nature portfolio

Peer Review File

Integration of multiple metabolic pathways supports high rates of carbon precipitation in living microbialites

Corresponding Author: Professor Rosemary Dorrington

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This paper has many major flaws, unfortunately. Although it is an interesting paper in which 13C-incorporation and microbial diversity was measured, in my opinion, major flaws include:
1) These are not stromatolites; maybe thrombolitic microbialtes. No thin sections are shown (or other evidence) that the surface mats shown in Fig 1 actually contribute to the structure underneath;
2) the shallow water overhead appears to be flowing, so there is degassing, which impacts the alkalinity - in addition to the metabolism considered, this must play a role in precipitation. In fact, Fig 1D is a prime example of a travertine deposit
3) the claim that the rates measured here are uniquely high is not true. I used Pace et al. 2018 (Geobiology) and found that assuming 10-12 hr daylight, the C-fixation rates reported there are 7.2-14.4 g/m2.d; there are numerous studies that report net photosynthetic rates in microbialites and microbial mats; Jorgensen and coworkers, De Beer, Kuhl, Visscher, Wieland and others have used micro electrodes (which prevents the issue of depletion of the C-pool; see below)
4) a very selective, poor number of papers is referenced when it comes to comparisons with existing literature (of the last 5 years); also, there is more involved in carbonate precipitation than an alkalinity change (see Kaczmeirczak)
5) predicted values cannot be compared with measured ones (e.g., for "stromatolite" growth")
6) as the authors know, the cycling of element is very tight in microbial mats like the ones they report on; this makes isotopic studies a bit difficult, especially those using carbon isotopes, because there is no isotopic discrimination as the C pool is virtually depleted;
7) Van Gemerden 1993 (Marine Geology) exactly describes his model of photosynthesis and chemolithotrophy as the authors of this paper do; what the authors do not mention is that several metabolisms dissolve (by lowering the alkalinity) carbonates;
8) slurries (homogenates) have of course much higher metabolic areas;in fact, in real mats (like the superficial mats of this study), the DIC replenishment is limited (the exact reason for an increase in alkalinity - see papers by Kempe & Kazmeirczak);
9) is a four-hour incubation in the middle of the day (peak sunlight) representative for the daytime period? DIC measurement from a similar investigation cannot be used - there may be fluctuations, or other factors; this can cause a problem in the calculations; it is unclear what "SP:" stands for.
10) for accretion rates, perhaps thin sections should be viewed; most likely, the precipitate is micrite with a different density (not a major issue, though).

These structures are not stromatolites like the ones in the Bahamas and Shark Bay and certainly do not resemble any stromatolite found in the fossil record. There is no consideration for other metabolic processes that play a role (either stimulating precipitation or dissolution of carbonates), not that simply an increase in alkalinity would suffice (there are other critical factors involved, e.g., nucleation sites for minerals to form).

Reviewer #2

(Remarks to the Author)
Review of "Integration of multiple metabolic pathways supports unprecedented rates of carbon sequestration in living stromatolites"

Thank you for the opportunity to review this manuscript. In this study of South African stromatolites, the authors state that

they have measured rates of carbon sequestration using 13C labeled bicarbonate tracer experiments and linked these rates to the metabolic function and genetic diversity of the microbial communities assessed by 16S rRNA and shotgun metagenomic analysis. In their results, the authors highlight an exceptionally high rate of carbon sequestration (higher than tropical evergreen and deciduous forests) that is facilitated by non-photosynthetic mechanisms bolstering known carbon uptake by the light-dependent photosynthetic pathway. They attribute the high rates to a combination of mineralization through alkalinization, and chemoautotrophic metabolism. An interesting potential link was made to NH4 concentrations in some settings.

Overall, the manuscript text is clearly written. The approach used in this study relies on published methods, and the authors use conservative estimates in their calculations based on previous published measurements of water chemistry in the study sites. In these calculations, I would suggest defining all variables in the rate calculations and including scale bars in the field photos.

However, I have concerns about the authors definition of sequestration and their accretion rate estimates, which are fundamental components of their analysis. If the conclusions are better supported and confirmed by measurement rather than estimate, this will be a high impact contribution that highlights the previously unrecognized importance of stromatolite environments in the suite of known global carbon sinks. I agree with the authors, this would be a highly significant observation if supported.

I am not a microbiologist, so my comments will be made on the aspects of sequestration and carbonate mineralization. I summarize those concerns in the following:

1. It seems the authors are using “uptake” and “sequestration” interchangeably. They interpret high uptake rates in the measurements of the 13C labeled experiments to reflect chemoautotrophy and carbonate precipitation, but how is this differentiated? How much precipitation occurred? How long is the carbon sequestered, or does it get quickly recycled through other microbial metabolisms?

2. To produce calcium carbonate, HCO3 needs to be broken into CO3 + H. The release of this proton can reduce pH and influence net carbonate production. How is this H+ shuttled away from precipitation to produce such high rates of proposed precipitation? If this is completed through the alternative metabolic processes, please clarify. Balanced reactions of each showing how this is expected to occur can be helpful to the reader.

3. The assumption that the density of stromatolite carbonate is equal in density to a non-porous pure calcite need further consideration. When viewed in thin section or SEM, it is apparent that carbonate in stromatolite is highly heterogeneous, with many voids that produce high porosity, in some instances high permeability. Depending on the environment and the stromatolite formation mechanism, multiple minerals can also be present in the stromatolite. I would encourage the authors to consider this in the context of their estimates of accretion rate before comparing to other settings.

4. The Bahamian stromatolites accrete via trapping and binding, while the accretion mechanism of these stromatolites in South Africa is not mentioned. Should it be precipitation as it might appear from the photos, Shark Bay stromatolites are a better analogue. It would be expected that trapped and bound carbonate accretes faster than microbial precipitation, but would not have the same magnitude of impact on bicarbonate uptake. This is an important area of comparison to discuss, and I encourage the authors to explain why the South African stromatolites behave so anomalously when it comes to carbon sequestration potential. This has implications for the applicability of the findings to the geological record.

5. The stromatolites in the field photos appear to be at least partially subaerially exposed. How do periods of subaerial exposure alter the continuity of the accretion rates proposed here? How does this impact estimates of carbon uptake and subsequent sequestration?

In its current form, I cannot recommend this manuscript for publication. Differentiation between uptake and sequestration is needed, and a mechanism to deal with the proton released during the transformation of bicarbonate to carbonate should be presented to warrant stromatolites as a carbon sink. Finally, I suggest the authors either measure precipitation rate (difficult in such short time spans) or produce a measured accretion rate curve for the samples. These results can be compared estimated accretion rate results calculated in the present study once the porosity and heterogeneity of stromatolites is accounted for. If this accretion rate curve agrees with the proposed revisions to the accretion rate estimates based on uptake, the conclusions of the paper would be strengthened.

Reviewer #3

(Remarks to the Author)
The work entitled "Integration of multiple metabolic pathways supports unprecedented rates of carbon sequestration in living stromatolites" offers new insights into these ecosystems. With a combination of fieldwork, bioinformatic analyses, and wet-lab experiments, the study proposes an unrecognized role for stromatolites. The study is well designed and the data support the conclusions. I only have minor remarks on the manuscript, but I have not received supplementary information: the zip file with these data had only another copy of the manuscript. I would not recommend publication before seeing the full data.

Some comments from the manuscript

Line 256: define NPP

Line 257-261: You compare predicted growth only based on your carbon sequestration measurements. Is this valid? Additional factors might be involved. For example, the Highbourne Cay and Shark Bay systems you cite have similar growth rates, but one order of magnitude differences in carbon sequestration rates.

Line 329: I understand ambient temperature can be variable and was not controlled, however, please provide at least a range of approximate temperature.

Lines 344-348: please define all abbreviations used in your formulas.

Line 370: "concentrations of between 2 – 4 µg of gDNA". Concentrations should be in µg/ml, I think that word should be changed. I understand 2 - 4 µg is the total amount of DNA you obtained.

In lines 384-385, please clarify which genes are used for each category.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The revisions to the manuscript "Integration of multiple pathways supports unprecedented rates of carbon sequestration in living microbialites" generally addressed many of the comments on the original manuscript.

The measurements document bicarbonate uptake rate, and the revised text reflects that in most of the text. The addition of microbial metabolisms to remove and recycle H was a useful addition.

However, carbon sequestration remains to be shown and is a central point of the manuscript's relevance and importance (see lines 102-105, 218-222, and 277-286). In addition to the release of the proton, the precipitation of carbonate minerals is known to reduce alkalinity, and thus may even release CO2 back to the atmosphere (see papers by Elderfield, Archer, Broecker and Peng, etc). This is fundamental to address in order to contextualize these results and support the author's proposal that the carbonate produced by microbialites are the most efficient carbon sink that sequesters carbon on geologically relevant time periods. The revised manuscript cites a review of carbon sequestration mechanisms (Fuss et al., 2018), and not measurements of sequestration via mineralization.

The authors also propose that microbialite mineralization could be an effective tool for mitigating carbon emissions. If sequestration as carbonate minerals can be demonstrated as described above, this idea should be supported further. Based on the distribution of modern peritidal stromatolites, what is the magnitude of the potential flux and sequestration period? For example, absolute rates of carbon uptake by microbialites were compared to NPP of tropical evergreen and deciduous forests, the areas of which are significantly different, and thus the flux of carbon is significantly different.

In its current form, the carbon sequestration potential has not been documented, and thus the data do not support the central relevance proposed for these observations.

Reviewer #3

(Remarks to the Author)
The revised manuscript of Professor Dorrington and colleagues addresses my concerns. Based mostly on carbon sequestration measurements and metagenomics data, the study proposes an unrecognized role for stromatolites/microbialites. The study is well designed and the data support the conclusions. Checking on the supplementary data now provided I have only a few inquiries/suggestions. I would recommend the publication of a revised version.

* Figure 3 requires the supplementary data to understand what specific genes are shown. I'd provide at least an abbreviation of the genes represented on each row.

* Following your discussion, on lines 232-235 you hypothesize on the role of CAs, which seem to be abundant in your dataset. Are there extracellular CAs in your data? How abundant are they?

* You mention the WL pathway for chemoautotrophic carbon fixation at night. And it's present in the OV745 samples as shown in Fig 3. However, which organisms would bear it? I can not tell only from the OTUs in Fig 2. Are these WL-bearing

organisms abundant? I doubt how much is this contributing to the observed sequestration, but defining the relative contributions is beyond this work.

Version 2:

Reviewer comments:

Reviewer #4

(Remarks to the Author)
Review of manuscript titled:

“Integration of multiple metabolic pathways supports unprecedented rates of carbon sequestration in living microbialites”

by Eric W. Isemonger, Rosemary A. Dorrington and collaborators

This manuscript addresses an important question about the carbonatogenesis potential of microbialites and the corresponding metabolic pathways. This study aims to link the relationships between the taxonomic diversity and functional capacity with C uptake and precipitation rates of microbialites. To this end, the authors performed DNA extraction from microbialites, 16S rRNA amplicon and metagenomic sequencing, together with laboratory-based experiments. The characterization of the microbial communities, and the metabolic pathways associated with microbialites is very valuable and the aim to combine both metabolic pathways and microbialite accretion rate and C uptake is novel. I recommend clarifying and modifying several points, in particular on clarifying the methods and on the discussion of the limits of the methods, before considering its publication to Nature Communications.

1. The core of the paper, i.e., the estimations of C uptake and the precipitation rates are both not robust enough. This is probably because the successive calculations are not provided in the manuscript nor in supplementary information. All the calculations must be provided in supplementary information as an excel file with all the calculations visible. In addition, please consider providing a workflow with the type of experiment/the samples used, the estimation from natural samples and the techniques/methods/calculations as well as the results obtained to better follow the study.

2. This study describes the accretion of existing carbonate grains, incubated as a slurry (crushed microbialite with water) in natural water collected from a natural site. The experiments do not demonstrate the in-situ formation of carbonate and lithification of these grains. However, this is now accepted by the community that microbialites form via different mineralization pathways, with the most prominent pathways being in situ precipitation of mineral (mainly carbonate) via (1) the metabolic activity of microorganisms (induced biomineralization) and (2) the presence of extracellular organic molecules (influenced biomineralization). An experiment never reproduces the complexity of Nature. The question is whether a solution with a microbialite slurry mimics the natural processes of growth of microbialite. I believe a point of discussion on the limits of the experiment conducted here regarding the “forced” accretion experiment applied here is needed.

3. The experimental approach conducted here leads to a very high accretion rate (i.e., 1 mm per week, figure 7 and lines 235-238) which I believe overestimates the reality. Accretion rates have been measured in situ by different groups (i.e., incubating substrates within lakes or by datation) for microbialites from both continental and marine settings and the studies show a range of accretion rate from 0.33 mm/year to up to actually 5 mm/yr (after Power et al., 2011). Again, the high accretion rate provided here from the experiments should be criticized in the manuscript.

4. Line 59: A point in the introduction on the role of physico-chemical conditions allowing the formation of microbialites is missing.

5. Lines 56, 81, 97: Different terms are used to refer to modern microbialites: extant, living, contemporary: please uniformize the nomenclature along the manuscript

6. For the uptake measurement: What was the 13C content of the collected microbialite used in the experiment? It is expected to have a consequent mass of 13C already coming from the slurry (the microbialites grains incubated within the bottle). This data is not mentioned in the method section nor in the result and is crucial to consider in the rate of 13C uptake.

7. There is very likely an overestimation of 13C on the filters after the experiments linked to the saturation of the filter with 13C-rich water. The filters from 2018 and 2019 experiments collected before the mass spectrometer analyses need to be rinsed with DI water to be reflective of the real 13C uptake by biomass and carbonate. This step is not mentioned in the method section.

8. Figure 7: under which type of laboratory culture?

9. Lines 45, 219, 233, 471, 721: Ca-carbonate is not necessary calcite. Ca-carbonate has different polymorphs. Ca-carbonate can be aragonite, calcite, vaterite, monohydrocalcite. This manuscript and the past study (Buttner et al., 2021) do

not decipher about the type of Ca-carbonate. Please remove the name calcite and replace by “Ca-carbonate”.

10. Line 144: write as “microbialite”.

11. Line 232: How the accretion of 9.20 to 16. 41 kg CO2/m2/yr was determined?

12. Lines 392-402: DIC is not only HCO3- this is also CO32- and CO2. Moreover, it is necessary to mention that the released CO2 during the formation of CaCO3 from 2(HCO3-) has not been measured and considered in the calculation of the C rate calculation.

13. Line 474: What are precisely the 7 selective domains?

14. Line 474: What are the proportions of Ca-carbonate in the 4 samples?

15. Lines 476-477: Provide the calculations (equations) made by the software RockMaker to calculate C contents of microbialites. Using which data? There is a lack of clarity on how the Ca-carbonate was quantified within the CR microbialite.

16. Line 475: After checking the link, different threshold plugins exist on ImageJ. What is exactly the name of the used threshold plugin?

17. A method paragraph is missing for the electron microscopy observations: how the samples were prepared? Under which analytical conditions?

Version 3:

Reviewer comments:

Reviewer #4

(Remarks to the Author)
The revisions to the manuscript “Integration of multiple metabolic pathways supports unprecedented rates of carbon sequestration in living microbialites” by Dorrington et al. generally addressed many of the comments on the last version of the manuscript. The authors provided the requested details on the methods and on the calculations.

However, considering the equation of carbonate precipitation 2HCO3- + Ca2+ --> CaCO3 + CO2 + H2O, no robust carbon sequestration is demonstrated if the released CO2 is not tracked and quantified during the precipitation of CaCO3. The authors demonstrate the transfer of carbon between the dissolved (HCO3-) and particulate fraction (CaCO3) and do not account for CO2 release. This crucial aspect must be clearly stated in i) the abstract ii) the results section iii) the discussion. This transfer of carbon between HCO3- and CaCO3 should not be referred as carbon sequestration since CO2 is released during the process. In this frame, the title must be replaced by “Integration of multiple metabolic pathways supports unprecedented rates of carbon transfer in living microbialites”.

This aspect on the CO2 release during the precipitation must be discussed regarding 1) the dissolved C speciation, 2) the bacterial communities populating the biofilm and 3) the type of carbonate precipitating. On this last point, Raman analyses is an important addition. However, the quality of the 3 spectra provided now in supplementary is particularly low and it is quite difficult to distinguish the peaks of calcite from the aragonite peaks for instance. In addition, whether the Raman analyses were done on all the studied samples or only on one sample is unclear. I suggest to do additional Raman analyses on the studied samples from the different studied sites, to decipher about the mineralogical composition of the microbialites, which is a crucial aspect to quantify the carbon transfer during carbonate precipitation. I also suggest combining in situ Raman analyses and SEM with bulk XRD and/or FTIR, to correlate microscopy (SEM and Raman) with the bulk mineralogy of the studied samples and highlight the presence of potential additional carbonate minerals. Last, the accretion rate determination is not clearly explained. I recommend more details on this result which is one of the important results of the study.

Specific comments:

1. Line 82: Mineralogy
Gypsum is a Ca-sulfate mineral (does not contain carbon).

2. Lines 57-59/Figure 1: Physico-chemical conditions promoting microbialites
In addition to pH, a(CO3-2) and a(Ca2+) are key parameters explaining the presence of microbialites (i.e., Zeyen et al., 2021, GCA and Caumartin et al., 2023 GPL). Specifically (over-)saturation of solutions with respect to monohydrocalcite/ACC is necessary to form microbialites. More integration of this recent research is needed to fully cover this question of the role of the physico-chemical conditions on microbialite formation.

3. Lines 445-465: Definition needed on specific vs. absolute uptake rates

Hama et al. did not use the terms “specific” and “absolute” for the C uptake rates. Please define each C uptake rate by a few words.

4. Uniformization of the figures
Uniformize the axis of your figures with “C specific uptake rate” and “C absolute uptake rate” to be coherent with the “methods” section (lines 455-460).

5. Line 455-462: Definition needed for the rate calculation
Please define what is “C_substrate_atom%” and define what is the meaning of “excess” in the text. Refer also here to the excel spreadsheet.

6. Line 457: Clarification needed on the calculation
On the spreadsheet the specific rate was calculated as C_atom%_XS / (C_Substrate_atom%_XS*time) and this does not correspond to the formulae written in the text.

7. Line 458: Clarification needed on the calculation of the absolute rate
Is [PC] the proportion of the core? Please detail each term of the equation in the text.

8. Lines 465-471: Clarification needed on the determination of the accretion rate
Is the unit of the accretion rate correct (i.e., mm m-2)? In the abstract the accretion rate is expressed as mm/year. After carefully checking the cited paper (Komad et al), I was not able to see the link between δ13C and accretion rate in mm/yr and to find the calculations resulting to the result “20-30 mm of vertical calcium carbonate accumulation per year” from the abstract. Please detail the method on your manuscript with the equation and excel table if possible to help the reader to follow better the reasoning of the result section (i.e., lines 235-257)

9. Line 467: “Fumigation” instead of “fugation”?

10. Lines 470-471: Details on the calculation of the maximal annual growth estimates
As requested previously, please provide the equation and the calculations for the estimates.

11. Suggestion on the methods organization.
Perhaps add the section “C accretion in mineralized parts of stromatolites” right after “rate calculations”.

12. Discussion: A point of comparison of the uptake rates and the physico-chemistry
How the 4 different uptake rates compared to the water chemistry (determined previously in the literature)? Do you have a correlation between uptake rate and alkalinity and Mg/Ca as described by Zeyen et al., 2021 for the Mexican lakes?

13. Line 236 and line 242: Clarification on detrital minerals
What do you refer as “mineral detritus”? Detrital minerals such as feldspar? Some studies have shown that silicate phases (similar to stevensite) can be authigenic and microbially mediated (e.g.,Suosaari et al., 2022) and are not always detrital.

14. Lines 296-309: CO_2 release during carbonate precipitation
A point of discussion is necessary on the CO_2 release during carbonate precipitation, with respect to the metabolisms;- and the physico-chemistry of the water. On this last point see for instance the recent article from Many et al., 2024 Science advances.

15. Line 319: Clarification on the ideal conditions
What are precisely the “ideal conditions” necessary for vertical layer growth?

16. Lines 328-332: Conditions and quantification of CO_2 release during carbonate formation
The conditions leading to CO_2 release must be understood and the carbon balance will need to be done.

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RESPONSE TO THE REVIEWER COMMENTS

We thank the reviewers for their insightful comments and suggestions. We have done a careful review of their comments and have edited the manuscript accordingly or have provided a detailed response as to why changes were not made. Our responses are in bold below each comment.

Reviewer #1 (Remarks to the Author):

This paper has many major flaws, unfortunately. Although it is an interesting paper in which 13C-incorporation and microbial diversity was measured, in my opinion, major flaws include:

1) These are not stromatolites; maybe thrombolitic microbialtes. No thin sections are shown (or other evidence) that the surface mats shown in Fig 1 actually contribute to the structure underneath;

Response: Two of the sites in this study (CR and SK) have been identified in the literature as stromatolites (Buttner et al. 2021[21], Smith et al. 2011[19]; Smith et al. 2018[20], Rishworth et al. 2020[22]). However, while the OV745 and TP formations display the characteristic layered structure, they still need to be confirmed as true stromatolites. Therefore, we have changed the term “stromatolites” in the title to “microbialites” and edited the text where appropriate (Introduction: additional references cited lines 87-89, Results lines 111-119).

2) the shallow water overhead appears to be flowing, so there is degassing, which impacts the alkalinity - in addition to the metabolism considered, this must play a role in precipitation. In fact, Fig 1D is a prime example of a travertine deposit

Response: Yes, the water is flowing over the sampling stations at all four formations. To determine if the carbon uptake observed was via abiotic reactions, we performed mercuric chloride (HgCl2)-killed controls and found that carbon uptake in these treatments accounts for a relatively small proportion ~15% of the rates observed in both day and night time treatments. If formation is solely, abiotically facilitated by degassing then one would expect the carbon uptake rates within the HgCl2 killed controls to account for a larger proportion of the total carbon uptake found in the live incubations. This was not the case, please see Figure 4.

3) the claim that the rates measured here are uniquely high is not true. I used Pace et al. 2018 (Geobiology) and found that assuming 10-12 hr daylight, the C-fixation rates reported there are 7.2-14.4 g/m2.d; there are numerous studies that report net photosynthetic rates in microbialites and microbial mats; Jorgensen and coworkers, De Beer, Kuhl, Visscher, Wieland and others have used micro electrodes (which prevents the issue of depletion of the C-pool; see below)

Response: We agree with the reviewer that some microbial mats have higher photosynthetic rates and have therefore made the appropriate edits to address this

recommendation. However, our study is unique in that the shotgun metagenomic data provides insights into the potential mechanisms that enable high rates of light independent (night time) uptake. The literature recommended by the reviewer has been helpful and we have cited some of the relevant papers.

Line 45-48: Abstract
"Through the integration of these mechanisms, stromatolites can sequester inorganic carbon over a full 24-hour cycle at a rate (6.87-12.26 gC m-2 24h-1) among the most productive microbial mats and exceeding the most productive terrestrial systems."

Lines 82-86: Introduction
"Unlike microbial mats, microbialites precipitate carbonate through biological alkalinization, potentially aided by the trapping and release of cations by exopolymers to form C-containing minerals, such as calcite, aragonite and gypsum[2,16,17]. This process can sequester carbon for extended periods on the order of 10^6 years[18]."

Line 235-248: Discussion
"The presence of gene indicators of chemoautotrophic metabolism, such as the WL pathway, raise the possibility that carbon is being actively taken up at night for chemoautotrophic carbon fixation. In addition, various metabolic guilds of microorganisms such as sulphur-cycling bacteria, methanogens, fermenters and heterotrophs, can promote either precipitation or dissolution of carbonate by changing alkalinity[16,17]. In stromatolites, these metabolisms can also be linked in a variety of syntrophic relationships[34]. Thus, it is probable that night-time carbon uptake is driven by both active HCO_3^- uptake to fill biological carbon demand, and biologically driven mineralization, as a by-product of a system where net metabolic activity is balanced in favour of alkalinisation. It is also possible that various genes related to H^+ transport (Na^+:H^+ antiporters), H_2 production (bidirectional hydrogenases), and electron bifurcation which can be linked to H^+ import, may aid carbonate precipitation by removing H^+ [35]."

Line 256-260: Discussion
"These rates are consistent with photosynthetic rate estimates in previous studies using intact stromatolite material[28]. These rates are significantly higher than a number of extremely productive microbial mats such as those of Tengchong and Little Hot Creek (4.6 mgC $gTOC^{-1}$ and 1.82 mgC $gC^{-1}h^{-1}$ for non-lithifying and lithifying mats, respectively)[26,2]."

4) a very selective, poor number of papers is referenced when it comes to comparisons with existing literature (of the last 5 years); also, there is more involved in carbonate precipitation than an alkalinity change (see Kaczmeirczak)

Response: We concur that there is more to carbonate precipitation than alkalinity alone. Therefore we explored the roles of various metabolisms and carbon concentrating pathways that may facilitate/ promote precipitation. We also conducted HgCl2 "kill" control incubations to assess abiotic precipitation. We assessed light dependent (day) and light independent (night) metabolisms and analysed shotgun metagenomic data sampled from each site. We also described the potential role of EPS in concentrating cations and increase precipitation with references to the work of Kaczmierczak. We have also added additional references.

Line 82-86
“Unlike microbial mats, microbialites precipitate carbonate through biological alkalinization, potentially aided by the trapping and release of cations by exopolymers to form C-containing minerals, such as calcite, aragonite and gypsum[2,16,17]. This process can sequester carbon for extended periods on the order of 10^6 years8[8].”

Line 238-248
“In addition, various metabolic guilds of microorganisms such as sulphur-cycling bacteria, methanogens, fermenters and heterotrophs, can promote either precipitation or dissolution of carbonate by changing alkalinity[16,17]. In microbialites, these metabolisms can also be linked in a variety of syntrophic relationships[34]. Thus, it is probable that night-time carbon uptake is driven by both active HCO_3^- uptake to fill biological carbon demand, and biologically driven mineralization, as a by-product of a system where net metabolic activity is balanced in favour of alkalinisation. It is also possible that various genes related to H^+ transport (Na^+:H^+ antiporters), H_2 production (bidirectional hydrogenases), and electron bifurcation which can be linked to H^+ import, may aid carbonate precipitation by removing H^+ [35].”

5) predicted values cannot be compared with measured ones (e.g., for "stromatolite" growth")

Response: We agree with the reviewer and have clarified the text to show that our calculation is at the lower range of possible accretion rates based on porosity and composition of each individual formation.

Line 269-274
“BBased upon daytime rates of $CaCO_3$ precipitation reported by du Plooy et al (2020)[32], the SK stromatolites may be sequestering more than 90% of their carbon uptake. Therefore, the uptake rates measured in this study over 24 hours could be equivalent to approximately 10 mm m^{-2} of pure calcite growth per year, although the true accretion rate is likely higher given the known heterogeneity and porosity of microbial carbonates.”

6) as the authors know, the cycling of element is very tight in microbial mats like the ones they report on; this makes isotopic studies a bit difficult, especially those using carbon isotopes, because there is no isotopic discrimination as the C pool is virtually depleted;

Response: The volume (water) to mass (microbial community) ratio was intentionally high in our incubations to account for rapid removal and replenishment of bicarbonate. Using the known bicarbonate additions, calculated rates and total mass of particulate carbon in each incubation, we can confirm that our samples did not run out of available bicarbonate and thus complete removal of ^{13}C during the 4 hour bioassay was not an issue.

Line 316-319
“To ensure that the microbial community did not run out of bicarbonate during the incubation, 1 mL of the slurry (100-500 µg microbialite carbon) was added to 500 mL of filtered station water in acid washed (10% HCl) 500 mL PETG incubation bottles.”

7) Van Gemerden 1993 (Marine Geology) exactly describes his model of photosynthesis and chemolithotrophy as the authors of this paper do; what the authors do not mention is that several metabolisms dissolve (by lowering the alkalinity) carbonates;

Response: We appreciate the reviewer's comment and drawing our attention to van Gemerden 1993. This is an interesting perspective, however if dissolving metabolisms are dominant without a tightly coupled counter metabolism or reaction, that should lead to a net release of bicarbonate and dilution of the total bicarbonate pool, which in turn would cause an underestimation of actual bicarbonate uptake rates. This supports the contention that our work represents a conservative estimate of carbon uptake.

Line 241-248: Discussion
"In microbialites, these metabolisms can also be linked in a variety of syntrophic relationships[34]. Thus, it is probable that night-time carbon uptake is driven by both active HCO_3^- uptake to fill biological carbon demand, and biologically driven mineralization, as a by-product of a system where net metabolic activity is balanced in favour of alkalinisation. It is also possible that various genes related to H^+ transport (Na^+:H^+ antiporters), H_2 production (bidirectional hydrogenases), and electron bifurcation which can be linked to H^+ import, may aid carbonate precipitation by removing H^+ [35]."

8) slurries (homogenates) have of course much higher metabolic areas;in fact, in real mats (like the superficial mats of this study), the DIC replenishment is limited (the exact reason for an increase in alkalinity - see papers by Kempe & Kazmeirczak);

Response: We agree with the reviewer that slurries can display enhanced metabolic rates therefore we were conservative with all calculations. That said, our observed rates are consistent with daytime primary production estimates conducted on intact material from site SK (Du Plooy et al, 2020[32]).

Line 256-260
"These rates are consistent with photosynthetic rate estimates in a previous study using intact stromatolite material from SK[32] and are significantly higher than a number of extremely productive microbial mats such as those of Tengchong and Little Hot Creek (4.6 mgC $gTOC^{-1}$ and 1.82 mgC $gC^{-1}h^{-1}$ for non-lithifying and lithifying mats, respectively)[2,28]"

9) is a four-hour incubation in the middle of the day (peak sunlight) representative for the daytime period? DIC measurement from a similar investigation cannot be used - there may be fluctuations, or other factors; this can cause a problem in the calculations; it is unclear what "SP:" stands for.

Response: We purposely targeted the highest light during the day and the darkest time at night to isolate the carbon uptake mechanisms of the diel cycle. By targeting the peak daylight and middle of the night, we were better able to isolate the processes and rates of light dependent and light independent processes. We used an estimate of 12hrs of daylight and 12 hours of night in our estimates to account for

twilight periods. Running longer incubations (24 hours) may have resulted in a depletion of the bicarbonate pool and our inability to determine an accurate rate, as the reviewer indicated in point 6. Combining peak daylight and night time rates is frequently done for diel studies that are not able to run the full 24 hours.

It is truly unfortunate that the bicarbonate concentration samples we collected were compromised during processing at a national laboratory, but we used the lowest observed bicarbonate concentration from published representative samples from this region (Dodd et al, 2020), although all sites had relatively similar concentrations. Therefore, while not ideal, using the lowest observed bicarbonate concentration would result in potential underestimations not over estimations of actual rates. We have been as conservative as possible with our findings and again while not ideal, using the lowest reported value of these relatively consistent DIC numbers enables us to show the minimal potential impact of these systems.

Line 367
SP is specific carbon uptake rate changed to v for clarity.

$$\boldsymbol{\rho = \frac{PC\ at\%\ xs}{v\ at\%xs\ \times\ Time}\ \times\ [PC]}$$

10) for accretion rates, perhaps thin sections should be viewed; most likely, the precipitate is micrite with a different density (not a major issue, though). These structures are not stromatolites like the ones in the Bahamas and Shark Bay and certainly do not resemble any stromatolite found in the fossil record. There is no consideration for other metabolic processes that play a role (either stimulating precipitation or dissolution of carbonates), not that simply an increase in alkalinity would suffice (there are other critical factors involved, e.g., nucleation sites for minerals to form).

Response: We respect the reviewer's point of view, but it seems that they are unfamiliar with the well documented South African Tufa stromatolite formations that are, as the review points out, very different from those found in marine and hypersaline systems and also, not travertine deposits (Rishworth et al. 2020[22]). While outside the scope of this study, which focuses on biologically driven bicarbonate uptake, we have cited our recent paper in *Geobiology* (Buttner et al. 2021[21]) showing that the CR formations are in fact phosphatic stromatolites that are directly comparable to those in the fossil record. Further validation can be found in Rishworth et al. 2020[22], Smith *et al.* 2011[19] Smith *et al.* 2018[20]. Smith *et al.* 2018 specifically identifies features native to these formations which are shared with stromatolites in the fossil record.

Reviewer #2 (Remarks to the Author):

Review of "Integration of multiple metabolic pathways supports unprecedented rates of carbon sequestration in living stromatolites"

Thank you for the opportunity to review this manuscript. In this study of South African stromatolites, the authors state that they have measured rates of carbon

sequestration using 13C labeled bicarbonate tracer experiments and linked these rates to the metabolic function and genetic diversity of the microbial communities assessed by 16S rRNA and shotgun metagenomic analysis. In their results, the authors highlight an exceptionally high rate of carbon sequestration (higher than tropical evergreen and deciduous forests) that is facilitated by non-photosynthetic mechanisms bolstering known carbon uptake by the light-dependent photosynthetic pathway. They attribute the high rates to a combination of mineralization through alkalinization, and chemoautotrophic metabolism. An interesting potential link was made to NH4 concentrations in some settings.

Overall, the manuscript text is clearly written. The approach used in this study relies on published methods, and the authors use conservative estimates in their calculations based on previous published measurements of water chemistry in the study sites. In these calculations, I would suggest defining all variables in the rate calculations and including scale bars in the field photos.

However, I have concerns about the authors definition of sequestration and their accretion rate estimates, which are fundamental components of their analysis. If the conclusions are better supported and confirmed by measurement rather than estimate, this will be a high impact contribution that highlights the previously unrecognized importance of stromatolite environments in the suite of known global carbon sinks. I agree with the authors, this would be a highly significant observation if supported.

I am not a microbiologist, so my comments will be made on the aspects of sequestration and carbonate mineralization. I summarize those concerns in the following:

1. It seems the authors are using "uptake" and "sequestration" interchangeably. They interpret high uptake rates in the measurements of the 13C labeled experiments to reflect chemoautotrophy and carbonate precipitation, but how is this differentiated? How much precipitation occurred? How long is the carbon sequestered, or does it get quickly recycled through other microbial metabolisms?

Response: We thank the reviewer for pointing this out and have edited the text accordingly, using "uptake" when referring to our incubations and the term "sequestration" in the literature review and in the discussion where appropriate.

2. To produce calcium carbonate, HCO3 needs to be broken into CO3 + H. The release of this proton can reduce pH and influence net carbonate production. How is this H+ shuttled away from precipitation to produce such high rates of proposed precipitation? If this is completed through the alternative metabolic processes, please clarify. Balanced reactions of each showing how this is expected to occur can be helpful to the reader.

Response: We appreciate the reviewer's suggestion to expand on this connection. The comment has encouraged us to look deeper into the mechanisms by which this could be accomplished. Based on the presence of genes encoding bidirectional hydrogenases, it is possible that cyanobacteria or other bacterial species could be producing H_2 during dark periods. Based on the presence of H^+ Na^+ antiporters and

heterodisulfide reductases it is possible that H^+ can be shuttled into cells to maintain pH homeostasis in an alkaline environment or as a means of energy conservation in bacteria utilizing the WL pathway. There may also be a pairing of contrasting metabolisms to take advantage of various metabolic by-products. This combination supports our observations of diverse metabolisms across formations.

Line 245-248
"It is also possible that various genes related to H^+ transport (Na^+:H^+ antiporters), H_2 production (bidirectional hydrogenases), and electron bifurcation which can be linked to H^+ import, may aid carbonate precipitation by removing H^+ [35]."

3. The assumption that the density of stromatolite carbonate is equal in density to a non-porous pure calcite need further consideration. When viewed in thin section or SEM, it is apparent that carbonate in stromatolite is highly heterogeneous, with many voids that produce high porosity, in some instances high permeability. Depending on the environment and the stromatolite formation mechanism, multiple minerals can also be present in the stromatolite. I would encourage the authors to consider this in the context of their estimates of accretion rate before comparing to other settings.

Response: This point was also made by Reviewer 1. We have clarified the text to show that our accretion calculation is at the lower range of possible accretion rates based on porosity and composition of each individual formation. We have also included $CaCO_3$ precipitation data reported by du Plooy et al (2020)[32] for the SK stromatolites that supports our estimation of accretion rates.

Line 260-276
"Based upon daytime rates of $CaCO_3$ precipitation reported by du Plooy et al (2020)[32], the SK stromatolites may be sequestering more than 90 % of their carbon uptake. Therefore, the uptake rates measured in this study over 24 hours could be equivalent to approximately 10 mm m^{-2} of pure calcite growth per year, although the true accretion rate is likely higher given the known heterogeneity and porosity of microbial carbonates. This is double that estimated for the microbialites of Clinton Creek[40] and orders of magnitude higher than the Highbourne Cay and Shark Bay systems (0.33 mm and 0.4 mm per year, respectively)[41-43].

4. The Bahamian stromatolites accrete via trapping and binding, while the accretion mechanism of these stromatolites in South Africa is not mentioned. Should it be precipitation as it might appear from the photos, Shark Bay stromatolites are a better analogue. It would be expected that trapped and bound carbonate accretes faster than microbial precipitation, but would not have the same magnitude of impact on bicarbonate uptake. This is an important area of comparison to discuss, and I encourage the authors to explain why the South African stromatolites behave so anomalously when it comes to carbon sequestration potential. This has implications for the applicability of the findings to the geological record.

Response: Identifying the type of accretion was outside of the scope of this study but the reviewer makes an important point that should be the focus of future work for use in interpreting the geological record. We don't believe that the South African stromatolites are anomalous but likely represent dynamics in peritidal stromatolites elsewhere (Rishworth et al. 2020)[22]. The difference between pertidal (supratidal) and

marine/hypersaline systems may be the community composition (Waterworth et al. 2021) and source water that result in differences in carbon uptake metabolisms and rates. We are hoping to broaden this study to compare our findings to microbialites in other systems in the near future. We have included mention of Buttner et al. 2021 who found few detrital grains in material from CR indicating that precipitation is most likely the primary form of accretion in that system.

Line 260-268
"The rates measured for OV745 were also higher than stromatolites, such as the Hamelin pool, predominantly formed through carbonate precipitation and Bahamian formations that are formed through trapping and binding (113 mgC m^{-2} h^{-1} and ~10 mgC m^{-2} h^{-1} respectively)[12,29]. When night-time rates are taken into account, and the process is extrapolated to a 24-hour day, the absolute rates of carbon uptake by the microbialites examined in this study (6.87-12.26 gC m^{-2} day^{-1}) are significantly higher than the average net primary production of tropical evergreen and tropical deciduous forests (3.83-3.89 gC m^{-2} day^{-1})[37-39]"

5. The stromatolites in the field photos appear to be at least partially subaerially exposed. How do periods of subaerial exposure alter the continuity of the accretion rates proposed here? How does this impact estimates of carbon uptake and subsequent sequestration?

Response: This is a very insightful observation and one that we are currently devising systems to test experimentally. For this study, we only collected samples that were under constant flow and not subaerially exposed.

In its current form, I cannot recommend this manuscript for publication. Differentiation between uptake and sequestration is needed, and a mechanism to deal with the proton released during the transformation of bicarbonate to carbonate should be presented to warrant stromatolites as a carbon sink. Finally, I suggest the authors either measure precipitation rate (difficult in such short time spans) or produce a measured accretion rate curve for the samples. These results can be compared estimated accretion rate results calculated in the present study once the porosity and heterogeneity of stromatolites is accounted for. If this accretion rate curve agrees with the proposed revisions to the accretion rate estimates based on uptake, the conclusions of the paper would be strengthened.

Response: We have addressed the uptake vs sequestration issue and have proposed mechanisms for proton release. While an interesting point, specifically identifying the accretion rate is outside of the scope of this manuscript, which focuses on linking carbon uptake to metabolic potential (genomics). We used a very narrow view of accretion rate to establish a baseline minimal increase in depth per formation to determine if our estimated rates could be close to realistic and to set the stage for future studies of accretion. We are currently devising experimental approaches to test true accretion rates in the field, which as the reviewer points out could take some time.

Reviewer #3 (Remarks to the Author):

The work entitled "Integration of multiple metabolic pathways supports unprecedented rates of carbon sequestration in living stromatolites" offers new insights into these ecosystems. With a combination of fieldwork, bioinformatic analyses, and wet-lab experiments, the study proposes an unrecognized role for stromatolites. The study is well designed and the data support the conclusions. I only have minor remarks on the manuscript, but I have not received supplementary information: the zip file with these data had only another copy of the manuscript. I would not recommend publication before seeing the full data.

We appreciate the reviewer's kind words and will make sure that the supplementary data is uploaded correctly.

Some comments from the manuscript

Line 25: define NPP

Line 266-268
"…are significantly higher than the average net primary production of tropical evergreen and tropical deciduous forests (3.83-3.89 gC m^{-2} day^{-1})[37-39]"

Line 257-261: You compare predicted growth only based on your carbon sequestration measurements. Is this valid? Additional factors might be involved. For example, the Highbourne Cay and Shark Bay systems you cite have similar growth rates, but one order of magnitude differences in carbon sequestration rates.

Response: Thank you for raising this point, which has been addressed in the text. Please see below.

Line 255-263
"The daytime uptake rates measured in OV745 averaged 11.5 mgC gC h^{-1} or 408 mgC m^2 h^{-1}. These rates are consistent with photosynthetic rate estimates in a previous study using intact stromatolite material from SK[32] and are significantly higher than extremely productive microbial mats such as those of Tengchong and Little Hot Creek (4.6 mgC gTOC^{-1} and 1.82 mgC g$C^{-1}h^{-1}$ for non-lithifying and lithifying mats, respectively)[28,2]. The rates measured for OV745 were also higher than stromatolites, such as the Hamelin pool, predominantly formed through carbonate precipitation and Bahamian formations that are formed through trapping and binding (113 mgC m^{-2} h^{-1} and ~10 mgC m^{-2} h^{-1} respectively)[12,29]

Line 269-274
"Based upon daytime rates of $CaCO_3$ precipitation reported by du Plooy et al (2020)[32], the SK stromatolites may be sequestering more than 90 % of their carbon uptake. Therefore, the uptake rates measured in this study over 24 hours could be equivalent to approximately 10 mm m^{-2} of pure calcite growth per year, although the true accretion rate is likely higher given the known heterogeneity and porosity of microbial carbonates."

Line 329: I understand ambient temperature can be variable and was not controlled, however, please provide at least a range of approximate temperature.

Response: We thank the reviewer for their suggestion. We have added this information to the text as outlined below.

Lines 291-293
"Daytime temperature in freshwater microbialite pools typically average between 23˚C and 24˚C during these months[19]."

Lines 351-352
"Temperatures fluctuated in the barrage pool by approximately 5 ºC over the full diel cycle"

Lines 34-348: please define all abbreviations used in your formulas.

Response: We thank the reviewer for catching this error. SP is specific carbon uptake rate changed to v for clarity.

Line 367

$$\boldsymbol{\rho = \frac{PC\ at\%\ xs}{v\ at\%xs\ \times\ Time} \times [PC]}$$

Line 370: "concentrations of between 2 – 4 µg of gDNA". Concentrations should be in µg/ml, I think that word should be changed. I understand 2 - 4 µg is the total amount of DNA you obtained.

Response: This has been changed.

Line 394-397
"DNA was extracted from approximately 500 mg of stromatolite material, yielding between 2 – 4 µg of gDNA. PCR amplification of template DNA was performed as described in Waterworth *et al*[26]."

In lines 384-385, please clarify which genes are used for each category.

Response: Thank you for your comment, the Kegg orthology numbers are contained in supplementary data.

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RESPONSE TO THE REVIEWER COMMENTS/ REBUTTAL

The reviewers have provided insightful comments and suggestions and after a careful review, we performed additional experiments. We have introduced the additional data and edited the manuscript accordingly. Our responses are in blue font below each comment.

Reviewer #2 (Remarks to the Author):

The revisions to the manuscript "Integration of multiple pathways supports unprecedented rates of carbon sequestration in living microbialites" generally addressed many of the comments on the original manuscript.

The measurements document bicarbonate uptake rate, and the revised text reflects that in most of the text. The addition of microbial metabolisms to remove and recycle H was a useful addition.

However, carbon sequestration remains to be shown and is a central point of the manuscript's relevance and importance (see lines 102-105, 218-222, and 277-286). In addition to the release of the proton, the precipitation of carbonate minerals is known to reduce alkalinity, and thus may even release CO2 back to the atmosphere (see papers by Elderfield, Archer, Broecker and Peng, etc). This is fundamental to address in order to contextualize these results and support the author's proposal that the carbonate produced by microbialites are the most efficient carbon sink that sequesters carbon on geologically relevant time periods. The revised manuscript cites a review of carbon sequestration mechanisms (Fuss et al., 2018), and not measurements of sequestration via mineralization.

Response: We understand the concerns that Reviewer #2 presents. By using the stable isotope tracer method, we would not be detecting any carbon uptake that is released as CO_2, we are only able to detect carbon incorporated into the particulate fraction. The tracer method is a well-established and diversely applied approach for assessing carbon fixation because the dissolved source of carbon (liquid or gas) is not retained. Therefore, if the “fixed” bicarbonate is released as CO2 that would not impact the rates we observed because that carbon would pass through the process undetected making our rates a conservative estimate of actual carbon cycling but a realistic assessment of carbon captured. In the follow up experiments, we confirm that the carbon taken up by the microbialites and was being bound as particulate inorganic carbon and not as particulate organic carbon by acidifying replicate samples to remove the inorganic carbon. The loss of particulate inorganic carbon was then compared to the decrease in 13C enrichment (amount of isotopically labelled 13C that was incorporated into the particulate mass). The loss in enrichment mimicked the loss of particulate inorganic carbon.

The assertion that we make is that biological activity facilitates carbon precipitation as calcium carbonate, which we deem as sequestered due to its longevity in the fossil record. When we remove the biological activity via the addition of mercuric chloride, the carbon capture and sequestration potential plummets as shown in Figure 4. Therefore, with the new data showing that up to 90% of the total carbon

taken up is retained as particulate inorganic carbon (calcium carbonate) we have clarified that previous estimates of calcium carbonate production by du Plooy et al (2020) are accurate.

The authors also propose that microbialite mineralization could be an effective tool for mitigating carbon emissions. If sequestration as carbonate minerals can be demonstrated as described above, this idea should be supported further. Based on the distribution of modern peritidal stromatolites, what is the magnitude of the potential flux and sequestration period? For example, absolute rates of carbon uptake by microbialites were compared to NPP of tropical evergreen and deciduous forests, the areas of which are significantly different, and thus the flux of carbon is significantly different.

Response:
Our study has demonstrated that these supratidal microbialites represent cooperative microbial communities that display highly efficient carbon capture and mineralization capabilities. Therefore, knowing which species or communities are most efficiently fixing and storing carbon in this form could have a global benefit. We have changed the text accordingly.

Lines 295-301
"By continuing C fixation after sunset with the ability to sequester fixed C into a geologically stable form that can persist for billions of years, the South African supratidal microbialite are among the most efficient, biological C sinks on Earth. Our study raises intriguing new questions about the mechanisms controlling diel C fixation in these systems and whether environmental conditions can be manipulated to further increase the rates of C fixation as a tool for mitigating C emissions and combatting global environmental change."

In its current form, the carbon sequestration potential has not been documented, and thus the data do not support the central relevance proposed for these observations.

Response:
We have addressed the reviewer's concerns by providing additional experimental data including the measurements of accretion rates over 24 hr (Lines 199 – 208; Supplementary Figure 3)

Reviewer #3 (Remarks to the Author):

The revised manuscript of Professor Dorrington and colleagues addresses my concerns. Based mostly on carbon sequestration measurements and metagenomics data, the study proposes an unrecognized role for stromatolites/microbialites. The study is well designed and the data support the conclusions. Checking on the supplementary data now provided I have only a few inquiries/suggestions. I would recommend the publication of a revised version.

* Figure 3 requires the supplementary data to understand what specific genes are shown. I'd provide at least an abbreviation of the genes represented on each row.

Response:
The supplementary data has been provided (Supplementary 2)

* Following your discussion, on lines 232-235 you hypothesize on the role of CAs, which seem to be abundant in your dataset. Are there extracellular CAs in your data? How abundant are they?

* You mention the WL pathway for chemoautotrophic carbon fixation at night. And it's present in the OV745 samples as shown in Fig 3. However, which organisms would bear it? I can not tell only from the OTUs in Fig 2. Are these WL-bearing organisms abundant? I doubt how much is this contributing to the observed sequestration, but defining the relative contributions is beyond this work.

Response:

We have addressed this query, providing additional information in Supplementary Data 4 and in the text as follows:

Lines 165 – 177

"Taxonomic classification of bacterial contigs showed that genes encoding CAs were primarily from Cyanobacteria (Coleofasciculaceae Oscillatoriaceae and Chamaesiphonaceae), Proteobacteria (Desulfobulbaceae) and Bacteriodetes (Cytophagales) phyla (Supplementary Data 4). There was a similar high abundance of a gene predicted to encode trimethylamine-corrinoid protein Co-methyltransferase (K14083) assigned to the Proteobacteria (Rhodobacteraceae and Desulfovibrionaceae) and Chloroflexi in all formations except for CR that could be indicative of the potential for some methanogenic capacity. The presence of genes associated with both branches of the WL pathway, mainly from Desulfobulbaceae, Cytophagales, the cyanobacterial Chamaesiphonaceae and Leptolyngbyaceae families as well as unclassified Chloroflexi (Supplementary Data 4) highlight the capacity for chemoautotrophic C fixation in all formations and in particular, at OV745 (Fig. 3B)."

Manuscript ID: NCOMMS-21-27695B-Z
"Integration of multiple metabolic pathways supports unprecedented rates of carbon sequestration in living microbialites" by Sipler, Isemonger, Waterworth et al.

RESPONSE TO REVIEWER 4

Reviewer #4 (Remarks to the Author):

This manuscript addresses an important question about the carbonatogenesis potential of microbialites and the corresponding metabolic pathways. This study aims to link the relationships between the taxonomic diversity and functional capacity with C uptake and precipitation rates of microbialites. To this end, the authors performed DNA extraction from microbialites, 16S rRNA amplicon and metagenomic sequencing, together with laboratory-based experiments. The characterization of the microbial communities, and the metabolic pathways associated with microbialites is very valuable and the aim to combine both metabolic pathways and microbialite accretion rate and C uptake is novel. I recommend clarifying and modifying several points, in particular on clarifying the methods and on the discussion of the limits of the methods, before considering its publication to Nature Communications.

1. The core of the paper, i.e., the estimations of C uptake and the precipitation rates are both not robust enough. This is probably because the successive calculations are not provided in the manuscript nor in supplementary information. All the calculations must be provided in supplementary information as an excel file with all the calculations visible. In addition, please consider providing a workflow with the type of experiment/the samples used, the estimation from natural samples and the techniques/methods/calculations as well as the results obtained to better follow the study.

Response: This has been done. We have expanded the methods section to better represent the workflow and as requested, provide an excel file with all the data and calculations used in this study. In light of the additional information, we believe a workflow is unnecessary.

2. This study describes the accretion of existing carbonate grains, incubated as a slurry (crushed microbialite with water) in natural water collected from a natural site. The experiments do not demonstrate the in-situ formation of carbonate and lithification of these grains.

Response: We have addressed the question of whether experiments conducted with crushed microbialites can be directly related to *in situ* (intact) microbialite by providing additional data referred to in the methods (lines 412-416) as follows:
"To confirm that the use of a slurry did not artificially inflate or deflate rate evaluations, we compared the daytime rates of whole (intact) versus slurry microbialite preparations. This effort revealed no significant difference in specific uptake rates between crushed-slurry and whole microbialite source material (p=0.4; Supplementary Figure 5)"

However, this is now accepted by the community that microbialites form via different mineralization pathways, with the most prominent pathways being in situ precipitation of mineral (mainly carbonate) via (1) the metabolic activity of microorganisms (induced biomineralization) and (2) the presence of extracellular organic molecules (influenced biomineralization). An experiment never reproduces the complexity of Nature. The question is whether a solution with a microbialite slurry mimics the natural processes of growth of microbialite. I believe a point of discussion on the limits of the experiment conducted here regarding the "forced" accretion experiment applied here is needed.

Response: The reviewer makes an important point. The contribution of induced biomineralization versus influenced biomineralization is shown Figure 5A and B, where the mercury chloride "killed" controls represented approximately 15% of C uptake during the day and at night. These controls account for both influenced biomineralization and abiotic mineralisation, therefore in our experimental system approximately between 85 92% of C uptake can be attributed to induced biomineralization.

To emphasise this, we have revised the text in the results section as follows:
Lines 193-196: "Mercuric chloride (HgCl2) was used in parallel control treatments to halt biological (induced) incorporation to determine the fraction of observed uptake that was due to the presence of extracellular organic molecules (influenced) and abiotic precipitation."
Lines 207-211: "Furthermore, HgCl2-treated control experiments placed abiotic incorporation/precipitation at only 8 to 12% of the daytime rates and 11 to 15% of the night-time rates (Fig 5A; Fig. 5B), significantly lower ($p<0.00506$) than all 13C uptake measurements, indicating that the rates observed are biologically driven."

3. The experimental approach conducted here leads to a very high accretion rate (i.e., 1 mm per week, figure 7 and lines 235-238) which I believe overestimates the reality. Accretion rates have been measured in situ by different groups (i.e., incubating substrates within lakes or by datation) for microbialites from both continental and marine settings and the studies show a range of accretion rate from 0.33 mm/year to up to actually 5 mm/yr (after Power et al., 2011). Again, the high accretion rate provided here from the experiments should be criticized in the manuscript.

Response: Our experimental assessment of C uptake and accretion rates of 86% concur with *in situ* measurements of daytime primary productivity and Ca accretion by du Plooy et al (2020). In the laboratory, the growth of laboratory cultures of SK microbialites at a rate of 1 mm per week, at an accretion rate of 86%, would equate to growth of approximately 44 mm per year under ideal, laboratory conditions. The growth rate in natural, field formations is lower influenced by environmental factors such as seasonal fluctuations in freshwater inflow and nutrient concentration, desiccation, marine overtopping, weathering, invertebrate grazing etc. This has been stated in the text (see lines 312-315).

The average daytime C uptake rate at OV745 is not that much higher that what has been measured at Hamelin Pool - 396 mg C m^{-2} h^{-1} vs.113 mg C m^{-2} h^{-1} (Bauld et al., 1976), respectively and the growth rate of the Hamelin pool microbialites in turn, is an order of magnitude higher than the Bahaman formations (Steppe et al., 2001), indicating significant variation in the growth rate of different microbialite formations. What sets this study apart is our finding that C uptake in the South African microbialites continues at almost the same rate at night and precipitate at high precipitation rates (Supplementary Fig. 3). Ours is the first diel study of microbialite primary production. It is entirely possible that other microbialites also continue C uptake at night.

Finally, the taxonomic and functional diversity of supratidal microbialite bacterial communities assessed in this study are distinct from other formations (e.g. Shark Bay, Highborne Cay, Pavillion lake and Little Creek). The composition of the bacterial communities together with environmental factors specific to their location in the supratidal zone, likely combine to support the exceptionally high rates of C uptake and C accretion observed for the microbialites in this study.

4. Line 59: A point in the introduction on the role of physico-chemical conditions allowing the formation of microbialites is missing.

Response: We have addressed this comment by adding in a new figure (Fig. 1) summarising the known physico-chemical conditions of microbialite formations (temperature and pH) including those in this study. Ambient DIC in the South African microbialite formations as reported by Dodd et al 2018, are referred to in the methods section (Lines 423-428).

5. Lines 56, 81, 97: Different terms are used to refer to modern microbialites: extant, living, contemporary: please uniformize the nomenclature along the manuscript

Response: This has been done.

6. For the uptake measurement: What was the 13C content of the collected microbialite used in the experiment? It is expected to have a consequent mass of 13C already coming from the slurry (the microbialites grains incubated within the bottle). This data is not mentioned in the method section nor in the result and is crucial to consider in the rate of 13C uptake.

Response: Filtered site water and microbialite samples were used to determine the natural proportion of ^{13}C in the microbialites at each site. The ambient ^{13}C atom % for each site has been provided in the methods and the supplementary Data Excel Spreadsheet where they are used to obtain the atom % excess used in rate calculations. We also conducted parallel control incubations that had no ^{13}C added and incubated in bottles under the same conditions for the same incubation time. These control samples showed little difference from the ambient (field) atom % and negligible impact on calculated rates (-0.0001 hr-1) in comparison to enriched treatments that averaged 0.0112 h-1 and 0.0090 h-1 for day and night incubations, respectively.

We have addressed this issue in the methods section as follows:
Lines 398-408: "The atom percent (atom %) enrichment, the proportion of the total carbon mass that is ^{13}C, of the microbialite mass used in the incubations is 1.0975 for CR, 1.0975 for SK, 1.0970 for OV 745 and 1.0981 for TP. For reference, ^{13}C exists in nature at 1.11%, therefore all samples showed clear and consistent concentrations similar to the expected natural abundance. Any bicarbonate released from the microbialite slurry would have diluted our additions, deflating the actual rates and thus making our estimates even more conservative. This is shown through the use of no-addition controls, prepared from the same slurry, incubated for the same amount of time in the same water but did not receive any $H^{13}CO_3^-$. The average specific uptake rate for controls treatments was a negligible -0.0001 hr^{-1}."

7. There is very likely an overestimation of 13C on the filters after the experiments linked to the saturation of the filter with 13C-rich water. The filters from 2018 and 2019 experiments collected before the mass spectrometer analyses need to be rinsed with DI water to be reflective of the real 13C uptake by biomass and carbonate. This step is not mentioned in the method section.

Response: We agree that the methods need to be more detailed and have included this information in the methods.
With specific reference to this point, we have included the following text in the methods:
Lines 391-395: "Once on the filter, samples were rinsed with unamended, filtered site water (under vacuum) to wash away unincorporated isotope. Separate dedicated funnels and filtration systems were used to produce filtered site water and to collect samples to determine the natural proportion of ^{13}C in the microbialites at each site."

8. Figure 7: under which type of laboratory culture?

Response: The title of Fig.7 (now Fig.8) was misleading and has been changed to read: "Nascent microbialites cultured in the laboratory." We have also introduced new text into the methods (Lines 359-365).

9. Lines 45, 219, 233, 471, 721: Ca-carbonate is not necessary calcite. Ca-carbonate has different polymorphs. Ca-carbonate can be aragonite, calcite, vaterite, monohydrocalcite. This manuscript and the past study (Buttner et al., 2021) do not decipher about the type of Ca-carbonate. Please remove the name calcite and replace by "Ca-carbonate".

Response: We have provided MS. Ramen Spectroscopy data that indicates that indicates that the C is accreted as calcite (New Supplementary Fig. 4)

10. Line 144: write as "microbialite".

Response: This has been done

11. Line 232: How the accretion of 9.20 to 16. 41 kg CO2/m2/yr was determined?

Response: The methods section has been expanded to explain how the CO_2 and C concentrations were determined.

12. Lines 392-402: DIC is not only HCO3- this is also CO32- and CO2. Moreover, it is necessary to mention that the released CO2 during the formation of CaCO3 from 2(HCO3-) has not been measured and considered in the calculation of the C rate calculation.

Response: The reviewer is correct that HCO_3^- is not the only form of DIC in aquatic systems, however, it is the most abundant form of DIC in most natural waters (85-95%) including our sample sites (Fig. 1). This high proportion in natural waters is why HCO_3^- is often used in aquatic carbon stable isotope tracer studies. The reviewer is also correct that CO_2 release and transformations via chemical and biological reactions is expected. However, the goal of this work was to investigate and compare the carbon uptake between microbialite sites and light cycles. Movement of carbon between forms during the incubation period does not negate the carbon uptake and precipitation (movement from dissolved to particulate) by the termination of the incubation. We added ^{13}C tracer based on the most dominant form of DIC and assessed how much of that carbon was in the particulate form at the end of the incubation. We propose several pathways that may contribute to this precipitation but the key finding is that the precipitation occurred through predominantly biological processes (Fig. 5) and into particulate inorganic forms (Supplementary Fig. 3) at rates that are similar to accretion rates observed *in situ*. We take the reviewers comment as an important next step on the path to exploring the factors underpinning the proposed mechanisms further, but that was outside the scope of this initial study as it was designed and executed.

13. Line 474: What are precisely the 7 selective domains?

Response: The ones listed in Fig 7B (and shown in 7A). We have added the reference to the revised text in this section.

14. Line 474: What are the proportions of Ca-carbonate in the 4 samples?

Response: We have this information only for SK and CR and the calcite proportions for the relevant samples are listed in Fig. 7B. We do not have data for TP or OV745, Since the CR microbialites had the higher C uptake rates compared to OV745. We are likely underestimating the accretion rates at CR.

15. Lines 476-477: Provide the calculations (equations) made by the software RockMaker to calculate C contents of microbialites. Using which data? There is a lack of clarity on how the Ca-carbonate was quantified within the CR microbialite.

Response: More detailed experiments have been added to the methods section and as a new Supplementary Data 5 and the old Supplementary Data 5 becomes Supplementary data 6.

16. Line 475: After checking the link, different threshold plugins exist on ImageJ. What is exactly the name of the used threshold plugin?

Response: There is only one “Threshold” tool. The other one is called “Colour Threshold", but since we are working with grey scale images there was no point in using this tool. Explanations on the use of “ImageJ” have been added into the Methods section.

Nature Communications manuscript NCOMMS-21-27695C

Response to Reviewer's Comments

Note: Line numbers refer to the track changes version of the manuscript

Reviewer #4 (Remarks to the Author):

The revisions to the manuscript "Integration of multiple metabolic pathways supports unprecedented rates of carbon sequestration in living microbialites" by Dorrington et al. generally addressed many of the comments on the last version of the manuscript. The authors provided the requested details on the methods and on the calculations.

However, considering the equation of carbonate precipitation 2HCO3- + Ca2+ --> CaCO3 + CO2 + H2O, no robust carbon sequestration is demonstrated if the released CO2 is not tracked and quantified during the precipitation of CaCO3. The authors demonstrate the transfer of carbon between the dissolved (HCO3-) and particulate fraction (CaCO3) and do not account for CO2 release. This crucial aspect must be clearly stated in i) the abstract ii) the results section iii) the discussion. This transfer of carbon between HCO3- and CaCO3 should not be referred as carbon sequestration since CO2 is released during the process. In this frame, the title must be replaced by "Integration of multiple metabolic pathways supports unprecedented rates of carbon transfer in living microbialites".

Response:

We have carefully considered the points raised by the reviewer and respond in full below. However, based on the context provided below, we do not agree that our abstract should be amended, since our findings have not changed.

We have toned down the final assertion in the abstract to read: -"Hence, contemporary microbialites provide a highly effective biological mechanism to precipitate dissolved CO_2 as geologically stable carbonate mineral deposits.". (Lines 46-48).

We have also added text to the Results (lines 177- 179) and Discussion (lines 303 – 311) sections as articulated below.

This aspect on the CO2 release during the precipitation must be discussed regarding 1) the dissolved C speciation, 2) the bacterial communities populating the biofilm and 3) the type of carbonate precipitating. On this last point, Raman analyses is an important addition. However, the quality of the 3 spectra provided now in supplementary is particularly low and it is quite difficult to distinguish the peaks of calcite from the aragonite peaks for instance. In addition, whether the Raman analyses were done on all the studied samples or only on one sample is unclear. I suggest to do additional Raman analyses on the studied samples from the different studied sites, to decipher about the mineralogical composition of the microbialites,

which is a crucial aspect to quantify the carbon transfer during carbonate precipitation. I also suggest combining in situ Raman analyses and SEM with bulk XRD and/or FTIR, to correlate microscopy (SEM and Raman) with the bulk mineralogy of the studied samples and highlight the presence of potential additional carbonate minerals. Last, the accretion rate determination is not clearly explained. I recommend more details on this result which is one of the important results of the study.

Response:

In aqueous solutions, dissolved inorganic carbon (DIC) exists primarily as HCO_3^-, CO_3^{2-}, with very little CO_2. H_2CO_3 is also present but as a rapidly cycled intermediate. Bicarbonate is the dominant species in most natural systems (typically > 90% of DIC) and accounts for ~98% of DIC in our microbialite system. This balance is maintained based on the following well-established carbonate equilibrium reactions:

$$CO_2(aq) \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- \leftrightarrow CO_3^{2-}$$

As the reviewer describes, when $CaCO_3$ precipitates it consumes HCO_3^- and produces CO_2. However, we point out that in buffered aqueous systems such as the one in this study, $CaCO_3$ precipitation does not necessarily result in net CO_2 release into the atmosphere. The removal of HCO_3^- and the subsequent release of CO_2 drives equilibrium reactions that strongly favour the production of HCO_3^-, meaning that most of the produced CO_2 quickly re-equilibrates with water into H_2CO_3 which dissociates to produce HCO_3^-.

$$CO_2(aq) + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$$

The reviewer is justified in highlighting the release of CO_2 during the $CaCO_3$ precipitation reaction. However, this process needs to also be considered in relation to equilibrium reactions and the buffering capacity of the system. In our system the pH remains relatively stable within a pH range of 8.5 – 8.7 along the freshwater to marine gradient (Dodd et al. 2018), indicating a highly buffered system that does not dramatically change with exposure to actively growing microbialites. Thus, even though $CaCO_3$ formation liberates CO_2 into the aqueous phase, the equilibrium chemistry ensures that most, if not all of that CO_2 is quickly converted back into HCO_3^- (Beer et al. 2020).

The capture of any additional CO_2 is also particularly likely in the presence of microbial communities actively fixing carbon (Beer et al. 2020), like those shown in our study, further reducing the likelihood of substantial CO_2 release from the system. If ^{13}C-labeled CO_2 is released into the water via the carbonate precipitation reaction and recycled back into the HCO_3^- pool or taken up in its CO_2 form, that does not negate our finding in that we are measuring and reporting the amount of ^{13}C taken up and precipitated as calcite from a HCO_3^- source. Our data therefore documents not just transfer, but biologically mediated stabilization of carbon into solid mineral form. While direct CO_2 fluxes were not measured *in situ*, the strong coupling between bicarbonate uptake (this study), O_2 production (du Plooy et al. 2020), and accretion rates (this study) demonstrates that these microbialites likely act as efficient carbon sinks.

We also point out that the strategic use of ^{13}C stable isotopes allowed us to trace/ track carbon in a manner that is not possible using less precise, bulk or flux methods, (Cullen et al. 2001). We used this well-established approach (> 41,400 studies for primary production alone based on a perfunctory Google Scholar search) to measure the proportion of the C supplied as $H^{13}CO_3^-$ that was directly captured (Figure 6). We were then able to show that 87% of the C captured was precipitated as inorganic carbon (lines 235-240 in the text). We also show that the microbialite systems used in this study primarily precipitate this carbon as calcite instead of other forms (revised manuscript lines 246-260, Figure 7; supplementary Figure 4), the growth/C accretion of nascent colonies over time (mass and volume of microbialites increased over time, Figure 8) and we explore the microorganisms (Figure 3) and metabolisms (Figure 4, 5) that likely support these rates. This work represents substantial evidence, multiple analytical approaches and reproducibility of these efforts that support our assertions that carbon is in fact being biologically captured (see comparison to abiotic assimilation in Figure 5) and then precipitated in a stable mineral form as calcite (see below).

We agree with the reviewer that our Raman spectra may be insufficiently robust to conclusively identify the $CaCO_3$ polymorph (calcite vs. aragonite, vaterite) may be insufficiently robust. We therefore replaced the Raman spectra with powder XRD spectra, which provide unequivocal evidence (Supplementary Figure 4; lines 246-250). Following the reviewer's suggestion, we analysed multiple samples from each of three sampling sites (Cape Recife, Schoenmakerskop and Cape St Francis OV745), all of which yielded identical results - the precipitating phase is calcite. In order to avoid redundancy, we removed the Raman data from the manuscript.

Finally, we are not in agreement with the reviewer about the use of sequestration in the title, but are willing to accommodate their view by using the term "precipitation", which we think is appropriate, instead. We have revised the title to read "Integration of multiple metabolic pathways supports unprecedented rates of carbon **precipitation** in living microbialites".

Specific comments:

1. Line 82: Mineralogy
Gypsum is a Ca-sulfate mineral (does not contain carbon).

Response:
"Gypsum" has been removed

2. Lines 57-59/Figure 1: Physico-chemical conditions promoting microbialites
In addition to pH, a(CO3-2) and a(Ca2+) are key parameters explaining the presence of microbialites (i.e., Zeyen et al., 2021, GCA and Caumartin et al., 2023 GPL). Specifically (over-)saturation of solutions with respect to monohydrocalcite/ACC is necessary to form microbialites. More integration of this recent research is needed to fully cover this question of the role of the physico-chemical conditions on microbialite formation.

Response:
We thank the reviewer for highlighting this important aspect of microbialite formation. We have revised the introduction and cited this research. We have added the text in Lines 82-88: "Notably, in addition to pH (Fig. 1), the chemical activities of carbonate

(CO_3^{2-}) and calcium (Ca^{2+}) also strongly influence microbialite formation. Recent work shows that microbialites often develop in waters oversaturated with respect to metastable $CaCO_3$ phases such as amorphous calcium carbonate or monohydrocalcite, highlighting that these and other permissive physicochemical conditions may also be required for biogenic precipitation to proceed[24, 25]." as suggested by the reviewer.

3. Lines 445-465: Definition needed on specific vs. absolute uptake rates
Hama et al. did not use the terms "specific" and "absolute" for the C uptake rates. Please define each C uptake rate by a few words.

Response:
This has been done in the methods section (see lines 508-513).

4. Uniformization of the figures
Uniformize the axis of your figures with "C specific uptake rate" and "C absolute uptake rate" to be coherent with the "methods" section (lines 455-460).

Response:
We agree that consistency is important, so we have made sure that all y-axis' are consistent and have clarified specific C uptake rates or absolute C uptake rates in the figure legends and captions.

5. Line 455-462: Definition needed for the rate calculation
Please define what is "C_substrate_atom%" and define what is the meaning of "excess" in the text. Refer also here to the excel spreadsheet.

Response:
This has been done (see methods, lines 515 – 530 and Supplementary Spreadsheet 1).

6. Line 457: Clarification needed on the calculation
*On the spreadsheet the specific rate was calculated as C_atom%_XS / (C_Substrate_atom%_XS*time) and this does not correspond to the formulae written in the text.*

Response:
This has been done (see methods, lines 519-530 and Supplementary spreadsheet 1).

7. Line 458: Clarification needed on the calculation of the absolute rate
Is [PC] the proportion of the core? Please detail each term of the equation in the text.

Response:
This has been done (see methods, lines 519-530 and Supplementary spreadsheet 1).

8. Lines 465-471: Clarification needed on the determination of the accretion rate
Is the unit of the accretion rate correct (i.e., mm m-2)? In the abstract the accretion rate is expressed as mm/year. After carefully checking the cited paper (Komad et al), I was not able to see the link between δ13C and accretion rate in mm/yr and to find the calculations resulting to the result "20-30 mm of vertical calcium carbonate accumulation per year" from the abstract. Please detail the method on your manuscript with the equation and excel table if possible to help the reader to follow better the reasoning of the result section (i.e., lines 235-257)

Response:
We are somewhat confused by this question. We do not report or use δ13C and we have provided all calculations for the reviewer in the Supplementary Spreadsheet 1. We used the method of Komada et al. (2008) to determine what fraction of the C taken up was incorporated into the particulate inorganic fraction and not the particulate organic fraction.

The extrapolation to mm yr^{-1} was based on direct conversions of uptake by a known mass that corresponded to a known area and time (incubation time). All steps of these conversions are shown in the Supplementary Spreadsheet 1 (columns Q to AG in the 2019 Assessment worksheet) as was requested by the reviewer. Since these conversions are straightforward, based on clear unit conversions (mm^{-2} to m^{-2}, days to years, grams to kilograms) and fully actionable equations are available in the supplementary spreadsheet, we do not feel that these equations need to be provided in the text as well. Further detail was added about the accretion rate in lines 571–577 and in columns AH and AI in Supplementary Spreadsheet 1, 2019 Assessment.

9. Line 467: "Fumigation" instead of "fugation"?

Response:
This has been corrected.

10. Lines 470-471: Details on the calculation of the maximal annual growth estimates
As requested previously, please provide the equation and the calculations for the estimates.

Response:
This has been provided. See lines 571-577 in the text.

11. Suggestion on the methods organization.
Perhaps add the section "C accretion in mineralized parts of stromatolites" right after "rate calculations".

Response:
This has been done.

12. Discussion: A point of comparison of the uptake rates and the physico-chemistry How the 4 different uptake rates compared to the water chemistry (determined previously in the literature)? Do you have a correlation between uptake rate and alkalinity and Mg/Ca as described by Zeyen et al., 2021 for the Mexican lakes?

Response:
We believe this is outside the scope of this study. We understand that there are many possible connections and comparisons but this paper focuses on carbon uptake/ precipitation and the microbial communities and the metabolisms that may facilitate these processes. We agree that the proposed work would be interesting to include in future efforts to explore other geochemical relationships. However, we also caution that there are some key distinguishing factors, including that the formations in our study are in very shallow flows (mm to cm deep) and not in lakes or inundated marine systems.

Dodd et al., 2018, report that during the winter and spring, the average Mg/Ca ratios in the freshwater inflows of six sites in the Gqeberha area, including Schoenmakerskop and Cape Recife, range from 0.15 to 0.21, based on their mg/L calculations, which is equivalent to ~ 0.25-0.35 based on our molar conversions). The salinity of the inflow water used in this study was <1. The microbialites that were the subject of our study thrive in perpetually-flowing fresh water with low Mg/Ca ratios and low salinity that distinguishes them from the lake microbialites in the Zeyen et al., 2021 study. To emphasise these differences, we have added a new section in the methods ("Study Site description", lines 369-383 in the text) and included additional visual images of the sites in Supplementary Fig. 1.

13. Line 236 and line 242: Clarification on detrital minerals
What do you refer as "mineral detritus"? Detrital minerals such as feldspar? Some studies have shown that silicate phases (similar to stevensite) can be authigenic and microbially mediated (e.g.,Suosaari et al., 2022) and are not always detrital.

RESPONSE:
This is a good point, but there is no textural evidence for the authigenic growth of clay minerals and we have added a statement in that regard (line 260-261). The few detrital grains that are present appear to be subrounded or rounded, indicating abrasion during transport by wind or water.

14. Lines 296-309: CO2 release during carbonate precipitation
A point of discussion is necessary on the CO2 release during carbonate precipitation, with respect to the metabolisms;- and the physico-chemistry of the water. On this last point see for instance the recent article from Many et al., 2024 Science advance.

Response
We have thoroughly reviewed the Many et al. 2024 manuscript, including the models and findings within. Their work specifically applies to large, monomictic lakes over annual/seasonal time scales. Key factors to interpreting these interactions are water column stratification and seasonal mixing cycles. By contrast, our systems are entirely and directly fed by constant groundwater seep inflows and the water flows along a vertical gradient of microbialite (rock) at flow rates up to 28 L min-1 (Dodd et al. 2018) and the depth of the water over the microbialites varies from millimetres to centimetres. Our systems are inherently well mixed and lack the large scale, multi-metre stratification that sets up the seasonal CO_2 dynamics inferred by the Many et al 2024 model. Images of our exact sample sites are provided in Fig. 2 and Supplementary Fig.1.

Samples used in this study were collected from the shallow, flowing region as the seep water enters the system. For more clarity, as indicated above in our response to point 12, we have added more detailed descriptions of each site in a new section of the methods (see text lines 369-383) and additional images in the Supplementary data (Supplementary Figure 1 B-E). Taken together, we believe that the dynamics described in the Many et al paper are a better comparison for other large lakes, like those described in Zeyen et al. 2021, or in marine systems such as those of the Bahamas, rather than for the microbialite sites used in our study.

The relationship to CO_2 release and likely capture is described in lines 303– 311 directly before the proposed mechanisms for H+ production that facilitates these reactions (lines 311-320).

15. Line 319: Clarification on the ideal conditions
What are precisely the "ideal conditions" necessary for vertical layer growth?

Response:
Clarifying text was added (lines 274-276) "…conditions, where macro- and micro-nutrients are non-limiting and the water supply is consistent"

16. Lines 328-332: Conditions and quantification of CO2 release during carbonate formation
The conditions leading to CO2 release must be understood and the carbon balance will need to be done.

Response:
We respectfully disagree with the reviewer that CO_2 quantification is required in the context of our findings. We have contextualised the process within the carbon cycle and we show that large components of the DIC pool are captured and precipitated in a stable (long-term) form as calcite using established tracer methods that are more sensitive than CO_2 flux calculations. Even if part of the CO_2 in the water is not

captured, this does not question our findings, based on the methods applied and number of different conformational analyses and investigations performed. This being said, we did highlight the lack of CO_2 measurements and carbonate equilibrium potential throughout the manuscript, at the reviewer's request (lines 177-179 and 303-311). We have also added the equilibrium equation to Figure 1.