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Interactive comment on "Warming effect on nitrogen fixation in Mediterranean macrophyte sediments" *by* Neus Garcias-Bonet et al.

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GENERAL COMMENTS

RC 1: The ms by Garcias-Bonet et al. addresses a relevant novel scientific question, which is the response of N2 fixation in vegetated marine sediments to warming. It is also valuable that the paper includes the sediments of, not only the well-studied seagrass species P. oceanica, but also the less studied seagrass species C. nodosa and the green macroalgae C. prolifera. The scientific question fits well in the scope of BG since N2 fixation is a relevant metabolic process in marine sediments and, in the particular case of the Mediterranean Sea, it supports the primary production of seagrass P. oceanica.

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AC 1: We thank the reviewer for her thorough review and constructive comments which will help us improve the manuscript.

RC 2: The conclusions of the ms are based on N2 fixation rates measured in triplicate sediment samples taken from one single location in the Mediterranean Sea and exposed to five meaningful experimental temperatures, from 25 to 33oC. The authors reach two clear and relevant conclusions: first, N2 fixation is higher in the sediment colonised with marine macrophytes than unvegetated sediments, and second, warming up to 31oC is expected to increase the N2 fixation rates in the sediment of marine macrophytes, but above 33oC the rate will decrease. The methods and experimental design are sound, but authors should encompass the limitations of the study when interpreting the results: first, rates were measured in sediments collected in just one site and, second, the study does not account for synergic or antagonistic effects with other environmental drivers.

AC 2: We agree with the reviewer on the limitations of our work and the need to carefully interpret our results.

In the revised version of the manuscript, we will acknowledge that our results are limited to one location where the three macrophytes coexisted and therefore the drawn conclusions need to be carefully put in perspective. Similarly, we will discuss the synergic or antagonistic effects with other environmental drivers as well as a possible adaptation to warming.

We propose to include the following text in the discussion: "The forecasted warming could affect as well other biogeochemical processes in coastal sediments, such as sulfate reduction (Robador et al., 2016), anaerobic ammonium oxidation and denitrification (Garcias-Bonet et al., 2018;Nowicki, 1994), among others, and, therefore, potential synergic or antagonistic effects may occur. Responses of sediments colonized by different macrophyte species may also differ due to differences in the lability of their OM and nutrient stocks, associated to differences in C:N:P ratios (Enríquez et al., 1993;Lanari et al., 2018). Similarly, losses of seagrass coverage by heatwaves could lead to an increase in CO2 emissions by increased remineralization of C stocks (Arias-Ortiz et al., 2018). The thermal dependence of N2 fixation in vegetated sediments found here might have important consequences for primary production in coastal ecosystems in the context of warming. This may not be the case for P. oceanica, as this species is projected to be critically compromised, to the extent that functional extinction is possible, with projected Mediterranean warming rates by 2050-2100 (Chefaoui et al., 2018;Jordà et al., 2012). However, in order to draw general conclusions on the effect of warming on N2 fixation in coastal ecosystems, the thermal-dependence found here needs be tested for a diversity of seagrass ecosystems. Similarly, our results from experimental temperature treatments did not account for potential acclimation and adaptation of microbial communities to warming, which should also be tested. Moreover, N2 fixation is likely to be subjected to other environmental controls that may change, either in an additive, synergistic or antagonistic manner, with warming, so predicting N2 fixation rates in a future warmer coastal ocean remains challenging."

RC 3: The overall presentation of the ms is clearly structured and ideas and paragraphs are presented in a logical way. Language is fluent and precise, with only some suggested corrections to improve readability and clarity (see technical comments). In general, methods are clearly outlined and described, but I have a relevant comment regarding the inclusion or not of the vegetation (above- and/or below-ground) during the cores extraction and later on in the sediment incubations (see specific comments). This should be clearly stated throughout the ms because in the introduction they explain that endophytic nitrogen-fixing bacteria have been detected in association to P. oceanica roots and leaves, so it is not clear if the N2 rates measured are solely due to the sediment behind or close to the marine macrophytes or if they are also due to the bacteria found on the surface of the macrophytes. I have other specific questions listed below.

AC 3: We thank the reviewer for pointing this out as we have realized that the text regarding the sediment sampling was not clear enough. The N2 fixation rates reported here were measured on slurries of sediment containing any belowground biomass collected with the sediment cores but did not contain aboveground biomass. Although we measured N2 fixation in sediments, we provided a detailed background of published literature regarding N2 fixation in Mediterranean seagrasses in the introduction. Therefore, we highlight the importance of N2 fixation in this seagrass species by mentioning the already reported rates of N2 fixation in tissues and in situ incubations (Agawin et al. 2016 and 2017; Lehnen et al. 2016) and our previous work on the detection of endophytic N-fixing bacteria (Garcias-Bonet et al. 2012 and 2016).

In the reviewed manuscript we will clarify the sediment sampling and we will improve clarity on how the Acetylene Reduction Assay was performed to avoid any confusion.

The new text will read as follow: "We collected 16 sediment cores for each type of sediment. The vegetated sediment cores were collected from the center of the macrophyte patches between shoots or blades, collecting belowground plant material but avoiding the collection of aboveground biomass. The bare sediment cores were collected about 5 m away from the edge of the vegetated patches."

SPECIFIC COMMENTS

Introduction.

RC 4: Authors should reinforce the background on the effects of warming on C. prolifera and C. nodosa, since most of the information given in the present version is focus on P. oceanica.

AC 4: We thank the reviewer for pointing this out and we will expand the introduction focusing on the effect of warming on the other macrophytes.

In the reviewed manuscript we will include the following information in the introduction: "...these coastal vegetated ecosystems are threatened by climate change at global scale (Duarte et al., 2018) and at the Mediterranean Sea scale (Marbà et al., 2015).

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In particular, warming increases the mortality rates of P. oceanica (Marba and Duarte, 2010), which is predicted to be functional extinct by 2049 to 2100 due to warming (Chefaoui et al., 2018;Jordà et al., 2012). Mesocosm experiments showed that C. nodosa is more resistant to warming than P. oceanica (Olsen et al., 2012), concurrent with thermal niche models (Chefaoui et al., 2016;Chefaoui et al., 2018), however a loss of 46.5 % in C. nodosa extension is predicted by 2100 under the worst-case warming scenario (Chefaoui et al., 2018). Although C. prolifera thrives well in warm waters, its photosynthesis is inhibited at temperatures above 30 oC (Lloret et al., 2008;Vaquer-Sunyer and Duarte, 2013), compromising its survival at temperatures above this threshold.

RC 5: Also, they should explain their hypothesis behind the comparison of the sediments of the three marine macrophyte types (L83-86).

AC 5: The rationale behind measuring N2 fixation rates in P. oceanica, C. nodosa and C. prolifera is that these three macrophytes are the key macrophyte species most commonly found in the Mediterranean Sea.

In the new version of the manuscript we will clearly state our hypothesis and the reason why we focus on these macrophytes.

The text will read as follow: "Here, we test the hypothesis that N2 fixation rates in coastal ecosystems is temperature dependent and will increase with the forecasted warming. We do so by experimentally assessing the response N2 fixation rates in coastal Mediterranean ecosystems to warming. We focus specifically on the key macrophyte species most commonly found in the Mediterranean Sea: two seagrass species (P. oceanica, C. nodosa) and one green macroalgae species (C. prolifera)…"

Materials and Methods.

RC 6: Regarding the sediment samples: a) how many cores were taken in the field for each vegetation type and in which part of the patch (edge or centre)?; b) did the sediment cores included the above- and/or below-ground vegetation or not?; c) Why were the top 10-cm selected for each core (rhizosphere depth varies between the three species)?

AC 6: We collected 16 cores for each sediment type. For the vegetated sediment, the cores were collected from the center of the patches. For the bare sediment, the cores were collected at least 5 m away from the vegetated patches. The cores from the vegetated sediments were collected between shoots or blades avoiding the aboveground material but containing the belowground rhizosphere. Despite the difference in rhizosphere depth among the macrophytes, we standardize our measurements to the first 10 cm of sediment in order to be able to compare rates among the 4 types of sediment, including bare sediment.

In a newer version of the manuscript, we will include more detailed information on the sediment sampling in the methods section. The new text will read as follow: "We collected 16 sediment cores for each type of sediment. The vegetated sediment cores were collected from the center of the vegetated patches between shoots or blades, collecting belowground plant material but avoiding the collection of aboveground biomass. The bare sediment cores were collected about 5 m away from the edge of the vegetated patches."

RC 7: The text explaining how the experimental temperatures were selected (L124-133) is confusing, in particular when comparing how values are given in figure 1 ("average summer median", L331), table 1 (many statistical descriptors) and the text ("average summer mean", L126). Also, the 29_C and 31_C treatments were selected as the current summer mean SST (26.54 +/- 0.17_C) plus the projected mean SST increase (2.8 +/- 1.1 _C) (L127-130). How did you yield the 29 and 31_C? My best guess is based on the errors reported, but this should be confirmed and explained: 26.54 + 0.17 + 2.8 + 1.1= 30.61_C and: 26.54 - 0.17 + 2.8 - 1.1= 29.34_C.

AC 7: We agree with the reviewer that the information provided in Table 1, fig 1, and text may lead to confusion. Following the reviewer comment, we will indicate the mean SST in fig. 1 (instead of the median SST) to keep consistency with the values mentioned in the text regarding how the experimental temperatures were calculated. We considered the 29 and 31 oC treatments as the range of the projected summer mean SST by end of the century calculated by adding the 2.8 $\pm$ 1.1 oC increase to the current summer mean SST registered in 2017 (when the experiment was performed): 27.03 + 2.8 + 1.1 = 30.93 oC and 27.03 + 2.8 - 1.1 = 28.73 oC. We will clarify that we used the last summer mean SST in the methods section.

Moreover, following the advice of the second reviewer, we will move the fig 1 and table 1 and 2 into the supplementary section.

RC 8: Authors explained that negative controls were run (L160-163), so they should report somewhere the results of the controls and if they were used to correct the rates calculated in the sample incubations.

AC 8: We thank the reviewer for pointing this out. We did not detect ethylene production in our negative controls. We will add this information in the methods section.

RC 9: Was the Arrhenius function fitted with a linear regression? (L179-180).

AC 9: Yes, we fit a linear regression between the ln of N fixation rates and the inverse of the temperature multiplied by the Boltzmann's constant.

In the revised version of the manuscript, we will clearly indicate this.

Results.

RC 10: Results are presented by 3 groups of temperatures (e.g. Fig. 2), although in L176 authors explain that statistical differences were tested by temperature treatment (5 levels). Please, check that figures, statistical tests and text should report results in the same way.

AC 10: We thank the reviewer for this comment as we realized that the statistical analysis considering 3 temperature ranges was missing in the statistical analysis section.

In the reviewed manuscript we will amend it.

RC 11: In figure 3, authors pooled the 3 types of vegetated sediment to see how the fixation rate varies with temperature. However, the rates, when expressed in a dw basis as it is in Fig. 3, differed statistically among the 3 types (L200-202). I think it would be more appropriate to assess this relationship using fixation rates by unit of area, because no differences were found among vegetated sediment types (L202-203).

AC 11: We have considered the option of plotting the rates by area in former Fig. 3 as suggested by the reviewer, however, we think that the best way to show the thermal dependence of the rates in this particular case is by sediment dry weight. Following the advice of reviewer 2, we will update the former Fig. 3 (current Fig. 2) in order to show the individual replicate measurements for each type of vegetated sediment.

Discussion.

RC 12: Authors should include in the discussion section the limitations of doing estimations based on results from just one location. Also, there could be many other environmental factors acting in synergy or antagonistically with temperature and affecting N2 fixation rates in the sediments.

AC 12: We agree with the reviewer on the limitations of our work and the need of carefully interpret our results.

In the newer version of the manuscript, we will acknowledge that our results are limited to one location where the three macrophytes coexisted and therefore the drawn conclusions need to be carefully put in perspective. Similarly, we will discuss the synergic or antagonistic effects with other environmental drivers as well as a possible adaptation to warming.

The new text will read as follows: "The forecasted warming could affect as well other biogeochemical processes in coastal sediments, such as sulfate reduction (Robador et al., 2016), anaerobic ammonium oxidation and denitrification (Garcias-Bonet et al., 2018;Nowicki, 1994), among others, and, therefore, potential synergic or antagonistic effects may occur. Responses of sediments colonized by different macrophyte species may also differ due to differences in the lability of their OM and nutrient stocks, associated to differences in C:N:P ratios (Enríquez et al., 1993;Lanari et al., 2018). Similarly, losses of seagrass coverage by heatwaves could lead to an increase in CO2 emissions by increased remineralization of C stocks (Arias-Ortiz et al., 2018). The thermal dependence of N2 fixation in vegetated sediments found here might have important consequences for primary production in coastal ecosystems in the context of warming. This may not be the case for P. oceanica, as this species is projected to be critically compromised, to the extent that functional extinction is possible, with projected Mediterranean warming rates by 2050-2100 (Chefaoui et al., 2018;Jordà et al., 2012). However, in order to draw general conclusions on the effect of warming on N2 fixation in coastal ecosystems, the thermal-dependence found here needs be tested for a diversity of seagrass ecosystems. Similarly, our results from experimental temperature treatments did not account for potential acclimation and adaptation of microbial communities to warming, which should also be tested. Moreover, N2 fixation is likely to be subjected to other environmental controls that may change, either in an additive, synergistic or antagonistic manner, with warming, so predicting N2 fixation rates in a future warmer coastal ocean remains challenging."

RC 13: Authors should include in the conclusion paragraph (L308-312) that there is a reversal in the thermal response of N2 fixation rate in vegetated sediments after 31 oC, and state briefly that the conclusions have limitations.

AC 13: Following the reviewer's suggestion we will include the reduction in N fixation rates at 33 oC in the vegetated sediments and we will as well acknowledge the limitation of our study.

TECHNICAL COMMENTS

RC 14: L27. Should be "on N2 fixation rates in the sediment of three key marine macrophytes". L28-31. Authors should include the experimental temperature values in or at least the tested range (25-33oC). L35. Write "showed" for consistency with the tense used before. L39. Explain the meaning of the lower values of Q10 and activation energy in the vegetated sediments (e.g. parameters of temperature dependence of N2 fixation was...). Otherwise remove that sentence. L79. Add space after semi-colon in the citation. L88. Add "vegetated" before "ecosystems to warming" to be specific. L91-92. Without reading the M&M section, it is surprising to find two temperature values for a summer mean. This needs clarification (e.g. by saying "summer mean range" or similar). L100. Add "macrophytes" before "community" to be specific. L117. Say which years are "the last years". L147. Remove "The incubation lasted 24h", it is already said in L145. L140-147. Text explaining how the temperature treatments were attained in the lab would fit better at the end of the first paragraph of this subsection (L133), since it seems logical to present the temperature methods after the explanation of the temperatures chosen, and then follow up with the detailed explanation of the acetylene reduction assay. L168-170. It is written that rates "were standardised to surface area integrated over 10 cm sediment depth", but they later show the results in terms of mol N gDW-1 h-1. Include the standardisation in terms of dw in these lines as well. L182. The Q10 parameter is not properly explained and I think that authors should show the formula for the calculation (it may be not be a familiar concept for the BG readers). L193. Should be "table 3". L195-205. For which temperature level are the values given in this paragraph? L197-200. Give also the values of fixation rate for P. oceanica, as it is given for the other two types of vegetated sediments. L204. Statistical information given for the correlation of OM and N2 rates is incomplete. Authors should give the Pearson's coefficient, df, and p. L209. Refer to Fig. 2 at the end of the sentence. L235-236. Check the text "... supported 3 to 4-fold higher H2 fixation rates", because in L196 is written "3-fold" and in L208 "twice". L264. Delete the extra parenthesis. L266. Delete the extra parenthesis. L376. "Sediment" is repeated twice in the sentence, remove one of them. L377. Add "Mallorca" after Pollença Bay, for consistency with the other legends. L378. Write the sample size of the OM and DBD values. Table 1.

I suggest to remove the lines of years 2014 and 2015 in the table and explain in the legend that data are not available for those years. Then remove the * footnote as well. Table 2. Write the sample size for the temperature measurements. Figure 2. Explain what the box plots show (box, whiskers, "+" symbol, in-box line, dots), since it may vary among statistical softwares. Explain what asterisks on top of the box plots mean. Figure 4. Explain what solid lines mean. Add units in the x-axis.

AC 14: We really thank the reviewer for the thorough review and in the reviewed manuscript we will amend all the highlighted typos and add the requested additional information.

We would like to answer specifically to the following comments: RC: L209. Refer to Fig. 2 at the end of the sentence. AC: The mean values provided in L209 are calculated pooling all temperature treatments, therefore we believe that referring to Fig 2 here is not appropriate. However, we will specify in the text how the mean values were obtained. RC: L235-236. Check the text "... supported 3 to 4-fold higher H2 fixation rates", because in L196 is written "3-fold" and in L208 "twice". AC: The differences pointed by the reviewer are due to the rates being expressed by different units or referring to specific temperature treatments. We will double check in the reviewed manuscript that the information is provided in a clearer way. RC: L182. The Q10 parameter is not properly explained and I think that authors should show the formula for the calculation (it may be not be a familiar concept for the BG readers). AC: We agree with the reviewer that more detailed information on the Q10 rate should be included here. We will describe the Q10 rate and include its formula.

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