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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 addresses changes in nitrogen fixation rates by diazotrophic bacteria in vegetated and unvegetated sediments in the Mediterranean Sea in face of the global warming. The topic is relevant considering the actual scenario of climate changes. The experimental methods are appropriate and the results are quite interesting, making a significant contribution to the study of biogeochemical cycles in seagrasses and macroalgae habitats. However, I think the MS would benefit from adding more information in the Introduction and Discussion. Some methods also need clarification. Finally, I have some doubts/suggestions regarding the statistical analyses and results presen-

tation. Please find my comments and corrections below.

AC 1: we thank the reviewer for his/her thorough review.

Specific comments:

RC 2: Lines 60-63 and 83-85: please add more information about the ecological role of C. nodosa, C. prolifera and P. oceanica as "key macrophytes" in the study area;

AC 2: Following reviewer suggestion, we will add the requested information.

Specifically we will include the following text in the introduction: " Seagrass ecosystems provide important ecosystem services, such as the increase in diversity, the reduction of wave action and the protection of coast, the increase in water clarity by trapping suspended particles, and climate change mitigation by acting as carbon sinks (Costanza et al., 1997;Duarte, 2017;Fourqurean et al., 2012). In the Mediterranean Sea, the most important seagrass species are Posidonia oceanica, an endemic long-living seagrass, and Cymodocea nodosa, commonly found in the Eastern Mediterranean Sea and the Northeastern Atlantic Coast. Similarly, benthic green macroalgae, such as the autochthonous Mediterranean Caulerpa prolifera, form highly productive ecosystems contributing to the atmospheric CO2 sequestration (Duarte, 2017). However, these coastal vegetated ecosystems are threatened by climate change at global scale (Duarte et al., 2018) and at Mediterranean Sea scale (Marbà et al., 2015)..."

RC 3: Line 100: Plant communities? Benthic communities?

AC 3: We refer to benthic communities. We will specify it in the reviewed manuscript.

RC 4: Lines 107-108: I wonder if benthic macrofauna was also sampled, which could influence microbial community (?). Please also provide more information whether samples were obtained from homogeneous patches or some kind of abundance quantification;

AC 4: We sampled the sediment by coring and therefore samples might include microand macro-fauna. Following the advice of the reviewer, we will add relevant information regarding the sediment sampling and we will provide available data on shoot density estimates for P. oceanica and C. nodosa in the study site.

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."

RC 5: Lines 111-119: I feel that these temperature results are just background information for your experimental set up. Although I recognize their importance, I think they (both Figure 1 and Tables 1 and 2) should go as supplementary material;

AC 5: Following the reviewer comment, we will provide Fig 1 and Tables 1 and 2 as supplementary material and amend the text accordingly.

RC 6: Lines 122-124: so you used a full factorial experimental design testing the factors "sediment type" and "temperature" and their interactive effects?

AC 6: Yes, in order to test the effect of temperature on N fixation rates, we performed a full factorial two-way ANOVA considering temperature (as a categorical explanatory variable with 3 levels: current SST, projected SST, and projected SSTmax) and type of sediment (as a categorical explanatory variable with 2 levels: vegetated and bare sediments) on log transformed N2 fixation rates to meet normality. Finally, we tested the thermal dependence analysis by fitting the Arrhenius function in order to provide activation energy and Q10 for N2 fixation in our coastal sediments.

In the revised manuscript, we will clarify the statistical analyses performed.

RC 7: Lines 134: were seagrass plants "transplanted" along with sediments to the laboratory? As you are talking about endophytic bacteria, I believe at least the belowground biomass was present

AC 7: 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."

RC 8: Lines 122-159: I would recommend first describing sediment sampling/preparation, then the experimental treatments followed by the description of N2 determination procedures;

AC 8: We thank the reviewer for addressing this comment and we will restructure the text following the suggested order.

RC 9: Line 147: as the incubations lasted only 24 h, I think you are investigating the impacts of heatwaves (short-term events) rather than warming per se (long-term warming) as your MS tittle suggests;

AC 9: The aim of this work is to address the thermal dependence of N fixation rates by measuring rates at different experimental temperatures. As pointed by the reviewer, we don't account for acclimation or adaptation of sediment microbial communities to warming. We will acknowledge the limitation of the study in the discussion. We suggest the following text: "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 10: Lines 166-170: please provide more information on why measuring the sedimentary OM (a proxy for belowground biomass?). Also clarify the difference between using sediment dry weight and standardized fixation rates in your results;

AC 10: We measured sediment OM content to test if differences in N fixation rates were explained by differences in sediment OM as it has been reported previously that increase in OM content enhance N fixation rates (i.e. Herbert 1999, Tibbles et al. 1994). We reported N fixation rates by sediment dry weight and in an aerial base as we believe both units provide valuable information. In literature, rates are mostly reported only in one of both forms and then comparisons among works are not always possible if sediment bulk densities are not reported.

RC 11: Lines 168-170: Speaking somewhat naïvely here but it seems to me that, if you have nutrient stocks in mind, you should focus on results standardized by sediment bulk

density. If dry bulk density is the best choice here, results shouldn0t be expressed in mg N m-3?

AC 11: We report N2 fixation rates as the transformation of atmospheric N2 into NH3, but we don't report N stocks. Therefore, we believe the best way to express our rates is using both nmol N gDW-1 h-1 and mg N m-2 d-1

RC 12: Lines 173-178: it appears to me that a factorial two-way ANOVA for all variables measured is the most informative analysis in your case. Has the data gone through some transformation? Or have you considered using a generalized linear model model with gamma or lognormal data distributions? I also could not understand why using a Friedman test (a non-parametric alternative for repeated-measures ANOVA) followed by a Mann-Whitney U test (non-parametric comparison between two populations). I think maybe you have an unbalanced design in the latter case (N = 9 for vegetated and N = 3 for bare sediment). If possible, try a one-way ANOVA partitioning the sum of squares into an a priori contrast between the unvegetated sediments and all pooled vegetated sediments (see more details in Bruno et al., 2005 for instance). My main point here is: whenever possible, use a more robust and informative single analysis where you can determine the interactive effects sediment types X temperature;

AC 12: We tested if N fixation rates differed among the 4 types of sediments (P. oceanica, C. nodosa, C. prolifera and bare sediments) matching by temperature treatment by running a Friedman test. Then, in order to test the effect of temperature on N fixation rates, we performed a full factorial two-way ANOVA considering temperature (as a categorical explanatory variable with 3 levels: current SST, projected SST, and projected SSTmax) and type of sediment (as a categorical explanatory variable with 2 levels: vegetated and bare sediments) on log transformed N2 fixation rates to meet normality. Then we separately, analyzed the differences between vegetated and bare sediment for each temperature range by a Mann-Whitney U test.

In the revised manuscript, we will clarify the statistical analyses performed.

RC 13: Lines 181: please provide more information on Q10;

AC 13: We thank the reviewer for pointing this out, as we realized this was not fully explained. In the newer version of the manuscript we will expand on Q10 and how it was calculated.

RC 14: Line 192: Table 3 instead of 2;

AC 14: We will amend the text.

RC 15: Lines 195-202: could you please provide a figure?

AC 15: We will include the measured N2 fixation rates expressed by sediment dry weight for each sediment type at each temperature treatment in Fig.3.

RC 16: Lines 195-217: Maybe I missed it, but you performed an experiment under five different temperatures, determining N2 fixation rates at 12,17, 20 and 24 h. I couldn0t find all these results in Figure 2.

AC 16: It seems that the reviewer understood that N fixation rates were measured at different time points. However, what we measured at different time point was the ethylene concentration in the headspace of each incubation bottle in order to calculate the rate of production of ethylene using the change in ethylene concentration over time.

RC 17: Lines 218-221: I dont think a linear regression is the best choice here, as highlighted by your low R2 value. Have you tried to fit your data to distinct models (e.g., a polynomial one)?

AC 17: we agree with the reviewer that the R2 value of the linear regression is low for the vegetated sediments (R2 = 0.11 and 0.51 for vegetated sediments and bare sediments, respectively). However, the linear regression is the best fit model compared to the second order polynomial model for both vegetated and bare sediments. We provide here the output of our model comparison: Bare sediment Vegetated sediment Null hypothesis Straight line Straight line Alternative hypothesis Second order polynomial

Second order polynomial Conclusion (alpha = 0.05) Other fit is ambiguous Other fit is ambiguous Preferred model Straight line Straight line

RC 18: Lines 209-215: this sentence is too long;

AC 18: We thank the reviewer for pointing this out and we will divide the sentence in two shorter ones. The new sentence will read as follow: "At current summer SST (25-27 oC), N2 fixation rates in vegetated sediments (3.15 $\pm$ 0.48 mg N m-2 d-1) were significantly higher (U= 13, p < 0.01) than those in bare sediments (1.14 $\pm$ 0.3 mg N m-2 d-1) (Fig. 1A). Similarly, at projected summer mean SST (29-31 oC), N2 fixation rates in vegetated sediments (5.25 $\pm$ 1.17 mg N m-2 d-1) were significantly higher (U = 23, p < 0.05) than the rates measured in bare sediments (2.18 $\pm$ 0.2 mg N m-2 d-1) (Fig. 1B)."

RC 19: Lines 227-233: please revise this sentence;

AC 19: We will shorten this sentence, following the reviewer comment.

RC 20: Lines 244-248: Do you mean variability in seagrasses habitats among sites?

AC 20: With this sentence, we meant that the differences in N2 fixation rates between our work and previously reported rates for the same seagrass species might reflect spatial variability in N2 fixation rates.

RC 21: Lines 248-259: seagrasses and macroalgae have distinct C/N ratios and, consequently, different biomass turnover rates and consumption susceptibility. Such differences determine their role as carbon (e.g., Krause-Jensen et al. 2018) and nutrient stocks (e.g., Lanari et al. 2018). Increasing temperatures may also enhance organic matter remineralization, which may counteract increasing N fixation rates in an ecosystem functioning perspective. These topics could be further explored here;

AC 21: Following the reviewer comment we will enrich the discussion accordingly. In the reviewed manuscript we will 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).

RC 22: Lines 274-278: please clarify this sentence;

AC 22: We thank the reviewer for pointing this out as we realized that our message was not clear enough.

In a newer version of the manuscript we will improve the readability of this sentence.

We propose the following new text: "We experimentally demonstrate that N2 fixation in coastal sediments is thermal dependent, both in vegetated and bare sediments. Despite a formal experimental demonstration was lacking, the N2 fixation thermal dependence reported here is in agreement with the higher rates typically measured in warm tropical and subtropical meadows compared to the rates reported in temperate and cold seagrass systems (Herbert, 1999;McGlathery, 2008;Welsh, 2000)."

RC 23: Lines 274-305: considering your MS tittle, I think the discussion on warming effects on N2 fixation can be enriched. For instance, effects of warming are also reported for other biogeochemical cycles, such as carbon stocks (e.g., Arias-Ortiz et al. 2018);

AC 23: We agree with the reviewer and we will discuss the potential effect of warming on other bioogechemical processes. We propose the addition of the following text: " 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. 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)."

RC 24: Lines 302-305: this argument was also presented in the Introduction. If P. oceanica is predicted to be extinct in 2049, why test it under scenarios expected by the end of the century?

AC 24: The functional extinction of P. oceanica is predicted to happen by 2050 (Jorda et al. 2012) or 2100 (Chefaoui et al. 2018). However, we believe that the response of N2 fixation in P. oceanica sediments, as well as other marine coastal vegetated and bare sediments to warming, is a relevant scientific question.

RC 25: Lines 307-308: I think here (and in your Discussion) you could explore more the significance of your results on local biogeochemical cycles. Moreover, in the lines 311-312, it was found that N fixation rates in vegetated sediments decreased at 33_C.

AC 25: We thank the reviewer for this comment and we will improve the conclusion paragraph. The conclusion section in the new version of the manuscript will read as follow: "Mediterranean macrophyte meadows are sites of intense N2 fixation rates, twice as high as those in adjacent bare sediments. As these rates increased with warming, realized warming of the Mediterranean Sea is expected to have led to enhanced sediment N2 fixation rates, with future warming leading to further increase in N2 fixation rates up to 33 oC in bare sediments and 31 oC followed by a decrease at higher temperatures in vegetated sediments. However, more work covering a larger area is needed to confirm a generalized warming effect on sediment N2 fixation."

Technical corrections:

RC 26: Although I am not a native English speaker, I think the MS would benefit from a further English revision (e.g. Lines 124-125: please be consistent with the use of the past tense in the methods description);

AC 26: Following the reviewer comment, the manuscript will go through an English native speaker check.

RC 27: Lines 238-239: correct the citation Agawin et al.

AC 27: We thank the reviewer for pointing this typo and we will amend the text accordingly.

RC 28: Figure 2: in my opinion, such results should be presented as in Figure 3 (i.e., temperature values in x-axis and distinct lines representing different sediment types);

AC 28: Our aim was to show differences between sediment types at different temperature ranges according to the forecasted warming by the end of the century. The XY plot showing the relation of N fixation rates and temperature is actually shown in figure 3. Therefore, we believe that our message will be clearer showing the results in the current way than repeating the XY plot.

RC 29: Figure 3: also present the results from distinct vegetation types (maybe panel A and B in the figure?)

AC 29: Following the reviewer comment we will add the rates measured for each sediment type at each temperature on Figure 3.

RC 30: Figures 2 and 3: please insert in the legend whether results refer to dry weight or bulk density. Insert N values. Please highlight the meaning of the asterisks. Some redundant information (e.g., ": : :under RCP6.0 scenario") can be removed. Explain the boxplots (mean, quartiles, etc).

AC 30: We thank the reviewer for his/her input and in the reviewed manuscript we will address these technical corrections.

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