We appreciate the critical comments from the two referees.

Below we would like to address the two problems raised by both referees first, and then would like to respond to each comment.

(1) Page 16627, Line 4 and Table 3, the high yield (> 100%) of N2O from NO

We prepared the NO solution by purging 30-mL ethanol with pure NO gas using a 69-mL vial sealed with butyl rubber stopper and needles at room temperature (25°C). We calculated the resulting concentration of NO in ethanol using the solubility at 25°C. However, at the time of adding the solution (about 42 micro liter) to the reaction vial, the NO concentration might have increased because the solution was stored in a refrigerator (4°C) before use. We re-evaluated the concentration by estimating the amount of NO in the gas phase of the solution vial and using the NO solubility at 4°C. Our careful check also revealed a minor error in translation of the solubility coefficient. As a result, Table 3 has been revised as follows. The yield can be interpreted as minimum value because NO concentration in the ethanol solution should have been less than the concentration expected at 4°C.

Table 3.

Experiment	NOR	Yield(%)	$\delta^{15}N^{bulk}$(‰)	$\delta^{15}N^{\alpha}$(‰)	$\delta^{15}N^{\beta}$(‰)	$\delta^{18}O$(‰)	SP(‰)
A	+	129.4	17.1	14.3	19.9	24.0	-5.7
A	+	127.9	17.1	15.1	19.1	24.1	-4.0
A	+	149.2	17.1	14.6	19.6	22.9	-5.0
A	-	7.6	14.5	22.0	6.9	-7.5	15.1
C	+	65.0	11.0	6.5	15.5	18.7	-9.0
C	-	7.4	11.7	19.9	3.5	13.5	16.4
D	-	8.9	16.3	24.7	8.0	-6.5	16.8
Average		117.9±36.6	15.6±3.0	12.6±4.1	18.5±2.1	22.4±2.5	-5.9±2.1

As seen in the revised table, however, the yield still exceeds 100% in the first three lines. The only reason we could imagine is that the pressure of the gas inside the solution vial might have been higher than 1 atm. This could happen if the needle that vented the purge gas (NO) from the vial was partially clogged or if there was time lag between the removal of venting needle and the removal of NO-supplying needle. If this was the case, more NO could have been dissolved in ethanol. We cannot validate this possibility because we did not monitored the inside pressure of the vial (even if we were aware of it, it would have been difficult). But this might explain the difference between the results obtained in experiment A and C. In the experiment C, concentration of the NO solution might have been identical to what we expected.

In summary, we cannot fully explain the high yield, but it is safely concluded that the N2O is produced by reduction of NO with NOR from the comparison with the

control experiments without NOR and that isotopomer ratios corrected for the blank N2O show the characteristic value for the reaction. We would like to revise the paragraph in Page 16628 Line 14-21 as follows.

"The yield of N_2O produced by the reduction of NO with NOR exceeded 100%, which could be caused by underestimation of initial NO concentration. Since the NO-saturated ethanol solution was prepared by purging the ethanol with pure NO gas in a sealed vial at room temperature (25℃), we calculated the NO concentration based on NO solubility at 25℃ and 1 atm. However, there is a possibility that the vial inside was pressurized and the excess NO dissolved in ethanol during the preservation at 4℃."

(2) Page 16627, Line 13, Table 4, and Fig. 2, the increase of N2O production by *N. oceani* with the increase in O2 concentration

The oxygen concentration wasn't measured or maintained in the experiment vials and the concentration certainly decreased as suggested. However, we can estimate the oxygen concentration in the vials after the incubation with 21% initial concentration at 18% based on the produced amount of nitrite and N2O and stoichiometry of the reactions below (note that AOB is chemoautotroph and oxygen is not used by other metabolism reactions). The relatively high retention of oxygen after long incubation (>14% after 28-day incubation) is also reported by previous study (Poth and Focht, 1985). This estimate implies that the medium in the incubation vial wasn't anaerobic. In addition, it is unlikely that part of the medium (e.g., bottom) became anaerobic enough to enhance nitrifier-denitrification because the medium in the incubation vials were well-mixed by shaking. Even if nitrifier-denitrification could have occurred, the SP value of N2O (> 20‰) obtained at initial oxygen concentration of 21% is distinct from the value expected for N2O produced by nitrifier-denitrification (about 0‰, Sutka et al., 2003;2004;2006). Thus, the effect of possible oxygen consumption on our conclusion is small.

$$NH_3+1.5O_2=HNO_2+H_2O$$

$$NH_3+O_2=0.5N_2O+1.5H_2O$$

Even so, it is true that our experiment is not an accurate representation of N2O production as a function of O2 as suggested by the referees. We'd like to add the following sentence in the discussion section.

"During the incubation, oxygen must have been partly consumed by the bacteria. However, a quantitative estimate based on the stoichiometry of ammonia oxidation to nitrite or N2O and amount of the products indicates that the oxygen consumption was relatively small (e.g., oxygen concentration after the incubation with 21% initial O2 was estimated at 18%)"

The fact that higher N2O production at 21% O2 could be caused by the difference of strains. To discuss this, we would like to add following figure showing a

two-step model of HAO reaction (Fig. 4) and sentences citing the recent structural analysis of the HAO from *N. europaea* in the substrate-binding state (Maalcke et al., 2013) in Page 16631 (line12, after "… respectively.") Maalcke et al. have proposed that the side-chain of Tyr (it places near the P460 molecule and forms active center of *N. europaea* HAO) is significant for the second step of HAO reaction. The Tyr is, however, not conserved in the HAO of gamma -AOB by replacing to Asn. We added discussion on the possibility that the result shown in Fig 3 (In contrast to the *N. europaea*, *N. oceani* NS58 generates N2O in the aerobic condition) maybe explainable by the structural deference between the active center of *N. europaea* HAO and that of *N. oceani* NS58 enzyme.

The higher N2O yield with higher initial O2 concentration observed in this study (Table 4) could be explained as follows. As initial O2 increase, the rate of ammonia oxidation is increased and the first product, NH2OH, is increased. Because the reactivity of HAO of *N. oceani* is more sensitive than that of *N.europaea* as explained above, higher N2O production was observed at aerobic condition.

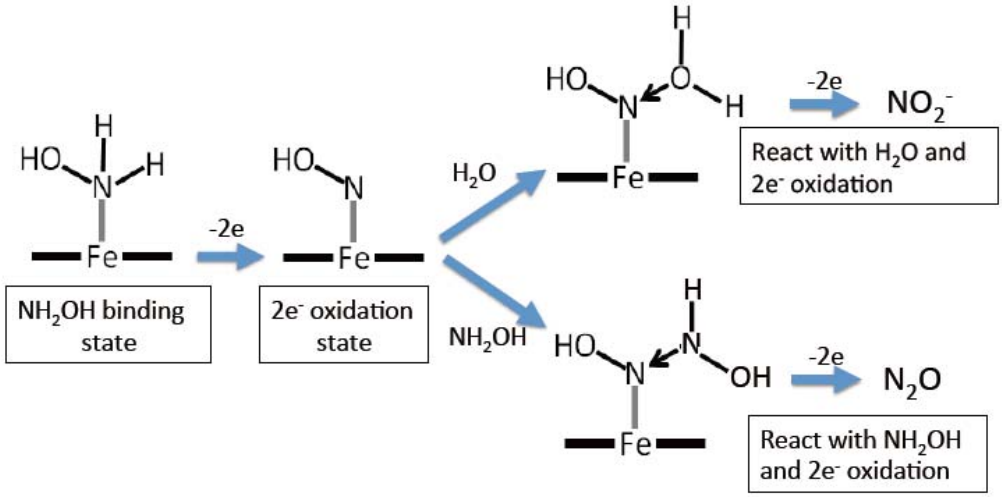

Fig. 4. Two-step model of NH_2OH oxidation with HAO

Sentences to be added to Page 16631 line12 after "… respectively"

"Catalytic center of the N. europaea HAO for hydroxylamine oxidation is formed by the P460 molecule and the side-chains of three amino acids (Asp291, His292, Tyr358; N. europaea HAO numbering) that surround the distal side of the heme (Igarashi et al. 1996). Although two of the three residues, Asp291 and His292, are completely converved among all the HAOs, another Tyr358 is replaced to Asn in the enzymes of the gamma-AOBs including the NS58. Recently, crystal structure of the N. europaea HAO in the substrate-binding state was reported (Maalcke et al. 2013). In this

paper, the authors has proposed that the side-chain of Tyr358 would participate in the addition of water molecule to the intermediate species bound on the P460, that is namely the second step of the two-step reaction shown in Fig. 4. The replacement of the Tyr to Asn (Asn368, N.oceani NS58 HAO numbering) may affect obstructively on addition of H_2O to the intermediate in the HAO reaction. For that reason, it is probable that the N_2O generating process revealed in the Fig. 4 would occur more easily in the N. oceani NS58 HAO rather than in the N. europaea HAO. In addition, this explanation is consistent with the present result that, in contrast to the N. europaea, N. oceani NS58 also generates N2O in the aerobic condition, as shown in Fig 3."

Reference:
Maalcke, W.J., Dietl, A., Marritt, S.J., Butt, J.N., Jetten, M.S.M., Keltjens, J.T., Barends, T.R.M., and Kartal, B.: Structural basis of biological NO generation by octaheme oxidoreductases, J. Biol. Chem. published online December 3, 2013.

Response to Dr. Ostrom's comments

Line 22: Details of enzyme purification missing.

We are not sure which part the reviewer mentions because page number is not specified, but we would like to add following details in section 2.2, 3.1, and supplement figure.

Page 16620 section 2.2 line19-21 (replacement of the two sentences)

"HAO was purified from the cultivated NS58 cells through three preparative steps, including $(NH_4)_2SO_4$ fractionation, gel-filtration and hydrogen-bonding chromatography according to the previous report (Hozuki et al. 2010)."

Page 16623 line 19-23 following sentences are deleted

~~The washed cell suspension of 10 ml in volume was put into the glass vial, then the vial was sealed by a butyl rubber stopper and an aluminium cap. After sealing, the headspace (58 mL in volume) of the vial was replaced with pure N_2 or O_2/N_2 mixture that contained 2% (v/v) O_2 (Shizuoka Sanso Co., Shizuoka, Japan) by gently bubbling for 15 min.~~

Page16623 line24

"Cell density of the medium in the reaction vial was estimated as 5.8 x 10^6 cells/ml by direct counting of the fixed and 4',6-diamidino-2-phenylindole (DAPI)-stained cells using epifluorescent microscope according to Takenaka et al. (2007).After sealing, the headspace (58 mL in volume) of the vial was replaced with

pure N_2 or O_2/N_2 mixture that contained 2% (v/v) O_2 (Shizuoka Sanso Co., Shizuoka, Japan) by gently bubbling for 15 min."

References:

Takenaka, T., Tashiro, T., Ozaki, A., Takakubo, H., Yamamoto, Y., Maruyama, T., Nagaosa, K., Kimura, H., and Kato, K. :Planktonic bacterial population dynamics with environmental changes in coastal areas of Suruga Bay, Microbe. Environ., 22, 257-267, 2007.

Section 3.1

"Purity of the HAO from N. oceani NS58 was confirmed by SDS-PAGE. As shown in Supplementary Fig. 1, SDS-PAGE of the purified enzyme gave single protein band whose molecular weight appeared to be 182,000 when the sample was pretreated by SDS (lane 2). By treating the enzyme with SDS and β-mercaptoethanol, accompanying the major band, minor protein band of which the molecular weight were 140,000 and 60,000 appeared in gel (lane 1). Chemical cleavage of thioester bonding between heme c and Cys residue in the enzyme resulted the appearance of protein band whose molecular weight was 60,000 (lane 3), suggesting that the enzyme molecule would be composed of homotrimer of the subunit proteins which were covalently bound each other that were mediated by prosthetic heme c molecules as previously reported in N. europaea HAO (Terry & Hooper 1981, Igarashi et al. 1997). Two minor protein bands appeared in lane 2 probably correspond to the monomeric and dimeric configuration of the subunit molecule of the HAO. Generally, AOB possesses two types of hydroxylamine oxidizing enzyme; one is the present HAO having trimeric configuration of octaheme c subunit, and another is cytochrome P460 which is a monoheme protein of which the molecular weight is only about 18,000 (Numata et al. 1990). Gel-filtration was applicated for purification of the N. oceani NS58 HAO, therefore, contamination of the cytochrome P460 in the enzyme preparation should be improbable. The result of electrophoretic analysis is also consistent with the absence of the components that would interfere the correct interpretation of the present experiments (lanes 1 and 2 in Supplementary Fig. 1)."

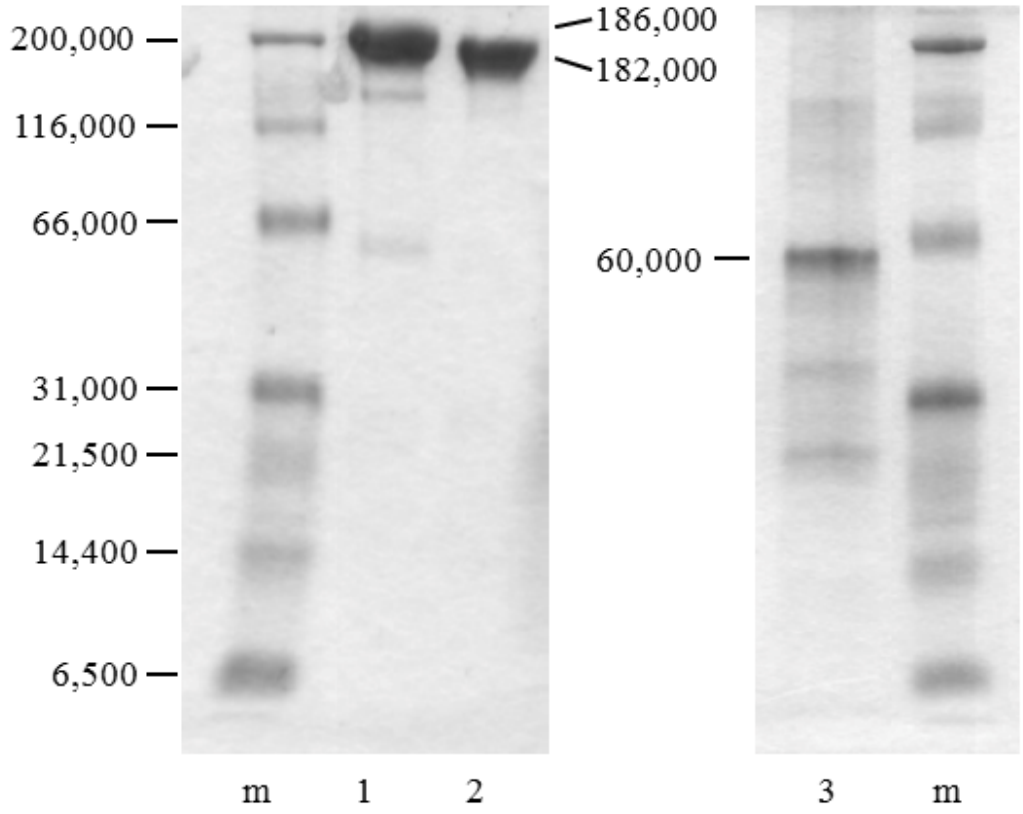

Fig. S1. SDS-PAGE of the purified HAO from *N. oceani* NS58. The purified enzyme (5 μg protein) was mixed with 4 % SDS with (lane 1) or without (lane 2) 4 % β-mercaptoethanol. After boiling for 5 min, the resulting samples were loaded on the 8 % polyacrylamide gel. Electrophoresis of the heme-cleavaged sample was also carried out in lane 3. Standard proteins, with their molecular weights shown in the left side of the gel, are in lanes marked by m. Minor protein bands that appeared at lane 3 may due to the chemically-fragmented and aggregated polypeptides generated from the subunit molecule of the HAO.

Line 13: Greatest N2O production under highest O2 level? Not what I would have expected.

Line 23: Need for improved writing I believe these were "batch" cultures which means that the O2 level likely dropped during the course of the experiments. This might explain why there was more N2O production with 21% O2; O2 may have rapidly been exhausted at the lower O2 level and declined to low levels in the culture at 21% O2. Thus this experiment is not likely an accurate representation of N2O production as a function of O2.

See above response (2).

Page 28, Lines 17 and 20. I believe you mean to indicate "preservation" and not "reservation".

Thank you. We correct the word as suggested.

Line 21. I am not certain if solubility constants for NO in ethanol are known but it is well known that cold solutions hold more gas than warm solutions. Thus the authors' speculation that colder temperatures might have resulted in a higher NO concentration in solution than expected is reasonable. However, there would have to have been a source of NO in storage for the ethanol to obtain a new and higher equilibrium concentration. As NO is very reactive this seems unlikely. The only reasonable possibility would be if they stored the ethanol with NO in the headspace but this also seems unlikely to me. The authors need a better explanation of these results.

See above response (1).

Page 29, Line 5: In Ostrom and Ostrom (2011), we suggested that the SP for N2O production is more likely controlled by the specific enzyme involved rather than by the "species". Do the authors have any indication that the specific enzyme or enzyme functional group (metal co-factor) differed between the species?

We agree that SP for N2O production is controlled by the specific enzyme. However, recent study reported a slight structural difference in the active center of HAO between different species (see below response to your other comment). We consider this could also be the case for NOR and it might cause slight difference between SP. We modify the sentence as follows.

"This implies that SP might depend on a small structural difference in the active center of NOR between the studied species or that previously reported SP for N2O from NO3-/NO2- reduction could have been affected by other processes like NH2OH oxidation."

Line 7-8: As written, the sentence suggests that N2O can be produced directly from nitrite via NIR. The authors should indicate that N2O is produced from nitrite by the sequential reduction of nitrite to NO (using NIR) followed by reduction of NO (NOR).

As suggested by the reviewer, we'd like to revise the sentence as follows.

"It is known that AOB produce N2O as a byproduct during the oxidation of NH2OH to NO2- with HAO and that some species of AOB can also produce N2O from nitrite by the sequential reduction of nitrite to NO (using NIR) followed by reduction of NO (NOR)."

Page 29, Lines 6-23: In all actuality, N2O production by nitrifiers is enhanced under low O2 conditions not complete anaerobic conditions (see Poth and Focht,

1980). N2O production by nitrifiers decreases markedly at no O2. It is very likely that the production of N2O as O2 levels drop in cultures of nitrifiers is the result of nitrifier-denitrification. The authors should describe N2O production during nitirification more accurately.

What is most surprising, however, is that the greatest degree of N2O production is at 21% O2; which is in contrast to prior studies of nitrifiers in culture which show little N2O production at these O2 levels (Poth and Focht, 1980 for example but there are others). I think it is very likely that as the O2 levels in the headspace of these experiments dropped over the course of the experiment. If the O2 levels were not maintained, or at least monitored over the course of the incubations, then the authors cannot relate N2O production or SP to the O2 level. This is a significant detriment to the study.

Further, I find the relationships based on only three levels of O2 are not very strong. Lastly, if O2 levels were not controlled there is little point to quantifying the relative contribution of hydroxylamine oxidation and nitrifier-denitrification to N2O production on the basis of SP. Thus the statement that even under aerobic conditions nitrifier-denitrification occurred cannot be supported. It is quite likely that O2 levels dropped over the course of the experiment. Given this, the calculations of the relative importance of N2O production by the two processes (Eq. 4) are not valid. Overall, this was too simplistic approach.

See above response (2). We agree that our assumption that N2O is produced only by the two processes is simple, and would like to add the following sentences after the first sentence in Line 24 and before Eq (4) in Page 16629.

"Here we assume that only NH_2OH oxidation and nitrifier-denitrification were the N_2O production pathways although there was a possibility that N_2O was produced by other processes such as anaerobic ammonia oxidation because N2O and nitrite were detected even under anaerobic condition (0% O_2). This assumption allows a simple interpretation that the relative contribution of hydroxylamine oxidation and nitrifier-denitrification to N_2O production on the basis of SP as follows."

Page 31: This is a very simplistic explanation of production mechanisms and difficult to follow. A diagram or figure would be quite helpful.

We add the Fig. 4 (see above) and the sentences as follows (Page 16631 line 6).

"To explain the high SP value of the N_2O generated by HAO reaction, two-step reaction mechanism was proposed as indicated in Fig. 4."

I find the structure of Table 3 confusing; why average by day? If letters indicate different days why average across days.

Table 3 is revised as shown above.

Response to Dr. Stein's comments

I would particularly concur with the problematic calculations leading to greater than 100% yield of N2O from NO in Table 3, particularly since the authors argue that this difference was likely from a problem with temperature of the NO preparation. If this is the case, the authors should re-do the experiments at the appropriate temperatures for accurate comparison.

See above response (1).

1) **Characterization of purified enzymes. What data did the authors collect to validate that they had purified the correct enzymes? There are two enzymes that can oxidize NH2OH to nitrite in ammonia oxidizing bacteria – HAO and cytochrome P460 (which also requires NO as a substrate). The authors should show some analysis – protein size, absorbance spectra, etc. – that validates purification of the proper homotrimeric enzyme complex that is HAO.**

Purity of the NS58 HAO was discussed based on the result of SDS-PAGE analysis where the profile of the protein bands on the gel was shown in the supplementary data (Fig. S1, see above). The result indicates that another hydroxylamine-oxidizing enzyme, cytochrome P460, was absent in the purified HAO sample used in the present study.

2) **I am quite surprised that the authors attained a cell pellet from 70 mL culture of N. oceani! How many cells were present in the culture? How does the density correspond to that of other studies?**

This is our mistake. The two sentences (p11623 L19~23) is removed from the manuscript. Cell density of the suspension used in this *in vivo* study was adjusted to that of the NS58 culture in the late-exponential ~early-stationary growth phase (5.8 x 10^6 cells/ml). Method for determining the cell density in the reaction solution was added. The cell density was comparable to high cell density experiments (1.5×10^6-1.5×10^5Cell mL^{-1}) reported by Frame et al., 2010.

The reported amount of N2O from ammonia oxidation (Fig. 2) is extremely high for N. oceani, even when compared to other AOB like N. europaea that are known as high producers.

The measured N_2O yields($1.5\text{-}9.5 \times 10^{-5}$) were comparable to *Nitrosococccus.ocenus* (2×10^{-4}) reported by Goreau et al.(1980) and *N.marina* (1.3×10^{-4} at high cell density experiments) reported by Frame et al.(2010).

Furthermore, it is quite surprising that more N2O would be produced at higher than at lower O2 levels. This is different from other AOB with the exception of a few studies on N2O production by N. europaea cultivated under very high concentrations of nitrite.

Since nitrite was initially absent from the cultures, it doesn't make sense that so much N2O would be produced particularly at the given ammonium concentrations.

The authors should explain the deviation of this result from other studies of AOB. Is the difference physiological or methodological? And if the authors conclude that the difference is physiological, what is the mechanistic underpinning of this conclusion?

See above response (2). The fact that more N_2O was produced at higher than at lower O2 concentration could be derived from the physiological differences.

3) How is it that zero nitrite was measured with 10 or 30 micromol NH_2OH in assays with HAO? The data in Fig. 1 should be discussed in light of prior studies describing the biochemical and structural details of HAO enzymes. This result, again, is different from others in the literature; hence, it is important to put into context how, based on enzyme structure and biochemistry, N_2O is generated by HAO. Most of the key structural and biochemical studies on HAO are not discussed or referenced in this paper.

As explained in the discussion paper (page 16628 line3-10), it could be explained by the relative amount of NH_2OH and electron acceptor (potassium ferricyanide). As reported in previous studies of HAO of *N.europaea* (Yamanaka and Sakano, 1980), nitrite formation occurred efficiently when the ratio of electron acceptor to NH_2OH added was more than 4. To explain the reason, they proposed that nitrite production hardly occur when the electron acceptor was completely reduced during the first oxidation of hydroxylamine to NOH (Figure added above was helpful). Therefore, at 10 or 30 micromol NH_2OH in assays with HAO, NH_2OH could not be oxidized to nitrite because of the lack of electron acceptor.

4) It seems a bit strange to estimate the contribution of NOR to N2O production by N. oceani based on few experiments and then state that the estimations are in line with prior experimental values.

In the previous study by Frame et al. (2010), the number of replicate experiments incubating AOB under different oxygen concentration was 5 under 0.5%, 2%, 20% (at same cell density). In this study, the number of replicates was 6, 2, 5 under 0%, 2%, 21%, respectively. As for the experiment using NOR and HAO related to SP measurement, it was 4 and 7, respectively, whereas it was 3 in the work by Sutka et al. (2006). Therefore, we consider the number was not so much different from the previous study.

The authors go on to state that, using these estimations, that NIR and NOR activities were not enhanced in their experiments. Such statements absolutely require validation as the regulation and activity of specific enzymes are critical to understanding whether they actually contribute to N_2O production in nature.

As suggested, we cannot validate the statement about activities of enzymes. We revise the sentence in Line 9-13 (the last sentence in the first paragraph in Page 16630) as follows.

"This might indicate that activity of NIR or NOR was not enhanced in this study although we did not directly measured the regulation and activity of the enzymes".

There is a major difference between substrate conversion in vitro by purified enzymes versus substrate conversion through a pathway in vivo. The authors must greatly clarify how they can reasonably and accurately combine in vitro and in vivo data as the calculations in Table 4 essentially refute what is known about experimentally validated results in other AOB strains; that being the relative contribution of NH3-N to the nitrite-N and N2O-N pools by either NH2OH oxidation or nitrite reduction.

In this study, site preference of N_2O produced by denitrifying bacteria (in vivo) was nearly the same as that of N_2O produced by the reaction with NOR (in vitro) extracted from the same strain. Based on this fact, we consider we can combine in vitro and in vivo data using SP value of N2O.

4) There is no known enzymatic pathway for N2O production by ammonia-oxidizing archaea.

Although we are not sure the exact sentence or paragraph which the referee mentions, we would like to revise the last sentence in the conclusion section as follows.

"Although enzymatic pathway for N2O production by AOA is still uncertain, combined analysis of genome sequence, physiology, and isotopomer ratios would promise further understanding of microbial N2O production mechanisms."

5) The following reference presents the argument that nitrifierdenitrification is not about detoxification of nitrite, but rather is a co-respiratory mechanism to allow AOB to respire under low oxygen levels. Co-respiration of oxygen and alternative electron acceptors, particularly nitrogen oxides, is well characterized in other bacterial (and fungal) lineages. Hence, there IS a defined and referenced physiological purpose for nitrifierdenitrification, even though the physiology must be confirmed using a wider spectrum of AOB strains. Reference: Stein, L.Y. 2011. "Heterotrophic nitrification and nitrifierdenitrification. In Nitrification. ASM Press, Washington DC. pp. 95-114.

As suggested, we revise the sentence in Line 10-11, Page 16629 as follows.

"Although the reason why those AOB have a function of nitrifier-denitrification must be confirmed using a wider spectrum of strains, detoxification of accumulated nitrite, co-respiration of oxygen and nitrite, and other mechanisms have been proposed (Stein, 2011)."