

## ***Interactive comment on “Fungal loop transfer of N depends on biocrust constituents and N form” by Zachary T. Aanderud et al.***

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Received and published: 14 March 2018

Authors response-general comment: First off, we strongly believe as authors that the major concerns outlined by reviewer 2 are not fatal flaws. . Hopefully we have addressed all your concerns adequately.

1. 1. There are no controls, where unlabeled  $\text{NH}_4^+$  and  $\text{NO}_3^-$  were applied and analyzed.

Authors response: Why is this a concern? Yes, there are some thermodynamic and enzymatic complications associated with isotopic additions, as heavier isotopes do fractionate, but we are just using the  $^{15}\text{N}$  as a tracer. We always refer to increases of  $^{15}\text{N}$  as  $^{15}\text{N}$  from  $^{15}\text{NH}_4^+$ , not  $^{15}\text{NH}_4^+$ . Further to help clarify any concerns over our

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isotope use we added the following sentence in the discussion, Unfortunately, based on our design, we were unable to distinguish the form of N captured or translocated by biocrust constituents. More information is needed to identify the importance of N form and the movement of organic N within fungal loops." We are using enriched  $^{15}\text{N}$  as a tracer as many of the authors have done multiple times in the past. We are happy to provide a list of our papers using  $^{15}\text{N}$  as a tracer without unlabeled controls. Again, we are unclear why a control is needed. We are happy to discuss this point further.

1. 2. The number of true replicates per crust type is only 3 and thus rather small. The authors calculate with an  $n$  of 14 and 15 for cyanobacteria- and moss-dominated biocrusts; however, most of the samples are not independent (since related to the same source, where  $^{15}\text{N}$  was applied). Thus, calculations have to be conducted accordingly, distinguishing between pseudo - and true replicates.

Authors response: A precedent for the design was established by Green et al. 2008 in the Journal of Ecology to capture movement of tracers in biocrusts (see citation below). Our design/linear regression stats mirror their methods. I am not sure how we could even reduce our data to  $n=3$  since all the biocrust soil and naturally occurring *A. hymenoides* samples are randomly distributed across many different distances. We are primarily interested in distance the isotope may travel. We decided that labeling a 5 cm diameter soil circle, via a rainfall event, and randomly selecting soils and plants up to almost 2 m away would more than serve as an indicator of movement. The potential area for the isotope to move was 1600-times (area of the entire circular plot/area of the rainfall addition) that of the area with the label. We are excited that we captured the translocation of the label and are confident in our data and analyses.

Green LE, Porrás-Alfaro A, Sinsabaugh RL. 2008. Translocation of nitrogen and carbon integrates biotic crust and grass production in desert grassland. Journal of Ecology 96:1076-1085.

1. 3. A 2.5 mm rainfall equivalent was only applied to the central 5 cm diameter circle,

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whereas the area outside remained dry. As grasses and crusts at a distance of 29-120 cm and 22-97 cm, respectively, were collected, the amount of water may have been not sufficient to activate such a large area around the central biocrust. The result, that a transport was observed in cyanobacteria- but not in moss-dominated biocrusts, supports this assumption, as moss- dominated biocrusts are known to need by far more water to be activated than cyanobacteria- dominated biocrusts. The experiments need to be analyzed in an adequate form (considering 3 replicates per crust type). It also is necessary to reassure that the different results obtained for different biocrusts are caused by functional differences and not by water limitation.

Authors response: Our intention was never to activate the entire biocrust and outside the 5 cm diameter circle of soil. We only activated the biocrust within the minor, localized rainfall event. We are testing a portion of the fungal-loop hypothesis. To help clarify this misconception, we added the word "dry" to the abstract and first sentence of the last paragraph in the introduction, "Minor rainfall events may allow fungi to act as conduits and reservoirs for N. To investigate the potential for biocrust constituents and N form to influence the movement of N through the putative fungal loops, we created minor, localized rainfall events and measured  $^{15}\text{N}$ , from  $^{15}\text{N-NH}_4^+$  and  $^{15}\text{N-NO}_3^-$ , within the surrounding dry cyanobacteria- and moss-dominated crusts, and grass, *Achnatherum hymenoides* (Indian ricegrass)." Further, the rainfall event was adequate in activating the cyanobacteria and mosses in that 5 cm diameter and to stress that the rainfall event was localized we added the words "minor, localized rainfall event," which was stated in the introduction to be included in the methods, results, and discussion sections. We also state in the discussion that *Syntrichia caninervis* is stimulated by minor rainfall events and cite papers published by us. Here is what we now say in the discussion, "Mosses, including *S. caninervis*, are stimulated by minor rainfall events (Wu et al. 2014), with events as small as 1 mm activating moss photosynthesis (Coe et al. 2012). Our moss, *S. caninervis*, became photosynthetically active following the 2 mm rainfall event, changing in color from brown to green. Thus, *S. caninervis* may have absorbed the N applied in our simulated rainfall event preventing most of the isotopic

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label from reaching other biocrust constituents."

Wu N, Zhang YM, Downing A, Aanderud ZT, Tao Y, Williams S. 2014. Rapid adjustment of leaf angle explains how the desert moss, *Syntrichia caninervis*, copes with multiple resource limitations during rehydration. *Functional Plant Biology* 41:168-177.

Coe KK, Belnap J, Sparks JP (2012) Precipitation-driven carbon balance controls survivorship of desert biocrust mosses. *Ecology* 93: 1626-1636

We have also integrated another reasoning for the lack of movement in moss-dominated crusts to help clarify our message. Line 342 states, "Additionally, moss-dominated biocrusts contain far more biomass than cyanobacteria-dominated crusts. The higher levels of biomass alone may have retained the label contributing to the lack of N movement." Again, we expected water limitations to occur outside the minor rainfall even and were testing for movement of N out of the localized rainfall event.

The presence of organisms (determined by qPCR) does not allow conclusions on their functional roles.

Authors response, please see the explanation for this concern in the next paragraph

Other major comments 2. 1. The authors show that in cyanobacteria crusts, the gene copy number of Ascomycota decreases parallel to the  $\delta^{15}\text{N}$  from  $15\text{NO}_3$ . However, this does not proof that there is a functional relation between both parameters. In moss-dominated biocrusts, gene copy numbers of Ascomycota are similarly high, but no relationship to  $\delta^{15}\text{N}$  was found. In the discussion (line 303ff.) it is suggested, that fungi, likely dark septate endophytes, rapidly translocate N at a rate of 40 mm h<sup>-1</sup>. I did not find any proof for this assumption and thus it needs to be phrased VERY carefully, showing that this is mainly speculation. There are fungi within the biocrust, but a proof for their functional role was not given here. How can one be sure that fungi and not e.g. bacteria or just diffusion are relevant for the transport of nutrients in this experiment?

Authors response: We agree that an estimation of biomass does not indicate function.

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In the introduction, last paragraph, we state our intentions, "In tandem with  $^{15}\text{N}$  analyses, we estimated the biomass of two major division of fungi (Ascomycota and Basidiomycota) and bacteria, and characterized fungal communities by sequencing the 18S rRNA gene to identify potential links between fungal taxa and  $^{15}\text{N}$  movement." To address your concern and be more explicit in the text we made the following edits: line 263, 272 in the results, added proxy or estimate when referring to biomass; line 307 in the discussion, added "may stimulate" to "rainfall event stimulated"; and added the following sentence to discussion section line 376, "However, to verify the movement of N through Ascomycota and the role of biomass in translocation, a more direct approach is needed. For example, quantum dots (fluorescent nanoscale semiconductors) have tracked the flow of organically derived N into arbuscular mycorrhizae and into *Poa annua* in less than 24 hours (Whiteside et al. 2009)."

2. 3Line 350 f.: A dominance of dark septate fungi does not allow any conclusion on their role in transport processes. This needs to be shown in an experimental approach or phrased much more carefully. 2. 4. Line 376: The theory that Pleosporales are the most likely conduits is based on theory and speculation, as their functional role has not been tested. This needs to be made clear.

Author response: We were sensitive to any concrete statement about Pleosporales role. For example, in the discussion the first sentence of the paragraph on Pleosporales states, "Our results support the idea that Pleosporales are the most likely conduits for N. However, to address your concern we have revised/rephrased the statements concerning Pleosporales involvement throughout the manuscript. We understand that it is a theory but it is not speculation but a rational conclusion based on one of the first high-throughput sequencing efforts of eukaryotes in biocrust soils. Specifically, we altered the concluding sentence in the abstract to now read, "Our findings suggest that minor rainfall events may allow dark septate Pleosporales to rapidly translocate N in the absence of a plant sink." Instead of "allowed;" and we added the following statement in the discussion section on Pleosporales that direct states Pleosporales

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participation in fungal loops as a theory, "Further research is needed to address the theory of Pleosporales conduits in biocrusts and the ecological importance of the dark septate endophytes in desert systems."

2. 2. In line 333 f. it is stated that "mosses may be effective scavengers for N and outcompete fungal endophytes for newly fixed N". This indeed is very speculative, as there have been several publications on mosses, showing that N compounds are strongly leached during major rainfall events (Coxson, 1991; Coxson et al., 1992).

Coxson DS (1991) Nutrient Release from Epiphytic Bryophytes in Tropical Montane Rain -Forest (Guadeloupe). *Canadian Journal of Botany- Revue Canadienne De Botanique* 69:2122-2129.

Coxson DS, McIntyre DD, Vogel HJ (1992) Pulse release of sugars and polyols from canopy bryophytes in tropical mountain rain forest (Guadeloupe, French West Indies). *Biotropica* 24(2a): 121-133.

Authors response: Thank you for the references and the insight. We have altered the text substantially to better convey/explain our intent. We no longer refer to mosses competing with fungi for N. We now state, "The ability of mosses to scavenge atmospheric deposited N is well recognized in other systems (Liu et al. 2013, Fritz et al. 2014). Most mosses acquire N from either wet and dry atmospheric deposition (Yuanming et al. 2016) or as biogenic sources from cyanobacterial associations on their leave (Rousk et al. 2013). Mosses, including *S. caninervis*, are stimulated by minor rainfall events (Wu et al. 2014), with events as small as 1 mm activating moss photosynthesis (Coe et al. 2012). Our moss, *S. caninervis*, became photosynthetically active following the 2 mm rainfall event, changing in color from brown to green. Thus, *S. caninervis* may have absorbed the N applied in our simulated rainfall event preventing most of the isotopic label from reaching other biocrust constituents." Authors response: am not sure that our minor, localized rainfall events qualifies as a major rainfall event. Further, both of the Coxson papers are on epiphytic bryophytes/canopy bryophytes in tropical

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systems. I know that this goes without saying but cold deserts are extremely different from rainforest. Most importantly, the amount of N compounds moving through aerial mosses on tropical trees is very different that the N compounds in classically N-poor, arid systems. We definitely need more research on moss physiology across biomes.

In addition, mosses are frequently associated with cyanobacteria, which also fix and deliver nitrogen (Rousk et al., 2013, 2017).

Rousk K, Jones DL, DeLuca TH (2013) Moss-cyanobacteria associations as biogenic sources of nitrogen in boreal forest ecosystems. *Frontiers in Microbiology*. Doi : 10.3389/fmicb.2013.00150.

Authors response: The predominant, and really only moss at our site, *Syntrichia caninervis*, most likely receives N from cyanobacteria that live on its leaves and dry and wet deposition, but very little from the soils due to not possessing true roots. Thus, during our wet deposition event, rainfall event, we hypothesize that *Syntrichia* was effectively holding N from the rainfall event in our N-limited, semi-arid system. We now include Rousk et al. 2013 in our manuscript, see sentence in the discussion "Most mosses acquire N from either wet and dry atmospheric deposition (Yaunming et al. 2016) or as biogenic sources from cyanobacterial associations on their leave (Rousk et al. 2013)."

Thus, it is hard to believe that the mosses actively scavenge and hold the N compounds. I consider it as much more likely, that the amounts of water/liquid were not enough to activate the moss-dominated biocrusts at a large enough diameter.

Author response: We definitely activated the biocrusts/mosses in our 5 cm diameter rainfall event. Previous studies show 1mm is sufficient (Coe et al. 2012 ); in addition, they turned green with our water addition. For more explanation, see above our response to 1.3

5. In the manuscript, gene copy numbers are seen equivalent to biomass. This is not really correct. By means of qPCR one can get an idea regarding the relevance of the

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different organism groups but this information is by no means equal to biomass. For example, qPCR also does not distinguish between genetic material of living and dead organisms. Thus, it is much more appropriate to speak of gene copy numbers.

Authors response: we never said that it was equivalent to biomass and talk of it as a proxy or estimate of biomass. We are well aware of the potential artifacts associated with PCR but our division specific primers to estimate specific fungi in complex matrices is a wonderful technique and gene copy number of an organism is a common proxy for biomass in microbial ecology. But to more explicitly clarify any misconceptions we made the following edits: line 263, 272, 358, and 364 added proxy or estimate when referring to biomass. For example line 359 states, "We found that  $\text{NH}_4^+$ , but not  $\text{NO}_3^-$ , was rapidly translocated within crusts. The enrichment of  $^{15}\text{N}$ , from  $^{15}\text{NH}_4^+$ , in cyanobacteria biocrusts was related to our proxy for Ascomycota biomass and potentially dark septate fungi due to their dominance in our sequencing effort." We are happy to provide references of gene copy number being used as a proxy for biomass.

Minor comments: Line 64: *Bouteloua* sp. (instead of *Bouteloua* species)

Authors response: Thanks

Line 80 ff.: "In such loops, minor rainfall events may stimulate  $\text{N}_2$  fixation by free or lichen-associated cyanobacteria (Belnap et al. 2003), N mineralization by bacteria and fungi (Cable and Huxman 2004, Yahdjian and Sala 2010) and nitrification and possibly denitrification (Wang et al. 2014) all increasing the levels of  $\text{NH}_4$  or  $\text{NO}_3$ : I think one cannot say exclusively that levels of  $\text{NH}_4$  and  $\text{NO}_3$  will increase during all of these processes. During nitrification for example,  $\text{NO}_3$  is expected to decrease, but  $\text{NH}_4$  will decrease and during denitrification  $\text{NO}_3$  amounts are expected to decrease. Thus, I think one can say that that  $\text{NH}_4$  and  $\text{NO}_3$  levels will be affected by these processes, but the overall direction of change depends on the relevance of the different processes involved.

Authors response: we have altered the sentence to the following, In such loops, minor

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rainfall events may stimulate N<sub>2</sub> fixation by free or lichen-associated cyanobacteria (Belnap et al. 2003), N mineralization by bacteria and fungi (Cable and Huxman 2004, Yahdjian and Sala 2010), and nitrification and possibly denitrification (Wang et al. 2014) potentially altering levels of NH<sub>4</sub><sup>+</sup> or NO<sub>3</sub><sup>-</sup>." Thanks for the catch

Line 329 ff.: The authors state that "When *S. caninervis* was lost from this system, a dramatic increase in NH<sub>4</sub>, which ultimately nitrifies to NO<sub>3</sub>, was observed". I had a look in the publication of Reed et al. (2012), and there a decrease of NH and an increase of NO was reported.

Authors response: Thanks, I can see how what we stated was a little confusing but I believe we are saying the same thing. One of our authors was also an author on this paper. We have now changed the text to read, "When *S. caninervis* was lost from our system, a dramatic increase in nitrification rates were observed (Reed et al. 2012). The higher levels of nitrification were most likely supported by the decomposition of dead moss biomass and subsequent release of new NH<sub>4</sub><sup>+</sup>; however, after the moss mortality, inorganic N pooled as NO<sub>3</sub><sup>-</sup>, in the remaining cyanobacteria-dominated biocrust." We believe the edits address your concern.

43&Aacute;Line 336: There are no "stem cells" in bryophytes as they are thallophytes and not cormophytes.

Authors response: Thanks, we removed "stem cells" from the sentence

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Interactive comment on Biogeosciences Discuss., <https://doi.org/10.5194/bg-2017-433>, 2017.

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