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Interactive comment on "Stable isotopes of nitrate reveal different nitrogen processing mechanisms in streams across a land use gradient during wet and dry periods" *by* Wei Wen Wong et al.

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We thank the reviewer for the constructive comments.

Reviewer #3 GENERAL COMMENTS In the manuscript Stable isotopes of nitrate reveal different nitrogen processing mechanisms in streams across a land use gradient during wet and dry periods, Wong et al present natural abundance nitrate isotopes from five streams across a land use gradient during wet and dry seasons, allowing them to elucidate the controls on sources and transformations of nitrate. This is an interesting dataset and the authors have been resourceful and knowledgeable in their presentation and interpretation of the data. However, prior to publication the

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manuscript would benefit from a clear and concise definition of terms, and a clearer explanation of the isotope effects and there subsequent implications, as currently it seems hard to follow in places for the none expert reader. An important aspect of the interpretation of this dataset is that there is a tight coupling of mineralisation and nitrification, resulting in no isotope effect being expressed and hence the 15N of organic matter / ammonium and nitrate are similar. Currently this is not fully explained until Page 9 Line 24, making it difficult to understand the authors interpretation of the data prior to this, explaining this earlier on in the discussion will enable the reader to follow your thoughts / interpretation. A good example of this is Page 6 Line 15/16, break this thought down and explain to the reader here the tight coupling between mineralization and nitrification and hence no isotope effect being observed. We will add a few sentences to explain the minimal isotope effect of the combined reaction of mineralisation and nitrification as suggested by the reviewer. Page 6 Line 31: Nitrogen isotope of the NO3- produced from these end members usually retains the signature of the δ15N-TN as a result of tight coupling between mineralisation (production of ammonium from organic matter) and nitrification (oxidation of ammonium to NO3-) as well as the minimal isotopic fractionation of both processes. It is well documented in the literature that in soil environment, mineralisation causes a small isotopic fractionation (±1‰ $Kendalletal.2007)totheproducedNH4+.InagriculturalareaswhereNH4+ israpidlyconsumedorassimilatedbycrops, nitrificationrateisusuallylowandwouldalsoexertasmallisotopicfractionationto$
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SPECIFIC COMMENTS Page 2 Line 10: it would be valuable here to state that you are talking about kinetic isotope effects and not equilibrium. This will be made clearer in the revised manuscript

Page 2 Line 23 to 25: for the none expert, please explain why rainfall patterns are different in the southern hemisphere and its subsequent effects. This will be explained in the revised manuscript. Page 2 Line 30: The southern hemisphere tends to have more sporadic and variable rainfall patterns compared to the northern hemisphere and

Australia is an example of this. The variable rainfall patterns can modulate different efficiencies of denitrification in soils and thus different fractionation effects to the residual NO3- pool.

Page 2 Line 32 to 33: start preparing your reader here, why are denitrification and assimilation more prevalent in wet periods. Study area: throughout this section you refer to the gradient in land use across the catchment, a map of this would be a great addition to the manuscript (or could maybe be added to Figure 1). Following sentences will be added to the revised manuscript to explain why denitrification can be more prevalent in wet periods. A land use map will be added as supplementary material (Figure S2). Page 3 Line 7: In some studies (e.g. Riha et al. 2014; Kaushal et al. 2011), denitrification and assimilation by plants and algae have been reported to be more prominent during the dry seasons compared to the wet seasons but in other studies (e.g. Murdiyarso et al. 2010; Enanga et al. 2016) denitrification appeared to be more prevalent during the wet seasons as precipitation induces saturation of soils resulting in oxygen depletion and thereby low redox potentials that favour denitrification.

Page 3 Line 32: how do the authors think using an integrated signal could of biased their interpretation of the results? We think the integrated signal could potentially bias the interpretation of the results; however, the integrated signal was the best representation of the percentage agriculture area in the catchment.

Page 5 Lines 9 to 10: the term total nitrogen needs to be defined here, as it is important for the mineralization discussion later on. Would particulate organic nitrogen not be a more suitable term? Also, please add in that the values are relative to AIR and the precision of the measurements. Particulate organic matter is a more suitable term however this was not specifically measured in our study. We used δ15N of total nitrogen of the soil to directly represent the soil organic portion as most of the nitrogen in soils is generally bound in organic forms. This will be explained more thoroughly in the revised manuscript.

Page 5 Lines 24: Please add in nitrite concentrations, to confirm for the reader that the values are less than 1% of the nitrate (as stated in the methods). Nitrite concentrations will be added to the results section of the revised manuscript. Page 6 Line 6: Nitrite concentrations ranged between 0.1μmol/L and 0.4μmol/L.

Page 6 Line 3: Be clear that you are talking about 15N values here. This sentence will be corrected to reflect more clearly on the δ15N values. Page 6 Line 18: Overall, δ15N of the riverine NO3- spanned a wide range (+4 to +33‰.

Page 6 Line 4: The enriched 15N-nitrate values seem to be constrained to a thin band between 70 to 85% agriculture, but then drop away again at higher percentage agriculture, do the authors have any hypotheses for this? We hypothesised that the drop off of δ15N-NO3- at > 85% agriculture was due to recent and possibly over-fertilisation of NH4+ fertiliser resulting in active nitrification. As a large amount of NH4+ was available, oxidation of NH4+ to NO3- became the rate-determining step resulted in large fractionation (-38‰ to -14‰ $Casciottietal.2003)anddepleted\delta$15N-NO3- in the residual NO3- pool. Unfortunately we could not test this hypothesis as we did not have the information on the rates of fertiliser application and nitrification. This would be a good avenue for future research.

Page 6 Line 5: Surely the same is true for the Bass. We agree with the reviewer and Bass will be included as exhibiting the same effect as Bunyip in the revised manuscript. Page 6 Line 20: Among all sites, δ15N-NO3- values in the Bunyip and Bass were relatively depleted (+4 to +12‰ for Bunyip and +10 to 12‰ for Bass), with the lower range found at upper Bunyip (+4 to +8‰.

Page 6 Line 16 to 28 and Equation 1: It would be valuable to explain to the readers the value of using both N and O isotopes i.e. N is recycled between fixed N pools and the O atoms are removed and then replaced by nitrification and thereby sensitive to internal processing (this could come here or in the introduction). The authors need to discuss the more recent literature when introducing and determining the oxygen isotope signal

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imparted by nitrification, the work of Carly Buchwald is particularly pertinent here. We will add a few sentences in the introduction to discuss the value of using both N and O isotopes. We will also discuss the calculation used to estimate δ18O-NO3- imparted by nitrification in more detail. All the texts and figures in the manuscript have been revised to reflect on these changes. Page 2 Line 7: To date, the most promising tool to investigate the sources and sinks of NO3- are the dual isotopic compositions of NO3- at natural abundance level (expressed as δ15N-NO3- and δ18O-NO3- in ‰. Preferential utilisation of lighter isotopes (14N and 16O) over heavier isotopes (15N and 18O) leads to distinctive isotopic signatures that differentiate the various NO3- sources/end members (e.g. inorganic and organic fertiliser, animal manure, atmospheric deposition) and the predictable kinetic fractionation effect when NO3- undergoes different biological processes (e.g. nitrogen fixation and denitrification). For instance, denitrification and phytoplankton assimilation fractionate N and O isotopes in a 1:1 pattern. Simultaneous measurement of δ15N-NO3- and δ18O-NO3- also provides complementary information on the cycling of NO3- in the environment. δ18O-NO3- is a more effective proxy of internal cycling of NO3- (i.e. assimilation, mineralisation and nitrification) compared to δ15N-NO3-. This is because during NO3- assimilation and mineralisation, N atoms are recycled between fixed N pools and the O atoms are removed and replaced by nitrification (Sigman et al. 2009; Buchwald et al. 2012). Page 7 Line 6: The δ18O of NO3- generated by nitrification of these sources is decoupled from δ15N-NO3- but relies on the oxygen isotope of water (δ18O-H2O), oxygen isotope of dissolved oxygen (δ18O-O2) as well as the kinetic and equilibrium isotope effects during the sequential oxidation of NH4+ to NO2- then NO3- (Casciotti et al. 2010; Buchwald et al. 2012). Previous culture studies (Casciotti et al. 2010; Buchwald and Casciotti 2010; Buchwald et al. 2012) and observations in various marine systems (Sigman et al. 2009; Granger et al. 2013; Rafter et al. 2013) have found that δ18O values for nitrified NO3- were within a few ‰ of the δ18O-H2O. Hence, -5.3‰ $theaveragevalueof\delta$18O-H2O is adopted to represent the lower estimate of δ18O of the nitrified NO3- in this study. In a system where equilibrium exchange of oxygen between H2O and NO2- is negligible but respiration and denitrification are prevalent/co-occurring, δ18O-NO3- can be much greater than that of δ18O-H2O. In this study, the δ18O-NO3- values were all more enriched than -5.3‰ suggesting the co-occurrence of a fractionating process, most likely denitrification (this is discussed in the following section). Based on this reason, using -5.3‰ can potentially underestimate the δ18O of the nitrified NO3-. The conventional 2:1 (δ18O-H2O:δ18O-O2) fractional source contribution model (Equation 1) is therefore used to calculate the maximum estimate of δ18O of the nitrified NO3- in our study which is +4.3‰ by using -5.3‰ for the average δ18O-H2O and +23.5‰ for δ18O-O2.

Page 6 Line 29: Is this value for cow manure similar to the literature to date? Yes these values are similar to the literature to date.

Page 7 Line 14: ‘terrestrial’ what are the authors referring to here, fertilizer, manure, leaf litter, please be consistent with the use of terms throughout. This term refers to a combination of sources and this will be made clearer in the revised manuscript. Page 8 Line 22: …..in stream NO3- comprised mainly of terrestrially derived NO3- (i.e. inorganic fertiliser, manure and soil organic matter) entered the streams through surface runoff…

Page 7 Lines 15 onwards: a slight restructure here would be beneficial, you are presenting your conclusions before the evidence, discussing your isotope data first in this section would make it easier to follow. This section will be restructured as suggested by the reviewer. Page 8 Line 2: Agricultural land use (i.e. market gardens and cattle rearing) appeared to influence NO3- concentrations in our study sites. As shown in Fig. 4(a), during the wet periods, high NO3- concentrations (> 40 μM) were particularly observed at sites with more than 70% agricultural land use. During the dry periods, although NO3- concentrations were generally lower than 36μM, the outliers were observed at sites with more than 70% agricultural land use. Similarly, enriched δ15N-NO3- in the streams were mainly found at sites with high percentage agricultural land use (between 75 to 85%) for both dry and wet periods suggesting that enriched δ15N-NO3- in the stream were originated from agricultural activities. In fact, the most enriched δ15N-NO3- values (>30‰ were observed at the most downstream site of Watson Creek which has the largest percentage of market gardens (although the total agricultural area is not the highest amongst all the studied sites). We also observed a significant positive relationship between δ15N-NO3- and percentage agriculture during the wet periods (Fig. 4b) which further supports the contention that agricultural activities were the main control of the δ15N-NO3- in the streams. Other researchers have also documented similar trends of enriched δ15N-NO3- with increasing percentage agriculture. For example Harrington et al. 1998, Mayer et al. 2002 and Voss et al. 2006 observed highly significant positive relationships between percentage agriculture land area and δ15N-NO3- with r2 ∼ 0.7. However, these studies showed comparatively narrower and more depleted ranges of δ15N-NO3- with 2.0 to 7.3‰ $4to8\ and -0.1to8.3\ respectively, suggestingmoresubtlechangesin\delta$15N-NO3- over a large span of agriculture land areas in these studies compared to our study. Given that none of the predicted sources of NO3- in the Western Port catchment exhibited an initial δ15N-NO3- of more than +6‰ the isotopically-enriched NO3- as well as the variability of NO3- concentrations observed in this study were consequences of a series of transformation processes. Hence, we propose the following factors to explain the heavy isotopes and the different NO3- concentrations across different periods observed in our study: (1) During the wet period when surface runoff was conspicuous and residence time of the water column was low, in-stream NO3- comprised mainly of terrestrially derived NO3- (i.e. fertilisers, manure and soil organic matter) and there was limited in-stream processing of these NO3-. The high NO3- concentrations and the heavy δ15N-NO3- values reflect the occurrence of mineralisation, nitrification and subsequent preferential denitrification of the isotopically lighter NO3- source/s in either the waterlogged soil or in the soil zone underneath the market gardens before transport to the streams through surface runoff. (2) During the dry periods when surface runoff was negligible and residence time of the water column was high, there was minimal introduction of terrestrial NO3- into the streams and in-stream processing of NO3- was more apparent than during the wet periods. In addition to mineralisation and nitrifica-

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tion, volatilisation and assimilation by plant and algae was highly likely to occur in the stream further reducing the NO3- concentration and further fractionating the isotopic signature of NO3-.

Page 7 Line 21: I think the authors are referring to Table 2 here. This will be corrected

Page 8 Line 15 and 32: I strongly suggest the authors cite and discuss the implications of the outcomes from the work of Granger and Wankel, 2016 (Isotopic overprinting of nitrification on denitrification as a ubiquitous and unifying feature of environmental nitrogen cycling; PNAS) and how this may influence your interpretation of N turnover in your catchment. The study by Granger and Wankel (2016) will be discussed in the revised manuscript as suggested by the reviewer. Page 10 Line 5: It is worth noting that although the dual isotopic composition of δ18O-NO3- and δ15N-NO3- deviates from a trajectory of 1 (trajectory of 1 indicates denitrification), it is still a salient trend indicating the occurrence of denitrification and is consistent with the δ18O-NO3-:δ15N-NO3- recurrently observed in freshwater systems (Kendall et al. 2007). This deviation in our study could be explained by concurrent NO3- production catalysed by nitrification and/or annamox (Granger and Wankel 2016) although the significance of annamox is still disputable. Based on the multi-process model developed by Granger and Wankel (2016), the two most important factors in the nitrification pathway that govern the δ18O of the newly produced NO3- are δ18O of the ambient water and the flux of NO2- oxidation (Granger and Wankel 2016). Deflation of δ18O-NO3-:δ15N:NO3- trajectory below 1 observed in this study was likely to be associated with the low δ18O-H2O values which contributed to lower δ18O values for nitrified NO3-. Higher NO3- reduction rate versus NO2- oxidation rate which contributed to the δ15N-enriched pool of nitrified NO3-, greater than the denitrified NO3- also drives the δ18O-NO3-:δ15N-NO3- trajectory to values below 1 (see Granger and Wankel 2016 for explanation). All in all, this highlights the significant contribution of nitrification along with denitrification in the WP catchment. Page 11 Line 5: NO3- in group B has variable δ15N and δ18O values as shown by Bunyip and Toomuc. This could be attributed to isotopic fractionation ei-

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ther during plant and/or algae uptake or denitrification as substantiated by the parallel increase of δ18O-NO3- versus δ15N-NO3- (Fig. 9). Based on Fig. 9, the large uncertainties in the δ18O-NO3- of the nitrified end members have resulted in overlapping of isotopic signatures of the three major sources (nitrified cow manure, nitrified inorganic fertiliser and nitrified SOM). All three sources appeared to have influenced the δ15N and δ18O of the residual NO3- in the stream. This scenario reinstates the sensitivity and the importance of accurately determining the δ18O-NO3- of the initial NO3- in the effort to apportion the relative contribution of different sources.

Page 10 section (3): an earlier introduction of the different behaviors of N and O isotopes during internal processing of nitrate will make this section easier to understand for the none expert reader. I would not put denitrification under the heading recycling, if the authors are referring to nitrate reduction, followed by reoxidation please say so. We will include a paragraph in the introduction to briefly discuss about the different behaviours of nitrate isotopes during internal processing of nitrate. We have also changed the term ‘recycling’ to ‘internal processes’. Page 2 Line 7: To date, the most promising tool to investigate the sources and sinks of NO3- are the dual isotopic compositions of NO3- at natural abundance level (expressed as δ15N-NO3- and δ18O-NO3- in ‰. Preferential utilisation of lighter isotopes (14N and 16O) over heavier isotopes (15N and 18O) leads to distinctive isotopic signatures that differentiate the various NO3- sources/end members (e.g. inorganic and organic fertiliser, animal manure, atmospheric deposition) and the predictable kinetic fractionation effect when NO3- undergoes different biological processes (e.g. nitrogen fixation and denitrification). For instance, denitrification and phytoplankton assimilation fractionate N and O isotopes in a 1:1 pattern. Simultaneous measurement of δ15N-NO3- and δ18O-NO3- also provides complementary information on the cycling of NO3- in the environment. δ18O-NO3- is a more effective proxy of internal cycling of NO3- (i.e. assimilation, mineralisation and nitrification) compared to δ15N-NO3-. This is because during NO3- assimilation and mineralisation, N atoms are recycled between fixed N pools and the O atoms are removed and replaced by nitrification (Sigman et al. 2009; Buchwald et

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al. 2012).

Page 10 Line 11: Do the authors know when fertilizer is applied in this catchment, how does this align with your runoff / turnover hypotheses? Unfortunately we do not have the information on when fertiliser was applied in the catchment hence no further conclusion could be drawn on the relationship of fertiliser application and the runoff processes.

Figure 1: Please mark on the map of Australia where southern Victoria is. Figure 3: Mark on upper / lower Bunyip. Figure 5: Where have the authors taken these isotope effects from? Please cite the relevant literature in the caption. A positive / inverse isotope effect for nitrification? Figure 7: More details are needed in the figure caption, what do the crosses and dashed line mean? I also assume that it is the y intercept values determined in Figure 6 that have been plotted. Figure 9: define what starting values you have used and where they have come from, particularly for the oxygen isotopes. All the figures will be revised according to the reviewer’s comments.

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