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1 Title: Microbial associates of an endemic Mediterranean seagrass enhance the access of the host  
2 and the surrounding seawater to inorganic nitrogen under ocean acidification

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15

16 **Abstract**

17 Seagrasses are important primary producers in oceans worldwide. They live in shallow coastal

18 waters that are experiencing carbon dioxide enrichment and ocean acidification. *Posidonia*

19 *oceanica*, an endemic seagrass species that dominates the Mediterranean Sea, achieves high

20 abundances in seawater with relatively low concentrations of dissolved inorganic nitrogen. Here

21 we test whether microbial metabolisms associated with *P. oceanica* and surrounding seawater

22 enhance seagrass access to nitrogen. Using stable isotope enrichments of intact seagrass with

23 amino acids, we show that ammonification by free-living and seagrass-associated microbes

24 produce ammonium that is likely used by seagrass and surrounding particulate organic matter.

25 Metagenomic analysis of the epiphytic biofilm on the blades and rhizomes support the ubiquity

26 of microbial ammonification genes in this system. Further, we leveraged the presence of natural

27 carbon dioxide vents and show that microbial ammonification was reduced at lower pH. The  
28 presence of *P. oceanica* enhanced the uptake of nitrogen by water column particulate organic  
29 matter, increasing carbon fixation by a factor of 8.6 to 17.4 with the greatest effect at CO<sub>2</sub> vent  
30 sites. Seagrass and its microbial associates thus enhanced water column productivity and were a  
31 locus for nutrient cycling.

## 32 **Introduction**

33 Associations between marine species and microbes have been increasingly identified with  
34 genomic methodologies, revealing many hidden species interactions between eukaryotic and  
35 prokaryotic partners<sup>1-3</sup>. In addition to affecting host fitness, these interactions may affect global  
36 biogeochemical cycles, through the use and cycling of carbon, nitrogen and other elements<sup>4,5</sup>.

37 Seagrasses are marine angiosperms and are foundational species in coastal ecosystems that are  
38 critical in structuring ecosystems and modulating the flux of nutrients and energy. They provide  
39 habitat for other species<sup>6,7</sup>, are key primary producers<sup>8,9</sup>, and also act as carbon sinks by  
40 sequestering carbon<sup>10,11</sup>. Marine angiosperms are some of the most widely distributed plants on  
41 the planet<sup>12</sup>, and recent studies suggest they host a diversity of microbial taxa<sup>13,14</sup>, with some  
42 functional partnerships demonstrated<sup>15,16</sup>.

43 Among seagrasses, a number of microbial taxa have been identified as important to nutrient  
44 cycling, including microbes capable of nitrogen fixation<sup>15</sup>, ammonification<sup>16</sup> and sulfur  
45 oxidation<sup>13,14</sup>. *Posidonia oceanica*, the seagrass species that dominates the Mediterranean Sea,  
46 achieves high abundances in coastal habitats with relatively low seawater dissolved inorganic  
47 nitrogen concentrations. Microbial partnerships may partly explain its productivity. First, the low  
48 oxygen environment surrounding the roots provides a microenvironment for nitrogen fixation

49 <sup>15,17</sup> and other reducing metabolisms. Second, seagrasses might have access to the dissolved  
50 organic nitrogen from the surrounding seawater. A related species of *Posidonia* in Australia has  
51 microbial associates that ammonify amino acids - resulting in dissolved inorganic nitrogen that is  
52 taken up as ammonium <sup>16</sup>. Although it has been claimed that seagrasses can directly take up  
53 dissolved organic nitrogen sources (e.g <sup>18</sup>), we know of no studies that show this while  
54 controlling for the role of bacteria.

55 Bacteria that deaminate amino acids to produce ammonium are likely abundant in nature, given  
56 that there is a diversity of enzymes that cleave carbon-nitrogen bonds producing<sup>14</sup>. Whether these  
57 bacteria are consistently associated with hosts is relatively little studied, including what  
58 determines their abundance. Studies that focus only on the taxonomy of microbes (e.g. amplicon  
59 sequencing) do not allow strong inference about the functional capacity of microbes. Information  
60 on amino acids in seawater in the environments where marine macrophytes are abundant are also  
61 lacking and infrequently quantified. Nonetheless, dissolved organic compounds can be abundant  
62 in coastal waters and can be a key part of nutrient recycling <sup>19</sup>.

63 While the discovery of host-microbe interactions is still nascent, populations of many marine  
64 macrophytes are in decline <sup>6,20,21</sup>. For seagrasses, global environmental change, including ocean  
65 acidification and warming, pollution, and physical disturbances such as increased turbidity  
66 represent key threats <sup>22</sup>. Future ocean conditions will include a continued decrease of pH and a  
67 change in carbonate system parameters. While marine angiosperms such as *P. oceanica* may  
68 benefit from increased bicarbonate concentrations<sup>23</sup>, little is known about how their associations  
69 with microbes may be altered. If increased bicarbonate access improves carbon fixation and  
70 fitness, host fitness might increase and microbial associates may benefit. If the availability of

71 inorganic carbon increases while nitrogen remains the same, the release of excess carbon via  
72 stoichiometric overflow, could increase <sup>24</sup>, also perhaps benefitting host-associated microbes.  
73 However, if low pH is a stressor to the host and host health, this may be important to host-  
74 associated microbes, and microbial function and diversity might change. Microbes may also be  
75 resistant or indifferent to any changes in host biogeochemistry. The seagrass *Cymodocea nodosa*  
76 showed taxonomic differences in rhizome microbes but not leaf microbes across a pH gradient  
77 driven by the natural release of carbon dioxide (CO<sub>2</sub>) at vents in the Mediterranean <sup>25</sup>, though the  
78 functional differences were unexplored.

79 At CO<sub>2</sub> vent sites in the Mediterranean the seagrass *P. oceanica* shows a number of differences  
80 compared to areas without the influence of venting CO<sub>2</sub>. Benthic communities near vents show a  
81 decline in calcified species <sup>26,27</sup>, and accordingly, calcareous epiphytes of *P. oceanica* are much  
82 reduced in proximity to the vents <sup>8,28</sup>. Additionally, herbivorous fish are more abundant near the  
83 vents <sup>29</sup> and *P. oceanica* has a shorter stature, fewer epiphytes, increased tissue nitrogen content  
84 <sup>30</sup>, and increased shoot density <sup>28,31</sup>. Seawater pH is variable in the areas surrounding CO<sub>2</sub> vent  
85 sites and *P. oceanica* is often in pH conditions around 7.7 <sup>27</sup>. Proximity to vents can increase *P.*  
86 *oceanica* productivity at the scale of individual leaves, but shows variable results at the  
87 community level <sup>8</sup>.

88 The foundational role that *P. oceanica* plays in the Mediterranean <sup>29</sup>, its economic importance <sup>32</sup>,  
89 and concern about its health status <sup>33</sup> makes this species an important one to investigate host-  
90 associated microbial function in a global change context, particularly under ocean acidification.  
91 We thus investigated microbial function at natural CO<sub>2</sub> venting areas and reference areas with no  
92 venting and ambient pH. We focused on nitrogen dynamics, a relatively little studied aspect of

93 ocean acidification (but see <sup>34</sup>). Populations of *P. oceanica* may have spent generations in  
94 association with decreased pH, allowing tests of community interactions and functional changes,  
95 including host-microbe interactions. We quantified whether ammonification, or the ability of  
96 microbes to make dissolved organic nitrogen available as ammonium, occurred in *P. oceanica*  
97 and if it differed in areas of low pH influenced by the vents versus areas of ambient pH.  
98 Ammonification involves deamination of amino acids to liberate ammonium; we quantified  
99 ammonium production in situ with stable isotope enrichments of amino acids and in the presence  
100 of *Posidonia* plants and the surrounding seawater. We tested carbon fixation rates, dissolved  
101 organic carbon release rates, and tissue nitrogen values as possible plant traits that differed  
102 among sites. We used metagenomics to describe the microbial taxa in association with *Posidonia*  
103 blades and roots, and probe their functions, both at a site with CO<sub>2</sub> venting with low pH and at a  
104 nearby site unaffected by vent activity with ambient pH.

## 105 **Results**

106 The daylight carbon fixation rates per unit tissue mass of *P. oceanica* did not differ whether  
107 *Posidonia* was in proximity to CO<sub>2</sub> vents at Castello South (CS) or in control areas with ambient  
108 pH (Sant' Anna, SA) (p=0.646, Figure 1a, Table S3). At night, respiration rates too were  
109 indistinguishable (Figure 1a). The nitrogen content in *Posidonia* tissue did not differ between the  
110 vent and control site, either in the meristem (t=0.993, p=0.360) or midblade (t=0.517, p=0.623),  
111 and averaged 2.1% in the meristem and 1.5% in the midblade region overall (Suppl Fig 1a). The  
112 C:N ratio of *P. oceanica* tissue did not differ among sites, whether we considered the meristem  
113 tissue or the midblade underneath epiphytes (Suppl Fig 1b). Initial conditions for the incubation  
114 bottles are in Table S1; statistics and all measurements of *P. oceanica* are in Supplementary

115 Tables 2 and 3, respectively.

116 There were differences in the fate of the  $^{15}\text{N}$  amino acids that were incubated with *P. oceanica*  
117 versus seawater only, with enrichment of seawater ammonium greatest at the control site of SA  
118 and greatest when in association with *P. oceanica* (Figure 2a). The  $^{15}\text{N}$  in ammonium in seawater  
119 ( $\delta^{15}\text{N}$  of  $\text{NH}_4$ ) increased during the first 7 daylight hours ('day') and increased again overnight  
120 ('night').

121 The ammonification rate in nmol per hour across the two locales, quantified with equation (1),  
122 revealed greater ammonification rates at the site with ambient pH compared with the vent site,  
123 both in seawater and when *P. oceanica* was present (Figure 2b,  $p=0.002$ ); overall,  
124 ammonification rates were more than 4 times greater at ambient sites. *P. oceanica* was not  
125 significantly associated with greater ammonification rates than seawater (ANOVA,  $p=0.060$ ),  
126 though ammonification with seagrass was 2.5 times greater when both sites were combined.  
127 Rates did not differ between night and day ( $p=0.732$ ). Comparing the ammonification rates  
128 where *P. oceanica* was in the incubation bottles, there was no diel pattern, while seawater  
129 particulate organic matter (POM) was associated with greater ammonification during the day.

130 *P. oceanica* plants showed  $^{15}\text{N}$  enrichment in their tissues in the meristem and the midblade.  
131 Enrichments were higher on the blade under epiphytes (Table S3). While all tissue types showed  
132 a similar pattern, we relied on the very newest tissue growth in the meristem for analysis. There  
133 was almost 3 times greater uptake of  $^{15}\text{N}$ -enriched ammonium in the meristem during the day  
134 compared to night ( $p=0.002$ ), but the increase seen at the vent during the day switched at night to  
135 greater uptake in control sites (interaction between time of the experiment and locale,  $p=0.020$ ,

136 Figure 2c). Rates of ammonium uptake by *Posidonia* reached up to 6-8  $\mu\text{mol N}$  per g dry mass  
137 per hour based on estimates of  $^{15}\text{N}$  enrichment and using equation (2) (Figure 2c, Table S3).

138 The dynamics of dissolved organic nutrients was affected by *P. oceanica*, locale and time of day.  
139 Dissolved organic carbon (DOC) was elevated when *P. oceanica* was present, with a nearly  
140 significant interaction between time of day (day or night) and locale ( $p=0.059$ ) but no significant  
141 main effect of locale ( $p=0.726$ ) or time of day ( $p=0.171$ ) (Figure S2a). DON release in *P.*  
142 *oceanica* showed no difference between locales ( $p=0.580$ ) but was significantly elevated during  
143 the day when *P. oceanica* was fixing the most carbon ( $p=0.001$ , Figure S2b). The pattern of  
144 dissolved organic release in seawater only showed that DOC was greater at the vent site  
145 ( $p=0.005$ ) and greater at night ( $p=0.041$ ). DON release was also greater at the vent site, though  
146 the interaction between locale and time of day ( $p<0.001$ ) indicated that DON release was greater  
147 during the day at the vent site and greater at night at the control site ( $p<0.001$ ). DOC and DON  
148 tended to have net production during the day and net uptake at night. The use of inorganic  
149 nutrients during daylight hours is illustrated by decreased concentrations of nitrite, nitrate and  
150 silica (Figure S2).

151 The presence of *P. oceanica* was associated with increased uptake of  $^{15}\text{N}$  in POM during  
152 nighttime hours, an effect that was greatest in the control site of SA, based on the interaction  
153 between locale and treatment ( $p=0.033$ , Figure 2d). Combining the day and night interval, POM  
154  $^{15}\text{N}$  uptake was always greater with *P. oceanica* (Table S2), resulting in 1.6 times more nitrogen  
155 uptake in chambers with *P. oceanica* compared with seawater at SA, and 2.5 times higher at the  
156 CS vent site.

157 Carbon uptake by POM was also enhanced with *P. oceanica* though only during the day; POM

158 carbon uptake decreased with *P. oceanica* at night (Fig 1b). Over all estimates of POM, the C:N  
159 ratio was 5.09 at SA and 5.97 at the CS vent site (Table S3). If the carbon:nitrogen uptake by  
160 POM is relatively constant, water column carbon uptake was increased 8.6 times (1.6 x 5.09) at  
161 SA and 17.4 times (2.5 x 5.97) at the vents due to the contribution of bacteria that metabolize  
162 amino acids in association with *P. oceanica*.

### 163 Metagenome analyses

164 After quality control and filtering, we obtained an average of 33.7 million sequence reads per  
165 sample (range 28.6 to 43.8 million). Metagenomic short reads were assembled into contigs of at  
166 least 1,000 nucleotides in length, resulting in 26,460 to 123,697 contigs per sample with a mean  
167 of 68,852 across all 6 samples (Table S4). Across all the metagenomes in our study, the  
168 taxonomy of *Posidonia* blades had the greatest alpha diversity, with rhizomes at the vent having  
169 the fewest (Figure 3, Table S4), though we are precluded from statistical tests of diversity, due to  
170 lack of replication in each tissue type and locale.

171  
172 The metagenomic reads allowed us to construct metagenome-assembled genomes (MAGs),  
173 ranging from only a single MAG on rhizomes at the vent site to 21 MAGs on a blade  
174 metagenome at the control site. We used Bowers et al <sup>35</sup> criteria to define a MAG as high quality  
175 if it had a competition score of >90% and a redundancy (or contamination) of <10% (Table S5),  
176 yielding 22 high quality MAGs. A remaining 42 were medium quality MAGs that had  
177 completion scores between 42% and 90% and redundancy scores between 0% and 11% (Table  
178 S5). All MAGs were bacterial and represented 5 phyla (Figure 3a). A notable difference among  
179 blades versus rhizomes was the presence of the Cyanobacteria and Bacteroidota phyla on blades  
180 but not on rhizomes. In three cases, we were unable to taxonomically resolve a metagenome-

181 assembled genome. Taxonomic diversity at the Class and Family level was higher on rhizomes  
182 compared with blades (Figure 3b,c).

183

184 The metabolism of these metagenomes was diverse, with some functional differences (Figure 4).

185 Photosynthetic and anoxygenic photosystem II were present only on blades, an expected result

186 given the presence of Cyanobacteria only on blades. Nitrogen fixation was indicated only on

187 rhizome tissue and at the control site (Figure 4). Ammonification was a prevalent metabolism

188 across all bacterial MAGs on *P. oceanica*. Of the 67 MAGs, all had enzymes classified as

189 EC:1.4.\* or EC:3.5.\*, while 61 MAGs had enzyme function classified as EC:4.3.1\* (Table S6).

190 Nitrate reduction capability was present in the rhizome of seagrass from control areas (MAG 1

191 and MAG4), including denitrification in control rhizomes (MAG4).

192

193 Other microbial metabolisms that could be beneficial to *P. oceanica* included the presence of B

194 vitamin metabolism on both tissue types at both vent and control areas. Vitamin B6 (pyridoxal)

195 biosynthesis was only present in control rhizomes, while thiamin biosynthesis, vitamin B1, was

196 present in all samples except vent rhizomes and vitamin B7 (biotin) biosynthesis was in multiple

197 MAGS in both tissue types and locales. Vitamin B12 (cobalamin) synthesis was suggested in

198 multiple MAGs at both sites, with aerobic biosynthesis only on the blades and anaerobic

199 biosynthesis suggested in MAGs from all samples. Multiple MAGs on blades and rhizomes at

200 both locales had sulfur oxidizing capability.

201

## 202 **DISCUSSION**

203 *Posidonia* in proximity to CO<sub>2</sub> vents with low pH did not differ in carbon fixation rates from

204 those that were in ambient pH areas. Carbon fixation rates were similar to the rates estimated at  
205 the same location previously<sup>8</sup> for blades without epiphytes. The NPP values reported by  
206 Berlinghof et al<sup>8</sup> in  $\mu\text{mol O}_2$  convert to  $\sim 0.7$  to  $0.9$  mg C per g dry mass per hour, assuming a  
207 photosynthetic quotient of 1.0 (a 1:1 molar ratio of oxygen release to carbon uptake), a range  
208 nearly identical to what we estimated, though they show increased net primary production in  
209 proximity to vents. Here, our estimates of primary production included the rhizome tissue which  
210 may have obscured carbon fixation differences due to respiration. Ambient DIN values were  
211 similar between the two sites at the single timepoint when we estimated them and previously  
212 published values indicate also cite similar low nitrate concentrations regardless of venting that  
213 are  $<1 \mu\text{M}$ <sup>36,37</sup>. Estimates of ammonification were greater in control areas, with microbes at both  
214 ambient and vent sites capable of increasing nitrogen access locally to the host. Thus, despite  
215 differences in plant morphology and grazing pressure, *P. oceanica* productivity was similar.  
216 Water column processes did differ between ambient and vent area, possibly due to microbial  
217 nitrogen processing.

218 The presence of seagrass enhanced water column nitrogen uptake to 2.5 times at the vent site and  
219 1.6 times at the ambient site when we integrated over both day and night intervals (Figure 2d).  
220 With a POM C:N ratio was 5.09 at SA and 5.97 at the CS vent site (Table S3), this yields 8.6  
221 times more carbon uptake at the control site and 17.4 times more carbon at the vent site due to  
222 the contribution of bacteria that metabolize amino acids in association with *P. oceanica*. Direct  
223 measurements of carbon uptake by POM further indicated increased carbon fixation, but only  
224 during the day (Figure 1b). A possible reason for enhanced POM carbon and nitrogen uptake is  
225 the increased dissolved organics available to heterotrophic bacteria when POC is present (Figure  
226 S2). Nitrogen cycling in association with *P. oceanica* has been shown previously to be elevated

227 near vents <sup>34</sup>.

228 Benthic-pelagic coupling and enhancement of pelagic productivity through the host-associated  
229 microbes on *P. oceanica* have implications due to global declines in seagrass cover <sup>38</sup> and the  
230 prevalence of seagrass wasting diseases <sup>39</sup>. Marine macrophytes have increasingly been shown to  
231 host a species rich microbiome <sup>40</sup>, with diverse functions that include not only ammonification,  
232 but also nitrogen fixation <sup>8,34,41</sup>, nitrification <sup>42</sup> and nitrate reduction <sup>14,43</sup>. Seagrasses are  
233 important to carbon sequestration due to carbon fixation <sup>44</sup> and the carbon storage in the root  
234 phyllosphere <sup>45</sup> and sediment. Seagrasses also ameliorate pH decreases <sup>46</sup>. Here we show that *P.*  
235 *oceanica* enhances carbon fixation in the water column, suggesting that the contribution of  
236 seagrass on the carbon cycle is likely greater than previously recognized due to water column  
237 effects. *P. oceanica* mirrors the findings for other foundational species, such as corals and  
238 sponges <sup>4,47,48</sup> where there are indirect effects through a food web due to the nutrient  
239 provisioning by particular species. Our findings with *P. oceanica* suggests that its role as a host  
240 for an active microbiome amplifies its role in these shallow Mediterranean systems.

241 Ammonification occurred during day and night and with or without *P. oceanica*, suggesting that  
242 there is an abundance of microbes that metabolize amino acids and enhance carbon fixation and  
243 primary productivity in seagrass ecosystems. Further, every genome that we assembled had the  
244 functional capacity for deamination of amino acids to ammonium (Table S6). The use of  
245 unfiltered, in situ seawater allowed us to quantify ammonification in the water column, both with  
246 and without *P. oceanica*. Bacteria in the water column was likely a significant contributor to  
247 ammonification. <sup>15</sup>N uptake to water column POM was detected in all incubation bottles, though  
248 an unknown amount of this uptake could be due to bacteria or eukaryotic phototrophs.

249

250 Regardless of whether the  $^{15}\text{N}$  went into bacteria first, then into water column phytoplankton, the  
251 presence of *Posidonia* enhanced the uptake of  $^{15}\text{N}$  into POM, suggesting that seagrass-associated  
252 bacteria, or even fungi, enhance water column productivity both at vent sites and controls. The  
253 comparison of light versus dark uptake of  $^{15}\text{N}$  into POM demonstrated that at least some of this  
254 uptake was likely bacteria given the continued incorporation of  $^{15}\text{N}$  into POM at night (Figure  
255 2d).

256

257 One enigmatic aspect of our results is ammonification rates in units of nmol per hour, while  
258  $^{15}\text{NH}_4$  uptake rates could be in  $\mu\text{mol}$  per hour. We can think of several explanations for this,  
259 including that amino acid concentrations that were greater than the  $1\mu\text{M}$  that we assumed would  
260 have overestimated enrichment ( $R_{\text{source}}$ ) and underestimated ammonification in equation (1). A  
261 second reason is that these processes could be spatially restricted, where microbial production in  
262 a biofilm could be immediately followed by host use, causing us to underestimate  
263 ammonification in the water column. Thirdly, if amino acid metabolisms are rapid and amino  
264 acid concentrations have high flux, we may have underestimated the processing and recycling of  
265 nitrogen during incubation.

266

267 Ammonium concentrations are often not measured to the extent that nitrate concentrations are  
268 measured, despite preferential use of the former by macrophytes<sup>49,50</sup>. Estimates of ammonium  
269 use and turnover in nature are high<sup>51</sup>, though bacterial ammonification has been measured only  
270 rarely<sup>52</sup>. Also measured rarely is the concentration of DON, including any component parts such  
271 as amino acids. Amino acid concentrations have been estimated at 0.5-1.9  $\mu\text{M}$  in other areas in

272 the Mediterranean<sup>53,54</sup>, but a single flux estimate in the Caribbean suggests the flux could be  
273 high<sup>52</sup> and the importance of DON as a persistent supply of ammonium could be even greater.

274

275 Our demonstration of increased  $\delta^{15}\text{N}$  in seagrass tissue does not conclusively prove that these  
276 plants are taking up  $^{15}\text{NH}_4$  from microbial processes. Only pulse-chase experiments with more  
277 precise imaging, for example using NanoSIMS<sup>15,16</sup>, could show that microbial metabolism was  
278 the intermediate between  $^{15}\text{N}$ -amino acids and the higher  $\delta^{15}\text{N}$  of macrophyte tissue. However,  
279 ammonification genes were ubiquitous across MAGs here (Table S4) and in other studies<sup>14,55</sup>,  
280 and suggests that microbes may mediate the availability of ammonium. Several previous studies  
281 that suggest seagrasses directly take up DON did not control or account for microbial activity<sup>18</sup>,  
282 and thus direct DON uptake by marine angiosperms remains unknown.

283

284 Amino acid concentrations in the ocean are rarely reported, though there are several studies in  
285 the Mediterranean in proximity to *P. oceanica* beds that report 0.5 to 1.9  $\mu\text{M}$  dissolved free  
286 amino acids (DFAA) concentrations<sup>53,54</sup>, several orders of magnitude greater than those reported  
287 in open ocean settings<sup>56,57</sup>. DFAA can be a reduced source of nitrogen for both phytoplankton  
288 and bacteria<sup>19</sup>, and may be rapidly renewed via the release from animals<sup>58,59</sup>, phytoplankton<sup>60</sup>,  
289 and possibly the host themselves, such as *P. oceanica* here<sup>53</sup>. Concentrations can be greater in  
290 the sediment surrounding *P. oceanica* when compared to the water column<sup>53</sup>. Although  
291 estimates are that DFAA is only from 1 to 10% of DON in coastal areas<sup>61</sup>, the estimates are few  
292 and DFAA and DON may have rapid turnover rates which make their contribution to overall  
293 nitrogen use difficult to estimate.

294 Our estimates of ammonium uptake by *P. oceanica* during the day from the addition of  $^{15}\text{N}$

295 enriched amino acids ranged from 1.17 to 15.33 umol per g dry mass per hour, comparable to the  
296 2.77 umol rate estimated in <sup>51</sup> for *P. oceanica* in the Mediterranean and suggesting amino acids  
297 from deamination may be significant to ammonium uptake rates here and in other areas.

298 *P. oceanica* has other means of acquiring dissolved inorganic nitrogen from microbial associates.  
299 Nitrogen fixation genes were found in a MAG from the rhizome at the control site and is  
300 consistent with other studies that have determined that nitrogen fixation <sup>62</sup>, *nifH* genes <sup>63</sup>, or  
301 nitrogen-fixing taxa in association with *P. oceanica* <sup>15</sup> in low-oxygen environment of the  
302 rhizomes. Mohr et al. <sup>15</sup> assembled a MAG for a novel taxon that fixes nitrogen within the cells  
303 of *P. oceanica* roots. We did not find the '*Candidatus Celerinatantimonas neptuna*' they  
304 described in any of the MAGs we assembled, nor in the family Celerinatantimonadaceae,  
305 perhaps due to either incomplete DNA sequencing or to our use of surface swabs rather than  
306 grinding the root tissue of *P. oceanica*, as they did. Our analysis of *P. oceanica* blades did not  
307 reveal *nifH* genes. Dissimilatory nitrate reduction to ammonium is an additional bacterial  
308 metabolism that may help the seagrass host access nitrogen. We found genes for this function in  
309 rhizomes in control areas, consistent with previous studies of denitrification in association with  
310 seagrass<sup>64</sup>.

311  
312 Estimates of ammonification were greater in control areas, though *P. oceanica* carbon fixation  
313 did not differ between the control area and the CO<sub>2</sub> vent area. Ambient nitrate values were  
314 similar between the two sites at the single timepoint when we estimated them; previously  
315 published values also cite similar low nitrate concentrations regardless of venting that are <1uM  
316 <sup>36,37</sup>. Both vent and ambient sites had microbes that could increase nitrogen to enhance *P.*  
317 *oceanica* primary production. In addition to the abundance of enzymes with deaminating

318 function (Table S4), nitrate reduction genes were indicated, as was nitrogen fixation.

319

320 There were microbial metabolisms that were unique to *P. oceanica* tissue types, such as  
321 cyanobacteria and anoxygenic photosynthesis only on seagrass blades. B vitamin synthesis was  
322 also prevalent, but the rhizomes in vent sites lacked Vitamin B1 and B6 synthesis. Vitamin B12,  
323 suggested to be auxotrophic for marine eukaryotic hosts<sup>65,66</sup> was in some MAGs in both tissue  
324 types and locales. Whether it plays a critical role in host fitness that has been demonstrated for  
325 other marine macrophytes (e.g.<sup>1</sup>) remains to be investigated. In general, alpha diversity and the  
326 number of high quality MAGs was low in vent samples. Whether this reflects lower bacterial  
327 abundance in these areas is unclear, though certain functions were absent.

328

329 Our finding that *P. oceanica* and its microbial associates stimulate water column carbon fixation  
330 adds an important dimension to the role of seagrass beds in the carbon cycle. Seagrasses are  
331 suggested to alter the dissolved organic nitrogen<sup>52</sup> and seaweeds can influence water column  
332 microbes<sup>67</sup>. Understanding whether there are general mechanisms by which the seagrass enhance  
333 the productivity of the surrounding water column are a necessary aspect of quantifying the role  
334 of seagrass in the marine carbon cycle.

335

## 336 **Methods**

### 337 Study System

338 Underwater CO<sub>2</sub> vent systems are a powerful tool for exploring the effects of low pH on the  
339 features of species and biological systems. In Ischia, Italy, CO<sub>2</sub> vent-influenced *P. oceanica*

340 meadows (here, Castello South (CS)) is separated by less than a km from areas unaffected by  
341 CO<sub>2</sub> vents and (Ambient pH site, Sant' Anna (SA)). The vent sites at Castello South have a mean  
342 pH near 7.7, while the Ambient pH site at Sant' Anna has ambient pH ~7.95<sup>27</sup>. Seawater  
343 temperature and other environmental variables (e.g. depth, light, salinity) are similar in CO<sub>2</sub> vent  
344 and in ambient pH sites<sup>27</sup>. While carbon dioxide is the main gas from the vents, other gasses  
345 include N<sub>2</sub> (3.2–6.6%), O<sub>2</sub> (0.6–0.8%), Ar (0.08–0.1%), and CH<sub>4</sub> (0.2%–0.8%) but no sulfur gas  
346<sup>31</sup>. Trace metal concentrations can be elevated near vents, although detrimental effects on *P.*  
347 *oceanica* have not been recorded<sup>68</sup>.

#### 348 <sup>15</sup>N amino acid incubation with *P. oceanica*

349 *P. oceanica* was collected *in situ* at the Ambient pH site SA at 1000h and the CO<sub>2</sub> vent site CS at  
350 1040h on 14 Sept 2021. At each site, blades attached to a single rhizome unit were selected via  
351 SCUBA to have both a new blade developing as well as a corresponding older blade that was  
352 generally free of epiphytes. The rhizome was removed so that the blades (from 4-6) remained  
353 within a sheath. *P. oceanica* wet mass was quantified with a Pesola and ranged from 2.0 to 3.5 g  
354 (mean=2.75 g). At each site, 8 *P. oceanica* shoots were collected. While we collected plants that  
355 were approximately a half meter distant from each other, we do not know if individuals were  
356 genetically distinct.

357 Simultaneously, gas-tight 500 ml polycarbonate bottles (actual volume is 660 ml) were filled at  
358 1-2 m depth at each site, holding the bottle underwater above the *P. oceanica* beds, filling to  
359 eliminate all bubbles, and capping underwater. For each site, *P. oceanica* was immediately added  
360 to 8 of the bottles, while 4 held seawater only to quantify water column processes. Oxygen and  
361 temperature were recorded immediately with 3 mm optical fiber probe (#OXROB3, Pyroscience,

362 Germany) and temperature sensor (Firesting FS02-4, Pyroscience, Germany) assuming 38 ppt  
363 Salinity.

364 We amended each of the 16 bottles with *P. oceanica* and the 8 with seawater only with amino  
365 acids that were enriched in  $^{15}\text{N}$  (98%, Cambridge Isotope, NLM-2151, Lot#PR-24163). We  
366 assumed a concentration of dissolved free amino acids (DFAA) of 1  $\mu\text{M}$  based on previous work  
367 in Mediterranean *Posidonia* beds that ranged from 0.5 to 1.9  $\mu\text{M}$ <sup>53,54</sup>. We added 50  $\mu\text{L}$  of a .05M  
368 solution to an assumed existing concentration of 1  $\mu\text{M}$  to achieve an enrichment of  $\sim 0.76$  at %.  
369 The bottle was gently inverted 5 times to distribute the isotope, then attached to a line with dive  
370 weights at either end and were deployed at 2 m depth at noon. All 32 bottles were deployed  
371 across 4 lines in the same embayment (Cartaromana Bay) where both sets of seagrasses  
372 originated, though in waters that were unaffected by the vent activity. The bottles rested on the  
373 benthos and thus received natural light and some gentle water surge throughout the experiment.

374 To quantify nitrogen metabolic processes during the daylight versus darkness, we analyzed half  
375 of the *P. oceanica* and seawater contained within the bottles after 6 hours at 1800h; the  
376 remaining 16 experimental units were removed from the water immediately after sunrise the  
377 following day on 15 Sept 2021 (0720h).

378 For each of the end of day and end of night censuses, the oxygen concentration in each bottle  
379 was measured and seawater was preserved for dissolved organic matter concentration, inorganic  
380 nutrient concentrations, including the concentration of  $^{15}\text{N}$ -ammonium. We also filtered 360-420  
381 ml of seawater through a 0.7  $\mu\text{M}$  gf/f filter (25 mm, Whatman) to quantify the  $^{15}\text{N}$  of particulate  
382 organic matter (POM) in the seawater and dried at 50°C for 48 h. Dissolved inorganic nutrient  
383 samples were filtered through a 0.2  $\mu\text{m}$  PE filter and frozen at -20°C. Seawater samples for

384 dissolved organic C and N analysis were filtered through pre-combusted GF/F filters into acid-  
385 washed HDPE vials, immediately fixed with 160 uL of 18.5% HCl and stored at 4°C until  
386 analysis on a total organic carbon analyzer (TOC-L with TNM-L Unit, Shimadzu Corporation,  
387 Japan). DON was obtained from TDN subtracting total dissolved inorganic N (nitrate, nitrite and  
388 ammonium), determined on seawater samples preserved frozen and analyzed on a Continuous  
389 Flow Analyzer (Flowsys, SYSTEA SpA., Italy).

390 We analyzed the changes to both organic nutrient (DOC and DON) and inorganic nutrients  
391 (nitrate, nitrite, ammonium, phosphorus, silica) when incubation bottles had seawater only or *P.*  
392 *oceanica*, using a univariate two-way ANOVA with locale and time of day and their interaction.  
393 All statistical analyses were performed in R (www.R-project.org), version 4.2.2 (2022-10-31)  
394 with modules *ggplot* and *dplyr*.

### 395 Quantifying Nitrogen dynamics

396 We quantified the amino acid mineralization rate (ammonification) using the source-sink model  
397 from Lipschultz<sup>69</sup>. The model quantifies the transfer from the source (<sup>15</sup>N amino acids) at the  
398 beginning of the experiment to the 'sink', which is the <sup>15</sup>NH<sub>4</sub> in the seawater, either at the end of  
399 the day or the end of the night. The rate from  $T_0$  to  $T_f$  was based on the following difference  
400 equation:

$$401 \text{ Amino Acid Mineralization Rate} = \frac{R(t)_{sink} - R(t)_{source}}{(R_{source} - R(0)_{sink}) * t} * [NH_4] \quad (\text{equation 1})$$

402 where  $R$  is the isotopic ratio of the sink or source,  $t$  is the length of the incubation, and  
403  $[NH_4]$  represents the average concentration of ammonium over the course of the experiment. By  
404 quantifying the metabolism of <sup>15</sup>N from amino acids (the source) to ammonium in the water  
405 column (the sink), we estimated the hourly rate in nmol for the approximately 7 hour daytime

406 incubation, as well as the entire 20 hours until the next morning. We then separated the  
407 ammonification rate for the night only by assuming the  $R(0)_{sink}$  was the mean value at the end of  
408 the day. We quantified equation (1) for all 8 bottles with unfiltered seawater and 16 incubation  
409 bottles with both *P. oceanica* and unfiltered seawater. We used unfiltered seawater from  
410 immediately adjacent to the *P. oceanica* used in the experiment to quantify activity by water  
411 column versus host-associated microbes.

412 Because we needed a concentration of  $^{15}\text{NH}_4$  in seawater that we could detect, given the  
413 relatively low concentrations of  $[\text{NH}_4]$ , we added 77.6 $\mu\text{L}$  of .05M  $\text{NH}_4\text{Cl}$ , effectively adding 1.4  
414 mg/L of  $\text{NH}_4$ . The  $\delta^{15}\text{NH}_4$  value of the  $\text{NH}_4\text{Cl}$  was known, allowing for reliable detection of  
415 ammonium without diluting the  $^{15}\text{NH}_4$  signal <sup>55</sup>.

416

417 We tracked the uptake of  $^{15}\text{N}$  to *P. oceanica* tissue to estimate ammonium uptake rates,  
418 following methods in <sup>70</sup>. This method uses the following equation:

419 
$$\text{NH}_4^+ \text{ uptake rate} = \frac{\text{particulate } ^{15}\text{N at\% excess}}{(R * t)} * N_T \quad (\text{equation 2})$$

420

421 where tissue  $^{15}\text{N}$  atom% excess is the final  $^{15}\text{N}$  atom% minus the initial  $^{15}\text{N}$  atom%,  $R$  is the  
422 mean  $^{15}\text{N}$  atom% enrichment in the  $\text{NH}_4^+$  pool,  $t$  is the duration of the incubation, and  $N_T$  is the  
423 amount of nitrogen in *P. oceanica* in  $\mu\text{mols}$ . We also used equation (2) to estimate  $^{15}\text{N}$  uptake by  
424 particulate organic matter (POM); for POM the  $^{15}\text{N}$  could have been in the form of ammonium or  
425 amino acids.

426

427 The percent carbon, nitrogen and  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  of *P. oceanica* tissue was measured from a 1 cm  
428 section of the midblade where we scraped off all epiphytes, as well as new meristematic tissue at

429 the base that was always free of epiphytes. *P. oceanica* tissue was sampled prior to enrichment,  
430 at the end of the daylight hours, and following the nighttime period. The tissue was dried, ground  
431 to a fine powder in a GenoGrinder Spex (Metuchen, NJ), and analyzed on an elemental-  
432 analyzer–isotope-ratio mass spectrometer at Northwestern University Stable Isotope  
433 Biogeochemistry Laboratory (NUSIBL).

434

#### 435 Metagenome Analyses

436 The surface microbiome of *P. oceanica* was sampled with a sterile cotton swab on the upper  
437 rhizome tissue; the mid blade region (corresponding to 2 above) and the older tissue with  
438 epiphytes (3. above) was sampled with both a cotton swab and Dentek brush to ensure a  
439 complete surface sample on blade tissue. All were placed in -20°C and shipped to storage at -80°  
440 C, then shipped frozen to the University of Chicago. DNA was extracted with a Qiagen  
441 PowerSoil Kit and multiple samples were pooled for each of 6 metagenome samples to increase  
442 DNA quantity and possible discovery. The concentration of DNA was less on Castello South  
443 (CS) vent site samples compared with the non-venting site (SA), so we pooled all 6 sampled  
444 individuals into a single sample for each of the vent blade and vent rhizome. Thus, *P. oceanica*  
445 blade samples were pooled from 6 plants at CS and 3 plants each at SA; rhizome samples were  
446 also pooled from 6 plants at CS and 3 plants each at SA. We thus had duplicated samples for  
447 control blades and rhizomes but only a single sample for CS vent tissue type.

448

449 The above 6 samples were run over 1 lane on a HiSeq 2500 (2x150) with TruSeq DNA library  
450 preps at Argonne National Laboratory. For each sample, resulting DNA sequences were first  
451 quality filtered <sup>71</sup>, then assembled with IDBA-UD v1.1.3 <sup>72</sup> with a minimum scaffold length of 1

452 kbp. Metagenomic short reads from each sample were then recruited back to their corresponding  
453 assembled contigs using Bowtie2<sup>73</sup>. Samtools<sup>74</sup> was used to generate sorted and indexed BAM  
454 files. Anvi'o v7.0<sup>75</sup> was used as the command line environment for all downstream analyses.  
455 'anvi-gen-contigs-database' was used to generate anvi'o contigs databases, during which  
456 Prodigal v2.6.3<sup>76</sup> identified open reading frames, and 'anvi-run-hmms' was used to identify  
457 genes matching to archaeal and bacterial single-copy core gene collections using HMMER<sup>77</sup>.  
458 We quantified overall alpha diversity with 'anvi-estimate-scg-taxonomy'. We then reconstructed  
459 genomes from the assembled metagenomes, we used a combination of automatic binning via  
460 CONCOCT v1.1.0<sup>78</sup>, followed by a manual curation of each MAG that had greater than 50%  
461 completion, following guidance by<sup>79</sup> Genome taxonomy was determined using the GTDB  
462 (v.1.3.0,<sup>80</sup>) database and 'anvi-run-scg-taxonomy'. We also inferred gene-level taxonomy using  
463 Centrifuge v1.0.4<sup>81</sup> to aid manual curation.

464

465 We examined the metabolic capability of the bacterial MAGs associated with *P. oceanica* using  
466 'anvi-estimate-metabolism' on the manually refined bins that were >50% complete and <10%  
467 redundancy<sup>82</sup>. We classified a metabolic module as complete if 75% percent of the genes within  
468 the module were complete.

469

470 We further quantified the presence of genes that could increase host access to nitrogen. Our  
471 experiment specifically tested whether amino acid deamination occurred, so we searched within  
472 our assembled MAGs for ammonification hydrolases, including ureases and ammonia-lyases that  
473 cleave the C-N bonds in amino acids and make ammonium available to the host. We identified  
474 these enzymes through the Enzyme Commission numbers, and searched for EC numbers

475 classified as acting on CH-NH<sub>2</sub> bonds (EC:1.4.\*), acting on carbon-nitrogen bonds other than  
476 peptides (EC:3.5.\*), or ammonia lyases (EC:4.3.1\*), where \* indicates any subset of these  
477 classifications, and determined whether a MAG had each of these 3 classes of enzymes (e.g. <sup>14</sup>).  
478 The Kegg Orthologs associated with each of these EC numbers is shown in Supplemental Table  
479 5. We searched for evidence of nitrogen fixation genes (*nifH*), as well as other nitrogen cycling  
480 genes and functions that could benefit the host.

481

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690

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697 the inorganic and organic nutrient analyses and J. Berlinghof for comments.

698

## 699 **Author Contributions**

700 CAP, NT, UC, AM, JPG designed the study and CAP, NT, UC, AM, LM carried out all field and  
701 laboratory analyses. CAP, UC, IV analyzed data. All individuals contributed to the writing of the

702 manuscript.

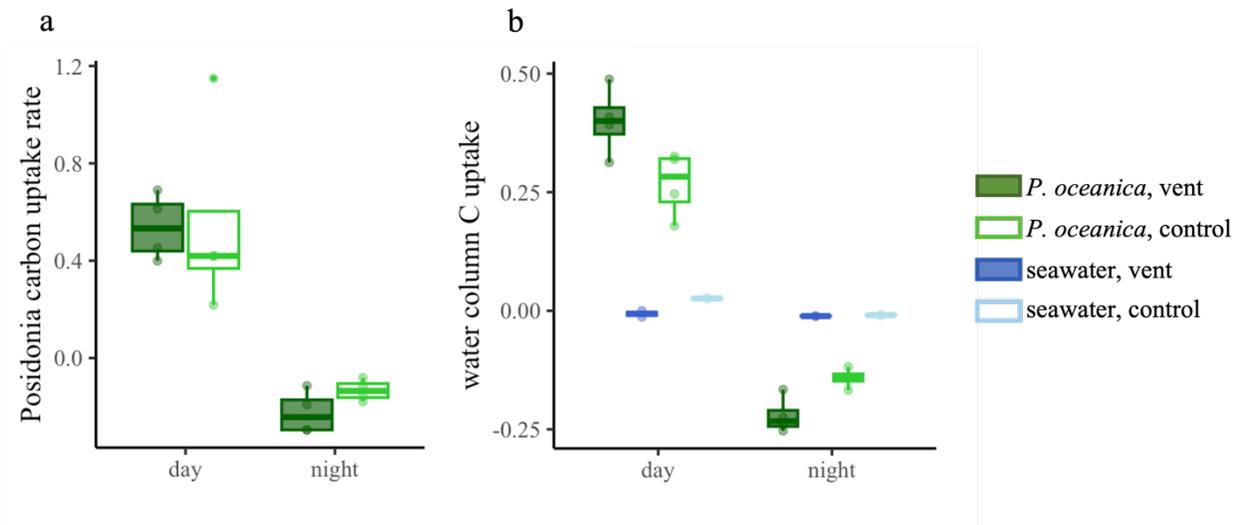
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704 **Data Availability**

705 Environmental data have been archived at Zenodo, doi: 10.5281/zenodo.7834043, while

706 metagenomic data are at NCBI, #SUB13085574.

Figure 1

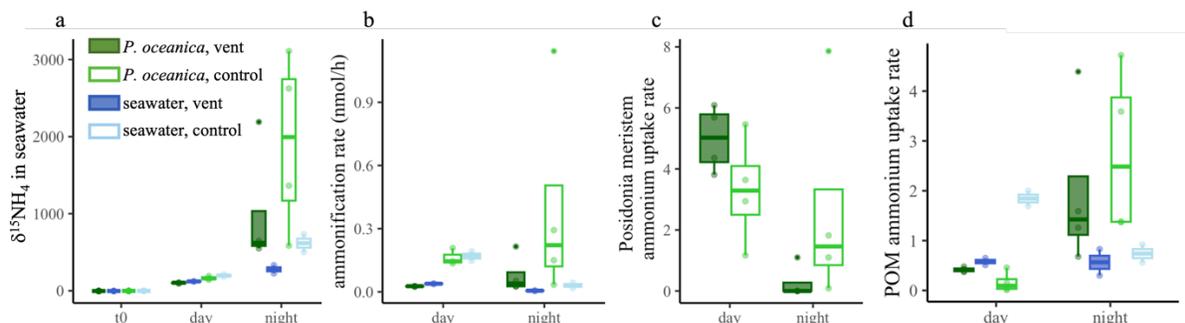


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711 Figure 1. Carbon uptake rates by a. *P. oceanica* and b. seawater. In a, carbon fixation or  
712 respiration, in mg C per hour per g *P. oceanica* dry mass in incubation bottles during daylight  
713 and nighttime hours. *P. oceanica* carbon fixation was based on oxygen change, following  
714 subtraction of water column rates. *P. oceanica* carbon fixation was greater during the day, but  
715 did not differ between control and vent sites. Two-way ANOVA (site effect 0.646, diel effect  
716  $p < 0.001$ , interaction  $p = 0.728$ ). In b, carbon uptake by unfiltered seawater (in mg C per L per  
717 hour) is shown for both sites and in incubation bottles with and without *P. oceanica*. POM  
718 carbon uptake differed between day and night ( $p < 0.001$ ) and with *P. oceanica* ( $p = 0.002$ ); there  
719 were significant interactions with time of day and locale ( $p = 0.004$ ) and time of day and treatment  
720 ( $p < 0.001$ ). Boxplots show mean values and delimit 25th and 75th percentiles; all points are  
721 shown, statistical tests in Table S2.

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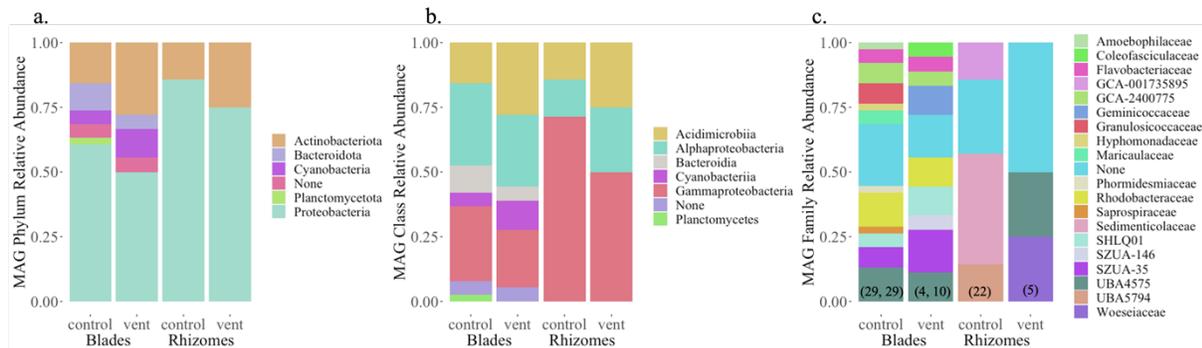
Figure 2



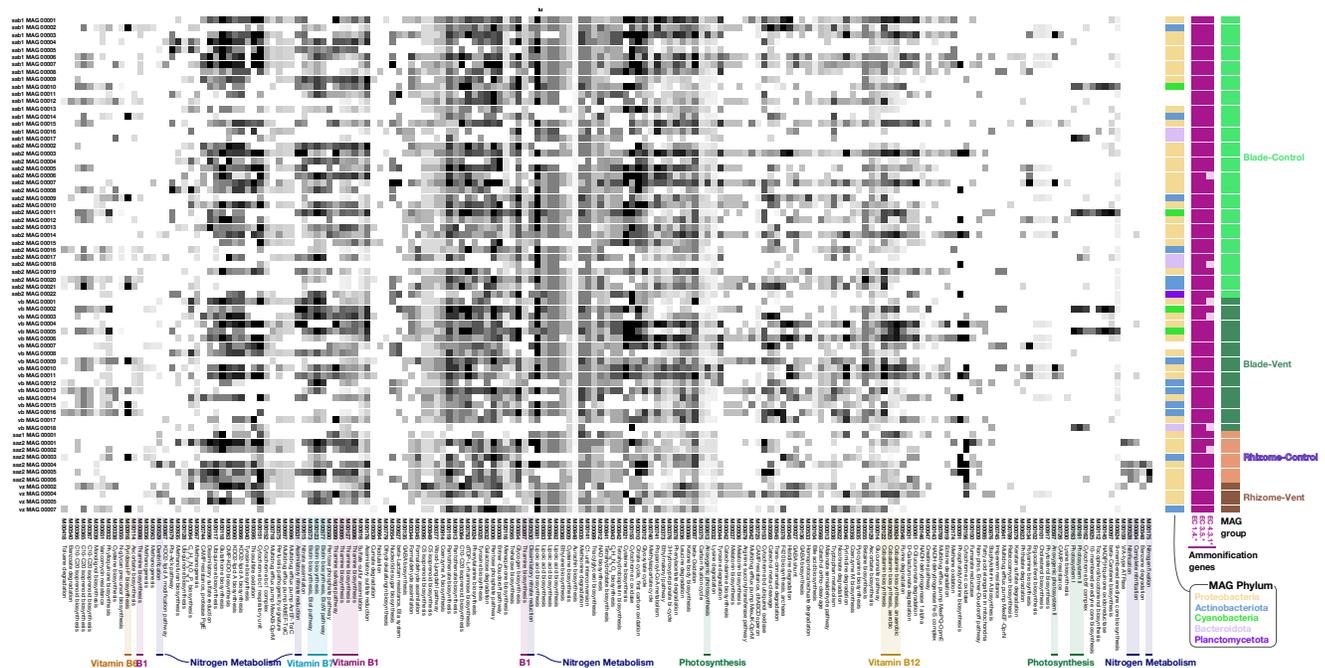
724  
 725 Figure 2. The fate of  $^{15}\text{N}$  amino acids added to incubation bottles with and without *P. oceanica*.  
 726 In a,  $\delta^{15}\text{N}$  ammonium (‰) in seawater in incubation bottles with *P. oceanica* or seawater only. A  
 727 comparison of the values during the end of day census and the end of experiment census, showed  
 728 a significant increase in the bottles at the end of the night, with increased values at SA and with  
 729 *P. oceanica*. There was also an interaction between time and treatment; the increase with time  
 730 was highest with *P. oceanica*. In b. The ammonification rate in nmol per hour across the two  
 731 locales and attributed to *P. oceanica* and seawater versus seawater only based on equation (1).  
 732 The control site had greater ammonification compared with the vent sites ( $p=0.002$ ) and *P.*  
 733 *oceanica* was nearly associated with greater ammonification rates than seawater ( $p=0.060$ ), while  
 734 rates did not differ overall between night and day ( $p=0.732$ ). Examining *P. oceanica* separate  
 735 from seawater during day versus night, *P. oceanica* has no diel pattern, while the POM in  
 736 seawater is associated with greater ammonification during the day, perhaps due to the greater  
 737 supply of DOC. In c, ammonium uptake rate in  $\mu\text{mol N per g dry mass per hour}$  in the *Posidonia*  
 738 meristem during both day and night periods using equation (2). Uptake was greater during the  
 739 day ( $p=0.002$ ), with a significant interaction ( $p=0.020$ ), indicating a relatively greater uptake of  
 740  $^{15}\text{N}$  ammonium at night in the control site. In d., the uptake of ammonium by POM was greater  
 741 when *P. oceanica* is absent from the incubation bottles during the day, and this effect was  
 742 greatest in the control SA site, based on the interaction between locale and treatment ( $p=0.033$ ).  
 743 At night, the situation is reversed and the incubation bottles with *P. oceanica* resulted in a greater  
 744 incorporation of  $^{15}\text{N}$  into the POM in the seawater compared with bottles that had no *P. oceanica*  
 745 ( $p=0.013$ ). Statistical analyses are in Table S2.

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747  
748 Figure 3  
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751  
752 Figure 3. The taxonomic assignments of 67 metagenome-assembled genomes (MAGs) in the 6  
753 samples from the blade or rhizome surface of *Posidonia* as a. Phylum, b. Class and c. Family.  
754 There were 22 high quality MAGs and 42 medium quality MAGs, while taxonomy was not  
755 found for 3. In c, alpha diversity in each sample is shown and based on ‘anvi-estimate-scg-  
756 taxonomy’. See Supplemental Table S4 and S5.  
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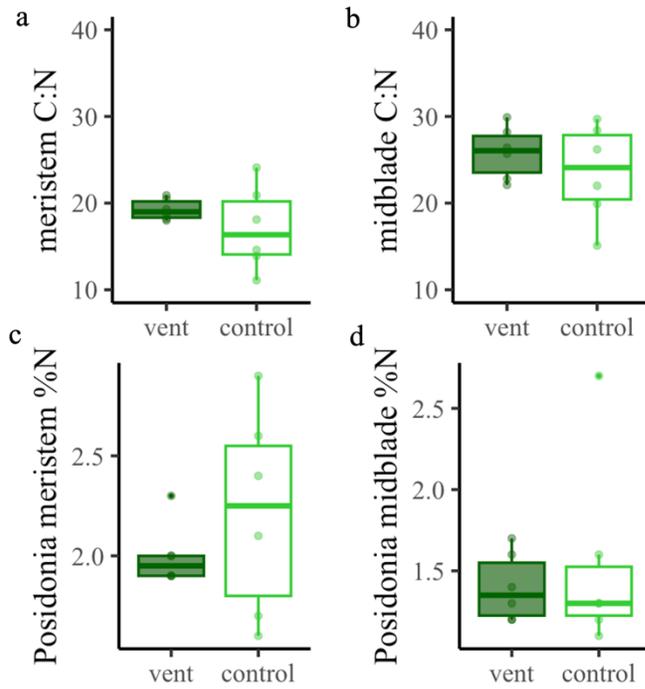
760  
761 Figure 4. The metabolisms in 67 microbial metagenome assembled genomes (MAGs) assembled  
762 on *P. oceanica* at Ischia, Italy across 5 Phyla and on four different tissue types. The completion  
763 (black=complete, white=absent) in each microbial MAG for metabolic modules that showed  
764 differences across many of the MAGs. A full list of MAG metabolisms are in Table S7. Every  
765 MAG had some ammonification genes (Table S6). The figure was generated from anvi'o<sup>83</sup>.

766

767 **Supplementary Figures**

768

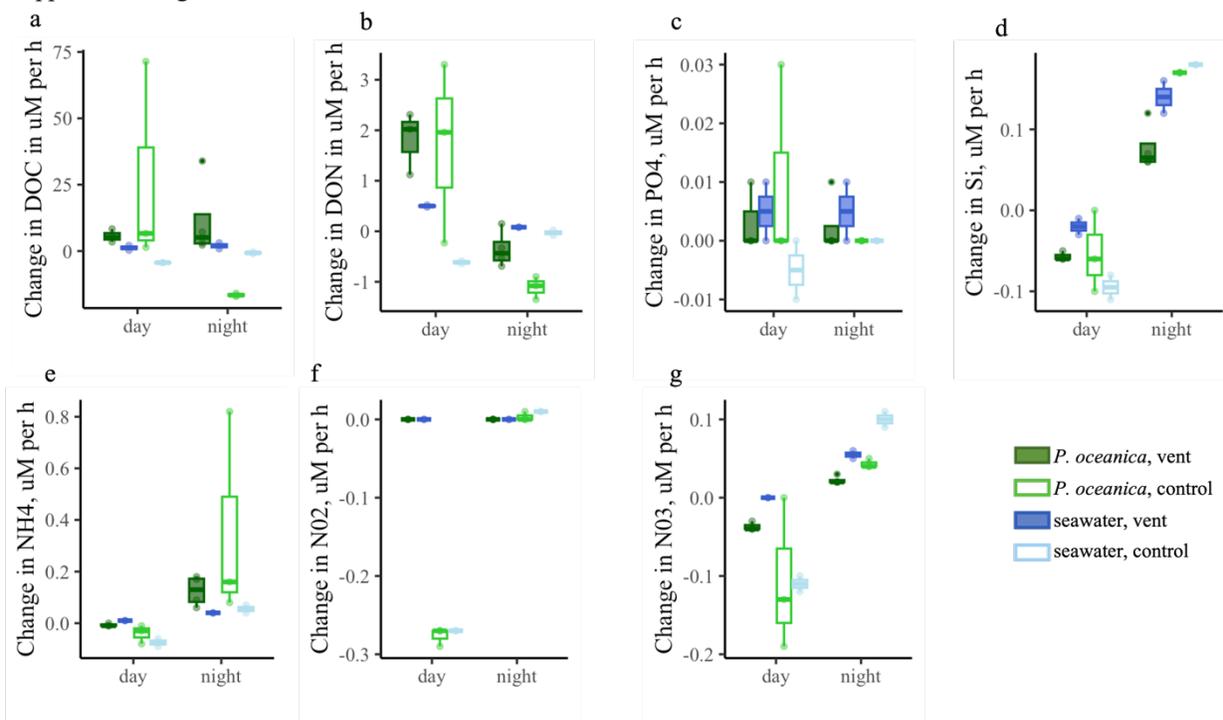
Supplemental Figure 1



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770 Supplementary Figure 1. *P. oceanica* tissue and POM carbon and nitrogen content. The ratio of  
771 tissue carbon to nitrogen did not differ in the a. meristem (n=6, t=1.057, p = 0.334) or b. the  
772 midblade region (t = 0.888, p= 0.401), nor were there differences in the overall nitrogen content  
773 for either c. meristem or d. midblade. All data in Table S3.

Supplemental Figure 2



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Supplementary Figure 2. Patterns of organic and inorganic nutrients in the incubations throughout the experiment, quantified as the change in uM per hour. Initial values are in Table S1.

Supplementary Tables 1-7 are combined in a single Excel file.