

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

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

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19   **Key words:** Nitrogen fixation, Coastal sediments, Macrophytes, Seagrasses, Macroalgae,  
20   Warming, Thermal dependence, Activation Energy, Q<sub>10</sub>, Mediterranean Sea.

21 **Abstract**

22           The Mediterranean Sea is warming faster than the global Ocean, with important  
23 consequences for organisms and biogeochemical cycles. Warming is a major stressor for key  
24 marine benthic macrophytes. However, the effect of warming on marine N<sub>2</sub> fixation remains  
25 unknown, despite the high productivity of macrophytes in oligotrophic waters is partially  
26 sustained by the input of new nitrogen (N) into the system by N<sub>2</sub> fixation. Here, we assess the  
27 impact of warming on N<sub>2</sub> fixation rates of three key marine macrophytes: *Posidonia oceanica*,  
28 *Cymodocea nodosa*, and *Caulerpa prolifera*. We experimentally measured N<sub>2</sub> fixation rates in  
29 vegetated and bare sediments at temperatures encompassing current summer mean (25 and 27  
30 °C), projected summer mean (29 and 31 °C) and projected summer maximum (33 °C) seawater  
31 surface temperatures (SST) by the end of the century under a scenario of moderate greenhouse-  
32 gas emissions. We found that N<sub>2</sub> fixation rates in vegetated sediments were 2.8-fold higher than  
33 in bare sediments at current summer mean SST, with no differences among macrophytes.  
34 Currently, the contribution of N<sub>2</sub> fixation to macrophytes productivity could account for up to 7  
35 %, 13.8 % and 1.8 % of N requirements for *P. oceanica*, *C. nodosa*, and *C. prolifera*,  
36 respectively. We showed the temperature dependence of sediment N<sub>2</sub> fixation rates. However,  
37 the thermal response differed for vegetated sediments, where rates showed an optimum at 31 °C  
38 followed by a sharp decrease at 33 °C, and bare sediments, where rates increased along the range  
39 of the experimental temperatures. The activation energy and Q<sub>10</sub> were lower in vegetated than  
40 bare sediments, indicating the lower thermal sensitivity of vegetated sediments. The projected  
41 warming is expected to increase the contribution of N<sub>2</sub> fixation to Mediterranean macrophytes'  
42 productivity. Therefore, the thermal dependence of N<sub>2</sub> fixation might have important  
43 consequences for primary production in coastal ecosystems in the context of warming.

## 44 **1. Introduction**

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

60 Seagrass ecosystems provide important ecosystem services, such as the increase in  
61 diversity, the reduction of wave action and the protection of coast, the increase in water clarity  
62 by trapping suspended particles, and climate change mitigation by acting as carbon sinks  
63 (Costanza et al., 1997; Duarte, 2017; Fourqurean et al., 2012). In the Mediterranean Sea, the most  
64 relevant seagrass species are *Posidonia oceanica*, an endemic long-living seagrass, and  
65 *Cymodocea nodosa*, commonly found in the Eastern Mediterranean Sea and the Northeastern  
66 Atlantic Coast. Similarly, benthic green macroalgae, such as the autochthonous Mediterranean

67 *Caulerpa prolifera*, form highly productive ecosystems contributing to the atmospheric CO<sub>2</sub>  
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 °C (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<sub>2</sub> 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<sub>2</sub>) fixation plays a fundamental role in balancing nutrient budgets at the basin  
92 scale in the Mediterranean Sea, with most of this N<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub>  
98 fixation. However, the magnitude of N<sub>2</sub> fixation rates in the rhizosphere of *P. oceanica* is still  
99 unknown, as well as N<sub>2</sub> 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<sub>2</sub> fixation rates is still unknown.

102 Here, we test the hypothesis that N<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> fixation rates in vegetated and bare sediments at  
108 temperatures encompassing current summer mean SST range (25 and 27 °C), projected summer  
109 mean SST range (29 and 31 °C), and projected summer SST<sub>max</sub> (33 °C) 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<sub>2</sub> 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 ( $\pm$  SE) shoot density estimates were  $699 \pm 444$  and  
123  $604 \pm 136$  shoot m<sup>-2</sup> 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 ( $\pm$ SE) SST  
125 was  $26.4 \pm 0.08$  °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 ( $\pm$ SE) SST  
139 varied from  $26.29 \pm 0.05$  °C in 2013 to  $27.03 \pm 0.04$  °C in 2017, with an average summer mean  
140 SST of  $26.54 \pm 0.17$  °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<sub>2</sub> 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  $26.54 \pm 0.2$  °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  $2.8 \pm 1.1$  °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<sub>max</sub> by the end of the century under a  
155 scenario of moderate GHG emissions equivalent to RCP6.0, by applying the projected SST<sub>max</sub>  
156 increase of  $3.4 \pm 1.3$  °C (Jordà et al., 2012) over the summer SST<sub>max</sub> 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  $\pm 0.05$  °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  $\times$  0.53 mm  $\times$  20  $\mu$ m, 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<sub>2</sub> fixation rates by applying the common ratio of 3 mol of acetylene:1 mol of N<sub>2</sub>  
191 (Welsh, 2000).

192 At the end of the incubation, we dried the sediment samples at 60 °C 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<sub>2</sub>  
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<sub>2</sub> 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<sub>max</sub> (33 °C)) and  
206 type of sediment (as a categorical explanatory variable with 2 levels: vegetated and bare  
207 sediments) on sediment N<sub>2</sub> 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<sub>2</sub> 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<sub>max</sub>  
212 (33 °C). Moreover, we tested the thermal dependence of sediment N<sub>2</sub> 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<sub>2</sub> fixation rates and the  
215 inverse of the temperature multiplied by the Boltzmann's constant (Dell et al., 2011), and Q<sub>10</sub>,  
216 the relative rate of increase in N<sub>2</sub> fixation expected for a 10°C temperature increase (Raven and  
217 Geider, 1988). The Q<sub>10</sub> was calculated using the following equation (Raven and Geider, 1988):

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

219 where R is the gas constant (8.314472 mol<sup>-1</sup>K<sup>-1</sup>), T is the mean absolute temperature across the  
220 range over which Q<sub>10</sub> was measured (K), and *Ea* is the activation energy (J mol<sup>-1</sup>). The activation  
221 energy and Q<sub>10</sub> of N<sub>2</sub> 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.

226

### 227 3. Results

228 Sediment OM content was significantly different in the sediments colonized by different  
229 macrophyte species ( $\chi^2_{3,56} = 50.33$ ,  $p < 0.0001$ ). *Posidonia oceanica* sediments had the highest  
230 OM content ( $13.34 \pm 0.56$  %), whereas bare sediments had the lowest OM content ( $0.44 \pm 0.50$   
231 %, Table 1). Sediment bulk density differed among sediment types ( $\chi^2_{3,56} = 46.02$ ,  $p < 0.0001$ ,  
232 Table 1).

233 Average  $N_2$  fixation rates in bare sediments was  $0.06 \pm 0.01$  (range from 0.01 to 0.09)  
234  $nmol\ N\ gDW^{-1}\ h^{-1}$ ; while, average  $N_2$  fixation rates in vegetated sediments was three-fold greater  
235 at  $0.19 \pm 0.03$  (range from 0.05 to 0.9)  $nmol\ N\ gDW^{-1}\ h^{-1}$ , pooling all temperature treatments  
236 together. Within the vegetated sediments, the maximum mean  $N_2$  fixation rate was detected in *C.*  
237 *prolifera* ( $0.22 \pm 0.05\ nmol\ N\ gDW^{-1}\ h^{-1}$ ), whereas the minimum mean  $N_2$  fixation rate was  
238 measured in *C. nodosa* ( $0.15 \pm 0.04\ nmol\ N\ gDW^{-1}\ h^{-1}$ ). Mean  $N_2$  fixation rate in *P. oceanica*  
239 was  $0.21 \pm 0.06\ nmol\ N\ gDW^{-1}\ h^{-1}$ . Nitrogen fixation rates differed among the four different  
240 sediment types (i.e. bare, *P. oceanica*, *C. nodosa*, and *C. prolifera* sediments) ( $\chi^2_{3,56} = 10.68$ ,  $p =$   
241  $0.005$ ) when expressed by sediment dry weight. However, once the rates were converted into  
242 aerial basis, these differences were no longer significant ( $\chi^2_{3,56} = 6.12$ ,  $p > 0.05$ ), due to high  
243 variability in sediment bulk densities. Sediment  $N_2$  fixation rates were independent of OM  
244 content (linear regression,  $dfN = 1$ ,  $dfD = 58$ , Pearson's  $r = 0.19$ ,  $p > 0.05$ ).

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

249 considering all temperature treatments. Temperature and type of sediment (vegetated and bare  
250 sediments) had a significant effect on N<sub>2</sub> fixation rates (two-way ANOVA; sediment type  $F_{1,59} =$   
251 10.40,  $p < 0.01$ ; temperature  $F_{2,59} = 4.89$ ,  $p < 0.05$ ), with no significant interaction between  
252 them. Specifically, at current summer SST range (25-27 °C), N<sub>2</sub> fixation rates in vegetated  
253 sediments ( $3.15 \pm 0.48$  mg N m<sup>-2</sup> d<sup>-1</sup>) were significantly higher ( $U = 13$ ,  $p < 0.01$ ) than those in  
254 bare sediments ( $1.14 \pm 0.3$  mg N m<sup>-2</sup> d<sup>-1</sup>) (Fig. 1A). Similarly, at projected summer mean SST  
255 range (29-31 °C), N<sub>2</sub> fixation rates in vegetated sediments ( $5.25 \pm 1.17$  mg N m<sup>-2</sup> d<sup>-1</sup>) were  
256 significantly higher ( $U = 23$ ,  $p < 0.05$ ) than the rates measured in bare sediments ( $2.18 \pm 0.2$  mg  
257 N m<sup>-2</sup> d<sup>-1</sup>) (Fig. 1B). However, N<sub>2</sub> fixation rates did not differ between vegetated and bare  
258 sediments at projected summer SST<sub>max</sub> (33 °C), with N<sub>2</sub> fixation rates of  $2.49 \pm 0.248$  and  $2.21 \pm$   
259  $0.15$  mg N m<sup>-2</sup> d<sup>-1</sup>, respectively (Fig. 1C).

260 In vegetated sediments, N<sub>2</sub> fixation rates increased linearly with temperature up to 31°C  
261 ( $N_2$  fixation (nmol N g DW<sup>-1</sup> h<sup>-1</sup>) =  $-0.63 + 0.03 * \text{Temperature}$ ,  $R^2 = 0.11$ ,  $p < 0.05$ ), with a  
262 marked decrease from  $0.32 \pm 0.09$  nmol N g DW<sup>-1</sup> h<sup>-1</sup> at 31 °C to  $0.11 \pm 0.01$  nmol N g DW<sup>-1</sup> h<sup>-1</sup>  
263 at 33 °C (Fig. 2). Nitrogen fixation rates in bare sediments increased linearly with temperature up  
264 to 33 °C ( $N_2$  fixation (nmol N g DW<sup>-1</sup> h<sup>-1</sup>) =  $-0.11 + 0.01 * \text{Temperature}$ ,  $R^2 = 0.51$ ,  $p < 0.005$ , Fig.  
265 2). The associated activation energies were  $0.91 \pm 0.39$  and  $1.25 \pm 0.39$  eV for vegetated and  
266 bare sediments, respectively (Fig. 3). Associated Q<sub>10</sub> values were  $3.84 \pm 2.22$  and  $6.41 \pm 2.97$  for  
267 vegetated and bare sediments, respectively.

#### 268 4. Discussion

269 The overall average N<sub>2</sub> fixation rate found in Mediterranean vegetated sediments at  
270 current summer mean SST ( $3.15 \pm 0.48$  mg N m<sup>-2</sup> d<sup>-1</sup>) is within the range of those rates reported  
271 for sediments colonized by temperate seagrass species (from 1.2 to 6.5 mg N m<sup>-2</sup> d<sup>-1</sup> 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<sup>-2</sup> d<sup>-1</sup> in *Zostera noltii* in the Northeast Atlantic coast  
274 (Welsh et al., 1996)). However, N<sub>2</sub> fixation rates are lower than the rates reported for tropical  
275 and subtropical seagrass species (see references in Welsh, 2000). The overall N<sub>2</sub> 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<sub>2</sub> fixation rates (Capone, 1988). Specifically, the vegetated  
278 sediments supported 3 to 4-fold higher N<sub>2</sub> 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<sub>2</sub> fixation rates we measured in *P. oceanica* sediments at current summer mean  
281 SST (2.86 ± 1.26 mg N m<sup>-2</sup> d<sup>-1</sup>) 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<sup>-2</sup> d<sup>-1</sup>). 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<sub>2</sub> fixation rates in bare sediments at current  
288 summer mean SST (1.14 ± 0.31 mg N m<sup>-2</sup> d<sup>-1</sup>) 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<sup>-2</sup> d<sup>-1</sup>) (Agawin  
290 et al., 2017), suggesting that these differences in N<sub>2</sub> fixation rates in *P. oceanica* sediment might  
291 also be due to variability among sites. The N<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> fixation  
298 rates at current summer mean SST in *C. prolifera* sediments found here ( $0.17 \pm 0.04$  nmol N  
299 gDW<sup>-1</sup> h<sup>-1</sup>) are similar to the sediment N<sub>2</sub> fixation rates associated to the invasive *C. taxifolia* in  
300 Monaco ( $0.12 \pm 0.09$  nmol N gDW<sup>-1</sup> h<sup>-1</sup>) but 20-fold lower than the N<sub>2</sub> fixation rates reported for  
301 *C. taxifolia* in France ( $3.96 \pm 1.99$  nmol N gDW<sup>-1</sup> h<sup>-1</sup>) (Chisholm and Moulin, 2003).

302         Although the sediments colonized by these three macrophyte species hold similar rates,  
303 the contribution of sediment N<sub>2</sub> 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<sup>-2</sup> d<sup>-1</sup> for *P. oceanica* and *C. nodosa*,  
305 respectively (Duarte and Chiscano, 1999) and  $5.16$  g DW m<sup>-2</sup> d<sup>-1</sup> 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<sub>2</sub> fixation rates detected at current summer  
309 mean SST (25 and 27 °C) 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<sub>2</sub> fixation to seagrass growth requirements fall within the range of the N<sub>2</sub>  
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<sub>2</sub> fixation to fulfill the  
314 macrophytes' growth requirements points N<sub>2</sub> fixation as partially supporting the high  
315 productivity of these primary producers in the Mediterranean oligotrophic waters.

316         We experimentally demonstrate that N<sub>2</sub> fixation in coastal sediments is thermal  
317 dependent, both in vegetated and bare sediments. Despite a formal experimental demonstration

318 was lacking, the N<sub>2</sub> 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<sub>10</sub>, for N<sub>2</sub> 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<sub>2</sub> fixation in vegetated sediments ( $0.91 \pm 0.4$  eV or  $87.8 \pm 37.6$  KJ mol<sup>-1</sup>) and in  
326 bare sediments ( $1.25 \pm 0.4$  eV or  $120.6 \pm 37.6$  KJ mol<sup>-1</sup>) are within the range of the activation  
327 energy reported for sediment sulfate reduction (range from 36 to 132 KJ mol<sup>-1</sup> (Robador et al.,  
328 2016;Westrich and Berner, 1988)) and for sediment organic matter degradation (range from 54 to  
329 125 KJ mol<sup>-1</sup> (Middelburg et al., 1996)). The Q<sub>10</sub> values associated to sediment N<sub>2</sub> fixation ( $3.84$   
330  $\pm 2.22$  and  $6.41 \pm 2.97$  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<sub>2</sub> fixation rates  
334 showed an optimum at 31 °C followed by a sharp decrease at 33 °C, and bare sediments, where  
335 N<sub>2</sub> fixation rates increased along the range of experimental temperatures tested here. The thermal  
336 response of N<sub>2</sub> fixation in vegetated sediments found here is similar to the thermal response  
337 reported for N<sub>2</sub> 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<sub>2</sub> fixation rates by 36.7% in vegetated sediments and 46.8% in bare

341 sediments. However, the decrease in N<sub>2</sub> fixation rates in vegetated sediments at 33 °C would  
342 imply a reduction of a third in the contribution of N<sub>2</sub> 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<sub>2</sub>  
350 emissions by increased remineralization of C stocks (Arias-Ortiz et al., 2018).

351         The thermal dependence of N<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub>  
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<sub>2</sub> fixation rates in a  
362 future, warmer coastal ocean remains challenging.

363



364 **Conclusion**

365 Mediterranean macrophyte meadows are sites of intense N<sub>2</sub> fixation rates, twice as high  
366 as those in adjacent bare sediments. As these rates increased with warming, realized warming of  
367 the Mediterranean Sea is expected to have led to enhanced sediment N<sub>2</sub> fixation rates, with future  
368 warming leading to further increase in N<sub>2</sub> fixation rates up to 33 °C in bare sediments and 31 °C  
369 followed by a decrease at higher temperatures in vegetated sediments. However, more work  
370 covering a larger area is needed to confirm a generalized warming effect on sediment N<sub>2</sub>  
371 fixation.

372

373 **Author contribution**

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

378

379 **Conflict of interests**

380 Authors declare that they have no conflict of interest

381

382 **Acknowledgements**

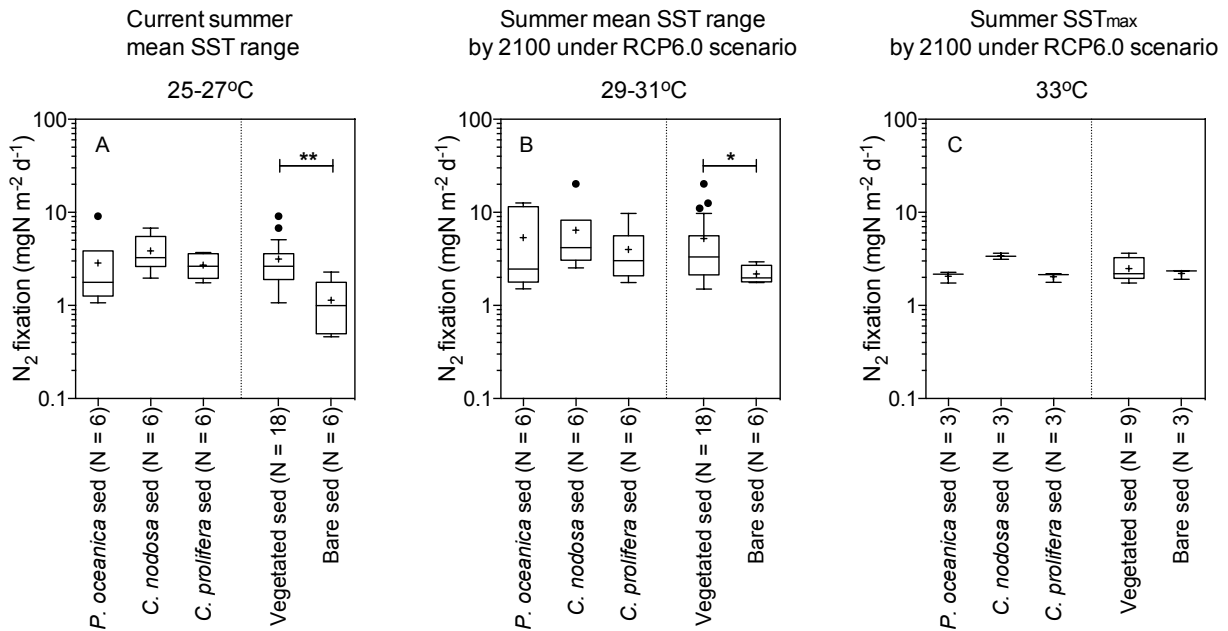
383 This work was funded by the Spanish Ministry of Economy, Industry and Competitiveness  
384 (Medshift project, CGL2015-71809-P) and base-line funding allocated by King Abdullah  
385 University of Science and Technology to CMD. We thank Carlos Alex Morell Lujan Williams

386 for field assistance and Maria Trinidad García Barceló for lab support. RVS was supported by a  
387 Juan de la Cierva incorporación contract (ref. IJCI-2015-23163).

388 **Figures**

389 Fig. 1. Box plot of the N<sub>2</sub> fixation rates (expressed by area) of sediments colonized by different  
 390 macrophytes (as well as grouping all vegetated sediments together) and bare sediment measured  
 391 at current summer SST range (25-27 °C, (A)), projected summer mean SST range by 2100 under  
 392 RCP6.0 scenario (29-31 °C, (B)), and projected summer SST<sub>max</sub> by 2100 under RCP6.0 (33 °C,  
 393 (C)). Boxes extend from 25<sup>th</sup> to 75<sup>th</sup> percentiles, whiskers are calculated using the inter-quartile  
 394 distance (IQR) according to the Tukey method, lines inside boxes represents the median, “+”  
 395 represents the mean, and dots represent individual values greater than the 75<sup>th</sup> percentile plus 1.5  
 396 X IQR. Statistically significant differences are indicated by asterisks where \* indicates p < 0.05,  
 397 and \*\* indicates p < 0.01. The sample size (N) is also indicated.

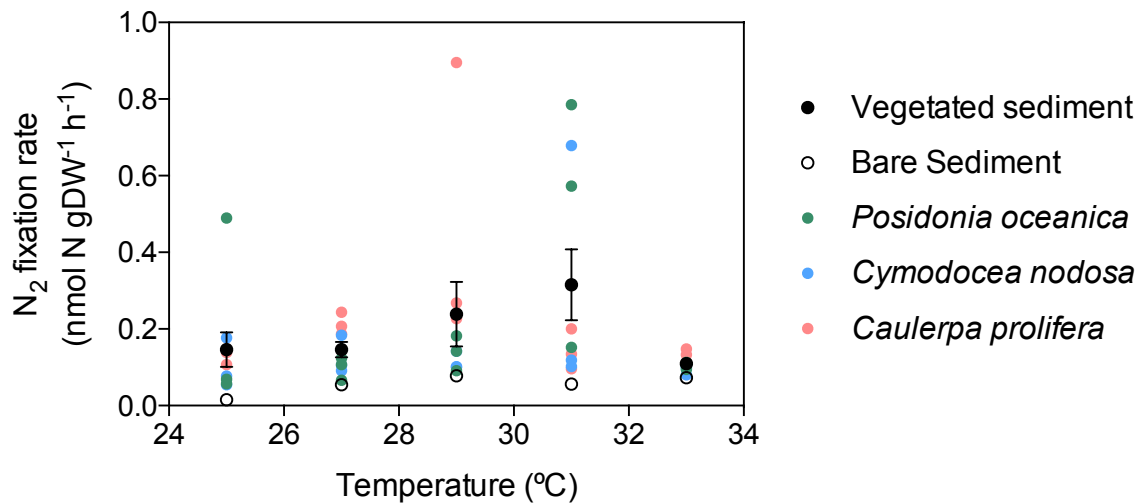
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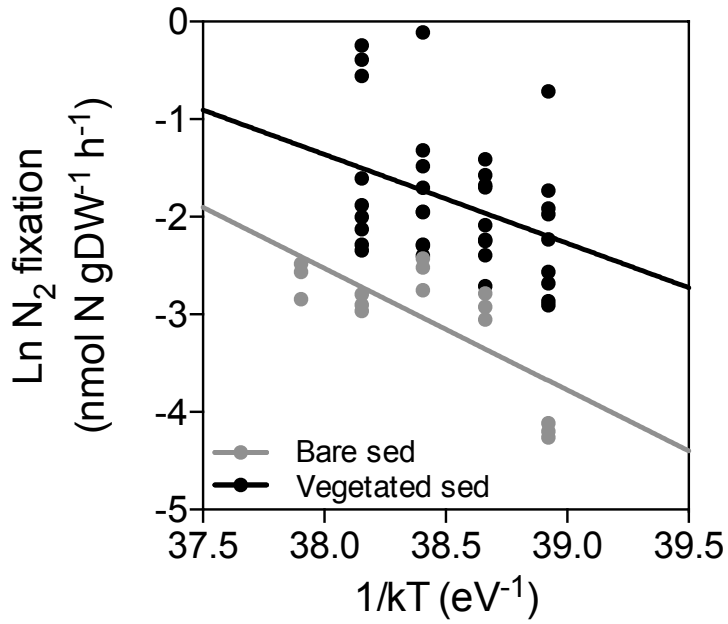
401 Fig. 2. Relationship of experimental incubation temperature and mean sediment N<sub>2</sub> fixation rates  
402 (expressed by sediment dry weight) in vegetated (black dots) and bare sediments (clear dots).  
403 Black and clear dots indicate mean values, and errors bars indicate standard error of the mean.  
404 Individual replicate measurements of N<sub>2</sub> 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.  
408



409  
410  
411

412 Fig. 3. Arrhenius plot for N<sub>2</sub> fixation rates in vegetated (black dots) and bare sediments (grey  
413 dots), showing the linear regression between ln N<sub>2</sub> 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.

416



420 **Tables**

421

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

425

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

426

427

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