CSIRO PUBLISHING

Environ. Chem. **2013**, *10*, 281–284
http://dx.doi.org/10.1071/EN13021

Rapid Communication

Is site preference of N_2O a tool to identify benthic denitrifier N_2O?

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Environmental context. The greenhouse gas nitrous oxide is produced by bacteria and emitted from terrestrial and aquatic environments; the origin of this compound can be determined by its ^{15}N intramolecular distribution (site preference). The site preference of nitrous oxide was characterised experimentally in bacterial denitrifying communities under controlled conditions. This study shows the importance of the last step of denitrification on the site preference values, and that complementary methods are necessary to identify the sources of nitrous oxide.

Abstract. Site preference values of nitrous oxide emitted during different steps of benthic denitrification were determined. Compared to that of nitrous oxide as end product, the site preference during complete denitrification presents a large variation, due to the final step, and is highly correlated with nitrate reduction rate. The nitrous oxide reduction step appears decisive on the site preference values.

Received 30 January 2013, accepted 25 June 2013, published online 16 August 2013

Nitrous oxide is a potent greenhouse gas that participates in ozone layer destruction.[1] Atmospheric N_2O concentrations have increased ~20 % with the rise of anthropogenic activities, such as the intensive use of fertilisers in agriculture areas.[2] In soils and sediments, the two main processes responsible for N_2O emissions are nitrification[3] and denitrification.[4] Mediated by denitrifying bacteria under anoxic conditions, denitrification emits localised and instantaneous N_2O as an intermediate product by reduction of nitrate (NO_3^-) to dinitrogen (N_2).[5] The linear asymmetric N_2O molecule has a site preference (SP) that is defined by the intramolecular ^{15}N difference between N^{α} (central N) and N^{β} (terminal N).[6] Determined by natural stable isotope ratios ($\delta^{15}N$ and $\delta^{18}O$), the N_2O site preference (N_2O-SP) signature has been proposed and used as an emerging tool to define the origin of N_2O.[7,8] Pure culture studies, using denitrifying bacteria that reduce NO_3^- to N_2O, have shown that the N_2O-SP signature for denitrification ranged between −5 and 0 ‰.[7,8] In addition to this, several denitrification studies have investigated the N_2O-SP signature originating from soil and water incubations either with N_2O as an end product, substrate or intermediate and found a large variation between −8.5 to 81 ‰.[9,10] Part of this variation has been shown to be due to the fact that the N_2O consumption during denitrification generates an increase of the SP values.[10,11]

Even though these investigations have increased the knowledge regarding the N_2O-SP signature associated with denitrification, several questions remain unanswered. The N_2O-SP signature of environmental denitrifying communities is, for example, lacking. In order to bridge the gap between the investigations on denitrification in pure cultures, the focus of this study was to determine N_2O-SP signatures of environmental benthic denitrifying communities under controlled conditions. More specifically, we determined SP values of N_2O produced by an environmental benthic denitrifying community during the different steps of benthic denitrification (either the production of N_2O or during complete denitrification) under denitrifying conditions (anoxia, nitrate supply).

The study was conducted in an open system using flow-through reactor (FTR) experiments.[12] The use of these reactors allows the determination of denitrification-derived N_2O by nitrate supply and creating anoxic conditions, and thereby exclude nitrification as an additional N_2O source. Nitrate reduction rates and the accompanying N_2O-SP signatures were determined simultaneously, allowing direct comparison.

During benthic denitrification in two types of sediments, nitrate reduction rates and the N_2O-SP values were determined in an open system using FTRs (Fig. 1). The FTRs are described in detail in Laverman et al.[12] Sediments were collected from the Charmoise River (48°36′37.80″N, 2°9′19.54″E, Essonne, France), which is 7.5 km long, in October and November 2011 and in the Manche-à-Eau lagoon (16°16′38.04″N, 61°33′27.93″W, Guadeloupe, France), which has an area of 260 000 m^2, in March 2012. In FTR experiments, denitrifying conditions were obtained by supplying anoxic (N_2 bubbled) nitrate solutions (infinite reservoir) to the sediments.

In order to determine the SP value of N_2O as end product during NO_3^- reduction to N_2O, by the environmental benthic denitrifying community, acetylene (C_2H_2) was used to block N_2O reductase, the enzyme responsible for the N_2O reduction to N_2.[13] In addition to the determination of the SP of N_2O as an

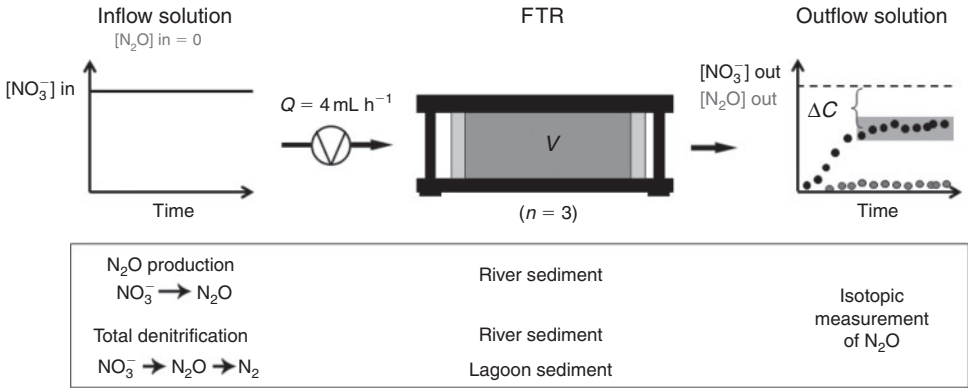

Fig. 1. Overview of the experimental 'flow-through reactor' system; containing sediment of known volume ($V = 13.85\ cm^3$), supplied with an anoxic inflow solution containing nitrate ($[NO_3^-]$ in), at a known flow rate (Q). Nitrate reduction rates are determined from the measured concentration difference between inflow and outflow nitrate (ΔC), once the outflow concentration of NO_3^- has stabilised ('steady state' conditions). Liquid inflow and outflow solutions were sampled during 3 or 6-h intervals for 3 (river sediment) and 4 days (lagoon sediment) to determine N_2O site preference (N_2O-SP) values or NO_3^- concentrations.

end product, the SP of N_2O appearing as an intermediate was measured during different microbial denitrification rates in two types of sediments (river and lagoon). In order to obtain different nitrate reduction rates, diverse nitrate concentrations were supplied to the sediment by the inflow solutions (Fig. 1). During NO_3^- reduction to N_2O (acetylene block) 5 mM nitrate was supplied, while a range of concentrations was applied during benthic denitrification in river sediment (1, 1.5, 3 and 5 mM) and in lagoon sediment (0.83, 1, 3 and 5 mM), in order to achieve different nitrate reduction rates. The denitrification rate depends not only on NO_3^- concentrations, as substrate, but also on organic carbon (C_{org}) content. Determined by the difference between the total carbon and inorganic carbon contents, the C_{org} content was on average 6.3 ± 0.07 % ($n = 5$) in the river sediment and 12.1 ± 0.04 % ($n = 6$) in the lagoon sediment. These amounts of carbon are sufficient to allow denitrification over the time the sediments were incubated in our experiments.[14]

Nitrate concentrations were determined by high-performance liquid chromatography (HPLC, Dionex, AS12 column; Thermo Scientific, Sunnyvale, CA, USA) for samples of benthic reduction of NO_3^- to N_2O and by colourimetric measurements using an autoanalyser for samples of complete denitrification within river sediment (Quaatro, Bran & Luebbe, Plaisir, France) and within lagoon sediment (Gallery; Thermo Fisher Scientific, Cergy-Pontoise, France).

Prior to the determination of benthic denitrification derived N_2O, the N_2O-SP signature of the nitrate reduction to nitrous oxide by azide was tested with a laboratory standard. This method, which allowed the isotopic composition of nitrate and nitrite to be obtained, consists of nitrate reduction to nitrite through a column containing activated cadmium; consequently, nitrite is reduced to nitrous oxide by sodium azide solution (NaN_3, $\delta^{15}N = -2.9 \pm -0.4$, $n = 10$) and the last step (N_2O to N_2) is blocked by the addition of sodium hydroxide.[15]

All liquid samples were analysed using an isotope ratio mass spectrometer (IRMS, DeltaVplus; Thermo Scientific, Bremen, Germany) in continuous-flow with a purge-and-trap system and coupled with a Finnigan GasBench II system (Thermo Scientific), in order to obtain the stable isotope composition of N_2O ($\delta^{15}N$–N_2O, $\delta^{18}O$–N_2O, $\delta^{15}N$–NO and $\delta^{18}O$–NO (N^{α}). The GasBench II allows sampling of the gases and removal of the water contained in the liquid samples, and finally separation of the interfering gases of N_2O using a chromatographic column (CP-poraPLOT U; Thermo Scientific). The N_2O-SP values were calculated based on the raw values of $\delta^{15}N$–bulk and $\delta^{31}NO$ ($\delta^{15}N^{\alpha}$–N_2O), which were measured by the IRMS from the same N_2O reference gas. Nitrate standards were used to calibrate the isotopic composition of N_2O (USGS34, $\delta^{15}N = -1.8$ ‰, $\delta^{18}O = -27.9$ ‰, USGS35, $\delta^{15}N = +2.7$ ‰, $\delta^{18}O = +57.5$ ‰ and USGS32, $\delta^{15}N = +180$ ‰, $\delta^{18}O = +25.7$ ‰). The precision is 0.5 ‰ for $\delta^{15}N$ and 1 ‰ for $\delta^{18}O$. Statistical analyses were conducted using *SigmaStat* with Spearman's correlation; $P < 0.05$ was inferred as statistically significant.

Nitrate reduction to N_2O by azide yielded an average N_2O-SP value of 2.2 ± 0.9 ‰ ($n = 35$). Considering the small variation (±0.9 ‰) of the N_2O-SP values of the laboratory standard, the NO_3^- reduction to N_2O by azide is a safe technique to determine the isotopic composition of nitrate and nitrite.

The $\delta^{15}N$ and $\delta^{18}O$ of N_2O as end product or as intermediate ($\delta^{15}N = -44.3$ to -21.5 ‰; $\delta^{18}O = -10.6$ to 30.2 ‰), obtained for 5 mM of nitrate supply, presented a value range consistent with that found in previous studies on soil denitrification ($\delta^{15}N = -41$ to 20 ‰; $\delta^{18}O = -55$ to 55 ‰).[16,17] However, considering these large ranges, the N_2O-SP value determination may bring a stronger proof that denitrification occurs only in these sedimentary studies.

The results concerning the N_2O-SP values for the different experiments at 5 mM of nitrate supply are shown in Fig. 2. Assuming that the acetylene block was complete and N_2O was not further reduced to N_2, the results during benthic denitrification indicated a low N_2O-SP value (6.3 ± 1.0 ‰; $n = 16$), which was, however, higher than those from pure cultures (−5 to 0 %, Fig. 2).[7,8] The N_2O-SP value derived from *Pseudomonas chlororaphis* and *P. aureofaciens* of 0 ‰[8] and of -5.1 ± 1.8 ‰ for *P. denitrificans*[7] suggest that these values vary among denitrifying species. Our data show that the environmental benthic denitrifying community generates N_2O with a SP value different from those found in pure cultures.[7,8] Most likely the enzyme implicated in the transformation of NO to N_2O (nitric oxide reductase, NOR) constrains the N_2O-SP values[7] due to enzyme differences of the environmental benthic denitrifying community and may result in N_2O-SP values deviating from the pure cultures tested so far. Despite the difference compared to pure cultures, the environmental benthic denitrifying community showed N_2O-SP

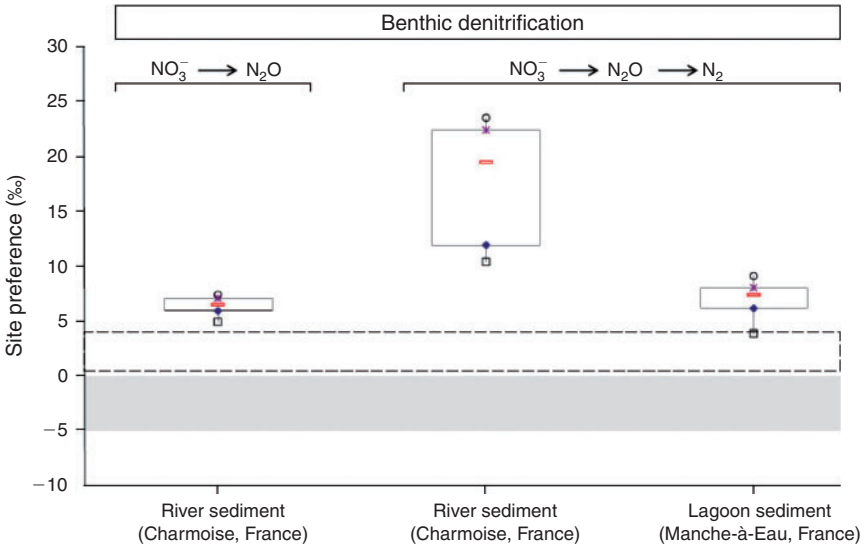

Fig. 2. Boxplots of N_2O site preference (N_2O-SP) values during benthic denitrification to N_2O (C_2H_2 block) and complete benthic denitrification for a 5 mM nitrate supply. The N_2O-SP values founded in the NO_3^- reduction to N_2O by azide (dotted area) and in the pure denitrifying culture studies (grey area).[7,8] Lower limit (i; white squares; i = Q1–1.5(Q3–Q1)), high limit (s; white circles; s = Q3+1.5 (Q3–Q1)), quartile 1 (Q1; blue diamonds; n/4 value), quartile 3 (Q3; purples crosses; 3*(n/4) value) and median (red bars).

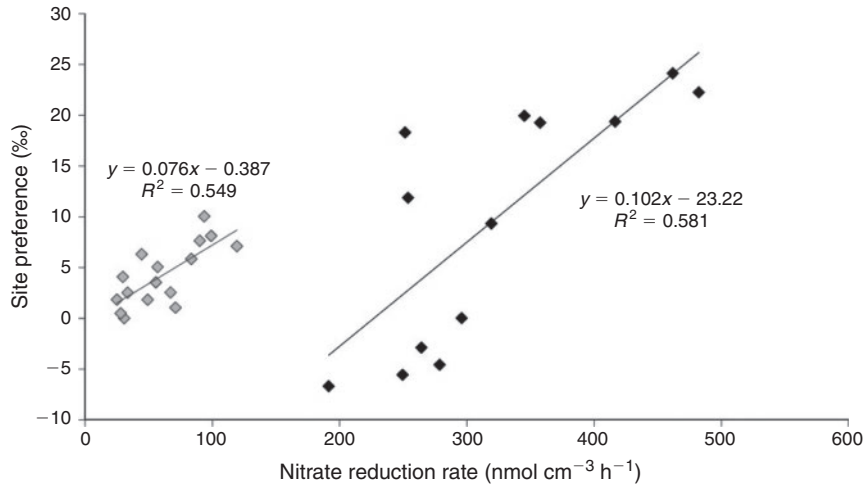

Fig. 3. N_2O site preference (N_2O-SP) values as a function of the NO_3^- reduction rates during denitrification in river (black diamonds, $P = 2.00 \times 10^{-7}$, $r = 0.835$) and lagoon sediments (grey diamonds, $P = 1.66 \times 10^{-3}$, $r = 0.712$).

values in a narrow range, which confirms that the SP value of N_2O as end product is constant during this process (NO_3^- to N_2O).[18]

In the river sediments, N_2O emitted during benthic denitrification showed higher average N_2O-SP values, together with a large variability, (17.4 ± 6.5 ‰, $n = 5$) compared with those of N_2O as an end product (6.3 ± 1.0 ‰, $n = 16$, Fig. 2). During the reduction step of N_2O to N_2, an enrichment of ^{15}N in the central N-position (N^{α}) of the residual N_2O occurs[19] and leads to an increase of the N_2O-SP values.[10,11] This has been explained by the preferential consumption of $^{15}N^{\beta}{}^{14}N^{\alpha}O$ relative to $^{14}N^{\beta}{}^{15}N^{\alpha}O$ because the $^{15}N^{\alpha}$–O bond is stronger than the $^{14}N^{\alpha}$–O bond.[6] Thus, the increase of the N_2O-SP values during the progress of denitrification will lead to a large variability of the N_2O-SP values.

During benthic denitrification, the SP of N_2O produced in the lagoon sediment, exhibiting an average of 6.8 ± 2.8 ‰ ($n = 6$), was lighter and less variable than that emitted from the river sediment (17.4 ± 6.5 ‰, $n = 5$, Fig. 2). This difference in N_2O-SP values from the two sediments during complete denitrification is most likely due to the presence and activity of distinct denitrifying communities, similar to the differences between N_2O-SP values obtained during NO_3^- reduction to N_2O between environmental and pure cultures.

During biological NO_3^- reduction to N_2O, the small variation in N_2O-SP values (varying between 4.2 and 7.8 ‰) corresponded to a wide range of nitrate reduction rates (100 to 300 nmol cm^{-3} h^{-1}). Thus, this result confirms that the step of NO_3^- reduction to N_2O results in a specific low N_2O-SP value independent of the nitrate reduction rate. On the contrary, in the two sediments investigated, the N_2O-SP values exhibited a large variation during complete denitrification (Figs 2, 3).

Fig. 3 shows the N_2O-SP values for the two sediments, obtained for the entire range of nitrate supply, indicating a

significant correlation between nitrate reduction rates and the N_2O-SP values ($P = 0.0086$; $r = 0.48$) during complete denitrification with N_2O appearing as intermediate. In the lagoon sediment, the NO_3^- reduction rates are lower than those found in the river sediment. This statement consolidates the idea of the presence and the activity of distinct species of denitrifying communities in the two sediments. Moreover, the variation of N_2O-SP values related to the variation of the NO_3^- reduction rate, during benthic denitrification in the two sediments, is in good agreement with previous studies that showed that the reduction of N_2O to N_2 leads to an increase of N_2O-SP values.[10,11] In addition, the denitrification potential by the denitrifying bacteria is enhanced with the higher nitrate concentration and leads to a N_2O reduction to N_2. Thus, the last step of denitrification is crucial in the determination of the N_2O-SP values in field studies where production and reduction of N_2O occur simultaneously. On the contrary, without this last step of denitrification the values of N_2O-SP will be invariant and close to those found by Toyoda et al.,[7] Sutka et al.[8] and this study for N_2O production only.

This study showed that the SP of N_2O as an end product of the benthic denitrifying communities is independent of the nitrate reduction rate and constant during the NO_3^- reduction to N_2O step of denitrification. However, the SP value of N_2O tends to increase during complete denitrification, with N_2O as intermediate, and shows a strong positive correlation with the nitrate reduction rate. In fact, a high nitrate concentration enhances the denitrifier activity and increases the N_2O-SP values by ^{15}N enrichment of residual N_2O during the last step in the denitrification process. Consequently, in field studies, during which production and reduction of N_2O occur simultaneously, the SP of N_2O can show a wide range of values, which does not depend on the composition of the denitrifying community only. This study underscores the importance of the last step of denitrification for the determination of the N_2O-SP values and that care should be taken using the SP of N_2O as the origin for denitrification in the case of field studies. In the identification of nitrous oxide sources, the determination of N_2O-SP values should be associated with other methods such as the use of the natural abundances of isotopes and the isotopic enrichment techniques or the identification of the denitrifying species involved.

Acknowledgements

The authors acknowledge A. Boc and O. Gros for hosting us in their laboratory in Guadeloupe, O. Gros and C. Yan for assistance in the field and E. Aubry and E. Huché-Pillet for chemical analysis and isotopic measurements.

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