

Response to reviewers

Anonymous Referee #1

The manuscript by Qixing Ji and colleagues investigates the controls on nitrous oxide concentrations in Chesapeake Bay. It is a topic that the authors are very familiar with and this expertise is reflected in the experimental design investigating the effect of nutrients and oxygen on nitrous oxide. The datasets are useful and should be published, but I recommend restructuring the manuscript to focus on the strengths of the work and dealing with the issues raised.

Major comments

[Referee] As can happen with studies which conduct repeated experiments at different time intervals with varying measurements, it is difficult at times to track all the activity. I believe there is a discrepancy between the Methods and Results, as Figure 2 shows N₂O production rates for July, November, and May, yet in the Methods you state that the experiments were only conducted in July. Instances like this make it very difficult for the reader to follow.

[Response] The N₂O production rates shown in figure 2 are measured in control incubations, which were performed on all three dates. In control incubations, samples received 5 μmol L⁻¹ ¹⁵N-nitrate or ¹⁵N-nitrite, respectively; and oxygen was removed from samples by helium flushing. The DIN manipulation experiments were only conducted in July. We explained this in page 4 line 99 and revised table 1 to minimize confusion.

[Referee] I had a hard time relating the experiments with the estuarine environment. If you want to measure nitrous oxide emissions from Chesapeake Bay, the work conducted in this study is not what needs to be conducted. You would need high resolution surveys of key hydrographic parameters coupled with nitrous oxide measurements, either continuously or at targeted times. I recommend the authors focus more on the experiments as a means to better understand the controls on nitrous oxide production and use Chesapeake Bay as the contextual background, rather than attempting to explain nitrous oxide dynamics in this estuary.

[Response] We agree with the reviewer's suggestion, and this focus on control is exactly what we intended, with the title stating explicitly that this manuscript is about examination of the control of N₂O production, rather than emissions, from the Chesapeake Bay. Previous study suggested the Chesapeake Bay is a N₂O source, which motives our study (line 47 – 56). We mention briefly that water column N₂O the may be emitted due to disruption of water column stratification (page 12, line 291 – 294), which points to further research directions. We'll revise accordingly in the next version.

[Referee] Two examples of the mis-match between the datasets reported and the contextual description provided in the Introduction are (1) The abstract talks about intense N₂O efflux from estuaries, but the results show nitrous oxide concentrations close to air-saturation values. (2) The title mentions seasonal anoxia, but this is not shown in the results.

[Response] For (1), this is a more of a “general” statement that estuaries can be sites of intense N₂O efflux, and previous studies have shown that the Chesapeake Bay is a N₂O source. This context motivated our research. The measurements and experiments showed that despite the fact that the water column in July was close to N₂O saturation level (line 171 – 175), N₂O production can occur (line 196 – 200).

For (2) The oxygen profiles obtained from this study (and many others) show that water column anoxia occurs in summer. In spring and autumn, the water column is oxygenated. These results demonstrate the water column anoxia is a seasonal event, as clearly shown in the two papers by Lee et al., which are cited in the manuscript.

A revised abstract will be presented in the next version to minimize confusion to the readers.

[Referee] I am not sure if the May 2017 dataset is necessary in Figure 1. It is helpful in Figure 2 only.

[Response] The May dataset illustrates seasonal variation in the usual parameters, and thus supports the seasonal nature of N₂O production and consumption. In May 2017, low oxygen condition was forming, along with higher availability of nitrate. These conditions are becoming favorable for denitrification in the water column.

[Referee] You need to include a description of how you calculate N₂O production in the Methods section.

[Response] The rate calculation is presented in section 2.4, equation 1

[Referee] The N₂O profiles puzzle me in the context of the other results. It looks like July 2016 is the only profile which has significant differences with depth, decreasing in concentration between 3 and 13 m. However, this time point is associated with the highest rates of N₂O production (Figure 2). Therefore, N₂O consumption is very important yet hardly mentioned in the manuscript. In context of your comment that estuaries are emitting large quantities of N₂O, the consumption processes deserve more attention.

[Response] N₂O consumption is indeed important; however it was not quantified nor addressed directly in the current manuscript. The work here focuses on the controls of N₂O production via denitrification. One of the future research direction is to examine the N₂O reduction during anoxic events. The fact that significant N₂O production was detected in the month when N₂O concentration was lowest implies that N₂O consumption was also occurring and probably minimizing efflux to the atmosphere (line 294 – 297).

[Referee] You should explain to the reader why you focused on the nirS gene and not other relevant genes

[Response] Good point. The nirS gene encodes the genetic material for nitrite reductase, the enzyme responsible for nitrite reduction to nitric oxide. NirS is often used as a proxy for the abundance and diversity of denitrifying bacteria (which was our application here) and is the gene in the denitrification sequence that is most reliably associated with a complete denitrification pathway (Graf et al. 2014). We explain in line 37 – 39.

Minor comments

[Referee] Page 1 Line 16 Change reducing to decreasing

[Response] Corrected.

[Referee] Page 2 Line 11 Agriculture such as paddy fields?

[Response] Is the reviewer referring to “agricultural land” in line 13? If so, paddy fields are not common in the Chesapeake Bay watershed. Agriculture is the main land use in this region and major agricultural activities in the area include livestock farming, greenhouse and nursery products (flowers, ornamental shrubs, and young fruit trees), corn and soybeans, all of which depend heavily on industrial fertilizers.

[Referee] Page 4 Oxidized nitrogen. I think this refers to NO₃ and NO₂. I recommend you write NO₃ and NO₂ if this is correct, as it avoids NO or NO_x. Also on Page 7, Line 18

[Response] Corrected.

[Referee] Page 3 Line 21 It would help orientate the reader if you provide a short explanation for why you chose these 3 depths. For example, why did you only sample below the oxycline in anoxic waters and out of curiosity, why did you not compare anoxic and oxic?

[Response] As the stated in the introduction, the anoxic events in the Chesapeake Bay is of great environmental and economic concerns. One of the motivations of this work is to examine the control of N₂O production under anoxic condition. We sampled oxygenated condition in May and November because anoxic conditions were not detected at the depths where they would have been expected during the seasonal anoxia, thus the oxic conditions were sampled for comparison and, not surprisingly, had lower rates of denitrification. It is likely that nitrification is important during oxygenated conditions. Future work more focus more on the oxic waters.

[Referee] Page 4 Line 14 Why do you inject N₂O to detect N₂O production? Is it an issue of detection limit? Or were you looking for N₂O consumption?

[Response] It is an issue of detection limit of mass spectrometer, which requires > 2 nmol of nitrogen. See line 93 – 94.

[Referee] Page 4 Line 14 If you inject 1.2 nmol, how do you get 20 nmol L⁻¹? Presumably you verified these target concentrations on a few bottles and you should state the final concentrations achieved.

[Response] N₂O concentration in the incubation bottles was estimated as follows: The incubation bottle is 60 ml in volume. Considering only 3 ml of headspace in the bottle, 90 – 95 % will dissolve in water phase (57 ml, 0.057 L) under experimentally relevant conditions. Therefore, $1.2 \text{ nmol} \times 0.9 \div 0.057 \text{ L} \approx 20.0 \text{ nmol L}^{-1}$. N₂O concentration was directly measured in the time course samples on the mass spec, so no assumptions were necessary in the actual rate calculations.

[Referee] Page 5 Line 1. Again, presumably you checked the final concentrations of oxygen against your target concentrations. I suspect you also did the air-equilibration at a single temperature which you should state.

[Response] Oxygen saturated site water was made by vigorously shaking an open-capped bottle with water collected at depth. Concentration was calculated using formula from Gordon and Garcia, 1992, at measured temperature (< 0.5 degC difference of in situ) and salinity. Oxygen concentrations were not measured directly in these experiments. We have, however, used an optical sensor to measure concentrations directly in the same kinds of bottles in similar experiments and the agreement between estimated target concentration and measured is excellent. Thus it is not necessary to measure every bottle every time. See line 113 – 114.

[Referee] Page 5, Line 19 The alternative to injecting N₂O standards into crimp-sealed vials is to air-equilibrate at controlled temperatures. This might be easier?

[Response] The measurement of N₂O is using purge-and-trap technique, which analyze all of N₂O contained in the vials by a mass spectrometer. Thus, the total amount of N₂O is important, even the N₂O standards may not be in equilibrium between the water and headspace.

[Referee] Page 7, Line 13 Where is the intense efflux that you mentioned in the abstract?

[Response] As explained earlier, this refers to a previous study documenting the Chesapeake Bay as a N₂O source. Conditions in estuaries are highly variable, in both time and space, which is one of the motivations for investigating the control mechanisms on N₂O production. Failure to detect efflux at this time does not mean that intense efflux does not occur at this site at other times, or in other parts of the Bay.

[Referee] Page 7, Line 13 If you are going to talk about saturation, you have to provide the saturation value for each of the three sampling occasions.

[Response] The surface N₂O saturation values in July, November and May were: 6.6, 10.4 and 12.0 nmol L⁻¹, respectively. These values have been included in page 7, line 171.

Anonymous Referee #2

General Comments:

[Referee] The authors have presented a well-designed and well-executed experiment on N₂O production in Chesapeake Bay. While the experiments and data are worthy of publication, the manuscript itself needs major revisions before it is accepted for publication. In reading the manuscript the introduction and methods were written clearly and concisely, but the results and discussion needs a substantial reworking. The paragraphs jumped from one topic to the next and often I found the subtitled headings were not appropriate for the range of topics covered in the text below. In the abstract the authors suggest two potential impacts of what reducing N to the bay will have on N₂O production. I think the paper is missing a more detailed explanation of how the experiments done lead to the conclusions they make, and they should expand on the specific details of these impacts that they suggest. I also think the authors could elaborate on the limitations of their study and what could be done in the future.

[Response] The reviewer's major criticism concerns the structure of results and discussion, which we will explain later. The main objective of this work is to examine the control of water column N₂O production via denitrification in the Bay. The experiments were designed to suit our objective, by increasing the availability of oxygen and dissolved inorganic nitrogen through manipulation, as well as measuring N₂O production in different seasons. In doing so we concluded that increasing DIN availability and decreasing oxygen availability could stimulate denitrification during summertime anoxia. In fall and spring the N₂O production rates via denitrification were low, probably due to lack of anoxic conditions and concomitant low abundance of denitrifiers.

The major limitation of our study, like other tracer incubation studies in aquatic environments, is that the N₂O production rates measured here should be treated as potential rates because the experimental conditions were not 100% identical to in situ environments (see line 204 – 206). Despite these artifacts, the conclusions that DIN and oxygen availabilities control N₂O production during anoxic events are robust. The current work could be complemented by these future research directions, (1) measurement of nitrification rates in oxic waters and associated N₂O production and nitrification genes; (2) measurement of N₂O reduction rates within anoxic layer and characterization of N₂O dynamics in the Chesapeake Bay; (3) Elucidation of intracellular nitrite exchange during nitrate reduction in natural waters; (4) Investigation of the physical, chemical and biological controls of N₂O emission in the Chesapeake Bay.

See the revised “Conclusion and outlook” section that explained the above.

Minor Comments:

Abstract:

[Referee] Pg1 line15: “N₂O production was positively correlated with the ratio of nitrate to nitrite concentrations.” Please clarify if this was in situ concentrations or the nitrate and nitrite added to the incubation.

[Response] This statement describes N₂O production rates under manipulated nitrate and nitrite concentrations. All of rates reported in the manuscript were measured using ¹⁵N tracers, meaning the substrate concentrations (nitrate and nitrite) were higher than in situ levels. We revised the sentence in line 204 – 206.

[Referee] Pg1 line18-19: What do you mean by nitrogen deficiency? What lengths of time are your referring to when you write “short term” and “in the long-run”, can you approximate the time scale?

[Response] The term “nitrogen deficiency” refers to nitrate and nitrite concentrations below detection. This occurs during 4 months of summer from June to September. We use “short-term” to describe a time scale of months, and “long-run” of years. See line 19.

Methods:

[Referee] Pg4 line15: Why do you add 20 nM N₂O before the incubation? If it is so you can have enough N₂O to measure the N₂O production, could you add it after the incubation is killed? Can you clarify here in the text? Do you think adding it before, could affect the production of N₂O?

[Response] It is an issue of detection limit of mass spectrometer, which requires > 2 nmol of N (see line 93 – 94). We agree that the added tracer concentration is slightly higher than in situ (6 – 12 nM). It is our intention to add the N₂O before the samples were preserved so as to have a more similar condition to in situ. The effect of N₂O concentration on the rate of N₂O production should be investigated.

[Referee] Pg4 line17: What was the total concentration of nitrite+nitrate of tracer added?

[Response] Here is the description of DIN manipulation experiment, which was conducted in July 2016 only (see line 99 – 100). The total concentrations of nitrate and nitrite were 5 μM each, and all the substrate concentrations of every experiments conducted are listed in table 1.

[Referee] Pg5 line8: How does 5uM of tracer compare to the in situ amounts of nitrite and nitrate.

[Response] This was stated in page 7. In July 2016, in situ nitrate and nitrite concentrations were below detection (0.02 μM), and the experimental conditions are in no way representing the in situ. Hence we treated the rates as potential rates. In November 2016, in situ nitrate and nitrite concentrations at the depth of experiment (17 m) were 5.0 and 0.4 μM, respectively.

Section 3.2 Active N₂O production by denitrification

[Referee] Pg8 line3: In the title you suggest all N₂O is from denitrification, it would be good discuss why it is not from nitrification, if you are using that as the section title.

[Response] Indeed. We only measured N₂O production via denitrification. Unfortunately we did not measure N₂O production via nitrification. We changed the title to “Active N₂O production in the water column” to minimize confusion.

[Referee] Pg8 line16: I think it’s good you say here they are potential rates of N₂O production, because you are removing all the oxygen, even if that’s not the in situ value. I am curious why there are no potential rates in May. Can you add some discussion on why removing the oxygen does not stimulate N₂O production in May versus November?

[Response] We explained in the following paragraph (see line 209 – 217). This is likely due to low denitrifier abundance (indicated by nirS gene abundance) in May, during which the anoxic condition had not yet developed. In addition, our anoxic incubation lasted around 2 hours; it is unlikely for denitrifiers to reactivate within such a short time scale.

[Referee] Pg9 lines1-13: This section seems disjointed from the paragraph above and the title of the section. This paragraph refers more to removal of fixed nitrogen from the bay rather than N₂O production.

[Response] Indeed. We will remove this paragraph in the next version.

Section 3.3 N₂O production pathways regulated by availability of nitrogen substrate

[Referee] Pg9 line16: I was confused by this term “NO₂- (NO₃-), is this synonymous with “NO₂- or NO₃- ”? If so, I would suggest changing it to the latter.

[Response] This is simply a combination of two sentences in one: "... increasing nitrite availability favors N₂O production from nitrite reduction" and "... increasing nitrate availability favors N₂O production from nitrate reduction." We change the sentence to "This suggests increasing NO₂⁻ or NO₃⁻ availability favors N₂O production from the reduction of respective substrate." in line 220 – 221.

[Referee] Pg10 lines1-21, pg 11 lines1-8: This could use a different subheading? Could you reformat the equations to be easier to read? Also in general this section could use a little more description, as is, it is a little hard to follow. I would start off the section with the punch line (on pg 11) and then describe why the calculations back up that statement.

[Response] We think another sub-heading is probably not necessary but we will reorganize the section in the next version. We'll add the conclusion "the exchange between intracellular and ambient nitrite during nitrate reduction to N₂O is limited" in the beginning of the section. The calculation is to find the ¹⁵N fraction label of N₂O when nitrite is fully exchanged, and we think it is rather straightforward. We used a different font and size to better illustrate the calculation in line 232 – 248.

Section 3.4 Oxygen inhibits N₂O production by denitrification

[Referee] Title: I suggest changing the title to something more detailed.

[Response] The title states the major conclusion of this section and seems pretty specific.

[Referee] Pg11 lines11-19: These sentences seem to belong to the other section about N influx to the bay. They could be moved or deleted during restructuring of discussion sections because it is somewhat repetitive from before.

[Response] Indeed. This paragraph will be organized in the "conclusion and outlook" section.

[Referee] Pg12 lines9-15: The result that there is a different oxygen tolerance for nitrite vs. nitrate reduction is really interesting. I think here is where you should expand on more why you get this result. I know you touch on it more in a later section, but it might be best to put it all together (maybe in it's own section). Could it be from nitrifier denitrification? How would nitrite oxidation affect the production rate? At low levels of oxygen I would suspect there would be some nitrite oxidation to nitrate.

[Response] It is possible that nitrifier denitrification is responsible for part of N₂O production via nitrite reduction. This is because increasing oxygen availability will inhibit the activity of denitrifiers, but not nitrifiers (see line 277 – 285). It is not possible to have a complete explanation with the data presented here (see line 150 – 152).

We did not measure nitrite oxidation rates in the samples but agree that it is likely nitrite oxidation was also occurring. We expect nitrite oxidation to have a negligible effect on N₂O production rates, however, since N₂O is not a product or intermediate in that reaction and expected rates of nitrite oxidation would not significantly affect the concentration of the substrates directly involved in N₂O production. (Nitrite oxidations rates in nM/d would not affect the substrate concentrations, which were in the micromolar range.)

[Referee] Pg12 lines16-19: This paragraph seems out of place? Again, would you suspect some nitrite oxidation in these nitrate reduction to nitrite measurements? How would that affect results?

[Response] See above.

[Referee] Pg13 lines3-4: This line should go with the section on differences of nitrite-stimulated production vs. nitrate-stimulated N₂O production.

Pg13 lines10-18: This paragraph is out of place.

Pg13 lines19-22: Should this be in the conclusion and outlook?

[Response] The section 3.4 was re-organized to improve clarity and make tighter connections between the data and conclusions.

Section 4 Conclusion and outlook

Pg14 lines14-19: I would reorder this section and put these lines first.

Conclusion and outlook section as a whole: Could you add something connecting the two pathways of management (nitrogen influx vs. oxygenation)? Also, could you quantify how each change inhibits N₂O production?

[Response] Indeed. The current work will be complemented by these future research directions, (1) measurement of nitrification rates in oxic waters and associated N₂O production and nitrification genes; (2) measurement of N₂O reduction rates within anoxic layer and characterization of N₂O dynamics in the Chesapeake Bay; (3) Elucidation of intracellular nitrite exchange during nitrate reduction in natural waters; (4) Investigation of the physical, chemical and biological controls of N₂O emission in the Chesapeake Bay.

See “Conclusion and outlook” section that explained the above.

Anonymous Referee #3

Summary

The authors present an examination of N₂O dynamics studied in the Chesapeake Bay during three samplings. Since the Chesapeake Bay exhibits a strong seasonal shift in water column redox state, the study focused on trying to link these shifts with N₂O production mechanisms at and below the oxic/anoxic interface. The authors bring a range of chemical, molecular and isotopic tools to bear on these dynamics, with an emphasis on elucidating N₂O producing processes occurring in the bottom waters and the primary controls on them. This contribution is timely – as coastal and estuarine systems are dynamic and generally understudied with respect to their place in the global N₂O budget. Overall the data appear to be of high quality. The manuscript is generally well written, though parts could benefit from some reorganization. I have some questions about the data interpretation as outlined below. Overall I think this work is worthy of publication, but that the manuscript could be improved through some more careful consideration of clarifying some sections.

Major Comments

[Referee] Pg 9 Ln 15: I appreciate the use of targeted assays for NO₂⁻ and NO₃⁻ reduction, though it is unclear whether this was designed to constrain/target nitrifier denitrification specifically or explicit nitrite reducing denitrifiers (???). Clearly denitrifying organisms also use NO₂⁻ in their electron transport chain. In Section 3.3 the authors attempt to tackle this – and I appreciate the argument that they are making about NO₂⁻ transport across the membrane – but I feel that this section is confusing as written. Using calculations laid out here, and making a few key assumptions, the authors conclude that since the level of ¹⁵N label in the N₂O pool is much higher than if there had been full exchange, then exchange between the cellular and ambient NO₂⁻ is minimal. I acknowledge that this is a difficult aspect of N cycling to track, but I am not overly convinced that they have proven that this type of exchange is ‘minimal.’ Their calculation demonstrates that high levels of exchange are not occurring, but whether modest levels might be influencing the results is unclear. Perhaps this argument could be streamlined and clarified.

[Response] The reviewer acknowledges the difficulty of our attempt to estimate intracellular nitrite exchange; we appreciate it. The hypothesis is: nitrite is fully (100%) exchanged during nitrate reduction to N₂O. And the calculation result shows that ¹⁵N-fraction labeled of N₂O from the calculation does not match our measurements (see line 249). Therefore, we reject the hypothesis. Yes, it is possible that some level of exchange might occur, but they would be undetectable by this argument. We think it is impossible, and beyond the scope of this paper, to quantify the actual percentage of nitrite using the data presented here. More elaborate experiments can be conducted in the future to tackle this question.

[Referee] Additionally, perhaps the introduction needs some sort of clearer description of the types of metabolisms being targeted by the study (complete denitrifiers, nitrite denitrifiers, nitrifier denitrification). These classifications of microbes and processes are confusing even to those who regularly study N cycling.

[Response] We focus only the reductive pathways of N₂O production under anoxic conditions. Given the limitation of ¹⁵N tracer study, only the denitrification pathways “NO₂⁻ → N₂O” and “NO₃⁻ → N₂O” can be quantified (line 59 – 60). It is therefore very difficult to attribute the pathway to certain groups of microbes. To minimize confusion, we generalize the term “N₂O production during denitrification” throughout the text. The current data set does not support in-depth discussion of functional microbial groups responsible for N₂O production because we cannot differentiate among the different kinds of microbes that can perform nitrite reduction to nitrous oxide using tracer experiments.

Minor Comments

[Referee] Pg 1 Ln15: I believe nitrate and nitrite are reversed here (and many other times throughout – leading to some frustration/confusion).

[Response] We did not follow the reviewer’s suggestion for this sentence. The experimental data shows that, higher nitrate or nitrite availability positively correlates with N₂O production rates from respective substrates. The sentence itself is correct.

[Referee] Pg 1 Ln17: Since the field data demonstrate that there is no net flux to the atmosphere– it seems odd to emphasize N₂O efflux here.

[Response] To minimize confusion, we changed the word “efflux” to “production”

[Referee] Pg 3 Ln 19: Please clarify whether a headspace was left in the incubation bottle or not.

[Response] The headspace (3 mL) was left throughout the incubation.

[Referee] Pg 4 Ln 1: course not courses

[Response] Thanks for catching that error – it was in line 21.

[Referee] Pg 5 Ln 3: Please indicate whether oxygen concentrations were measured or calculated?

[Response] The concentrations were calculated, see line 110 – 113.

[Referee] Pg 6 Ln 1: Why were no other functional gene assays performed? I think this is justified later, but given that nirS only reflects denitrification – its linkage with N₂O from this pathway is clear, yet reveals little about the dynamics of the other pathways investigated as I understand. Why not include nirK? Or norB?

[Response] Good point. The nirS gene encodes the genetic material for nitrite reductase, the enzyme responsible for nitrite reduction to nitric oxide. NirS is often used as a proxy for the abundance and diversity of denitrifying bacteria (which was our application here) and is the gene in the denitrification sequence that is most reliably associated with a complete denitrification pathway (Graf et al. 2014). See line 37 – 39.

[Referee] Pg 7 Ln 19: I believe nitrate and nitrite are reversed here again.

[Response] Corrected.

[Referee] Pg 7 Ln 25: “positively correlates” – yes, but this is difficult to defend statistically with n=3.

[Response] The sentence is changed to “...nirS abundance increases with increasing measured rates of N₂O production.” See line 192.

[Referee] Pg 13 Ln 2: I would suggest “microbial groups” instead of microbial communities (which may imply the ‘greater community’ – not just N cycling organisms ?).

[Response] The word “groups” has replaced “communities”.

[Referee] Pg 14 Ln 5: It seems that if nitrifier denitrification and ammonia oxidation are implicated in N₂O production as discussed – then the nitrifier community dynamics would also play an important role and should be acknowledged?

[Response] Indeed. The reviewer points out one of the future research direction, that is to examine the link between nitrifying community and N₂O production via nitrification. See line 310 – 317.

Anonymous Referee #4

[Referee] The paper reports an experimental study of rates and pathways of nitrous oxide production in Chesapeake Bay waters. Water was sampled on three occasions (spring, summer, autumn) and incubated with N-15 labelled nitrate or nitrite under anoxic conditions. Additional incubations were made with oxygen added back to investigate the oxygen sensitivity of the processes. Based on the results, the authors draw conclusions about the controls of N₂O emissions from the Bay. The paper addresses an interesting subject and the experimental work is of good quality. However, the results do not provide strong support for the conclusions because the experimental conditions do not sufficiently reflect the environmental conditions in the Bay.

Also, although there are few previous experimental studies from comparable environments, the paper largely neglects the large number of previous studies on N₂O dynamics in estuaries, although these do provide some insight to the controls of N₂O emission.

Importantly, the literature points to nitrification (ammonium oxidation) as a major N₂O source in estuaries whereas the present study only investigates N₂O production through denitrification. Without data on the rates and controls of N₂O production by ammonia oxidation (i.e. experiments with N-15 labelled ammonia at different oxygen concentrations), no conclusions can be drawn about the controls of N₂O emissions from Chesapeake Bay.

Based on the mismatch between the experiments and the conclusions, I recommend that the paper be rewritten to focus on what the experiments can actually tell us, i.e. how N₂O production during denitrification is affected by oxygen, and how production from nitrate and nitrite seem to function independently, which is novel and interesting.

I also warn against trying to translate the results into understanding denitrification as a N₂O source in the Bay as a whole, and the role of anoxia in this, because denitrification is an interface process there, and the anoxic water body might serve as net sink for N₂O, drawing it down from the overlying oxycline. Here, fine scale profiling of N₂O across the interface might be more informative than the experimental approach.

[Response] The reviewer's major criticism is that the manipulated experimental conditions led to the conclusion of the Chesapeake Bay being a N₂O source, whereas the static concentration profile suggested the Bay as a N₂O sink. We shall explain as follows:

(1) Our work of environmental control of N₂O production is motivated by previous studies that identified the Chesapeake Bay as a N₂O source (Elkins et al., 1978; McElroy et al., 1978).

(2) Through incubation experiments, it is straightforward to draw the conclusion from the results: Adding nitrogen substrates and removing oxygen stimulate N₂O production in summer and autumn. Thus the Bay is potentially a N₂O source when pulses of nitrogen enters the water body that is experiencing summertime anoxia (see line 204 – 208). Conditions in estuaries are highly variable, in both time and space, which is one of the motivations for investigating the control mechanisms on N₂O production. Failure to detect efflux at this time does not mean that intense efflux does not occur at this site at other times, or in other parts of the Bay.

(3) Indeed there's a large body of literature reporting the variation of N₂O fluxes in estuaries around the world. Many of them applied the traditional methodology: By quoting surface N₂O supersaturation, wind speed, temperature, water turbulence, etc. and measure N₂O fluxes, which is then related to water column oxygen and nitrogen availability. A small number of work applied nitrogen isotopic approach to study the mechanism of N₂O production. The isotopic approach reveals the dynamic, potential N₂O production that is masked by the static

concentration profile. In addition, the isotopic approach allows to quantify the environmental controls of N₂O production in the water column.

We disagree with the reviewer about the lack of similarity between experimental and actual conditions. Our incubation experiments were designed to study the effects of oxygen and nitrogen availability on N₂O production, by changing experimental nitrogen (1 – 10 μM) and oxygen concentrations (0 – 10 μM) that often occur in the Chesapeake Bay (Lee et al., 2015).

As we have stated in the Introduction, nitrification is another important pathway for N₂O production. The focus of the manuscript is N₂O production under naturally occurring and laboratory anoxic condition. It is unlikely that nitrification could occur, and thus nitrification is beyond the scope of this manuscript. We thank the reviewer for pointing out one possible future research direction: study the N₂O production via nitrification and denitrification and their environmental controls with extended spatial and temporal coverage across the Chesapeake Bay. See line 310 – 317.

The scope of this manuscript is to examine the control of N₂O production by nitrogen and oxygen availability during denitrification under naturally occurring and laboratory anoxic condition. It is another future research direction that study the N₂O dynamics by fine scale profiling of N₂O across the interface so as to demonstrate whether the anoxic Chesapeake Bay serves as a net sink or source for N₂O.

Specific comments

[Referee] 3, 3: Why pilot? – to me this indicates preliminary results

[Response] We chose “pilot” because we are the first group to study N₂O production using 15N tracer at a single station in the Chesapeake Bay, and we examined the environmental control of denitrification pathway for N₂O production. This is a small scale study and the results are important for a larger scale, more comprehensive study in the future.

[Referee] 7, 10: The detection limit for H₂S by smell is ~ 10 μM. It seems strange if no sulphide was present at all, if all more favourable oxidants were depleted.

[Response] It would be more helpful if the reviewer list the reference.

First, the odor threshold of H₂S is ~ 0.5 ppb [1], and Henry’s Law constant of H₂S is ~ 0.1 mol/kg/bar [2]. Under atmospheric pressure with 0.5 ppb H₂S, the equilibrium concentration of a H₂S solution is

$$0.5 \times 10^{-9} \text{ bar} \times 0.1 \text{ mol/kg/bar} = 0.05 \times 10^{-9} \text{ mol/kg} \approx 0.05 \text{ nM.}$$

The calculation shows the detection limit for H₂S by smell is on the order of sub-nM range. Under such a low concentration, the statement “sulphide compounds were most likely not present” still holds.

[1] Iowa State University Extension (May 2004). The Science of Smell Part 1: Odor perception and physiological response. PM 1963a.

[2] NIST Chemistry WebBook, SRD 69.

<https://webbook.nist.gov/cgi/cbook.cgi?ID=C7783064&Mask=10>

[Referee] 9, 5: I would leave out this back-of-the-envelope estimate of denitrification. It does not add new, robust insight to N loss in C. B.

[Response] Indeed. We will remove this paragraph in the next version.

[Referee] 9, 23 and onwards: This is an important finding, which requires elaboration. I suggest calculating the direct contribution from nitrate to N₂O for all the different combinations of nitrate and nitrite concentrations instead of just one example. If rates are assumed to be constant

during the incubation, a simple model can describe the concomitant production and consumption of nitrite and hence how N-15 should accumulate in the extracellular nitrite pool if the intermediate nitrite were exchanging freely.

[Response] The reviewer points out the need to quantify intracellular nitrite exchange during nitrate reduction. We think it is impossible, and beyond the scope of this paper, to quantify the actual percentage of nitrite using the data presented here. We attempted to examine one hypothesis: nitrite is fully (100%) exchanged during nitrate reduction to N₂O. And the calculation result shows that ¹⁵N-fraction labeled of N₂O from the calculation does not match our measurements (see line 249). Therefore, we reject the hypothesis. More elaborate experiments can be conducted in the future to tackle this question.

[Referee] 10, 9: I don't understand this formula. How can the amount of ¹⁵N-nitrite produced depend on either the total nitrate and the total nitrite concentration? Shouldn't it simply be: Rate of NO₂⁻ production from NO₃⁻ × incubation time × initial fraction labelled of NO₃⁻?

[Response] The formula was incorrect. Now the formula has changed to “Rate of NO₂⁻ production from NO₃⁻ × incubation time × initial fraction labelled of NO₃⁻”. The result is 0.2 μmol-N L⁻¹ hr⁻¹ × 2 hr × 0.16 = 0.064 μmol-N L⁻¹. And the revised value will be inserted to the subsequent calculations. The resulting ¹⁵N fraction of N₂O will be 0.0052 (see line 248).

[Referee] 12, 1: don't understand this. Oxygenation will just shift the zone of denitrification and N₂O consumption to greater depth (until it reaches the sediment). It won't necessarily inhibit it.

[Response] Good point. The N₂O concentration profile in July indicates that N₂O consumption is occurring. Our incubation experiment showed N₂O production is occurring at the oxic-anoxic interface. These results demonstrate denitrification is responsible for N₂O production and consumption in different layers of water column. Whether the Chesapeake Bay is net N₂O source or sink requires further evaluation, and will be one of the future research directions. See line 287 – 299.