Neus Garcias-Bonet, Ph.D.
King Abdullah University of Science and Technology
Red Sea Research Center
Saudi Arabia

Dr Clare Woulds

Associate Editor, *Biogeosciences*

Dear Dr Clare Woulds,

We are pleased to submit the reviewed manuscript entitled "Warming effect on nitrogen fixation in Mediterranean macrophyte sediments" authored by N. Garcias-Bonet, R. Vaquer-Sunyer, C.M. Duarte, and N. Marbà.

We have constructively addressed all comments raised by the referees. Specifically, these are the relevant changes made in the manuscript:

- we reinforced the background information on the ecological role and the effect of warming on the three macrophyte species in the introduction section.

- we provided additional information in the Materials and Methods section.

- we discussed the limitations of our study.

- we moved former Fig 1 and Tables 1 and 2 to the supplementary materials.

In the following pages, we include the response to all reviewers' comments and the action taken in order to address them.

We thank the reviewers for their input which has helped improve the manuscript.

Sincerely,

Neus Garcias-Bonet

On behalf of all coauthors

Referee #1: C. B. de los Santos

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.

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 33°C. 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 31°C is expected to increase the N2 fixation rates in the sediment of marine macrophytes, but above 33°C 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 of carefully interpret our results.

In the revised version of the manuscript, we 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 discuss the synergic or antagonistic effects with other environmental drivers as well as a possible adaptation to warming.

The new text reads as follow: "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 CO_2 emissions by increased remineralization of C stocks (Arias-Ortiz et al., 2018).

The thermal dependence of N_2 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 N_2 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, N_2 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 N_2 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 N_2 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 N_2 fixation in sediments, we provided a detailed background of published literature regarding N_2 fixation in Mediterranean seagrasses in the introduction. Therefore, we highlight the importance of N_2 fixation in this seagrass species by mentioning the already reported rates of N_2 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 clarify the sediment sampling and we improve clarity on how the Acetylene Reduction Assay was performed to avoid any confusion.

The new text 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 expand the introduction focusing on the effect of warming on the other macrophytes.

In the reviewed manuscript we have included 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). 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 N_2 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 clearly state our hypothesis

The new text reads as follow: "Here, we test the hypothesis that N_2 fixation rates in coastal ecosystems is temperature dependent and will increase with the forecasted warming. We do so by experimentally assessing the response N_2 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 the reviewed manuscript we have included detailed information on the sediment sampling in the methods section. The new text reads 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 have indicated 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 °C treatments as the range of the projected summer mean SST by end of the century calculated by adding the 2.8 ± 1.1 °C 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.

Moreover, following the advice of the second reviewer we have 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 have added 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 clearly indicated 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 have amended 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. Moreover, following the advice of reviewer 2, we have updated 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 reviewed manuscript, we acknowledged that our results are limited to one location where the three macrophytes coexisted and therefore our conclusions need to be carefully put in perspective. Similarly,

we discuss the synergic or antagonistic effects with other environmental drivers as well as a possible adaptation to warming.

The new text reads 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 CO_2 emissions by increased remineralization of C stocks (Arias-Ortiz et al., 2018).

The thermal dependence of N_2 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 N_2 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, N_2 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 N_2 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 have included the reduction in N fixation rates at 33 ºC in the vegetated sediments and 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 have amended all the highlighted typos and added 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. We specified 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.

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: In the reviewed manuscript, we have described the Q10 rate and included its formula.

The text reads as follow: "The Q_{10} was calculated using the following equation (Raven and Geider, 1988):

$$Q_{10} = e^{\left(\frac{10\,Ea}{RT^2}\right)}$$

where R is the gas constant (8.314472 $mol^{-1}K^{-1}$), T is the mean absolute temperature across the range over which Q_{10} was measured (K), and *Ea* is the activation energy (J mol^{-1})."

Anonymous Referee #2:

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 presentation. 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 have added the requested information in the reviewed manuscript.

Specifically we included 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 CO_2 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 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 micro- and macro-fauna. Following the advice of the reviewer, we have added relevant information regarding the sediment sampling and provided available data on shoot density estimates for *P. oceanica* and *C. nodosa* in the study site.

The new text reads 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 moved Fig 1 and Tables 1 and 2 to supplementary material

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 SST_{max}) and type of sediment (as a categorical explanatory variable with 2 levels: vegetated and bare sediments) on log transformed N_2 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 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 N_2 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 N_2 fixation in sediments, we provided a detailed background of published literature regarding N_2 fixation in Mediterranean seagrasses in the introduction. Therefore, we highlight the importance of N_2 fixation in this seagrass species by mentioning the already reported rates of N_2 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 have clarified the sediment sampling and we have improved clarity on how the Acetylene Reduction Assay was performed to avoid any confusion.

The new text reads 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 have restructured 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 acknowledged the limitation of the study in the discussion of the reviewed manuscript.

The new text reads as follow: "The thermal dependence of N_2 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 N_2 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, N_2 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 N_2 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 shouldnot be expressed in mg N m-3?

AC 11: We report N_2 fixation rates as the transformation of atmospheric N_2 into NH_3, 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 SST_{max}) and type of sediment (as a categorical explanatory variable with 2 levels: vegetated and bare sediments) on log transformed N_2 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 clarified 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 reviewed manuscript we have expanded on Q10 and how it was calculated.

The new text reads as follow: "The Q_{10} was calculated using the following equation (Raven and Geider, 1988):

$$Q_{10} = e^{\left(\frac{10\,Ea}{RT^2}\right)}$$

where R is the gas constant ($8.314472\,mol^{-1}K^{-1}$), T is the mean absolute temperature across the range over which Q_{10} was measured (K), and Ea is the activation energy ($J\,mol^{-1}$)."

RC 14: Line 192: Table 3 instead of 2;
AC 14: We amended the text.

RC 15: Lines 195-202: could you please provide a figure?
AC 15: We have included 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 couldnot 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 ($R^2 = 0.11$ 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 (quadratic)	Second order polynomial (quadratic)
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.

The new text reads as follow: "At current summer SST (25-27 °C), N_2 fixation rates in vegetated sediments (3.15 ± 0.48 mg N m^{-2} d^{-1}) were significantly higher (U= 13, p < 0.01) than those in bare sediments (1.14 ± 0.3 mg N m^{-2} d^{-1}) (Fig. 1A). Similarly, at projected summer mean SST (29-31 °C), N_2 fixation rates in vegetated sediments (5.25 ± 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 ± 0.2 mg N m^{-2} d^{-1}) (Fig. 1B)."

RC 19: Lines 227-233: please revise this sentence;
AC 19: We shortened 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 N_2 fixation rates between our work and previously reported rates for the same seagrass species might reflect spatial variability in N_2 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 have enriched the discussion accordingly.
In the reviewed manuscript we have included 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 CO_2 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 the reviewed manuscript we improved the readability of this sentence.

The new text reads as follow: "We experimentally demonstrate that N_2 fixation in coastal sediments is thermal dependent, both in vegetated and bare sediments. Despite a formal experimental demonstration was lacking, the N_2 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 discussed the potential effect of warming on other bioogechemical processes.
The new text reads as follow: " 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 CO_2 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 N_2 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.
The conclusion section in the reviewed manuscript reads as follow: "Mediterranean macrophyte meadows are sites of intense N_2 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 N_2 fixation rates, with future warming leading to further increase in N_2 fixation rates up to 33 °C in bare sediments and 31 °C 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 N_2 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: We followed the reviewer advice.

RC 27: Lines 238-239: correct the citation Agawin et al.
AC 27: We thank the reviewer for pointing this typo and we have amended 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 have added 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: In the reviewed manuscript we have addressed all these technical corrections.

1 **Warming effect on nitrogen fixation in Mediterranean macrophyte sediments**

3 Garcias-Bonet, Neus[1]*; Vaquer-Sunyer, Raquel[2]; Duarte, Carlos M.[1] and Marbà, Núria[2]

5 [1] King Abdullah University of Science and Technology, Red Sea Research Center, Thuwal

6 23955-6900, Saudi Arabia

7 [2] Institut Mediterrani d´Estudis Avançats, IMEDEA (CSIC–UIB), Department of Global Change

8 Research, 07190 Esporles, Mallorca, Spain

11 ***corresponding author**: Garcias-Bonet, Neus.

12 Red Sea Research Center, Division of Biological and Environmental Sciences and Engineering,

13 King Abdullah University of Science and Technology (KAUST), 23955-6900, Saudi Arabia.

14 Phone: +966 (012) 8082564.

15 E-mail: neus.garciasbonet@kaust.edu.sa

17 **Running head:** Nitrogen fixation in Mediterranean macrophytes

19 **Key words:** Nitrogen fixation, Coastal sediments, Macrophytes, Seagrasses, Macroalgae,

20 Warming, Thermal dependence, Activation Energy, Q_{10}, Mediterranean Sea.

Abstract

The Mediterranean Sea is warming faster than the global Ocean, with important consequences for organisms and biogeochemical cycles. Warming is a major stressor for key marine benthic macrophytes. However, the effect of warming on marine N_2 fixation remains unknown, despite the high productivity of macrophytes in oligotrophic waters is partially sustained by the input of new nitrogen (N) into the system by N_2 fixation. Here, we assess the impact of warming on N_2 fixation rates of three key marine macrophytes: *Posidonia oceanica*, *Cymodocea nodosa*, and *Caulerpa prolifera*. We experimentally measured N_2 fixation rates in vegetated and bare sediments at temperatures encompassing current summer mean (25 and 27 ºC), projected summer mean (29 and 31 ºC) and projected summer maximum (33 ºC) seawater surface temperatures (SST) by the end of the century under a scenario of moderate greenhouse-gas emissions. We found that N_2 fixation rates in vegetated sediments were 2.8-fold higher than in bare sediments at current summer mean SST, with no differences among macrophytes. Currently, the contribution of N_2 fixation to macrophytes productivity could account for up to 7 %, 13.8 % and 1.8 % of N requirements for *P. oceanica*, *C. nodosa,* and *C. prolifera*, respectively. We showed the temperature dependence of sediment N_2 fixation rates. However, the thermal response differed for vegetated sediments, where rates showed an optimum at 31 ºC followed by a sharp decrease at 33 ºC, and bare sediments, where rates increased along the range of the experimental temperatures. The activation energy and Q_{10} were lower in vegetated than bare sediments, indicating the lower thermal sensitivity of vegetated sediments. The projected warming is expected to increase the contribution of N_2 fixation to Mediterranean macrophytes' productivity. Therefore, the thermal dependence of N_2 fixation might have important consequences for primary production in coastal ecosystems in the context of warming.

1. Introduction

Global mean surface temperatures have increased 0.85 °C from 1880 to 2012 and are projected to increase between 1 and 3.5 °C by the end of the 21st century, relative to pre-industrial times (IPCC, 2013). Similarly, heatwaves are more frequent since the second half of the 20th century in Europe, Asia and Australia (IPCC, 2013;Perkins et al., 2012). Oceans store most of the accumulated heat in the biosphere, warming at an average rate of 0.11 °C per decade at surface (up to 75 m depth) since 1970 (IPCC, 2013), with longer and more frequent marine heatwaves over the last century (Oliver et al., 2018). Warming is larger in small and enclosed basins such as the Mediterranean Sea (Vargas-Yáñez et al., 2008), which is warming at 0.61 °C per decade (Belkin, 2009), with rapid migration of marine isotherms (Burrows et al., 2011) and increased thermal extremes (Diffenbaugh et al., 2007). Specifically, the maximum surface seawater temperature (SST_{max}) in the Balearic Islands, in the western Mediterranean Sea, is projected to increase by 3.4 ± 1.3 °C by 2100 under a scenario of moderate greenhouse-gas (GHG) emissions (A1B scenario from the IPCC Special Report on Emissions Scenarios, equivalent to the RCP6.0 scenario of the IPCC Fifth Assessment Report) (Jordà et al., 2012), with important consequences for marine organisms and ecosystems.

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 relevant 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

67 *Caulerpa prolifera*, form highly productive ecosystems contributing to the atmospheric CO_2

68 sequestration (Duarte, 2017). However, these coastal vegetated ecosystems are threatened by

69 climate change at global scale (Duarte et al., 2018) and at the Mediterranean Sea scale (Marbà et

70 al., 2015). In particular, warming increases the mortality rates of *P. oceanica* (Marba and Duarte,

71 2010), which is predicted to be functional extinct by 2049 to 2100 due to warming (Chefaoui et

72 al., 2018;Jordà et al., 2012). Mesocosm experiments showed that *C. nodosa* is more resistant to

73 warming than *P. oceanica* (Olsen et al., 2012), concurrent with thermal niche models (Chefaoui

74 et al., 2016;Chefaoui et al., 2018); however a loss of 46.5 % in *C. nodosa* extension is predicted

75 by 2100 under the worst-case warming scenario (Chefaoui et al., 2018). Although *C. prolifera*

76 thrives well in warm waters, its photosynthesis is inhibited at temperatures above 30 ºC (Lloret et

77 al., 2008;Vaquer-Sunyer and Duarte, 2013), compromising its survival at temperatures above this

78 threshold.

79   Warming also affects metabolic processes driving biogeochemical cycles in coastal

80 benthic ecosystems. Warming enhances sediment sulfate reduction rates (Robador et al., 2016),

81 leading to an increase in sulfide accumulation in coastal bare sediments (Sanz-Lázaro et al.,

82 2011) and seagrass colonized sediments (Koch et al., 2007). In the Mediterranean Sea,

83 heatwaves and warming trigger sulfide intrusion in *P. oceanica* shoots (García et al., 2013),

84 which has toxic effects on plant meristems (Garcias-Bonet et al., 2008) and increases shoot

85 mortality (Calleja et al., 2007). Sanz-Lázaro et al. (2011) found that warming enhances sediment

86 oxygen uptake and CO_2 emissions in coastal sediments, boosted by the addition of labile organic

87 matter, in a mesocosm experiment. Similarly, warming together with eutrophication have been

88 identified as main drivers of hypoxia in a Mediterranean macroalgae *Caulerpa prolifera* meadow

89 (Vaquer-Sunyer et al., 2012). However, studies on the effect of warming on atmospheric

90 nitrogen fixation in coastal marine ecosystems are lacking.

91   Nitrogen (N_2) fixation plays a fundamental role in balancing nutrient budgets at the basin

92 scale in the Mediterranean Sea, with most of this N_2 fixation associated with *P. oceanica*

93 seagrass meadows (Béthoux and Copin-Montégut, 1986). Indeed, endophytic nitrogen-fixing

94 bacteria have been detected in roots of *P. oceanica* (Garcias-Bonet et al., 2012;Garcias-Bonet et

95 al., 2016) and N_2 fixation has been reported in leaves and roots of *P. oceanica* (Agawin et al.,

96 2016;Lehnen et al., 2016) and *in situ* incubations (Agawin et al., 2017). Therefore, the high

97 productivity of *P. oceanica* in the oligotrophic Mediterranean waters is partially supported by N_2

98 fixation. However, the magnitude of N_2 fixation rates in the rhizosphere of *P. oceanica* is still

99 unknown, as well as N_2 fixation associated to other key macrophytes commonly found in the

100 Mediterranean Sea, such as *C. nodosa* and *C. prolifera*. In addition, whether warming will affect

101 N_2 fixation rates is still unknown.

102   Here, we test the hypothesis that N_2 fixation rates in coastal ecosystems is temperature

103 dependent and will increase with the forecasted warming. We do so by experimentally assessing

104 the response of N_2 fixation rates in coastal Mediterranean vegetated ecosystems to warming. We

105 focus specifically on the key macrophyte species most commonly found in the Mediterranean

106 Sea: two seagrass species (*P. oceanica*, *C. nodosa*) and one green macroalgae species (*C.*

107 *prolifera*). We experimentally measured N_2 fixation rates in vegetated and bare sediments at

108 temperatures encompassing current summer mean SST range (25 and 27 ºC), projected summer

109 mean SST range (29 and 31 ºC), and projected summer SST_{max} (33 ºC) by the end of the century

110 under a scenario of moderate GHG emissions to assess: i) differences between vegetated and

111 bare sediments, ii) differences among macrophyte species and iii) the thermal dependence of

112 sediment N_2 fixation rates.

113

114 **2. Materials and methods**

115

116 **2.1. Study site**

117 The study was conducted with benthic communities sampled in Pollença Bay (Mallorca,

118 Spain), a bay located in the western Mediterranean Sea (39° 53.792' N; 3° 5.523' E). The study

119 site was selected based on the coexistence of the three most commonly found macrophyte

120 species in the region, including two seagrasses (*Posidonia oceanica* and *Cymodocea nodosa*) and

121 one green macroalgae (*Caulerpa prolifera*). The three macrophytes grow close to each other in

122 monospecific patches at 5-m depth. Mean (± SE) shoot density estimates were 699 ± 444 and

123 604 ± 136 shoot m^{-2} for *P. oceanica* and *C. nodosa*, respectively (Marbà and Vaquer-Sunyer,

124 unpublished). The study was conducted in mid-June 2017 when *in situ* daily mean (±SE) SST

125 was 26.4 ± 0.08 °C.

126 We sampled sediment colonized by these three macrophytes and the adjacent bare

127 sediment using sediment cores (50 cm length and 4.5 cm in diameter). We collected 16 sediment

128 cores for each type of sediment. The vegetated sediment cores were collected from the center of

129 the macrophyte patches between shoots or blades, collecting belowground plant material but

130 avoiding the collection of aboveground biomass. The bare sediment cores were collected about 5

131 m away from the edge of the vegetated patches. We collected the sediment samples by pushing

132 the cores down into the sediment with the help of a rubber hammer and carefully extracting at

133 least 15 cm of undisturbed top sediment. The cores were transported immediately to the

134 laboratory. We measured seawater salinity using a calibrated conductivity meter (ProfiLine Cond

135 3310, WTW®, USA) and summer SST was monitored and recorded *in situ* every 2 hours from

136 2012 to 2017 using a Hobo logger (Onset Computer Corporation®, MA, USA). Full summer

137 SST records are available for 2012, 2013, 2106, and 2017. No data is available for 2015, and

138 only partial temporal coverage is available for 2014 (Fig. S1). The summer mean (±SE) SST

139 varied from 26.29 ± 0.05 °C in 2013 to 27.03 ± 0.04 °C in 2017, with an average summer mean

140 SST of 26.54 ± 0.17 °C from 2012 to 2017. Average summer minimum and maximum SST were

141 22.92 and 29.08 °C, respectively. The highest maximum SST was 29.67 °C and was registered in

142 August 2017 (Table S1).

143

144 **2.2. Nitrogen fixation rates**

145   We measured sediment N_2 fixation rates by the Acetylene Reduction Assay (Capone and

146 Taylor, 1980) in *P. oceanica*, *C. nodosa,* and *C. prolifera* vegetated sediments and the adjacent

147 bare sediment at five incubation temperatures: 25, 27, 29, 31, and 33 °C. The 25 and 27 °C

148 temperature treatments represent the current summer mean SST, covering the *in situ* recorded

149 average summer mean SST of 26.54 ± 0.2 °C (25 % percentile = 25.81 °C and 75 % percentile =

150 27.61 °C) from 2012 to 2017. The 29 and 31 °C temperature treatments represent the range of the

151 projected summer mean SST by the end of the century under a scenario of moderate GHG

152 emissions equivalent to RCP6.0, applying the projected mean SST increase of 2.8 ± 1.1 °C in the

153 region (Jordà et al., 2012) over the summer mean SST registered in 2017 (27.03 °C) . The 33 °C

154 temperature treatment represents the projected summer SST_{max} by the end of the century under a

155 scenario of moderate GHG emissions equivalent to RCP6.0, by applying the projected SST_{max}

156 increase of 3.4 ± 1.3 °C (Jordà et al., 2012) over the summer SST_{max} of 29.67 °C already

157 recorded in 2017. The sediment incubations were run in five water baths (i.e. one per

158 temperature treatment) equipped with thermometers and heaters located in a stable temperature

159 room. The target temperature for each water bath was maintained using an IKS-AQUASTAR

160 system which controlled and recorded the temperature every 10 minutes. During the incubations,

161 the temperature oscillation around the target temperatures ranged from 0.3 to 0.7 °C, and the

162 temperature accuracy was ± 0.05 °C on average (Table S2).

163 Once in the laboratory, the sediment from the cores was extruded carefully using a

164 plunger, and the first 10 cm of sediment below surface were collected, the rest of the sediment in

165 the core was discarded. For each replicate, 80 ml of sediment together with the belowground

166 biomass present was placed in a 500 ml glass bottle. No aboveground biomass was included in

167 the incubation glasses. Then, we added 200 ml of autoclaved seawater and the bottles were

168 closed with a lid fitted with a gas-tight valve. Finally, we added 20 ml of acetylene-saturated

169 seawater through the gas-tight valve of each bottle in order to achieve a final acetylene

170 concentration of 4 mM. The acetylene-saturated seawater was prepared according to Wilson et

171 al. (2012). We ran the sediment incubations in triplicate for each type of sediment and each

172 temperature treatment under dark conditions. The incubations lasted 24h, starting after the

173 addition of acetylene-saturated seawater. We sampled the headspace five times: at the start of the

174 experiment and at 12, 17, 20, and 24 h since the onset of the experiment. Specifically, we

175 withdrew 3 ml of air from the headspace with a gas-tight syringe. The headspace air sample was

176 immediately injected into a 3 ml vacuum vial for further analysis of ethylene concentration on a

177 gas chromatographer equipped with a flame ionization detector (FID-GC, Agilent 5890) using a

178 PoraPLOT U GC column (25 m × 0.53 mm × 20 μm, Agilent Technologies, USA). We built a

179 calibration curve using three ethylene standards of known concentration (1.02, 10.13 and 99.7

180 ppm) and Helium as a balance gas, supplied by Carburos Metálicos S.A. (Palma de Mallorca,

181 Spain). We estimated the concentration of dissolved ethylene, from the ethylene concentration in

182 the equilibrated air as described previously (Wilson et al., 2012) and applying the solubility

183 coefficient of ethylene according to Breitbarth et al. (Breitbarth et al., 2004) as a function of

184 temperature and salinity.

185   We ran negative controls consisting in sediment without addition of acetylene-saturated

186 seawater in order to confirm that ethylene was not naturally produced by our samples, and

187 autoclaved seawater used in the preparation of the incubations with addition of acetylene-

188 saturated seawater in order to confirm that ethylene was not produced in the seawater. No

189 ethylene was produced in any the negative controls. The ethylene production rates were

190 converted into N_2 fixation rates by applying the common ratio of 3 mol of acetylene:1 mol of N_2

191 (Welsh, 2000).

192   At the end of the incubation, we dried the sediment samples at 60 ºC and recorded the dry

193 weight for further calculations. Moreover, we calculated the sediment organic matter (OM)

194 content of each replicate sediment sample by loss on ignition (Dean Jr, 1974). The sediment N_2

195 fixation rates were first calculated by sediment dry weight and then standardized to surface area

196 integrated over 10 cm sediment depth by taking into account the sediment bulk density.

197

198 **2.3. Statistical analysis**

199   Differences in sediment OM content and bulk density among *P. oceanica*, *C. nodosa,* and

200 *C. prolifera* vegetated sediments and bare sediment were tested with the non-parametric Kruskal

201 Wallis test. Differences in sediment N_2 fixation rates among the four types of sediment (*P.*

202 *oceanica*, *C. nodosa,* and *C. prolifera* vegetated sediments and bare sediment) were tested by

203   Friedman test matching by temperature treatment. Then, we tested the effect of temperature (as a

204   categorical explanatory variable with 3 levels: current summer mean SST range (25 and 27 °C),

205   projected summer mean SST range (29 and 31 °C), and projected summer SST_{max} (33 °C)) and

206   type of sediment (as a categorical explanatory variable with 2 levels: vegetated and bare

207   sediments) on sediment N_2 fixation rates (our response variable) after a log transformation to

208   meet normality requirements by a full factorial two-way ANOVA. Finally, differences in

209   sediment N_2 fixation rates between vegetated and bare sediments were tested by non-parametric

210   Mann-Whitney U test at three different temperature ranges: current summer mean SST range (25

211   and 27 °C), projected summer mean SST range (29 and 31 °C), and projected summer SST_{max}

212   (33 °C). Moreover, we tested the thermal dependence of sediment N_2 fixation rates in vegetated

213   and bare sediments by fitting the Arrhenius function to estimate the activation energy (Ea),

214   derived from the linear regression between the natural logarithm of N_2 fixation rates and the

215   inverse of the temperature multiplied by the Boltzmann's constant (Dell et al., 2011), and Q_{10},

216   the relative rate of increase in N_2 fixation expected for a 10°C temperature increase (Raven and

217   Geider, 1988). The Q_{10} was calculated using the following equation (Raven and Geider, 1988):

218
$$Q_{10} = e^{\left(\frac{10\ Ea}{RT^2}\right)}$$

219   where R is the gas constant (8.314472 $mol^{-1}K^{-1}$), T is the mean absolute temperature across the

220   range over which Q_{10} was measured (K), and Ea is the activation energy (J mol^{-1}). The activation

221   energy and Q_{10} of N_2 fixation in vegetated sediments were calculated using the increasing rates

222   measured at four temperature treatments (25, 27, 29, and 31 °C), while the declining rates

223   measured at 33 °C were not included. The full range of temperature treatments was used for bare

224   sediments since no decline was detected. All statistical analyses were performed using JMP

225   (SAS Institute Inc., USA) and PRISM (GraphPad Software Inc., USA) statistical software.

3. Results

Sediment OM content was significantly different in the sediments colonized by different macrophyte species ($\chi^2_{3,56}$ = 50.33, p < 0.0001). *Posidonia oceanica* sediments had the highest OM content (13.34 ± 0.56 %), whereas bare sediments had the lowest OM content (0.44 ± 0.50 %, Table 1). Sediment bulk density differed among sediment types ($\chi^2_{3,56}$ = 46.02, p < 0.0001, Table 1).

Average N_2 fixation rates in bare sediments was 0.06 ± 0.01 (range from 0.01 to 0.09) nmol N gDW^{-1} h^{-1}; while, average N_2 fixation rates in vegetated sediments was three-fold greater at 0.19 ± 0.03 (range from 0.05 to 0.9) nmol N gDW^{-1} h^{-1}, pooling all temperature treatments together. Within the vegetated sediments, the maximum mean N_2 fixation rate was detected in *C. prolifera* (0.22 ± 0.05 nmol N gDW^{-1} h^{-1}), whereas the minimum mean N_2 fixation rate was measured in *C. nodosa* (0.15 ± 0.04 nmol N gDW^{-1} h^{-1}). Mean N_2 fixation rate in *P. oceanica* was 0.21 ± 0.06 nmol N gDW^{-1} h^{-1}. Nitrogen fixation rates differed among the four different sediment types (i.e. bare, *P. oceanica*, *C. nodosa*, and *C. prolifera* sediments) ($\chi^2_{3,56}$ = 10.68, p = 0.005) when expressed by sediment dry weight. However, once the rates were converted into aerial basis, these differences were no longer significant ($\chi^2_{3,56}$ = 6.12, p > 0.05), due to high variability in sediment bulk densities. Sediment N_2 fixation rates were independent of OM content (linear regression, dfN = 1, dfD = 58, Pearson's r = 0.19, p > 0.05).

Nitrogen fixation rates in aerial basis were significantly higher in vegetated sediments compared to bare ones (U = 154, p < 0.002) when pooling all temperature treatments together, with sediments colonized by macrophytes supporting, on average, twice the nitrogen fixation rate than bare sediments (mean ± SE = 3.86 ± 0.53 and 1.77 ± 0.20 mg N m^{-2} d^{-1}, respectively).

 considering all temperature treatments. Temperature and type of sediment (vegetated and bare

sediments) had a significant effect on N_2 fixation rates (two-way ANOVA; sediment type $F_{1,59} =$

10.40, p < 0.01; temperature $F_{2,59} = 4.89$, p < 0.05), with no significant interaction between

them. Specifically, at current summer SST range (25-27 ºC), N_2 fixation rates in vegetated

sediments (3.15 ± 0.48 mg N m^{-2} d^{-1}) were significantly higher ($U= 13$, p < 0.01) than those in

bare sediments (1.14 ± 0.3 mg N m^{-2} d^{-1}) (Fig. 1A). Similarly, at projected summer mean SST

range (29-31 ºC), N_2 fixation rates in vegetated sediments (5.25 ± 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 ± 0.2 mg

N m^{-2} d^{-1}) (Fig. 1B). However, N_2 fixation rates did not differ between vegetated and bare

sediments at projected summer SST_{max} (33 ºC), with N_2 fixation rates of 2.49 ± 0.248 and 2.21 ±

0.15 mg N m^{-2} d^{-1}, respectively (Fig. 1C).

In vegetated sediments, N_2 fixation rates increased linearly with temperature up to 31ºC

(N_2 fixation (nmol N g DW^{-1} h^{-1}) = -0.63 + 0.03*Temperature, $R^2 = 0.11$, p < 0.05), with a

marked decrease from 0.32 ± 0.09 nmol N g DW^{-1} h^{-1} at 31 ºC to 0.11 ± 0.01 nmol N g DW^{-1} h^{-1}

at 33 ºC (Fig. 2). Nitrogen fixation rates in bare sediments increased linearly with temperature up

to 33 ºC (N_2 fixation (nmol N g DW^{-1} h^{-1}) = -0.11 + 0.01*Temperature, $R^2 = 0.51$, p < 0.005, Fig.

2). The associated activation energies were 0.91 ± 0.39 and 1.25 ± 0.39 eV for vegetated and

bare sediments, respectively (Fig. 3). Associated Q_{10} values were 3.84 ± 2.22 and 6.41 ± 2.97 for

vegetated and bare sediments, respectively.

4. Discussion

The overall average N_2 fixation rate found in Mediterranean vegetated sediments at

current summer mean SST (3.15 ± 0.48 mg N m^{-2} d^{-1}) is within the range of those rates reported

for sediments colonized by temperate seagrass species (from 1.2 to 6.5 mg N m^{-2} d^{-1} in *Zostera*

272 *marina* sediments in the North Sea (McGlathery et al., 1998) and the Northwest Atlantic coast

273 (Capone, 1982), and from 0.1 to 7.3 mg N m^{-2} d^{-1} in *Zostera noltii* in the Northeast Atlantic coast

274 (Welsh et al., 1996)). However, N$_2$ fixation rates are lower than the rates reported for tropical

275 and subtropical seagrass species (see references in Welsh, 2000). The overall N$_2$ fixation rates in

276 vegetated sediments are higher than in bare sediments, consistent with the long-recognized role

277 of marine plants in enhancing N$_2$ fixation rates (Capone, 1988). Specifically, the vegetated

278 sediments supported 3 to 4-fold higher N$_2$ fixation rates than bare sediments did at current

279 summer mean SST range when expressed by area and by sediment dry weight, respectively.

280   The N$_2$ fixation rates we measured in *P. oceanica* sediments at current summer mean

281 SST (2.86 ± 1.26 mg N m^{-2} d^{-1}) are higher than the rates reported in summer, at similar seawater

282 temperature, by Agawin et al. (2017) using benthic bell-jar chambers containing *P. oceanica*

283 shoots and the underlying sediment (ranging from 0.06 to 1.51 mg N m^{-2} d^{-1}). However, the

284 different methodological approaches make comparisons difficult: while sediment slurries might

285 slightly overestimate rates due to sediment structure disturbance and increase in organic matter

286 availability, incubation chambers might underestimate rates due to poor diffusion of acetylene

287 into the sediment (Welsh, 2000). Nevertheless, the N$_2$ fixation rates in bare sediments at current

288 summer mean SST (1.14 ± 0.31 mg N m^{-2} d^{-1}) are very similar to those measured by benthic bell-

289 jar in bare sediment adjacent to a *P. oceanica* meadow (from 0.01 to 1.99 mg N m^{-2} d^{-1}) (Agawin

290 et al., 2017), suggesting that these differences in N$_2$ fixation rates in *P. oceanica* sediment might

291 also be due to variability among sites. The N$_2$ fixation rates in *C. nodosa* and *C. prolifera*

292 sediments reported here are the first reports, to the best of our knowledge, for these two

293 important Mediterranean macrophyte species. Indeed, the analysis of sediment N stocks in a *C.*

294 *nodosa* meadow in the Mediterranean Sea suggested that N$_2$ fixation might be contributing to

295 enhance the N stocks compared to bare sediments (Pedersen et al., 1997). The similar stable N

296 isotope composition of *C. nodosa* tissues and those of *P. oceanica* in the Mediterranean

297 (Fourqurean et al., 2007) also suggests that they use similar sources of nitrogen. The N_2 fixation

298 rates at current summer mean SST in *C. prolifera* sediments found here (0.17 ± 0.04 nmol N

299 gDW^{-1} h^{-1}) are similar to the sediment N_2 fixation rates associated to the invasive *C. taxifolia* in

300 Monaco (0.12 ± 0.09 nmol N gDW^{-1} h^{-1}) but 20-fold lower than the N_2 fixation rates reported for

301 *C. taxifolia* in France (3.96 ± 1.99 nmol N gDW^{-1} h^{-1}) (Chisholm and Moulin, 2003).

302   Although the sediments colonized by these three macrophyte species hold similar rates,

303 the contribution of sediment N_2 fixation to the productivity of each plant is different. Taking into

304 account the average net production (2.63 and 1.47 g DW m^{-2} d^{-1} for *P. oceanica* and *C. nodosa*,

305 respectively (Duarte and Chiscano, 1999) and 5.16 g DW m^{-2} d^{-1} for *C. prolifera* (Marbà

306 unpublished)) and the tissue nitrogen content (from 1.55 to 1.63 % for *P. oceanica*, from 1.91 to

307 2.28 % for *C. nodosa* (Duarte, 1990;Fourqurean et al., 2007) and from 3 to 4.9 % for *C. prolifera*

308 (Morris et al., 2009)), the mean measured sediment N_2 fixation rates detected at current summer

309 mean SST (25 and 27 ºC) could account for 6.7 to 7 %, 11.5 to 13.8 % and 1.1 to 1.8 % of the

310 nitrogen requirements for *P. oceanica*, *C. nodosa,* and *C. prolifera*, respectively. The calculated

311 contribution of N_2 fixation to seagrass growth requirements fall within the range of the N_2

312 fixation contributions reported for temperate seagrasses, ranging from 5 to 12 % for *Z. marina*

313 and *Z. noltii*, respectively (Welsh, 2000). The calculated contribution of N_2 fixation to fulfill the

314 macrophytes' growth requirements points N_2 fixation as partially supporting the high

315 productivity of these primary producers in the Mediterranean oligotrophic waters.

316   We experimentally demonstrate that N_2 fixation in coastal sediments is thermal

317 dependent, both in vegetated and bare sediments. Despite a formal experimental demonstration

318   was lacking, the N$_2$ fixation thermal dependence reported here is in agreement with the higher

319   rates typically measured in warm tropical and subtropical meadows compared to the rates

320   reported in temperate and cold seagrass systems (Herbert, 1999;McGlathery, 2008;Welsh, 2000).

321   The thermal dependence, as reflected by the activation energy and Q$_{10}$, for N$_2$ fixation rates was,

322   however, higher in bare sediments than in vegetated sediments, possibly due to different

323   bacterial communities. Westrich and Berner (1988) also found that sulfate reduction exhibited a

324   more pronounced thermal dependence in sediments supporting lower rates. The activation

325   energies for N$_2$ fixation in vegetated sediments (0.91 ± 0.4 eV or 87.8 ± 37.6 KJ mol^{-1}) and in

326   bare sediments (1.25 ± 0.4 eV or 120.6 ± 37.6 KJ mol^{-1}) are within the range of the activation

327   energy reported for sediment sulfate reduction (range from 36 to 132 KJ mol^{-1} (Robador et al.,

328   2016;Westrich and Berner, 1988)) and for sediment organic matter degradation (range from 54 to

329   125 KJ mol^{-1} (Middelburg et al., 1996)). The Q$_{10}$ values associated to sediment N$_2$ fixation (3.84

330   ± 2.22 and 6.41 ± 2.97 for vegetated and bare sediments, respectively) are higher than those

331   reported for sediment sulfate reduction (from 1.6 to 3.4 (Robador et al., 2016)), but still similar

332   to those values associated to organic matter degradation (from 2.2 to 6.3 (Middelburg et al.,

333   1996)). Moreover, the thermal response differed for vegetated sediments, where N$_2$ fixation rates

334   showed an optimum at 31 °C followed by a sharp decrease at 33 °C, and bare sediments, where

335   N$_2$ fixation rates increased along the range of experimental temperatures tested here. The thermal

336   response of N$_2$ fixation in vegetated sediments found here is similar to the thermal response

337   reported for N$_2$ fixation in soil crusts (Zhou et al., 2016) and seagrass rhizosphere (Garcias-Bonet

338   et al., 2018), with an increase in rates up to 30 and 29 °C, respectively, and a marked decrease in

339   rates at temperatures above the optimum. The forecasted warming by the end of the century

340   could potentially increase N$_2$ fixation rates by 36.7% in vegetated sediments and 46.8% in bare

341 sediments. However, the decrease in N_2 fixation rates in vegetated sediments at 33 °C would

342 imply a reduction of a third in the contribution of N_2 fixation to the macrophytes' productivity

343 during heatwaves. The forecasted warming could affect as well other biogeochemical processes

344 in coastal sediments, such as sulfate reduction (Robador et al., 2016), anaerobic ammonium

345 oxidation and denitrification (Garcias-Bonet et al., 2018;Nowicki, 1994), among others, and,

346 therefore, potential synergic or antagonistic effects may occur. Responses of sediments colonized

347 by different macrophyte species may also differ due to differences in the lability of their OM and

348 nutrient stocks, associated to differences in C:N:P ratios (Enríquez et al., 1993;Lanari et al.,

349 2018). Similarly, losses of seagrass coverage by heatwaves could lead to an increase in CO_2

350 emissions by increased remineralization of C stocks (Arias-Ortiz et al., 2018).

351   The thermal dependence of N_2 fixation in vegetated sediments found here might have

352 important consequences for primary production in coastal ecosystems in the context of warming.

353 This may not be the case for *P. oceanica*, as this species is projected to be critically

354 compromised, to the extent that functional extinction is possible, with projected Mediterranean

355 warming rates by 2050-2100 (Chefaoui et al., 2018;Jordà et al., 2012). However, in order to

356 draw general conclusions on the effect of warming on N_2 fixation in coastal ecosystems, the

357 thermal-dependence found here needs be tested for a diversity of seagrass ecosystems. Similarly,

358 our results from experimental temperature treatments did not account for potential acclimation

359 and adaptation of microbial communities to warming, which should also be tested. Moreover, N_2

360 fixation is likely to be subjected to other environmental controls that may change, either in an

361 additive, synergistic or antagonistic manner, with warming, so predicting N_2 fixation rates in a

362 future, warmer coastal ocean remains challenging.

363

Conclusion

Mediterranean macrophyte meadows are sites of intense N_2 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 N_2 fixation rates, with future warming leading to further increase in N_2 fixation rates up to 33 °C in bare sediments and 31 °C 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 N_2 fixation.

Author contribution

NG-B, RVS, CMD and NM designed the study. NG-B and RVS performed the field work, and sample and data analysis. NG-B, RVS, CMD and NM interpreted the results. NG-B wrote the first draft of the manuscript. All authors contributed substantially to the final manuscript.

Conflict of interests

Authors declare that they have no conflict of interest

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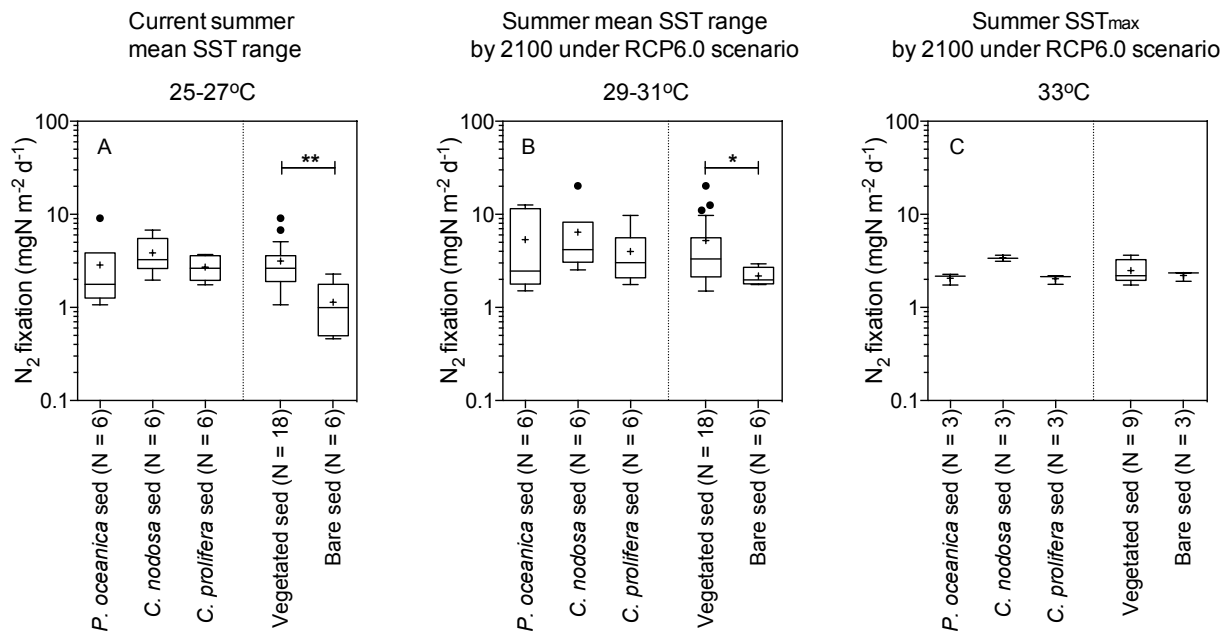
Figures

Fig. 1. Box plot of the N_2 fixation rates (expressed by area) of sediments colonized by different macrophytes (as well as grouping all vegetated sediments together) and bare sediment measured at current summer SST range (25-27 °C, (A)), projected summer mean SST range by 2100 under RCP6.0 scenario (29-31 °C, (B)), and projected summer SST_{max} by 2100 under RCP6.0 (33 °C, (C)). Boxes extend from 25th to 75th percentiles, whiskers are calculated using the inter-quartile distance (IQR) according to the Tukey method, lines inside boxes represents the median, "+" represents the mean, and dots represent individual values greater that the 75th percentile plus 1.5 X IQR. Statistically significant differences are indicated by asterisks where * indicates $p < 0.05$, and ** indicates $p < 0.01$. The sample size (N) is also indicated.

401 Fig. 2. Relationship of experimental incubation temperature and mean sediment N_2 fixation rates

402 (expressed by sediment dry weight) in vegetated (black dots) and bare sediments (clear dots).

403 Black and clear dots indicate mean values, and errors bars indicate standard error of the mean.

404 Individual replicate measurements of N_2 fixation rates for each macrophyte species are also

405 shown in colored coded dots, where green dots represent measurements on *P. oceanica*

406 sediments, blue dots represent measurements on *C. nodosa* sediments, and pink dots represent

407 measurements on *C. prolifera* sediments.

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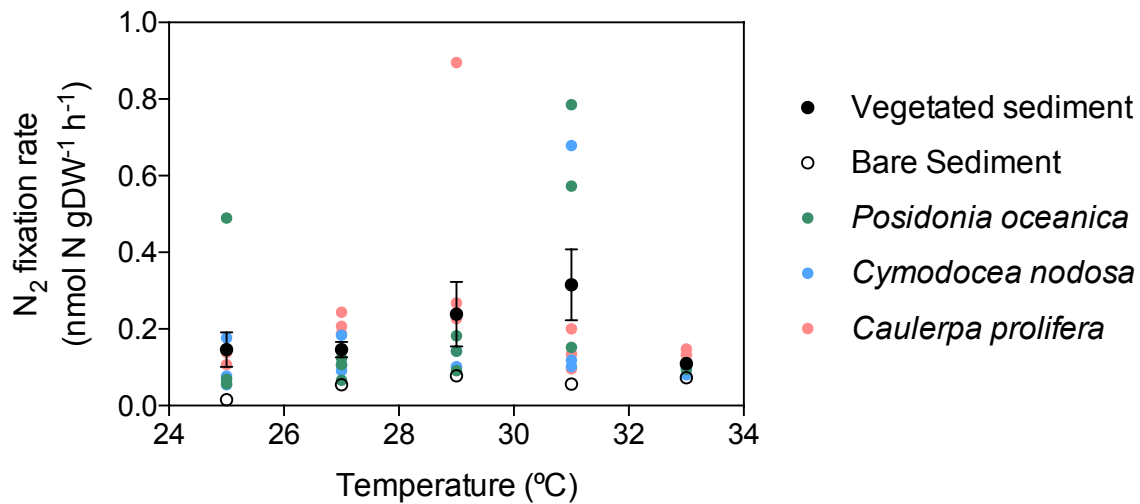
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412   Fig. 3. Arrhenius plot for N_2 fixation rates in vegetated (black dots) and bare sediments (grey

413   dots), showing the linear regression between ln N_2 fixation rates and the inverse of the

414   temperature multiplied by the Boltzmann's constant (1/kT) for vegetated (black solid line) and

415   bare (grey solid line) sediments.

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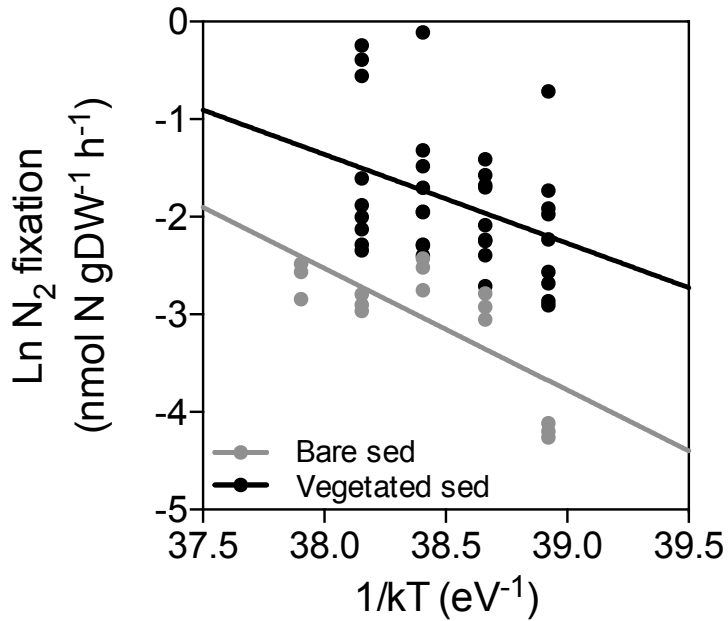

Tables

Table 1. Organic matter content and bulk density in sediments colonized by different macrophyte species and bare sediment in Pollença Bay (Mallorca) in June 2017. Mean values (±SEM), the ranges (minimum – maximum values), and the sample size (N) are shown.

	P. oceanica sediment	*C. nodosa* sediment	*C. prolifera* sediment	Bare sediment
Sediment organic matter content (%)	12.58 ± 0.95 (8.17 – 20.63) N = 15	6.14 ± 0.39 (4.49 – 10.22) N = 15	0.74 ± 0.12 (0.04 – 1.31) N = 15	0.44 ± 0.08 (0.02 – 1.41) N = 15
Sediment bulk density (g DW sed cm^{-3})	0.54 ± 0.03 (0.29 – 0.69) N = 15	1.01 ± 0.03 (0.74 – 1.24) N = 15	0.46 ± 0.02 (0.32 – 0.56) N = 15	0.96 ± 0.02 (0.84 – 1.09) N = 15

Supplementary material

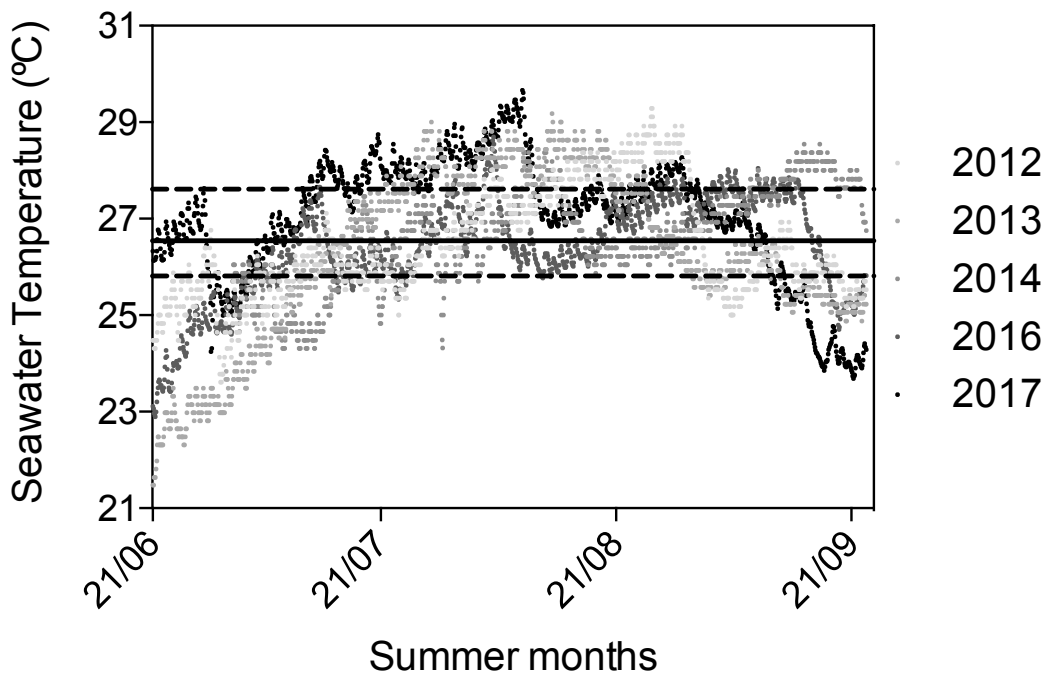
429

430 **Fig. S1.** Summer *in situ* SST (from June 21st to September 22nd) from 2012 to 2017 at the study

431 site. Solid line indicates average summer mean SST and dashed lines indicate the 25 and 75%

432 percentiles. Average summer mean SST and percentiles do not include data from 2014 due to

433 partial temporal coverage in temperature records. No data available for 2015.

434

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436

437 **Table S1.** Summary of *in situ* summer SST at the study site (Pollença Bay, Mallorca) from June

438 21st to September 22nd from 2012 to 2017, showing the mean ± SEM, minimum (Min) and

439 maximum (max), median, and 25 and 75% percentiles. Average calculated using data from 2012,

440 2013, 2016, and 2017 for which full summer records are available. Data not available for 2015

441 and partial temporal coverage available for 2014.

442

Year	Summer Surface Seawater Temperature (SST)				
	Mean (±SEM)	Min - Max	Median	25% percentile	75% percentile
2012	26.44 ± 0.03	23.61 – 29.29	26.23	25.70	27.28
2013	26.29 ± 0.05	21.48 – 29.18	26.28	25.23	27.98
2016	26.39 ± 0.03	22.90 – 28.69	26.43	25.82	27.24
2017	27.03 ± 0.04	23.69 – 29.67	27.21	26.50	27.92
Average 2012 – 2017	**26.54 ± 0.17**	**22.92 – 29.21**	**26.54**	**25.81**	**27.61**

443

444

445

446 **Table S2.** Experimental temperature set up used in the sediment incubations, indicating the five

447 target temperatures and the actual mean (±SEM) temperature recorded in each incubation water

448 bath during the 24h incubations every 10 minutes. The ranges (minimum – maximum values)

449 and number of observations (N) are shown.

450

Target temperature (ºC)	Incubation temperature (ºC)
25 ºC	25.07 ± 0.01 (24.9 – 25.2) N = 144
27 ºC	27.02 ± 0.01 (26.6 – 27.2) N = 144
29 ºC	29.05 ± 0.01 (28.8 – 29.3) N = 144
31 ºC	31.08 ± 0.01 (30.7 – 31.4) N = 144
33 ºC	33.05 ± 0.01 (32.7 – 33.2) N = 144

451

452

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