We thank the reviewer for their thoughtful comments on our manuscript. The reviewer's comments and suggested revisions will improve the final version of the manuscript. Our responses to reviewer comments are in blue.

This manuscript describes the results of several batch experiments performed with the objective of evaluating N2O emissions response to different environmental stressors. The tested stressors were: salinity, pH, temperature, moisture and Zn. The reported results include the measurement of accumulated N2O in the head-space of the reactors for the different tested treatments and the detection of nirS and nosZ genes expression. Also, the N2O to N2 ratio is estimated because acetylene is added in some reactors to stop N2O reduction. The methodological approach is well described and the obtained results and conclusions are clear. The findings allow an improved understanding on the role of environmental perturbations on N2O emissions.

Nevertheless, I would kindly ask the authors to address the following issues:

1. In the present study, three different approaches are employed to study the role of salinity changes on N2O emissions. Instead, a single approach is employed for the other tested parameters (pH, temperature, moisture and Zn). Which is the reason of using different methodologies for different parameters? Which has been the reason of choosing these parameters instead of others (e.g. pesticides)? Also, regarding Zn, why this metal instead of others? All the responses might be related to the study site but it is not clear enough in the manuscript.

   The initial experiment in which we tested multiple types of perturbation (pH, temperature, soil moisture, Zn, and salinity) was focused on understanding how the denitrifying community responds to various physicochemical perturbations. There are indeed additional parameters that could have been investigated such as pesticides or other toxic heavy metals, and it was simply a decision around keeping the experimental design feasible. Future studies might expand the tested parameters. The subsequent experiment investigated how denitrifying communities from environments experiencing a range in one of those parameters (salinity in estuarine sediments) respond to changes in that parameter, and the final experiment evaluated the long-term response of the denitrifying community to a change in that same parameter. We elected to focus on a single parameter in these sets of experiments for logistical reasons – we did not have the resources to investigate multiple parameters. We elected to focus on salinity for the additional experiments for several reasons, including 1) it is a parameter that changes over daily (tidal) and seasonal timescales in estuarine environments and is therefore ecologically relevant, 2) it is a parameter that will

be altered in some environments in response to climate change, 3) it is relatively easy to manipulate and to measure. We readily acknowledge these same arguments could be made in favor of other parameters such as temperature and pH, and that additional experiment using these (or other) parameters were beyond the scope of the current study. We will clarify these points in the opening paragraph of the methods section of the manuscript.

2. In the methodology section concerning the agricultural soils tests (2.1), you explain that you tested conventional and organic farming practices. However, nothing more is said about it on the results and discussion sections. Could you explain why? Also, to which type of soil correspond the results you are showing?

   The data shown in Figure 1 is for the conventionally farmed soil. It was an oversight that we did not include that information in the figure caption, and we will do so. The full data from both sets of experiments is included in the appendix, but we elected to show results from one soil type and did not address the differences between the conventional and organic soils because the response of both soil types to the various physicochemical perturbations is remarkably similar. We will make that point more clearly in the revised manuscript.

3. It is well stated that all experiments were performed under anoxic conditions. However, which is the gas replacing oxygen in the section 2.3 experiments? Why did you use N2 for the experiments described in 2.1 while He was used in the experiments described in 2.2? The same issue is found regarding the gas chromatography methodology employed for N2O measurements for each set of experiments. In 2.1, an Agilent GC is used, in 2.2 it is a Shimadzu GC, while no information is provided for 2.3 experiments.

   We used N_2 to create anoxic conditions in the section 2.3 experiment, and N_2O was analyzed on an Agilent GC in section 2.3. We will include that information in the revised manuscript. These differences are simply the result of working in different laboratories (Villanova University in Pennsylvania, USA for sections 2.1 and 2.3, and the Netherlands Institute of Ecology and the University of Georgia, USA for section 2.2) with different resources for different portions of the work described here, and we don't expect that the differences in headspace gas or gas chromatography influenced the results.

4. In several sentences along the manuscript (e.g. lines 83 and 160) the authors associate the nirS gene to a nitrate reductase. This is not correct since nirS gene

is associated to nitrite reductase (as well as nirK). In this context, why checking the nitrite reductase instead of the nitrate reductase or maybe both?

Thank you for catching this mistake. We define nir as nitrite reductase initially but then made the mistake of calling it nitrate reductase in several spots. We will correct this typo in the revised manuscript. We elected to focus on nir expression because it is downstream of the nar nitrate reductase enzyme and is the first step in the overall denitrification pipeline that produces a gaseous end-product (nitric oxide). The reduction of nitrate via the nar enzyme may lead to annamox, an alternate pathway of nitrite reduction. The measurement of nir nitrite reductase enzyme expression thus more fully isolates steps in the denitrification pathway.

In the following sentences, you will find some additional specific comment where numbers correspond to the manuscript lines:

1: It should maybe be mentioned in the title that the study focuses on nitrous oxide production from denitrification because other pathways of nitrous oxide production are not considered (e.g. nitrification or chemodenitrification).

This is good point of clarification. We will change the title to "Physicochemical Perturbation Increases Nitrous Oxide Production from Denitrification in Soils and Sediments"

46-48: Even I understand it's not the object of the study, it might be worth it to mention that N2O emissions can also be derived from the nitrification and chemodenitrification processes apart from denitrification. In fact, the contribution of chemodenitrification on N2O emissions is not still well known but could be a significant source. E.g. Robinson et al. (2021) https://doi.org/10.1039/D1EM00222H; Jones et al. (2015) https://doi.org/10.1021/es504862x; Cooper et al. (2003) https://doi.org/10.1128/AEM.69.6.3517-3525.2003 .

We mention abiotic denitrification in this section which is a synonym for chemodenitrificaiton, but will clarify that statement to include chemodenitrification.

51-52: How many decades exactly?

The rate of 0.26% per year increase in N_2O concentrations is based on data from 1980-2005 (Forster et al. 2007). We will clarify this in the revised manuscript.

62-63: What about the role of inorganic electron donors such as ferrous iron or sulfide?

Yes, this is a good point – we will include a statement about the availability of electron donors.

68: Due to incomplete denitrification?

Possibly, but the mechanism is unclear. Burgin and Groffman (2012) suggest that the presence of O_2 selects for microbes that produce more N_2O. It may also be inhibition of nitrous oxide reductase (Chen et al. 2023; https://doi-org.ezp1.villanova.edu/10.1021/acs.est.2c07081) in a mechanism somewhat similar to the more generalized physicochemical perturbation response we posit in this manuscript.

71-72: Why?

Lower pH has been linked to the posttranscriptional inhibition of nitrous oxide reductase (Liu et al. 2010). This will be clarified in a statement in the manuscript.

74: You have already explained the potential role of soil moisture in the previous paragraph. Try to avoid repeated information.

This will be addressed in the revision.

102-171: Even the methodology description is very clear, a table summarizing all tested conditions could be included in the revised version of the manuscript to facilitate following the results and discussion sections.

We will include the following table in the revised manuscript:

Table 1. Summary of the three experiments in which the response of denitrification and nitrous oxide production to physicochemical perturbation was investigated (numbers corresponding to the subsections of the Methods section).

Expt.	Brief Description	Type of Soil/Sediment	Perturbation
2.1	Short-term (pulse) effect of perturbation by various physicochemical parameters	Agricultural soils (Lancaster, Pennsylvania, USA)	Salinity, pH, Zinc, Temperature and Moisture

2.2	Short-term (pulse) effect of perturbation on sediments that experience various ranges in the perturbation parameter	Estuarine sediments (Scheldt River, Netherlands/Belgium)	Salinity
2.3	Long-term (press) effect and subsequent short-term (pulse) response together with gene abundance and expression	Estuarine sediments (Delaware River, New Jersey, USA)	Salinity

99: Gram soil/sediment per day --> dry or fresh soil/sediment?

All rates are per gram fresh (wet) soil/sediment. This will be clarified in the revised manuscript.

108+125: Units for salinity are missing

This will be clarified in revision.

119-120: Jars were incubated 12h except for the temperature treatment. Why did this treatment have a different incubation time?

Cooler temperatures were incubated for longer periods of time (24 hr) to allow for adequate biogeochemical activity, while warmer temperatures were incubated for short periods of time (8 hr) because of higher biological activity. This will be clarified in the paper.

177: For Zn this is not observed for the first 3 points, right?

Yes, this is true. We will amend the sentence to clarify the pattern of N_2O production with increasing zinc concentration.

188: Fig.3 --> Fig.2 ?

Correct. Thank you for catching that.

190: “above43” --> above

This will be corrected.

215: I think “moderate” is not enough specific here

We will reword this statement.

We propose that changes in physicochemical conditions can induce a generalized short-term perturbation response from the soil denitrifying community, with higher N_2O:DNF ratios and increased net N_2O production with reductions in N_2O production at higher levels of perturbation for some parameters (Fig. 5a).

231: “do not appear to” or “might”?

This will be amended.

248: Only across ecosystem types or also across stressor types?

Yes, good suggestion. We will amend this statement to “Further research to determine the generality of this finding across ecosystem types and forms of physicochemical perturbation is warranted.”

249-261: Could an isotope labelling approach (15N) be more appropriate than the acetylene block method?

Yes, an isotope labelling approach would be a good way to further address the role of physicochemical perturbation. That is what we had in mind in writing this section, though we don’t state it clearly. We will edit this paragraph to include the suggestion that isotope labeling methods would be an appropriate next step.

262-277: Concerning the succession of active denitrifying microorganisms, I think it is also worth having a look at the paper published by Liu et al., in 2019 (DOI: 10.3389/fmicb.2018.03208).

Agreed, this is a good study to reference in this section. We will amend this portion of the paper and include the citation to Liu et al. 2019.

284: No pattern or flat pattern?

We will clarify this statement. “We observed no correlation between nirS expression and either N_2O production or the N_2O:DNF ratio.”

292-229: In general, the information included in this paragraph seems somehow repetitive with respect to the statements made on the two previous paragraphs. Consider summarizing it.

We will attempt to shorten this paragraph in the revision. The purpose of this paragraph is to address the timeframe of the perturbation response, which is not addressed in the previous paragraphs. We will remove any redundancy with previous paragraphs.

319: ”nos” --> nosZ ?

We will correct this.

Figure 1 to 4: why did you choose to fit quadratic equations? In some cases, it does not reflect the evolution of the measured parameters and induces confusion on the results interpretation.

We agree that not all relationships require nonlinear fits (linear fits would work in some instances), but many of the relationships are nonlinear and we felt it would induce

more confusion to use different types of fits than to be consistent. We argue that the current approach causes the least confusion.

Figure caption of Figure 1: I don't understand to what refers the "1" for standard deviation.

This was meant this to clarify that we meant 1 standard deviation (rather than 2 or 3), but we agree that it creates confusion rather than reduces it, and we will remove the number. All error bars are a single standard deviation.

Figure 4: Why did you prefer to draw joint instead of separate lines for press and control experiments? Could it be useful to include the nirS results in this Figure?

In a revised Figure 4 (below), we have shown separate lines for the press and control experiments in the nosZ to N_2O:DNF figure, and we have included the nirS to N_2O:DNF figure as well showing no relationship. The separate lines in the nosZ portion of the figure show a significant relationship for the press data and a very similar but non-significant relationship for the control data. The updated figure will be included in the revision, and we will amend the text in the manuscript as appropriate to cite this figure.

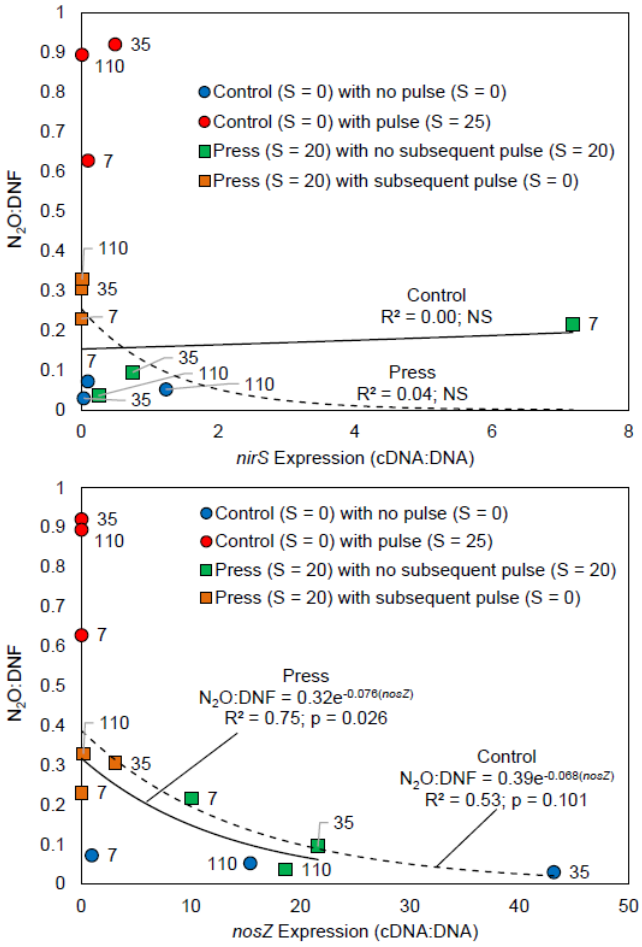
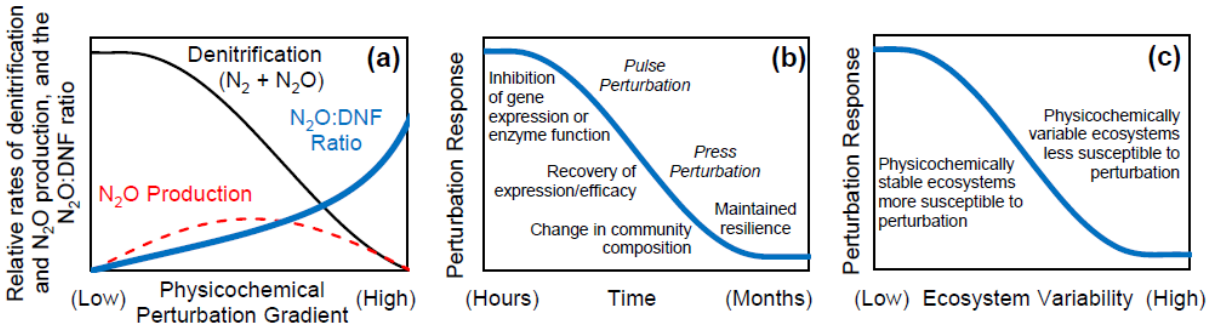


Figure 5: I think you should state in the figure caption that this model has been performed according to your results. Maybe you could also check if it fits what has been found by other authors to perform a scheme according to all literature available up to date (including your study)? In plots b and c, following your conclusions maybe the slope at the right side should be softened to clarify adaptation after time.

We have softened the slope in panels b and c in Fig. 5 to clarify maintained resilience in the N_2O perturbation response as suggested. The new figure, copied below, will be included in the revision. We will change the figure caption as follows:

Figure 5. Conceptual model based on the results of this study that shows (a) relative rates of total denitrification (DNF), nitrous oxide (N_2O) production, and the N_2O:DNF ratio in sediments and soils as a function of a physicochemical perturbation gradient, (b) response of the denitrifying microbial community to physicochemical perturbation over time, and (c) the hypothesized relationship between ecosystem physicochemical variability and the perturbation response.


Relative rates of denitrification and N2O production, and the N2O:DNF ratio
Denitrification (N2 + N2O)
(a)
N2O:DNF Ratio
N2O Production
(Low)
Physicochemical Perturbation Gradient
(High)
Perturbation Response
Pulse Perturbation
(b)
Inhibition of gene expression or enzyme function
Recovery of expression/efficacy
Press Perturbation
Change in community composition
Maintained resilience
(Hours)
Time
(Months)
Perturbation Response
(c)
Physicochemically variable ecosystems less susceptible to perturbation
Physicochemically stable ecosystems more susceptible to perturbation
(Low)
Ecosystem Variability
(High)