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Interactive comment on "Cyanobacterial species richness and *Nostoc* highly correlated to seasonal N enrichment in the northern Australian savannah" *by* Wendy Williams et al.

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Overall We thank all referees for providing their time and recognising the importance of this research that focuses on the critical role of cyanobacteria within biocrust communities and their contribution to soil nutrients, especially N-fixation.

We appreciate the constructive comments for improvements to the manuscript and have addressed each one below.

Referee 1 "There are some flaws in the methodology that are critical to the central findings of this article. These need to be explained more thoroughly or additional ex-

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periments conducted." Specific areas: R1: 'Identification of species "richness". The method by Anagnostidis is a very classical method of classifying cyanos based on morphology, but this is quite outdated and not a very reliable metric of species 'richness'. From this approach, how exactly is abundance and richness determined? Can you really differentiate the different species (of, for example, Microcoleus) using this visual approach? If samples are still available, the authors should consider 16S sequencing. At the very least, they should extract the pigment Scytonemin as another proxy for the abundance of N-fixers present in their sample.'

Response: The authors acknowledge that advances in molecular techniques have shown the limitation of morphology-based metrics due to cryptic diversity, however in this case we are interested in relative differences between treatments, not absolute measures of richness. Therefore, in this application, the use of morphology-based measures (by two cyanobacterial specialists as described below) of species richness is adequate. 16S sequencing might be explored in future works to fully understand the diversity of these mat communities. Both B. Büdel (for 43 years!) and W. Williams (for 10 years) are specialists in taxonomic identification of cyanobacteria using high resolution DIC microscopy (examples of scientific publications include: Aboal et al., 2016; Anagnostidis and Komarek, 2005; Büdel et al., 2009; Komárek, 2013; Ullmann and Büdel, 2001; Williams et al., 2014; Williams and Büdel, 2012). Microcoleus paludosus, M. lacustris are first described in Australia by Williams and Büdel, (2012) together with M. vaginatus and others. We recognise that there are differences when morphological and sequenced data is studied. For example: Almost non-detectable visual differences between M. vaginatus and M. steenstruppii however there is evidence that M. steenstruppii (often described in literature as M. vaginatus) may represent several different cryptic species (Boyer et al., 2002); or in the case of Nostoc commune, ecomorphs such as N. flagelliforme that clearly have different morphological features, yet cannot be separated from N. commune through sequencing (Aboal et al., 2016). On the other hand, species which are not necessarily actively found in the soil but found with 16s sequencing might just be present with their DNA. In a recent publication, we

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compared the use of both methods and found, expert knowledge presupposed, that reliable results can be achieved with both methods. Here is an extract of the Abstract from the manuscript submitted to Polar Biology now ready for resubmission after minor revision (Rippin et al., Algal biodiversity of Arctic and Antarctic biological soil crusts - Morphological vs. molecular approaches): Morphological identification using light microscopy and the annotation of ribosomal sequences taken from metatranscriptomes. The analyzed samples were collected at Ny-Ålesund, Svalbard, Norway, and the Juan Carlos I Antarctic Base, Livingston Island, Antarctica. This study is focused on the following taxonomic groups: Klebsormidiophyceae, Chlorophyceae, Trebouxiophyceae, Xanthophyceae and Cyanobacteria. In total, 143 and 103 genera were identified in the Arctic and Antarctic sample, respectively, via both approaches, while 15 and 7 taxa were determined concordantly. Hence, some genera were identified only by one of the two techniques. In general, the molecular analysis indicated a higher degree of microalgal and cyanobacterial diversity (about 11 times higher). In terms of eukaryotic algae, the two sampling sites displayed comparable genus counts while the cyanobacterial diversity was much higher in the BSC from Ny-Ålesund. Furthermore, the Arctic and the Antarctic BSC shared a total of 63 microalgal species. For the first time, the presence of the genera Chloroidium, Ankistrodesmus and Dunaliella in Polar Regions was determined. Overall, these findings illustrate that only the combination of morphological and molecular techniques, in contrast to one single approach, reveals a higher degree of biodiversity for complex communities such as polar BSCs. The description of species richness in this manuscript has been derived from the separation of different cyanobacterial species through morphological features and identified to a species level where possible (see p5, Section 2.3.3). Please note the reference list used for identification has been updated (see p5, Section 2.3.3) to include more recent taxonomic literature. We respectfully disagree that identifying cyanobacteria morphologically is outdated, inasmuch that many species from the Australian continent are undescribed both in literature and sequences not available in current gene libraries (e.g. (Chilton et al., 2017 and Williams, Chilton and Alchin, unpublished data). Samples have been

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preserved for future sequencing analysis (when funds are available) that will be part of a larger biogeographical study. If scytonemin has been used as a proxy for N-fixers it assumes that only diazotrophic cyanobacteria produce scytonemin which is not the case. The position of filaments within the mat profile (and their relative exposure to UV light) is a large determinant of scytonemin production. The authors respectfully disagree that additional data relating to scytonemin would add to the records presented here of known N-fixing species.

R1: The methods overall are a bit difficult to follow: Response – areas of concern are addressed below.

R1: 'Why were the samples reactivated in the glasshouse' and 'which samples were reactivated (just for ARA)?' Response – we have added a sentence into the methods and clarified for each section the treatment of each sample set for the different analyses. Added into methods Section 2.3.1, p5, L10 Full resurrection during the wet season (when humidity increases) was critical due to the inability of these cyanobacteria to reactivate during the dry season (see Williams et al. 2014).

R1: "Please summarise method used to measure bioavailable N

Response: Added into methods p4 Section 2.3.1 Cyanobacterial crusts (0–1 cm) and sub-surface soils (1–3 cm) were analysed for bioavailable N (NH4+ + NO3Âŕ) according to Method 4, (Gianello and Bremner, 1986) and Williams and Eldridge, (2011). The samples had been immediately dried in the field (>40°C) and returned to the laboratory where they were stored dry. Duplicates for each of nine-time periods and each depth (minimum four reps) were sieved (1.86 mm) and weighed (20 mg) for both hot and cold analysis (total across all time periods for each depth, 0–1 cm n=125, 0–3 cm n=99). This method determines ammonium-N (NH4+) produced from organic soil N when the soil is heated with 2M KCl in a stoppered tube at 95°C for 16 hours. NH4+ is determined by the difference between the NH4+ liberation during the hot distillation of 20 mg soil and the NH4+ present prior to heating (Gianello and Bremner, 1986) and

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provides an index for bioavailable (mineralizable) N at the time of sampling (also see Tongway and Ludwig, 1996; Williams and Eldridge, 2011).

R1: "How were the samples collected (were they cored) and how were the samples stored after collection? Were they stored at all or were they analysed immediately?

Response: Added to P4, Section 2.2 L16-18,: 'Cyanobacterial surface crusts and subsoils were removed using a 10 cm metal spatula to extract exactly 0–1 cm vertical depth with care to include the complete sample, followed up by 1–3 cm depth directly below the crust.' In Section 2.3.1 we have added the dry storage and time frame for when the samples were analysed. 'The samples had been immediately dried in the field (>40°C) and returned to the laboratory where they were stored dry and analysed after the end of the wet season (2010).'

R1: "More details are needed in the statistics section (section 2.3.4) especially pertaining to section 3.2."

Response: The following detail has been added to improve the description of the statistics and provides an explanation of the analysis of the results presented in section 3.2 2.3.4 Statistics 'We used linear regression models to examine potential relationships between bioavailable N and cyanobacterial richness separately, for N-fixing and non-N-fixing cyanobacteria (see Table 1). We examined differences in bioavailable N between the two depths across time with mixed-models ANOVA. Our model had two strata, one that accounted for the differences among the nine time periods, and a second stratum accounting for depth and its interaction with time. All of these analyses were run in Minitab Version 16.1.0 (2010). Least Significant Difference (LSD) testing was used to examine differences in means among the nine time periods. Tests for homogeneity of variance, independence and normality in the data, using Levene's test and other diagnostic tools in the Minitab (2010) statistical package, indicated that no transformations were necessary.'

R1: "In the first sentence of the discussion it says that isotopic signatures for 15N2

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clearly demonstrate that cyanobacteria were the primary source of bioavailable N. The results that are presented do not “clearly” demonstrate that. It says that nitrogen fixation is correlated with cyanobacterial richness (and with certain species) but it cannot be ruled out that other N-fixers are present. This is especially true for other biocrusts where cyanobacteria are not the primary N-fixers.”

Response: In the first sentence of the discussion (Section 4) we have changed the start of the first paragraph to read: ‘In this study, isotopic signatures for δ 15N across seven months of active N-fixation demonstrated cyanobacteria were likely to be the primary source of bioavailable N (Evans and Ehleringer, 1994), although it is now understood that microbial resource partitioning on a microscale takes place within the biocrust strata (Baran et al., 2015). Given the rapid wetting following the dry season, potential cell lysis (Williams et al., 2014; Williams and Eldridge, 2011) and the presence of other N-fixers (Hawkes, 2003), it is difficult to separate the exact source of bioavailable N (e.g. Baran et al., 2015; Dojani et al., 2011; Johnson et al., 2007). . ..’

R1: Technical corrections:

R1: P1, L12: Response: Added ‘Many’ R1: P1, L18: Response: Changed sentence to read: ‘Over the wet season cyanobacterial richness ranged from 6–19 species. N-fixing Scytonema accounted for seasonal averages between 51–93% of the biocrust.’ R1: P1, L20: Response: deleted ‘It was established. . .’ R1: P2, L3: Response: changed to ‘the northern Australian savannah. . .’ R1: P2: Response: Rossi et al. 2017 added as reference R1: P4, L5: Response: added the word ‘fixation’ R1: P5, L17: Response: Formatted heading to bold R1: P6, L5/6: Response: revised sentence to ‘Three species of the nostocalean N-fixing Scytonema accounted for 74% of the biocrust in varying proportions (range 55–93%) throughout the season (Table 1).’ R1: P6, L14: Response: changed to ‘. . .before changing to more than half across January to February. . ..’ R1: P8, L1/2: Response: Sentence changed to: ‘It had been previously shown that the biocrust had high enzymatic relative to the underlying soil (Chen et al., 2014).’ R1: P8, L9: Response: changed ‘reabsorbed’ to ‘consumed’ R1:

P8, L21: Response: added the word ‘biocrust’

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