Video Article

Estimating Sediment Denitrification Rates Using Cores and N_2O Microsensors

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Abstract

Denitrification is the primary biogeochemical process removing reactive nitrogen from the biosphere. The quantitative evaluation of this process has become particularly relevant for assessing the anthropogenic-altered global nitrogen cycle and the emission of greenhouse gases (*i.e.,* N_2O). Several methods are available for measuring denitrification, but none of them are completely satisfactory. Problems with existing methods include their insufficient sensitivity, and the need to modify the substrate levels or alter the physical configuration of the process using disturbed samples.This work describes a method for estimating sediment denitrification rates that combines coring, acetylene inhibition, and microsensor measurements of the accumulated N_2O. The main advantages of this method are a low disturbance of the sediment structure and the collection of a continuous record of N_2O accumulation; these enable estimates of reliable denitrification rates with minimum values up to 0.4-1 µmol N_2O m^{-2} h^{-1}. The ability to manipulate key factors is an additional advantage for obtaining experimental insights. The protocol describes procedures for collecting the cores, calibrating the sensors, performing the acetylene inhibition, measuring the N_2O accumulation, and calculating the denitrification rate. The method is appropriate for estimating denitrification rates in any aquatic system with retrievable sediment cores. If the N_2O concentration is above the detection limit of the sensor, the acetylene inhibition step can be omitted to estimate the N_2O emission instead of denitrification. We show how to estimate both actual and potential denitrification rates by increasing nitrate availability as well as the temperature dependence of the process. We illustrate the procedure using mountain lake sediments and discuss the advantages and weaknesses of the technique compared to other methods. This method can be modified for particular purposes; for instance, it can be combined with ^{15}N tracers to assess nitrification and denitrification or field *in situ* measurements of denitrification rates.

Video Link

The video component of this article can be found at https://www.jove.com/video/58553/

Introduction

Anthropogenic alteration of the nitrogen cycle is one of the most challenging problems for the Earth system[1]. Human activity has at least doubled the levels of reactive nitrogen available to the biosphere[2]. However, there remain large uncertainties regarding how the global N cycle is evaluated. A few flux estimates have been quantified with less than ±20% error, and many have uncertainties of ±50% and larger[3]. These uncertainties indicate the need for accurate estimations of denitrification rates across ecosystems and an understanding of the underlying mechanisms of variation. Denitrification is a microbial activity through which nitrogenous oxides, mainly nitrate and nitrite, are reduced to dinitrogen gasses, N_2O and N_2[4]. The pathway is highly relevant to the biosphere availability of reactive nitrogen because it is the primary process of removal[5]. N_2O is a greenhouse gas with a warming potential nearly 300 times that of CO_2 over 100 years, and it is the current major cause of stratospheric ozone depletion due to the large quantities being emitted[6,7].

In the following, we present a protocol for estimating sediment denitrification rates using cores and N_2O microsensors experimentally (**Figure 1**). Denitrification rates are estimated using the acetylene inhibition method[8,9] and measurements of the accumulation of N_2O during a defined period (**Figure 2** and **Figure 3**). We demonstrate the method by applying it to mountain lake sediments. This case study highlights the performance of the method for detecting relatively low rates with minimal disturbance to the physical structure of the sediments.

Denitrification is particularly difficult to measure[10]. There are several alternative approaches and methods, each with advantages and disadvantages. Drawbacks to available methods include their use of expensive resources, insufficient sensitivity, and the need to modify the substrate levels or alter the physical configuration of the process using disturbed samples[10]. An even more fundamental challenge to measuring N_2 is its elevated background levels in the environment[10]. The reduction of N_2O to N_2 is inhibited by acetylene (C_2H_2)[8,9]. Thus, denitrification can be quantified by measuring the accumulated N_2O in the presence of C_2H_2, which is feasible due to low environmental N_2O levels.

The use of C_2H_2 to measure denitrification rates in sediments was developed about 40 years ago[11], and the incorporation of N_2O sensors occurred about 10 years later[12]. The most widely applied acetylene-based approach is the "static core". The accumulated N_2O is measured

during an incubation period of up to 24 h after the C_2H_2 is added to the headspace of the sealed sediment core[10]. The method described here follows this procedure with some innovations. We add the C_2H_2 by bubbling the gas in the water phase of the core for some minutes, and we fill all the headspace with sample water before measuring the accumulation of N_2O with a microsensor. We also include a stirring system that prevents the stratification of the water without resuspending the sediment. The procedure quantifies the denitrification rate per sediment surface area (*e.g.*, μmol N_2O m^{-2} h^{-1}).

The high spatial and temporal variation of denitrification presents another difficulty in its accurate quantification[10]. Usually, N_2O accumulation is measured sequentially by gas chromatography of headspace samples that are collected during the incubation. The method described provides improved monitoring of the temporal variation of the N_2O accumulation, because the microsensor provides a continuous signal. The microsensor multimeter is a digital microsensor amplifier (picoammeter) that interfaces with the sensor(s) and the computer (**Figure 1a**). The multimeter allows several N_2O microsensors to be used at the same time. For instance, up to four sediment cores from the same study site can be measured simultaneously to account for the spatial variability.

The core approach barely disturbs the sediment structure compared to some other methods (*e.g.*, slurries). If the integrity of the sediments is altered, this leads to unrealistic denitrification rates[13] that are only adequate for relative comparisons. Higher rates are always obtained with slurry methods compared to core methods[14], because the latter preserves the limitation of denitrification by substrate diffusion[15]. Slurry measures cannot be considered representative of *in situ* rates[16]; they provide relative measures for comparisons made with the exact same procedure.

The method described is appropriate for estimating denitrification rates in any sediment type that can be cored. We particularly recommend the method for performing experimental manipulations of some of the driving factors. Examples are experiments that modify nitrate availability and temperature as needed for estimating the energy activation (E_a) of denitrification[17] (**Figure 2**).

Figure 1: Experimental setup. (**a**) General experimental setup to estimate sediment denitrification rates using cores and N_2O microsensors. The incubation chamber ensures darkness and controlled-temperature (±0.3 °C) conditions. Five intact sediment cores can be processed simultaneously using their respective N_2O sensors. (**b**) N_2O sensor calibration chamber. We adapted it with rubber stoppers and syringes to mix the N_2O water (see protocol step 3.4.3). There is a thermometer to control the water temperature. (**c**) Close-up of a sediment core sample with the sensor inserted into the central hole of the PVC cover and the joints sealed with adhesive tape. The stirrer is hanging in the water, and the electromagnet is close to it and fixed to the external part of the acrylic tube. (**d**) Close-up of the N_2O microsensor tip protected by a metal piece. (**e**) A sediment core that has just been recovered. It was sampled from a boat in a deep lake; the acrylic tube with the core is still fixed to the messenger-adapted gravity corer[19]. See the **Table of Materials** for all the items needed to perform this method. Please click here to view a larger version of this figure.

Protocol

1. Preparation

NOTE: Begin this on the day before the measurements are taken.

1. Assemble the measurement setup (**Figure 1a**, see the **Table of Materials**).
   NOTE: To ensure a constant and high-quality power supply, the measurement device is connected to the grip *via* an uninterruptible power supply (UPS) that can also act as a backup. In the case of a long-duration power failure, a car battery serve as an extra power source.
2. Start the sensor's software and apply a -0.8 V voltage to **polarize the N_2O microsensors**. The signal shows a rapid descent and a subsequent rise, then it finally decreases until it is low and stable.
   NOTE: The microsensor manufacturer recommends polarization at least overnight (or longer) to ensure the stability of the sensor's signal. Another recommendation is to keep the sensor polarized if measurements are planned for multiple or consecutive days[18].
3. Switch on the incubation chamber and **adjust the experimental conditions** (*e.g.*, selected light off and temperature set to be similar to that expected in the field). Place a container with deionized water inside the chamber so that water is available later at the measurement temperature for calibration of the sensors.
   NOTE: This step can be done the same day of the planned measurements, before the departure to collect the cores. For standard measurements, it is advisable to use dark conditions.
4. Pack the field core collection materials: corer device, sampling tubes, rubber stoppers, polyvinyl chloride (PVC) taps, screwdriver, global positioning system (GPS) unit, thermometer, handheld sounder, wader, and inflatable boat (see the **Table of Materials**). Use a checklist to ensure that all materials are included.

2. Sediment Core Collection

1. Depending on the water depth, follow 2.1.1 or 2.1.2.
   1. **For deep water bodies**
      1. Use a **messenger-adapted gravity corer**[19] from a boat or a platform (**Figure 1e**).
      2. Fix the sampling tube (acrylic, ø 6.35 cm, length ≥ 50 cm) to the corer with a screwdriver.
      3. Select the sampling point according to the investigation aims. Take note of the position (*e.g.*, using GPS coordinates) and measurement depth (*e.g.*, using a handheld sounder). If sampling from a boat, use an anchor (*e.g.*, a bag with stones) to avoid drifting during core collection.
      4. Deploy the coring system until the sampling tube is ~1 m from the sediment. Use a rope with regular marks (*e.g.*, intervals of 1 m) to control the depth position of the sampling equipment.
      5. Stabilize the sampling equipment for 60 s (*e.g.*, to minimize the movement of the boat). This will ensure the correct sediment penetration and recovery of a scarcely disturbed sediment core.
      6. Release ~1 m more rope so that the sampling tube penetrates the sediment. Be aware that if the sampling tube penetrates too much, it can disturb the water/sediment interface.
      7. Release the messenger while trying to keep tension in the rope so that the corer remains fixed and in a vertical position. When the messenger impacts the corer, a small difference can be felt in the tension of the rope. At that time, close the corer to generate the vacuum that allows for recovery of the sediment core.
      8. Recover the corer by pulling the rope constantly and gently.
      9. Once the core is close to the surface but still entirely submerged (including the rubber part of the corer that ensures the vacuum), place a rubber stopper at the bottom of the sampling tube. Inspect the water/sediment interface; it should be clear and not visibly disturbed (**Figure 1e**). If this is not the case, discard the core, clean the tube, and repeat steps 2.1.1.4-9.
      10. Uplift the entire coring system from the water. Release the sampling tube from the corer and place a PVC cover on the top. Seal it with adhesive tape. Avoid the formation of air space.

   2. **For littoral habitats and shallow water bodies**
      1. Dress in a **wader** for sampling in very shallow waters (<0.6 m).
      2. Use **snorkeling** or **scuba gear** for deeper sampling (up to 3 m).
      3. Select the sampling point according to the investigation aims. Take note of the position (*e.g.*, GPS coordinates). Manually, insert the sampling tube (*e.g.*, acrylic, ø 6.35 cm) into the sediment.
      4. Place a rubber stopper in the top side of the sampling tube to obtain a vacuum.
      5. Remove the core from the sediment and quickly introduce another rubber stopper at the tube bottom.
         NOTE: It is necessary to work with the tube underwater at all times; at very shallow sites, we recommend shortening the tube down to 20 cm. Sometimes the sediment has a high water content and drains when the tube is removed from the sediment bed. In this case, it is necessary to introduce the bottom stopper without uplifting the core outside the sediment. To do this, manually immerse the stopper in the sediment around the tube and place it carefully to close the bottom of the tube.
      6. Out of the water, substitute the topside rubber stopper with a PVC cover and seal the junction with adhesive tape.

2. Protect the core during its transfer to the laboratory by minimizing rotations and shaking.

3. Calibration of the Nitrous Oxide (N_2O) Microsensors

1. Using the computer (strip chart, sensor software), check that the sensor's signal is stable and low (<20 mV).
2. Create a new file (*e.g.*, with the date and the sampling site (130903_Redon_Lake)) to record the calibration values and sensor signals.

NOTE: The sensor signals are sensitive to temperature (**Figure 4**). Use the **same temperature** for the measurements and the sensor calibration. The sensor responds linearly between 0%-2.5% N_2O[20]. Therefore, a two-point calibration is sufficient[18].

3. For the calibration **value with zero nitrous oxide**, read the sensor signal keeping the sensor tip submersed in N_2O-free water (deionized).
4. Calibrate with N_2O water at the desired concentration.
   NOTE: Prepare water with a defined N_2O concentration, which will slightly exceed the maximum concentration expected during incubation. We use ~25 µM N_2O as the calibration value. Be aware of not exceeding the maximum sensor range concentration of 500 N_2O µM.
   1. Obtain N_2O-saturated water by bubbling N_2O in deionized water for a few minutes.
      NOTE: The N_2O water solubility depends on temperature and salinity[21]; see the table in the appendix of the sensor manual[18].
   2. Dilute the N_2O saturated water by adding a certain volume of saturated N_2O water to a volume of deionized water. For example, at 20 °C, add 0.3 mL of saturated N_2O water, which has a concentration of 28.7 mM N_2O, to a total of 375 mL of water to obtain a 22.9 µM N_2O concentration. Note that 375 mL is the total volume of the calibration chamber (**Figure 1b**).
   3. After gently mixing the N_2O saturated water with deionized water in the calibration vessel to dilute it to the desired concentration, read the sensor signal when it is constant. This reading is the **calibration value with X µM N_2O water**. When mixing the solution, be careful not to generate bubbles, as this would eliminate N_2O from the calibration solution.
      NOTE: Be aware that the N_2O in the water will slowly escape into the air; thus, the prepared calibration solution can only be used for a few minutes.

4. Core Preparation and Acetylene Inhibition

1. Change the PVC cover located at the top of each sediment core by another cover with a hole in the center and a hanging magnetic stirrer. Re-seal the junction with adhesive tape.
2. Reduce the water phase of each sample to an approximate height of 12 cm (volume ≈ 380 mL). For this, first insert a silicone tube in the central hole.Then, put the sediment core in a cylinder and push the bottom stopper to create pressure. The stopper and sediment sample go up, and the excess water passes through the tube. Collect the water in a recipient vessel.
   NOTE: Samples with coarse granularity can be problematic during this step. Sediment particles placed between the stopper and the tube can deform the stopper and open a hole through which air bubbles can pass and disturb the sample. To avoid this problem, put the cylinder in the center of the bottom stopper and try to push with a constant force. The joint between the silicone tube used to evacuate the excess water and the PVC cover consists of a solid part (*e.g.*, a 5 mL pipette tip without its narrowest end) inserted in the silicone tube.
3. Perform the acetylene inhibition by **bubbling with acetylene gas** in the water phase of the core for approximately 10 min . Avoid resuspending the sediment.
   NOTE: As a possible modification of the method, add a substrate (nitrate) through a concentrated liquid medium before bubbling acetylene for potential denitrification measurements (*e.g.*, as in **Figure 3b, c**).

5. Denitrification (N_2O accumulation measure)

1. Fill all the air space in the sample with the previous leftover water. Place the sensor in the sediment core through the central hole of the topside PVC cover. The tip of the sensor should be located in the water phase above the stirrer (**Figure 1c**).
   NOTE: All the joints of the acrylic sampling tube must be sealed to avoid gas and water leaks during the measurement (**Figure 1a, c**). In the bottom part of the tube, the rubber stopper is sufficient for this. Sealing the topside part is more difficult. The PVC cover must be tuned. It must be heated with a torch; then, when the material becomes flexible but is not scorched, the cover is placed in the tube so that its shape can be molded. After cooling, the cover needs more modifications (with the exception of the cover used to transport the samples to the laboratory in steps 2.1.1.10 or 2.1.2.6). The central hole where the sensor is inserted must be drilled. The stirrer can be held with a fishing line, which in turn is adhered with glue to the inside of the cover so that the stirrer hangs on the fishing line in the water (**Figure 1c**). Also, all the joints (PVC cover tube and PVC cover sensor) are sealed with adhesive tape. Place elastic adhesive tape to adjust the diameter of the sensor in order to seal the contact surface between the central hole of the PVC cover and the sensor (**Figure 1c**).
2. Switch on the electromagnetic pulse circuit that is part of the stirring system.
   NOTE: The stirring system prevents the stratification of the water phase without disturbing (resuspending) the sediment. The stirring system consists of a circuit that switches on/off the electromagnet that attracts/releases the magnetic stirrer (see the **Table of Materials** for a detailed description).
3. Move the electromagnet around the external part of the acrylic tube until the stirrer moves continuously, and then fix it in place using adhesive tape (**Figure 1c**).
4. Close the incubation chamber to ensure a constant temperature (*e.g.*, variation of ±0.3 °C).
5. Press the record button (sensor software) to **start recording the sensor signal**. Readings are typically recorded every 5 min.
6. Press the stop button at the **end of the measurement period**.

6. Final Measurement Steps

1. Wait at least ~10 min with the sensor's tip submerged in free-N_2O water (deionized) before reading the signal of the zero N_2O calibration measure.
2. Perform a **final sensor calibration**. For this, repeat the sensor calibration, following Section 3 but starting with step 3.3.
3. Save the file (sensor software).

7. Denitrification Rate Calculations

1. Start with the tabulated output file generated by the sensor software that contains the record of the sensor's signal in mV and µM N_2O, and the calibration data.

2. Plot the sensor signal against time to visualize the N_2O accumulation trend (*e.g.*, **Figure 2a**).
3. Use only the time range with a **linear accumulation**, excluding the initial acclimation period of the sample and a possible final saturation due to substrate limitation (*e.g.*, **Figure 2b**). Create a linear model of the sensor signal (µM) over time (h) .
   Note: The slope is the denitrification rate (µM N_2O $core^{-1}$ h^{-1}), which, if divided by the area of the core (πr^2), transforms into the rate in µM N_2O m^{-2} h^{-1}, and when multiplied by the water volume ($\pi r^2 h$, where h is the height of the water phase and r is the inner radius of the acrylic tube, in this case 0.12 m and 0.03175 m, respectively) transforms into the rate in µmol N_2O m^{-2} h^{-1}.

Representative Results

A total of 468 denitrification rates were estimated using the protocol above in sediments from Pyrenean mountain lakes over the period 2013-2014. We show some of these results to illustrate the procedure (**Figure 2** and **Figure 3**). In general, the linear model between the N_2O concentration and time has good correlation ($R^2 \geq 0.9$). The slope of the relationship provides an estimate of the denitrification rate (step 7.3; *e.g.*, **Figure 2d**). If the denitrification activity is very low, the sensor's electronic noise becomes more important and the goodness of fit declines (*e.g.*, sensors 4 and 5 in **Figure 2b** and **Figure 3a**). Although the baseline detection limit of N_2O is ~0.1 µM in water[22], which is an intermediate value concerning alternative methods[23], the possibility of accumulating thousands of continuous measurements to filter the noise permits estimates at relatively low denitrification rates, up to ~1 µmol N_2O m^{-2} h^{-1} (**Figure 2** and **Figure 3**). Lower rates (*i.e.,* ~0.4 µmol N_2O m^{-2} h^{-1}) can be estimated by narrowing the water phase of the core sample to a height of 8 cm (see protocol step 4.2).

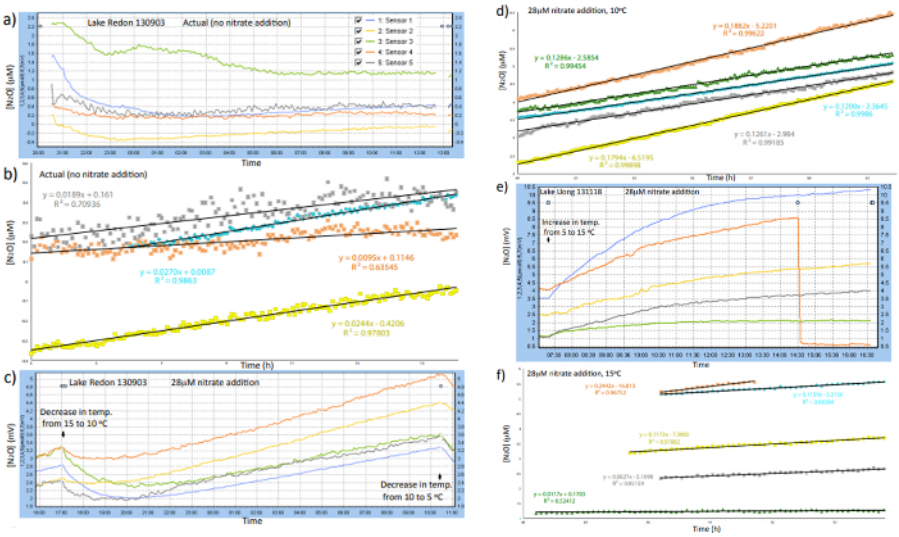

Figure 2: Denitrification rate calculations in a temperature dependence experiment. Actual (**a** and **b**) and potential denitrification measurements (**c-f**) are shown. When the temperature of the measurement is decreased (**c**), at first the sample cools and the sensor signal, which is temperature dependent, declines. (**a**) A similar event occurs at the start of the incubation in the actual denitrification measurement; the warmer laboratory environment with respect to the incubation conditions produces a cooling of the sample, again accompanied by a decline in the sensor signal. (**e**) When the temperature is increased, at first the samples warm and the sensor signal increases exponentially instead of linearly. When the samples reach a constant temperature, the sensor signal increases linearly as usual. In all cases, it is possible to calculate the denitrification rates just by using the period of linear N_2O accumulation (**b**, **d**, and f). (**b**) Inactive sample 3 is not shown. Please click here to view a larger version of this figure.

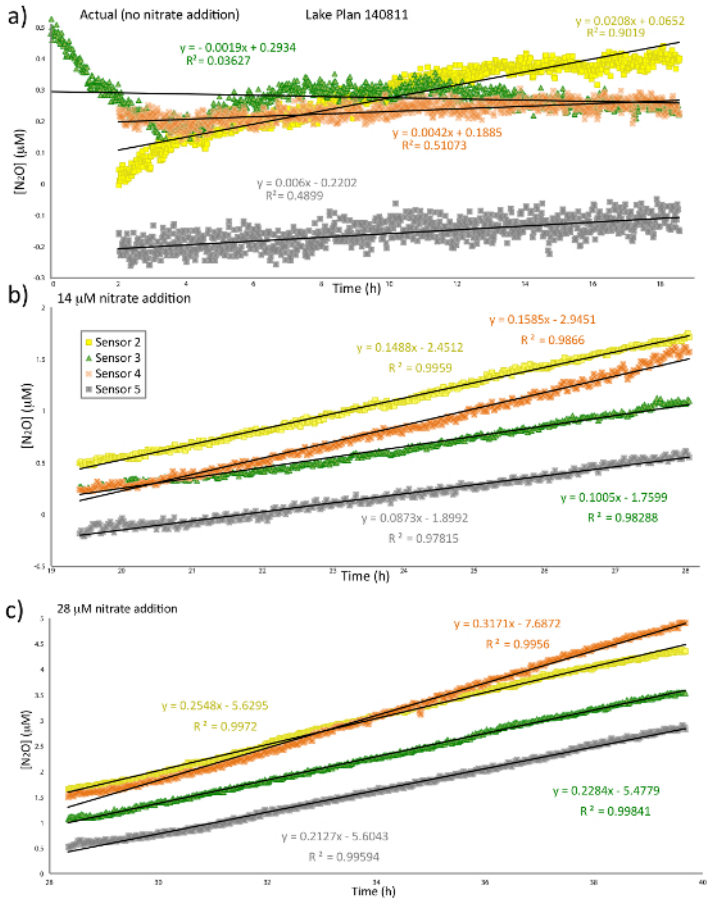

Figure 3: Examples of denitrification rate calculations. Actual (**a**) and potential (**b** and **c**) denitrification rates were estimated. We only used the time range with a linear N_2O accumulation to calculate the denitrification rate (slope of the linear model). However, in (**a**), for educational purposes, we show all the measurements (models) with more and less success; we would discard sample 3 due to the high instability of the sensor and sample 2 due to saturation in the N_2O accumulation. (**a**) Samples 4 and 5 with rates of 0.5 and 0.7 µmol N_2O m^{-2} h^{-1}, respectively, are cases of measurements near the detection limit of the method. Please click here to view a larger version of this figure.

Discussion

The main advantages of the described method are the use of minimally disturbed sediment core samples and the continuous recording of the N_2O accumulation. These allow estimation of relatively low denitrification rates that are likely similar to those occurring *in situ*. Nonetheless, some aspects concerning the coring, sensor performance, and potential improvements are discussed.

An apparently simple but critical step of the method is good core recovery. The sediment/water interface must satisfy three criteria: (i) no modification in its chemical or constituent composition, (ii) no alteration in the water content or void ratio, and (iii) no structure pertubation[24]. The fewer disturbances suffered by the sample during the entire protocol, the more realistic and closer to *in situ* conditions will the measured denitrification rate be. There are several devices/techniques for the sediment core collection[25], and their selection depends on the water depth. We use a messenger-adapted gravity corer[19] for deep samples (**Figure 1e**) because it is a reasonably light-weight device and can rapidly recover short cores[25] (a core sediment of ≥10 cm length is more than enough to encompass the oxic and denitrifying layers in the sediments[26,27,28]). In coring jargon, "feel" is often referred to as the ability to know the location of the corer (whether it is still in the water column or already in the sediment) and whether it is open or closed[25]. For intermediate water depths (5-50 m), usually there are no difficulties with feeling. A loss of feeling occurs in deeper water (>50 m) because the movements of the water column may mask the location of the corer[25]. Feeling may also be lost in shallow water (<3 m) due to lateral drift and wave action[25]; this is why we use a different method in shallow water, either direct manual coring by scuba diving or dressing in a wader. With this system, the person performing the sampling can see the sediment and choose the exact place before coring; this allows, *e.g.*, the sampling of a sediment core that contains a macrophyte. After sampling, the researcher must continue to work carefully to minimally disturb the sediment core sample during the rest of the protocol, especially when performing acetylene inhibition by bubbling.

Some details must be considered when using N_2O microsensors. The sensor software provides a continuous visualization (strip chart) of the sensor signal (background frequency of 1000 Hz)[29]. These raw data and the strip chart (*e.g.*, **Figure 2a**) can be saved. It is necessary to check the correct behavior of the sensor after its polarization (*e.g.*, when returning from field collection before step 4). In particular, a low (<20 mV) and constant base signal is expected when it is submerged in N_2O-free water. Recalibrate the sensor shortly (~2 h) after starting its use; if it has already been used for some days, the interval can be extended (~24 h)[18]. To minimize recalibrations, keep the sensor polarized unless it is not used for several days[18]. Over time, a change in the sensor signal may occur, up to 50% in months, which is due to a different permeability of its membrane[18]. The lower the electronic interference in the laboratory, the more constant and stable will be the sensor signal. In that sense,

using a UPS improves the quality of the electrical energy that reaches the measurement device by filtering the voltage fluctuations. The sampling interval, selected in the Logger tab, is different from the background frequency. Each registered point is generated from the average of many measurements. The sampling interval (up to 10 s) indicates the frequency with which a data point is recorded. The number of measurements per unit of time used in the average is defined by the background frequency[29]. For instance, if we set a sampling frequency of 5 s and a background frequency of 500 measurements per second, then the data points are recorded every 5 s and the average of the 500 samples per second is measured during the previous 5 s. We record the sensor signal every 5 min (sampling interval) and set the background frequency to 1000 measurements per second. The study system must be known to select the correct sampling interval without "averaging" expected fluctuations. In highly active systems, short sampling intervals are recommended, while longer intervals allow optimizing the computer's memory[29]. Some possible interfering substances (H_2S, NO, and CO_2) can affect the N_2O sensor's signal[22]. The sensor is calibrated with deionized water, but the samples can contain interfering substances and modify the sensor's reference signal. This situation could explain why negative values appear in samples 2 and 5 in **Figure 2b** and **Figure 3a**, respectively. However, when the objective is to estimate the denitrification rate, the exact level of N_2O is not the key parameter. What is key is the slope of the linear model (evidencing a linear accumulation of N_2O). Finally, it is necessary to work with a fixed temperature because the response of the N_2O sensor changes with temperature (**Figure 4**).

Simple modifications or additions to the protocol also enable (i) characterization of the environmental conditions controlling the measured denitrification rates, (ii) estimation of the potential denitrification rates by simulating the response to a driving gradient (*e.g.*, nitrate), and (iii) estimation of the sediment N_2O emission rates by skipping the C_2H_2 inhibition Depending on the study aims, several complementary measurements can be made: (i) just after recovering the core, *in situ* conditions, *e.g.*, temperature; (ii) before the measurement, samples of the water phase, *e.g.*, [NO_3^-]; and (iii) after the measurement, extrusions and slices of the core at different resolutions (mm-cm)[25,30], following the procedures explained by P. T. Schwing *et al.*[30].

To measure the potential denitrification rates, add nitrate to the water-phase of the core (*e.g.*, **Figure 2** and **Figure 3**) as described in C. Palacin-Lizarbe, L. Camarero and J. Catalan[17]. If doing so, add the nitrate before the C_2H_2 inhibition (step 4.3). Also, if nitrate is added, it is advisable to also add carbon (C; *e.g.*, acetate) and phosphorus (P) to maintain the *in situ* stoichiometric proportions of C, N, and P (*e.g.*, in the surface sediment). This will prevent the limitation of denitrification by these elements[31,32], and will also keep the C/N ratio that can influence the dominance of the nitrate consumption process (*i.e.,* denitrification *versus* dissimilatory nitrate reduction to ammonium (DNRA))[4]. Anoxia can be fixed by bubbling an N_2-CO_2 mixture for a few minutes after the nitrate addition, to prevent oxygen interference with denitrification; however, note that this leads to a blockage of nitrification. To calculate sediment N_2O emission rates, omit the C_2H_2 inhibition (step 4.3). However, keep in mind that, as far as it is currently known in aquatic ecosystems, N_2O emissions are proportionally low compared to N_2 emissions (0%-4.3%)[33], so it is possible that the accumulated N_2O will be below the detection limit. If this is the case, an option is to add nitrate to increase the emitted N_2O, calculating potential N_2O emissions.

The main weakness of the method is the inhibition of nitrification by C_2H_2[10,34]. During the incubation, this inhibition of nitrification and the incomplete inhibition of N_2O reduction may become apparent, as both are very time dependent. For instance, the starting N_2O accumulation rate must reveal the real denitrification rate and progressively decay as the nitrate availability drops and N_2O diffuses into the nitrate free zone, where it is reduced[35]. Therefore, an estimated denitrification rate can be considered valid only if the readings show a linear accumulation of N_2O[10].

The method described estimates a denitrification rate per area that integrates the entire sediment activity. In this respect, there is some uncertainty about the radius of action of the acetylene inhibition when bubbling the gas in the aqueous phase of the sample. It is assumed that, at least, inhibition of the surficial layer of the sediment occurs, which is the one with the highest denitrification rates[26,27].

Possible improvements to this method are its combined use with ^{15}N tracers and modifications that could allow the measurement of denitrification *in situ*. ^{15}N tracer methods can be used to determine the proportion of nitrification-denitrification coupling occurring in the samples[36], and it can also account for other N flux processes besides denitrification (*e.g.*, anammox and dissimilatory nitrate reduction to ammonium (DNRA))[13,37]. However, these methods have the drawback of changing the substrate concentration[10]. A. Behrendt, D. de Beer and P. Stief [26] use a method combining N_2O microsensors, C_2H_2 inhibition, and ^{15}N tracers to analyze the vertical activity distribution of dissimilatory nitrate reduction processes (denitrification and DNRA) in sediments. They made vertical profiles in the sediment by penetrating the sediment with the sensors. The main difficulty in measuring denitrification *in situ* is the ability to handle a nonconstant temperature environment. It is necessary to record the N_2O accumulation and temperature simultaneously and then correct the N_2O sensor's signal by the temperature dependence during the denitrification rate calculations. This correction requires a previous analysis of the temperature dependence of the N_2O signal for each sensor. The sensors are handmade, and each one responds differently to temperature (*e.g.*, sensor 1 shows a higher temperature dependence than the others in **Figure 2c, e**).

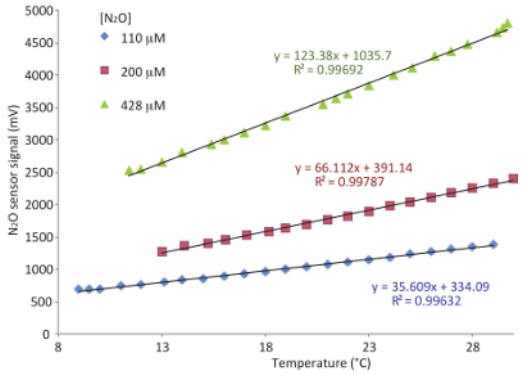

Figure 4: Temperature dependence of the N_2O microsensor response. The different slopes of the linear model of the sensor signal *versus* the temperature at each N_2O concentration shows the temperature effect on the sensor's signal. Please click here to view a larger version of this figure.

Disclosures

The authors have nothing to disclose.

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