Factors controlling nitrous oxide at the microbial community and estuarine scale

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ABSTRACT: This paper examines the effect of oxygen on nitrous oxide (N₂O) concentrations in estuarine waters. N₂O has been measured year-round in the Schelde estuary, a high-nitrogen, low-oxygen macrotidal system. N₂O concentrations were above atmospheric equilibrium levels indicating that this estuary represents a source to the atmosphere. The distribution of N₂O showed consistent and systematic relationships with distribution patterns of ammonium, oxygen, nitrite and nitrification activities. A controlled laboratory experiment with a natural bacterial community from the Schelde estuary revealed maximum N₂O production to occur at oxygen concentrations of about 5 μM. This production was inhibited by acetylene, a nitrification inhibitor. Maximum N₂O concentration in the field occurred at oxygen concentrations below 35 μM. The difference in the oxygen concentration that results in maximum N₂O may have arisen because low-oxygen environments present in the estuary were destroyed by stirring in our laboratory experiment. It appears that low oxygen concentrations in estuarine water trigger enhanced N₂O production if ammonium is present in sufficient amounts. This conclusion is further illustrated by data from the Thames, Loire and Gironde estuaries.

KEY WORDS: Nitrous oxide • Nitrification • Oxygen • Ammonium • Estuary • Scheldt • Thames • Loire • Gironde

INTRODUCTION

Nitrous oxide (N₂O) affects the global climate in 2 ways (Bange 2000). In the lower atmosphere, N₂O acts as a greenhouse gas that is more than 100 times more powerful in warming potential than CO₂. N₂O is chemically stable in the troposphere and reaches the stratosphere where it forms NO radicals in photochemical reactions that are involved in the destruction of ozone; hence, N₂O contributes indirectly to the destruction of the ozone layer. Atmospheric mixing ratios of N₂O have been increasing steadily over the past 100 yr (Battle et al. 1996). The accumulation of the gas in the atmosphere is the result of unbalanced sources and sinks of N₂O. Aquatic systems, both salt- and freshwater, contribute 25 to 30% of the total global N₂O emission (IPCC 1996). Estuaries are calculated to account for approximately 60% of total marine N₂O production/emission (Bange et al. 1996). To understand the biogeochemistry of N₂O in estuaries, both quantification of N₂O concentrations and fluxes, and understanding of factors controlling the production of N₂O are necessary.

In aquatic systems, N₂O can be produced during both nitrification and denitrification (Codispoti et al. 1992, 2001). The first step of nitrification, the oxidation of ammonia to nitrite, can be a source of N₂O, in particular under low oxygen concentrations (Goreau et al. 1980, Jorgensen et al. 1984, Anderson & Levine 1986, Stüven et al. 1992, Kester et al. 1997). The concentration range of oxygen, under which significant quantities of N₂O per unit volume are produced, is very narrow. At oxygen concentrations higher than this critical range, oxi-
oration to nitrite and subsequently nitrate is completed with only modest net production of N₂O, while the ammonium oxidation reaction does not occur at all at oxygen concentrations close to 0 and below the range for N₂O production (Codispoti et al. 1992). Besides this oxidative pathway, N₂O may also be formed in the reductive process of denitrification. During denitrification, nitrate is reduced to molecular nitrogen gas (N₂) via the intermediates NO₂, NO and N₂O. N₂O reduction is, at least in some denitrifying species, more sensitive to oxygen than the other reduction steps (Betlach & Tiedje 1981, Zunft & Kroncke 1990). This can lead to accumulation of N₂O at low oxygen concentrations, in particular during periods with alternating, transient conditions (Naqvi et al. 2000). Oxygen concentration apparently has a strong regulating influence on N₂O production. Additionally, ammonia concentration, nitrite concentration, pH, the physical environment and the physiological characteristics of the microbial community determine the amount and extent of the eventual emission of N₂O to the atmosphere. The overall factors controlling N₂O production and emission seem to be dependent on the specific conditions and the system studied, and may vary temporarily.

At the global scale, N₂O production and emission from riverine and coastal systems has been related to N loading (Seitzinger & Kroeze 1998). Similarly, the main factor controlling N₂O emission from estuarine sediments is N load (Seitzinger & Nixon 1985, Middelburg et al. 1995).

At the level of isolated populations (Goreau et al. 1980, Anderson & Levine 1986, Anderson et al. 1993) and natural communities (Jørgensen et al. 1984), oxygen is governing N₂O yield during nitrification and denitrification. Consistent with these laboratory studies, Yoh et al. (1983) reported accumulation of N₂O in oxygen-deficient layers of freshwater lakes, and Naqvi et al. (2000) reported enhanced N₂O production in a low oxygen zone of the Indian continental shelf. There appear to be different controlling factors of N₂O production at different scales, with nitrogen loading governing N₂O at the global and across ecosystem scales, and oxygen being the key factor in laboratory studies and within individual aquatic ecosystems. Moreover, the temporal variability in oxygen and ammonium and/or nitrate availability may be another factor in natural systems. Transient events rather than long-term steady-state conditions have been proposed to govern N₂O production (Naqvi et al. 2000).

In this study, we present a seasonal study of N₂O concentrations in the Schelde estuary, a high-nitrogen, low-oxygen macrotidal system. Furthermore, a natural community from the Schelde estuary was used in controlled laboratory experiments to identify the controlling factor of N₂O production. These field and environmental data will be used to argue that oxygen is the controlling factor provided that ammonium is abundant. Finally, we present dissolved N₂O data for a number of additional macrotidal European estuaries (Thames, Loire and Gironde) to strengthen our arguments.

**MATERIALS AND METHODS**

**Research areas.** Samples were collected in the context of BIOGEST, an EU-supported program on biogeochemical processes and trace-gas production in a number of European tidal estuaries (Middelburg et al. 2002). The Schelde estuary (also known as the Scheldt and Westerschelde) drains an estimated 21 000 km² of northern France, Belgium and The Netherlands, an area with approximately 10 million inhabitants and a nitrogen load of 5 x 10¹⁰ mol N yr⁻¹ (Soetaert & Herman 1995). Turbidity is high in the entire upper estuary, which is illustrated by the observed suspended matter concentrations (10 to 350 mg l⁻¹). The Thames estuary is a turbid, tidal estuary on the east coast of the UK, entering the North Sea at Southend on Sea. The drainage area of the river Thames is rather small (14 000 km²) but hosts a population of about 12 million, including London, and has a dissolved inorganic nitrogen load of 2.4 x 10⁹ mol N yr⁻¹ (Trimmer et al. 2000). The river Loire drains a major part of central France and its estuary is well-mixed and very turbid with suspended matter concentrations over 1 g l⁻¹. The rivers Garonne and Dordogne that drain a large part of SW France feed the Gironde estuary. It is a well-mixed, highly turbid estuary with suspended matter concentrations over 1 g l⁻¹. The Thames, Loire and Gironde estuaries were studied only once in February 1999, September 1998 and June 1997, respectively. The Schelde estuary was examined at monthly resolution from April 1997 to April 1998.

**N₂O measurements.** Dissolved N₂O concentrations were measured in surface waters while sailing or on station using a continuous flow gas equilibrator connected to a photoacoustic gas analyser (Brul & Jkaer type 1302). This method has been described elsewhere (Abril et al. 2000, De Wilde & De Bie 2000, Middelburg et al. 2002). Headspace gas concentrations were recalculated to water concentrations using the temperature- and salinity-dependent partitioning coefficient (Weiss & Price 1980). The precision of dissolved N₂O concentrations was better than 3 % and the accuracy is based on calibration with certified standards traceable to the US National Institute of Standards.

**Ancillary measurements.** Salinity, temperature and oxygen were measured either with a CTD system, equipped with a polarographic oxygen sensor (THIS-
HYDRO H2O) or obtained from Middelburg & Nieuwenhuize (2000) and Middelburg et al. (2002). Nutrients were measured using automated colorimetric techniques (De Wilde & De Bie 2000, Middelburg & Nieuwenhuize 2000). Nitrification activity was measured by the $^{14}$C-bicarbonate incorporation method (Somville 1978). Briefly, carbon fixation by autotrophic ammonia-oxidising bacteria is quantified by the difference of inorganic carbon incorporation during incubation with and without N-serve, a specific inhibitor of nitrification activity. These data have been discussed in detail by De Bie et al. (2002).

**Incubation experiments.** Estuarine waters with a salinity of 1 from the upper part of the Schelde estuary were collected in March to April 1998 and subsequently incubated in a 4 l glass incubation vessel (3.5 l water and 0.5 l headspace) that was kept at in situ temperature (10°C) by a cooling spiral connected to a water bath. The water was stirred by a magnetic stirrer and continuously bubbled with a gas mixture at 20 l h$^{-1}$, of which the composition was accurately controlled by mass flow control valves (Brooks Instruments) connected to N$_2$, air and acetylene bottles. Oxygen concentrations in the vessel were monitored by an oxygen electrode (ECOLAB, Maarssen, The Netherlands). Every 5 min, N$_2$O concentration was monitored in the headspace using the photoacoustic gas analyser that was connected in a closed circuit to the vessel. Water samples could be taken by means of a syringe in the vessel lid, where a gas-tight valve prevented gas exchange.

At the start of the experiment, ammonium was added to a final concentration of about 2 mM, and the vessel was flushed with air until 100% air saturation and subsequently incubated for 4 d while stepwise lowering imposed oxygen levels (Fig. 1). In the absence of acetylene, there was significant nitrification as reflected in increases in nitrate (6.7 ± 0.8 μM h$^{-1}$) and nitrite (6.2 ± 0.4 μM h$^{-1}$) and a proportional decrease in ammonium (~14.6 ± 1.3 μM h$^{-1}$). In the presence of 1% acetylene, there was no significant decrease in ammonium, while nitrite and nitrate were consumed (Fig. 1C). Fig. 1

![Fig. 1. Time evolution of oxygen (——), N$_2$O (-----), ammonium (▲), nitrate (X) and nitrite (○) during the laboratory incubations. (A) Incubation experiment without acetylene; (B) enlargement of the data presented in Panel A for the period of 0.6 to 0.9 d; (C) incubation experiment with 1% acetylene. The arrow in Panel A indicates the period of malfunctioning of the stirring system resulting in transient accumulation of N$_2$O](image-url)
clearly shows that $N_2O$ production depends on the oxygen level and that it was predominantly formed at low oxygen concentrations. After imposing a new oxygen level, dissolved $N_2O$ concentrations reached an approximate steady-state level within 2 to 8 h, which was dependent on conditions (Fig. 1A,B). Fig. 2A shows the steady-state $N_2O$ concentrations as a function of imposed oxygen levels. In the absence of acetylene, $N_2O$ concentrations were maximal at an oxygen concentration of about 5 μM (1.5% air saturation; Fig. 2A), but $N_2O$ production occurred at all oxygen levels. At lower oxygen concentrations, $N_2O$ concentration decreased, although some $N_2O$ was produced even when oxygen was completely absent. In the presence of 1% acetylene, there was no maximum in $N_2O$ production at oxygen levels of about 5 μM oxygen (1.5% saturation). However, high $N_2O$ concentrations were observed under anoxic conditions. Acetylene is an inhibitor of the first step of nitrification (ammonium oxidation) and the last step of denitrification ($N_2O$ reduction to dinitrogen). The absence of $N_2O$ production at low, non-zero oxygen in the presence of the nitrification inhibitor acetylene provides evidence that nitrification is the major source of $N_2O$ in this experiment. The importance of nitrification was further illustrated by the immediate increase in concentration of $N_2O$ upon addition of ammonium (Fig. 3).

Field observations

The Schelde estuary is a highly heterotrophic system with low oxygen levels and high N loading. Fig. 4 shows a detailed set of longitudinal profiles for April
1997. Ammonium concentrations were highest in the river Schelde (417 µM) and rapidly decreased with increasing salinity (Fig. 4A). This decrease in ammonium concentration was accompanied by a proportional increase in nitrate from 146 µM in the river to a maximum of 429 µM at salinity 7.6 (Fig. 4B). At higher salinities, nitrate concentrations decreased again due to dilution with relatively nitrate-poor North Sea water. Nitrification activity was highest (0.025 µmol C l⁻¹ h⁻¹) in the riverine samples and increased downstream to a maximum (0.046 µmol C l⁻¹ h⁻¹) at salinity 7.6. Nitrification activities rapidly dropped further downstream (Fig. 4C).

Oxygen saturation levels were below 20% in the low-salinity zone with high nitrification activities, while oxygen was supersaturated in the lower estuary due to a spring bloom. Nitrification of ammonium to nitrate in the low-salinity, low-oxygen zone was very likely incomplete because there was significant production of nitrite (uniform concentration of 15 µM; Fig. 4B) and N₂O (245 nM at salinity 7.6; Fig. 4A).

This basic pattern of oxygen and nitrogen species was consistently observed over the 13 mo period of observation (Fig. 5), although concentration levels and profile shapes varied in response to variability in river discharge, temperature and other environmental factors. Ammonium concentration in the upper estuary varied from 86 µM in September to 483 µM in November. Concentrations of N₂O in the tidal river varied between 8.7 nM in November and 1457 nM in August. N₂O showed prominent peaks in the period April until September with maximum concentrations during August. For all samples but the river in November, the estuary was a net source of N₂O to the atmosphere with a median saturation ratio (observed/equilibrium concentration) of 7.1 (atmospheric equilibrium levels varied from 6.9 to 14 nM). In accordance with the experimental results (Fig. 2A), N₂O concentrations were related to oxygen levels in a non-linear way with peak concentrations of N₂O at oxygen saturation levels between about 2 and 15%, and lower N₂O concentration above and below this oxygen level (Fig. 2B).

Ammonium concentrations in the Gironde estuary varied from 0.4 to 1.6 µM with no clear trend with salinity (Fig. 6A). Oxygen increased from 65% saturation in the river to close to saturation in the most saline part. N₂O concentrations are relatively low and varied from 9.8 to 36.7 nM, consistent with observations by Bange et al. (1996) and Abril et al. (2000). Ammonium concentrations in the Loire estuary were also relatively low (2 to 5.5 µM). Oxygen concentration in the Loire estuary were rather low with 2 oxygen minimum zones, one down to 21% saturation at salinity 7.5 and one down to 45% saturation at salin-

Fig. 5. Ammonium (■), oxygen (○) and N₂O (●) versus salinity in the Schelde estuary from May 1997 to April 1998. Notice the differences in scale
Fig. 6. Ammonium (■), oxygen (○) and N\textsubscript{2}O (●) versus salinity in the Gironde (A), Loire (B) and Thames (C) estuaries. Surface samples were taken in June 1997, September 1998 and February 1999 respectively.

ity 18 to 20 (Fig. 6B). N\textsubscript{2}O concentrations were rather low (7.3 to 21 nM), but showed a maximum in the oxygen minimum zone at salinity 18 to 20 (Fig. 6B). Ammonium concentrations in the river Thames and upper part of the estuary were high (30 to 43 pM) but decreased rapidly with increasing salinity (Fig. 6C). Oxygen concentrations were lowest in the upper estuary but were always above 50\% saturation. N\textsubscript{2}O concentration ranged from 49 nM in the river to a maximum of 93 nM at salinity 2.7 and then steadily decreased down to 11.2 nM in the mouth of the estuary (Fig. 6C).

**DISCUSSION**

Concentrations of dissolved N\textsubscript{2}O are the result of production as well as consumption in the water bodies, inputs from the sediments, outgassing to the atmosphere and dispersal. Our data clearly revealed distinct N\textsubscript{2}O maxima in the Schelde estuary indicating either that N\textsubscript{2}O is locally produced or that there are large local inputs from the sediments. Robinson et al. (1998) and Dong et al. (2002) have reported that benthic denitrification represents the major source of N\textsubscript{2}O in the nitrate-rich Colne estuary. Sediment release could be a major source of N\textsubscript{2}O in the Schelde estuary as well. However, N\textsubscript{2}O maxima move along with the tides consistent with a water-column source. Middebeg et al. (1995) reported N\textsubscript{2}O fluxes from exposed intertidal sediments of the Schelde estuary. These sedimentary fluxes are highly variable but at least about 1 order of magnitude too low to sustain the flux of N\textsubscript{2}O from the water (De Wilde & De Bie 2000). There are unfortunately no data for N\textsubscript{2}O release from inundated intertidal or subtidal sediment in the Schelde estuary.

High N\textsubscript{2}O concentrations in the Schelde estuary were consistently observed over a restricted range of salinity, which is also the zone of oxygen depletion (Figs. 4 & 5). This might suggest that salinity could be the controlling factor of N\textsubscript{2}O production. In a previous study, we discussed this possibility while comparing our data with N\textsubscript{2}O measurements from 1978 (De Wilde & De Bie 2000). At that time, the oxygen-depleted zone of the estuary was more extensive and the N\textsubscript{2}O peak was observed at a higher salinity (i.e. more downstream), but at the same oxygen concentration. This indicates that salinity is more an indicator of the re-aeration status of the estuarine water rather than that there is a direct effect of salt concentration on N\textsubscript{2}O production.

An oxygen control of N\textsubscript{2}O production is also consistent with most of the literature observations (Codispoti et al. 1992, 2001). Experimental studies with pure cultures (Goreau et al. 1980, Anderson et al. 1993, Kester et al. 1997) and suspended marine sediments (Jorgensen et al. 1984) have clearly shown maximum N\textsubscript{2}O production at a narrow range of low, but non-zero oxygen levels. We found similar results in the experiment with the natural estuarine population (Fig. 2A) as well as in our field observations (Fig. 2B). On the basis of these N\textsubscript{2}O-oxygen relationships alone, it is difficult to conclude whether N\textsubscript{2}O production in the natural population is the result of nitrification or denitrification. Addition of acetylene resulted in complete inhibition of N\textsubscript{2}O production above 1\% oxygen saturation, indicating that the N\textsubscript{2}O produced above this oxygen concentration is the product of nitrification. Moreover, addition of ammonium to natural communities resulted in higher N\textsubscript{2}O concentrations consistent with a nitrification source (Fig. 3). The importance of nitrification as a source of N\textsubscript{2}O has also been shown in the Humber estuary through quantification of the transformation of \textsuperscript{15}N-labelled ammonium into \textsuperscript{15}N-labelled N\textsubscript{2}O (Barnes & Owens 1998).

A consortium of ammonia- and nitrite-oxidising bacteria carries out the complete process of nitrification. Nitrite-oxidising bacteria usually have a lower affinity for oxygen- than ammonia-oxidising bacteria (Laanbroek et al. 1994), which may then lead to the transient accumulation of nitrite at low oxygen levels (Fig. 4B; Helder & De Vries 1983). Since nitrite and N\textsubscript{2}O can both be formed during nitrification at low oxygen levels, one might expect a correlation. They are indeed correlated (r\textsuperscript{2} = 0.42, n = 146), but interpretation of this correlation is not simple, because of the escape of the N\textsubscript{2}O gas into the atmosphere. Moreover, nitrite and
N₂O are also intermediates in denitrification, and with the data at hand, we cannot exclude some contributions by this process.

When comparing oxygen concentrations at which N₂O is produced in laboratory experiments (Figs. 1 & 2A) and in estuaries (Figs. 2B, 4, 5 & 6), it is clear that the N₂O peaks in the field are occurring at higher oxygen concentrations than in the laboratory experiment. Maximum N₂O levels in the Schelde estuary were observed at in situ oxygen concentrations in the range from 5 to 35 µM (2 to 15% air saturation; Figs. 2B, 4 & 5), while this range was more restricted and at a lower level in the laboratory experiment (2 to 7 µM; 0.5 to 2% air saturation). Maximum N₂O concentrations in the Thames, Loire and Gironde estuaries occurred at even higher oxygen saturation levels (Fig. 6).

In our incubations, we vigorously stirred our water samples to optimise gas exchange between water and the headspace that was analysed. This stirring also inhibited the development of suboxic microsites so that all micro-organisms most likely experienced nearly the same, imposed oxygen level. Oxygen concentrations measured in estuarine waters are not necessarily representative for the microsites at which N₂O formation might occur, e.g. in flocculate material or in fluid mud layers (Abril et al. 2000). Anoxic microsites in the abundant flocculate material have been proposed as the site for denitrification in the Schelde estuary (Soetaert & Herman 1995) and suboxic microsites may thus provide niches for N₂O production. The structure of flocculate material is readily destroyed by experimental manipulation and was certainly destroyed in our experiments. The Loire and Gironde estuaries are characterised by the temporal presence of dynamic, biogeochemical active fluid mud layers (Abril et al. 2000). These fluid mud layers are partly anoxic and suboxic, and have been shown to be a site of N₂O production, nitrification and denitrification (Abril et al. 2000). Erosion of these mud layers and dispersal of the produced N₂O would account for the observed N₂O distribution. Both the abundance of flocculate materials and fluid mud layers are maximal in the maximum turbidity zone of estuaries (Herman & Heip 1999) and could explain the correlation between N₂O and turbidity in the Humber estuary (Barnes & Owens 1998).

On the basis of our results, we propose the following mechanism for N₂O production in estuarine waters. Nitrifying bacteria travelling in the estuary may be oxygen-limited in the upstream part, where ammonium concentrations are high (De Bie et al. 2002). As a consequence of estuarine mixing there is aeration and oxygen concentrations increase. Once the oxygen concentration has reached a level at which ammonium oxidation can occur, nitrifying bacteria turn active (Billen et al. 1985) and nitrite accumulates in the water, because of the low affinity for oxygen of nitrite-oxidising bacteria (Helder & De Vries 1983). Since the oxygen concentration is still sub-optimal for ammonium oxidation, part of the oxidation products is released in the form of N₂O. More downstream in the estuary, when oxygen has further increased, the nitrification reaction is carried out more completely, as indicated by increasing nitrate concentrations, and decreasing ammonium and nitrite concentrations. N₂O formation is reduced, and the N₂O in the water is either emitted into the atmosphere or consumed by other processes in the water or sediment and diluted with seawater (Barnes & Owens 1998). This mechanism is perfectly illustrated by the Schelde data (Figs. 4 & 5) and may also apply to the Thames estuary (Fig. 6). When ammonium concentrations are too low, like in the Gironde and Loire, N₂O does not reach high levels. When oxygen concentrations are very low, i.e. during summer conditions in the upper Schelde estuary (Fig. 5) or in the fluid muds of the Gironde estuary (Abril et al. 2000), nitrification is oxygen-limited and denitrification of nitrate may cause additional N₂O production. Denitrification based N₂O production may be substantial given that maximum N₂O concentration occur during the summer period with low oxygen conditions.

Although our study has clearly revealed that oxygen is the major factor controlling N₂O in estuarine systems, it is not in conflict with the conceptual nitrogen loading model of Seitzinger & Kroeze (1998). Integrated over large spatial and temporal domains, there are always areas in or periods during which oxygen supply is limited and denitrification occurs, i.e. in estuarine and coastal sediments that are rich in labile carbon. In coastal sediments, denitrification, and consequently N₂O production, is primarily limited by bottom-water nitrate concentrations either directly (nitrate influxes) or indirectly via enhancement of the nitrification-denitrification coupling efficiency (Middelburg et al. 1996). N₂O in estuarine systems with oxygenated water columns then depends on the nitrate and/or nitrite concentration in the water that affects N₂O production processes in sediments (e.g. the Colne estuary in the UK, Robinson et al. 1998, Dong et al. 2002) or in fluid mud layers (e.g. the Gironde estuary in France; Abril et al. 2000). In estuarine systems with low water-column oxygen concentrations, there is additional N₂O formation resulting from nitrification, which is controlled primarily by oxygen and ammonium availability. Moreover, in the complete absence of oxygen, there may be denitrification and N₂O formation in the water column. There appear to be multiple controlling factors: nitrate availability for benthic N₂O production during denitrification and oxygen (ammonium and nitrate) for pelagic N₂O production during nitrification and denitrification, respectively. These processes are
not exclusive but additive and may alternate in their relative importance depending on the system, the season and tidal cycle. This may then explain why Cole & Caraco (2001) found only a weak correlation between average nitrate concentrations and N$_2$O fluxes across a number of estuarine systems.

In conclusion, this study has identified the non-linear relation between low oxygen concentration and N$_2$O production by nitrifying bacteria, both in the field and experimentally. The combination of low oxygen conditions with high nitrogen loads is not uncommon in estuaries and other dynamic coastal systems, which the importance of estuaries as a source of N$_2$O.

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