Qeios

Peer Review

Review of: "Nitrification in a Seagrass-Sponge Association"

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This study investigates the nitrogen and carbon trophic link between a seagrass (SG) and a sponge (SP). Fresh material was incubated alone or in co-incubation in locally-sourced seawater over 4 days under two contrasted environmental conditions, light and dark (achieved by wrapping dark plastic bags). Various C and N nutrients (DOC, DIN, DIN) were measured at the beginning and end of the incubation, and isotopically labelled ammonia (15N-NH4+) was used to specifically trace nitrification activity and the fate of its nitrate. Microbial communities were also characterized!

This is an interesting topic and system to be examining biotic interaction. The introduction seems rather exhaustive in tackling the objectives, and the methods are state-of-the-art, analytical techniques with up-to-date isotopic and genomic methodology. The global and putative environmental importance of the study is explained and tied to the context of increased human pressure on coastal areas in the discussion.

I enjoyed reading your manuscript, and you are presenting a very interesting design and dataset, but once I reached the discussion, I felt that the data were not used to their full potential and thus some discussion points could be better supported. Here are some suggestions to help improve the manuscript and discussion.

- The contribution you provide is simply the rate of PNR normalized over the total N requirement of the SG holobionts. But the SG is able to consume NO3- in spring in mono-incubation (negative flux), so it is not clear if sponge nitrification provides additional nutrients, or what share of the nitrate present in the seawater is coming from the sponge (for example, SG produces a net NO2 flux in the spring, but no microbiomes data for the SG was provided to check for nitrifiers or other N cyclers associated with the SG). In addition, other pathways can contribute to filling N requirements, as there is evidence of net NH4 production by the sponge in the spring, possibly indicating nitrogen fixation or DNRA. Could

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comparing PNR and NO3 seawater concentration give us an idea of how much nitrate the sponge might be responsible for in the systems (perhaps from a calculation of SP nitrification NO3 turnover time, or how long it will take the sponge to create that much nitrate)?

- The link between nitrification and the nutrient fluxes is hard to grasp, as fluxes are only net rates. A possible way to highlight changes in nutrient flux would be to compare the co-cultivation data to the weighted sum of the individual incubations to highlight how they are modified together vs. alone. Also, I wonder if tracer experiments can help tell apart some of the nutrient usage. For example, if the SG is preferentially uptaking NH4, you should see higher enrichment in SG tissue than in its epiphytes using NO3.
- The key to mutualistic association is that they benefit from each other (higher growth rate, higher activity...). This seems clear for the sponge, as PNR increases in co-cultivation, but I have not seen similar data for whether the SG is directly benefiting. Is there a direct link between higher PNR and higher growth (derived from oxygen demand) in the various conditions/time periods/replicates? Also, because you used isotopic tracer approaches, I was really expecting you were going to use these data to go a bit further. For example, nutrient rates, inferences from natural isotopic data, and contribution to N demands could perhaps be used to calculate what would be the expected 15N signal of enriched experiments for the different organisms (SG and epiphytes, SB). It might not be a strong proof in itself, but it can help evaluate if the N percent contributions you calculate are reasonable.
- Finally, a lot of the text is about data not presented in the main figure, such as natural abundance isotopic composition and biomass enrichment, oxygen demand, all of which directly relate to the trophic link of the association. I am not sure about the format and length requirements of this journal, but I would really like to see them in the main text/figure, as these are the key to most of the demonstrations you are making. Please consider showing the data in a way that directly supports your claims (PNR rate, comparison of rates of nutrient flux and oxygen demand in the weighted sum of individual incubations vs. co-incubation, actual biomass enriched 15N signal vs. expected from estimates, isotopic natural abundance in the tissue, ...) and perhaps move the net flux (harder to interpret but interesting in itself) to the SI.

Other specific comments (some might repeat key points)

Methods:

Here are some methodological inquiries I have. The manuscript would potentially benefit from discussing some or all of these limitations.

- Is the biomass used to standardize results for co-incubation data the sum of the mass of SP and SG? If yes, as the flux results for the association are often found in between the ones for SG and SP, I think it might be worth investigating comparing the result of SP+SG association to the weighted sum of the individual incubation fluxes to better understand and highlight the interaction effect. Plus, the absolute value of the fluxes is biased by the difference in density between sponge and SG and so only directionally informative.
- The samples of 15N-NO3 are filtered. How would NO3 direct uptake by microbial biomass influence the nitrification results and particularly the effect of light-dark difference? Could it be that the photosynthetic phytoplankton (which is clear from the genomic data) will scavenge NO3 more under light conditions, reducing the apparent rate? (In addition to the effect of light inhibition).
- You used the final 15N enrichment in the medium to calculate the concentration rate. Why not the initial enrichment for which you provide an estimate based on NH4, and why is this enrichment (96%) different from the one stated in the first method paragraph (60 and 76 atom%)? Please clarify.
- There is maybe a bit too much emphasis on the DNA extraction protocol with regard to the other methods, in my opinion (almost 1 page), considering it is using a commercial kit and is modified from an existing publication. It is customary to only highlight the difference between the most up-to-date published protocol and the present one (All the information could easily be added in SI to allow easier reproducibility). Please also state the name of the article for the Ti methods of 15-NO3 isotope measurement. (Page 7)
- Conversely, please provide a bit more detail on your N demand estimates for the holobionts. Right now, there are only 3 lines with limited explanation of how you actually calculated it. And perhaps on the Ti methods for NO3 analyses (just a brief sentence explaining the concept of the analyses).

Discussion

- It is stated at the beginning of the discussion that “this study is the first to show that DIN provided by nitrification can be taken up by the SG, supporting its N demand.” But the demonstration was not made yet, which is confusing. I think it is important to guide the reader to the evidence supporting this statement before making it.
- The discussion is also not always clear to me, as it brings important information that is not presented before: for example, SG prefers NH4+ over NO3-, so we are looking at a competition for NH4+, and only the associate microbiomes would benefit from NO3-? You discuss some 15N natural fractionation arguments that are not presented in the main figures. And I was waiting to see actual evidence of 15N

incorporation in SG biomass and epiphytes and calculations to demonstrate how it originates or not from nitrification.

- I really like that Fig 1 shows PNR increase in co-incubation, which is strong support that there is a beneficial interaction. However, the link to C or N from SG senescence (particularly in Autumn) is not clear. However, the net flux for C is not showing any difference in C production between spring and fall, and N is only produced as NO2 in the spring, while PNR are 10-fold higher in the fall. This needs to be discussed.
- I did not really see any direct link with PNR made in the figure. Isotopic data and presenting the flux/PNR data differently might help better represent some of the arguments you are using in your demonstration.
- How much nitrogen fixation or DNRA could contribute to the system? There are positive NH4 fluxes in the Sponge-only incubation, so this could certainly influence the results and estimates.

Finally, in addition to my general comments, the text can be improved with little adjustments.

- Please consider being more specific when referring to each treatment, such as « the sponge » or « in the association », as this is a bit unclear at times. Prefer « in sponge-only incubation » or « in Sponge-seagrass co-incubation », or better, introduce the SP SG, SP+SG abbreviations that are only briefly referred to in the M&M. Also, when referring to the SG epiphytes, please be specific about what type it is (Microbial epiphytes). Providing a picture of the SG and SP would be nice to visualize the association.
- Throughout the text, consider fixing your significant digits. It is customary to have only 1 digit for SD (as SD itself has large uncertainty) or 2 when the first digit is a 1, and to have the mean with the according position (30+-10, 31 +- 1.4, 31.5+-0.7, or 31.65 +-0.04). It will simplify reading! (In addition to being mathematically and statistically more accurate too)

Typo and Minor comments:

- The current nutrient flux calculation description in Material and Methods is not accurate and refers to concentration per g of biomass, not mol.g.d-1. Please correct this in the M&M.
- I think it is Prokaryote, not procaryote.
- RNA later is a brand, so it needs to be followed by ® =(r).
- In the incubation experiment with stable isotopes, I think you mean 5µmol, not µM (which is µmol.L-1). Else, the 15N-NH4+ addition of 5µM represents 0.04% of the total ammonium in the site water

based on table 1, while the natural abundance of 15N in nature is in the range of 0.4%.

Nice study overall!! (And I apologize for the long delay in completing this review.)

Declarations

Potential competing interests: No potential competing interests to declare.