The reply to the reviewer comments are shown in red italics.

Reviewer 1

The manuscript, “The Fractionation of nitrogen and oxygen isotopes in macroalgae during the assimilation of nitrate” by Swart et al., presents the results of a laboratory study of isotopic fraction by macroalgae over a range of nitrate concentrations. This topic is relevant to a broad audience in biogeochemistry and environmental science, which makes Biogeochemistry an appropriate journal for this work. The subject of nitrogen isotope fractionation during assimilation is an area of active research, and a more complete understanding of the process is critically important to interpreting nitrogen stable isotope data in the environment, therefore this study is a valuable contribution. The methods and tools used in the study are not particularly novel, however the presentation of fractionation data for macroalgae is new. The methods and results sections are clearly outlined. The results are appropriately interpreted in the context of recent work on microalgae and bacteria. Overall this is a well written paper.

Reply: We thank the reviewer for their comments and agree that such work has not been generally carried out with rigor using macroalgae. In addition the range of concentrations used in the study of microalgae in previous work has been generally at high values. Hence studies on the assimilation in both micro and macro algae has not been carried out at concentrations approaching those found in the normal marine or even coastal environments. Therefore we believe our paper presents valuable insight into the processes involved in assimilation.

Specific Comments

1. In the results section nitrogen isotopes of algae and NO_3^- are described separately for the two types of experiments. I would encourage combining these sections, so that the difference between the NO_3^- and the algal material can be understood quickly. I would suggest combining Tables 1, 2 and 3, into a single table. Or at a minimum adding the average initial $\delta^{15}N_{NO3-}$ values to the caption of Table 1.

Reply: Tables 1-3 have been now combined into one table.

2. In the discussion section of the Ulva experiments it is stated that the 15e values decrease toward zero with decreasing concentration. It seems more appropriate to say they decrease to a minimum of -3.2.

Reply: The text has been changed to say that the values decrease to -3.2‰.

3. Given the emphasis on concentration dependence- how do you interpret the result that the 15 in the syringe experiment at ~3 uM concentration and the free drift experiment at 500 μM are so similar for Ulva (2.1 ‰ vs 2.0 ‰ respectively).

Reply: In the syringe experiments during which a constant concentration of ~ 3 uM was maintained, the δ^{15}N *of the algae decreased from 3.1 to 1.3 equivalent to about 2 per mille fractionation. This decrease was similar to that observed in the 500 uM treatment. We have added a section to the text to discuss this, but it should be pointed out that the free drift showed a decreasing fractionation with decreasing concentration to that the extent that the values fell significantly below zero at low concentrations. While this may be real we are not as confident about the solid tissue data and we have added a small discussion of the pitfalls of using the tissue numbers.*

4. In the discussion section for Ulva it states that "although the quadratic equation predicts values less than zero at concentrations less than 1 uM, none of the experiments were actually performed at these low concentrations and there for this observation will need to be confirmed" Isn't it somewhat confirmed by the negative 15ε values measured at concentrations of 60 and 14 uM? Perhaps this effect begins a higher concentrations.

Reply: We did not want to over interpret the data (see response to previous query). The negative e values in the Ulva experiments certainly suggest the preferential incorporation of ^{15}*N, but such values have not been seen previously and were not observed in the case of the other algae. Hence we want to err on the side of caution. Nonetheless your statement is true so we have added something to the text here to explain the results of the apparent negative epsilon values for the solid tissues in the case of Ulva. See also the comments of reviewer 2.*

5. In the discussion section it also states that the syringe experiment yielded a 15ε value 1 ‰ higher than the ε values estimated from NO_3^-draw down and from the solid free drift experiments" What is the difference between draw down and free drift experiments? It seems only fair to compare solids from the syringe experiment to solids from the free drift experiments in which case the difference looks like nearly 6 ‰.

Reply: In the case of Ulva the ε values calculated from the solids seem to indicate ε values of less than unity. The discrepancy with the free drift and the syringe data may suggest that there is a problem with the solid Ulva data. This comment is also pertinent to issue 4 (see above). Therefore we trust the syringe experiment and the NO_3^-*data rather than the solid* δ^{15}N *values. We have emphasized this in the text and the reasons why we trust that data to a great extent.*

6. In general I think the discussion of negative values for 15ε could be more clear. It seems that the free drift experiment for Ulva yielded negative values at lower concentration and this is can be modeled with both the linear and quadratic approximations, therefore it is only the syringe experiment that is not consistent with this result. One thing that might help clarify the discussion would be to consistently use nomenclature that differentiates 15ε (quadratic) from 15ε (linear). The caption to Table 5 could specify 15ε (linear).

Reply: We have attempted to clarify the discussion here. This comment is also covered by the previous three comments of this reviewer and we thank them for pointing this out. The caption for Table 3 (old Table 5) no indicates the linear values.

7. For the discussion of oxygen isotopic composition and 18ε:15ε values it would be helpful to have 18ε values and the ratios presented in table form. Given that this a main conclusion of the paper it is odd not to see the values. Similarly the trend lines on Figure 2 should be labeled with their respective slopes.

Reply: I believe that 18ε values were already in the table (old Table 5, new Table 3). (Note that tables have been renumbered). Also slopes are now given in Figure 2.

Technical Corrections

1. In the caption for Table 5, add text to explain the calculated fractionation factors using eqns 3 and 4. To differentiate from 15ε calculated from the quadratic fit.

   Reply: This table presents the fractionation using the linear model. If the quadratic approach is used then the slope can be calculated using the first differential at a specific NO_3^- *concentration.*

2. Line 5, pg 6922 "a more refine of equation"

   Reply: This has been fixed.

3.Line 6-8, pg 6922, awkward repetition of "As in the case"

Reply: This has been fixed.

4.Figure 2. It appears that there was more analytical error in measurements of *Ulva* compared to Agardhiella- is that right or are the error bars just absent from *Agardhiella*?

Reply: Error bars have been removed for clarity. We have added this to the figure caption.

5. Figure 3. The black open box in the legend seems not to match the open grey box in the figure. Also it it would be helpful to label the trend lines with 15ε values.

Reply: Symbols changed and lines labeled.

6. For figure captions 4, 5, 6 it would be easier to grasp quickly if the caption include the experiment, species, etc. (currently Fig 6 says data in Fig 5, and Fig 5 says data in Fig 4 and so on.)

Reply: This has been changed

Reply to Reviewer 2

General comments:

Swart et al present data for the N and O isotopic fractionation imparted during NO_3^-- assimilation by a species of green and red macroalgae (*Ulva* sp. and *Agardhiella* sp., respectively). Similar studies have been conducted for years – but are an important component of a foundational understanding of the behavior of stable isotopes in the environment. Without such fundamental studies – field data can be severely confounded by factors that are overlooked or misunderstood. Overall – the paper is well presented (a few minor typos) and the data appear robust and of high quality. The general finding that the apparent isotope fractionation decreases at lower concentrations has been observed before in other microalgae and bacterial system. However, this work represents the first dual isotopic study of macroalgae fractionation during assimilation – and therefore fills an important gap in our overall understanding of nitrogen isotope dynamics in marine systems.

Reply: We thank the reviewer for their comments. In addition the range of concentrations used in previous work has been generally at the higher concentrations used in this study and studies have not been carried out at values approaching those found in the normal marine environment. The one study which we are aware of actually concluded that there was no concentration dependence of the fractionation factor, but they used fairly high concentrations in their experiments.

Specific comments:

1-The prediction of an inverse isotope effect at the lowest NO_3^- concentrations is interesting (although necessarily adequately supported by the data). There could be very important implications for this in oligotrophic ecosystems. Given the fact that the *Ulva* experiment with 3uM did not show this, however, I suspect that this is probably due to either error in sampling the new algal growth (giving the observed $\delta^{15}N$ values that were higher than $NO_3^-$and/or an artifact of the quadratic fit to the 'free drift' experiments additionally – the experiments in which the algal tissue $\delta^{15}N$ was higher than the NO_3^- were performed at 14 and 60uM – hardly 'low' levels – and so I find this to be perhaps a little bit overly speculative. What did the 'old growth' look like? Were its values measured?

Reply: We agree that the inverse isotope effect may be speculative and we did not want to emphasize it too much. However, reviewer 1 felt that we did not emphasize enough. We tend to agree with this reviewer(reviewer 2) that at this point the data only suggest a possibility. We have reworked our interpretation of the Ulva data in the text to cover possible artifacts.

2- Since isotopic fractionation factors were calculated using both $NO_3^-$and algal biomass – the authors argue that the two methods give 'statistically' the same results. This may be true – but I wonder about the validity of sampling 'new growth' – and indeed the results presented in Table 5 show a consistent offset between the fractionation calculated between the two approaches, with the biomass approach regularly giving lower results in the Ulva experiments (but not in the *Agardhiella*). I think this may be revealing something important – either about the fidelity of sampling 'new biomass,' nutrient translocation in algae (?), or perhaps some other physiological explanation?

Reply: This may be correct and we have added material to the discussion. The distinction between the new and old growth is clear, but it does not preclude some type of nutrient redistribution in the solid samples and therefore the data on the NO_3^- *is probably better.*

3- Apparently NH_4+ and NO_2- were also measured? Where are the data? Was any NH4+ or NO2- observed? This would be an important component to constrain. Pg 18 Ln 23: Granger et al 2010 actually revised this argument to consider that diffusion is likely not the major cause of the 2:1 slope – but rather transport effects are the primary driver. Karsh et al., 2014 of course elaborate on this as well.

Reply: All samples were screened for NO_2- and NH_4+ but we did not mention the data as none was detected. This has been added to the text.

Pg 19 Ln 11: While I think that the quadratic fit to the data may accurately represent the observations and be useful for predicting the isotope effects, I think it is inaccurate to state that the relationship between 15N and 18O is mathematically described by a quadratic equation. Indeed – the results indicate that a non-linear (or curved) evolution is predicted – I agree. Maybe consider replacing "quadratic" here with "curved?"

Reply: We are not sure why the relationship cannot be explained by quadratic equation. Just like the normal expression is described by a linear relationship Nevertheless the curved or non-linear relationship can be fitted with a quadratic equation, which when one takes the first differential provides the estimate of epsilon at any concentration of NO_3^-*. This is key as figure 3 clearly shows changing slopes with different initial concentrations.*

Pg 20 Ln 1: Is this in contrast to Cohen and Fong, 2005? If so – I think there needs to be a little bit of direct discussion for addressing the differences between the findings of the two studies.

Reply: We have added some discussion of Cohen and Fong here. Cohen and Fong did experiments in which only NO_3^-*was added at fairly high concentrations. These concentrations are above the level at which our experiments shows little relationship between concentration and fractionation. Hence it is not surprising that they did not find any relationship between the amount of fractionation and the concentration. We have added this to the discussion.*

Pg 20 Ln 6-9: Mentioning the Haber-Bosch process here, or the original composition of the NO_3^-, is irrelevant, right? You just stated that the effects should be seen "regardless of the 15N of the original NO_3^-.

Reply: We only mention the H-B process as most artificial N fertilizer is produced by this process and since it produces values close to zero. We have removed reference to the HB process from this version.

Pg 20 Ln 11: I don't follow this exactly. If fractionation is 5‰ and the NO_3^- $\delta^{15}N$ is 0‰ then the initial algal biomass would be -5‰ as NO_3^- is consumed in the open system described – the N NO_3^-would increase from 0 to +5‰ while the biomass would increase from -5 to 0‰W˙ here in this scenario would "isotopically positive algal material be formed?" I agree that isotopic fractionation and differential drawdown and mixing and convolute any simple source signatures – but I don't quite understand the argument as it's presented.

Reply: The point is that the water containing the nitrate is moving along a path and as the nitrate decreases its $\delta^{15}N$ increases to value far higher than +5. One can model this using Rayleigh distillation. For example if the fractionation is 5 per mille and one starts with a nitrate concentration of 20 uM with a $\delta^{15}N$ of zero, then by the time the concentration has been reduced to 2 uM the $\delta^{15}N$ of the $NO_3^-$is+11.5 per mille. Assuming that the fractionation decreases linearly as the concentration decreases, this increase would not be so dramatic. Starting with higher concentrations would produce larger enrichments in the $\delta^{15}N$ of the NO_3^-.

Minor comments:

1. Pg 2 Ln 17: It would be nice to define what is meant here by 'typical' – these algae are commonly found in coastal systems – and often these systems may see higher concentrations than coral reef, oligotrophic or open water systems.

   Reply: We have defined typical marine values. While coastal values are higher the abstract is probably not the place to discuss this.
2. Pg 2 Ln 17 and Ln 22. Sentence is repeated.

   Reply: Yes you are correct. This sentence has been deleted.
3. Pg 2 Ln 23: Again – being somewhat specific here about what constitutes 'higher'would be helpful in the abstract.
   Reply: Values higher than ~ 10 uM. This has been added.
4. Pg 4 Ln 7: Isotope ratios are expressed:

   Reply: This has been fixed.

5. Pg 4 Ln 9: VSMOW?
   Reply: This has been fixed.
6. Pg 6 Ln 8: investigations
   Reply: This has been fixed.
7. Pg 6 Ln 5: have been
   Reply: This has been fixed.

8. Pg 8 Ln 11: I assume it is well established that new macroalgal growth is comprised of the 'new' nitrogen – and that there is no translocation of internal N pools in these species? The results seem consistent with this – but maybe it should be stated for clarity.

   Reply: The justification is that previous work had found a concentration dependence in micro algae, yet Cohen and Fong said that there was none. This has been stated.

9. Pg 14 Ln 4: Equation 6. Since you've already defined 'f' – I feel this equation could/should be written in its more familiar form (e.g., f* ln(f)/(1-f)).

   Reply: That has been changed and the term 'f' used throughout.

11. Pg 14 Ln 6: "tend to equal" isn't quite accurate – maybe change to "approach"?

    Reply: This has been changed to approach rather than equal

12. Pg 15 Ln 9: " during the experiment as the concentration: : :"

    Reply: We are not sure what to change here

13. Pg 19 Ln 21: " have implications for the application: : :"

    Reply: This has been changed.

14. Pg 20 Ln 5: I wonder in estuaries about the role of NH_4^+ assimilation, since estuarine sediments typically support a substantial flux of NH_4^+ into the overlying water.

    Reply: We agree but NH_4^+ is another story.

15. Pg 21 Ln 8: I think “inverse” is the appropriate term.

Reply: This has been changed to inverse

16. Where is the 13C and C:N data?

Reply: As stated in the result section these data are included in the supplemental material. While interesting they were not considered to be integral to the message of the paper.

17. Figure 2: What is the slope of the line?

Reply: I think that was given the result section as being close to unity, but I have now put the exact value into the figure caption.

18. Figure 3: The 60uM treatment seems to be substantially pulled by a single point ($\delta^{15}N$ 9.5‰). The other 60uM data seems to line up well with the 103uM data –suggesting a similar mechanism at work at both of these concentrations? I guess this is revisited and addressed in Figure 5 and 6. Also in Figures 3, 5 and 6 – is this both algae or just Ulva?

Reply: The r2 is 0.99,albeit only for three points. Still it is not substantially pulled out by one point. There may be some confusion regarding the points so we changed the symbols to make each one more identifiable. The non-linear model was only applied to the Ulva data as we did not have enough time steps for the other species to make a difference between the non-linear and linear approach.