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 N2O 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 N2O 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 N2O 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 N2O 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 N2O 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 N2-CO2 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 15N 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 N2O concentration is above the detection limit of the sensor, the acetylene inhibition step can be omitted to estimate the N2O emission instead of denitrification.”

In the old version we already mention in the discussion the possibility than N2O emissions could be below the detection limit. In the revised version we also added the possibility to add nitrate to enhance the N2O emissions until are above the detection limit (lines 527-532: “To calculate sediment N2O emission rates, omit the C2H2 inhibition (step 4.3). However, keep in mind that, as far as it is currently known in aquatic ecosystems, N2O emissions are proportionally low compared to N2 emissions (0%–4.3%)33, so it is possible that the accumulated N2O will be below the detection limit. If this is the case, an option is to add nitrate to increase the emitted N2O, calculating potential N2O 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?

N2O has nearly 300 times more warming potential during 100 years than CO2. 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 N2O 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 C2H2 than enough for the N2O-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 (H2S, NO, and CO2) can affect the N2O sensor’s signal22. 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 N2O is not the key parameter. What is key is the slope of the linear model (evidencing a linear accumulation of N2O)”

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 N2O m-2 h-1 showing the lower rates we can measure with our method. (line 46 and lines 393-396)