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An experimental protocol to estimate sediment denitrification rates using cores and N2O microsensors

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Dear Editor,

Please find enclosed our manuscript entitled "An experimental protocol to estimate sediment denitrification rates using cores and N_2O microsensors" that we would like to be considered for publication in JoVE. This paper highlights a protocol for estimating sediment denitrification rates using sediment cores, the acetylene inhibition technique and measuring the accumulated N_2O with microsensors. We consider of value publishing these data in JoVE, as we explains how to collect the cores, calibrate the sensors, perform the acetylene inhibition, measure the N_2O accumulation, and calculate the denitrification rate. The techniques presented in this paper will be highly useful for researchers working in the field of global change, biogeochemistry, limnology, water chemistry and nutrient cycling.

CPL, LC and JC designed the procedures described in the manuscript. CPL performed the experiments and analyzed the data. Finally, CPL and JC wrote the manuscript.

During the preparation and submission of this manuscript, we have been kindly assisted by Lyndsay Troyer.

Thank you for your consideration of this manuscript. We look forward to hearing from you.

Sincerely yours,

Carlos Palacin-Lizarbe
Lluís Camarero
Jordi Catalan

TITLE:

Estimating Sediment Denitrification Rates Using Cores and N_2O Microsensors

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Biogeochemistry, limnology, marine chemistry, water chemistry, nitrogen, nitrous oxide, voltammetry, acetylene inhibition, temperature

SHORT ABSTRACT:

This method estimates sediment denitrification rates in sediment cores using the acetylene inhibition technique and microsensor measurements of the accumulated N_2O. 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.

LONG 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.

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 (**Figures 2** and **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**).

[Place **Figure 1** here]

PROTOCOL:

1. Preparation

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

1.1. 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.

1.2. 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].

1.3. 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.

1.3. 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

2.1. Depending on the water depth, follow 2.1.1 or 2.1.2.

2.1.1. For deep water bodies

2.1.1.1. Use a **messenger-adapted gravity corer**[19] from a boat or a platform (**Figure 1e**).

2.1.1.2. Fix the sampling tube (acrylic, ø 6.35 cm, length ≥ 50 cm) to the corer with a screwdriver.

2.1.1.3. 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.

2.1.1.4. 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.

2.1.1.5. 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.

2.1.1.6. 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.

2.1.1.7. 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.

2.1.1.8. Recover the corer by pulling the rope constantly and gently.

2.1.1.9. 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.

2.1.1.10. 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.

2.1.2. For littoral habitats and shallow water bodies

2.1.2.1. Dress in a **wader** for sampling in very shallow waters (<0.6 m).

2.1.2.2. Use **snorkeling** or **scuba gear** for deeper sampling (up to 3 m).

2.1.2.3. 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.

2.1.2.4. Place a rubber stopper in the top side of the sampling tube to obtain a vacuum.

2.1.2.5. 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.

2.1.2.6. Out of the water, substitute the topside rubber stopper with a PVC cover and seal the junction with adhesive tape.

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

3. Calibration of the Nitrous Oxide (N_2O) Microsensors

3.1. Using the computer (strip chart, sensor software), check that the sensor's signal is stable and low (<20 mV).

3.2. 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].

3.3. For the calibration **value with zero nitrous oxide**, read the sensor signal keeping the sensor tip submersed in N_2O-free water (deionized).

3.4. 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.

3.4.1. 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].

3.4.2. 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**).

3.4.3. 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

4.1. 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.

4.2. 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.

4.3. 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)

5.1. 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**). Fill all the air space with the previous leftover water before sealing the junction sensor PVC cover.

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**).

5.2. 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).

5.3. 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**).

5.4. Close the incubation chamber to ensure a constant temperature (*e.g.*, variation of ±0.3 °C).

5.5. Press the record button (sensor software) to **start recording the sensor signal**. Readings are typically recorded every 5 min.

5.6. Press the stop button at the **end of the measurement period**.

6. Final Measurement Steps

6.1. 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.

6.2. Perform a **final sensor calibration**. For this, repeat the sensor calibration, following Section 3 but starting with step 3.3.

6.3. Save the file (sensor software).

7. Denitrification Rate Calculations

7.1. 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.

7.2. Plot the sensor signal against time to visualize the N_2O accumulation trend (*e.g.*, **Figure 2a**).

7.3. 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 (**Figures 2** and **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 **Figures 2b** and **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} (**Figures 2** and **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).

[Place **Figure 2** and **3** here]

FIGURE AND TABLE LEGENDS:

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.

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.

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.

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.

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[26-28]). 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 **Figures 2b** and **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.*, **Figures 2** and **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**).

[Place **Figure 4** here]

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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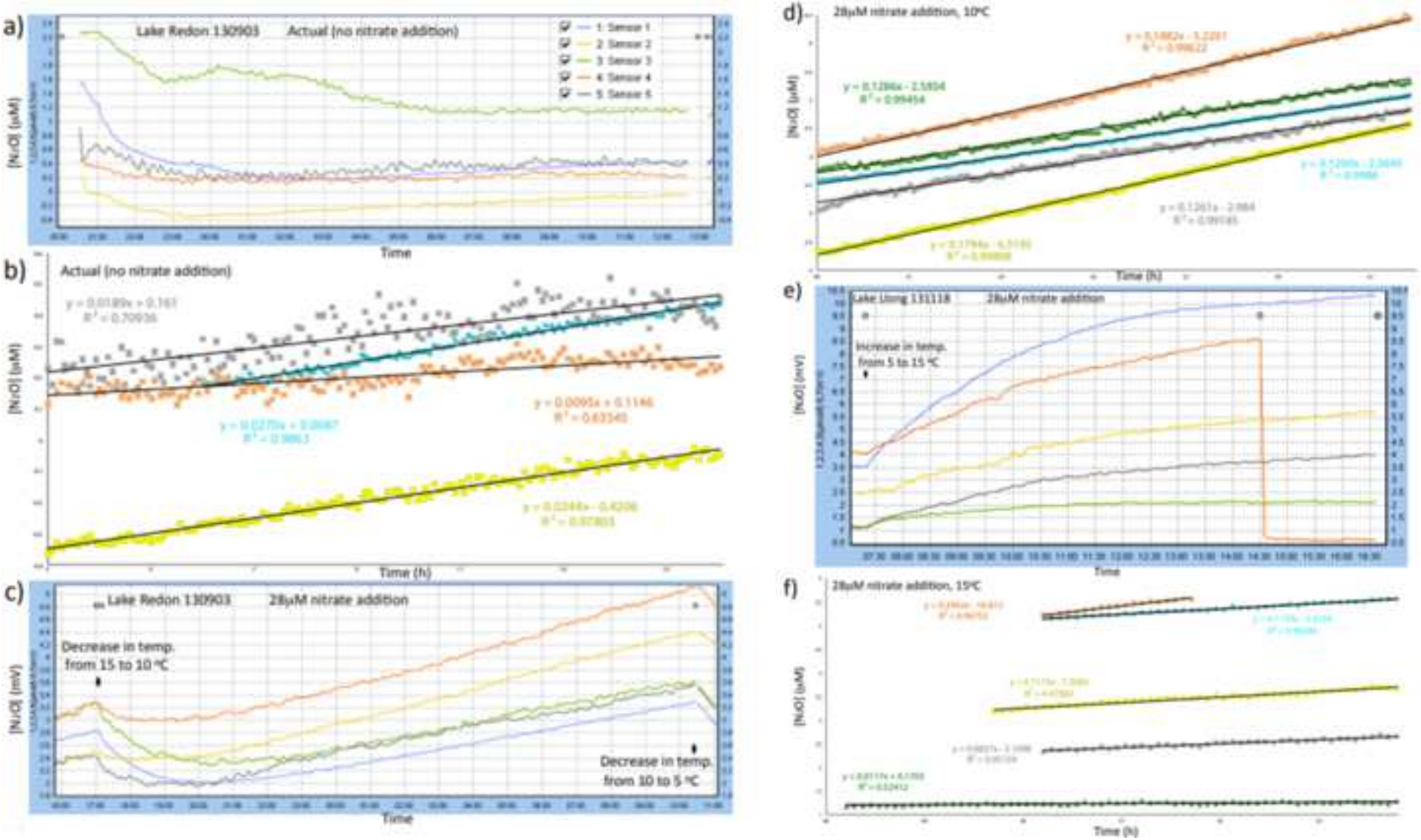


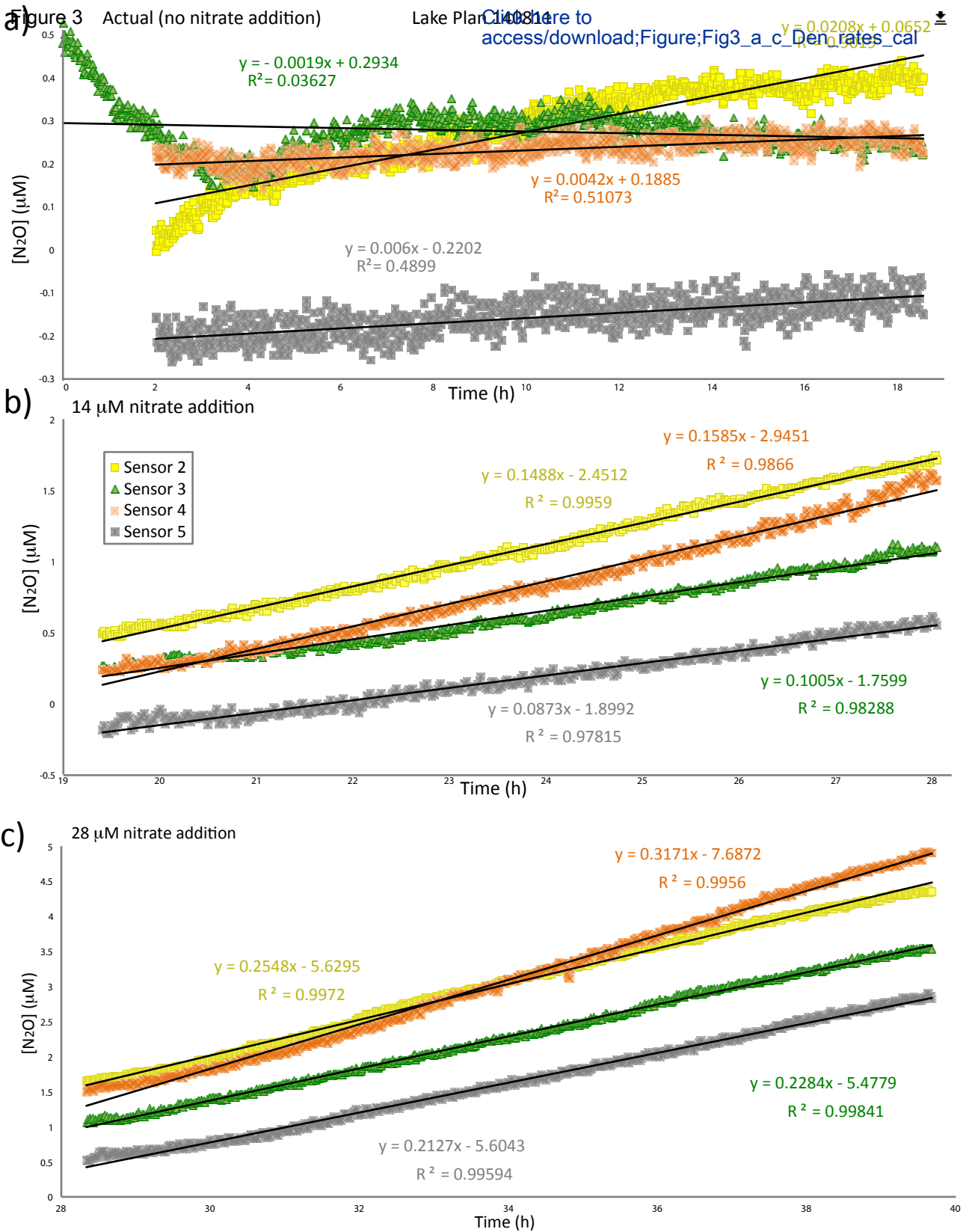
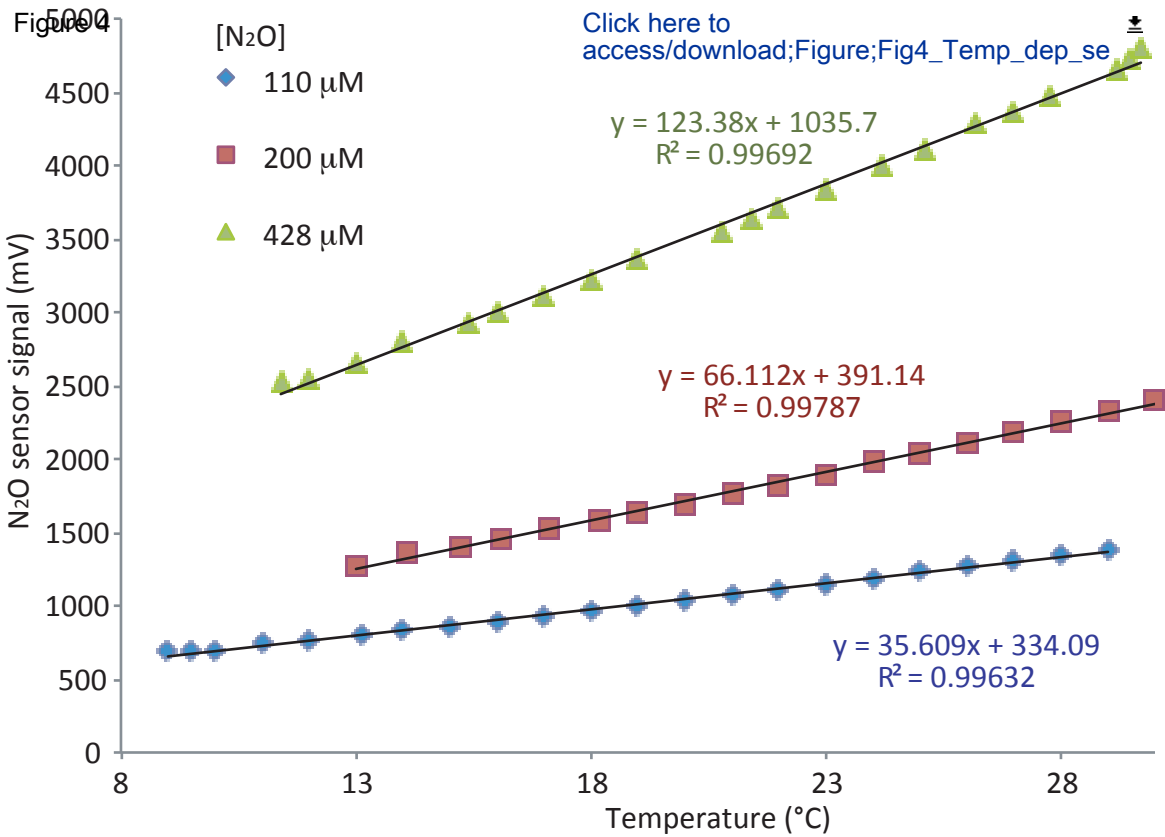


Figure 3 Actual (no nitrate addition) Lake Plan 140814
Click here to access/download;Figure;Fig3_a_c_Den_rates_cal
a)
y = 0.0208x + 0.0652
y = - 0.0019x + 0.2934
R²= 0.03627
y = 0.0042x + 0.1885
R²= 0.51073
y = 0.006x - 0.2202
R²= 0.4899
[N2O] (µM)
Time (h)
b)
14 µM nitrate addition
Sensor 2
Sensor 3
Sensor 4
Sensor 5
y = 0.1585x - 2.9451
R ² = 0.9866
y = 0.1488x - 2.4512
R ² = 0.9959
y = 0.1005x - 1.7599
R ² = 0.98288
y = 0.0873x - 1.8992
R ² = 0.97815
[N2O] (µM)
Time (h)
c)
28 µM nitrate addition
y = 0.3171x - 7.6872
R ² = 0.9956
y = 0.2548x - 5.6295
R ² = 0.9972
y = 0.2284x - 5.4779
R ² = 0.99841
y = 0.2127x - 5.6043
R ² = 0.99594
[N2O] (µM)
Time (h)

Figure 4


Click here to access/download;Figure;Fig4_Temp_dep_se
[N2O]
110 µM
200 µM
428 µM
y = 123.38x + 1035.7
R² = 0.99692
y = 66.112x + 391.14
R² = 0.99787
y = 35.609x + 334.09
R² = 0.99632
N2O sensor signal (mV)
Temperature (°C)
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
8
13
18
23
28

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Messenger-adapted gravity corer	-	-	Reference in the manuscript. Made by Glew, J.
Sampling tube	-	-	Acrylic. Dimensions: 100 cm (h)
Handheld sounder	Plastimo	38074	Echotest II Depth Sounder.
Rubber stopper	VWR	DENE1012114	With two holes, used to mix the N_2O-water in the calibration chamber. Dimensions: 20 mm (h) × 14 mm (d) × 18 mm (D) (3 mm hole (D)).
Rubber stopper	VWR	217-0125	To seal the bottom part of the methacrylate tube and to sample in shallow water bodies. Dimensions: 45 mm (h) × 56 mm (d) × 65 mm (D).
PVC cover	-	-	To seal the top side part of the acrylic tube. Dimensions: 45 mm (h) × 56 mm (d) × 65 mm (D). Dimensions: 65 mm (D).
Adhesive tape	-	-	Waterproof. To ensure all joints (PVC cover sampling tube and PVC cover sensor) and to avoid water leaks.

Thermometer	-	-	Portable and waterproof, to measure the temperature in the water overlying the sediment just after sampling the cores.
GPS	-	-	To save the location of a new sampling site or to arrive at a previous site.
Wader	-	-	For littoral or shallow site samplings.
Boat	-	-	An inflatable boat is the best option for its lightness if the sampling site is not accessible by car.
Rope	-	-	Rope with marks showing its length (e.g., marked with a color code to distinguish each meter).
N_2O gas bottle and pressure reducer	Abelló Linde	32768-100	Gas bottle reference.
C_2H_2 gas bottle and pressure reducer	Abelló Linde	32468-100	Gas bottle reference.
Tube used to evacuate the excess of water	-	-	Consists of a solid part (e.g., a 5 ml pipette tip without its narrowest end) inserted in a silicone tube.
Nitrous Oxide Minisensor w/ Cap	Unisense	N2O-R	We use 4 sensors at a time.

Microsensor multimeter 4 Ch. 4 pA channels	Unisense	Multimeter	Picoammeter logged to a laptop. The standard device allows for 2 sensor picoammeter connections (e.g., N_2O sensor), one pH/mV and a thermometer. We ordered a device with four picoammeter connections, allowing the use of 4 N_2O sensors simultaneously.
SensorTrace Basic 3.0 Windows software	Unisense		Sensor data acquisition software.
Calibration Chamber incl. pump	Unisense	CAL300	Calibration chamber. We tuned it with rubber stoppers and syringes to mix the N_2O-water without making bubbles.
Incubation chamber	Ibercex	E-600-BV	Indispensable equipment for working at a constant temperature (±0.3 °C). It also allows control of the photoperiod.
Electric stirrer	-	-	Part of the stirring system. It hangs in the water, overlying the sediment subject, by a fishing line that is hooked to the PVC cover.

Electromagnet	-	-	Part of the stirring system. It is fixed to the outside of the acrylic tube, approximately at the same level as the stirrer. It is activated episodically (ca. 1 on-off per s) by a circuit, attracting the stirrer when it is on and releasing it when it is off, thereby generating the movement that agitates the water.
Electromagnetic pulse circuit	-	-	Part of the stirring system. It is connected by wires to the electromagnet and sends pulses of current that turn the electromagnet on and off.
Uninterruptible power supply (UPS)	-	-	It improves the quality of the electrical energy that reaches the measurement device, filtering the highs and low of the voltage, thereby ensuring a more constant and stable N_2O sensor signal.

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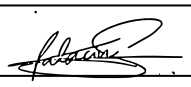
Signature:	
Date:	31 May 2018

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Dear Dr. Alisha D. Souza,
We are pleased to submit our revised manuscript entitled " An experimental protocol to estimate sediment denitrification rates using cores and N_2O microsensors " to JoVE [current code: JoVE58553_R2].

The reviewers have helped us a lot, but some of their comments already appeared elsewhere in the manuscript; if not, we have incorporated them.

Below we provide a detailed response to the suggestions made by you and by the reviewers, which we have generally followed. The original comments are in grey and our response in black characters.

Editorial Comments:

• The manuscript will benefit from thorough **language revision** as there are a number of grammatical errors throughout. Please thoroughly review the manuscript and edit any errors.
Done. The manuscript has been reviewed by an English native professional modifying the grammatical errors.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:
Done. We have clarified and improved some steps of the protocol, and adding more items to the table of materials (e.g., stirring system). Regarding the button clicks of the sensor software we have also clarified; and readers will also have the chance to take a look of the sensor software manual, which is already referenced in the manuscript (discussion paragraph about using N_2O sensors, lines 472, 488 and 496).

1) 1.1: How is the polarization performed?
We have clarified how to perform the polarization "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." (Lines 141-143)
Unclear what we can film here.
This part of the protocol will not be filmed.

2) 1.2: What sound the lighting and temperature be adjusted to?
Clarified: "selected light off and temperature set to be similar to that expected in the field". (Line 151)
What type of water (e.g. distilled, ultrapure etc.)?
Added: "Deionized".

3) 1.4: Please mention what the materials to be prepared are and how they are prepared.
Done. "Pack the field core collection materials: corer device, sampling tubes, rubber stoppers, PVC taps, screwdriver, GPS, thermometer, handheld sounder, wader, and inflatable boat (see the Table of Materials). Use a checklist to ensure all materials are included." (Lines 160-163)
This likely need not be highlighted for filming.
Yes, this part of the protocol will not be filmed.

4) 2.1a3: Are there any instructions to select the sampling point?
Changed. "Select the sampling point according to the investigation aims." (Line 177)

5) 2.1.a.4: How do you track the distance from the sediment?
Changed. We know the distance because we use a rope with regular marks to control the depth position of the sampling equipment, and previously we have measured the depth (water-column vertical length) of the site with a handheld sounder.
Unclear what we would show here.
It would be good to record a shot with the corer device out of the water showing how works the mechanism activated by the messenger, which generates the vacuum. The researcher could explain: "This is a messenger-adapted gravity corer. At first the rubber piece of the corer and the upper edge of the sampling tube are not in contact favoring a penetration in the sediment that minimizes the disturbances in the sample. Then, when the messenger arrives and hits the corer, releases the rubber piece, which contacts with the sampling tube generating the vacuum, which allows extracting the sediment core".
Also the cameraman will be filming by the shoreline how we make the core collection, and meanwhile a voice in off will read the highlighted part of the protocol…"Deploy the coring system until the sampling tube is ~1 m from the sediment. Stabilize the sampling equipment for 60 s. Release ~1 m more of 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. Release the messenger while trying to keep tension in the rope so that the corer remains fixed and in a vertical position. Recover the corer by pulling the rope constantly and gently. 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. 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."

6) 2.1.a9: Unclear how this is done, is the tube still submerged?
Filming from the shoreline, the tube is still submerged but close to the surface. Water is too transparent.
7) 2.1.a10 Note: We cannot film scuba diving portions.
No problem, film from the shoreline.

8) Sections 6, 7: All sections must have a set of substeps under them.
Done, united in same section "6. Final measurement steps"

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.
Done.

• **Protocol** **Highlight:**
1) We can usually only film within a laboratory and all filming would need to be completed within 1 day, please let us know if your filmable content does not meet these criteria.
I have already spoken with the JoVE editor (Lindsay Troyer) to film the protocol in 1 day… This is an extract of a previous e-mail: "To reduce the time of filming, we (the authors) have think to meet directly with the JoVE filming person/s in the sampling place (parking in the entrance of Aigüestortes National Park). Then we will sample in the closest lake, which it can be reached by car in 5 minutes. After record the field sampling (<2h) we will drive (1h) to our research center in the Pyrenees to record the rest of the protocol (lab procedures, 3h). This way we ensure to record everything within the supposed time of 1 day (6-8h)."

2) After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
Done. Entire protocol length = 6 pages.

a) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
Done.

b) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
Done.

c) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
Done. Highlighted protocol length = 2.5 pages.

d) Notes cannot be filmed and should be excluded from highlighting.
Done.

e) Please bear in mind that software steps without a graphical user interface/calculations/ command line scripting cannot be filmed and must be unhighlighted (e.g. unhighlight section 8).
The idea was to not film directly in the computer screen this part, if not show the plots (Figure. 2a (old 3a) and 2b (old 3b) and read the sentences highlighted (Lines 370-380).

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.
Done. Some parts moved to notes in the protocol, and the rest organized in paragraphs. This is the order of the discussion: (1) Critical steps within the protocol (good core recovery and some details must be considered when using N_2O microsensors) (2) possible modifications of the method, (3) main weaknesses/limitations, (4) future improvements/applications (here we also mention the utilities of 15N tracer methods, which can be combined with our method).

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Sensor Trace Basic, MilliQ.
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
Done.
2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.
Done.

• Please define all abbreviations at first use.
Done.

• Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.
Done.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."
Done. Part of figure 1, just panel a, is similar to a previous published one. During the day of film we can take another picture of the experimental setup (updated, just with 4 sensors and the Multimeter, without the *in situ* equipment) to avoid any future copyright problems.

Comments from Peer-Reviewers:

[Editor's Note: *Please note that the reviewers raised some significant concerns regarding your method and your manuscript. For each peer review comment, please provide either (1) a description of how the comment was addressed within the manuscript or (2) a rebuttal describing why the comment was not addressed if you feel it was incorrect or out of the scope of this work for publication in JoVE.]*

Reviewer #1:

Manuscript Summary:
The manuscript describes a method to quantify the denitrification rate in sediments using a novel modification of the classical acetylene inhibition technique. As the authors state correctly, there is currently no easy and at the same time 100% reliable method to measure this important process available. State of the art are probably the N2 method and the isotope pairing technique.

Major Concerns:
The acetylene assay suffers from a number of serious problems, which limits its application. In fact, the only advantage of the method is, that it is comparably easy. I would question that the acetylene inhibition method gives reliable whole sediment denitrification rates for a number of reasons:

1) denitrification in sediments is typically nitrate limited. Thus, coupled nitrification-denitrification at oxic-anoxic interfaces becomes very important. Since acetylene inhibits nitrification, the method will in most cases under-estimate the denitrification rate.
They are already discussed in the article (lines 533-540) and are aspects that may influence. But the limitation by nitrate/nitrification will be clearly as a non-linear behavior of the N_2O signal.

2) As the authors state correctly, the part of the sediment which is reached by the acetylene is not defined. Surely the inhibitor will reach only a part of the sediment - thus inhibition will probably be incomplete.
Again, they are already discussed in the article: (Lines 541-545) "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".

3) N2O is measured in the water. It has to diffuse from the sediment into the water. Thus, what you

measure is not the rate of denitrification in the sediment but the flux of N2O out of the sediment. This flux might be transport-limited and is not necessary equal to the denitrification rate in the sediment.
All methods that do not distort the sediment have this problem. By making direct measurements in the sediment, its conditions are modified. You change one problem for another.

There might be situations (no nitrate limitation, denitrification restricted to the very sediment surface,…) in which the proposed method gives the correct denitrification rate. However, before the method can be published I would ask for a comparison with an independent method, e.g. using 15N. The authors then have to show under which conditions the method is OK and under which conditions not.
All methods have advantages and weaknesses. We have already discussed it, a comparison of methods is out of the scope of this work.

It is not clear to me, why the cores are bubbled with N2. This will block nitrification and at the same time stimulates denitrification. Furthermore, bubbling with pure N2 rises the pH of the water. To keep the pH constant we usually bubble with a N2-CO2 mixture.
We have excluded from the protocol and added to the discussion including the suggestions of the reviewer (see the discussion paragraph about possible modifications of the method, lines 524-527: “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”).

The sediment-coring protocol depends very much on the corer used. The probably most widely used gravity corer from UWITECH (Mondsee, Austria) e.g. would require a modified procedure.
UWITECH is quite similar as the one we used in these work, we have been using this corer too, but there is not important differences in the protocol.
I think it does not make sense to give a general manual "how to get sediment cores" in this particular manuscript. There are several manuals for coring available and the coring technique is quite independent from the denitrification assay. Thus, I would remove the entire part about coring.
We do not agree. Not all denitrifying researchers are familiar with coring techniques. Yes, there are some manuals, but here you will have a video record.

Minor Concerns:
For a practical manual, the method description is not detailed enough. A technical drawing would help to make the construction of the stirrer more clear.
We have clarified some protocols steps. We have detailed the items used in the stirring system in the Table of Materials. We thing is not necessary the technical drawing, since in the video we will show the stirring system.
I wonder whether the temperature dependence of the N2O microsensor as shown in 4 has already been published by the Revsbech group or by Unisense.
The plot is based in our measurements. The temperature dependence of the microsensors is well known and maybe already published, but is a critical aspect and is worth to be clearly illustrated.

Reviewer #2:

Manuscript Summary:
This manuscript explain one method to estimate denitrification rates in sediment cores by the measurement of nitrous oxide changes using electrochemical sensors. The final denitrification step performed by the nitrous oxide reductase is inhibited with acetylene, resulting in measurable N2O concentrations. Denitrification rate is measured as the progressive accumulation of N2O in the water phase.

General Comments:
Although the novelty of the methodology is not a requirement for this journal, the authors should revise the extensive work done with acetylene inhibition and N2O sensors for the measurement of denitrification. The use of N2O sensors to measure denitrification in sediments has been done for 30 years already (Revsbech et al 1988 "Combined Oxygen and Nitrous Oxide Microsensor for Denitrification Studies." Applied and environmental microbiology 54: 2245-2249) and the use of acetylene was developed 10 years before (Sørensen 1978 "Denitrification Rates in a Marine Sediment as Measured by the Acetylene Inhibition Technique." Applied and Environmental Microbiolology 36: 139-143). In consequence, there are several manuscripts reviewing the advantages and disadvantages of the methods. The authors should include such revisions in the introduction and take into account the weaknesses of the method when it is described in the introduction and abstract.
We have included the historical references (lines 93-95). Regarding the weaknesses of the method, we have already mentioned in the discussion (one paragraph starting at line 533) according to JoVE instructions for authors. In the long abstract we also mention “Advantages and weaknesses compared to other methods are discussed”.

The authors should be more critical with this method and do not try to promote it by hiding or diminishing the benefits of other methodologies.

We are not aware of being doing that.

Microsensors has many advantages but some other methods provide much higher resolution and lower detection limit to measure N2O (by simple gas chromatography, for example) or measure denitrification rates.

You're right. We have exclude/modify the sentences talking about a lower denitrification rates than some alternative methods (e.g., in the long abstract, line 45).

Reagents and analysis of 15N cost a fraction of one single N2O microsensor and the sensitivity to measure denitrification rates might be as low as few nanomol l-1 day-1 (Bristow et al 2017 "N2 production rates limited by nitrite availability in the Bay of Bengal oxygen minimum zone." Nature Geosciences 10: 24-29). In addition, isotopic pair technique can be applied in intact cores without the significant modifications required in the proposed procedure (Risgaard-Petersen et al 2003 "Application of the isotope pairing technique in sediments where anammox and denitrification coexist." Limnology and Oceanography: Methods 1: 63-73).

Two references added when talking about the 15N tracer methods (lines 547-550). Ok the technique has a lower detection limit, as mention before we have delete the part talking about a lower detention limit. But 15N-tracer methods needs to modify the substrate availably (addition of ^{15}N reagent).

There is no suggested control. Indeed, the procedure produces significant modification of the natural conditions and therefore measured rates cannot be assumed to be "real Denitrification rates". Acetylene has been proved to inhibit other processes such as nitrification and anammox at the concentrations needed to inhibit the nitrous oxide reduction. The accumulation of N2O is estimating only a fraction of real denitrification rates. In addition, the artificial anoxic conditions and the addition of nitrate used in the present manuscript are both enhancing denitrification. Under anoxic and nitrate rich conditions, facultative denitrifiers shift their metabolism to use nitrate instead of oxygen. The modification of the natural conditions modify significantly the estimated rates. The use of the term "potential denitrification" in the title and throughout the text is more realistic for the proposed methodology.

We prefer to talk about potential denitrification rates only when nitrate is added. We have removed the artificial anoxia step from the protocol.

Specific comments:

-English must be revised thoroughly. There are numerous grammatical errors and the vocabulary is frequently wrong. I have spotted only some mistakes.

A native English professional has reviewed the manuscript to correct all grammatical errors.

Similarly, terminology must be revised and jargon excluded as it seems to be local jargon with no meaning for other groups.

We do not understand this comment; we do not know what the reviewer refers to.

-In the title and, in general, it should be noted that potential denitrification rates are measured, but not real or effective denitrification.

We prefer to keep the manuscript as it is talking about potential or actual denitrification rates respectively, depending on whether or not nitrate is added.

Abstract:

-Contrary to the stated in the manuscript, the method should not be applied without acetylene. The N2O sensor does not have the accuracy to measure N2O concentrations at natural levels in unpolluted environments. Natural saturation level is around 10 nM, two orders of magnitude lower than the limit of detection of the sensor.

We have modified the sentence (line 51): “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.”

In the old version we already mention in the discussion the possibility than N_2O emissions could be below the detection limit. In the revised version we also added the possibility to add nitrate to enhance the N_2O emissions until are above the detection limit (lines 527-532: “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”).

Introduction:

-As stated before, authors should include more reviews of the technique. The benefits and weaknesses of the technique should be more clear and realistic.

Same reply as before: We have included the historical references (line 89). Regarding the weaknesses of the method, we already mention in the discussion (one paragraph starting at line 533) according to JoVE instructions for authors (is the only proposed place).

-L65-66: Please rephrase "warming potential nearly 300 times that of CO2 over 100 years". It is not clear, is the warming potential lasting for 100 years?
N_2O has nearly 300 times more warming potential during 100 years than CO_2. We have not changed the sentence, because we think is clear, in fact the native English professional have not modify this part.

-L77: consider the manuscript: Sørensen, J. (1978). "Denitrification Rates in a Marine Sediment as Measured by the Acetylene Inhibition Technique." Applied and Environmental Microbiolology 36(1): 139-143
Reference incorporated (line 94).

-L92: why the acetylene is applied by direct bubbling? Effective concentration blocking the N2O-reductase is reached with only 10 kPa. Therefore, acetylene saturation is not needed and it might even inhibit other microbial processes.
To ensure the inhibition of N_2O reduction.

-L102: N2O microsensors does not have low detection limit, environmental concentrations are usually way below the limit of this sensor.
We have delete in the manuscript the mention of a low detection of our method compared to alternative methods.

-L108: Comparison with Isotopic pair technique is missing (and needed)
We mention in the discussion (line 546), the combined use of our method with 15N-tracer methods, as a future improvement of the method because of the advantages of the Isotopic pair technique. But a comparison of methods is out of the scope of this work.

-L122-123: these sensors are most likely not sensitive enough to measure N2O concentrations or fluxes in unpolluted areas.
Same as before, we already mention this possibility in the abstract (line51) and in the discussion (line 528).

List of materials:

-The in situ equipment is not needed. The authors used it for including one more sensor, but it is easier to include one more picoammeter. In addition, it depends of the number of replicates. If more than four replicates are needed, better use two multimeters.
We agree. We have already removed the *in situ* equipment from the Table of Material.

-"methacrylate" is not correct, it is poly methyl methacrylate (PMMA) or acrylic.
Changed to acrylic.

-"Downside"? I guess the authors meant the bottom part of the acrylic tube.
Done. Changed by “bottom”.

Protocol:
-L135 (and throughout the manuscript): "one" cannot be used as subject. Please, rephrase everywhere.
We have changed to clarify. Although exists https://en.wikipedia.org/wiki/One_(pronoun).

-L138: "temper" is not correct.
Changed, now line 152-154.

-L145-146: Unisense Multimeter can be connected to the grid, ensuring constant power supply. Also, an UPS unit can be used as a backup.
Done. Rewritten as a note after step 1.1. (lines 136-139). UPS also mentioned in discussion (line 482) and added to the Table of Material.

-L295-303: Rephrase, I cannot understand what the authors are suggesting to do. "Downside" means inconvenience or problem.
Done. Changed to “bottom”.

-L305: why induce anoxia? It is not justified.
Excluded the induction of anoxia in the protocol. Just mention as a possibility in the discussion for potential denitrification measurements (line 524).

-L321: is the space filled up with external water?
Is refilled with part of the leftover from the excess of water from the sample.
Then the anoxic and C2H2 saturated conditions change.

Yes but the change is minimal, because this water supposes a small part of the entire water volume. Furthermore, as the reviewer mention before, by bubbling we have more C_2H_2 than enough for the N_2O-inhibition.

-L324: what is the "electromagnetic pulse circuit"?

We have clarified the components of the stirring system in the Table of Material. E.g., This is the electromagnetic pulse circuit description: “Part of the stirring system. It is connected by wires to the electromagnet and sends pulses of current that turn the electromagnet on and off.” Furthermore, when filming the method, we will make close shots of all the components of the stirring system, including the circuit.

-L334: every 5 min.

Done.

-L356: Reference to Figure 3 appears before than Figure 2.

Yes, changed in order.

Results:

-Figure 1. It is the same than use in a previous publication. The details are not clear as the lid, tape and other things do not allow to see clearly inside the sediment core.

Is the same setting, but not the same picture. Anyway as we mention to the editor, we will substitute the picture of the figure 1a “During the day of film we can take another picture of the experimental setup (updated, just with 4 sensors and the Multimeter, without the *in situ* equipment) to avoid any future copyright problems.”.

Regarding the quality for the definitive manuscript we will send a file with a higher quality (We sent this “.jpg”, to not exceed the maximum weight of the file in the submission process)

-Figures 2 and 3:

- The use of screen captures does not look nice.

Yes, we have removed in old figure 2 (now figure 3), but we have kept it in old figure 3 (now 2) as an example of sensor software display.

- Addition of substrate (nitrate) is not explained in the method but used in the figures.

Added as a note in the protocol (line 312-314). Was already in the discussion in the old version. See the new version discussion paragraph about possible modifications of the method (starting at line 516).

- Negative concentrations are physically impossible. The authors did not perform correct zero calibrations or did not take into account the sensor signal drift. These data should not be used as they were drawn and even less used as example in a Figure.

Yes you are right, negative concentrations are impossible. As we mention in the manuscript (lines 496-503) “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 Figures 2b and 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)”

As we mention in the caption, new Figure 3a (line 434), samples 4 and 5 are examples of estimating denitrification rates near the detection limit of the sensors, and is because we want to show exactly this figure.

- Figures are messy and the numerous data are not needed. Please, select some nice data sets and simplify the figures. Indeed, one single figure is enough.

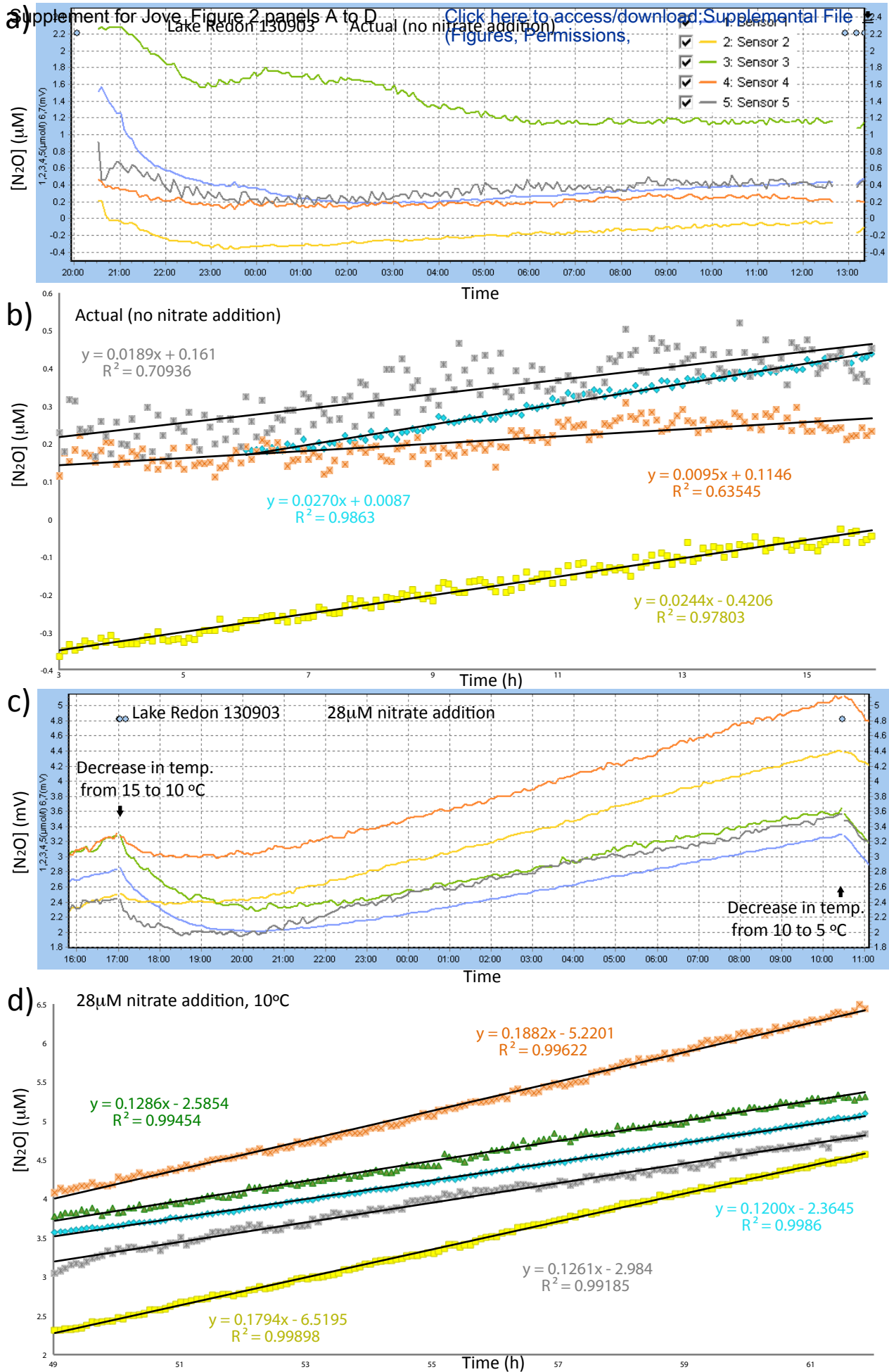
We have simplified old figure 2 (now 3) by deleting the sensor software images. We have kept both figures, one as an example of a simple denitrification measurement and the other as a denitrification temperature dependence experiment.

-L376-379: Other simple methods as Gas Chromatography reach detection limits orders of magnitude lower than the sensor. Same thing for the sensitivity of rates. The values measured with the sensors are not very low.

We have changed: “very low” to “relatively low” (line 393).

The important is that we give a range of values 0.4-1 µmol N_2O m^{-2} h^{-1} showing the lower rates we can measure with our method. (line 46 and lines 393-396)

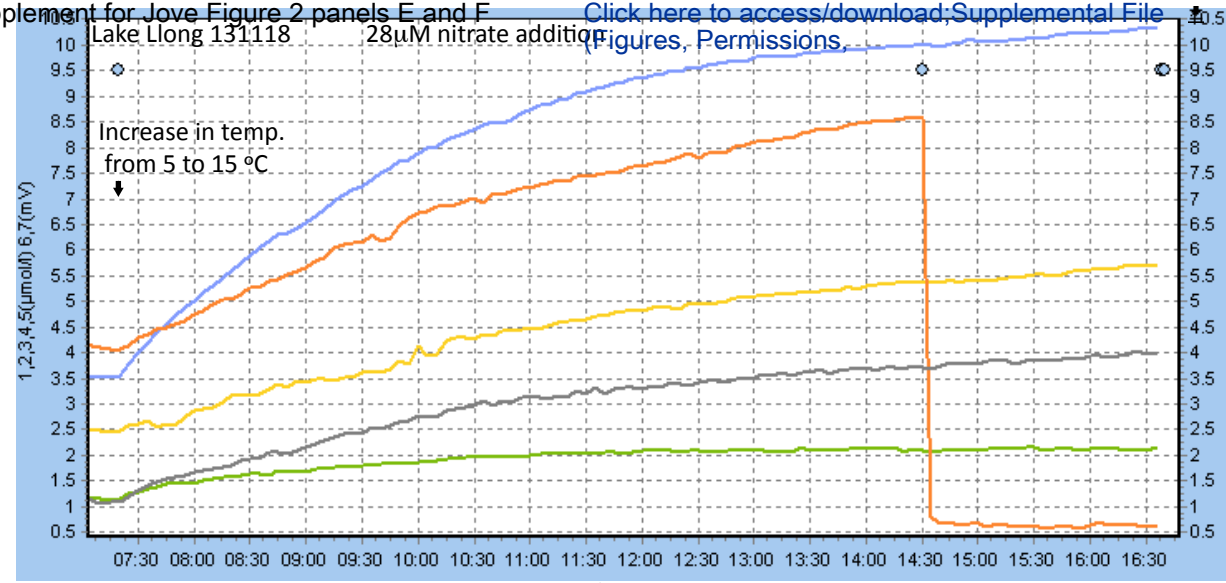
Supplement for Jove, Figure 2 panels A to D



Supplement for Jove Figure 2 panels E and F

Click here to access/download;Supplemental File (Figures, Permissions,

e)



f)