ to ~83 ‰ in the course of the batch experiment, due to kinetic N isotope fractionation during denitrification. The decrease in $\delta^{15}N\text{-}NO_3^-$ at 130 min may be explained by reduced N isotope fractionation due to NO_3^- limitation (< 0.2 $mgNO_3^-N/l$), or some admixture of another NO_3^- source, possibly from NO_2^- oxidation. From the $\delta^{15}N\text{-}NO_3^-$ to $[NO_3^-]$ relationship, we calculated an isotope effect of ~17 ‰ for nitrate reduction, which is within the range of reported literature values between 13 and 30 ‰ (compilation in Casciotti 2009). The calculated $\delta^{15}N$ of NO_2^- was significantly lower at the beginning of the experiment (-23.5 ‰) with respect to the substrate nitrate (5 ‰). As the reaction proceeds, NO_2^- becomes increasingly enriched in ^{15}N, reflecting the parallel $\delta^{15}N$ increase of the dwindling substrate (NO_3^-) pool.

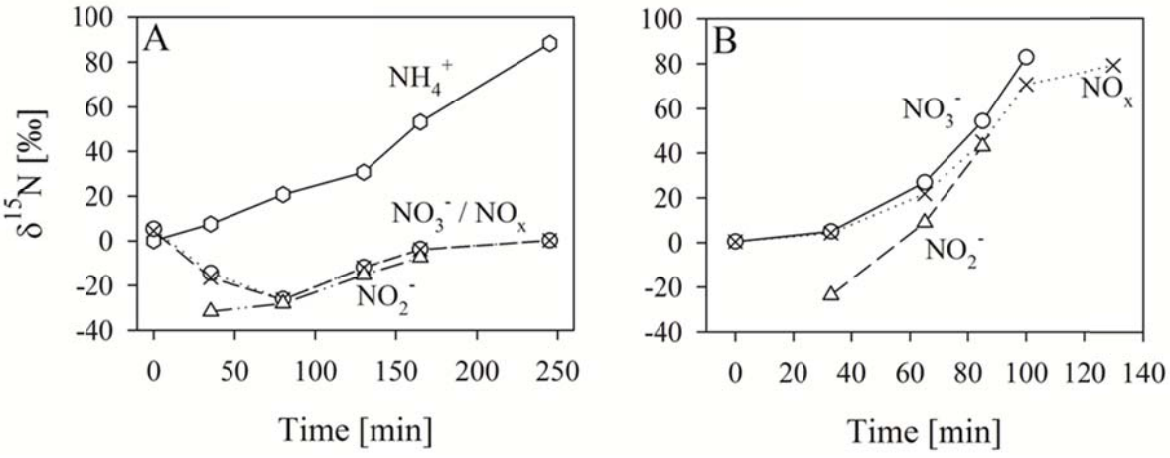

Figure S4. *Temporal trend of $\delta^{15}N$ of NO_3^-, NO_2^-, and NH_4^+ under (A) nitrifying conditions (at 2.1 mgO_2/l), and (B) denitrifying conditions (0 mgO_2/l). Note that $\delta^{15}N\text{-}NO_2^-$ and $\delta^{15}N\text{-}NH_4^+$ were both calculated from isotope mass balances using measured dissolved*

inorganic nitrogen (DIN) concentrations, and δ^{15}N-NO_x, δ^{15}N-NO_3^-, as well as the δ^{15}N-NH_4-substrate measurements (see Equations 1 and 2).

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Chapter 5

Pathway identification of N_2O production in biological wastewater treatment: Is the nitrogen isotopic signature a suitable tool?

This chapter has been submitted for publication

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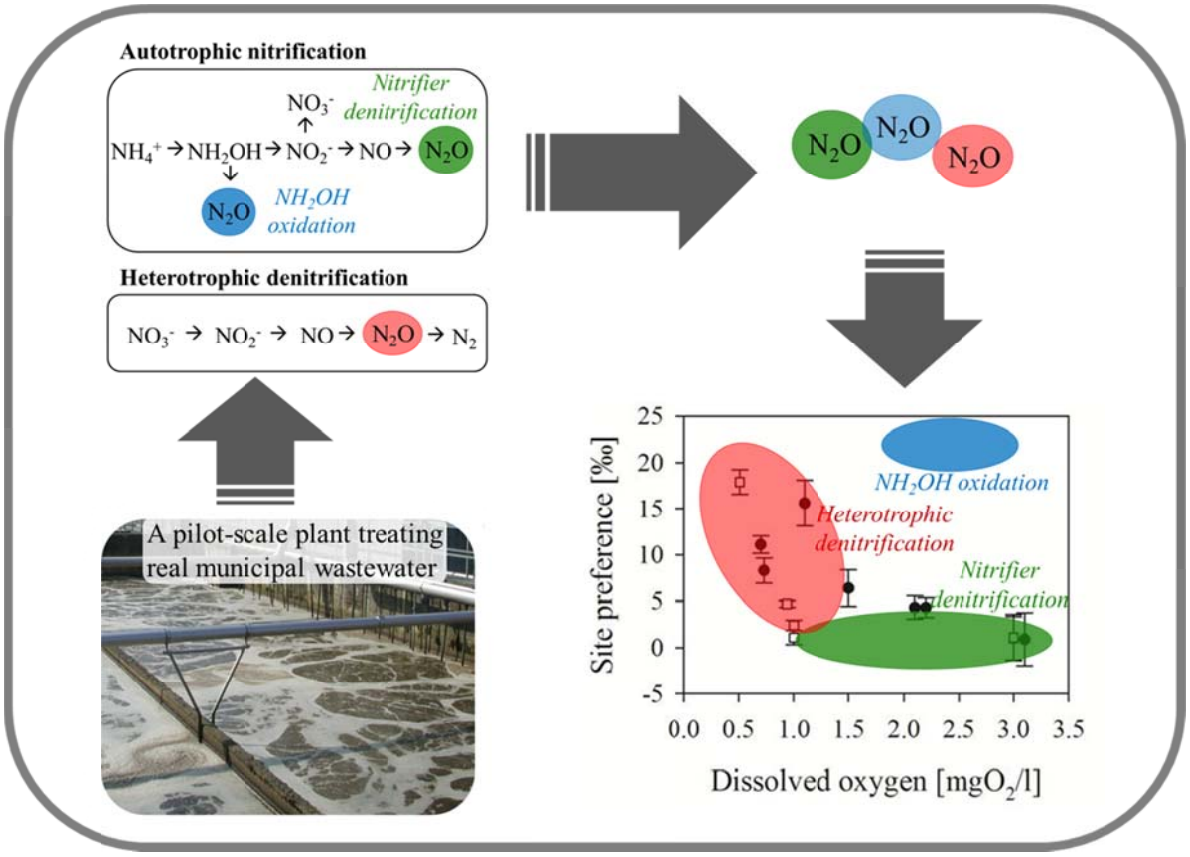
Graphical abstract



Abstract

Nitrous oxide (N_2O) is an important greenhouse gas and a major sink of stratospheric ozone. Microbial transformation processes, particularly autotrophic nitrification and heterotrophic denitrification, have been identified as major N_2O sources in biological wastewater treatment. The ^{15}N isotopic signature of N_2O is a novel and powerful technique to differentiate between these pathways: it is based on the site-specific distribution of ^{15}N (site preference, SP) as well as the average ^{15}N content ($\delta\,^{15}N^{bulk}_{N2O}$) of the asymmetric N_2O molecule.

In the present study, we investigate the applicability of N_2O isotopomer analysis to source allocation in a pilot-scale biological wastewater treatment plant. The ^{15}N isotopic signature was analyzed with respect to the dissolved oxygen concentration in the activated sludge tank, which is one of the most important controlling parameters for autotrophic nitrification and heterotrophic denitrification. Results show that under aerated conditions with 2 to 3 mgO_2/l, N_2O is produced from nitrite (NO_2^-) reduction by nitrifier denitrification (SP close to 0 ‰) or heterotrophic denitrification (SP close to 0 ‰, without N_2O reduction). Hydroxylamine (NH_2OH) oxidation cannot be excluded, but the SP data suggests that the contribution from this pathway is low. With decreasing dissolved oxygen (below 1 mgO_2/l), however, the increase in SP is most probably caused by heterotrophic denitrification due to N_2O reduction. On the other hand, $\delta\,^{15}N^{bulk}_{N2O}$ supports the findings from the SP analysis, but cannot be used as a quantitative measure in this study, since the isotopic composition of the substrates is unknown. In sum, this study illustrates the value of the ^{15}N isotopic signature of N_2O as a promising tool to further investigate N_2O production pathways in biological wastewater treatment.

Keywords

Biological wastewater treatment; nitrous oxide (N_2O); nitrifier denitrification; pathway identification; site preference

Nomenclature

^{15}N; ^{14}N:	Stable nitrogen isotopes
R:	Nitrogen isotope ratio, $^{15}N/^{14}N$
$\delta\,^{15}N^{\alpha}$, $\delta\,^{15}N^{\beta}$:	Relative differences of isotope ratios to the standard material (nitrogen, N_2) for the inner (α) and the outer (β) nitrogen atom in the asymmetric N_2O molecule
$\delta\,^{15}N^{bulk}_{N2O}$:	The average ^{15}N content in N_2O relative to nitrogen (N_2)
SP:	Site preference, the difference between the $\delta\,^{15}N^{\alpha}$ and $\delta\,^{15}N^{\beta}$

Introduction

Nitrous oxide (N_2O) is an important greenhouse gas (about 300 times more effective than CO_2) and a major sink for stratospheric ozone (IPCC 2007). These impacts on our environment make it an urgent requirement to limit anthropogenic N_2O emissions (Ravishankara et al., 2009). It is estimated that about two thirds of the overall N_2O is emitted by microbial processes occurring mainly in agriculture, but also in biological wastewater treatment (USEPA 2009). Depending on the operating conditions, N_2O emissions are estimated to represent a major part of the total greenhouse gas emissions from biological wastewater treatment (Daelman et al., 2012). Continuous online monitoring of N_2O is consequently helpful in reducing overall greenhouse gas emission from biological wastewater treatment (e.g. Wunderlin et al., 2013b).

N_2O production in biological wastewater treatment is mainly attributed to autotrophic nitrification and heterotrophic denitrification, which produce N_2O via different pathways (Kampschreur et al., 2009; Wunderlin et al., 2012): (i) during the oxidation of ammonia (NH_4^+) or hydroxylamine (NH_2OH) to nitrite (NO_2^-), (ii) via reduction of NO_2^- by ammonia-oxidizing bacteria (AOB), known as nitrifier denitrification, and (iii) during heterotrophic denitrification where N_2O is an obligate intermediate. The importance of each of these pathways remains unclear, but it is generally believed that nitrifier denitrification is dominant in biological wastewater treatment under aerobic conditions (Colliver and Stephenson 2000).

A promising way of apportioning N_2O production to nitrification and denitrification is to analyze the stable nitrogen (N) isotopes (Baggs 2008; Schreiber et al., 2012; Wunderlin et al., 2013a). This is done by measuring the isotope ratio of a sample (R_{sample}) against atmospheric nitrogen (N_2; $R_{standard}$) and expressing the result in the delta (δ) notation (Mariotti et al., 1981): $\delta\,^{15}N_{sample} = (R_{sample} - R_{standard}) / R_{standard} * 1000$. The difference in ^{15}N between the central oxygen-bound (position and the outer (position is known as the site preference (SP): $SP = \delta\,^{15}N^{\alpha} - \delta\,^{15}N^{\beta}$. In recent years, analytical procedures have been developed to determine the intramolecular distribution of ^{15}N within the asymmetric linear N_2O molecule by isotope-ratio mass-spectrometry (IRMS) (Brenninkmeijer and Röckmann 1999; Toyoda and Yoshida 1999) as well as by quantum cascade laser absorption spectrometry (QCLAS) (Waechter et al., 2008; Mohn et al., 2012; Köster et al., 2013).

The advantage of SP is its independence from the ^{15}N content of the substrates and its specificity to the processes involved in N_2O production and degradation (Sutka et al., 2006). Due to the complexity of the mixed population system used in this investigation, the characteristic SP values of specific pathways need to be studied on pure bacterial cultures. The following SP end-member values were consequently reported for N_2O production by both nitrifying and denitrifying bacterial strains (Sutka et al., 2006): (i) 31.4 to 33.5 ‰ for NH_2OH oxidation, (ii) 0.1 ‰ for nitrifier denitrification by AOB, and (iii) -0.5 to -0.6 ‰ for heterotrophic denitrification (for a more detailed overview, see Wunderlin et al., 2013a). Positive SP values in the range from 0 to 26.1 ‰, under anoxic or very low dissolved oxygen conditions, are attributed to a significant

heterotrophic N_2O reductase activity relative to N_2O production (Yamagishi et al., 2007; Koba et al., 2009; Wunderlin et al., 2013a). Conventional parameters, such as the dissolved O_2, the N species (NH_4^+, NO_2^-, NO_3^-), the organic carbon as well as the process control and the N_2O off-gas analysis, must be considered to interpret the isotope data more conclusively (e.g. to discriminate between NH_2OH oxidation and heterotrophic denitrification, since both pathways lead to a positive SP; Decock and Six 2013).

The SP of the N_2O emitted from an activated sludge lab-scale batch reactor was investigated in a recent publication (Wunderlin et al., 2013a): the results show that under aerobic conditions, the SP values were within the range for NO_2^- reduction, which is hypothesized to be primarily due to AOB nitrifier denitrification. However, the contribution of the NH_2OH oxidation to the total N_2O production was of minor importance under typical nitrifying process conditions. Furthermore, heterotrophic denitrification activity under anoxic conditions is supported by positive SP data (up to 26.1 ‰).

In this study, we investigated the nitrogen isotopic signature of N_2O emitted from a pilot-scale treatment plant fed with real municipal wastewater and operated at different concentrations of dissolved oxygen. The goal was to test the applicability of N_2O isotopic analysis for source allocation in a set-up that was closer to full-scale plants than that of previous lab-scale batch experiments. The tested hypothesis was that NO_2^- reduction is the dominant route for N_2O production under aerobic conditions in this system treating real municipal wastewater, i.e. the SP of N_2O is expected to be close to 0 ‰.

Materials and methods

A pilot-scale wastewater facility treating around 60 population equivalents and consisting of primary clarification followed by activated sludge treatment with pre-denitrification (Bio1), nitrification (Bio2) and secondary clarification was monitored (Figure 1). It was operated with a solids retention time of around 12 days. The concentration of mixed liquor suspended solids (MLSS) was about 3 g/l. The plant was controlled by a programmable logical controller (PLC) linked to an interface for direct access to data visualization and storage. It was fed with real municipal wastewater taken from the nearby sewer in the municipality of Dübendorf (CH) at a fixed flowrate, changing every 2 hours with a mean value of 27 m^3/day (Figures 2 and 3). Both reactors (Bio1 and Bio2) had a volume of 8 m^3 and were operated together as a completely stirred tank reactor. The return sludge flow was about 2.3 times higher than the influent flow. The dissolved oxygen concentrations in the activated sludge tanks were controlled at a given set-point (3, 2, 1, 0.5 or <0.5 mgO_2/l) based on the aeration rate adjustment (on / off control).

Plant operation during measuring campaign

In the first week of the measuring campaign (day 1 to 7; Figure 2), the off-gas of Bio2 was continuously measured at dissolved oxygen (DO) concentration set-points of 3, 2, 1, 0.5 and <0.5 mgO_2/l (during 24 hours each). The O_2 set-point was changed in the morning before the influent N peak (before 8 a.m.). In the second week (day 8 to 14; Figure 3), the exhaust gas of Bio1 was continuously analyzed, at DO set-points of 3, 2, 1, 0.5 and <0.5 mgO_2/l (during 24 hours each; changed in the morning), while Bio2 was continuously operated at 2 mgO_2/l (Figure 2). These two set-ups were chosen in order to investigate the relative contribution of heterotrophic denitrification (predominantly active in the pre-denitrification reactor) to total N_2O emission from the nitrification reactor.

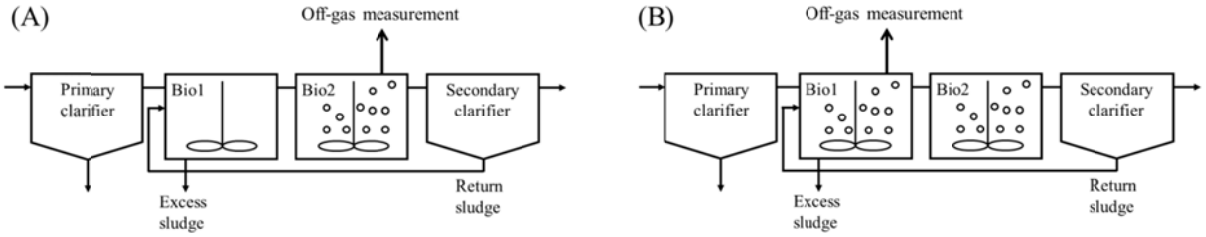


Figure 1. *Overview of the pilot-scale wastewater facility with off-gas measurement locations. (A) In the first week of the measuring campaign, Bio1 was operated under anoxic conditions while Bio2 was operated at different oxygen concentrations (range from minimum to 3 mgO_2/l; Table 1). (B) In the second week, Bio1 was operated at different oxygen concentrations (range from minimum to 3 mgO_2/l; Table 1), while the concentration of the dissolved oxygen in Bio2 was 2 mgO_2/l.*

N_2O concentration and isotopic analysis

To enable quantitative emission measurements, the individual reactors were sealed and the off-gas was directed through an exhaust pipe. The N_2O mixing ratios were analyzed on-line by FTIR spectroscopy (GASMET CX-400, Temet Instruments, Helsinki; Mohn et al., 2008). The N_2O emission loads were computed by multiplying the N_2O off-gas concentration by the air flowrate measured at the reactor inlet (t-mass S AT70, Endress + Hauser), with 1 ppm N_2O corresponding to 1.249 $mgN_2O\text{-}N/m^3_{air}$ under standard conditions (273.15 K, 1013 mbar). The N_2O emission factors per DO set-point (typically lasting over 24 hours) were then determined by dividing the total mass of the N emitted as N_2O by the total mass of the N in the inflow present as NH_4^+ (Table 1).

For the site-specific N_2O isotopic analysis, dry and CO_2-free off-gas samples integrating over 30 minutes were collected in Tedlar bags (sampling time indicated by arrows in Figures 2 and 3) and analyzed by quantum cascade laser absorption spectroscopy (QCLAS; Waechter et al., 2008). Standard reference gases with a known isotopic composition as analyzed by IRMS were used for the $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ calibration (Toyoda and Yoshida 1999). Samples with N_2O concentrations below 10 ppm were pre-concentrated prior to isotopomer analysis (Mohn et al., 2010; Mohn et al., 2012). The

performance achieved by high-precision analysis and its equivalence to IRMS were recently demonstrated in an inter-comparison study (Köster et al., 2013).

Wastewater sampling procedures and analysis

Laboratory measurements were carried out every weekday. Grab samples were taken six times per day at 7, 8, 10 and 12 a.m. and 2 and 4 p.m. The samples were immediately filtered through a 0.45 μ m syringe filter and stored at 4°C. The NH_4^+-N was analyzed by photometry using a Foss FIAstar (flow-injection 5000 analyzer). The NO_3^--N and NO_2^--N were determined by anion chromatography (761 compact IC, Metrohm). The measurements were carried out within 24 hours of sampling. The influent municipal wastewater was subjected to flow-proportional sampling for 2 hours in each case (WaterSam, Gerber Instruments) during the whole measuring campaign for the total organic carbon (TOC) analysis. The samples were homogenized (Ultra-turrax T25, Faust Laborbedarf AG) before TOC analysis (IL 550 TOC-TN, Hach Lange GmbH). The ratio of COD_{total} (total chemical oxygen demand) to TOC is 3.3 ± 0.5 (n = 39, measured over one year), while the COD_{total} to $COD_{soluble}$ ratio is 1.8 ± 0.2 (n = 6; measured over two months).

Results and discussion

Trends of dissolved nitrogen species and N_2O emissions

The trend data for the NH_4^+ influent concentrations, influent flows, aeration rates, dissolved oxygen concentrations, N_2O off-gas concentrations and dissolved N species (NH_4^+, NO_2^-, NO_3^-) are shown in Figures 2 and 3. The NH_4^+ influent loads tended to peak during the morning hours (between 8 to 11 a.m.) due to high NH_4^+ concentrations in the sewer system and in combination with the highest daily influent flow into the activated sludge plant (~1.4 m^3/h between 8 to 10 a.m.). The low N influent loads in the morning of days 3, 8, 11 and 12 were affected by rainfall in the catchment area leading to dilution in the sewer networks (pilot-plant influent flow with a fixed daily variation; see Figures 2 and 3). Under normal dry weather conditions, the NH_4^+ influent loads were typically in the range from 20 to 70 gN/h. The total organic carbon (TOC) concentrations of the influent wastewater were between 100 and 250 mg/l (data not shown), corresponding to about 330 to 850 mg/l total chemical oxygen demand (COD; i.e. about a factor of 3.3 higher than the TOC).

The N_2O emissions were highly dynamic but seemed to depend on the dissolved NH_4^+ and NO_2^- concentrations in the activated sludge tanks: for example, during day 2 (Figure 2), the N influent load was high, and both NH_4^+ and NO_2^- built up in the nitrification reactor, while the N_2O emissions rose in parallel. On the other hand, during low N loading situations, such as during rain events on days 8 and 14 (Figure 3), N_2O emissions were low or even below the limit of detection (< 0.5 ppm). This is in accordance with several studies: thus Lotito et al. (2012) reported a similar daily pattern of N_2O emissions correlating with the N influent load, and Ahn et al. (2010) identified dissolved NO_2^- as a relevant parameter for N_2O production in full-scale treatment plants.

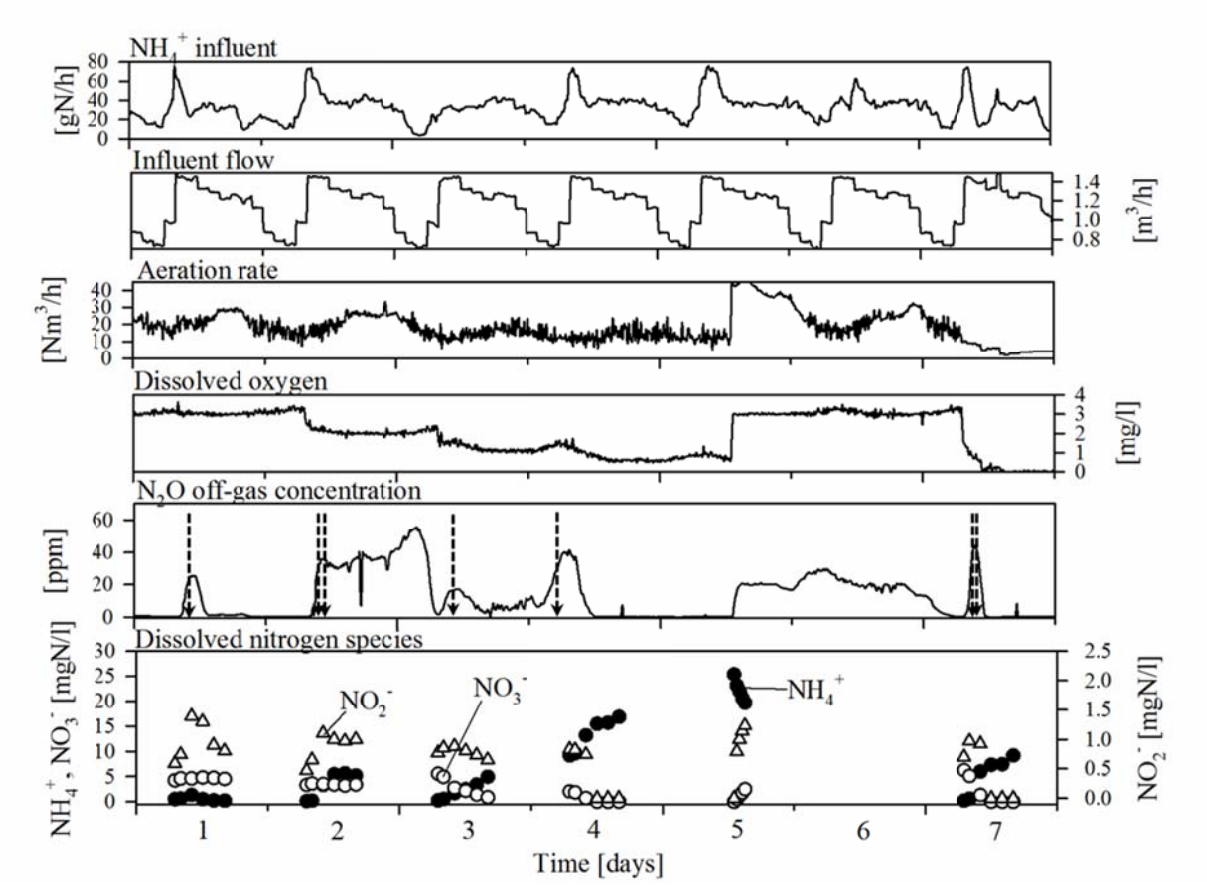


Figure 2. *Data overview of the measuring campaign from Bio2 (Bio1 was operated under anoxic conditions; see also Figure 2 A): NH_4^+ influent load and influent flow into Bio1, aeration rate, dissolved oxygen, N_2O off-gas concentration (average over 10 minutes) and dissolved N species (NH_4^+ (●), NO_2^- (Δ), NO_3^- (○); not available for day 6) of Bio2 (day 2 adapted from Houweling et al., 2011). Arrows indicate time of gas sampling for isotopic analysis (one arrow is equal to one sample integrated over 30 minutes).*

No clear correlation between DO and N_2O emissions could be observed in this study (Table 1). This might be partly due to incomplete nitrification, leading to NH_4^+ accumulation in the activated sludge tank, at DO below 1.5 mg/l (Figures 2 and 3). According to the literature, N_2O emissions are expected to be higher under oxygen-limiting conditions than in situations with DO in excess (Tallec et al., 2006; Wunderlin et al., 2012). In fact, the N_2O emissions from Bio2 were in the range from 0.1 to 2.6% with respect to the NH_4^+ influent load, and between 0.005 and 2.1% from Bio1, respectively (Table 1). These data are variable, but are within the range of values reported in the literature: thus (i) Ahn et al. (2010) reported a range from 0.01 to 1.8 ± 0.79% with respect to the N influent load, and (ii) in a recent investigation by Aboobakar et al. (2012) an average emission factor of 0.04% with respect to the NH_4^+ influent flow was measured, and (iii) N_2O emissions monitored on a full-scale treatment plant over one year yielded about 3% of the incoming N (Daelman et al., 2012).

As shown in this section, the reported N_2O emissions were dynamic and variable, and the dominant N_2O production pathway could only be approximately assessed. However, additional information is needed to obtain a better understanding.

Consequently, the N_2O nitrogen isotopic signature is analyzed and its applicability discussed in the following sections.

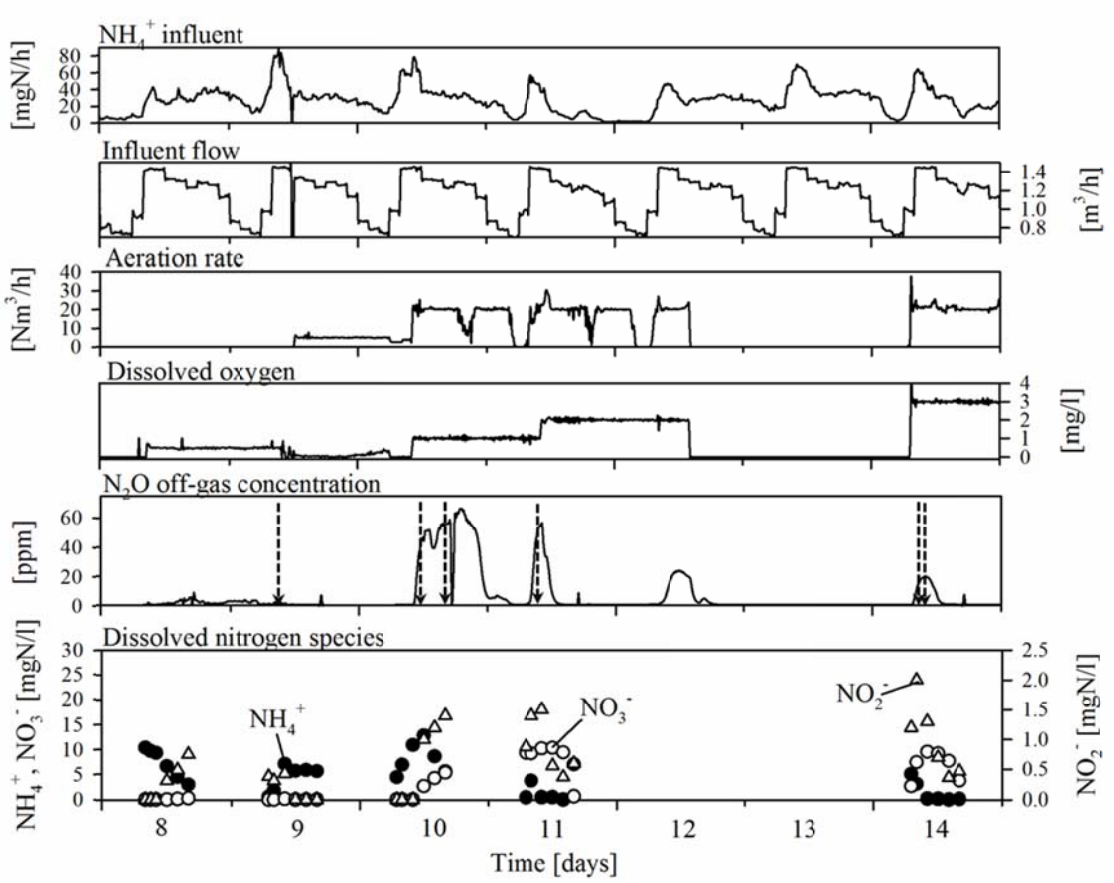


Figure 3. *Data overview of the measuring campaign from Bio1 (Bio2 was constantly operated at 2 mgO_2/l; see also Figure 1 B): NH_4^+ influent load and influent flow into Bio1, aeration rate (not detected during day 8 and during the morning of day 9), dissolved oxygen, N_2O off-gas concentration (averages over 10 minutes) and dissolved N species (NH_4^+ (●), NO_2^- (Δ), NO_3^- (○); not available for day 12 and 13) of Bio1. Arrows indicate time of gas sampling for isotopic analysis (one arrow is equal to one sample integrated over 30 minutes).*

Site preference (SP) of N_2O

In the previous section, we noted that N_2O emissions were highly dynamic and variable, which led to difficulties in drawing any reliable conclusions about dominant N_2O production pathways. However, additional information is provided by the analysis of the N_2O site preference (SP): the SP of N_2O emitted under different concentrations of dissolved oxygen varied between 0.9 and 17.9 ‰ (Figure 4 A and Table 1). Basically, the SP values were low at 2 to 3 mgO_2/l, and higher under low DO concentrations (<1 mgO_2/l; except for Bio1 operated at 1 mgO_2/l). This is consistent with a recent study by Wunderlin et al. (2013a), who reported SP values of -5.8 to 5.6 ‰ during NH_4^+ oxidation and values of up to 26.1 ‰ under anoxic conditions from activated sludge lab-scale batch experiments. In another publication reporting on the investigation of the nitrogen isotopic signature of N_2O emitted from a full-scale activated sludge treatment

plant, an SP value of 4.5 ‰ was estimated for N_2O emitted from the nitrification reactor (Toyoda et al., 2011). Moreover, SP values in the range from 0.1 to 16.9 ‰ were measured in river water below the effluents of wastewater treatment plants (Toyoda et al., 2009).

Table 1. *Overview of (i) the average dissolved oxygen (DO) in the activated sludge reactor (where N_2O was measured), (ii) the N_2O emissions with respect to NH_4^+-N influent loads, (iii) $\delta^{15}N^{bulk}_{N2O}$, and (iv) SP data with their interpretation of microbial processes (ND refers to AOB nitrifier denitrification, and NN to NH_2OH oxidation; adapted from Wunderlin et al., 2013). Basically, ND is dominant at DO above 1 mgO_2/l, while heterotrophic denitrification (N_2O reduction) is more active at lower DO. Please note that DO values refer to the period between DO set-point changes.*

	Day	Precipitation*	DO	N_2O emissions**	$\delta^{15}N^{bulk}_{N2O}$	SP	Interpretation of microbial processes based on SP
			[mgO_2/l]	[%]	[‰]	[‰]	
Monitoring Bio2 (Bio1 anoxic)	1	No	3.1 ± 0.1	0.4	(-30 ± 2.9)	0.9 ± 2.9	ND high, NN low
	2	No	2.1 ± 0.1	2.6	(-44.1 ± 1.1)	4.3 ± 1.3	ND high, NN low, or N_2O red.
	3	Yes	1.3 ± 0.2	0.7	(-34.2 ± 2)	6.4 ± 2	ND high, NN low, or N_2O red.
	4	No	0.8 ± 0.2	0.1	(-20.3 ± 2.5)	15.6 ± 2.5	NO_2^- red and strong N_2O red.
	5	No	3.0 ± 0.1	1.7	n.d.	n.d.	n.d.
	6	No	3.1 ± 0.2	1.2	n.d.	n.d.	n.d.
	7	No	~0.2	0.2	(-37.1 ± 1.4) (-31.6 ± 0.99	8.3 ± 1.4 11.1 ± 0.9	NO_2^- red and strong N_2O red.
Monitoring Bio1 (Bio2 at 2 mgO2/l)	8	Yes	0.5 ± 0.1	n.d.	n.d.	n.d.	n.d.
	9	No	~0.1	0.005	4.1 ± 1.4	17.9 ± 1.3	NO_2^- red and strong N_2O red.
	10	No	1.0 ± 0.1	2.1	(-39.4 ± 0.4) (-35.6 ± 0.5)	4.7 ± 0.4 2.4 ± 0.5	ND high, NN low, or N_2O red.
	11	No	2.0 ± 0.1	1.2	(-42.3 ± 0.8)	1.1 ± 0.8	ND high, NN low, or N_2O red.
	12	Yes	anoxic	no gas-flow	n.d.	n.d.	n.d.
	13	No	anoxic	no gas-flow	n.d.	n.d.	n.d.
	14	No	3.0 ± 0.1	0.4	(-33.3 ± 2.3) (-29.5 ± 2.5)	1 ± 2.3 1.1 ± 2.5	ND high, NN low

* Precipitation leads to lower influent N and COD loads

** With respect to NH_4^+-N influent load

The data presented in Figure 4 A exhibit a negative correlation between the SP and the dissolved oxygen concentration in the activated sludge reactor. This is hypothesized to be mainly due to the activity of heterotrophic N_2O reduction, which leads to an increase in the SP. In contrast, NH_2OH oxidation with an end-member SP of 31.4 to 33.5‰ (Sutka et al., 2006) is not assumed to contribute significantly to the total N_2O production under the given conditions: for example, a similar trend between the dissolved oxygen concentration and the SP was already shown (Sutka et al., 2006), and it was

demonstrated that decreasing concentrations of dissolved oxygen increase the importance of nitrifier denitrification relative to NH_2OH oxidation in a pure culture study of *N. europaea*. This is also in agreement with a recent publication showing that N_2O production is related to NH_4^+ oxidation in the case of imbalanced metabolic activity (Yu et al., 2010), a situation where N_2O might be produced as an intermediate of the NH_2OH oxidation pathway (e.g. via NO or HNO; Ritchie and Nicholas 1972). Since this would result in the opposite correlation to that shown in Figure 4 A (e.g. higher SP with higher oxygen concentration), it is concluded that NH_2OH oxidation only contributed marginally to the total N_2O production in this investigation (SP up to 6.4 ‰; Table 1). Similarly, in activated sludge lab-scale batch experiments, an SP close to 0 ‰ indicated a low contribution from NH_2OH oxidation under NH_4^+ oxidizing conditions and DO up to 3 mgO_2/l (Wunderlin et al., 2013a).

According to the current state of knowledge, nitrifier denitrification is hypothesized to be the dominant N_2O production pathway in biological wastewater treatment under aerobic conditions (Colliver and Stephenson 2000; Kampschreur et al., 2009; Wunderlin et al., 2012; Wunderlin et al., 2013a). This is supported by SP data (SP close to 0 ‰, at DO >1.5 mgO_2/l) and by the presence of dissolved NO_2^- during periods of N_2O emissions (Figure 2 and 3) as reported in our study. On the other hand, positive SP values, as detected under low dissolved oxygen (e.g. 8.3 to 17.9 ‰ below 1.5 mgO_2/l; Table 1), are interpreted as an indicator of heterotrophic N_2O reduction. As such, the shift to lower SP values with increasing oxygen concentrations is hypothesized to be due to (i) an increasing inhibition of the N_2O reductase or (ii) to reduced overall heterotrophic activity: according to the literature, N_2O reductase is assumed to be inhibited more efficiently than the other heterotrophic denitrifying enzymes in the presence of oxygen (von Schulthess et al., 1994; Kampschreur et al., 2009).

Preliminary studies show the meaning of the SP data: (i) SP values at 0 ‰ are an indicator of NO_2^- reduction, either by AOB nitrifier denitrification or heterotrophic denitrification, and (ii) positive SP values at high DO refer to NH_2OH oxidation, while heterotrophic N_2O reduction is the targeted process at low DO. As demonstrated in this section, SP data provide quantitative information about the relevant N_2O production pathway. Our data are interpreted as follows: SP data close to 0 ‰ and high DO are an indicator of NO_2^- reduction, while the increase in SP at low DO is attributed to increasing heterotrophic denitrification activity.

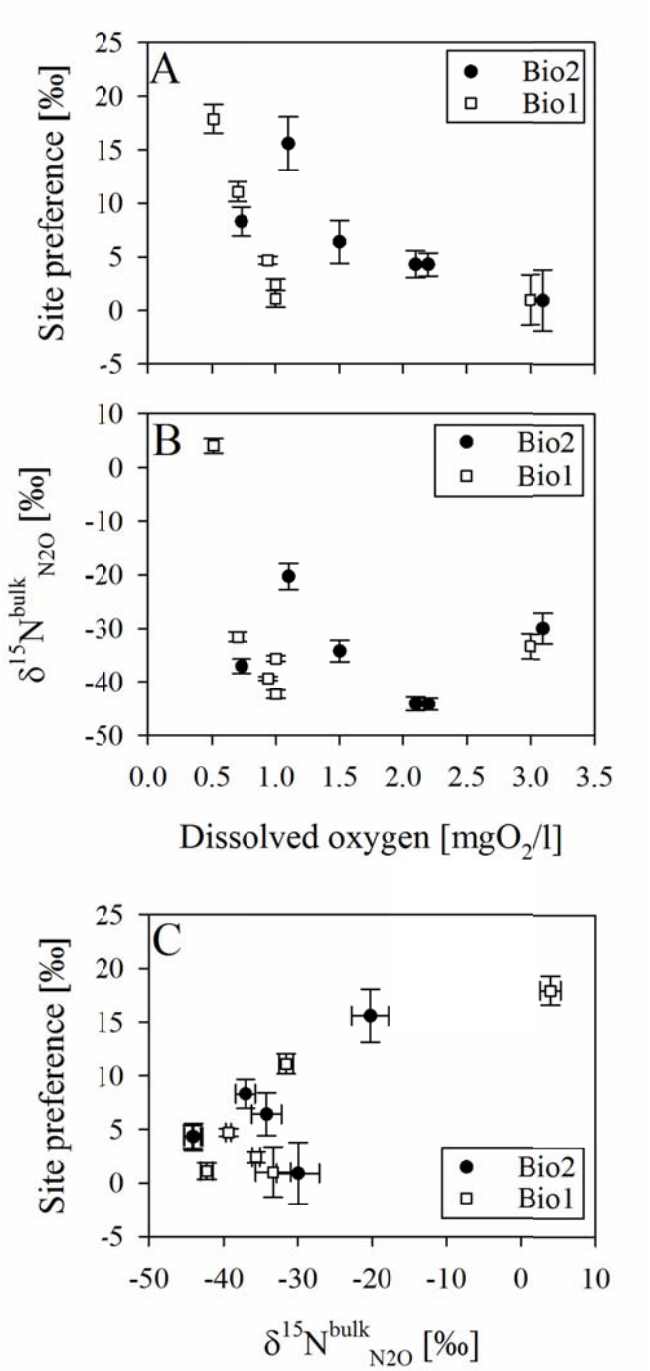


Figure 4. *(A) The site preference (SP) of the N_2O emitted from Bio1 and Bio2 is plotted against the dissolved oxygen concentration (DO values averaged over the off-gas sampling period). The shift in SP towards more positive values with decreasing dissolved oxygen concentrations is due to the increasing importance of the heterotrophic N_2O reductase activity relative to N_2O production (see also Table 1). (B) $\delta^{15}N^{bulk}_{N2O}$ (average ^{15}N content within N_2O) plotted against the dissolved oxygen concentration in Bio1 and Bio2 (DO values averaged over the off-gas sampling period). The $\delta^{15}N^{bulk}_{N2O}$ is strongly depleted in ^{15}N, which is an indicator of N_2O production without strong N_2O reduction activity. The two data points at -20.3 and 4 ‰ under low dissolved oxygen are less depleted in ^{15}N, thus supporting simultaneous heterotrophic denitrification activity (N_2O reduction). (C) The SP of N_2O plotted against the $\delta^{15}N^{bulk}_{N2O}$ emitted from Bio1 and Bio2. A positive trend*

between SP and $\delta^{15}N^{bulk}_{N2O}$ is due to heterotrophic N_2O reduction, which increases the SP and leads to a less negative $\delta^{15}N^{bulk}_{N2O}$. Differentiation between AOB and HET based on $\delta^{15}N^{bulk}_{N2O}$ alone is limited due to a lack of isotope signature data from the substrate (NH_4^+, NO_3^-). Error bars represent the 2 σ confidence level.

Average ^{15}N content in N_2O ($\delta^{15}N^{bulk}_{N2O}$)

In addition to SP, the average ^{15}N content of the N_2O molecule ($\delta^{15}N^{bulk}_{N2O}$) provides supplementary information about the N_2O production pathways involved. The range of the $\delta^{15}N^{bulk}_{N2O}$ values varied widely between -44 and 4 ‰, with no strong dependence on DO concentrations (Table 1; Figure 4 B). However, the values are similar to those in the literature: between -24.4 and 5.6 ‰ was reported in a study on N_2O in groundwater (Koba et al., 2009), while in river water, $\delta^{15}N^{bulk}_{N2O}$ values ranged between -18 to 9 ‰ (Toyoda et al., 2009). In a full-scale wastewater treatment investigation, a $\delta^{15}N^{bulk}_{N2O}$ value of -13.4 ‰ was estimated for N_2O emitted from the oxidation tanks (Toyoda et al., 2011). And in a recent activated sludge lab-scale investigation, $\delta^{15}N^{bulk}_{N2O}$ values of between -57.6 and -25.3 ‰ were reported for NH_4^+ oxidation experiments, and from -17 to 50.1 ‰ for NO_3^- reduction experiments with a similar initial substrate signature ($\delta^{15}N\text{-}NH_4^+$ = 1.15 ‰, $\delta^{15}N\text{-}NO_3^-$ = 2.5 ‰; Wunderlin et al., 2013a): isotopic signatures were most depleted in ^{15}N at the beginning, and continuously increased throughout the experiments, due to Rayleigh distillation kinetics (an increase of ^{15}N in the educt; refer to e.g. Mariotti et al. (1981) for more information about the basic principles of kinetic isotopic fractionation).

The data presented in Figure 4 B in general exhibit no significant correlation between dissolved oxygen concentration and $\delta^{15}N^{bulk}_{N2O}$. Except at low dissolved oxygen concentrations (<1 mgO_2/l), there is a slight correlation between $\delta^{15}N^{bulk}_{N2O}$ and O_2 in Bio1, mainly due to strong ^{15}N enrichment (around +4 ‰) in one experiment. This is explained by an increasing heterotrophic N_2O reductase activity relative to N_2O production under low dissolved oxygen concentrations (0.5 mgO_2/l), which is also in agreement with a positive SP value (17.9 ‰; see also Figure 4 C). The increase in $\delta^{15}N^{bulk}_{N2O}$ as DO increased from 2 to 3 mgO_2/l is congruent with the findings of Wunderlin et al. (2013a), where the lower isotopic fractionation during NH_4^+ oxidation was attributed to a high NH_4^+ oxidation rate in combination with a comparatively sluggish NO_2^- oxidation (NO_2^- accumulated up to 5 mgN/l). However, in the present study, NO_2^- accumulation was only slight (up to 2 mgN/l; see Figure 3 and 4). On the other hand, a reduced N fractionation can also be linked to low substrate concentrations (e.g. Lehmann et al., 2007; Granger et al., 2008), which might be partly consistent with a lower NH_4^+ concentration range at 3 mgO_2/l (0.9 to 4.1 mgN/l) compared to concentrations at 2 mgO_2/l (4.4 mgN/l).

Nevertheless, the wide variation in $\delta^{15}N^{bulk}_{N2O}$ and the lack of data concerning the isotopic signature of the relative substrates (NH_4^+ and NO_3^-) make it difficult to differentiate between N_2O production originating from AOB (nitrifier denitrification

and NH_2OH oxidation) and from heterotrophic denitrification. Assuming that (i) the isotope signature of influent ammonia is around 0 ‰ and that (ii) ammonia is in excess during nitrification (large reactant concentration; Figure 2 and 3), the isotopic signature of the N_2O produced via nitrification and heterotrophic denitrification is expected to be in the range of -47 to -68 ‰ and 0 to -39 ‰, respectively (e.g. Koba et al., 2009). Our $\delta^{15}N^{bulk}_{N2O}$ data are between -44 and 4 ‰, which can be interpreted to be within the range of both pathways, making it difficult to draw further conclusions. In fact, the isotopic signature of the influent ammonia may differ from 0 ‰, thus the ^{15}N enrichment due to NH_4^+ oxidation, or the isotopic fractionation in this study deviated from the values reported in the literature. This is supported by the findings of Toyoda et al. (2011) who measured $\delta^{15}N\text{-}NH_4^+$ up to ~10 ‰ and calculated a fractionation of -48.4 ± 0.3 ‰ for the NH_4^+ to N_2O step, which supports a range from 30 to 50 ‰ for nitrification.

Overall, we conclude that $\delta^{15}N^{bulk}_{N2O}$ data qualitatively support the findings from SP analysis, even though the quantitative interpretation is complex because it is not only influenced by the pathways involved (multiple reaction steps) but also by their individual enzymatic activities, substrate concentrations and isotopic compositions (see also Decock and Six, 2013).

Relationship between SP and $\delta^{15}N^{bulk}_{N2O}$

Figure 4 C shows the relationship between SP and $\delta^{15}N^{bulk}_{N2O}$. There is a slightly positive trend between SP and $\delta^{15}N^{bulk}_{N2O}$, which has already been documented by Koba et al. (2009), and is interpreted to be due to heterotrophic N_2O reduction: ^{15}N depletion during N_2O production is compensated by heterotrophic N_2O reduction, which leads to both an enrichment of ^{15}N in N_2O and a positive SP. The shift to higher SP and less negative $\delta^{15}N^{bulk}_{N2O}$ values in Bio1, shown in Figure 4 C, is explained by a high rate of heterotrophic N_2O reduction compared to N_2O production. Similarly, Ostrom et al. (2007) noted that the activity of N_2O reductase needs to be higher than 10% compared to N_2O production in order to significantly impact the SP. Therefore, a low SP, e.g. in the range from 0 to 5‰ in combination with $\delta^{15}N^{bulk}_{N2O}$ values between -30 to -42‰ is an indicator of either (i) sole nitrifier denitrification activity or (ii) a combination of nitrifier denitrification activity and heterotrophic N reduction, respectively (Figure 4 C). Nevertheless, $\delta^{15}N^{bulk}_{N2O}$ values of around -30 ‰ in combination with SP values of around 0 ‰ are out of line with the positive trend proposed above. Again, this might be due to a reduced kinetic N fractionation of the microbial transformation pathways, probably affected by high transformation rates at 3 mgO_2/l, as well as to substrate limitation ultimately resulting in reduced $^{15}N\text{-}N_2O$ depletion.

In sum, SP close to 0 ‰ in combination with negative $\delta^{15}N^{bulk}_{N2O}$ values point to N_2O production via NO_2^- reduction (N_2O as a side-product). On the other hand, positive SP data in conjunction with less depleted $\delta^{15}N^{bulk}_{N2O}$ values and low dissolved O_2 support N_2O production from denitrification with substantial N_2O reductase activity (N_2O as an intermediate).

Using ^{15}N isotope signature to understand N_2O emissions from biological wastewater treatment

If further studies corroborate the fact that N_2O reductase activity alone controls the SP signature of N_2O in conventional biological wastewater treatment and the numerical value can be confirmed, this measurement can be directly used to apportion the inhibition of the last step of the heterotrophic denitrification. Since an SP of 0 ‰ is expected in case of heterotrophic N_2O emissions with completely inhibited N_2O reductase, this measurement cannot be used directly to assess the N_2O emissions via heterotrophic denitrifiers because a similar SP value is also assumed for nitrifier denitrification (e.g. 0.1 ‰; Sutka et al., 2006). Nevertheless, SP values close to 0 ‰ are a strong indicator of N_2O production via NO_2^- reduction, either by nitrifier denitrification or heterotrophic NO_2^- reduction, a finding which will significantly impact future plant operating strategies. However, in situations with a significant contribution of NH_2OH oxidation to the total N_2O production, e.g. at high nitrification rates in combination with low NO_2^- concentrations (Wunderlin et al., 2012), positive SP values are expected at high dissolved oxygen concentrations. This would result in SP values close to the NH_2OH oxidation end-member signatures (31.4 to 33.5 ‰; Sutka et al., 2006), since they are unlikely to be biased by heterotrophic N_2O reduction. The latter is assumed to be inhibited in the presence of oxygen.

On the other hand, the interpretation of the $\delta^{15}N^{bulk}_{N2O}$ is more complex because the observed signature results from several processes (multiple reaction steps during nitrification and denitrification). Recent publications used the net isotope effect, which is the difference in the isotope values between N_2O and the substrate ($\Delta\delta^{15}N = \delta^{15}N_{substrate} - \delta^{15}N^{bulk}_{N2O}$), instead of the kinetic isotope fractionation to overcome this difficulty (e.g. Koba et al., 2009; Wunderlin et al., 2013a). Clearly, information about the isotopic signature of the substrates (NH_4^+, NO_2^-, NO_3^-) would enhance our understanding of N_2O production. We therefore suggest that the fractionation of the substrates be characterized in forthcoming studies as a function of different microbial activity levels and operating conditions. In addition, we recommend that the kinetic N fractionation be quantified with greater accuracy. The interpretation of the $\delta^{15}N^{bulk}_{N2O}$ signal obtained could then be used as a quantitative measure of the relative importance of AOB and HET in N_2O production.

Conclusion

The present study tested whether the nitrogen isotopic signature is a suitable tool for N_2O source allocation in a pilot-scale biological wastewater treatment plant. The hypothesis was that NO_2^- reduction is the dominant route of N_2O production under aerobic conditions in the studied system. The following conclusions can be drawn:

- The SP data, in combination with dissolved O_2, NO_2^- and N_2O off-gas analysis, confirmed that NO_2^- reduction was the dominant N_2O production pathway.

- Moreover, NH_2OH oxidation was of minor importance in this investigation, while heterotrophic denitrification was relevant under low concentrations of dissolved oxygen.
- The SP data illustrate the potential to discriminate between the different N_2O production pathways, in combination with conventional parameters such as dissolved O_2, nitrogen species, and N_2O off-gas concentrations.
- The $\delta\,^{15}N^{bulk}{}_{N2O}$ data qualitatively support the findings from the SP analysis, since quantitative interpretation requires information about individual enzymatic activities, substrate concentrations as well as their isotopic compositions.
- Further studies are required to confirm whether plant operating strategies at low concentrations of dissolved NO_2^- can significantly reduce N_2O emissions.

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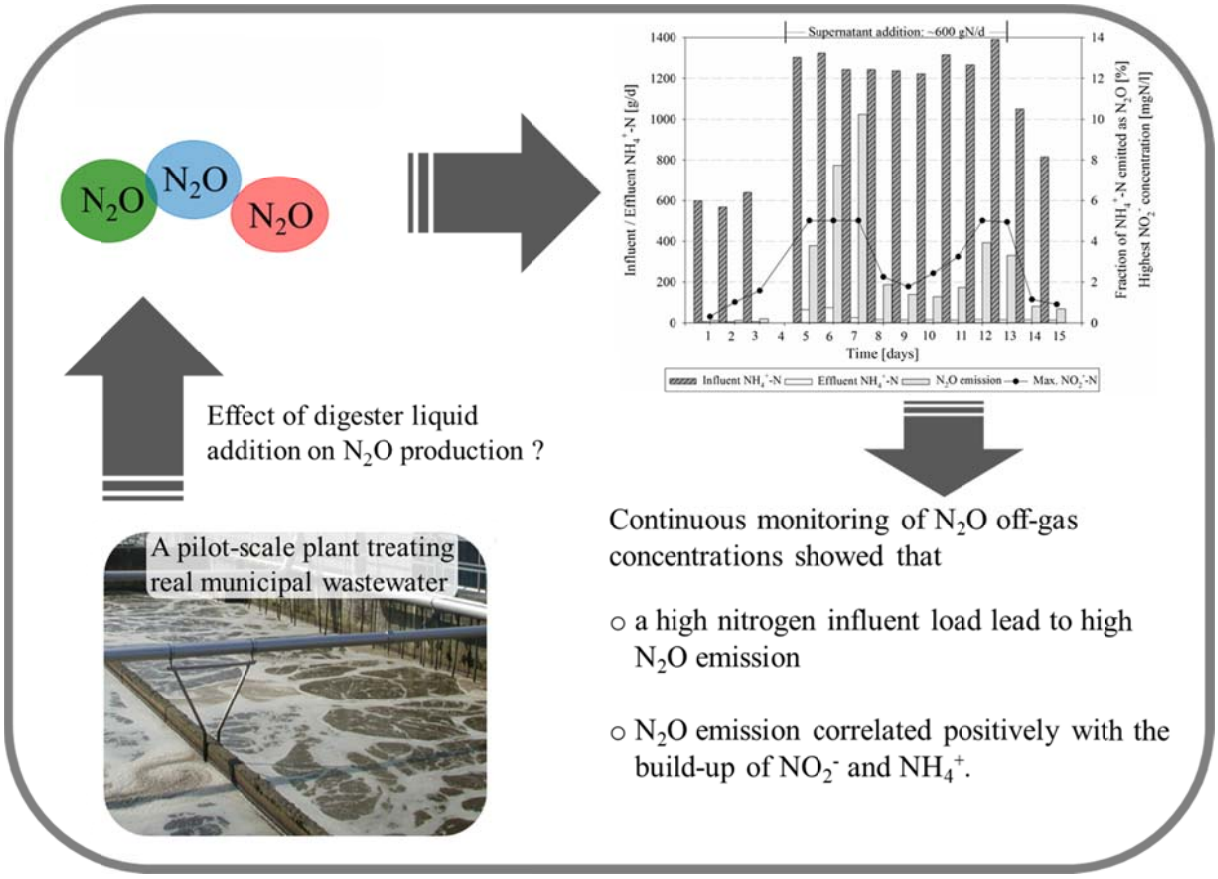
Chapter 6

Effect of digester liquid addition on N_2O production in a pilot-scale biological wastewater treatment plant

Thesis chapter

Wunderlin, P., Kipf, M., Lotito, A.M., Joss, A., Siegrist, H.

Graphical abstract



Abstract

Nitrous oxide (N_2O) is an important greenhouse gas, involved in the stratospheric ozone depletion. Its emission from biological wastewater treatment should therefore be avoided. With regard to on-going debate about energetic optimization and efficient conventional treatment of ammonia-rich digester liquid, a better understanding of N_2O dynamics as well as of emission levels is needed.

In the present study, we operated a pilot scale wastewater facility treating municipal wastewater and investigated the effect of peak as well as continuous digester liquid addition on N_2O emission. Under 'normal' operating conditions (without digester liquid dosing), 0.1 to 0.6 % of the ammonia influent load was emitted as N_2O, while being substantially increased at high nitrogen loads (during digester liquid peak addition), positively correlating with the build-up of ammonia (NH_4^+) and nitrite (NO_2^-) in the nitrification activated sludge tank. Results further show that continuous digester liquid addition (doubling 'normal' average nitrogen influent load) over 9 days, directly into the pre-denitrification reactor, resulted in high N_2O emissions during the first 3 days (up to 10 %). Even though N_2O emission decreased to a level of 1 to 4 % during the consecutive days, emission ranges were still high compared to 'normal' operating conditions. During a second continuous addition period, with variable influent loads, N_2O emission levels were lower. These findings indicate that transient nitrification overloading leads to elevated N_2O emission, and is therefore sensitive to digester liquid dosing. Moreover, nitrifier denitrification is hypothesized to be the dominant N_2O production pathway, due to the positive correlation between N_2O emission and dissolved NO_2^- concentrations. A continuous online N_2O off-gas measurement is deemed necessary for plant specific overall carbon footprint minimization.

Keywords

Biological wastewater treatment; denitrification; nitrification; nitrous oxide (N_2O); digester liquid addition

Introduction

Nitrous oxide (N_2O) is an atmospheric pollutant relevant as a greenhouse gas (about 300 times stronger than carbon dioxide (CO_2)) and a major sink of stratospheric ozone (Ravishankara et al., 2009). In the last decade, significant efforts have been made to reduce the energy consumption and to improve nitrogen (N) removal of wastewater treatment plants: since aeration is responsible for about 50 to 60 % of the total energy consumption, one of the most adopted solutions is to lower the aeration of the nitrification stage to the required minimum (Müller 2010; Wang et al., 2012). However, the resulting low dissolved oxygen (O_2) concentrations in the aerated reactors are currently discussed to be one of the key factors impacting N_2O production (Kampschreur et al., 2009). N_2O emissions higher than 0.5 to 1 % compared to converted N, result in greenhouse gas emission equivalent to aeration energy consumption (Law et al., 2012). Consequently, N_2O emissions have to be considered in energy optimization strategies, in order to avoid a net increase of greenhouse gas emission instead of the alleged reduction (Kampschreur et al., 2009).

In biological wastewater treatment, N_2O is produced either during nitrification by ammonia oxidizing bacteria (AOB) or during heterotrophic denitrification by heterotrophic denitrifiers (HET). There is increasing evidence of a dominant N_2O production pathway via nitrite (NO_2^-) reduction by AOB, called nitrifier denitrification, strongly correlating with the build-up of ammonia (NH_4^+) and NO_2^- (Tallec et al., 2006a; Kampschreur et al., 2009; Ahn et al., 2010b; Houweling et al., 2011; Wunderlin et al., 2012). Hydroxylamine (NH_2OH) oxidation to NO_2^- (via intermediates such as nitric oxide, NO, or nitroxyl, HNO; Law et al., 2012; Schreiber et al., 2012) is currently discussed to be active at high NH_4^+ oxidation rates in combination with high NH_4^+ and low NO_2^- concentrations, but is probably of minor importance within concentration ranges typically found in conventional municipal wastewater treatment systems (Sutka et al., 2006; Yu et al., 2010; Wunderlin et al., 2013). Similarly, the contribution from heterotrophic denitrification is considered negligible under normal operating conditions, such as low NO_2^- and no dissolved oxygen concentrations during denitrification (von Schulthess et al., 1994; Kampschreur et al., 2009).

The literature is currently inconsistent about the quantities of N_2O emitted during wastewater treatment. For example:

- Lotito et al. (2012) continuously measured N_2O emission from the oxidation tank of a pilot activated sludge plant, and reported ranges of <0.04 to 0.1 %, with respect to influent N, for solid retention times higher than 15 days, but up to 0.3 % for a solid retention time of approximately 11 days.
- von Schulthess and Gujer (1996) investigated N_2O emission from a continuously fed full-scale wastewater treatment plant. Total N_2O emissions were between 0.02 to 0.07 %, compared to total N input.
- N_2O emission of 12 full-scale treatment plants were monitored by Ahn et al. (2010b), and emission factors, in terms of N_2O emitted compared to total N

input, in the range of between 0.01 to 1.8 ± 0.79 % were reported (corresponding to 0.01 to 3.3 ± 1.5 % with respect to N removed).

- A highly variable N_2O emission range of 0.6 to 25.3 % (3.5 ± 2.7 % on average), with respect to N denitrified, has been calculated from an investigation across seven biological nutrient removal plants in Australia (Foley et al., 2010).

Likewise, no consensus has been achieved about the operating strategies to reduce N_2O emission. Discussion has been focused on some factors that have been recognized to be mainly responsible for N_2O production, such as (i) low dissolved O_2 concentration during nitrification as well as heterotrophic denitrification, (ii) the accumulation of NO_2^- and / or NH_4^+ during nitrification, or (iii) a low ratio of readily biodegradable organic compounds to NO_3^- during heterotrophic denitrification (Kampschreur et al., 2009; Ahn et al., 2010b; Desloover et al., 2012; Wunderlin et al., 2012). Recently, through the continuous operation of a pilot scale activated sludge plant combined with continuous N_2O off-gas measurement, it has been demonstrated that N_2O emissions follow a reproducible specific daily pattern: while emissions are quite negligible during night (at low N loads), it peaks in the morning during the hours of maximum N load, correlating with the build-up of NH_4^+ and NO_2^- in the activated sludge tanks (Lotito et al., 2012).

In this study we investigated the impact of digester liquid addition on the dynamics as well as on the level of N_2O production in a pilot-scale activated sludge plant, with the hypothesis that high N loading situations promote the build-up of NH_4^+ and NO_2^-, which in turn favors high N_2O emissions. As a first step, short-term effects of digester liquid peak addition on N_2O emission was evaluated. Then, the impact of continuous digester liquid addition over several days was investigated, in order to address potential biomass adaptation. To the best of our knowledge this is the first time that N_2O off-gas concentrations are continuously monitored over several weeks, in combination with continuous measurement of dissolved NO_2^- and NH_4^+ in the activated sludge tanks.

Materials and methods

Pilot plant set-up and operation

The pilot scale wastewater facility, treating around 60 to 80 population equivalents, consisted of a mechanical pre-treatment and a primary clarification, followed by an activated sludge treatment with pre-denitrification (Bio1) and two nitrification reactors (Bio2 and Bio3; each operated as a completely stirred tank reactor with a volume of about 7.5 m^3) and a secondary clarifier (Figure 1). The plant was fed with municipal wastewater from the sewer system of the city of Dübendorf (Switzerland) and was operated at a solid retention time (referred to the biological compartments) of approximately 11 to 12 days (7 to 8 days aerobic) by wasting excess sludge from Bio1. The concentration of total suspended solids (TSS) was in the range of 1.6 to 1.8 g/l. Aeration was controlled at a constant dissolved oxygen (DO) concentration in the reactor (set-point at 0.5, 1 or 2 mgO_2/l). Furthermore, an internal recirculation from the second nitrification tank into the pre-denitrification stage was applied at 1.5 m^3/h, in order to

maintain a sufficient NO_3^- load for denitrification. The influent flow followed a fixed diurnal pattern, changing every two hours, with an average value of 1.2 m^3/h (minimum influent flow of 0.7 m^3/h between 4 to 6 a.m.; maximum influent flow of 1.6 m^3/h between 8 to 10 a.m.). The return sludge, from the secondary clarifier to Bio1, varied proportionally to the influent flow.

The digester liquid from the municipal wastewater treatment plant Werdhölzli (Zürich, Switzerland), was stored in our experimental hall (neither aerated nor stirred) and was dosed directly into Bio1, either continuously or by discontinuous ('peak') addition (see below). The average ammonia concentration was 800 mgN/l, while NO_2^- and NO_3^- were below 1 mgN/l.

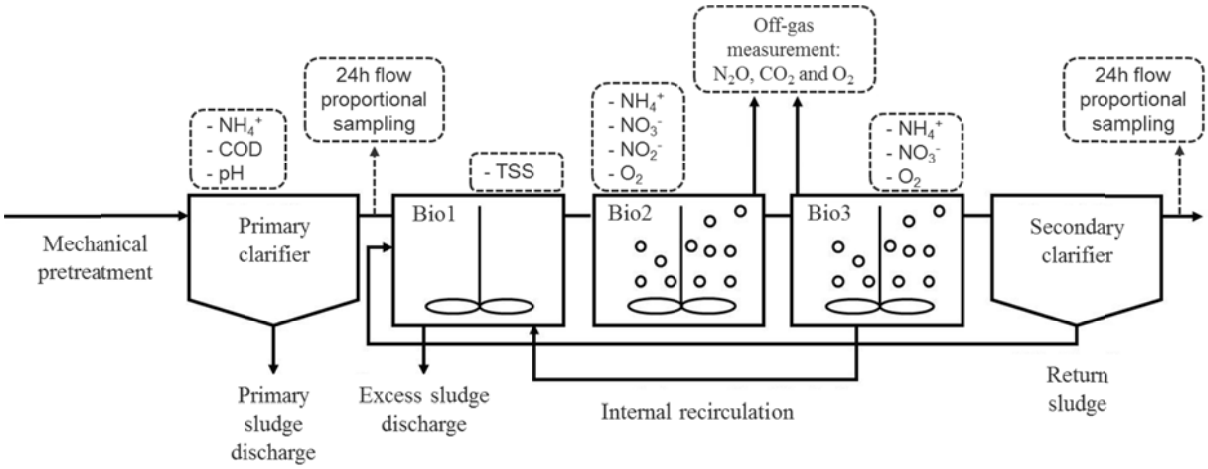


Figure 1. *Flow scheme of the pilot scale wastewater facility treating municipal wastewater. The plant was operated with one pre-denitrification reactor (Bio1), followed by two aerated reactors (Bio2 and Bio3). N_2O was analyzed in the off-gas of Bio2 and Bio3. Further online measurements are indicated by dotted lines.*

Analytical methods for off-gas measurement

Off-gas concentrations of N_2O (Rosemount Analytical X-Stream X2) were measured continuously. The sample gas was dried prior to analysis (PKE 521, Bühler Technologies). A 3-way valve was used in order to switch between the off-gas of Bio2 and Bio3 (every 36 minutes), and missing data were supplemented by linear interpolation. The analyzer was calibrated three times per week: dinitrogen (N_2) was used for zero point calibration, while a standard gas (Carbagas, Switzerland) with known concentration for N_2O (200 ppm) was used for the second calibration point. Then, N_2O emission loads were computed by multiplying the concentration by the air flow rate (1 ppm N_2O corresponds to 1.249 $mgN_2O\text{-}N/m^3_{air}$ under standard conditions at 273.15 K and 1013 mbar; further details about the accuracy of N_2O off-gas measurement is given in Lotito et al., 2012.), and were integrated over time to evaluate the total mass of N_2O-N emitted. N_2O emission factors per day were then calculated by dividing the total mass of N emitted as N_2O by the total mass of N in the inflow present as NH_4^+.

Analytical methods for dissolved species

The plant was equipped with various online sensors (Figure 1), such as (i) NH_4^+ (Ion selective electrode ISEmax CAS 40, Endress + Hauser), pH (Orbisinit CPS11D, Endress + Hauser), total and dissolved COD (chemical oxygen demand; spectrometric s::can) in the primary clarifier, (ii) TSS (Turbimax, Endress + Hauser) in Bio1, (iii) NH_4^+ and NO_3^- (ISEmax, Endress + Hauser), NO_2^- (Stamolys CA 70 analyzer, Endress + Hauser) and O_2 (Optical LDO, Hach Lange) in Bio2, and (iv) NH_4^+ and NO_3^- (ISEmax, Endress + Hauser) and O_2 (Optical LDO, Endress + Hauser) in Bio3. All data were logged to a supervisory control and data acquisition system. Ion selective electrodes were calibrated once a week. Additionally, 24-hour flow proportional composite samples of the influent and effluent were sampled three times a week (daily sampling during continuous supernatant addition), for analyzing the following species: total N (commercial photochemical test kits, Hach Lange GmbH, Düsseldorf, Germany), NH_4^+ (Foss FIAstar flow injection 5000 analyzer), NO_2^- and NO_3^- (ion chromatography, 761 compact IC, Metrohm), total and soluble COD (chemical oxidation demand; commercial photochemical test kits, Hach Lange GmbH, Düsseldorf, Germany), and TSS (standard methods). Mixed liquor suspended solids were measured according to standard methods (APHA 1998).

Operational schedule

Throughout the whole monitoring phase, the plant was operated under three different conditions:

(i) 'normal' operating condition without any digester liquid addition, defined as reference days, including both working and week-end days (discussed in section 'N_2O production during 'normal' operating conditions').

(ii) 'digester liquid peak' condition, where digester liquid was added directly into Bio1 at 8:30 or 9:00 a.m., at high rates (~120 gN within 1 minute, corresponding to about 20 % of the average daily NH_4^+ influent load), during three different days applying different DO set-points (0.5, 1 and 2 mgO_2/l in Bio3; discussed in section 'Effect of digester liquid peak addition on N_2O production under different dissolved O_2 concentrations').

(iii) 'continuous digester liquid' addition during 24 hours directly into Bio1 (over 9 days) at a constant flow rate of about 600 gN per day (doubling the 'normal' average ammonia influent load), as well as over 12 days during a second continuous digester liquid dosing period at more variable influent loads (from 790 to 1339 gN/d; discussed in section 'Effect of continuous digester liquid peak addition on N_2O production').

Results and discussion

N_2O production during 'normal' operating conditions

During 'normal' operation, the total N influent concentrations were in the range of 17.9 to 42.1 mgN/l, corresponding to an average N influent load of 35 gN/h and an NH_4^+ influent load of 25.3 ± 4.8 gN/h, respectively (Figure 2). The N removal rate was 72 ± 5.5 %, with an ammonia oxidation rate higher than 98 % at a DO set-point of 2 mgO_2/l in both nitrification reactors. The dissolved COD influent concentration was 124 ± 47 mgCOD/l, resulting in an average dissolved COD / NH_4^+-N influent ratio of approximately 11.3 ± 4. The total COD removal rate was higher than 80 %.

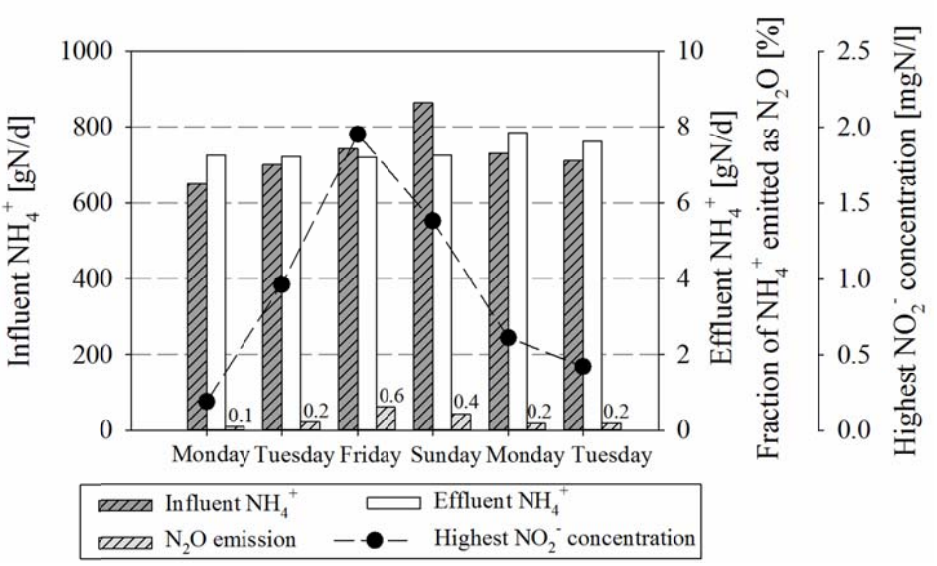


Figure 2. *Fraction of N_2O emitted with respect to influent ammonia during six reference days, without external digester liquid addition, including the daily highest NO_2^- concentrations.*

During the 'normal' operating conditions, N_2O emissions were 0.1 to 0.2 % with respect to an average NH_4^+-N influent load of 600 gN/d (Figure 2). During 'normal' days, there was usually no N_2O emission during night (except when digester liquid was added): then, N_2O started to increase in the morning hours (8 to 9 a.m.), paralleled by a build-up of NH_4^+ and NO_2^- in the nitrification reactor (maximum daily NO_2^- concentrations up to 2 mgN/l, as illustrated in Figure 2). Furthermore, N_2O remained at an elevated level throughout the day, and finally decreased again in the afternoon, in parallel with a reduction of NH_4^+ and NO_2^- concentrations, similarly to what observed by Lotito et al. (2012) and Ahn et al. (2010b). It has to be mentioned that N_2O emission during weekends were typically higher compared to work days which is hypothesized to be due to higher N influent loads during weekends (city with high ratio of commuter during work days). Looking at the data in Figure 2, it can be seen that 0.4 to 0.6 % of influent ammonia was emitted as N_2O during the exemplary Friday and Sunday (compared to normal values during working days of 0.1 to 0.2 %), and similarly, maximum NO_2^- concentrations were higher during these two days (up to 1.4 to 2

mgN/l). The N_2O emission ranges found for 'normal' operating conditions are in good agreement with data presented in literature (e.g. von Schulthess and Gujer 1996; Ahn et al., 2010a; Ahn et al., 2010b).

In this section it was shown that 'normal' days emitted relatively low amounts of N_2O, but is slightly accelerated during weekend days and higher N influent loads. N_2O emissions followed a typical pattern: low emissions during night, and high emissions during the day in combination with high N influent loads.

Effect of digester liquid peak addition on N_2O production under different dissolved O_2 concentrations

During digester liquid peak addition about 120 gN was added within 1 minute directly into Bio1, which accounted for about 20 % of the average daily ammonia influent load (Figure 3). This led to total ammonia influent loads between 750 and 810 gN/d, being in the range of 'normal' weekend days (Figure 2). Ammonia loss via effluent was highest at a DO set-point of 0.5 mgO_2/l in Bio3, which accounted for ~7 % with respect to the influent load, and was due to oxygen-limited nitrification.

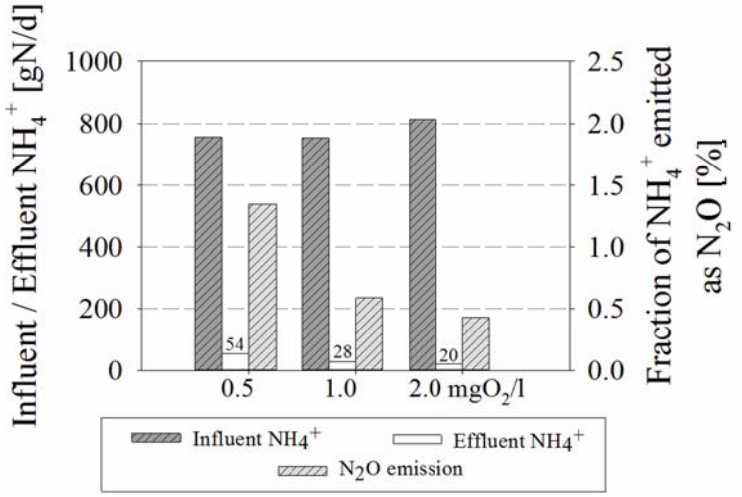


Figure 3. *N_2O emission during digester liquid peak addition in combination with three different DO set-points in Bio3 (0.5, 1 and 2 mgO_2/l, respectively; DO set-point in Bio2 was unchanged at 2 mgO_2/l).*

Figure 3 shows that N_2O emissions were substantially increased due to digester liquid peak addition, compared to normal weekdays. This is in agreement with literature, reporting a positive correlation between NH_4^+ shock loads and the N_2O off-gas concentrations (Burgess et al., 2002a; Lotito et al., 2012). Moreover, in our study, N_2O emissions were highest at the DO set-point of 0.5 mgO_2/l (~1.3 %), while being only about 0.6 and 0.4 % at the DO set-points of 1 and 2 mgO_2/l, respectively (Figure 3). Again, this is congruent with literature: for example, Wunderlin et al. (2012) reported highest N_2O emissions at low DO (0.5 mgO_2/l), or Tallec et al. (2006) measured the highest N_2O emission level at an oxygen concentration of 1 mgO_2/l. In another investigation, it was reported that a reduction of DO during high ammonia loads significantly increased N_2O emission in a pilot-scale municipal wastewater treatment

plant (Lotito et al., 2012). We interpret these correlations as indicative for nitrifier denitrification driven N_2O production, which is also supported by a recent study, based on isotopomeric measurements, where N_2O production in a pilot-scale treatment plant was dominated by NO_2^- reduction (Wunderlin et al., in preparation; Chapter 5).

Heterotrophic denitrification on the other side, is assumed to be of minor importance in our investigation, due to (i) anoxic conditions in the denitrification reactor, (ii) relatively low NO_2^- concentrations (not higher than 2 mgN/l in Bio 2), and (iii) COD / N influent ratios higher than 5 (ratios below 5 were identified as critical with respect to N_2O production; Alinsafi et al., 2008).

Based on the presented data in this section, it is concluded that digester liquid peak addition resulted in higher N_2O emission, compared to 'normal' week days, but being similar to 'normal' weekend days with higher N loads. Furthermore, N_2O emissions are accelerated by low dissolved oxygen concentrations during nitrification, which supports nitrifier denitrification as relevant N_2O production pathway.

Effect of continuous digester liquid addition on N_2O production

During the first two days of continuous digester liquid addition (doubling the 'normal' ammonia influent load), the NH_4^+ removal rate was reduced, due to probably a limiting nitrification capacity. But from the third day onward, NH_4^+ effluent loads were lower than 20 gN/d, which accounted for less than 5 % compared to NH_4^+ influent loads. NO_2^- concentrations were remarkably high, especially during the first three days (>5 mgN/l; Figure 4), which is assumed to be due to a temporary plant overload, leading to incomplete nitrification or inhibition of NOBs. Consequently, N_2O emission raised up to 7.5 to 10 % with respect to influent NH_4^+ (Figure 4), being significantly higher than during 'normal' operating conditions (e.g. 0.1 to 0.6 %, see Figure 2 and section 'N_2O production during 'normal' operating conditions') and compared to values reported in literature: for example, von Schulthess and Gujer (1996) published a range from 0.2 to 0.7 ‰ with respect to the total N input, and Ahn et al. (2010b) measured maximum values of 3.3 % compared to total N removed.

At the fourth day after the start of continuous digester liquid addition, N_2O emission decreased to values in the range of between 1.4 to 1.9 %, paralleled by a decline in dissolved NO_2^-. It is hypothesized that this reduction is due to lower N influent loads (1324 gN/d during day 5 and 6 compared to about 1233 gN/d during the consecutive days). The COD / N ratio is speculated to be another potential factor impacting N_2O emission: as during weekends the COD / N influent ratio is typically lower compared to week-days (due to industrial activities in the catchment area), this might result in lower heterotrophic activity, and consequently NO_2^- produced by incomplete nitrification is less efficiently removed in the denitrification tanks. Of course, heterotrophic denitrification cannot be completely ruled out as significant N_2O producers in this investigation, since high NO_2^- concentrations and low COD / N ratios (below 5; Alinsafi et al., 2008) are discussed to favor N_2O production by heterotrophic denitrifiers (von Schulthess et al., 1994; Wunderlin et al., 2012). However, just based on the presented

data, it is not possible to quantitatively differentiate between the individual contributions of the N_2O production pathways.

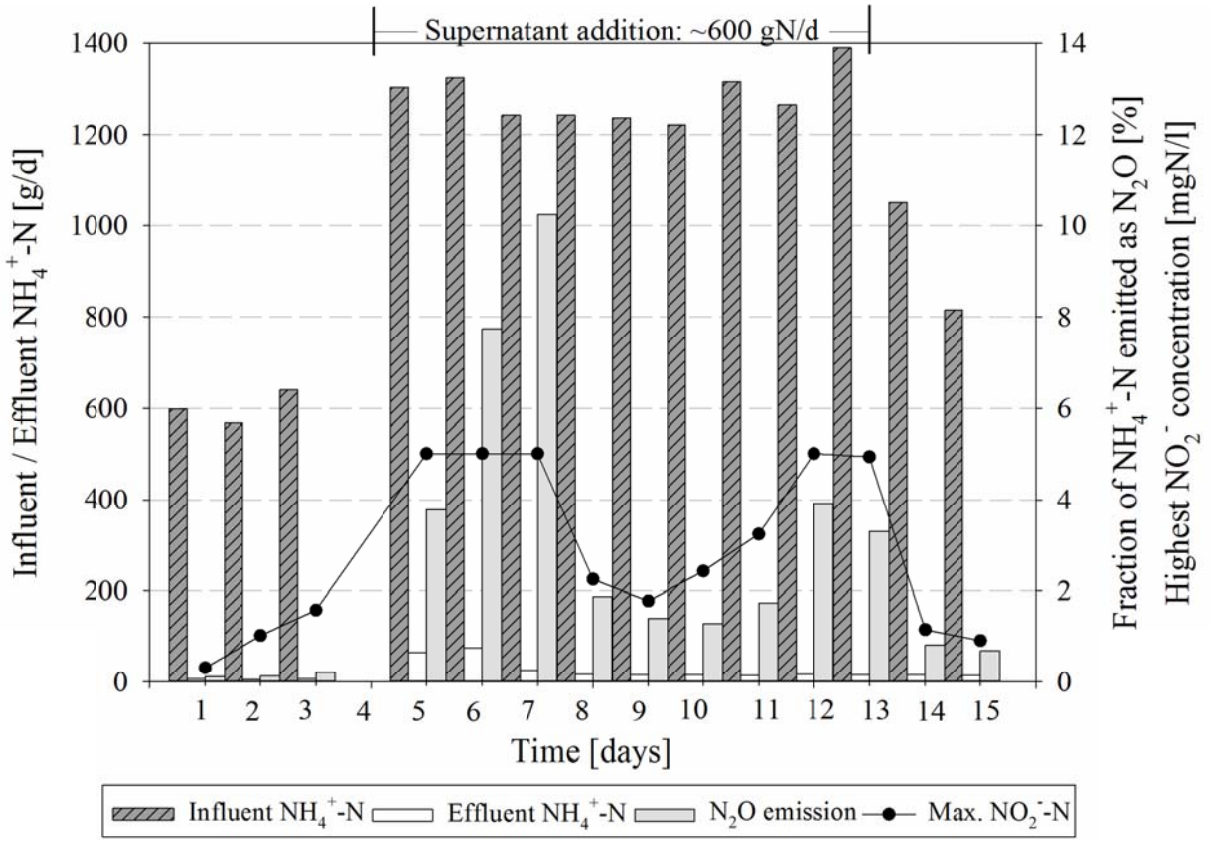


Figure 4. *NH_4^+ influent load (gN/d; dark grey bars), NH_4^+ effluent load (gN/d; white bars), fraction of NH_4^+ influent load emitted as N_2O (%; light grey bars) and the daily highest NO_2^- concentrations (mgN/l; bullets) during 9 days (day 5 to 13) of continuous supernatant addition (no data available for day 4 due to sensor calibration). Reverting to low N influent loads (day 14 and 15), results in a sharp decrease in both N_2O emission as well dissolved NO_2^-.*

During the second weekend of continuous digester liquid addition, N_2O emission decreased to values of maximum 4 %, which is significantly lower than during the first weekend. It is hypothesized that this reduction might be due to biomass adaptation (e.g. due to metabolic adaptation, or growth of the nitrifier community), since nitrification was able to better deal with the high ammonia influent loads (less ammonia in the effluent). Similarly, Chandran et al. (2011) observed a steady decrease in N_2O emission from *N. europaea* pure culture within the first 8 days upon repeatedly NH_4^+ pulse loadings, which was attributed to an adaptive metabolic response.

The significant reduction of N_2O emission at day 14 and 15 in our study (down to 0.7 to 0.8 %, Figure 4) is due to a cessation of continuous digester liquid addition, and reverting to 'normal' influent loads. Dissolved NO_2^- as well as N_2O emission reached similar levels as during the days before continuous digester liquid dosing. This situation nicely illustrates how to reduce N_2O emission by lowering N influent loads.

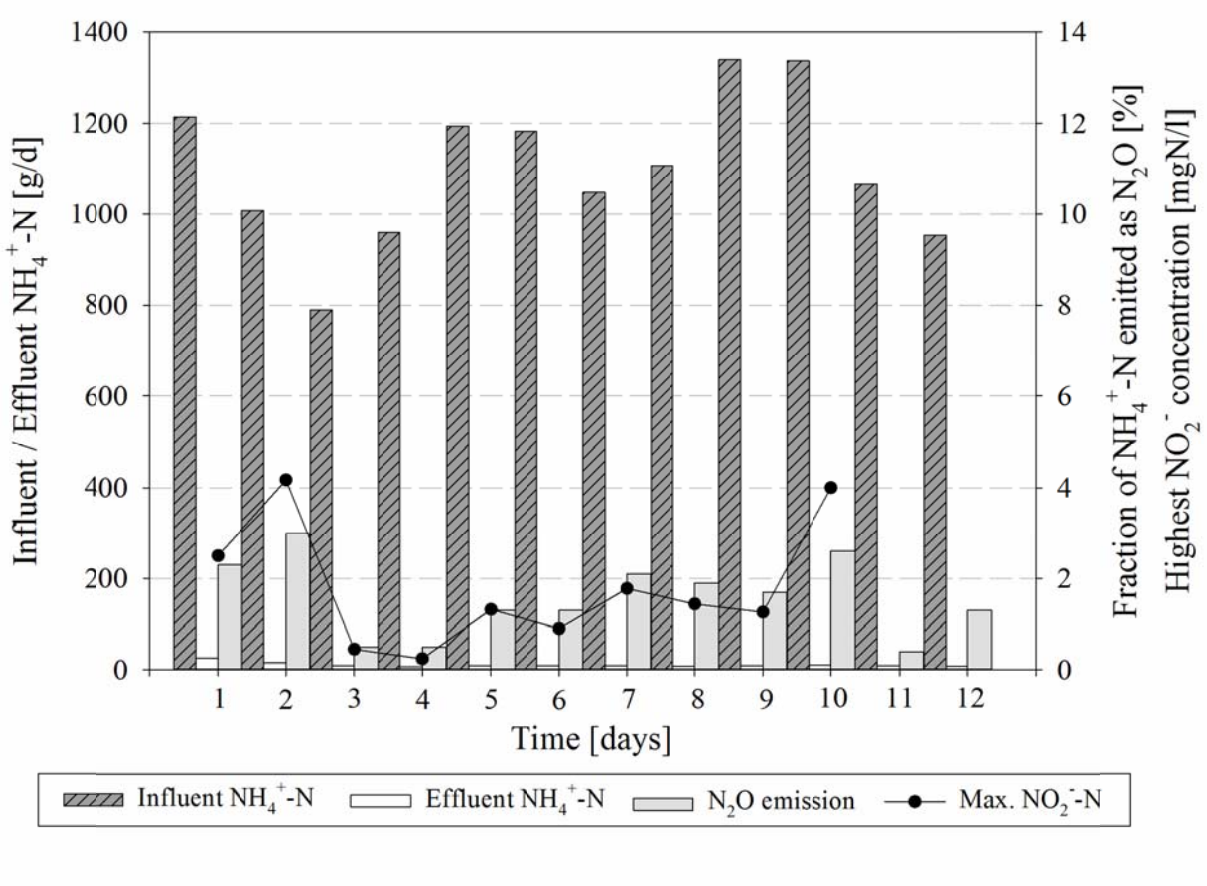


Figure 5. *NH_4^+ influent load (gN/d; dark grey bars), NH_4^+ effluent load (gN/d; white bars), fraction of NH_4^+ influent load emitted as N_2O (%; light grey bars) and the daily highest NO_2^- concentrations (mgN/l; bullets) during 12 consecutive days of variable continuous supernatant addition. High N influent loads result in higher N_2O emission rates as well as NO_2^- concentrations compared to low N influent days.*

In a second series of continuous digester liquid addition, it was tested whether a variable digester liquid dosing can keep NO_2^- low and in parallel also reduce N_2O emission peaks. In fact, the N influent loads varied between 781 and 1331 gN/d (Figure 5), and N_2O emission levels were in the range from 0.4 to 3 % with respect to NH_4^+ influent load. Highest daily NO_2^- concentrations were in between of 0.5 and 4.2 mgN/l. As hypothesized, both, N_2O emission rates as well as highest daily NO_2^- concentrations varied substantially, presumably depending on the N influent loads. Moreover, dissolved NO_2^- was significantly lower within the first three days, after the start of digester liquid dosing, compared to the data shown in Figure 4. This finding supports the assumption of nitrification overloading or (partial) NOB inhibition as a trigger for NO_2^- accumulation within the first days of continuous digester dosing illustrated in Figure 4. Nevertheless, under more variable influent loads, the N_2O emission dynamics remained in line with the above reported trends, where high N loads resulted in high NO_2^- concentrations as well as N_2O emissions, and vice-versa. This indicates that transient nitrification overloading leads to elevated N_2O emission, and is therefore sensitive to digester liquid dosing, which should be added preferentially during low NH_4^+ loading situations.

In this section, the effect of continuous digester liquid dosing over several days was investigated. Similar to the previous sections, (i) N_2O emission increased during high N influent loads, as well as (ii) in parallel to NO_2^- accumulation. Microbial adaptation, e.g. on a metabolic level or by an increase in the nitrifier population, might explain the lower N_2O emission levels during the second weekend after continuous digester liquid dosing, depicted in Figure 4.

Nitrogen influent load and dissolved NO_2^-: two key factors impacting N_2O emission

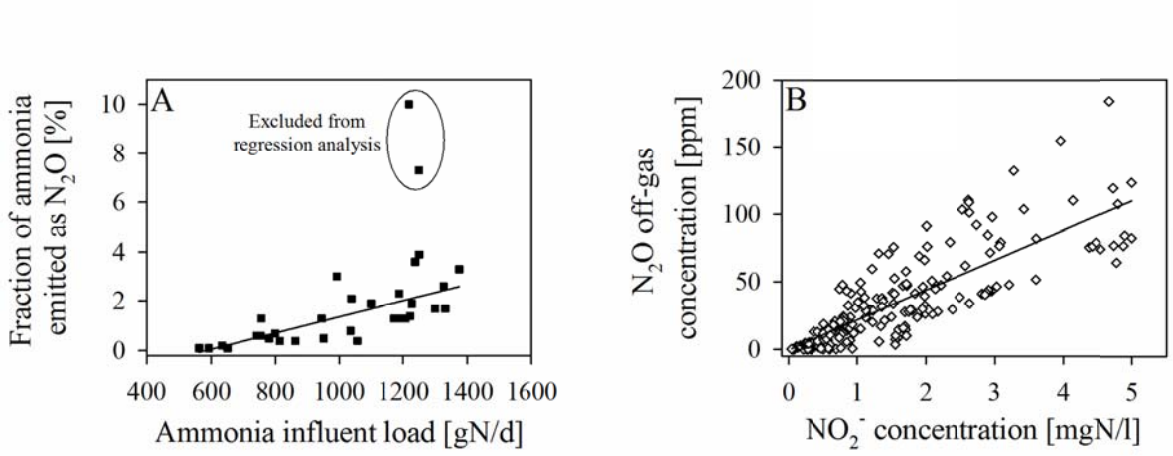


Figure 6. *(A) Correlation between N_2O emission (N_2O-N emitted with respect to NH_4^+-N influent load) and ammonia influent load: Emissions are low under 'normal' operating conditions (average NH_4^+ influent load of 600 gN/d), but are increasing proportionally to the ammonia influent load. The two unusually high N_2O emission days might be due to nitrification inhibition. (B) Correlation between bulk NO_2^- and N_2O off-gas concentration (average values over one hours) of Bio2 during 'normal' operation and continuous supernatant addition: high NO_2^- concentrations are indicative for elevated N_2O off-gas concentrations.*

A positive correlation between NH_4^+ influent loads and N_2O emissions is illustrated in Figure 6 A. At 'normal' influent loads (e.g. 600 gN/d), N_2O emissions were relatively low, compared to situations of higher influent loads (e.g. at 1400 gN/d). A similar trend has been described previously, but for lower nitrogen load situations (Lotito et al., 2012): N_2O emission were absent during low loaded nights but started to increase in the morning hours in parallel to the NH_4^+ peak in the inflowing wastewater and the build-up of NH_4^+ in the activated sludge tanks.

Chandran et al. (2011) proposed, that excessive nitrogen loads to a nitrification reactor are likely to trigger higher NH_4^+ oxidation rates, resulting in higher N_2O emissions, presumably produced via NH_2OH oxidation. Similarly, Law et al. (2012) observed an exponential correlation between N_2O production and NH_4^+ oxidation rates during partial nitrification of ammonia-rich wastewater, which is hypothesized to be due to chemical HNO decomposition. Additional methods, such as the analysis of the N_2O site-

specific isotopic signature, are needed to obtain a more accurate allocation of the emissions reported in this study.

The correlation between dissolved NO_2^- and the N_2O off-gas concentrations is shown in Figure 6 B: nitrification at high dissolved NO_2^- concentration, leads to high N_2O emissions. This is comparable to former investigations reporting increasing N_2O emissions upon NO_2^- build-up (Burgess et al., 2002a; Burgess et al., 2002b; Shiskowski and Mavinic 2006). Such a correlation is consistent with the general understanding of NO_2^- reduction as the dominant pathway for N_2O production in biological wastewater treatment (Colliver and Stephenson 2000), and leads to the conclusion that plants operated at low dissolved NO_2^- (and NH_4^+) emit only low amounts of N_2O, which was confirmed by ceasing continuous digester dosing (day 14 and 15 shown in Figure 4).

Implementation of continuous online N_2O measurement for reducing overall carbon footprint of biological wastewater treatment

A continuous N_2O off-gas monitoring is recommended in order to elucidate and adopt appropriate plant specific operating strategies, which is supported by a recent finding of Daelman et al. (2013) who showed that continuous long-term online monitoring is required to capture N_2O emission ranges accurately and emission dynamics more fully.

The application of continuous N_2O off-gas analysis in conventional biological wastewater treatment was already proposed by Burgess et al. (2002b), who came up with the idea of using N_2O off-gas emission measurement as an indicator for monitoring nitrification process stability: it was shown that nitrification failure (e.g. due to microbial inhibition) or during NH_4^+ shock loads resulted in NO_2^- build-up and in increasing N_2O off-gas concentrations (similar to our data). Their work resulted in an international patent application (number PCT/GB00/01094), which was further developed by Butler et al. (2009) who investigated N_2O off-gas measurement as a tool for early warning of biological nitrification failure: basically, an increase in N_2O was detected in parallel to an increase in NO_2^-. However, to our knowledge, this idea was not further developed nor applied in full-scale treatment plants.

The accumulation of NO_2^- is unwanted but difficult to measure so far. As such, based on the correlation between dissolved NO_2^- and N_2O emission, the latter could be used as an indirect measure for NO_2^- accumulation. Still, it is to be elucidated whether, beside nitrifier denitrification, also NH_2OH oxidation contributes substantially to N_2O production under specific operating conditions, as for example in a sequencing batch operating mode or during partial nitrification, since the NH_2OH pathway is not expected to correlated with dissolved NO_2^-. Nevertheless, by controlling activated sludge treatment processes at minimal N_2O emissions, the contribution from both pathways, NH_2OH oxidation as well as nitrifier denitrification, will be low.

Conclusions

The effect of digester liquid addition on N_2O production during conventional biological wastewater treatment was evaluated in this study, with the hypothesis that high N load situations promote the build-up of NH_4^+ and NO_2^- in the activated sludge tanks, which in turn favors high N_2O emission. The conclusion can be summarized as follows:

- The external addition of digester liquid (high N load situation) significantly increased the N_2O emission rate compared to 'normal' operating conditions. Consequently, ammonia rich digester liquid should be dosed preferentially during low ammonia loads.
- N_2O emissions were lower when activated sludge was adapted to continuously high N influent loads, presumably by an increase of the nitrifier population. Thus, ammonia rich digester liquid could be added to bridge low N load situations in order to maintain a 'strong' nitrifier community.
- N_2O emission correlated with the N influent load and the build-up of NO_2^- (and NH_4^+) in the nitrification reactor. As a consequence, plants facing high N influent load variations, should be operated at sufficient dissolved oxygen, in order to avoid incomplete nitrification and to keep NO_2^- low (< 1 to 2 mgN/l; e.g. aeration with NH_4^+ control).
- Energetic optimization of wastewater treatment by reducing aeration (and O_2 input) is critical, especially during high N loads. Therefore, it is recommended to reduce (or switching off) the aeration solely at low N load situations.
- A continuous online N_2O off-gas measurement is deemed necessary for plant specific overall greenhouse gas minimization.

Acknowledgements

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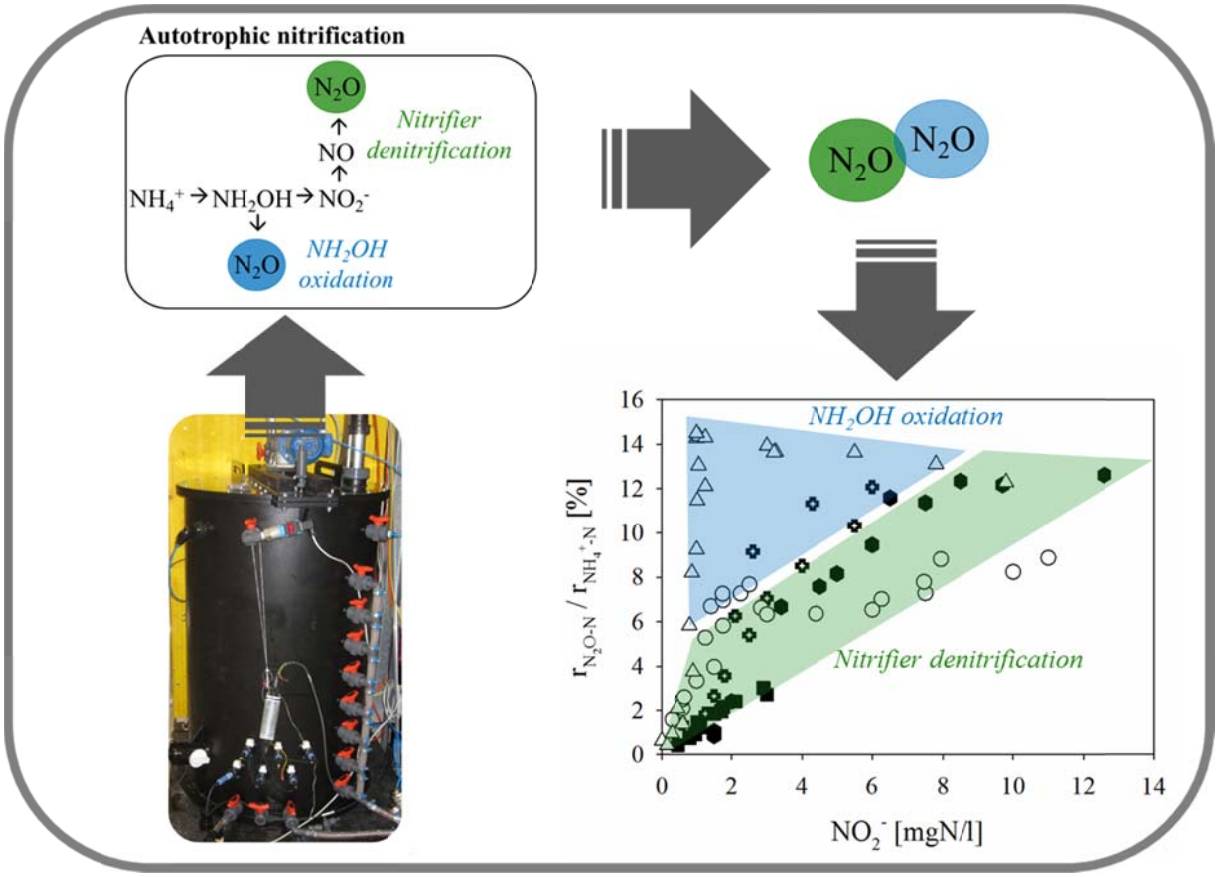
Chapter 7

Monitoring N_2O emission for indirect NO_2^- measurement and nitritation-anammox process control

Thesis chapter

Wunderlin, P., Joss, A., Kipf, M., Siegrist, H.

Graphical abstract



Abstract

Nitrous oxide (N_2O) is a strong greenhouse gas and involved in the destruction of the stratospheric ozone layer. N_2O production mechanisms of nitritation-anammox processes are not fully understood, but hypothesized to depend on dissolved NO_2^- concentrations. Latter accumulates if the process is not stable and promotes growth of unwanted nitrite oxidizing bacteria (NOB), but so far, NO_2^- is not easily measured online. Therefore, NO_2^- is expected to be monitored indirectly via N_2O off-gas analysis.

In this investigation, a single-stage pilot-scale nitritation-anammox process was fed with digester liquid. The reactor was temporary operated at high aeration rates, up to the double of the usually applied airflow rates, in order to accumulate NO_2^-, and thus to test the correlation between NO_2^- and N_2O. Results show that N_2O emissions were controlled by the airflow rate: high aeration rates resulted in high NO_2^- concentrations as well as high N_2O emissions. However, there was not always a clear correlation between dissolved NO_2^- and N_2O off-gas concentrations, which is hypothesized to be due to NH_2OH oxidation activity (another N_2O production pathway). Nevertheless, reactor operation controlled at minimal N_2O emission kept stable ammonia (NH_4^+) removal rates at low NO_2^- concentrations (<0.6 mgN/l). This is a promising finding and supports future directions incorporating continuous N_2O off-gas measurement in the automatic process control, for example to control the airflow rate.

Keywords

Aeration rate; Nitrous oxide (N_2O); Nitrite (NO_2^-); Nitritation-anammox process control

Introduction

Nitrous oxide (N_2O) is a strong greenhouse gas, involved in the destruction of the stratospheric ozone layer (Ravishankara et al., 2009). However, N_2O production and its relevance in biological wastewater treatment is still under debate. There is only a limited number of studies, investigating N_2O production mechanisms and emission levels from anammox-based nitrogen (N) removal processes. For example, Kampschreur et al. (2008) measured an N_2O emission rate of 2.3 % with respect to the N load during full-scale two-stage reject water treatment. And an emission level of 9.6±3.2 %, with respect to N removed, was reported for a lab-scale two-stage granular nitrification-anammox process (Okabe et al., 2011). In another study, 1.2 % of the N load was emitted as N_2O from a full-scale single-stage granular reactor (Kampschreur et al., 2009). Similarly, Joss et al. (2009) investigated N_2O emission from a single-stage nitritation-anammox reactor with suspended biomass, and measured an N_2O emission rate of 0.6 % with respect to removed N during intermittent aeration, and 0.4 % during continuous aeration, respectively.

These data reveal that N_2O emission ranges from anammox-based systems can be relevant, depending on the operating conditions. And in some situations, N_2O emission might even annihilate the avoided environmental impact achieved by lower energy consumption and organic carbon requirements compared to conventional treatment schemes.

In addition to greenhouse gas emission, process stability is another issue of nitritation-anammox processes. Currently, an active discussion concerning the selection of suitable sensors for process control, such as pH, conductivity, NH_4^+ or O_2, and on process control strategies is going on (e.g. Joss et al., 2011). A volumetric aeration control was recently proposed to be crucial for process stability in a single-stage nitritation-anammox reactor, since O_2 supply beyond depletion by AOB (e.g. during situations of over-aeration) can lead to anammox inhibition and thus favor NOB growth. Such process instability could be detected at an early stage by online monitoring of NO_2^-. However, this requires a suitable online electrode with sufficient resolution (measuring few mgNO_2^--N/l in digester liquid on the background of 0 to >100 mgNO_3^--N/l), which is, to the author's knowledge, currently not commercially available. However, based on the findings of enzymatic NO_2^- reduction as a dominant N_2O production pathway in biological wastewater treatment (e.g. Colliver and Stephenson 2000; Kampschreur et al., 2009; Wunderlin et al., 2012), and the confirmation of increased N_2O off-gas concentrations upon NO_2^- accumulation in a granular nitritation-anammox system operated at high dissolved oxygen (DO; >2 mgO_2/l) (Kampschreur et al., 2009), it is hypothesized that dissolved NO_2^- can be monitored indirectly via the measurement of N_2O off-gas concentration (see also Butler et al., 2009 and Colliver et al., 2002). Thus, N_2O emission rates, as a proxy for NO_2^- concentration, could be used to control the aeration rate, as well as to design process control strategies reducing environmentally relevant gaseous emissions of the nitritation-anammox systems.

Materials and methods

Set-up and operation of the nitritation-anammox pilot-scale sequencing batch reactor (SBR)

A 400 liter one-stage nitritation-anammox pilot-scale reactor was operated in sequencing batch mode with digester liquid from the full-scale wastewater treatment plant Werdhölzli (Switzerland). The SBR cycle always included the following phases: (i) feeding (15 to 20 % volume exchange) with mixing, (ii) aeration, (iii) mixing, (iv) sedimentation, and (v) discharge (a typical SBR cycle is illustrated in Figure 2). A complete cycle usually lasted between 5 and 7 hours, depending amongst others, on parameters like NH_4^+ degradation rate, sludge concentration, or aeration rate. The nitritation-anammox process was controlled by a programmable logical controller equipped with online sensors for the water level, NH_4^+, NO_3^-, DO concentrations, temperature, pH, and conductivity. Moreover, the reactor was also equipped with a stirrer, an aeration unit, as well as a temperature control (30±1 °C unless stated otherwise). Further details are given in Joss et al. (2011).

Unless stated otherwise, the reactor aeration rate was manually controlled with a rotameter, to keep NO_2^- concentration below 1 mgNO_2^--N/l. This typically resulted in DO concentrations, measured in the bulk, of <0.05 mgO_2/l. For experimental purposes, the reactor aeration rate was temporary increased to foster NO_2^- accumulation, since an increase in oxygen supply is known to trigger NO_2^- accumulation due to (i) anammox inhibition by DO, and (ii) a weak (or absent) NOB population (Joss et al., 2011) (see section 'Correlation between dissolved NO_2^- and N_2O emission rate'). The proposed control of the aeration rate according to N_2O emission, was done by manually adjusting the airflow rate, based on the N_2O off-gas concentration measurement, where high N_2O concentrations resulted in an airflow reduction, and vice-versa (see section 'Manual airflow adjustment based on continuous N_2O off-gas concentration measurement').

Analytical methods for dissolved species

The sequencing batch reactor was equipped with various online sensors, such as for NH_4^+ and NO_3^- (ISEmax, Endress+Hauser), pH (Orbisint CPS11D, Endress+Hauser), O_2 (Oxymax H COS61D, Endress+Hauser) and conductivity (Indumax CLS50D, Endress+Hauser). All data were logged to a supervisory control and data acquisition system. To test the accuracy of the online sensors, dissolved NH_4^+ and NO_3^- were measured with commercial photochemical test kits (Hach Lange GmbH, Düsseldorf, Germany, Test LCK 305 or 303; LCK 340). Dissolved NO_2^- was measured by anion chromatography (881 compact IC, Metrohm; detection limit 0.2 mgN/l), photochemically (Hach Lange GmbH, Düsseldorf, Germany, LCK 341) or with NO_2^- test strips (Nitrite-test, 0-24 mgNO_2^--N/l, Merck, Darmstadt, Germany).

Analytical methods for continuous N_2O off-gas concentration measurement

The reactor was closed for off-gas sampling purposes. However, it was not possible to seal it perfectly, due to reactor design reasons, like for example placement of stirrer and online sensors. Therefore, off-gas dilution rates were estimated based on O_2 off-gas concentration analysis, while the reactor was purged with dinitrogen (N_2) gas at different supply rates. O_2 off-gas concentrations were continuously measured (Rosemount Analytical Binos 100 2M Dual-Channel Gas Analyzer; N_2 gas (Carbagas, Switzerland) and ambient air were used for zero and second point calibration). The dilution rates were computed by dividing the measured O_2 off-gas concentrations by the atmospheric O_2 content (21 %). Results are illustrated in Figure 1: it is shown that dilution rates are less than 10 % at N_2 flow rates higher than 500 l/h, while being less than 25 % at flow rates in between of 300 to 500 l/h. At N_2 flow rates below 300 l/h, dilution rates are continuously increasing.

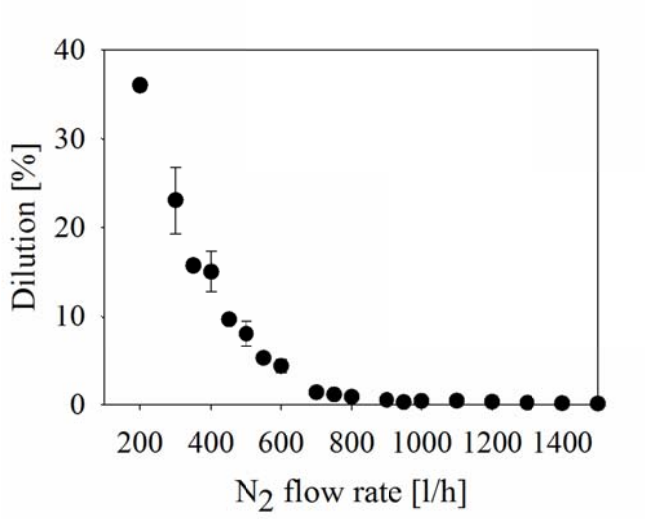


Figure 1. *Dilution rates, based on O_2 off-gas concentration analysis. Error bars indicate exemplarily the standard deviation for three individual measurements carried out on different days (flow rates at which multiple measurements have been carried out).*

N_2O off-gas concentrations were measured continuously (Rosemount Analytical X-Stream X2). The sample gas was dried prior to analysis (PKE 521, Bühler Technologies) in order to avoid condensation in the analyzer. The N_2O gas analyzer was calibrated three times a week with N_2 for the zero point, and with a 200 ppm standard gas (Carbagas, Switzerland) for the second calibration point. For N_2O off-gas concentrations higher than 200 ppm, the second point was calibrated by using a 5510 ppm standard gas (Carbagas, Switzerland), which was diluted with N_2 gas to the required N_2O concentration by using two mass flow controllers (Red-y Smart series, Vögtlin Instruments, Switzerland). N_2O emission loads were computed by multiplying the N_2O off-gas concentrations by the air flow rate measured at the reactor inlet, and integrating over time to evaluate the total mass of N_2O-N emitted (1 ppm corresponds to 1.249 $mgN_2O\text{-}N/m^3_{air}$ at 1013 mbar and 273 K). Emission factors were then calculated by dividing the nitrogen load emitted as N_2O by the NH_4^+ depletion rate, which was

evaluated by linear regression of the NH_4^+ online signal during the aeration phase, typically with simultaneous AOB and anammox activity.

Results and discussion

Reactor performance and N_2O emission under normal operating conditions

During regular and stable nitritation-anammox process operation, the aeration rate was adjusted to keep dissolved NO_2^- below 0.5 mgN/l. The NH_4^+ concentration was typically about 120 to 150 mgN/l, at the beginning of the aeration phase. At 20 mgNH_4^+-N/l the aerated period is ceased and switched to the stirring phase (depletion of residual NO_2^-) to be followed by sedimentation. Reactor temperature was controlled at 30±1 °C (except during one week where temperature was lowered to about 23 °C), and pH was between 7 and 8 (no pH control). There was no significant NOB activity, since NO_3^- production was in the typical range for anammox activity (Figure 2). Total N removal rates were in the range from 250 to 400 mgN/l/d, depending amongst other factors, on aeration rate, microbial activity and sludge concentration. DO concentrations were below 0.05 mgO_2/l, and thus below the limit of detection.

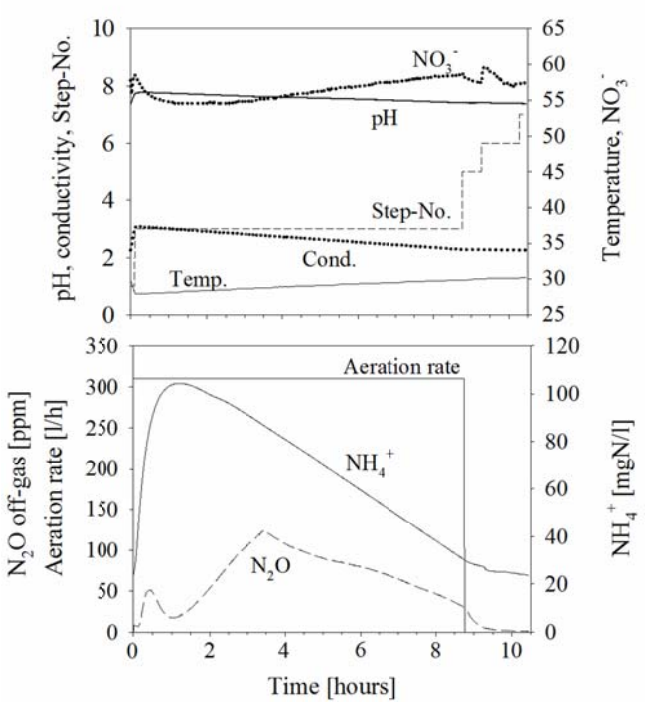


Figure 2. *A typical SBR-cycle consisted of (i) feeding with mixing, (ii) aeration, (iii) mixing, (iv) sedimentation and (v) discharge. NH_4^+, NO_3^-, pH, volume, conductivity, temperature and N_2O off-gas concentration was continuously monitored. N_2O emission usually peaked right after the aeration is switched on, and reached a maximum concentration during the first half of the aeration phase, before continuously decreasing in the second half of the aeration phase (according to Figure 1, N_2O concentrations during this cycle are underestimated by about 23 % due to off-gas dilution).*

N_2O emissions were in between of 0.2 and 1 % with respect to converted N. This emission range is comparable to other investigations: for example, Joss et al. (2009) measured an N_2O emission rate of 0.4 % with respect to N removed from a single-stage nitritation-anammox full-scale reactor. N_2O emissions in our investigation were highly dynamic, usually peaking in the first part of the aeration phase, followed by a continuous decrease in parallel with the NH_4^+ concentration (Figure 2). An emission delay, caused by the residence time in the headspace, is of minor importance, since being lower than 10 minutes at aeration rates higher than 300 l/h. In another investigation it was reported that N_2O emission levels decreased by more than 10-times between the start and the end of the aerated period in a full-scale nitritation reactor (Kampschreur et al., 2008). An initial N_2O peak was also reported for a partial nitrification system treating synthetic high strength NH_4^+ wastewater: here it is assumed that under low DO concentrations of around 1 mgO_2/l (during feeding) nitrifier denitrification was induced, while the continuous consumption of alkalinity during the second half of the aeration phase seemed to slow down AOB activity as well as N_2O production (Kong et al., 2013). We hypothesize that our observed N_2O emission peak at the beginning of the cycle and in combination with high NH_4^+ concentrations, might, in addition to AOB nitrifier denitrification, presumably be due to a contribution from NH_2OH oxidation to total N_2O production (discussed in the next section). However, this needs to be confirmed in upcoming investigations. Isotopomeric analysis is considered a suitable tool to quantitatively apportion between these two pathways (please see Wunderlin et al., 2013 for more details about this method).

Correlation between dissolved NO_2^- and N_2O emission rates

As demonstrated in the previous section, both NO_2^- concentrations as well as N_2O emissions were low under regular and stable nitritation-anammox process operation. In this section, the purpose was to investigate the short-term effect of high aeration rates on NO_2^- and N_2O emission levels, because an increase in the airflow as well as in dissolved O_2 is expected to accelerate AOB activities and to inhibit anammox bacteria.

Figure 3 A illustrates the correlation between dissolved NO_2^- and the N_2O emission rates. It is shown that a build-up of dissolved NO_2^-, upon a manual elevation of the airflow rate, leads to a linear increase of N_2O emissions. Similarly, Kampschreur et al. (2009) reported higher N_2O emissions upon NO_2^- accumulation in a single-stage nitritation-anammox full-scale granular reactor, and also showed a linear correlation between NO_2^- and N_2O off-gas concentrations under non-limiting dissolved O_2 concentrations (>2 mgO_2/l). We hypothesize that such a correlation, illustrated in Figure 3 A, is due to AOB nitrifier denitrification activity, since switching off the oxygen supply or adding allylthiourea, a specific AOB inhibitor, ceased N_2O production instantaneously (data not shown). Moreover, this is in agreement with the current assumption that N_2O does not play a role in the anammox metabolism (Kartal et al., 2007; Kampschreur et al., 2009).

The proportionality constants, shown in Figure 3 A, are variable and do not allow to quantitatively correlate dissolved NO_2^- with N_2O off-gas concentrations (e.g. an N_2O emission rate of about 3 % was measured at NO_2^- concentrations in between of 1 to 3 mgN/l). Thus, it is proposed to control the aeration rate according to an acceptable target N_2O emission, for example by the use of a proportional-integral-derivative (PID) controller: N_2O emissions below a defined threshold would lead to increasing aeration rates, while emissions above this threshold would result in lower airflow rates. According to Figure 3 A, N_2O emission rates below 1 % would keep NO_2^- below 1 mgN/l. NH_4^+ oxidation rates of this study were not optimal, since experiments were carried out during an nitritation-anammox recovery-phase. Therefore, the here presented correlations need to be reproduced and automatic process control to be implemented and tested under higher activities.

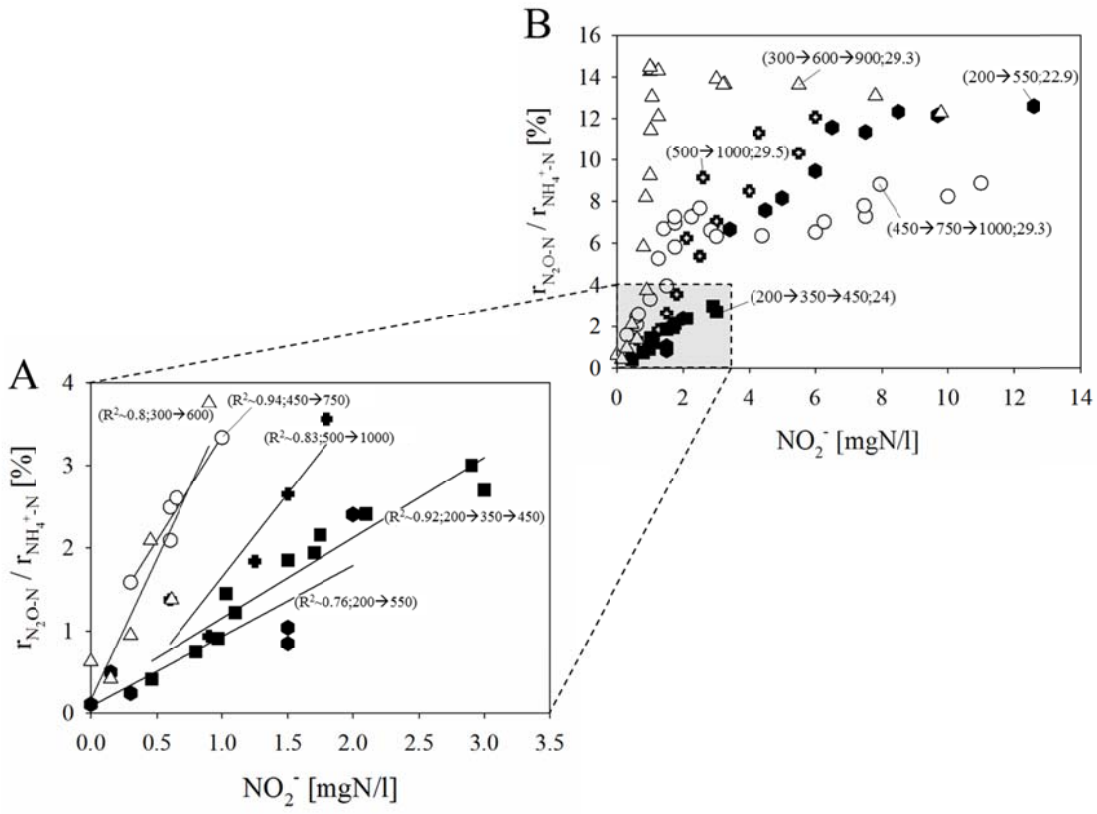


Figure 3. *(A) Correlation between dissolved NO_2^- and the N_2O emission rate (in terms of N_2O-N emitted with respect to NH_4^+-N removed): at low NO_2^- also N_2O emission is low, while increasing NO_2^- concentrations fosters N_2O emission. In parenthesis: R^2 and reactor aeration rates (l/h; adapted from Wunderlin et al., 2013b). (B) Correlation between dissolved NO_2^- and the N_2O emission rate (in terms of N_2O-N emitted with respect to NH_4^+-N removed). There was always N_2O production in the presence of NO_2^-. On the other side, high N_2O emission rates must not necessarily be accompanied by high dissolved NO_2^- concentrations, which is interpreted to be an indicator for a significant contribution of NH_2OH oxidation to total N_2O production (e.g. triangles). In parenthesis: reactor aeration rates (l/h) and reactor operating temperature (°C).*

In some experiments, N_2O strongly increased, even though NO_2^- did not accumulate substantially (e.g. triangles in Figure 3 B). We hypothesize that this characteristic behavior was due to a significant contribution of NH_2OH oxidation, which is another N_2O production pathway of AOB. Accordingly, for nitrification, it was reported that N_2O production via NH_2OH oxidation seemed to be favored at high NH_4^+ concentrations in combination with a high nitrification activity, and low NO_2^- concentrations (Yu et al., 2010; Law et al., 2012; Wunderlin et al., 2013a). In our experiments, N_2O emission, at high AOB activities and low NO_2^- concentrations, correlated with the NH_4^+ concentration (data not shown). Moreover, in combination with a temporary airflow increase, this might have induced imbalanced metabolic activities, as proposed by Yu et al. (2010), and consequently might have fostered N_2O production via NH_2OH oxidation. Again, N_2O production mechanisms in the (single-stage) nitritation-anammox process needs to be further investigated.

Manual airflow adjustment based on continuous N_2O off-gas concentration measurement

Based on the results illustrated in Figure 3 and discussed in the previous sections, it is concluded that (i) under normal operation, both NO_2^- as well as N_2O were kept at low levels, while (ii) an increase in the airflow rate resulted in a built-up of NO_2^- and in high N_2O emission rates. Consequently, in this section, it is investigated, whether process operation can be optimized by manual aeration rate adjustment, solely based on N_2O off-gas concentration data, which is considered a necessary stepping stone implementing this signal in automated process control.

Figure 4 shows a typical nitritation-anammox cycle, where the aeration rate was manually adjusted based on N_2O off-gas data. In general, N_2O off-gas concentrations decreased upon decreasing airflow rates, and vice-versa: for example, in Figure 4, a strong N_2O emission increase at the beginning of the aeration period (within the first 30 minutes), was counteracted by lowering the airflow from 500 to 400 l/h. Then, a stepwise airflow increase (from 400 up to 550 l/h) accelerated N_2O emissions again. However, there was usually a threshold aeration rate, below which the N_2O emission did not correlate anymore with the airflow rate (when dissolved NO_2^- was below the limit of detection). These emissions are attributed to NH_2OH oxidation, and are presumably impacted by the presence of toxic compounds or high NH_4^+ concentrations.

The above reported responses in N_2O emissions upon changing airflow rates are interpreted to be primarily due to changing microbial activities: at high aeration rates, AOB activity is higher with respect to anammox bacteria, since latter are partly inhibited by the presence of oxygen. Therefore, aeration rates controlled according to microbial activities lead to stable and more balanced nitritation-anammox process operation. This argumentation is also supported by the effect of reducing process temperature: for example, lowering the temperature from 30 to about 23 °C resulted in decreasing microbial activities (data not shown; in agreement with the Arrhenius equation describing the dependency of reaction rate constants on temperature), and

consequently, the airflow rate needed to be reduced in order to prevent over-aeration and NO_2^- accumulation (data not shown).

Off-gas dilution with ambient air as well as physical mass-transfer characteristics are two additional factors potentially impacting N_2O off-gas concentrations as a result of changing aeration rates. However, off-gas dilution, as illustrated in Figure 1, was not significant, since changes in N_2O off-gas concentrations were higher than assumed solely based on dilution rates. Physical mass-transfer characteristics would typically lead to increasing off-gas concentrations upon airflow reduction, and vice-versa, opposing to what we observed in our experiments (Figure 4).

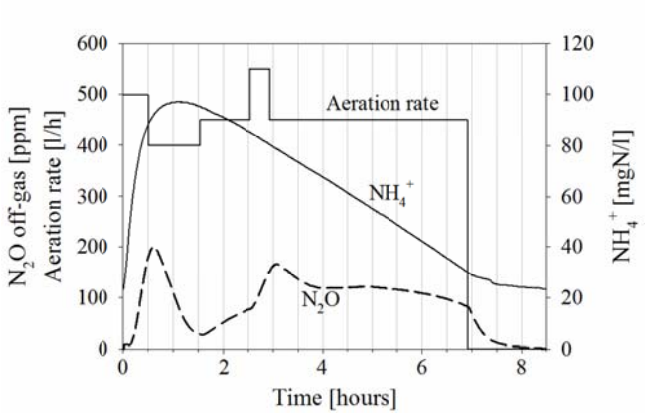


Figure 4. *An exemplified nitritation-anammox cycle with manual airflow control, and the effect on N_2O off-gas concentrations: a reduction in the airflow resulted in lower N_2O off-gas concentrations, while an increase in airflow again fostered N_2O emission.*

Monitoring N_2O emission for indirect NO_2^- measurement and process control: The potential for future applications

In this study, it has been shown that continuously monitoring N_2O off-gas concentrations is promising to significantly improve nitritation-anammox process control and stability. Data reveal that NO_2^- accumulation was successfully detected via N_2O off-gas analysis, and is hypothesized that reactor operation controlled at minimal N_2O emission also keeps stable NH_4^+ removal rates and low NO_2^- concentrations. However, an increase in N_2O was not always due to a substantial NO_2^- increase, since probably related to NH_2OH oxidation, and linked to high AOB activities (e.g. due to relatively high NH_4^+ concentrations). Therefore, to reduce N_2O emission as well as for improving its significance for controlling dissolved NO_2^- concentrations, process operating strategies minimizing NH_2OH oxidation activity need to be elucidated. One promising approach is to reduce NH_4^+ concentrations by changing to continuous feeding during aeration, since a temporary build-up of NH_4^+ is known to trigger N_2O emission. Moreover, a combination with the (online) analysis of the N_2O site-specific nitrogen isotopic signature (site preference; for more information see e.g. Wunderlin et al., 2013a) is considered an excellent tool for further pathway identification and nitritation-anammox process optimization with respect to minimize overall environmental impact of gaseous emissions.

Conclusions

In the present study, it was tested whether the continuous monitoring of N_2O off-gas concentrations can be used as an indirect measure for dissolved NO_2^- in a single-stage nitritation-anammox process, which is based on the hypothesis that NO_2^- dependent N_2O emission by AOB is the dominant cause for the observed off-gas concentrations. The conclusions can be summarized as follows:

- N_2O emissions have proven to correlate with dissolved NO_2^- concentrations: temporary high airflow rates resulted in both, increasing NO_2^- concentrations as well as increasing N_2O emissions, while aeration rates adequate to microbial activities kept NO_2^- and N_2O at low levels.
- Operating conditions at high N_2O emission and in combination with relatively low NO_2^- is assumed to be biased by NH_2OH oxidation activity, another independent pathway for N_2O formation.
- Reactor operation controlled at minimal N_2O emissions, give stable NH_4^+ removal rates and low NO_2^- concentrations.
- In sum, N_2O off-gas concentration is a promising parameter for nitritation-anammox process control and supports future directions incorporating this approach in full-scale.

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Chapter 8

Online N_2O measurement: The next standard for controlling biological ammonia oxidation?

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N_2O emission carries information about AOB activity: N_2O formation during biological wastewater treatment is complex because it is produced via different pathways and influenced by many factors. At an earlier stage of the research, heterotrophic denitrifiers were considered the dominant N_2O producers, a situation which changed significantly in recent years, the focus having moved to ammonia-oxidizing bacteria (AOB). The latter can generate N_2O via (so far) two different pathways: (i) by reducing nitrite (NO_2^-), and (ii) via intermediates formed during the oxidation of hydroxylamine (NH_2OH) to NO_2^-, known as nitrifier denitrification and NH_2OH oxidation, respectively.

Nitrification was recently proposed to be the dominant N_2O source in full-scale biological wastewater treatment, which is mainly attributed to sub-optimal process operating conditions, such as the accumulation of NO_2^-, the presence of low concentrations of dissolved oxygen or peak ammonia loads (Ahn et al., 2010). Monitoring N_2O off-gas concentrations combined with a better understanding of its dynamics and triggers is consequently helpful for improved AOB process control.

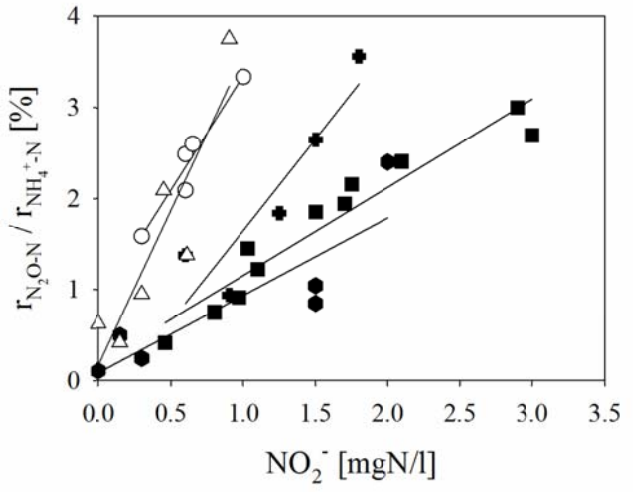

Figure 1. *Correlation between N_2O emissions and NO_2^- levels of different experimental runs (adapted from chapter 7). Measurements were carried out in a 400 liter one-stage nitritation-anammox sequencing batch reactor fed with digester liquid from a municipal sludge digester, with continuous online N_2O off-gas analysis (Rosemount Analytical X-Stream X2).*

Online N_2O is suitable for controlling AOB: We are currently working on the implementation of online N_2O off-gas analysis for the process control of full-scale nitritation-anammox treatment. Process stability and control is still a big issue here, involving an ongoing discussion about suitable sensors. Toxic influents, activity loss or over-aeration, all of which supply oxygen beyond the point of immediate AOB consumption, thus usually lead to microbial inhibition and NO_2^- accumulation. As a result, N_2O emission rates have increased substantially (Figure 1). N_2O off-gas analysis is consequently suitable for automated aeration rate control, leading to stable and more balanced operation of the nitritation-anammox process.

In nitritation-anammox systems, N_2O production is driven by AOB activity, either via nitrifier denitrification or NH_2OH oxidation, since heterotrophic denitrification activity is negligible due to the low availability of degradable organic substrate, and anammox bacteria are considered not to produce N_2O. Therefore, suitable process control strategies need to be clarified in order to (i) minimize N_2O emissions, (ii) maintain low concentrations of dissolved NO_2^- and (iii) keep NH_2OH oxidation activity low. In this context, the continuous analysis of the N_2O site-specific nitrogen isotopic signature is considered an excellent tool for quantitative pathway investigation. One promising approach is to feed the digester liquid continuously during aeration: at low NH_4^+ concentrations, N_2O production via NH_2OH oxidation is also low, and the N_2O emissions can therefore be taken as proxy for the NO_2^- concentrations in the reactor (Figure 1).

It was recently concluded that long-term continuous N_2O off-gas analysis is required for the accurate recording of emission dynamics and levels of conventional wastewater treatment plants (Daelman et al., 2013). As such, nitrification (AOB) process control based on N_2O emissions is considered helpful, because multiple factors impact on N_2O production, resulting in a complex, dynamic and plant-specific pattern of N_2O emissions (Burgess et al., 2002). Operating strategies minimizing overall greenhouse gas (GHG) emissions thus need to be tested individually in combination with online N_2O emission analysis.

N_2O emission is environmentally relevant and must be considered in energy optimization scenarios: N_2O is an environmentally harmful substance: it is a GHG and is involved in the destruction of the stratospheric ozone layer. Indeed N_2O is estimated to play the biggest role in depletion of stratospheric ozone during the 21st century (Ravishankara et al., 2009). Therefore, N_2O (and other GHG) emissions from the wastewater treatment sector must be minimized.

On the basis of Siegrist et al. (2008), it is estimated that the average net energy consumption of Swiss wastewater treatment plants, including biogas use from sludge digestion, is in the range from 40 to 50 watt hours per person and day (Wh/p/d). This value can be reduced by about 50% by introducing the nitritation-anammox process for digester liquid treatment while keeping nitrogen removal at the same level. The resulting savings of 20 to 25 Wh/p/d correspond to about 150 $gCO_{2,equivalents}$/p/d, assuming 700 $gCO_{2,equivalents}/kWh_{electrical}$, which refers to emissions of about 0.5 gN_2O/p/d. This accounts for approximately 3% of the daily nitrogen load of one person. Consequently, any discussion of potential future treatment schemes, must necessarily include N_2O (and other GHG) emissions. Analogously, aeration energy-saving concepts for conventional treatment must include N_2O emissions, which should not be significantly increased by them: for example, emission levels in the range of 0.5 to 1% with respect to oxidized nitrogen are already in the same order of magnitude as GHG emissions from the production of energy for aeration.

Online N_2O off-gas measurement is cost-effective, robust and requires little maintenance: Today's N_2O off-gas analyzers are stable and robust, enabling automated calibration procedures. Monitoring off-gas concentrations avoids direct contact with

activated sludge, hence significantly reduces cleaning, matrix interferences and the overall maintenance effort compared to probes inserted in the liquid phase. Investment costs are estimated to be higher, but still in the same order of magnitude, than those for the conventional commercially available ion selective electrodes usually applied for online NH_4^+ and NO_3^- measurement in the liquid. Alternatively, monitoring dissolved NO_2^- would require a suitable online sensor with sufficient resolution, which needs, to the authors' knowledge, considerably more maintenance. In addition, extending the N_2O off-gas analyzer by an oxygen sensor in combination with an airflow measurement would allow the oxygen consumption to be continuously monitored and thus the aeration to be controlled via the online nitrification rate.

A win-win situation for the environment and the plant operators: The implementation of a financial GHG crediting system, as suggested by Wang et al. (2011), is considered a powerful incentive to promote the broad application of a continuous N_2O off-gas monitoring concept. We are convinced that the efforts described here have an enormous potential to reduce air and water pollution substantially and thus contribute significantly to sustainable development within the global wastewater treatment sector.

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Chapter 9

General Conclusions

In this doctoral thesis, the production and emission of N_2O in biological wastewater treatment was investigated with a specific focus on pathway identification, plant operating reduction strategies and the potential use of continuous N_2O off-gas real-time measurement for process control.

The general conclusion section is structured as follows: first, the highlights of the presented work are summarized, followed by an extended conclusion of the individual chapters. A specific focus is put on the role of NO_2^-. Moreover, it is discussed whether biomass might potentially adapt to N_2O promoting factors, such as N influent peaks, which could lead to a reduction of N_2O emission. Finally, the implications on wastewater treatment plant operation are addressed.

Highlights of this thesis

The overall objective of this thesis was to identify the mechanisms of N_2O production in biological wastewater treatment and how the resulting emissions can be reduced. The work can be summarized with the following highlights:

- NO_2^- reduction to N_2O was found to be a key pathway in the investigated conventional municipal wastewater treatment systems.
- Isotopomeric analysis has been confirmed to be a powerful method to quantitatively differentiate between the three most important N_2O production routes, e.g. nitrifier denitrification, hydroxylamine (NH_2OH) oxidation pathway and heterotrophic denitrification in presence of low dissolved O_2 concentrations.
- Continuous N_2O off-gas analysis is considered a necessary stepping stone for overall carbon footprint minimization of wastewater treatment.
- Implementation of continuous N_2O off-gas analysis in full-scale has the potential to further improve nitritation-anammox process stability of supernatant treatment.

Main conclusions of the individual chapters

Lab-scale batch experiments with mixed microbial culture indicated that both, NH_2OH oxidation as well as NO_2^- reduction contributed to N_2O production during NH_4^+ oxidation: the former was interpreted to be active at the beginning of the aeration phase, when NH_4^+ concentration was high, but NO_2^- still low, while in the course of nitrification a shift to nitrifier denitrification driven N_2O production was observed (chapter 3). This trend is supported by N_2O isotopomeric analysis, a novel method in the field of biological wastewater treatment. These data confirm that under aerobic conditions, NO_2^- reduction seemed to be the dominant N_2O production pathway. The contribution from NH_2OH oxidation, however, cannot be completely excluded, but is deemed of minor importance in our investigation (chapter 4). Moreover, N_2O isotope measurements on a pilot-scale treatment plant (SP close to 0 ‰), indicate that NO_2^- reduction was dominant at dissolved oxygen concentrations higher than 1.5 mgO_2/l. An

increase in SP at low dissolved oxygen is attributed to heterotrophic denitrification activity, with a substantial N_2O reductase activity compared to N_2O production (chapter 5).

The effect of digester liquid addition, equivalent to a temporary increase of the nitrogen load, to a pilot-scale activated sludge plant showed that high N loads accelerated N_2O emission significantly, correlating positively with the NO_2^- build-up in the nitrifying activated sludge tanks (chapter 6). This underscores the assumption that an operating strategy at low dissolved NO_2^- (<2 mgN/l) contributes to keeping N_2O emissions at low levels.

The application of N_2O as a potential indirect measure for dissolved NO_2^- was the focus of chapter 7, where a nitritation-anammox reactor was operated at different aeration rates in order to experimentally operate at various degrees of NO_2^- accumulation. Latter is feasible due to the absence of nitrite oxidizing bacteria (NOBs), and anammox inhibition at elevated dissolved oxygen concentrations. Data indicate that the correlation between dissolved NO_2^- and the N_2O off-gas concentration seems to be biased by a (so far) unknown contribution from NH_2OH oxidation. In future investigations, the application of the isotopomeric analysis is promising to quantitatively differentiate between the two pathways. Nevertheless, the here presented results are judged to be prospective for further implementation in full-scale plants, and point to future directions incorporating continuous N_2O off-gas measurement in the process control (chapter 8).

The role of NO_2^- within N_2O production pathways

A main conclusion of this thesis is, that NO_2^- reduction is a major N_2O production pathway in the investigated activated sludge systems. Consequently, low NO_2^- concentrations during aeration are expected to reduce overall N_2O emission. This is in line with several studies reporting a positive correlation between dissolved NO_2^- and N_2O emission (e.g. Kampschreur et al., 2009a; Kampschreur et al., 2009b; Ahn et al., 2010; Foley et al., 2010). Moreover, data of this study also indicate that a shift from one dominating production route to another might occur over time within the same system, or that all of the known pathways are contributing simultaneously to total N_2O production, depending on operating and environmental conditions, respectively. This implies that critical operating parameters, optimally combined with isotopomer data, need to be considered when discussing N_2O emission dynamics, when testing N_2O reducing operating strategies or when applying mathematical models. The latter currently includes either NH_2OH oxidation or nitrifier denitrification, but not both at the same time due to parameter identifiability problems (e.g. Ni et al., 2011; Ni et al., 2012; Ni et al., 2013). Another important point to consider in upcoming studies is whether the correlation between NO_2^- and N_2O holds also for high NO_2^- concentrations, since this might have severe implications on nitritation systems (e.g. two-stage nitritation-anammox process), which are usually operated at high dissolved NO_2^- concentrations (see e.g. Law et al., 2013).

Adaptation of the biomass might influence N_2O production: Short-term vs. long-term effects

The present study is based on short-term effects, such as NH_4^+ or NO_2^- peak addition, on N_2O production, with activated sludge taken from a continuously operating pilot plant fed with municipal wastewater. Additionally, pilot-scale monitoring was conducted over several weeks, and continuous digester liquid dosing was performed over 9 days. An adaptive response, in terms of lower NO_2^- concentrations and lower N_2O emissions over the time, at simultaneously high N influent loads, was observed. This is in agreement with a recent publication, where daily NH_4^+ feed-pulses were conducted over 18 days, and 'stable' N_2O emissions were reached after about 8 days (Chandran et al., 2011). However, in another study it took about 80 days to stabilize N_2O emission after transition from full nitrification to nitritation (Ahn et al., 2011).

In this thesis, microbial long-term adaptation to changing conditions e.g. high N loads, high NO_2^- concentrations, or low dissolved oxygen, was not systematically investigated. This is necessarily to be done in upcoming studies. The trend within the N_2O research community goes towards long-term on-line monitoring to measure and control N_2O emission levels, in order to better capture daily, weekly and seasonal N_2O emission dynamics (Daelman et al., 2013), as well as to improve aeration and load control minimizing N_2O emission. Therefore, upcoming long-term investigations, addressing this topic, are expected.

Implications for wastewater treatment plant operation

This study supports the current understanding, that NO_2^- reduction is the dominant N_2O production pathway in biological wastewater treatment (Colliver and Stephenson 2000). Moreover, it is hypothesized that process perturbations, such as sudden increases of NH_4^+ or NO_2^- or drops in dissolved oxygen (at high N influent loads), leads to immediate N_2O increases (Desloover et al., 2012; Law et al., 2012). In future, such N_2O responses upon process perturbations might be used as an early warning for process failure in full-scale plants, as already proposed by e.g. Butler et al. (2009), Colliver et al. (2002), or Burgess et al. (2002).

With respect to strategies minimizing N_2O emissions, it is concluded that biological wastewater treatment plants should be operated at low NH_4^+ and NO_2^- concentrations, which means (i) a high solid retention time and thus a large population with nitrifying activity to handle nitrogen-peak-loads, (ii) sufficient dissolved oxygen at high N influent loads in order to avoid incomplete nitrification and to keep NO_2^- low (<1 to 2 mgN/l), (iii) an extended denitrification preventing soluble oxygen (to avoid inhibition of the N_2O to N_2 reduction step) and operated with sufficient readily biodegradable organic carbon (to avoid competition for reducing equivalents among denitrifying enzymes), and (iv) that ammonia rich digester liquid should be dosed preferentially during low ammonia loads (equalization of load variations; adapted from Wunderlin et al., 2012). However, these recommendations have yet to be verified in full-scale plants.

Energetic optimization of wastewater treatment by reducing aeration (and O_2 input) is critical, especially during high N loads. Therefore, it is recommended to reduce oxygen set-point (or partly switching off) the aeration solely at low N load situations. Based on the current understanding, it is postulated that plants designed for a high degree of N removal and operated under stable conditions emit only low amounts of N_2O: high nitrogen removal and low N_2O emission rates are therefore not considered as opposing targets (Chandran et al., 2011; Law et al., 2012).

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Chapter 10

Outlook

The outlook chapter is structured as follows: first, the remaining open questions for future investigations are addressed, followed by a specific focus on future applications of N_2O isotopic analysis in combination with microbiological tools and mathematical modeling. This chapter is then completed with relevant aspects from a practical point of view, such as discussing the potential of implementing continuous N_2O off-gas analysis on full-scale plants, and how to provide incentives to reduce overall carbon footprint of wastewater treatment plants.

Further application of N_2O nitrogen isotopic signature in combination with pure culture studies and mathematical modeling: A need for an integrated approach

In this study, the N_2O nitrogen isotopic signatures of mixed cultures from a pilot-scale plant treating real municipal wastewater were compared to published pure-culture investigations where the active pathways are known. A mixed population system was chosen as a necessary compromise between pure culture and full-scale studies, since it more closely resembles a full-scale plant, as compared to pure culture investigations and most of the microorganisms are barely cultivable as pure cultures. Yet, at the same time, a lab or pilot scale system is more easily controllable compared to a full-scale treatment plant required to discharge according to legal constraints.

Nevertheless, pure culture investigations are considered an important approach complementing mixed culture studies determining N_2O production mechanisms, and is also relevant for novel pathway identification. For example, Schreiber et al. (2012) concluded that, combining the genomic/proteomic approach, as demonstrated by Yu et al. (2010), with isotopomeric analysis, as investigated in this thesis (chapter 4; Wunderlin et al., 2013), could be a promising future direction for characterizing the relationship between transcriptional response of the AOB *Nitrosomonas europaea* and N_2O production.

Isotopomeric analysis has been demonstrated to be powerful for quantitatively apportioning N_2O production from nitrifier denitrification, NH_2OH oxidation and heterotrophic denitrification, respectively, in mixed microbial activated sludge systems, especially when combined with parameters such as the concentrations of dissolved O_2, N species and COD (Wunderlin et al., 2013). Thus, combining this method with conventional N_2O emission monitoring, is expected to further improve overall process understanding. Moreover, using $\delta^{15}N^{bulk}_{N2O}$ as an additional quantitative measure, it is beneficial to further characterize the fractionation of the substrates depending on different microbial activity levels and operation conditions. Otherwise, the interpretation of the $\delta^{15}N^{bulk}_{N2O}$ signal obtained from mixed culture remains, beside SP, limited to a qualitative information of the relative importance of AOB and heterotrophic denitrification to N_2O production (for more details please see chapter 5; Wunderlin et al., in preparation).

Finally, improved process understanding needs to be incorporated into existing mathematical models, which is a powerful tool for better dealing with N_2O emission dynamics. Especially, including NH_2OH oxidation, nitrifier denitrification, as well as heterotrophic denitrification in a unified model would allow more general conclusions. Current mathematical models do not yet allow robust quantitative prediction of N_2O emission without local parameter calibration. This is interpreted as an indicator for the model structure not yet having reached a generally applicable form.

Implementation of continuous N_2O off-gas measurement in full-scale: A win-win situation for plant operators and the global climate

The dynamic N_2O emission pattern observed in this thesis and reported in literature, and its correlation with multiple factors, make it challenging to define general plant operating strategies for reducing N_2O emission. Therefore, it is concluded that the implementation of continuous on-line N_2O off-gas measurement at full-scale is favorable to achieve plant operation optimized with respect to its overall carbon footprint (comprehensive energy optimization strategies). On the long-term this might lead to sustainable operation strategies where online N_2O measurement is not required anymore (e.g. conservative operation for small-scale facilities).

Basically, N_2O production is related to process instabilities leading for example to incomplete nitrification. In this context, Butler et al. (2009) came up with the idea of using N_2O off-gas measurement as a tool for early warning of biological nitrification failure: for example, an increase in N_2O was detected in parallel to an increase in NO_2^-, similar to our data. Moreover, for a single-stage nitritation-anammox process, NO_2^- accumulation induced by sudden activity loss could be easily detected online. Thus process monitoring via N_2O off-gas analysis is expected to improve overall process stability of ammonia oxidizing processes. However, up to now, this idea was not further pursued nor applied in full-scale treatment plants, which might also be due to the only recent development of affordable and low maintenance off-gas monitoring. It is therefore considered an important step to further develop a robust method enabling representative measurements (e.g. to adequately deal with the spatial N_2O emission variability; Ahn et al., 2010), with only low maintenance efforts required from the operator (Wunderlin et al., 2013).

Providing financial incentive for reducing N_2O emissions from biological wastewater treatment

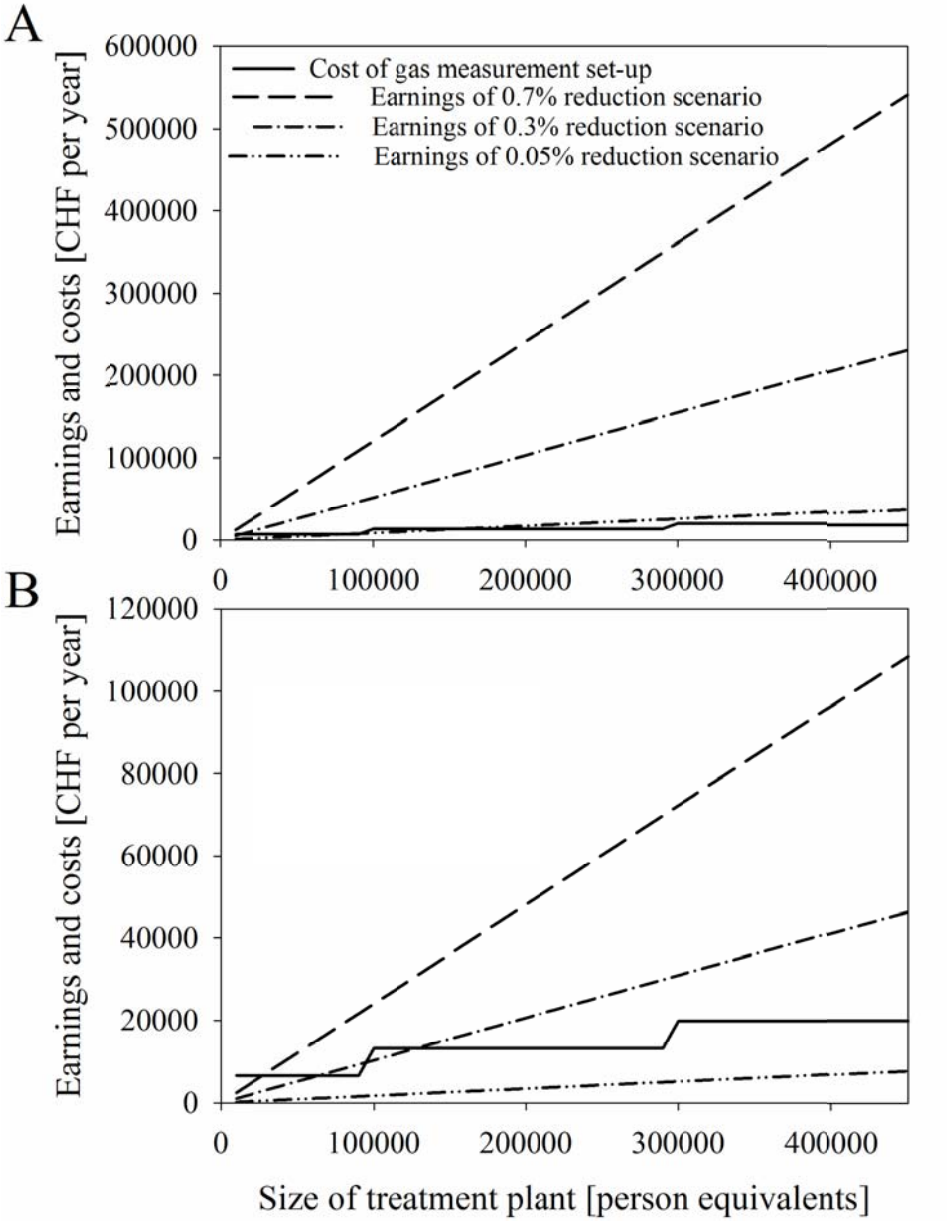

Figure 1. Comparison of the earnings and the costs of the 'N_2O implementation approach' and three different emission reduction scenarios. One ton of reduced carbon dioxide equivalents ($CO_{2,equiv}$) is assumed to be equal to 100 (A) and 20 CHF (B), respectively.

The implementation of a continuous N_2O off-gas measurement is considered a helpful stepping stone to monitor and optimize full-scale plants (e.g. aeration energy efficiency) towards a high degree of nitrogen removal with minimal greenhouse gas emissions. Moreover, online N_2O measurement can be considered as carbon off-setting strategy, since total greenhouse gas emissions are aimed to be reduced. Therefore, a financial greenhouse gas crediting system, as proposed by Wang et al. (2011), could be

an additional incentive, beside improving process stability, to promote widespread adoption of a continuous N_2O off-gas monitoring concept. For example, N_2O analytical equipment could be financed over greenhouse gas certificates: then, an N_2O emission baseline scenario has to be defined first (as-is state), followed by the implementation of N_2O reduction strategies resulting in lower overall emissions compared to the baseline scenario (according to the Swiss CO_2 act, this reduction is called 'additionality'; http://www.bafu.admin.ch/klima/12325/index.html?lang=de, September 2013).

It is roughly estimated the 'N_2O implementation approach' might lead to total costs of about 6'625 CHF per year, including the analyzer, the hood for gas sampling, the implementation and starting-up, as well as the maintenance (Table 1). Moreover, it is assumed that wastewater treatment plants (WWTPs) smaller than 100'000 person equivalents (PE) require one N_2O monitoring set-up (6'625 CHF per year), WWTPs smaller than 200'000 PEs need two of them (13'250 CHF/a), and plants bigger than 300'000 PEs require three set-ups (19'875 CHF/a) or even more, in order to get representative measurements.

Table 1. *Estimated costs of the 'N_2O implementation approach' per year. The investment costs of the N_2O analyzer is in the range of 40'000 CHF and has an estimated lifetime of 10 years (personnel communication with MBE AG, Wetzikon, Switzerland). Both, the hood for off-gas sampling as well as the costs for implementation and starting-up are estimated to 10'000 CHF each (amortized over 10 years).*

Time horizon	5 years
Costs of analyzer	20'000 CHF
Costs of hood	5'000 CHF
Cost of implementation and starting-up	5'000 CHF
Cost of maintenance (0.5 h per week at 25 CHF)	3'125 CHF
Total estimated costs per year	**6'625 CHF**

Figure 1 compares the costs from Table 1 with the estimated earnings (resulting from the financial crediting system) of three exemplifying (conservative) N_2O reduction scenarios: an N_2O emission reduction of (i) 0.05 %, (ii) 0.3 %, and (iii) 0.7 % with respect to influent nitrogen (e.g. a reduction of 0.3 % means that N_2O emissions are reduced from 0.8 to 0.5 % of the influent nitrogen). Data show that an absolute reduction of 0.3 % leads to positive cash flows at a price of only 20 CHF per ton reduced $CO_{2,equiv}$ for plants bigger than 130'000 PEs (Figure 1 B). At a price of 100 CHF per ton $CO_{2,equiv}$ emission offset, however, an absolute reduction of 0.05 % already leads to positive cash flows for plants bigger than 170'000 PEs, while a reduction of 0.3 % is already cost efficient for WWTPs bigger than 20'000 PEs (Figure 1 A).

It is estimated that, with this approach, about 10'800 tons of $CO_{2,equiv}$ emissions can be offset per year, when only 1/3 of the Swiss wastewater is treated in plants equipped with online N_2O sensors, and resulting optimized operation is attributed to an absolute

emission reduction of about 0.3 % (there are probably additional emission savings due to a higher aeration efficiency, which is not considered in this calculation). Consequently, about 70'000 tons of $CO_{2,equiv}$ could be offset till 2020, which is about 1 % of the amount that has to be reduced by the Swiss mineral oil industry (in case they do not fulfill this target, they are obligated to pay a penalty of 160 CHF per ton $CO_{2,equiv}$ that has not been offset; www.klik.ch, September 2013). Even if this seems to be a small contribution, it still has to be considered potentially relevant, because Swiss offsetting projects are currently rare. For example, myclimate, one of the leading global provider of voluntary carbon offsetting solutions, presents just four Swiss offsetting projects on their webpage, reducing between 200 and 9'700 tons $CO_{2,equiv}$ over 7 to 10 years (http://www.myclimate.org/en/carbon-offset-projects/international-projects.html, September 2013). Therefore, wastewater treatment plants might become an attractive partner in Switzerland for carbon offsetting projects in the near future and thus contributing substantially to a further sustainable development.

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Appendix A

N_2O emission of sludge incineration

Thesis chapter

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Abstract

Nitrous oxide (N_2O) is a strong greenhouse gas (GHG) and involved in the destruction of the stratospheric ozone layer. During sludge incineration, significant N_2O emissions may occur due to the high nitrogen content of sewage sludge.

Literature studies as well as data from two Swiss fluidized bed furnaces display lower N_2O emissions when operated at high freeboard temperature, compared to incinerators operated at lower temperatures. On the other side, literature studies indicate that NO_x production is influenced by the bed temperature, where high temperatures favor NO_x formation. Due to these opposing trends, it is important to note that focusing solely on N_2O reduction strategies, e.g. by increasing the freeboard temperature, might result in increasing NO_x emissions. New operating strategies have therefore to be tested at individual plants since N_2O and NO_x production and emissions are highly variable and plant-specific.

To lower NO_x emissions, fluidized bed furnaces can be equipped with a secondary DeNOx system (e.g. selectively non-catalytic reduction, SNCR) where NO_x is reduced to N_2 by injection of a reducing agent, such as ammonia or urea. On the other hand, the addition of nitrogen compounds (especially urea) encloses the risk of additional N_2O formation. During standard system operation, however, it is difficult to distinguish between N_2O produced during the primary incineration process (e.g. within the freeboard) and the secondary DeNOx system, respectively.

Sewage sludge incineration in waste to energy plants (WTE) may lead to additional N_2O emissions because of the relatively high nitrogen content within the sewage sludge. However, at low sewage sludge fraction, differentiation between N_2O from waste and sewage sludge incineration, respectively, is not feasible, and thus can only roughly be estimated.

N_2O emission during sludge incineration in Swiss fluidized bed furnaces were in the range of 1.2 to 3.8 % with respect to the nitrogen content of sewage sludge. These emission levels indicate that N_2O production during sludge incineration is relevant, since being around one fifth of total GHG emissions of wastewater treatment, but not the most important source.

Zusammenfassung

Lachgas (N_2O) ist ein starkes Treibhausgas und hat einen wesentlichen Einfluss auf die Zerstörung der stratosphärischen Ozonschicht. Bei der Klärschlammverbrennung können relevante N_2O-Emissionen auftreten, da der Brennstoff einen relativ hohen Stickstoffanteil aufweist.

Verschiedene Literaturstudien, wie auch Messungen an zwei Schweizer Wirbelschichtöfen deuten darauf hin, dass bei hohen Brennraumtemperaturen die N_2O-Emissionen tiefer sind, als bei Anlagen, die bei geringeren Temperaturen betrieben werden. Auf der anderen Seite wird die NO_x-Bildung stark durch die Betttemperatur beeinflusst. So deuten Literaturstudien darauf hin, dass eine erhöhte Betttemperatur die NO_x-Bildung fördert. Vor diesem Hintergrund muss unbedingt beachtet werden, dass durch N_2O-Reduktionsmassnahmen die NO_x-Emissionen nicht wesentlich erhöht werden. Da die N_2O- und NO_x-Bildung jedoch sehr variabel und anlagenspezifisch ist, müssen allgemeinformulierte Betriebsstrategien mit Vorsicht betrachtet, und im Einzelfall getestet werden.

Zur Senkung der NO_x Emissionen können Wirbelschichtöfen zur Klärschlammverbrennung mit einer sekundären Entstickungsanlage (meist selektive nicht-katalytische Reduktion, SNCR) ausgerüstet werden. Bei diesen Systemen kann es jedoch zu erhöhten N_2O Emissionen kommen, insbesondere bei der Verwendung von Harnstoff als Reduktionsmittel. Eine Unterscheidung der Emissionen aus dem primären Verbrennungsprozess und der sekundären Entstickungsstufe ist im regulären Anlagenbetrieb jedoch nicht möglich.

Bei Klärschlamm-Mitverbrennung in Kehrichtverbrennungsanlagen können auf Grund des N Gehaltes im Klärschlamm erhöhte N_2O Emissionen auftreten. Ist der Anteil des mitverbrannten Klärschlamms jedoch gering, so ist eine Unterscheidung der N_2O Emissionen aus Kehrrichtverbrennung bzw. Klärschlammverbrennung nicht bzw. nur näherungsweise möglich.

Die N_2O-Emissionen bei den untersuchten Schweizer Anlagen waren im Bereich von 1.2-3.8 % bezogen auf den im Schlamm enthaltenen Stickstoff. Der Vergleich mit anderen Treibhausgas-Quellen in der Abwasserreinigung zeigt, dass die Klärschlammverbrennung nicht den Hauptanteil der Emissionen ausmacht, mit einem Beitrag von rund einem Fünftel aber als relevant einzustufen ist.

Einleitung

Lachgas (N_2O) ist ein 300-mal stärkeres Treibhausgas als CO_2 (IPCC 2007). Anthropogene Aktivitäten haben seit der Industrialisierung einen Anstieg der N_2O Konzentration um etwa 20 % verursacht, nicht zuletzt auf Grund der langen Halbwertszeit in der Atmosphäre von 120 Jahren. Der N_2O Abbau findet primär in der Stratosphäre statt, wobei das dabei gebildete Zwischenprodukt, NO, den Ozonabbau massiv beschleunigt (Kramlich and Linak, 1994). Aus diesem Grund ist N_2O die bedeutendste anthropogen-emittierte ozonabbauende Substanz des 21. Jahrhunderts (Ravishankara et al., 2009).

Die globalen anthropogenen N_2O Emissionen machen schätzungsweise 8 % der Treibhausgasemissionen aus, wobei der Grossteil der Lachgasemissionen aus der Landwirtschaft stammt (intensive Bodenbewirtschaftung/Stickstoff-Düngung) (IPCC, 2007). Weitere Quellen sind industrielle Prozesse und stationäre Verbrennungsanlagen, deren Anteil an den globalen N_2O Emissionen zirka 5 % beträgt (Svoboda et al., 2006; Vitovec 1991a; Vitovec 1991b). Der Beitrag der biologischen Abwasserreinigung bezogen auf die gesamten anthropogenen N_2O Emissionen ist noch unklar (BAFU, 2011).

Im Rahmen des Forschungsprojekts *‚N_2O Emissionen in der Abwasserreinigung: Biologische Nährstoffelimination und Schlammverbrennung‘* werden die N_2O Emissionen und insbesondere deren Bildungsmechanismen in der biologischen Abwasserreinigung untersucht und kritische Betriebszustände identifiziert. Zudem sollen die N_2O Emissionen der Klärschlammverbrennung abgeschätzt werden, da Verbrennungsprozesse ebenfalls zur N_2O Bildung beitragen können. In einigen Literaturstudien wurde die Verbrennung von kommunalem Klärschlamm in Wirbelschichtfeuerungen bereits als bedeutende N_2O Quelle identifiziert, wobei der hohe Stickstoffgehalt des Brennstoffs und die tiefen Brennraumtemperaturen (verglichen mit einer konventionellen Feuerungsanlage) Gründe für die relativ hohen N_2O Emissionen zu sein scheinen (Vitovec 1991a; Vitovec 1991b; Korving et al., 2010; Sänger et al., 2001; Wether et al., 1999). Vor diesem Hintergrund soll die N_2O Bildung an zwei Schweizer Schlammverbrennungsanlagen exemplarisch untersucht werden.

Im Rahmen dieser Untersuchung wird anhand einer vertiefen Literaturstudie auf die Mechanismen der N_2O Bildung bei Verbrennungsprozessen eingegangen (siehe Kapitel ‚Mechanismen der N_2O Bildung‘). In Kapitel 2 (‚Fallstudie Schweiz‘) werden die N_2O Emissionen an zwei Klärschlamm-Monoverbrennungen im Vollmassstab (Anlage A und B), sowie einer Kehrichtverbrennungsanlage mit Klärschlamm-Mitverbrennung (Anlage C) quantifiziert, sowie der Einfluss verschiedener Prozessparameter abgeschätzt.

Mechanismen der N_2O Bildung in der Schlammverbrennung

Lachgas wird bei der Klärschlammverbrennung in Wirbelschichtöfen hauptsächlich aus dem im Brennstoff enthaltenen Stickstoff gebildet (eine Reaktion von Luftstickstoff zu N_2O ist bei Temperaturen <1000°C nicht zu erwarten). Die Verbrennung von Klärschlamm hat ein hohes N_2O-Bildungspotential, da der Stickstoff-Anteil im

Klärschlamm relativ hoch ist (40-60 g/kg Trockensubstanz; Korving et al., 2010). Ein Blick in die Literatur zeigt, dass N_2O einerseits durch Reaktionen in der Gasphase (homogener Mechanismus), aber auch durch oberflächen-katalysierte Prozesse (heterogener Mechanismus) gebildet werden kann (Vitovec 1991a; Vitovec 1991b).

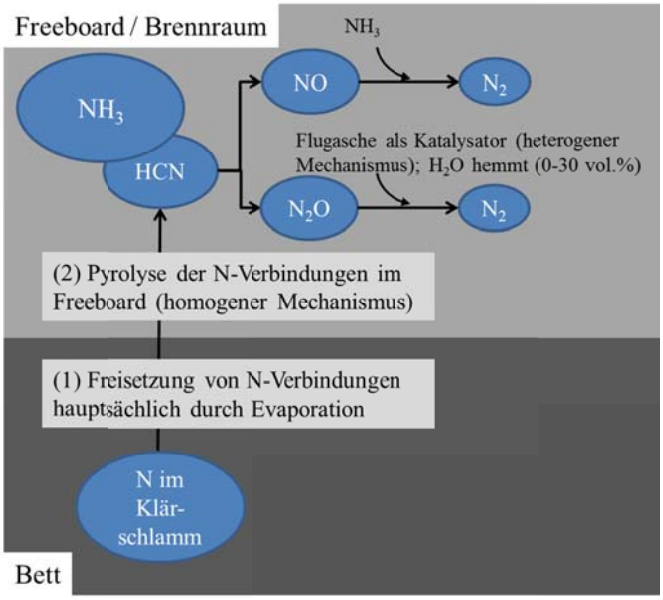

Abbildung 1. *Übersicht über mögliche N_2O Bildungsmechanismen bei der Schlammverbrennung in Wirbelschichtöfen (nach Korving et al., 2010).*

Abbildung 1 gibt eine Übersicht über mögliche N_2O-Bildungmechanismen. Die im Klärschlamm enthaltenen Stickstoffverbindungen (liegt in Biomasse hauptsächlich als Amin-Struktur vor) werden durch Verdampfung in die Gasphase freigesetzt, und anschliessend zu HCN und NH_3 umgesetzt (Vitovec 1991a; Vitovec 1991b). Nach aktuellem Stand des Wissens wird vor allem HCN als zentrale Substanz für die N_2O Bildung betrachtet. So hat Kramlich et al. (1989) gezeigt, dass durch eine externe Zugabe von HCN die N_2O Bildung signifikant erhöht wurde, weniger stark aber durch die Zugabe von NH_3.

Neben der N_2O Bildung ist auch der N_2O-Abbau ein wichtiger Prozess in der Gasphase, wobei hauptsächlich H-, O- oder OH-Radikale beteiligt sind. H-Radikale zerstören N_2O in einer sehr schnellen Reaktion, OH-Radikale reagieren etwas langsamer. Gemäss Literatur sind H-Radikale dominierend unter brennstoffreichen Bedingungen (tiefes Luft/Brennstoff-Verhältnis, siehe weiter unten), wohingegen O- und OH-Radikale bei Sauerstoffüberschuss (hohes Luft/Brennstoff-Verhältnis) den Hauptanteil ausmachen (Vitovec 1991a; Vitovec 1991b). Grundsätzlich gilt aber, dass hohe Brennraumtemperaturen die Bildung der N_2O-abbauenden Radikale begünstigen (bei Temperaturen über 1000°C ist der N_2O-Abbau vorherrschend; Vitovec 1991a; Vitovec 1991b). Zudem wird durch hohe Temperaturen auch ein weiterer Abbauprozess gefördert, die sogenannte thermische Dissoziation, worauf hier aber nicht weiter eingegangen wird.

Neben den Reaktionen in der Gasphase können in Wirbelschichtfeuerungen aber auch Reaktionen an Oberflächen (heterogener Mechanismus) eine wichtige Rolle spielen: zum Beispiel kann Flugasche den N_2O-Abbau katalysieren, wie in Abbildung 1 dargestellt. Grundsätzlich sind diese Prozesse aber noch wenig verstanden, und Gegenstand aktueller Forschung (Vitovec 1991a; Vitovec 1991b).

Wirbelschichtfeuerung und N_2O Emissionen

Wirbelschichtfeuerungen werden grundsätzlich bei tieferen Brenntemperaturen betrieben als andere stationäre Feuerungsanlagen (z.B. KVAs, Holzfeuerungen), welche überwiegend geringe N_2O Emissionen aufweisen (Vitovec 1991a; Vitovec 1991b). Diese tieferen Brennraumtemperaturen begünstigen einerseits geringe NO_x-Emissionen, was ein Vorteil der Wirbelschichtfeuerung darstellt, wirkt sich aber andererseits, neben den hohen Stickstoffgehalten im Brennstoff, negativ auf die N_2O-Bildung aus (siehe weiter unten).

In Abbildung 2 ist der Querschnitt eines Etagen-Wirbelschichtofens dargestellt: es wird unterschieden zwischen Wirbelbett und Brennraum, dem sogenannten ‚Freeboard'. Häufig wird die Schlammentwässerung direkt über dem Brennraum durchgeführt, wozu die aus dem Brennraum aufsteigende Wärme genutzt werden kann. Das Bett weist tendenziell tiefere Temperaturen auf als die Brennkammer, um einerseits eine ‚Bettschmelze' zu verhindern, und andererseits die Bildung von NO_x zu minimieren (Dräger und Vosteen, 2004).

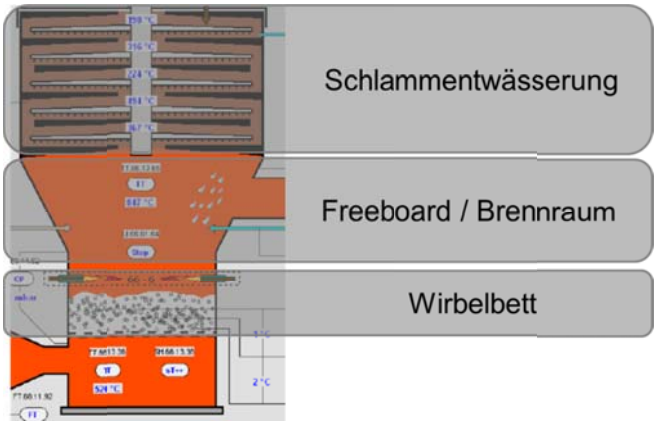

Abbildung 2. *Aufbau eines Etagen-Wirbelschichtofens. Es wird unterschieden zwischen Wirbelbett und Brennraum (Freeboard). In dem gezeigten Etagen-Wirbelschichtofen wird der Schlamm direkt über dem Brennraum getrocknet, und mittels Wurfbeschickung durch den Brennraum in das Wirbelbett gegeben (Bild aus Prozessleitsystem der Anlage B).*

Gemäss Korving et al. (2010) besteht eine Korrelation zwischen der Brennraumtemperatur und der N_2O-Bildung (Abbildung 3 links), insbesondere wenn die Temperatur knapp über dem Sand-Bett gemessen wird (Vitovec 1991a; Vitovec 1991b). So wurde gezeigt, dass eine tiefe Brennraumtemperatur (um 880°C) relativ hohe N_2O Emissionen begünstigt, während durch eine Temperaturerhöhung auf 930°C die Emissionen um rund 70% gesenkt werden konnten. Dieser Trend wird durch N_2O-Emissionsdaten anderer Klärschlammverbrennungsanlage bestätigt (z.B. Keller, 2010;

Sänger et al. 2001); in einer japanischen Anlage konnten die Emissionen durch eine Erhöhung der Brennraumtemperatur von 800 auf 850°C um rund 70% gesenkt werden (Abbildung 3 rechts). Die Autoren nehmen an, dass tiefe O_2-Konzentrationen, eingestellt über ein tiefes Luft/Brennstoff-Verhältnis, einerseits zu höheren Brennraumtemperaturen führen (da auf Grund des geringeren Luftdurchsatzes der Brennraum weniger stark abgekühlt wird) und andererseits die H-Radikal-Bildung fördert, was sich positiv auf einen schnellen N_2O-Abbau auswirkt (Abbildung 3 links; und siehe weiter oben). In anderen Untersuchungen wurde jedoch kein Zusammenhang zwischen Sauerstoffgehalt im Abgas und N_2O Bildung beobachtet (Sänger et al. 2001).

Tiefe Betttemperaturen scheinen einen geringeren Einfluss auf die N_2O-Bildung zu haben (Abbildung 4 links), jedoch, ähnlich wie im Brennraum, mit einer Tendenz zu abnehmenden Emissionen bei höheren Temperaturen. Zudem ist ersichtlich, dass ein höheres Luft-/Brennstoff-Verhältnis zu tendenziell höheren Emissionen führt.

Ein stärker ausgeprägter Einfluss hat die Betttemperatur aber auf die NO_x-Bildung: Abbildung 4 rechts zeigt, dass bei höherer Betttemperatur die NO_x-Emissionen ansteigen.

Aus diesen Untersuchungen geht hervor, dass (i) in Betriebsstrategien zur Reduktion der Lachgas-Emissionen ebenfalls die NO_x-Bildung berücksichtigt werden muss, und (ii) eine Erhöhung der Brennraumtemperatur möglichst bei konstant (tief) bleibender Betttemperatur erfolgen soll.

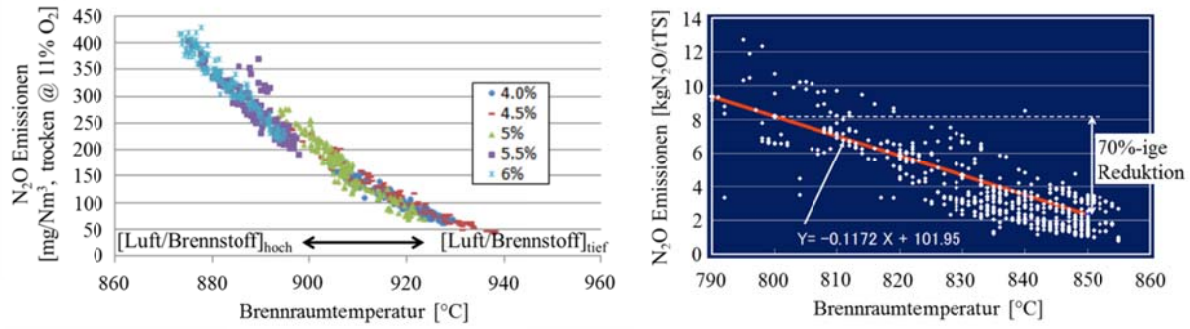

Abbildung 3. *Links: Einfluss der Brennraumtemperatur auf die N_2O Emissionen: höhere Temperaturen im Brennraum reduzieren die N_2O Emissionen. Die Prozentangaben im Kasten entsprechen den O_2-Konzentrationen im (feuchten) Rauchgas: tiefe O_2 Konzentrationen resultieren aus einem geringen Luft/Brennstoff-Verhältnis, welches auch zu hohen Brennraumtemperaturen führt (Quelle: Korving et al., 2010). Rechts: Daten aus Japan zeigen eine starke Brennraumtemperatur-Abhängigkeit der N_2O-Emissionen: eine Erhöhung der Temperatur reduziert die Emissionen signifikant (Keller, 2010).*

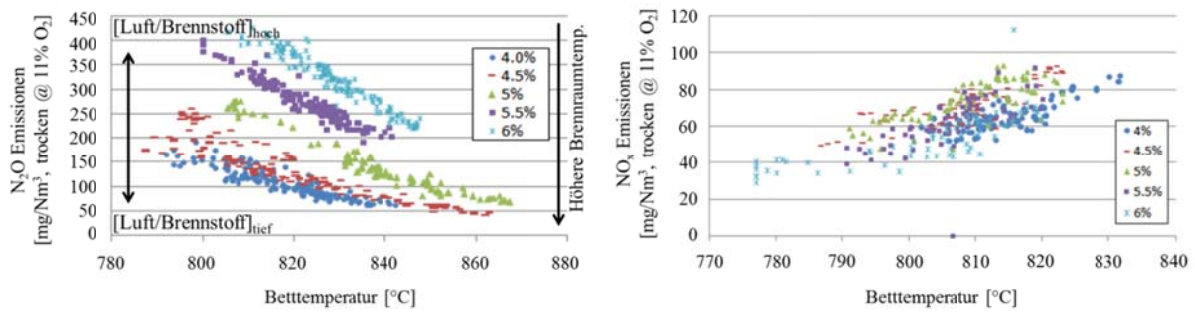

Abbildung 4. *Links: Einfluss der Betttemperatur auf die N_2O Emissionen: eine höhere Betttemperatur führt tendenziell zu geringeren N_2O Emissionen. Die Prozentangaben im Kasten entsprechen den O_2-Konzentrationen im (feuchten) Rauchgas, wobei tiefe O_2 Konzentrationen aus einem geringen Luft/Brennstoff-Verhältnis resultieren, was ebenfalls zu höheren Brennraumtemperaturen führt (Quelle: Korving et al., 2010). Rechts: Einfluss der Betttemperatur auf die NO_x Emissionen, welche bei höheren Betttemperaturen ansteigen. Eine Veränderung des Luft/Brennstoff-Verhältnisses bzw. der O_2 Konzentration im (feuchten) Rauchgas hat nur einen geringen Einfluss auf die NO_x Emissionen. Die Betttemperatur wurde über den Schlammvortrocknungsgrad sowie die Temperatur der zugegebenen Luft reguliert (Quellen: Korving et al., 2010).*

N_2O Emissionen der Klärschlamm-Mitverbrennung in Kehrichtverbrennungsanlagen (KVAs) und Effekte der Entstickung

Die beiden am häufigsten eingesetzten sekundären Entstickungs-Verfahren in Verbrennungsanlagen sind (i) die selektive katalytische Reduktion (SCR), und (ii) die selektive nicht-katalytische Reduktion (SNCR). Bei beiden Verfahren werden NO_x-Verbindungen mit NH_3 oder Harnstoff zu N_2 und H_2O umgesetzt.

Beim SCR-Verfahren wird gasförmiger Ammoniak oder eine wässrige Ammoniak-Lösung verwendet, üblicherweise in einem Temperaturbereich zwischen 270 und 450°C. Messungen an österreichischen Anlagen haben gezeigt, dass die SCR-Verfahren eine vernachlässigbare Wirkung auf die N_2O Emissionen haben (Vitovec 1991a; Vitovec 1991b).

Beim SNCR-Verfahren wird häufig Ammoniak oder Harnstoff in den 850 bis 1000°C heissen Brennraum dosiert. Hierbei treten vor allem bei der Verwendung von Harnstoff höhere N_2O-Zusatzemissionen auf, da davon auszugehen ist, dass Harnstoff zu HNCO, einer wichtigen N_2O-Vorgängersubstanz zersetzt wird. Die N_2O Emissionen nehmen dabei linear mit der Menge des zugegebenen Reduktionsmittels zu, wobei die Höhe der Emissionen anlagenspezifisch ist und daher sehr unterschiedlich sein kann (Vitovec 1991a; Vitovec 1991b).

Fazit N_2O Bildung

Die Lachgas-Bildung bei der Klärschlammverbrennung in Wirbelschichtfeuerungen wird hauptsächlich durch den hohen Stickstoffgehalt im Brennstoff und die tiefen

Verbrennungstemperaturen verursacht. Neben der Bildung haben aber auch N_2O abbauende Reaktionen einen Einfluss auf die N_2O Emissionen.

Gemäss Literaturangaben begünstigen tiefe Brennraumtemperatur die N_2O Bildung. Im Weiteren wurde beschrieben, dass der Einsatz einer sekundären Entstickungsstufe (SCR) nicht per se einen Einfluss auf die N_2O-Bildung haben muss, wobei insbesondere die Verwendung von Ammoniak als wenig problematisch einzustufen ist. Hingegen können bei der Eindüsung von Harnstoff als Reduktionsmittel in den Brennraum (SNCR), erhöhte Emissionen auftreten, welche möglicherweise durch die Bildung von HNCO, eine Vorläufersubstanz von N_2O, aus Harnstoff verursacht werden.

Es gilt festzuhalten, dass bei Schlammverbrennungsanlagen die N_2O-Emissionen stark variieren können, da verschiedene Faktoren, wie Brennstoffzusammensetzung, Feuchte (Entwässerungsgrad), Art der Schlammentwässerung oder Betrieb des Ofens (insbesondere Temperatur) zeitlichen Schwankungen unterworfen sind.

Im folgenden Kapitel wird (i) die Auswirkung der Bett- und Brennraumtemperatur auf die N_2O-/NO_x-Emissionen von zwei Klärschlamm-Monoverbrennungsanlagen (A und B) betrachtet, und (ii) die Effekte einer sekundären Entstickungsanlage (DeNOx) einer KVA untersucht (C).

Fallstudien Schweiz

Von Schweizer Klärschlammverbrennungsanlagen liegen noch keine systematisch erhobenen Daten vor. Das Handbuch Emissionsfaktoren für stationäre Quellen des BAFU geht aber von vergleichbaren N_2O Emission wie bei KVAs aus (Bafu, 2000). Im Folgenden werden die Emissionen von zwei grosstechnischen Klärschlamm-Monoverbrennungsanlagen (Anlage A und B) und einer Kehrichtverbrennungsanlage mit Klärschlamm-Mitverbrennung (Anlage C) präsentiert. Insbesondere wird die Auswirkung der Brennraumtemperatur beim Wirbelschichtofen (Anlage A und B), sowie die Wirkung eines DeNOx Systems (SCR-Verfahren mit NH_3-Eindüsung) auf die N_2O-Emissionen einer KVA diskutiert (Anlage C).

Anlage A ist ein Wirbelschichtofen, in dem rund 40‘000 Tonnen Klärschlamm pro Jahr (entspricht 280‘000 Einwohnergleichwerten), mit einem mittleren Trockenmaterialgehalt (TS) von 25% verbrannt werden. Dies ergibt rund 9‘000 Tonnen TS pro Jahr. Die Anlage wird bei einer Brennraumtemperatur von rund 850°C betrieben und die NO_x Emissionen werden mittels SNCR Verfahren, durch Zudosierung des Klärschlammfiltrats, reduziert (Angaben des Anlagenbetreibers).

Anlage B verfügt über drei Etagen-Wirbelschichtöfen mit einer Nachbrennkammer, einer Wärmerückgewinnung und einer Entstickungsanlage (SNCR Verfahren mit Ammoniak Zugabe). Die Brennraumtemperatur liegt im Mittel bei rund 800°C. Es werden durchschnittlich etwa 9‘600 Tonnen Trockenmaterial pro Jahr verwertet (8‘500 Tonnen eigener und zirka 1‘100 Tonnen fremder Klärschlamm; 25-26% TS-Anteil im entwässerten Klärschlamm; gemäss Angaben des Anlagenbetreibers).

Anlage C ist eine Kehrrichtverbrennungsanlage mit einem Durchsatz während der Messkampagne von rund 99 bis 103 Tonnen Müll pro Tag und etwa 4.7 Tonnen Klärschlamm (TS) pro Tag. Der Klärschlammanteil, auf TS-Basis, liegt somit bei rund 5%. Der mittlere Trockensubstanzanteil lag bei 29%, und die Brennraumtemperatur zwischen 830 und 870°C (Lödel und Sköries, 2012).

Im Folgenden werden die gemessenen N_2O-Emissionen in Abhängigkeit der Einflussparameter wie der Brennraum-, und Betttemperatur, der O_2-Abgaskonzentration sowie der Ammoniak-Eindüsung diskutiert. Die N_2O-Emissionsfaktoren werden in Tabelle 2 präsentiert und im Kapitel ‚N_2O Emissionsfaktoren der untersuchten Anlagen' diskutiert.

Anlage A

Entsprechend Abbildung 5 links ist ein linearer Zusammenhang zwischen den N_2O-Emissionen und der Brennraumtemperatur zu beobachten. Es ist erkennbar, dass die Emissionen bei einer Erhöhung der Temperatur von 800 auf 870°C signifikant tiefer sind. Diese Abnahme ist in Übereinstimmung mit verschiedenen Literaturangaben, in denen ein wesentlicher Einfluss der Brennraumtemperatur auf die N_2O Emissionen diskutiert wird (siehe Einleitung; Vitovec 1991a, Vitovec 199b, Korving et al., 2010).

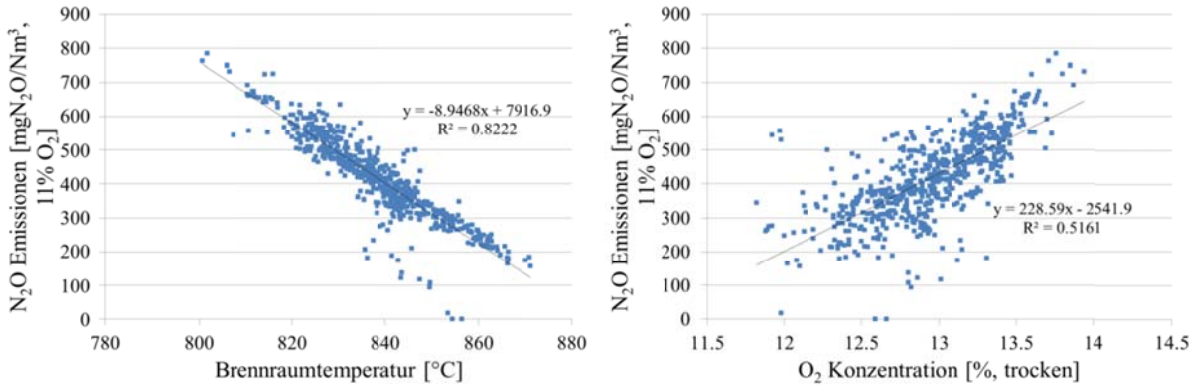

Abbildung 5. *Links: Abhängigkeit der N_2O-Emissionen von der Brennraumtemperatur: eine höhere Brennraumtemperatur führt zu tieferen N_2O-Emissionen. Rechts: Abhängigkeit der N_2O-Emissionen von der O_2-Konzentration im Reingas: bei höherer O_2-Konzentrationen sind die N_2O-Emissionen ebenfalls höher; jedoch wurde das Rauchgas vor der Messung mit Umgebungsluft abgekühlt (und somit verdünnt). Die Messungen wurden im Zeitraum vom 1.2.2012 bis 29.2.2012 durchgeführt (gezeigt sind die Stundenmittelwerte).*

Ausserdem ist ein Zusammenhang zwischen den N_2O-Emissionen und der Sauerstoff-Konzentration im Reingas zu beobachten (Abbildung 5). Es fällt auf, dass mit zunehmender O_2-Konzentration die N_2O-Emissionen ansteigen. Auch dieser Trend ist in Übereinstimmung mit anderen Untersuchungen: z.B. wurde durch Korving et al. (2010) gezeigt, dass bei einem Anteil von 6% O_2 die N_2O Emissionen signifikant höher sind als bei einem Anteil von 4% (siehe auch Abbildung 3 links). Dieser Zusammenhang ist jedoch vermutlich nicht nur auf die O_2-Konzentration zurückzuführen, da parallel auch eine Veränderung der Brennraumtemperatur aufgetreten ist. Vitovec (1991a) erwähnt

in seinem Bericht über ‚Pyrogene N_2O Emissionen', dass die N_2O-Emissionen mit zunehmendem Luftüberschuss aber gleichbleibender Temperatur ansteigen, und begründet dies mit einem langsameren radikal-bedingten N_2O-Abbau bei O_2-Überschuss, verglichen mit O_2-limitierenden Bedingungen. Es muss hier allerdings angemerkt werden, dass die in Abbildung 5 rechts dargestellten O_2 Konzentrationen im Reingas nach vorheriger Verdünnung mit Umgebungsluft gemessen wurden. Diese Verdünnung wird durchgeführt, um das Gas vor dem Eintritt in den Abgaswäscher abzukühlen. Aus diesem Grund sind die Sauerstoff-Konzentrationen deutlich höher als beispielsweise bei Anlage B, dargestellt in Abbildung 7.

Anlage B

In Abbildung 6 links ist der Zusammenhang zwischen der Brennraumtemperatur und den N_2O- und NO_x-Emissionen der Anlage B dargestellt. Analog zu Anlage A wird auch auf Anlage B bei höherer Brennraumtemperatur tendenziell weniger N_2O emittiert. Ein ähnlicher Zusammenhang ist auch für die beiden anderen Öfen der Anlage B 67 und 86 zu beobachten (siehe Abbildungen A1 und A3 im Anhang). Zwischen der Wirbelbetttemperatur und den N_2O-Emissionen ist ebenfalls ein leicht negativer Trend zu erkennen (Abbildung 6 rechts), was ebenfalls in Übereinstimmung ist mit Literaturwerten (vgl. Abbildung 4 links).

Die NO_x Emissionen bleiben bis zu einer Brennraumtemperaturen von etwa 850°C relativ konstant; zwischen 850 und 900°C steigen sie leicht an. Die konstant tiefen Emissionen sind jedoch vermutlich auf eine effiziente sekundäre Entstickung (SNCR mit Ammoniak Zugabe) zurückzuführen. Ein Anlagenbetrieb bei höheren Brennraum- und Betttemperaturen sowie nachgeschaltetem SNCR Katalysator ist somit ohne signifikant höhere NO_x Emissionen möglich.

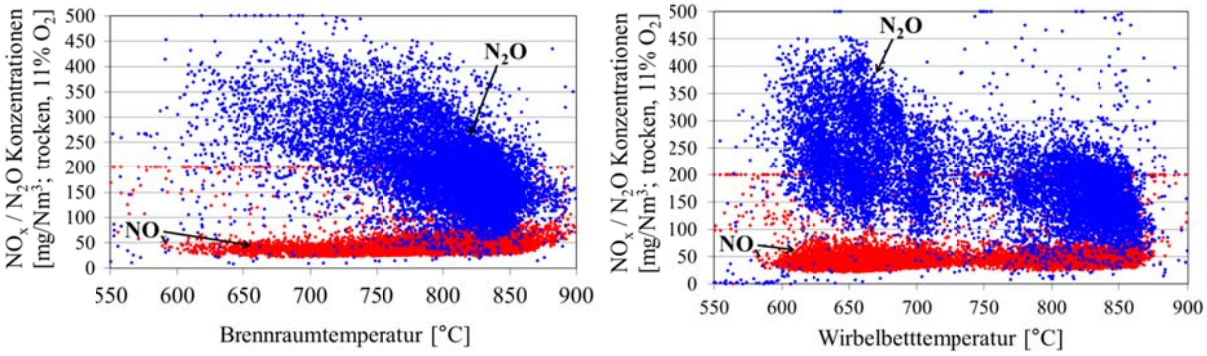

Abbildung 6. *N_2O (blau) und NO_x (rot) Emissionsdaten von Ofen 66 im Zeitraum vom 1.7.2009 bis 31.12.2011 (gezeigt sind Stundenmittelwerte). Links: in Abhängigkeit von der Brennraumtemperatur; Rechts: in Abhängigkeit der Wirbelbetttemperatur. Die Rauchgastemperatur vor der Nachbrennkammer war konstant bei 880°C.*

Die vorliegenden Daten in Abbildung 7 links zeigen, dass bei Anlage B die Sauerstoff-Konzentration keinen direkten Einfluss auf die N_2O-Bildung zu haben scheint (auch bei der Betrachtung von kürzeren Zeitintervallen). Dies ist im Gegensatz zu Anlage A und auch einigen Literaturangaben (z.B. Korving et al., 2010), wobei andere Untersuchungen

ein gegensätzliches Bild zeigen (z.B. Sänger et al. 2001). Anhand der vorliegenden Daten ist es leider nicht möglich, zu beurteilen, warum dieser Zusammenhang nicht bei allen Anlagen beobachtet werden kann. Dies zeigt, dass die N_2O Emissionen in der Schlammverbrennung zu einem gewissen Grad anlagenspezifisch sein können, und allgemeinformulierte Betriebsstrategien (und beeinflussende Parameter) im Einzelfall getestet werden müssen.

In Abbildung 7 rechts ist zu sehen, dass eine höhere Ammoniak-Dosierung tendenziell zu tieferen N_2O Emissionen führt (wobei die NO_x Emissionen nicht weiter reduziert werden konnten). Dies entspricht grundsätzlich nicht den Erwartungen erhöhter N_2O Emissionen bedingt durch eine Steigerung der Ammoniakzugabe (siehe Kapitel 1.3). Eine mögliche Erklärung könnte sein, dass bei einer höheren Brennraumtemperatur nicht nur weniger N_2O gebildet wird (Abbildung 6 links), sondern auf Grund einer erhöhten NO_x-Bildung auch mehr Ammoniak dosiert werden muss. Folglich würde kein direkter Zusammenhang zwischen der Ammoniak-Dosierung und den N_2O Emissionen bestehen.

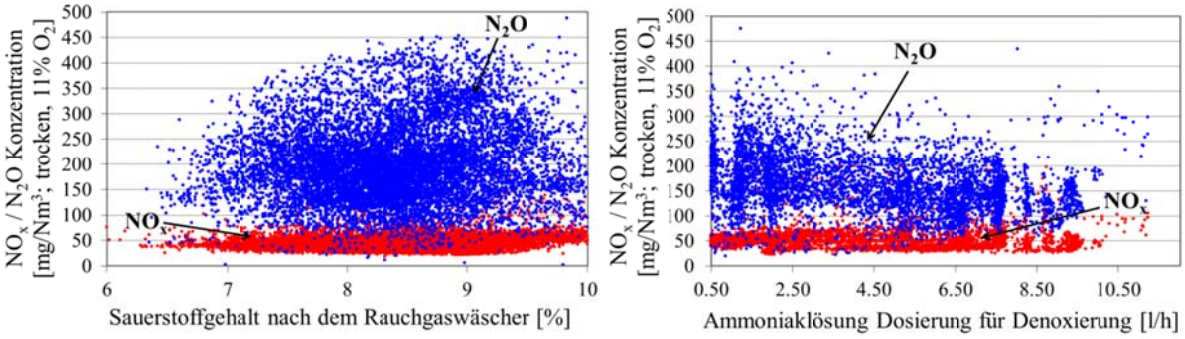

Abbildung 7. *N_2O (blau) und NO_x (rot) Emissionsdaten von Ofen 66 im Zeitraum vom 1.7.2009 bis 31.12.2011 (gezeigt sind Stundenmittelwerte). Links: in Abhängigkeit von der O_2-Konzentration; Rechts: in Abhängigkeit von der Ammoniak-Dosierung. Die Rauchgastemperatur vor der Nachbrennkammer war konstant bei 880°C.*

Anlage C

Bei einer Kehrrichtverbrennungsanlage wurden die N_2O Emissionen bei Mitverbrennung von Klärschlamm sowie der Einfluss eines SCR Katalysators mit NH_3-Eindüsung auf die N_2O-Bildung an zwei aufeinander folgenden Messtagen untersucht. Hierfür wurden nacheinander Messungen an den beiden Ofenlinien vor dem Katalysator und im vereinigten Abgas nach dem Katalysator durchgeführt. Zudem wurden die N_2O-Emissionen mit und ohne Mitverbrennung von Klärschlamm verglichen.

In Tabelle 1 ist ersichtlich, dass die N_2O-Konzentrationen vor dem Katalysator tendenziell höher sind wenn Klärschlamm mitverbrannt wird (2.1-4.3 mgN_2O/Nm^3), verglichen mit dem Betriebszustand ‚ohne Klärschlamm' (1.5-2.1 mgN_2O/m^3), was möglicherweise auf einen höheren Stickstoffanteil im Brennstoff mit Klärschlamm zurückzuführen ist. Nach dem Katalysator wurden sowohl mit als auch ohne

Klärschlamm vergleichbare Werte von 2.1-3.9 mgN_2O/Nm^3 gemessen. Damit kann kein wesentlicher Einfluss des Katalysators, auf die N_2O-Emissionen nachgewiesen werden. Dies ist in Übereinstimmung mit Literaturwerten (Vitovec 1991a; Vitovec 1991b)

Tabelle 1. *N_2O Messungen einer KVA mit und ohne Klärschlamm-Beimischung vor und nach dem SCR Katalysator. Bei der Klärschlamm-Mitverbrennung wurden dem Müll rund 5% (TS-Anteil) Klärschlamm beigemischt. Die Daten wurden am 11./12.4.2012 erhoben, mittels mehreren jeweils über eine Stunde dauernden Messungen (Lödel und Sköries, 2012).*

Messort	N_2O-Konzentrationen* [mg-N_2O/m^3]	N_2O Massenstrom** [kg-N_2O/h]	Betriebszustand
Vor Kat., Linie 1	3.5-4.3	0.107-0.134	mit Klärschlamm
Vor Kat., Linie 2	2.1-4.3	0.054-0.111	mit Klärschlamm
Nach Kat., Linie 1 &2	2.1-2.3	0.11-0.12	mit Klärschlamm
Vor Kat., Linie 1	1.8-2.1	0.056-0.067	ohne Klärschlamm
Vor Kat., Linie 2	<1.5-1.5	0.044	ohne Klärschlamm
Nach Kat., Linie 1 &2	2.3-3.9	0.132-0.228	ohne Klärschlamm

* bezogen auf 11 Vol.-% O_2, trocken, 273K, 1013hPa

** trocken, 273K, 1013hPa

N_2O Emissionsfaktoren der untersuchten Anlagen

Die Untersuchungen an den Schweizer Anlagen haben gezeigt, dass die N_2O-Emissionen der Klärschlamm-Monoverbrennung (Anlage A und B) gegenüber anderen Verbrennungsanlagen deutlich erhöht und bezüglich ihres Anteils an den Treibhausgasemissionen der Abwasserreinigung relevant sind (Tabelle 2). Die N_2O Emissionen einer KVA mit Klärschlamm-Mitverbrennung sind nicht signifikant höher als ohne Klärschlamm-Mitverbrennung (Tabelle 1). Im Weitern zeigt Tabelle 2, dass die Emissionen sehr variabel und anlagenspezifisch sind (Emissionsfaktoren im Bereich von 1.2-3.8 % bezogen auf den verbrannten Stickstoff). Dies verdeutlicht, dass allgemeinformulierte Betriebsstrategien mit Vorsicht zu betrachten sind, und im Einzelfall getestet werden müssen.

Bei den untersuchten Klärschlamm-Monoverbrennungsanlagen lagen die durchschnittlichen Brennraumtemperaturen im Bereich von 750 bis 870°C. Durch eine Vermeidung tiefer Brennraumtemperaturen konnten die N_2O Emissionen nachhaltig gesenkt werden. Zudem könnte durch ein tieferes Luft/Brennstoff-Verhältnis die Betttemperatur auf einem für die NO_x-Bildung optimalen tieferen Niveau gehalten werden. Eine O_2-Limitierung, ebenfalls bedingt durch ein tiefes Luft/Brennstoff-Verhältnis, könnte zusätzlich die effiziente, für den N_2O-Abbau wichtige Radikal-Bildung fördern. Inwieweit diese Massnahmen aus anlagentechnischer Sicht umsetzbar sind müsste abgeklärt werden.

Tabelle 2. *N_2O Emissions-Faktoren der drei untersuchten Verbrennungsanlagen.*

		Anlage A	Anlage B	Anlage C	
		Klärschlammmono-verbrennung		Kehrichtverbrennung	
		(mit KS)	(mit KS)	(mit KS; vor Kat)	(mit KS, nach Kat)
Brennraum-temperatur	[°C]	795-865	750-870	835-861	835-861
N_2O Emissionen	[mgN_2O/Nm^3]	420(1)	153-199(1)	2.1-4.3(1)	2.1-2.3(1)
	[mgN_2O-N/Nm^3]	267(1)	97-127(1)	1.3-2.7(1)	1.3-1.5(1)
O_2 Abgas	[%]	13(2)	8.8	8.1-10.3	7.7-8.0
Anteil TS	[%]	30	26	29.1(5)	29.1(5)
TS-Umsatz	[tTS/h]	1.1	1.1	0.057(3,5)	0.057(3,5)
Luftvolumen-strom	[Nm^3/h]	7852	7360	20100-27400	47900(4)
	[Nm^3/tTS]	7138	6691	-	-
N-Umsatz (N-Gehalt ~5%)	[kgN/h]	55	58	2.96(5)	2.96(5)
N_2O Massen-strom	[kgN_2O/h]	3.3(1)	1.1-1.5(1)	0.054-0.134	0.11-0.12
	[kgN_2O-N/h]	2.1(1)	0.7-1.0(1)	0.034-0.085	0.07-0.076
N_2O Emissionen	**[%]**	**3.8**	**1.2-1.7**	**k.A.(6)**	**k.A.(6)**

(1) bezogen auf 11 Vol.-% O_2, trocken, 273K, 1013hPa
(2) vor Messstelle verdünnt mit Umgebungsluft
(3) betrifft Klärschlamm (~4.7 t/d), zusätzlich wurde 98.91 (Linie 1) bzw. 99.35 (Linie 2) t/d Müll verbrannt
(4) Abluft Linie 1 und 2
(5) N-Umsatz bezieht sich lediglich auf den im Klärschlamm enthaltene Stickstoff
(6) keine Angabe möglich, da der Anteil der N_2O Emissionen aus der Klärschlammverbrennung nicht berechnet werden kann.
KS: Klärschlamm; Kat: Katalysator/sekundäre Entstickung

Relevanz der N_2O Emissionen der Klärschlammverbrennung

Die Lachgasemissionen in der Schlammverbrennung müssen in Relation zur Treibhausgase-Freisetzung bei der eigentlichen Abwasserreinigung (ARA) gesetzt werden. Abbildung 8 gibt einen Überblick der abgeschätzten Emissionswerte und deren Anteile an den Gesamtemissionen. Mit dieser Gegenüberstellung soll insbesondere die Relevanz der N_2O Emissionen der Schlammverbrennung verdeutlicht werden.

Methan (CH_4) Emissionen stammen hauptsächlich aus den Abwasserkanälen. Der geschätzte Anteil liegt im Bereich von 5% bezogen auf die Kohlenstoff (CSB)-Fracht im Abwasser, wobei angenommen wird, dass die übrigen Emissionen aus der Faulung stammen. Insgesamt schätzen wir die CH_4 Emissionen auf knapp einen Drittel (30 $gCO_{2,äquiv}$/EW*Tag) der totalen Treibhausgasemissionen in der Abwasserreinigung (die

Autoren gehen davon aus, dass in der Schweiz somit insgesamt etwa 10% der CSB-Fracht als CH_4 emittiert wird).

Lachgas kann ebenfalls in der biologischen Reinigungsstufe gebildet werden, hauptsächlich durch mikrobiologische Prozesse wie die Nitrifikation und die Denitrifikation: die Emissionen sind vor allem abhängig von der Stickstoffbelastung, und daher sehr dynamisch und anlagenspezifisch. Verschiedenste Untersuchungen zeigen, dass die N_2O Freisetzung durchaus im Bereich von 0.5 % bezogen auf den Stickstoffumsatz liegen kann (Wunderlin et al., 2013). Das Treibhauspotential dieser N_2O Emissionen ist in einer vergleichbaren Grössenordnung wie dasjenige des Energieverbrauchs für die Belüftung der biologischen Abwasserreinigung (24 $gCO_{2,äquiv}$/EW*Tag verglichen mit 40 $gCO_{2,äquiv}$/EW*Tag aus der Belüftungsenergie).

Das Treibhauspotential der N_2O Emissionen der Schlammverbrennung kann rund 20% der total Treibhausgasemissionen der Abwasserreinigung betragen (20 $gCO_{2,äquiv}$/EW*Tag; Abbildung 8). Dieser Vergleich zeigt, dass die Treibhausgasemissionen der Klärschlammverbrennung nicht den Hauptanteil des Treibhauspotentials der Abwasserreinigung ausmacht, mit einem Beitrag von rund einem Fünftel aber als relevant einzustufen ist.

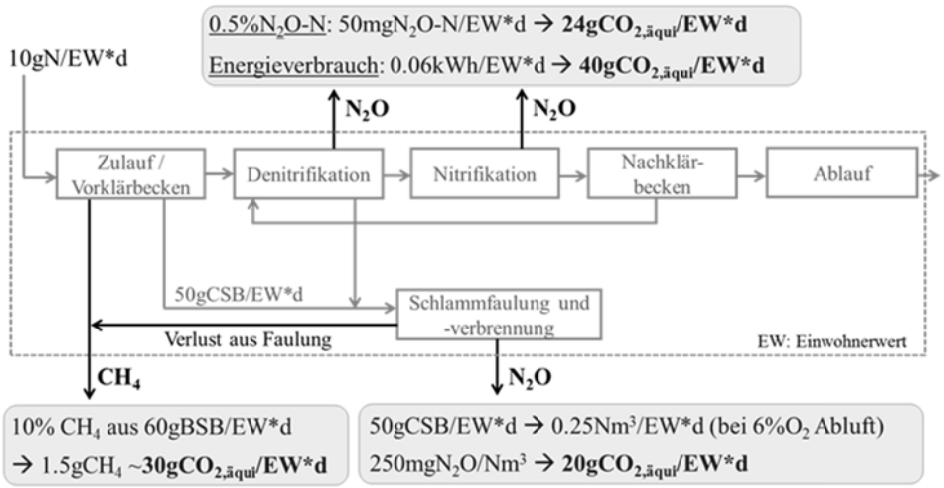

Abbildung 8. *Treibhausgasemissionen in der Abwasserreinigung: Methan wird hauptsächlich im Kanal gebildet und im Kläranlagenzulaufbereich in die Atmosphäre abgegeben (CH_4 Emissionen aus der Faulung sind ebenfalls realistisch), N_2O kann sowohl in der biologischen Reinigungsstufe und der Klärschlammverbrennung produziert werden (aus Wunderlin et al., 2013).*

Schlussfolgerungen und Empfehlungen

Im Rahmen dieser Untersuchung wurden anhand einer vertiefen Literaturstudie die Mechanismen der N_2O Bildung bei Verbrennungsprozessen diskutiert. Ausserdem wurden N_2O Emissionsdaten von zwei Schweizer Klärschlamm-Monoverbrennungen im Vollmassstab (Anlage A und B), sowie punktuelle Messungen an einer Schweizer

Kehrichtverbrennungsanlage, mit Klärschlamm-Mitverbrennung (Anlage C) ausgewertet und der Einfluss relevanter Faktoren betrachtet.

Die Messungen haben ergeben, dass bei der Klärschlamm-Monoverbrennung eine Korrelation zwischen der Brennraumtemperatur und den N_2O Emissionen besteht, wobei hohe Temperaturen tendenziell tiefere Emissionen begünstigen. Dieser Zusammenhang ist in Übereinstimmung mit Literaturstudien, in denen die Brennraumtemperatur als einer der wichtigsten Faktoren für die N_2O-Bildung diskutiert wird. Im Weiteren muss darauf hingewiesen werden, dass durch Erhöhung der Brennraumtemperatur auch die NO_x-Emissionen ansteigen können. Insbesondere, wenn dadurch die Betttemperatur erhöht wird. Erhöhte NO_x Emissionen können jedoch durch eine nachgeschaltete sekundäre Entstickung vermieden werden. Gemäss Korving et al. (2010) ist eine Minimierung der N_2O Emissionen durch ein tiefes Luft-/Brennstoffverhältnis möglich, wobei eine grössere Temperaturdifferenz zwischen dem Bett und dem Brennraum resultiert.

Aufgrund punktueller Messungen vor bzw. nach dem SCR Katalysators einer KVA mit bzw. ohne Klärschlamm-Mitverbrennung, konnte kein signifikanter Einfluss des SCR Katalysators bzw. der Klärschlamm-Mitverbrennung beobachtet werden.

Gemäss Vitovec (1991a) sind drei Strategien zur Reduktion der N_2O Bildung denkbar: (i) Optimierung der Verbrennungstemperatur, (ii) thermische Nachbehandlung (durch Zugabe eines zusätzlichen stickstoffarmen Brennstoffs, z.B. Erdgas, wird eine Zone hoher Temperatur und hoher Radikalkonzentrationen geschaffen), oder (iii) katalytische N_2O-Entfernung.

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Übersicht der N_2O-Emissionen aus Ofen 67

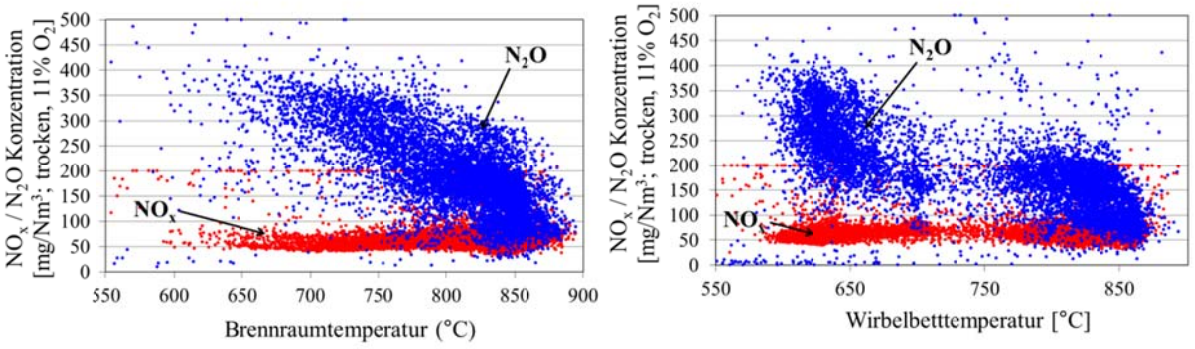

Abbildung A1. *N_2O (blau) und NO_x (rot) Emissionsdaten von Ofen 67 im Zeitraum vom 1.7.2009 bis 31.12.2011 (gezeigt sind Stundenmittelwerte). Links: in Abhängigkeit von der Brennraumtemperatur; Rechts: in Abhängigkeit der Wirbelbetttemperatur. Die Rauchgastemperatur vor der Nachbrennkammer war konstant bei 880°C.*

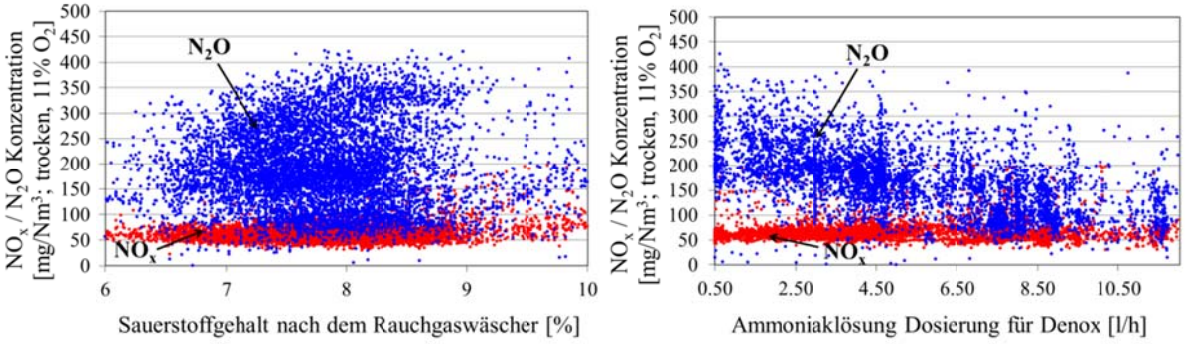

Abbildung A2. *N_2O (blau) und NO_x (rot) Emissionsdaten von Ofen 67 im Zeitraum vom 1.7.2009 bis 31.12.2011 (gezeigt sind Stundenmittelwerte). Links: in Abhängigkeit von der O_2-Konzentration; Rechts: in Abhängigkeit von der Ammoniak-Dosierung. Die Rauchgastemperatur vor der Nachbrennkammer war konstant bei 880°C.*

Übersicht über die N_2O-Emissionen aus Ofen 86

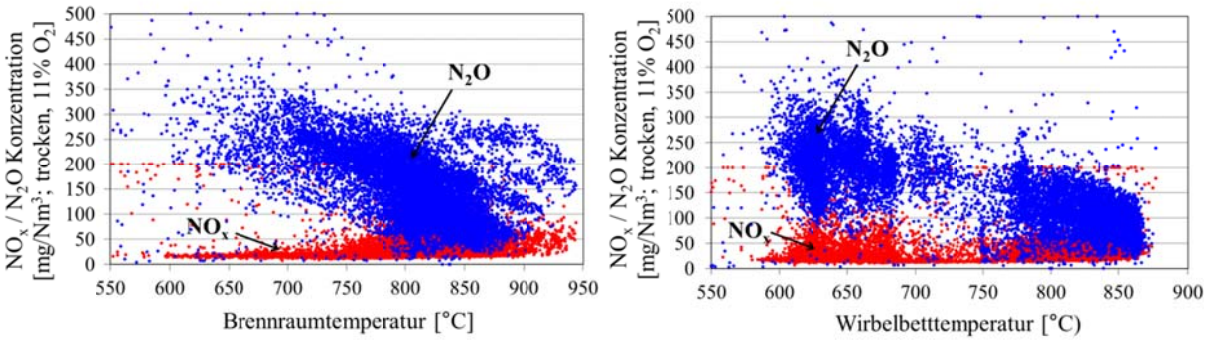

Abbildung A3. *N_2O (blau) und NO_x (rot) Emissionsdaten von Ofen 86 im Zeitraum vom 1.7.2009 bis 31.12.2011 (gezeigt sind Stundenmittelwerte). Links: in Abhängigkeit von der Brennraumtemperatur; Rechts: in Abhängigkeit der Wirbelbetttemperatur. Die Rauchgastemperatur vor der Nachbrennkammer war konstant bei 880°C.*

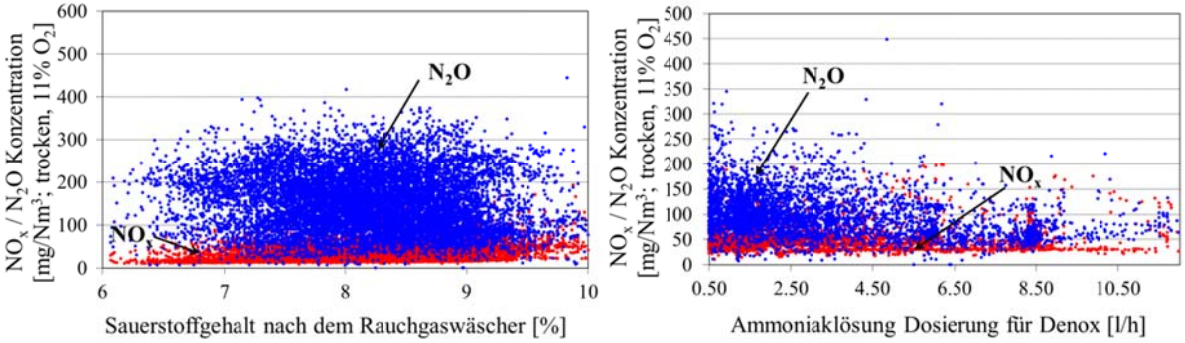

Abbildung A4. *N_2O (blau) und NO_x (rot) Emissionsdaten von Ofen 86 im Zeitraum vom 1.7.2009 bis 31.12.2011 (gezeigt sind Stundenmittelwerte). Links: in Abhängigkeit von der O_2-Konzentration; Rechts: in Abhängigkeit von der Ammoniak-Dosierung. Die Rauchgastemperatur vor der Nachbrennkammer war konstant bei 880°C.*

Appendix B

N_2O emission of biological wastewater treatment

This chapter has been published as:

Wunderlin, P., Mohn, J., Joss, A., Emmenegger, L., Siegrist, H. Lachgas-Emissionen aus ARA - Relevanz, Bildungswege und Reduktionsstrategien. Aqua & Gas 2013, 2, 54-59.

Zusammenfassung

Lachgas (N_2O) ist ein starkes Treibhausgas und bedeutend an der Zerstörung der stratosphärischen Ozonschicht beteiligt. Seine Bildung und Freisetzung in die Atmosphäre hat deshalb eine grosse Umweltrelevanz. In der biologischen Abwasserreinigung kann N_2O sowohl während der Nitrifikation (Oxidation von Ammonium zu Nitrat), wie auch durch die heterotrophe Denitrifikation (Reduktion von Nitrat zu Luftstickstoff) gebildet werden. Diese Prozesse laufen meist gleichzeitig ab. Daher lässt sich ihr individueller Beitrag zur N_2O Bildung nur schwer abschätzen. Für diese Studie wurde ein neuartiger Ansatz angewandt, der eine bessere Beurteilung der N_2O Bildung auf Abwasserreinigungsanlagen erlaubt. Anhand der Analyse der stabilen Stickstoffisotopen wurde gezeigt, dass unter aeroben Bedingungen die Nitrifikation die N_2O Bildung dominiert. Dabei fördern hohe Ammonium (NH_4^+) und Nitrit (NO_2^-) Konzentrationen die N_2O Bildung. Ausserdem ist zu beachten, dass die Entstehung von N_2O räumlich und zeitlich sehr variabel ist und durch viele Parameter beeinflusst wird. Die aktuellen Emissionsschätzungen sind daher mit einer grossen Unsicherheit verbunden, was eine Bestimmung von präzisen Emissionsfaktoren schwierig macht. Untersuchungen aber zeigen, dass die N_2O Emissionen die Treibhausgasbilanz der gesamten Anlage dominieren können, welche ansonsten vor allem durch die indirekten Emissionen der Belüftung der biologischen Reinigungsstufe beeinflusst wird.

In Anbetracht der negativen Umweltauswirkungen von N_2O und der langen Aufenthaltszeit in der Atmosphäre (rund 120 Jahre) sollte unbedingt eine möglichst weitgehende Reduktion der Emissionen angestrebt werden. Das gegenwärtige Verständnis der relevanten Bildungsmechanismen deutet darauf hin, dass bei einer möglichst vollständigen Nitrifikation und Denitrifikation gleichzeitig auch die N_2O Bildung minimiert werden kann. Aus diesem Grund sollte sowohl bei der Auslegung wie auch bei der Optimierung und beim Betrieb von Belebtschlamm-Anlagen (z.B. Regelung der Belüftung) die N_2O Bildung mitberücksichtigt werden.

Stichwörter

Betriebsstrategien, biologische Abwasserreinigung, Denitrifikation, Lachgas (N_2O), Nitrifikation, Treibhausgas, Zerstörung der Ozonschicht

Einleitung

Lachgas (N_2O) ist ein zirka 300-mal stärkeres Treibhausgas als Kohlendioxid (CO_2; IPCC 2007). Anthropogene Aktivitäten haben seit der Industrialisierung einen Anstieg der N_2O Konzentration in der Atmosphäre um 18% verursacht, nicht zuletzt wegen seiner langen Halbwertszeit von 120 Jahren. Der Abbau findet primär in der Stratosphäre statt, wobei das dabei gebildete Zwischenprodukt Stickstoffmonoxid (NO) den Ozonabbau massiv beschleunigt (Kramlich and Linak 1994). Aus diesen Gründen ist Lachgas die wichtigste durch den Menschen freigesetzte ozonabbauende Substanz des 21. Jahrhunderts (Ravishankara et al., 2009).

Die anthropogenen N_2O Emissionen machen schätzungsweise 6% der gesamten Treibhausgasemissionen aus, wobei ein Grossteil davon durch die Stickstoff-Düngung landwirtschaftlicher Flächen verursacht wird (BAFU 2011). Weitere relevante Quellen sind die chemische Industrie und Verbrennungsprozesse. Der Beitrag der biologischen Abwasserreinigung ist noch unklar.

In der Schweiz ist der überwiegende Teil der kommunalen Abwasserreinigungsanlagen (ARA) mit einer biologischen Reinigungsstufe ausgerüstet. In Anbetracht des hohen Energieverbrauchs für die Belüftung (50 bis 70%; Müller 2010) sind zukünftige Optimierungsbestrebungen zu erwarten. So gibt es Bestrebungen den Energieeinsatz durch einen reduzierten Gebläse-Betrieb zu senken. Da hierdurch tiefere O_2-Konzentrationen in der Nitrifikationsstufe verursacht werden, müssen die Mechanismen der N_2O Bildung sowie die Einflüsse der relevanten Parameter (z.B. O_2) besser verstanden werden.

Treibhausgaspotential der N_2O Emissionen in der Abwasserreinigung

Die N_2O Emissionen in der Abwasserreinigung sind von vielen Prozessparametern abhängig und daher zeitlich und räumlich sehr variabel. In den USA wurde in einer umfangreichen Messkampagne gezeigt, dass zwischen 0.01 bis 3.3% des umgesetzten Stickstoff als N_2O freigesetzt werden (Ahn et al., 2010). In einer einjährigen Messkampagne auf einer ARA in den Niederlanden war der relative Anteil der N_2O Emissionen bei rund 3% bezogen auf die Stickstofffracht (Daelman et al., 2012). Diese Werte liegen zum Teil deutlich über dem bis anhin vom Weltklimarat (IPCC) verwendeten Emissionsfaktor von 7 gN_2O/Einwohnerwert(EW)/Jahr, was etwa einer Freisetzung von 0.1% des umgesetzten Stickstoffs als N_2O bei einer Stickstofffracht von 3.7 kgN/EW/Jahr entspricht (IPCC 2007).

Fig. 1 zeigt eine grobe Abschätzung und Gegenüberstellung der Treibhausgasemissionen durch die Abwasserreinigung. Es ist aktuell davon auszugehen, dass im Mittel etwa 0.5% des umgesetzten Stickstoffs als N_2O emittiert werden. Das dadurch verursachte Treibhausgaspotential entspricht somit etwa demjenigen, welches durch den Energieverbrauch für die Belüftung der biologischen Reinigungsstufe verursacht wird. Neueste Untersuchungen deuten darauf hin, dass während der Schlammverbrennung ebenfalls N_2O gebildet werden kann (Korving et al.,

2010). Untersuchungen auf Schweizer Klärschlammverbrennungsanlagen zeigen, dass die N_2O Emissionen je nach Brennraumtemperatur zwischen 0.2 und 1%, bezogen auf die Stickstofffracht im ARA Zulauf, betragen können. Generell werden für tiefere Brenntemperaturen höhere Emissionen nachgewiesen. Diese Emissionen entsprechen 10 bis 47 $CO_{2,äqui}$/EW*Tag. In der Schlammfaulung, der Kanalisation oder im Vorklärbecken kann zusätzlich Methan (CH_4) gebildet werden. CH_4 ist ein rund 25-mal stärkeres Treibhausgas als CO_2 und hat einen ähnlichen Anteil an den totalen Treibhausgasemissionen in der Abwasserreinigung wie N_2O.

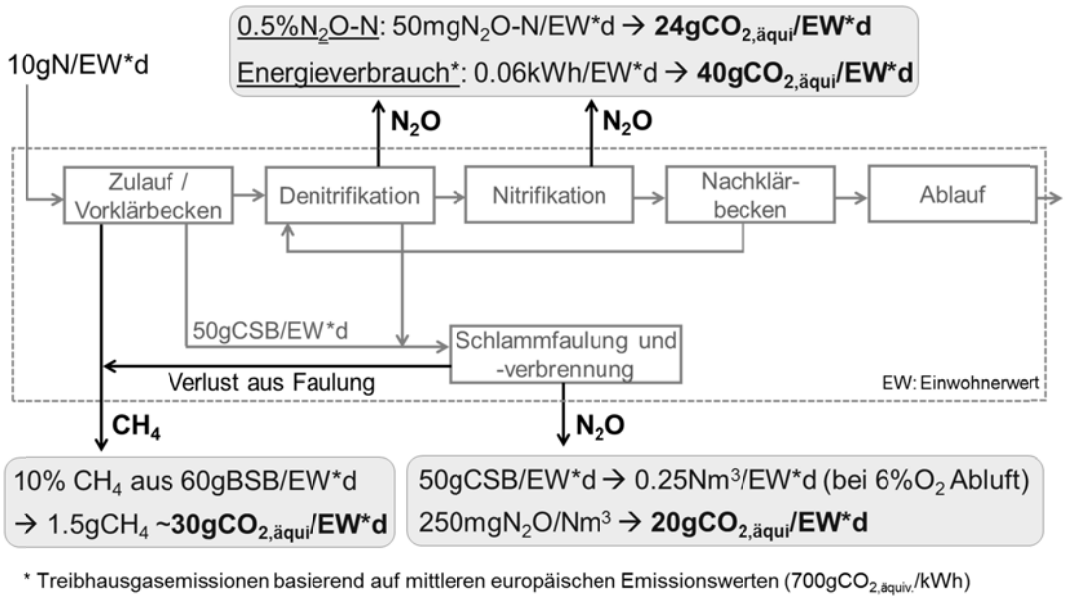

Fig. 1: *Abschätzung der Treibhausgasemissionen in der Abwasserreinigung: N_2O Emissionen aus der Belebung und der Schlammverbrennung, sowie CH_4 Emissionen aus der Kanalisation und dem Vorklärbecken. Die Schlammfaulung kann ebenfalls einen signifikanten Beitrag leisten, insbesondere wenn die Nachfaulung nicht abgedeckt ist und das produzierte CH_4 somit direkt in die Atmosphäre entweichen kann (Daelman et al., 2012).*

Mikrobielle N_2O Bildungswege

Die wesentlichen N_2O Bildungswege sowie die relevanten Prozessparameter in der biologischen Reinigungsstufe sind noch nicht vollständig identifiziert. Aus diesem Grund sind die Emissionsfaktoren noch relativ unsicher und eine Extrapolation der Messergebnisse auf andere Anlagen ist mit Vorsicht zu betrachten. In den letzten Jahren hat sich aber insgesamt das Prozessverständnis deutlich verbessert: In den 1990er Jahren wurde beispielsweise noch davon ausgegangen, dass N_2O hauptsächlich als Zwischenprodukt der heterotrophen Denitrifikation gebildet wird. Heute weiss man, dass zusätzlich auch während der Nitrifikation (Ammoniumoxidation) N_2O gebildet werden kann (Fig. 2). N_2O wird grundsätzlich unter suboptimalen Wachstumsbedingungen produziert. Dazu gehören etwa tiefe O_2 Konzentrationen, hohe NH_4^+ und NO_2^- Konzentrationen, oder ein tiefes Kohlenstoff (CSB) zu Stickstoff-Verhältnis (Kampschreur et al., 2009).

Um die N_2O Bildungswege in der kommunalen Abwasserreinigung identifizieren zu können wurde im Rahmen des in diesem Artikel vorgestellten Forschungsprojekts N_2O Emissionen in der Abwasserreinigung: Biologische Nährstoffelimination und Schlammverbrennung an Eawag und Empa eine neue Methode entwickelt und erfolgreich angewendet. Diese Methode basiert auf der Messung der stabilen Stickstoffisotopen (durchschnittlicher ^{15}N-Gehalt in N_2O) sowie deren Verteilung im N_2O Molekül ($^{15}N^{14}NO$ bzw. $^{14}N^{15}NO$, Fig. 3, siehe auch weiter unten; Mohn et al., 2012; Wunderlin et al., 2013): da über die einzelnen Bildungswege N_2O mit unterschiedlichem ^{15}N Gehalt und einer unterschiedlichen ^{15}N Verteilung gebildet wird, kann diese Messung für eine Prozessidentifikation verwendet werden.

In der biologischen Abwasserreinigung kann grundsätzlich zwischen autotropher Nitrifikation und heterotropher Denitrifikation unterschieden werden, wobei N_2O in beiden Prozessen gebildet werden kann (Fig. 2). Obwohl diese Prozesse meistens räumlich oder zeitlich voneinander getrennt ablaufen, ist nicht auszuschliessen, dass der jeweils andere Prozess ebenfalls aktiv ist. Für die Erarbeitung von Massnahmen und von Betriebsstrategien zur Reduktion der Emissionen ist es aber essentiell, den jeweils für die N_2O Produktion relevanten Bildungsweg zu kennen.

N_2O Bildung unter nitrifizierenden Bedingungen

In der Nitrifikation wird NH_4^+ unter aeroben Bedingungen über NO_2^- zu Nitrat (NO_3^-) oxidiert. Dabei kann N_2O durch die Ammonium oxidierenden Bakterien (AOB) über zwei mögliche Wege gebildet werden (Fig. 2): (i) via Reduktion von NO_2^- (Nitrifikanten-Denitrifikation), oder (ii) über das Zwischenprodukt Hydroxylamin (NH_2OH), d.h. während der Oxidation von NH_4^+ zu NO_2^-. Es wird davon ausgegangen, dass in der kommunalen Abwasserreinigung die Nitrifikanten-Denitrifikation der dominante Prozess ist, und hauptsächlich bei erhöhten NO_2^- und tiefen O_2 Konzentrationen aktiv ist. Die N_2O Bildung über NH_2OH ist möglicherweise von Bedeutung bei einer hohen NH_4^+ Oxidationsrate (bei hohen O_2 Konzentrationen), z.B. in einem sequentiellen biologischen Reinigungsverfahren (SBR) am Anfang der Belüftungsphase, bei hohen NH_4^+ und tiefen NO_2^- Konzentrationen.

N_2O Bildung unter denitrifizierenden Bedingungen

In der heterotrophen Denitrifikation wird das zuvor gebildete NO_3^- unter anoxischen Bedingungen zu Luftstickstoff (N_2) reduziert. Dabei ist N_2O ein obligates Zwischenprodukt (Fig. 2, unten). Insbesondere die Hemmung des letzten Schritts (von N_2O zu N_2), z.B. durch Sauerstoff oder Nitrit, kann zu hohen N_2O Emissionen führen. Ausserdem kann ein zu geringes Verhältnis von abbaubarem organischem Substrat zu Nitrat (ungleichmässige Aktivität der reduzierenden Enzyme) die N_2O-Bildung erhöhen.

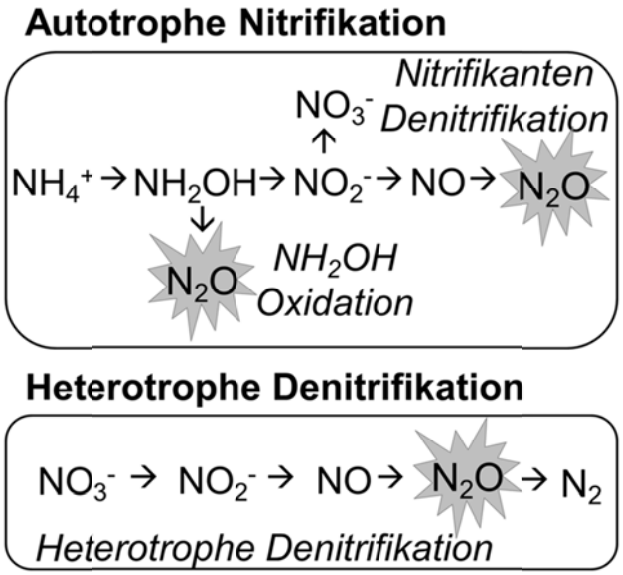

Fig. 2: *Mögliche N_2O Produktionswege in der biologischen Abwasserreinigung Wunderlin et al. (2012). Während der Nitrifikation (oben) kann N_2O durch AOB über zwei Stoffwechselwege gebildet werden: Nitrifikanten-Denitrifikation, oder Hydroxylamin (NH_2OH) Oxidation. Während der heterotrophen Denitrifikation (unten) ist N_2O ein obligates Zwischenprodukt und dessen Akkumulation vor allem davon abhängig wie gut die einzelnen Reduktionsschritte aufeinander abgestimmt sind.*

Identifikation der N_2O Bildungswege anhand der N_2O Isotopensignatur

Die in dieser Untersuchung angewandte Methode erlaubt es aufgrund der Messung der stabilen Stickstoffisotope im N_2O Molekül zwischen den unterschiedlichen N_2O Bildungswegen zu unterscheiden. Hierfür werden sowohl der durchschnittliche ^{15}N-Gehalt, sowie dessen Verteilung im N_2O Molekül ($^{15}N^{14}NO$ bzw. $^{14}N^{15}NO$, genannt Site preference; Fig. 3) bestimmt. Wesentlich für dieses Verfahren ist, dass die drei möglichen N_2O Bildungswege (Nitrifkanten-Denitrifikation, NH_2OH Oxidation, heterotrophe Denitrifikation; Fig. 2) über ihre jeweilige charakteristische Isotopenzusammensetzung, analog eines Fingerabdrucks, identifiziert werden können.

Laborexperimente mit Belebtschlamm zeigen, dass unter aeroben Bedingungen die NO_2^- Reduktion (vermutlich grösstenteils durch die Nitrifikanten-Denitrifikation) den dominanten N_2O-Bildungsweg darstellt, wohingegen die NH_2OH Oxidation lediglich geringfügig beiträgt (graue Flächen in Fig. 3). Andererseits ist unter anoxischen Bedingungen erwartungsgemäss die heterotrophe Denitrifikantion der dominierende Prozess.

Weitere Messungen im Pilotmassstab bestätigen diesen Trend (Fig. 3): unter aeroben Bedingungen (O_2 >2 mg/l) ist die Nitrifikanten-Denitrifikation (NO_2^- Reduktion) der wesentliche Bildungsprozess, während bei tiefen O_2 Konzentrationen (<1.5 mg/l) zunehmend die heterotrophe Denitrifikation aktiviert wird. Der Beitrag der NH_2OH Oxidation bleibt auch unter diesen Bedingungen gering.

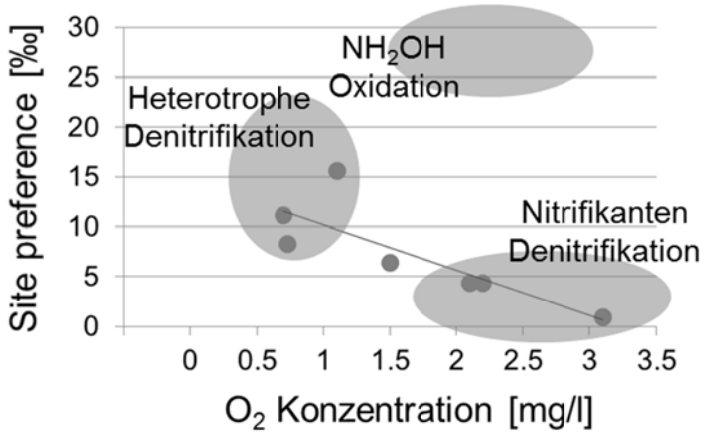

Fig. 3: *Die Messungen im Pilot-Massstab (8 m^3 Reaktoren) bestätigen eine dominante Nitrit-Reduktion (Nitrifikanten-Denitrifikation) bei O_2 Konzentrationen von grösser 2 mg/l, wohingegen die heterotrophe Denitrifikation bei tiefen O_2 Konzentrationen zunehmend aktiv wird (aus Wunderlin et al., in preparation). Die N_2O Bildungswege wurden über ihre Stickstoff-Isotopensignaturen (Site preference) identifiziert, welche zuvor in Laborexperimenten bestimmt wurden und als graue Flächen dargestellt sind (aus Wunderlin et al., 2013).*

Insgesamt konnte durch die Anwendung dieser neuartigen Methode das Prozessverständnis der N_2O-Bildung verbessert werden, wobei der Fortschritt hauptsächlich darin besteht, dass die NH_2OH Oxidation als relevante Quelle in der biologischen Reinigung von kommunalem Abwasser kaum eine Rolle spielt. Die Reduktion von NO_2^- als dominanter Stoffwechselweg rückt somit weiter in den Fokus, und deshalb kommt im Anlagenbetrieb der NO_2^- Konzentration zunehmend eine wichtige Bedeutung zu.

Typische Tagesgänge der N_2O Emissionen und relevante Prozessparameter

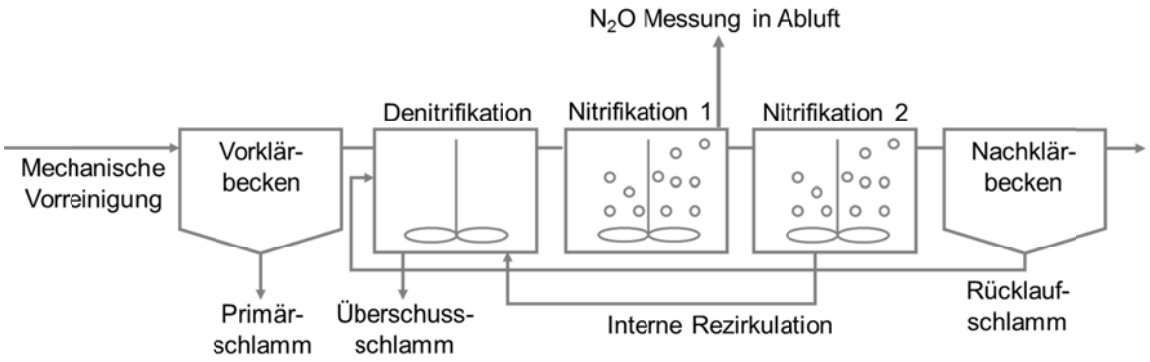

Fig. 4: *Fliessschema der Pilotanlage mit einer der Nitrifikation vorgeschalteten Denitrifikationsstufe. Die N_2O Emissionen wurde kontinuierlich in der Abluft des ersten Nitrifikationsreaktors gemessen.*

Um die Bedeutung der NO_2^- Konzentration für die N_2O Bildung zu überprüfen wurden Untersuchungen im Pilotmassstab unter verschiedensten Betriebsbedingungen durchgeführt. Für diese Messkampagne wurde eine Pilotanlage mit einer vorgeschalteten Denitrifikationsstufe betrieben (siehe Fig. 4). Für die Nitrifikation wurden zwei einander nachgeschaltete Reaktoren verwendet, wobei die N_2O Konzentration kontinuierlich in der Abluft des ersten Nitrifikationsreaktors gemessen wurde. Das Schlammalter lag bei etwa 11 Tagen und die O_2 Konzentration in der Nitrifikationsstufe war auf 2 mgO_2/l geregelt (Fig. 4). Die N_2O Emissionen der beiden in Fig. 5 dargestellten Tagesgänge lagen bei rund 0.5% während des ersten Tages, und bei etwa 0.2% während des zweiten Tages (bezogen auf die NH_4^+-Zulauffracht und Emissionen aus dem ersten Reaktor). Die Emissionsfaktoren einer Anlage im Vollmassstab könnten auf Grund der grösseren Beckentiefe jedoch etwas geringer sein.

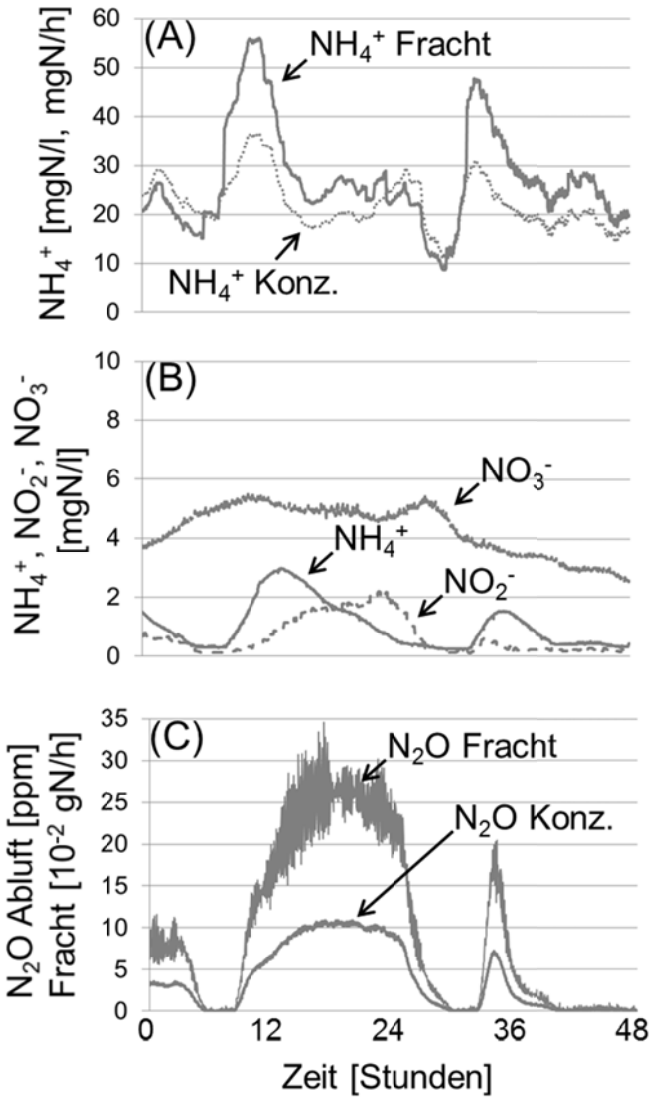

Fig. 5: *N_2O Emissionen und Verläufe der gelösten Stickstoff Komponenten an zwei aufeinander folgenden Messtagen. (A) NH_4^+ Zulauffracht in die biologische Reinigungsstufe, sowie deren Konzentration im Vorklärbecken, (B) NH_4^+, NO_2^- und NO_3^- Konzentrationen im ersten Nitrifikationsreaktor, (C) kontinuierlich aufgezeichnete N_2O Abluftkonzentrationen und berechnete Frachten der ersten Nitrifikationsstufe. Der Zulauf*

ist nach einem fixen Tagesgang geregelt (jeweils konstant über 2 Stunden; aus Lotito et al., 2012).

In der durchgeführten Messkampagne wurden typische Tagesgänge beobachtet, welche die grosse Dynamik der Emissionen illustrieren (Fig. 5): während der Nacht nimmt die NH_4^+ wie auch die NO_2^- Konzentration in der Nitrifikationsstufe ab, da auch die Stickstoff Zulauffracht sinkt, während des Tages (ab ca. 8 Uhr morgens) steigen ihre Konzentrationen, bedingt durch eine erhöhte Stickstoff-Zulauffracht, wieder an. Die N_2O Emissionen (Konzentrationen in der Abluft) folgen diesen Trends: bei tiefen Stickstoff-Konzentrationen während der Nacht sind auch die N_2O Emissionen gering, wobei während des Tages, bei erhöhten NH_4^+ und NO_2^- Konzentrationen, auch die N_2O Emissionen hoch sind.

In Fig. 5 (B) ist ersichtlich, dass neben NO_2^- möglicherweise auch die NH_4^+ Konzentration in der Belebung einen wichtigen Parameter für die N_2O Produktion darstellt. Gemäss Fig. 2 (oben) kann N_2O theoretisch bei hoher Nitrifikationsaktivität über die NH_2OH Oxidation gebildet werden. Mit der Isotopenmethode (Fig. 3) konnte aber gezeigt werden, dass der NH_2OH Oxidationsweg in der kommunalen Abwasserreinigung keinen wesentlichen Beitrag leistet. Somit scheint die Bedeutung von NH_4^+ hauptsächlich darin zu liegen, dass es das Ausgangsprodukt der Nitrifikanten-Denitrifikation ist (N_2O Bildung über die NO_2^- Reduktion). Zudem begünstigt eine hohe $NH4^+$ Konzentration die NO_2^- Akkumulation in der Nitrifikation.

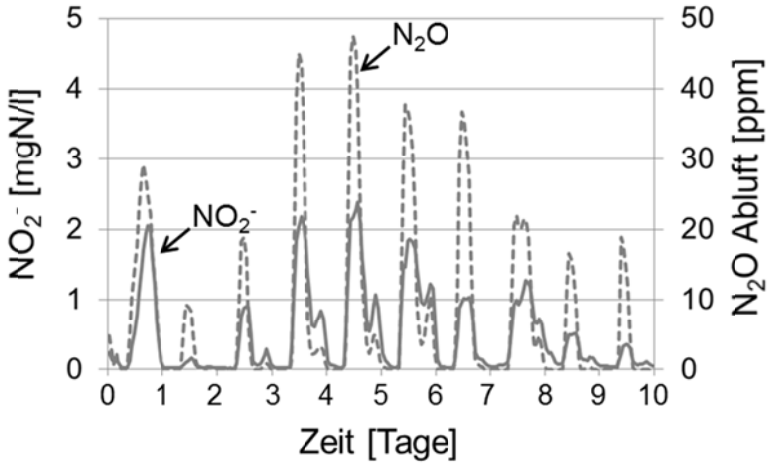

Fig. 6: *Typische Tagesgänge der gelösten NO_2^- und N_2O Abluft-Konzentrationen (Stundenmittelwerte) der ersten Nitrifikationsstufe, an zehn aufeinander folgenden Messtagen im Normalbetrieb.*

Die gelöste NO_2^- Konzentration stellt somit einen zentralen Parameter für die Beurteilung der N_2O Bildung dar. In Fig. 6 werden NO_2^- Konzentration und N_2O Abluftkonzentration für 10 aufeinander folgende Messtage dargestellt. Es ist ersichtlich, dass die Konzentrationsverläufe der beiden Spezies gut übereinstimmen: in der Nacht sind die Konzentrationen erwartungsgemäss tief, während sie am Tag, bei hohen Stickstoff Zulauffrachten, erhöht sind. Vor diesem Hintergrund hat sich die Hypothese bestätigt, dass bei tiefen NO_2^- Konzentrationen auch die N_2O Bildung gering

gehalten werden kann, was impliziert, dass die biologische Reinigungsstufe bei möglichst tiefen NO_2^- Konzentrationen betrieben werden sollte.

Neben erhöhten NH_4^+ und NO_2^- Konzentrationen ist auch die O_2-Konzentration ein relevanter Parameter für die N_2O Bildung. Dies soll hier nochmals kurz aufgegriffen werden, denn im Zusammenhang mit der N_2O Bildung über die NO_2^- Reduktion ist bekannt, dass bei tiefen O_2 Konzentrationen (<1 mgO_2/l) die N_2O Emissionen tendenziell höher sind als bei 2 bis 3 mgO_2/l (Kampschreur et al., 2009). Bei der energetischen Optimierung der biologischen Reinigungsstufe, insbesondere wenn bei der Belüftung angesetzt wird, ist daher besondere Vorsicht geboten. Die N_2O-Bildung ist hoch komplex und weist eine grosse Dynamik auf. Daher kann an dieser Stelle keine abschliessende Empfehlung zur Optimierung der biologischen Abwasserreinigung präsentiert werden, um die N_2O Emissionen aus ARA möglichst tief zu halten. Vielmehr sollte vor dem Hintergrund der in diesem Artikel diskutierten Einflussparameter, wie NO_2^-, NH_4^+ oder O_2, jede Anlage für sich betrachtet werden, und optimierte Betriebsstrategien anhand einer fix installierten N_2O Abluftmessung getestet und überwacht werden (empfohlene Betriebsstrategien, siehe unten). Die Messgeräte für eine kontinuierliche Abluftmessung sind standardmässig verfügbar und können bei verschiedenen Anbietern gekauft werden. Bei abgedeckten Reaktoren mit einer gefassten Abluft ist die Implementierung relativ einfach. Bei offenen, ungedeckten Reaktoren liegt die Schwierigkeit bei der Fassung der Abluft. In diesen Fällen muss eine auf der Wasseroberfläche aufliegende ‚Haube' eingesetzt werden, was aktuell noch nicht routinemässig angewendet wird. Es existieren aber Ansätze wie dies in Zukunft umgesetzt werden kann. Die Investitionskosten für eine online N_2O-Abluftmessung liegen höher als beispielsweise für konventionelle ionenselektive Sonden. Die Betriebskosten sind jedoch wegen des geringeren Wartungsaufwands tiefer.

Die anschliessend diskutierten Betriebsempfehlungen können aus den präsentierten Daten und dem daraus resultierenden Prozessverständnis abgeleitet werden. Sie stellen einen ersten Ausgangspunkt für weitere Optimierungen dar, die anhand von direkten Emissionsmessungen für die jeweilige Anlage überprüft und verifiziert werden sollten:

- Es wird als wichtig erachtet, dass die Nitrifikation wie auch die Denitrifikation möglichst vollständig ablaufen können, um die NO_2^- Bildung tief zu halten. Insbesondere sollten bei einer vorgeschalteten Denitrifikation zu kurze anoxische Phasen vermieden werden. Andererseits muss bei einer NH_4^+ geregelten Belüftung genügend Zeit für die NO_2^- Oxidation zur Verfügung stehen.
- Generell sollte die NH_4^+ Konzentration in der Belebung möglichst tief gehalten werden, was eine ausreichende Nitrifikationskapazität voraussetzt, d.h. ein aerobes Schlammalter mit genügend Sicherheit zur Verarbeitung von Stickstoff-Frachtspitzen. Zudem sollte Faulwasser nicht während Phasen mit hohen Stickstoff-Frachten im Zulauf dosiert werden.
- Während Phasen mit hohen Stickstoff-Frachten sollte die Nitrifikation bei ausreichend Sauerstoff betrieben werden, da bei tiefen O_2 Konzentrationen die

N_2O Bildung gegenüber der NO_2^- Reduktion begünstigt wird. Somit sind tiefere O_2-Konzentrationen während der Nacht bei geringen Stickstoff-Frachten sinnvoll und sparen Energie ohne viel N_2O zu produzieren.

- Anderseits sollte die Denitrifikation anoxisch betrieben werden. Daher ist es wichtig, einen O_2-Eintrag aus der Nitrifikation (Rücklauf, interne Rezirkulation) oder durch das Rührwerk möglichst zu vermeiden.

Schlussfolgerungen

Aus dem vorliegenden Artikel geht hervor, dass das Treibhausgaspotential der N_2O Emissionen in der biologischen Abwasserreinigung relevant ist und eine vergleichbare Grössenordnung aufweist wie die indirekten Emissionen der Belüftung. Aus diesem Grund sollten die N_2O Emissionen möglichst reduziert werden, und sowohl bei der Auslegung, der Optimierung sowie beim Betrieb der Anlage beachtet werden.

Anhand der präsentierten Daten wurde gezeigt, dass der mikrobiologischen Reduktion von NO_2^- zu N_2O in der kommunalen Abwasserreinigung eine grosse Bedeutung zukommt. Im Weitern ist ersichtlich, dass die N_2O Emissionen einer grossen zeitlichen und räumlichen Dynamik unterliegen, was es schwierig macht, von einem Tagesgang auf den nächsten zu extrapolieren, oder gar Aussagen über das Emissionspotential von anderen, nicht untersuchten Anlagen zu machen. In diesem Sinne wird als Schlussfolgerung in Aussicht gestellt, dass für eine gezielte Optimierung des Anlagenbetriebes eine kontinuierliche N_2O Emissionsmessung unerlässlich ist. Denn zusätzlich zur Emissionsüberwachung kann anhand der N_2O Bildung die Prozessstabilität relativ gut überwacht werden, gilt doch die Akkumulation von NO_2^- als Indiz für eine (vorübergehende) Überlastung der Anlage, eine O_2-Unterversorgung durch ineffiziente Belüftung oder gar Hemmung der mikrobiologischen Prozesse (Burgess et al., 2002; Butler et al., 2009).

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