

Reassessment of the previous decision on Puntin et al. 2024 preprint

I have carefully examined the previous reviews, decision letters, additional information provided by the previous recommender, the authors' appeal letter and the current draft (<https://www.biorxiv.org/content/10.1101/2023.08.23.554380v3>). I acknowledge that the previous review rounds appear to have clearly contributed to the preparation of the current improved draft, and have pinpointed the shortcoming of the data that the study presents. However, I identified that some of the suggestions that were made and incorporated along the way have slightly diverged the focus of the paper away from the main topic and the strongest potential of the existing data. Some of these suggestions from the previous reviewer and recommender unfortunately appear to be stemming from misunderstanding of the study procedures and misconception of the main message that the study was designed to deliver by the author. As such, I am in support for reversing the decision of rejecting this article from the consideration for recommendation. You can also refer to my comments on the review history, appeal letter, and the previous recommender's comments attached, showing how I came to this conclusion.

Here I present my views on the main three areas; (1) the effect of menthol-induced coral bleaching on coral associated microbiota, (2) the effect of geographic locations and aquarium facilities on the coral microbiota, and (3) the effect of long-term aquarium-based captivity on the coral microbiota; and where this study can be placed in each area. The preprint at the current form can merit a revision before it is recommended for publication. Therefore I also provided my review of the current manuscript and made recommendations for changes. I hope these strengthen the article's clarity along the authors' intentions, as well as its scientific contributions.

1) The menthol-bleaching effect on the coral microbiota: The main study focus, and the only one topic with barely enough supporting data in my view, is the effect of menthol-induced coral bleaching on coral-associated bacterial community compositions. Observations were made using 5 coral colonies (3 sourced from Red Sea and kept in a German aquarium for 7 months, and 2 sourced from Hong Kong and kept in a HK aquarium for 3 months), each of which were physically separated to generate 6 clonal polyps. From each colony, 3 polyps were kept in the aquarium without menthol, and 3 were subjected to menthol bleaching to remove most (if not all) algal symbionts. Microbial 16S rRNA gene sequences were then obtained from these corals, with $n = 3$ polyps per treatment per colony, times 5 colonies examined. However, a few polyps did not generate enough data (Fig 1), thus there were only 2 colonies that allowed between-treatment comparisons on 3 samples-vs.-3 samples statistically, resulting in most treatment-based comparisons being on average pictures across all colonies (Fig2, Fig3B), which in my opinion were not very convincing due to the colony-level variability in the microbial compositions responded to menthol bleaching. Considering the lack of sufficient data, I believe the previous reviews were fair to characterise this study to be a preliminary result (as inserted in the abstract as well). Nevertheless, when we look within each colony, while non-bleached polyps showed similar microbial compositions, microbiomes of bleached polyps were in general more diverse. Comparing across the 5 colonies, this non-bleached vs. bleached difference (i.e. apparent 'shift'; noting that microbiomes before the experiment was not obtained) appear in a non-directional, stochastic manner. I agree that this follows patterns expected from dysbiosis, as the authors interpreted. As data presented are barely minimal due to missing samples, this

interpretation should however be presented as suggestion that requires verification through follow-up studies. This should be reflected in the statements on this topic throughout the paper.

2) The location effect on the stochastic shift of microbiome upon menthol bleaching: Authors may point out that the shifting patterns of microbiota induced by menthol bleaching differ across colonies; however in my view this difference cannot scientifically be attributed to the location or facility difference without having sufficient replicate colonies in Hong Kong (currently n=2 colonies; a third colony may change the whole picture, which we cannot know). There are also other factors to be considered and examined than the difference in the locations and facilities, which include host coral phylogenetic lineages, as the studied coral species is known to be polyphyletic containing cryptic lineages (e.g. Wepfer et al. 2020; cited in the manuscript though missing from the reference list; please check the reference list for other items, too). As such, the abstract statement “response of the bacterial microbiome to menthol bleaching varied between the two facilities”, the paragraph “Microbial community dissimilarity patterns differed by geographic origin of colonies” (L187-) and the paragraph starting with “Changes in community compositions between symbiotic states differed for coral colonies from the two regions.” (L196-) are questionable; I recommend these to be removed, as highlighting this aspect diverges the focus from the main objective of the study ((1) above). Specifically for the result paragraph L196-, it targets to address the apparent ‘changes’ between treatments, which in my opinion should best consider within each colony given the inter-colony variability, but all bleached and all non-bleached polyps across colonies were examined together to point out the difference patterns between locations (i.e. presence/absence of treatment-based clustering; Fig3C, Fig3D); this is an inappropriate approach to address the main question, as all polyps were not independent samples. I see the non-directional ‘shifts’ across 5 colonies, and a treatment-based clustering in Hong Kong may well not have formed if a third colony showed another change direction, which we cannot know. Similarly in the discussion paragraph starting from L301, location-based contrast can be speculative - Pointing out the HK responses being more ‘uniform’ needs to be backed up by more than two sample colonies. The discussion on location-based differences overall should reflect the speculative nature cautiously - the discussion paragraph L301- should be removed (or substantially reduced its weight), also L406-407.

3) The ‘captivity’ effect: Authors may note that the microbiome of aquaria-kept corals (without menthol bleaching) are less diverse than what is reported from field-based studies from the same taxonomic coral species; however this should not be their scientific claim without examining wild-vs-captivity corals in the same study using the identical methodology, performing due scientific rigor. PCR primers, sequencing depths, identification of OTUs or ASVs, assignment of taxonomic affiliations differ among studies, making the microbial diversity based on reported numbers of taxa being difficult to compare directly. In addition, comparing microbiome of the studied coral species from different regions may be problematic due to the potential of cryptic species as mentioned above, which may well form host lineage-specific microbiomes. While I agree with the previous reviews that the lack of wild coral data in this study prevents proper examination in this aspect, it appears that this was never the authors’ intended area of interest in this manuscript. Therefore I do not support that the lack of new wild coral data in this study to form the absolute reason to reject this paper, but rather suggest this aspect to be significantly ‘toned down’. Accordingly, I suggest removing the abstract sentence “the coral-associated microbiomes were composed of relatively few bacterial taxa, when compared to literature reports from wild corals.”, sentences in L326-334 (“In fact...”), and a

concluding sentence L404- "gain initial insights into the potential effects of long-term captivity in this coral species."; also rephrase L408 "composed of fewer taxa than reported for the wild corals" to fit better with statements on the potential advantage of simple multipartite symbiosis study models.

Again, the last two aspects (the location and captivity effects) should be handled as speculative notions and removed or toned down clearly as such, certainly not to be claimed as established observations in the abstract or the conclusions section, for the reasons highlighted above. By doing so, I hope that the focus of the study, i.e. the pattern of bleaching effect on coral microbiota, is further emphasised and comes clearer. I found the finding of stochastic changes upon menthol-bleaching insightful and representing a step forward in manipulative experiments in coral symbiosis, and thus I believe there is a merit to publish this dataset.

In addition, compositions of algal symbionts data were further limited (8 polyps out of 15 non-bleached polyps studied, and 2 out of the 8 samples have 20~30 sequences each, cf. 1,085~1,669 in other), and were not able to contribute to decipher the effect of algal symbiont loss on bacterial community differences (in terms of what initial algal symbionts the bleached corals may have had). In fact, no statistical testing was conducted on this aspect above general pattern description (L253-). While I acknowledge that algal composition could have formed an important facet to discuss the menthol-bleaching effect, the data presented were preliminary at most. I'd recommend removing this aspect from the main body (methods/results) and cite the existing data in a supplementary material while speculate this effect within the discussion, as one of the area that need further studies on (such that is written currently).

Minor and more specific points:

L24 "tripartite interactions"; seeing that bacteria here refers to diverse communities consisted of many species, it is not technically a 'tripartite' relationships, but more like a highly complex multipartite interactions. The same applies to L51 "three partners".

L59: What seems missing here is what is known about the direct effect of menthol on bacteria in general. Menthol is known to inhibit wide variety of bacteria, and can select certain members in the community, such that known in cigarette associated microbiome. It is worth mentioning potential direct effects of menthol on bacterial communities associated with corals, not only by indirect impact through the lack of symbiotic algae, for a more balanced view.

L69-: It would be a good courtesy to outline the volume and water filtration systems in the Ocean2100 facilities, in comparisons to HK, as these may be important information regarding microbial environments.

L71: The unit for salinity is missing. E.g. ppt.

L113: it is worth mentioning how the corals appeared after 13 days post the menthol treatment day, as this can be critical to interpret bacterial compositions data.

L121: Was the tissue dissociated from the skeleton before proceeding to DNA extraction? How was this performed?

L281- "This surprisingly included Symbiodiniaceae-associated bacteria that we were expecting to be reduced after the physical removal of Symbiodiniaceae (Fig. S5; Supplementary Materials and Methods)." This seems to come out of blue in the discussion, and should be mentioned in Results. Also, menthol-induced bleaching is not 'physical removal' of the algal symbionts.

Around L300: Again, I feel that one missing aspect to discuss here is about the direct effect of menthol on bacterial communities. The text so far implies bacterial shift is solely due to the loss of algal symbionts, but this may well be rather indirect impact of menthol, as I indicated above. How bacterial composition may have affected by menthol-'selection' and how long the impact to last etc., would be an important area to discuss.

L350: In my opinion this suggests the presence of cryptic species (spp.) within a region; as Wepfer et al. (2020) demonstrates.

Comments on the appeal

This document outlines and explains the reasons behind our appeal against the decision to reject the manuscript titled "The bacterial microbiome of symbiotic and menthol-bleached polyps of *Galaxea fascicularis* in captivity".

During the review process, we have identified factual errors and unfair treatment that significantly impacted the decision to reject our work. These are outlined and expanded below.

Please note that text in italics in quotation marks are direct quotes from the recommender and reviewers.

To briefly summarize, the manuscript focuses on the emerging model organism *Galaxea fascicularis* for coral symbiosis research, in the context of further establishing it as model system. The *Galaxea* model represents a useful tool to unravel the complexity of the coral holobiont through the 'disassembling' of its components (animal host, algal symbiont, bacteria, etc., as demonstrated in previous publications). As a first step, the removal of the algal symbiont (Symbiodiniaceae) can be effectively achieved through menthol bleaching. However, the effects of menthol on other essential components of the holobiont (i.e., bacteria) have not yet been investigated. We therefore replicated the menthol bleaching protocol in two facilities and characterized the bacterial microbiome of both symbiotic (untreated) and menthol bleached polyps (clonal replicates) of *G. fascicularis* that were maintained in captivity (aquaria) for several months. This study therefore provides valuable first insights into the bacterial microbiome of menthol bleached corals of this aquarium reared model species. The results showed that menthol treatment was generally associated with random changes in the bacterial communities, however the response differed between the two facilities, highlighting the importance of small but relevant differences in rearing conditions. The study did not aim to rigorously test the effect of captivity itself (e.g., by drawing direct comparison between wild and captive corals). In addition, a pattern of microbial simplification was observed supporting reports that showed similar effects in other coral species and captive animals more broadly.

But biological replication for different facilities are n=3 vs n=2.. Also this could come from different genetic lineages (cryptic species).

Yui Sato
29/04/2024 14:28

Without a test of tank vs. wild corals, there was no observation in my view.

Yui Sato
29/04/2024 14:30

While we appreciated the time and effort that the reviewers and recommender dedicated to this evaluation, we argue that they did not provide a factually correct and unbiased assessment of our work. Below is the breakdown of each point:

Factual errors made by the reviewers or recommender that had a major impact on the decision:

The recommender used incorrect information with regards to a) the time frames of captivity vs. duration of the experiment, b) the bacterial taxa identified, and c) the current state of the literature.

- a) The recommender and reviewer 2 wrongly understood the **duration** of time spent in **captivity** (aquarium-rearing) of our corals before the experiment took place, which is used to justify their decision to reject our manuscript.

Specifically, the recommender finds our 'captivity effect' hypothesis and relative discussion "*highly speculative*", while he finds the alternative hypothesis proposed by Reviewer 2 as "*more compelling: that the 10-14 days of captivity sufficiently impacted and weakened the bacterial community associated with symbiotic Red Sea polyps to make the effects of bleaching barely perceptible.*". However, the corals in the facility were **maintained in captivity for several months** before the start of the experiment (see manuscript at lines 68-70, and abstract: "*G. fascicularis* [...] that were **long-term aquarium-reared** [...]"). The recommender confused the total duration of the captivity at the time of the experiment (several months) with the time allowed to recover from fragmenting (10-14 days)—both

described in the Materials and Methods section. Importantly, all coral colonies used for the experiment are still alive and well in the long-term rearing facility four years later. In fact, that is the whole purpose of establishing a model system that can be investigated under strictly constrained conditions. The hypothesis that captivity itself severely weakened the coral before the menthol treatment appears therefore unsupported.

- b) Speculative interpretation of the role of associated bacteria: “*This idea gains support from the significant presence of a putative coral pathogen (Alteromonas spp.) within the core microbiome of captive Galaxea.*”. This is also factually incorrect, as members of the genus *Alteromonas* may provide broad functions in the coral holobiont: members of *Alteromonas* are common coral-associates found in almost every coral-associated microbiome, some members are also candidate probiotics with suspected beneficial functions for the coral, and others are suspected pathogens, as we reviewed in our manuscript well supported by literature (see lines 372-374 and 384-389). The presence of *Alteromonas* spp. therefore cannot be used as evidence for the alleged “*impacted and weakened*” state of the corals’ bacterial community prior to menthol bleaching.

Additionally, the recommender’s interpretation that *Alteromonas* spp. abundance should be taken as in **indicator of underlying stress** in our corals because of its presence in the core microbiome is **not supported by our data**: if *Alteromonas* was positively correlated with stress, then its abundance should have been higher in the menthol bleached samples (even assuming that captivity itself caused significant stress, menthol bleaching would have unquestionably caused additional stress). However, as visible from figure 4 (lines 241:244), the abundance of *Alteromonadaceae* both increased (RS1, RS3) and decreased (RS2, HK1, HK2) in bleached samples. A similar pattern can be seen in Figure 5 (lines 272-276) for the abundance of the core members of the microbiome ASV_003 and AVS_008, both *Alteromonas* spp. Hence, the role of *Alteromonas* spp. in the coral holobiont, and in the *Galaxea* holobiont, remains to be clarified.

- c) Reviewer 2 suggests a new interpretation of what drives the bacteria-Symbiodiniaceae association, which is however based on a misinterpretation of the studies that we referenced. The statement that “*the 3 external studies were based on non-bleached corals and clearly confirmed this assumption*” is plain wrong, as these are based on Symbiodiniaceae algae cultures, as explicitly reported in our Supplementary Material and Methods section.

Other sections of the review also lack clarity or misinterpret the current state of the literature (other peer-reviewed studies as well as our data), showing a lack of attention or expertise in coral microbial ecology throughout the review. For instance, the sentence “*Unfortunately, the authors have already dismissed this notion (Line 349), arguing that the reduction or simplification of the microbiome is not an issue associated with captive corals’ simplified microbiomes*” is self-contradictory and does not correctly interpret our point of discussion. Captive animals have simplified microbiomes, and corals are no exception.

Unfair treatment and/or bias in the review process

Our concerns regarding the fairness of the review process are based on the following points: d) the reviewers and recommender do not point out any objective methodological flaw regarding the

primary objective of our study; e) neither of the reviewers clearly recommends a rejection (indeed one says that the work in its current form could be published); f) unclear arguments from the recommender effectively misled our efforts.

- d) There are **no standing critiques regarding** the methodology, the analytical approach, the transparency and reproducibility of the analysis, and the presentation of the **primary objective** of this study (i.e., to investigate the bacterial microbiome of symbiotic and menthol bleached polyps of the emerging coral model *Galaxea fascicularis*, as per the title). We present our results in the most clear and transparent way. The criticisms rather focused on possible alternative interpretations and on secondary aspects (the 'captivity effect') which however cannot be substantiated with the present data.

- e) **Neither of the reviewers recommend a rejection.** Such a decision seems therefore based primarily on the recommender's opinion and interpretation of our work, which we showed above to be based on false premises.
In summary: **Reviewer 1** is satisfied with how we addressed the points raised in the first round of revision, and gives the green light for publication. **Reviewer 2** points out how the study limitations "*are not completely insurmountable ; it is not impossible that authors overcome them, by continuing to dig the interpretation of their data and polish the main message.*".

- f) The rejection decision is based on our inability to provide additional data to address a **secondary** aspect of our investigation (the 'captivity effect'). Hence completely dismissing the primary objective of our work: to describe the bacterial microbiome of symbiotic and menthol bleached polyps of captive *Galaxea fascicularis* (see: **title**). **We did not conceive this experiment to investigate how captivity affects the bacterial microbiome (the 'captivity effect')**. For that, samples from wild and captive corals need to be sequenced together. As we cannot travel in time, the recommender basically requests that we run a completely new experiment **to address a different question**.

To summarize, not a single one of the arguments put forward in this round of revisions is valid on closer inspection, and we hope that you will reverse the decision to reject our manuscript.

Comments on the previous recommender decision

Regarding Puntin's request, here are some facts that may help identify factual errors that had a major impact on the decision:

- The article underwent 3 reviews, one by myself, another one by a member of my group (which make the two reviews not completely independent) and a anonymous reviewer (XXXXXXXXXX).
- None reviewer specializes in corals
- The anonymous review was brief and suggested minor revisions, such as polishing and better explaining the sampling design.
- However, subsequent reviews, including mine, indicated a need for major revisions. I emphasized in the first round that the study lacked replication and clarity in measurement replication. I suggested a second round only if additional results were included, especially comparisons with **wild communities, which are critical.**
- The main problem at this point was that the study suffered from limited number of replicates, sampling from different locations, analysis in different laboratories with **slight yet seemingly significant variations in rearing methods,** and **the absence of comparisons with natural colonies.**
- The paper's conclusion originally attributed differences between bleached and untreated communities to a rather vague "destabilization and loss of structure." In the revised version, authors clarified this by emphasizing stochastic factors. However, they did not include fresh data on wild corals from the original sampling sites, which is crucial for drawing clear conclusions given the stochastic nature of coral microbiome responses.
- Menthol bleaching induces stochastic changes in the microbiome of Galaxea, as discussed in Part 1 of the discussion, which constitutes one-third of the discussion. The remaining two-thirds of the discussion dig into the only significant finding of the study, which is the simplification of the microbiome in captive-reared polyps compared to those in the wild (i.e. compared to literature). Initially seeming secondary due to the experimental design focusing on bleached versus symbiotic states, this finding holds significant weight. But in my view, the authors were unable to revise the manuscript to prioritize the secondary result—simplification of the microbiome—and to make assumptions/hypothesis about the core microbiota simply because the study was not originally designed in this way. That's why I asked additional results in my first round of review.

In the appeal, authors claim that the wild-vs-captivity aspect was secondary to the study object of the menthol bleaching effect on microbiome; however.

Yui Sato
29/04/2024 21:43

I'd argue that the data on the wild corals is critical for pointing out that corals kept in tanks for a long time have lower diversity in bacterial communities than in the corals in the wild; but NOT to draw "clear conclusions" on the effect of bleaching. These are two separate aspects. For the captivity effect, authors compare with other studies, but this lacks the support that other studies were looking at the same coral species, considering cryptic spp. (e.g. <https://www.sciencedirect.com/science/article/pii/S1055790320301779>).

Yui Sato
29/04/2024 13:37

As to the stochastic responses to menthol bleaching, in my opinion, data would be sufficient if multiple polyps from the same colonies are showing stochastic responses, while non-bleached corals were consistent across polyps (n=3 polyps per treatment per colony; 5 colonies tested). Claiming that the stochastic modes are different between locations needs more samples in HK (currently n=2).

Yui Sato
29/04/2024 13:44

I do not share this view, as the supporting data is missing.

Yui Sato
03/05/2024 11:31

Comments of the peer-review history

Revision round #2

Decision for round #2 : **Rejected**

Unable to recommend

First and foremost, I want to extend my sincere appreciation to the authors for their diligent work on this manuscript. The revisions to the experimental protocol have notably enhanced clarity, aligning the message more closely with the results.

However, there remain areas where I diverge from the authors' conclusions. A case in point is the section titled "The microbiome of long-term aquarium-reared *Galaxea fascicularis*," where the authors propose their hypothesis that captivity leads to the streamlining of the microbiome. This terminology, though technically accurate, may be unnecessarily complex. It essentially refers to the simplification of the microbiome in captive-reared polyps compared to those in the wild. The authors attribute this simplification to the more controlled and stable environmental conditions and the reduced structural complexity of the polyp. While this section also delves into the potential advantages and opportunities presented by this simplified microbiome for experimental manipulation and understanding holobiont functioning, it ventures into discussing the influence of host genotype and environmental conditions on microbiome composition, suggesting avenues for further research to explore these relationships.

However, without a direct comparison of both the microbiome and genomes of the hosts with wild corals, these assertions remain highly speculative.

A reviewer has proposed an alternative hypothesis, which I find more compelling: that the 10-14 days of captivity sufficiently impacted and weakened the bacterial community associated with symbiotic Red Sea polyps to make the effects of bleaching barely perceptible. This idea gains support from the significant presence of a putative coral pathogen (*Alteromonas* spp.) within the core microbiome of captive *Galaxea*. Unfortunately, the authors have already dismissed this notion (Line 349), arguing that the reduction or simplification of the microbiome is not an issue associated with captive corals' simplified microbiomes.

Both hypotheses stem from the observation that coral microbiota is simplified in captivity but approach this from different angles. However, without direct comparison data with wild corals and measurements on inputs such as microbiota associated with the corals' diet, etc., it is challenging to ascertain which hypothesis holds greater validity.

Given these circumstances, I anticipate difficulty in crafting a letter of recommendation unless I am personally convinced of the validity of the interpretations drawn from the study's results. I thereby regret to say that despite recognizing the authors' commendable efforts and the significant amount of work they have invested in revising the manuscript, I find myself unable to wholeheartedly recommend the article for publication.

Sincerely,
Cédri Hubas

I agree with the recommender that the 'simplification' of microbiome ('captivity effect') in this study should remain a speculation without data, as each study may differ in sequencing depths (translates to apparent species richness) and corals' phylogenetic lineages considering cryptic species between and within locations (e.g. Wepfer et al. 2020; <https://doi.org/10.1016/j.mpev.2020.106905>). I also see that the investigation of the captivity effect was never the main focus of the study. These should clearly reflect in the tone of the discussion clearly indicate that further studies are needed as a conclusion. If this is appropriately done, I believe this should not form a basis for the rejection.

Yui Sato
30/04/2024 12:52

As the authors' appeal pointed out, this is incorrect.

Yui Sato
30/04/2024 14:52

This seems out of context: It reads to point out that simple microbial community offers an opportunity of studies of a less complex, better handled system.

Yui Sato
30/04/2024 14:56

Here, the recommender is referring to the ideas by the reviewer, which is based on an incorrect understanding of the study; thus it should not form a reason for rejection.

Yui Sato
30/04/2024 14:58

by **Cédric Hubas**, 22 Mar 2024 10:32

Manuscript: <https://doi.org/10.1101/2023.08.23.554380>

version: 2

Review by anonymous reviewer 1, 26 Feb 2024 09:41

In the revised ms the authors satisfactorily covered issues raised by the reviewers,
For this reason I believe the manuscript could be accepted

Review by Tony Robinet, 03 Mar 2024 18:28

Dear authors,

I sincerely acknowledge that changes were made by the authors, mostly in the discussion, and answers brought to my different comments were clear and correct. However it appears that the main correction awaited by the editor ("the inclusion of additional results") was not fulfilled.

During the time since my last reading of this manuscript, I realized a couple of considerations :

1) that Symbiodiniaceae-associated bacteria in coral polypes are triggered by two main parameters : the coral symbionts themselves *and* the surrounding environment. In the Figure S5, the 3 external studies were based on non-bleached corals and clearly confirmed this assumption. In a same way, a majority of the Symbiodiniaceae-associated bacteria present in these external studies were not found in the non-bleached *Galaxea* ("symbiotic polypes") of the present study.

2) the dispersion of bacterial communities of symbiotic corals from Red Sea in the NMDS (Fig 3) was higher in symbiotic polypes than in bleached polypes (this was not observed for Hong Kong corals). This symptom, a dysbiosis, may be a direct effect of captivity, already affecting symbiotic polypes before bleaching.

Figure 3 and specially the panel B (Bray-Curtis dissimilarities within symbiotic and within bleached colonies), although somehow contradicting this view if not accompanied by statistical tests brouhgt by the authors in the text, was interpreted as indicating "random changes in the communities of the menthol-bleached polyps" (or loss of structure). I agree. In other words, if bleached polyps were dying, the bacterial niche they constituted when Symbiodiniaceae were there continued to destructure and started to collapse. So if the dysbiosis was already there for Red Sea polypes, the bleaching event may just have accentuate an ongoing process. An hypothesis could be that the 10-14 days of captivity has impacted and weakened sufficiently the bacterial community associated with Red Sea symbiotic polypes to make barely perceptible the effects of bleaching.

To my opinion, the weakness in the protocol (effects of captivity couldn't be assessed due to the absence of wild corals and food supply analysis) are not completely insurmountable ; it is not impossible that authors overcome them, by continuing to dig the interpretation of their data and polish the main message.

Experimental microbiology is a steep path, go on and good luck.

The authors appeal letter indicated that this recommendation was not aligned well with their intention of the paper (main focus). In my opinion, the authors argument in the letter is agreeable.

Yui Sato
30/04/2024 09:19

As the authors' appeal letter, the cited papers are not coral associated microbiome, but ones associated with clutres of symbiotic algae (i.e. not environmental either). Besides, reviewer's point here is unclear to me.

Yui Sato
30/04/2024 11:10

The dispersion is also capturing inter-colony variability, not only the effect of bleaching... This point appears not valid, as there are no data on inter-colony differences before captivity.

Yui Sato
30/04/2024 11:15

Not a valid assumption, as the authors pointed out that these corals survived for multiple years after the study.

Yui Sato
30/04/2024 11:19

As the authors' pointed out, this is incorrect.

Yui Sato
30/04/2024 11:21

I feel that suggestions made by the reviewer and recommender were not particularly designed to polish the main message, which in my opinion is that menthol-induced coral bleaching leads to stochastic changes (breakdown of symbiotic

Yui Sato
30/04/2024 12:21

Revision round #1

Author's Reply, 29 Jan 2024 13:45

We express our gratitude to the recommender and reviewers for their assessment of our study and constructive comments, which we have carefully addressed in our authors' reply letter (please refer to the attached PDF).

We have updated the manuscript to incorporate the suggested improvements, involving comprehensive editing to clarify the preliminary nature of our study and underscore its limitations. Specific additions include a new visual summary of the experimental design and analysis, within-colony statistical testing for differences in alpha diversity between symbiotic and bleached polyps (when possible), and a re-plotted Figure S2 displaying raw data points.

We are confident that these adjustments not only align with the constructive feedback received but also improve the quality of our work. We appreciate the time and effort invested in evaluating our manuscript.

With best wishes on behalf of the authors,
Giulia Puntin

Decision for round #1 : *Revision needed*

I trust this letter finds you in good health.

I, along with two different reviewers, have had the opportunity to thoroughly review your study and provide feedback on your submission. Reviewers have articulated several concerns about your preprint, and I wholeheartedly concur with their assessments. The reviewers have highlighted issues such as the relatively low level of replication and the lack of clarity in how measurements were replicated. Additionally, Reviewer 2 has offered valuable insights regarding sequencing and assignment.

The primary concerns raised is about the preliminary nature of the study. While your research presents intriguing insights into the core microbiota associated with the *Galaxea fascicularis* coral model, the methodology used appears to hinder the generation of clear, conclusive results. The abstract, for instance, implies an expectation of a decisive conclusion regarding the impact of bleaching on the bacterial microbiome of *G. fascicularis*. However, due to various issues such as the limited number of replicates, sampling from different locations, analysis in different laboratories with slight yet seemingly significant variations in rearing methods, and the absence of comparisons with natural colonies (as pointed out by Reviewer #2), the results come across as over-interpreted. Thus, in my opinion, the study's validity and potential impact must be significantly enhanced with additional results.

After a comprehensive review of your work, I have arrived at the conclusion that I am unable to recommend it for publication in its current state. Below, you will find specific comments that I provide in addition to reviewers comments. Given the methodological limitations, a thorough revision may require the inclusion of additional results.

This aspect of the 'captivity effect' is a separate issue from the bleaching effect.

Yui Sato
29/04/2024 22:19

This suggestion is not to the main focus ('bleaching effect'), but on secondary points (i.e. 'captivity effect' and 'location effect'), which should be toned down, not enhanced, in my opinion. I see that this comment diverged the study more into unnecessary non-supported directions, than strengthen the main story.

Yui Sato
30/04/2024 12:30

It seems to be a leap from this statement, to the next paragraph essentially saying no new data, no recommendation...

Yui Sato
29/04/2024 22:24

I cannot make assumptions regarding your ability to supply the requested additional data, so I have opted to request a significant revision rather than an immediate desk rejection. If you are confident in your capacity to furnish additional results and address the queries raised by the reviewers and myself, kindly submit a revised edition of your work. In the event that this proves unfeasible, you will be given the option to withdraw your submission from PCI Microbiology.

Specific comments :

Materials & Methods:

Experimental Design: The experimental design in your study appears to be quite complex, but it is not sufficiently explained. The number of replicates is unclear, which is a critical aspect of any research study. For instance, in the section titled "Sampling for microbial analysis," you mentioned that "n = 15" for both bleached and symbiotic polyps, but it is not clear how this number was determined. Furthermore, the number of polyps collected seems inconsistent with the mentioned number.

Fig. 1: The statement in Fig. 1 that "Alpha diversity remained similar between symbiotic and menthol-bleached samples across all diversity and richness indices tested" is contradicted by the significant difference in Shannon and Simpson indices in RS1 (Red Sea colony #1). This contradiction should be addressed and clarified.

Results:

Fig. 2A and PERMANOVA: In Fig. 2A, the microbial communities from the Red Sea and Hong Kong colonies appear to be very similar, raising doubts about the significance of the PERMANOVA results. It is essential to reconsider this analysis and possibly perform ANOSIM. Considering the clear heterogeneity of multivariate dispersion in Fig 2A, I doubt that betadisper (PERMDISP2) gave a p-value > 0.05.

Discussion:

The main takeout is that bleaching induce a very different response in bacterial communities in « HK » compared to « Red Sea ». This is probably linked to the fact that HK and Red Sea experiments were conducted in different places with slightly different conditions. Unfortunately, it is impossible to test the laboratory/feeding procedure/aquarium effect because this factor has not been replicated which makes it difficult to draw a conclusion. This limitation should be emphasized to provide a more balanced interpretation of your findings.

Conclusion:

The conclusion section in your paper appears to contrast with the abstract. The abstract fails to clearly convey that the difference between bleached and untreated communities is apparently due to stochastic factors. Instead, it suggests "destabilization and loss of structure of the communities," which comes across as vague and overly wordy.

I hope you find these comments helpful in improving the quality of your research.

Sincerely,

Cédric Hubas

by *Cédric Hubas*, 16 Oct 2023 18:46

Manuscript: <https://doi.org/10.1101/2023.08.23.554380>

version: 1

I'd argue that the main message is that the loss of symbiotic algae causes stochastic changes in coral associated microbial communities among colonies, but not about their differences by locations... If the recommender's point here aligns with authors', then the recommender's point of needing additional HK data is a valid point. Note that this is a separate topic from having wild coral data to examine the 'captive' effect.

Yui Sato
29/04/2024 22:32

I do not think this is less "vague" than just saying "stochastic factors" in my opinion.

Yui Sato
29/04/2024 23:18

Review by anonymous reviewer 1, 02 Oct 2023 17:12

In their manuscript, Puntin et al. present very interesting results from their study focusing on the bacterial microbiome of symbiotic and menthol-bleached polyps of *Galaxea fascicularis*. However some I have some concerns mostly on the way results are presented.

- 1.l. 5-6: please rephrase
- 2. l. 150 (and elsewhere): What is Simpson evenness? I only know Simpson diversity index.
- 3. Figure 2B: Where is HK1 symbiotic and HK2 bleached in Fig.2B? Apparently you had only two replicates. Did you mention that earlier because I cannot find it.
- 4. L. 215: You sampled 30 polyps, and excluded 1 from RS3 symbiotic (which one???) due to low seq. depth, so that makes us 29 polyps. Right? In Fig. 2b above you miss two more polyps. You need a table that will show all your samples with proper encoding and will explicitly explain which was used for every analysis. Also you refer to your samples according to origin (i.e. RS: Red Sea) and a number which indicates the colony (1,2,3). However this is confusing since you do not separate the triplicates you sampled from each colony. I would suggest you add a simple encoding (l.e.a,b,c) since it is confusing (e.g. which RS3 sample was excluded????).

These are all addressed

Yui Sato
29/04/2024 21:02

Review by Tony Robinet, 25 Sep 2023 14:08

Comments on the MS from Giulia et al. sent to PCI microbiol

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The authors aimed at evaluating the behaviour of microbial community in the tropical coral *Galaxea fascicularis* after polyps from the same wild colonies were kept in captivity under controlled conditions only, or under controlled conditions and bleached with menthol.

Bleaching, corresponding to the disappearance of the photosymbiotic Symbiodiniaceae from the polypes, induced a disorganisation in microbiomes in the way that the structure formed by core taxa in symbiotic polypes vanished, turning into a kind of stochastic assemblage of taxa. Authors did not notice any typical signature of bleaching, like would have been the systematic death of some key-taxa.

Authors discussed that, in this study, captivity did reduce the diversity of microbiomes in polypes, compared to those living in non-captive ones, but there were no assessment of wild polypes microbiomes in this study. The comparison relied on data from literature only. However, the captivity effect, i.e. the lack of exogenous wild bacterial flow into bleached polypes, and the potential bacterial flow from food, were appropriately proposed to explain the observed convergence of microbiomes of Red Sea and Hong Kong due to their captivity in similar conditions.

Results are presented by a scientific team who is experienced in coral microbiology, as we can read it in introduction and discussion. Concepts are well defined, literature is recent and abundant, questions are clearly addressed, scripts are clean and working.

A complete study of the bleaching effect would probably have gathered more samples (here only 14 from 5 colonies), sequenced "wild" samples in coral colonies of the same locations where captive ones have been collected, developed a correct sequencing protocol for the Symbiodiniaceae ITS2 (this axis is unfortunately under-explored), and analyzed the unknown microbial contamination brought by feeding (L103: "polyps were fed daily with one small frozen adult *Artemia* each"). We can

Hence recommending to tone it down, in my view. This point is legitimate due to differences in methods and the potential of cryptic species that other studies may have been looking at different coral lineages from different locations.

Yui Sato
29/04/2024 21:56

I agree that the data on algal symbiont compositions were dismissingly limited, and thus were not contributing to the analysis as to the loss of what symbiont types contributed to the bleached bacterial compositions.

Yui Sato
29/04/2024 21:59

understand all the reasons explaining why these elements are lacking, but in their absence, I think that this study can be worth to be shared with the scientific community if authors present their results as preliminary, or with these gaps expressed in the abstract, before a complete study can be lead. As well, the title should be clarified by mentioning the fact that "symbiotic" and "bleached" corals were both captive.

I have no specific comments, the manuscript is well written, only a specific question : Why did you assigned taxa only to genus rank, and then numbered the ASV ? (= the unique sequences, i.e. all variants found on this marker in each species), given that (1) Silva database assignation is quite good down to species rank ; (2) if assignation with qiime is not robust for a given taxa, species is named "unassigned species" ; (3) 16S marker is known to be prone to an unknown number of copies in a same organism, with possible nucleotidic variation between copies, and therefore with the possibility to over-estimate the effective number of different organisms, and thus the reality of some of them ? Or maybe you know that **Symbiodiniaceae have only one 16S copy** ? Did you try the same analyses at the species level (97% of similarity between sequences)?

This indicates that this reviewer thought the eukaryotic algal symbiont of corals as a bacterial species, which is incorrect.

Yui Sato
29/04/2024 22:07

This indicate that the reviewer may not be very familiar with QIIME analyses of 16S data. Working with ASVs (no similarity clustering) does not prevent species level annotation. I believe that the species level annotation in SILVA can cause problem as a host organisms's name is at times listed in the species classification, not the species name of bacteria. This is a reported known issue using QIIME and SILVA. Consequently, users commonly opt out from species level classification.

Yui Sato
29/04/2024 22:12