Interactive effects of global warming and eutrophication on a fast-growing Mediterranean seagrass

AUTHORS

Yaiza Ontoria[a,*], Eva González-Guedes[a], Neus Sanmartí[a], Jaime Bernardeau-Esteller[b], Juan M Ruiz[b], Javier Romero[a], Marta Pérez[a]

Affiliations

[a]Department of Evolutionary Biology, Ecology and Environmental Sciences, University of Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain

[b]Seagrass Ecology Group, Oceanographic Center of Murcia, Spanish Institute of Oceanography, C/ Varadero, 30740, San Pedro del Pinatar, Murcia, Spain

Email addresses

Yaiza Ontoria: yaizaontoria@ub.edu

Eva González-Guedes: egonzalez360@hotmail.com

Neus Sanmartí: neussanmarti@ub.edu

Jaime Bernardeau-Esteller: jaime.bernardeau@ieo.es

Juan M Ruiz: juanm.ruiz@ieo.es

Javier Romero: jromero@ub.edu

Marta Pérez: maperez@ub.edu

Corresponding author: Yaiza Ontoria

Email address: yaizaontoria@ub.edu

ABSTRACT

Coastal ecosystems, such as seagrasses, are subjected to local (e.g. eutrophication) and global (e.g. warming) stressors. While the separate effects of warming and eutrophication on seagrasses are relatively well known, their joint effects remain largely unstudied. In order to fill this gap, and using *Cymodocea nodosa* as a model species, we assessed the joint effects of warming (three temperatures, 20°C, 30°C and 35°C) with two potential outcomes of eutrophication: (i) increase in nutrients concentration in the water column (0,50 and 300 μM), and (ii) organic enrichment in the sediment). Our results confirm that temperature in isolation clearly affects plant performance; while plants exposed to 30°C performed better than control plants, plants exposed to 35°C showed clear symptoms of deterioration (e.g. decline of photosynthetic capacity, increase of incidence of necrotic tissue). Plants were unaffected by high ammonium concentrations; however, organic enrichment of sediment had deleterious effects on plant function (photosynthesis, growth, demographic balance). Interestingly, these negative effects were exacerbated by increased temperature.

Our findings indicate that in addition to the possibility of the persistence of *C. nodosa* being directly jeopardized by temperature increase, the joint effects of warming and eutrophication may further curtail its survival. This should be taken into consideration in both predictions of climate change consequences and in local planning.

Keywords: global warming, eutrophication, *Cymodocea nodosa*, nutrients, anoxia, ammonium, interactive effect

1. INTRODUCTION

Coastal ecosystems are facing multiple anthropogenic stressors that adversely affect their biodiversity and functioning (Vinebrooke et al., 2004). Such stressors are generated at a range of spatial scales, from the global to the most local. Global stressors are mostly related to climate change and include rising sea level, seawater acidification, warming and an increased frequency of heat waves (IPCC, 2013). The most prominent and pervasive stressor generated locally is probably eutrophication: increased loading of nutrients and organic matter from human activities (Nixon 2009). This has come to be considered one of the major threats confronting coastal ecosystems (Bricker et al., 2008; Hemminga and Duarte, 2000). The knowledge accumulated to date on the effects of individual stressors on key species is impressive. However, stressors rarely occur in isolation in the environment and, when acting together, they can be synergistic, additive or antagonistic (Todgham and Stillman, 2013). The interaction between stressors is now viewed as a crucial issue, to the point that it is recognized that single-factor experiments are of limited use for assessing the effects of climate change on coastal marine ecosystems subjected to other disturbances, such as eutrophication (Wernberg et al., 2012). Undoubtedly, experiments with single stressors can help us gain knowledge of the intrinsic, basic response mechanisms involved. However, the results should only be extrapolated to nature with great caution, not only due to problems associated with scaling up, but also due to potential interactions with concurrent stressors (Gunderson et al., 2016).

Seagrasses are widespread habitat-forming species of great ecological value that are exposed to multiple threats and are currently suffering declines worldwide (Waycott et al., 2009). The effects of climate change (including increased temperature and acidification) or eutrophication on the distribution, abundance and vitality of seagrasses are relatively well known (see reviews by Koch et al., 2013, for climate change; and

71 Burkholder et al., 2007, for eutrophication), and even though their effects have, for the
72 most part, been assessed separately (but see Campbell and Fourqurean, 2014, 2018; Ow
73 et al., 2016). Thus, it is well known that eutrophication has two main consequences for
74 seagrasses. On the one hand, the effects of increased nutrient concentrations are generally
75 considered detrimental, although they strongly depend on species-specific features and
76 on local conditions (Kilminster et al., 2015; Romero et al., 2006; Ruiz et al., 2001). Thus,
77 while a moderate supply of nutrients to plants adapted to nutrient-poor environments can
78 stimulate growth (Alcoverro et al., 1997; Pérez et al., 1991; Short, 1987), once a threshold
79 is reached, it may cause negative effects on plant photosynthesis and may even curtail
80 survival (Brun et al., 2002, 2008; Hauxwell and Valiela, 2003; van der Heide et al., 2008).
81 These negative effects can be caused directly, mainly by ammonium toxicity (Touchette
82 and Burkholder, 2000; van Katwijk et al., 1997); or indirectly, by stimulating
83 phytoplanktonic, epiphytic and macroalgal overgrowth, and enhancing negative biotic
84 interactions such as macro herbivore activity (Campbell et al., 2018; Ruiz et al., 2009;
85 Wear et al., 1999). On the other hand, an increased supply of organic matter to the
86 seagrass sediment, such as that caused by eutrophication, stimulates its oxygen demand,
87 eventually leading to hypoxic or anoxic conditions (Frederiksen et al., 2008; Pérez et al.,
88 2007). This oxygen shortage not only blocks metabolic function in seagrass roots,
89 including respiration, growth and nutrient acquisition (Smith et al., 1988), but it also
90 stimulates microbial sulphate reduction, which leads to belowground seagrass organs
91 (rhizomes and, specially, roots) being exposed to sulphide, a strong phytotoxin (Holmer
92 and Bondagarrd, 2001). Despite seagrass having evolved a number of adaptations which
93 increase its chances of surviving in naturally organic-rich sediments (Hasler-Sheetal and
94 Homer, 2015), additional deposition of organic C can exceed the seagrass response
95 capacity, and have negative effects such as reduced photosynthesis, impaired growth or,

96 in some cases, mass mortality (Collier and Waycott, 2014; Frederiksen et al., 2008; Koch
97 et al., 2007; Olivé et al., 2009).

98 Temperature affects seagrass physiology in a number of ways. It is known that increased
99 temperature usually stimulates both photosynthesis (Campbell et al., 2006; Winters et al.,
100 2011) and respiration (Schulze et al., 2015); but beyond some threshold, it generally
101 increases the latter more than the former, thus leading to an impaired C balance and
102 reduced growth (Lee et al., 2005, 2007; Marín-Guirao et al., 2016, 2018; Pérez and
103 Romero, 1992). Temperature also affects other processes, such as for instance nutrient
104 uptake (Borum et al., 2004; Bulthuis, 1987) or protein synthesis (Campbell et al., 2006;
105 Marín-Guirao et al., 2017). Overall, when the temperature exceeds a given threshold,
106 which is largely species specific, thermal stress leads to a reduction in growth (Lee et al.,
107 2007), deterioration of shoot status and eventually shoot mortality (Marbà and Duarte
108 2010). The responses of seagrasses to increased temperature are relatively well
109 documented; however, little is known of the potential distortion of these responses caused
110 by eutrophication.

111 Global warming is expected to increase in the coming decades and will affect the surface
112 waters of almost all of the world's oceans. Meanwhile, a large part of the planet's coastal
113 areas are subjected to different degrees of eutrophication (Halpern et al., 2007), which is
114 especially notable in industrialized countries. Consequently, many cases, thermal stress
115 will have an impact on meadows already affected by chronic or acute eutrophication,
116 whose responses to thermal stress will probably differ from that of unaffected plants, thus
117 limiting our ability to make reliable and realistic predictions for future warming scenarios.
118 To date, only a few studies have focused on the combined effects of warming and other
119 stressors, such as anoxia (Koch et al., 2007, with *Halodule wrightii* and *Thalassia*
120 *testudinum*), nutrients (Kaldy 2014, with *Zostera marina*) or light (York et al., 2013 with

121 *Zostera muelleri*). These works seem to suggest that synergistic effects are more the rule
122 than the exception. If this is the case, the consequences of global warming may be worse
123 than expected based solely on studies of thermal effects. In fact, a synergistic interaction
124 between eutrophication and seawater warming has already been suggested for the
125 Mediterranean seagrass *Posidonia oceanica* to forecast trajectories in abundance and
126 distribution of this seagrass species in the context of the different global climate change
127 scenarios (Jordà et al., 2012). However, a considerable gap exists in our knowledge of the
128 combined effects of warming and other stressors; and research is needed to confirm (or
129 refute) the potential synergies in seagrasses, especially in species that dominate areas that
130 are particularly sensitive to climate change. The present study attempts to help fill this
131 gap, by evaluating the joint effect of warming and eutrophication on a Mediterranean
132 seagrass (*Cymodocea nodosa*). The Mediterranean is one of the regions that are expected
133 to be most affected by warming, and the sea surface temperature rise, already in evidence
134 (Burrows et al., 2011; Jordà et al., 2013) may reach 3°C by the end of the 21st century
135 (Jordà et al., 2012); while the frequency of heat waves is also expected to increase (IPCC,
136 2013). Moreover, eutrophication has been identified as one of the major environmental
137 threats to seagrass habitats in coastal areas, mainly due to loading from urban, agricultural
138 and aquaculture wastes, particularly in the more confined environments where *C. nodosa*
139 is dominant (Boudouresque et al., 2009).

140 *C. nodosa* is widely distributed across a broad variety of shallow Mediterranean
141 environments, from open coastal areas to coastal lagoons, and extends into the Atlantic,
142 from the south of the Iberian Peninsula to the Canary Islands and Mauritania (Green and
143 Short 2003; Mascaró et al., 2009b; Reyes et al., 1995a). Its ecological value and its
144 capacity to survive relatively eutrophic conditions (Oliva et al., 2012), as well as its
145 considerable phenotypic plasticity (Pérez et al., 1994; Sandoval-Gil et al., 2014), make it

146 an interesting model species to evaluate the joint effects of increased temperatures and
147 eutrophication.

148 The aim of this study is thus to explore the combined effect of a global stressor (warming)
149 and a local stressor (eutrophication) on functional traits of *C. nodosa*. We partition the
150 eutrophication effects into an increased nutrient concentration in the water column and
151 an increase of organic matter loading of the sediment. We then determine the response of
152 the plant to each one of the three stressors (elevated temperature, nutrient increase and
153 increased organic matter loading) separately; and also to the combined effects of
154 temperature and each of the other two. The main hypothesis we wish to evaluate is that
155 temperature and eutrophication act synergistically, with deleterious consequences for the
156 seagrass. To this end, we perform two fully factorial experiments on indoor mesocosms
157 in which plants are exposed to three levels of temperature and, on the one hand, to three
158 different nutrient concentrations and, on the other hand, to two different levels of organic
159 matter in the sediment.

160

161 ## 2. MATERIAL AND METHODS

162 We explored the interactive effects of eutrophication and temperature in two separate
163 experiments. In the first experiment (TNUT experiment, hereinafter), temperature
164 increase and nutrient (ammonium) addition were applied; while in the second (TANOX
165 experiment, hereinafter) the stressors were temperature increase and addition of labile
166 organic C to the sediment.

167 ### 2.1 Plant and sediment collection

168 Undamaged healthy *C. nodosa* shoots (including their rhizomes and roots) were carefully
169 collected by hand from a shallow, undisturbed meadow (0.5 m deep) in Alfacs Bay (NW

Mediterranean) in late April. Only shoots less than one year old (less than 12 scars on the vertical rhizome, Mascaró et al., 2014) were selected to reduce the effects of physiological and morphological variability between shoots of different ages (Pagès et al., 2010; Pérez and Romero, 1994). Sediment was collected from the same area, extracting the surface layer (up to 10 cm deep), and immediately sieved (1 mm pore) to exclude macroinvertebrates and detritus. Sediment and plants were then transported separately in aerated tanks to the laboratory, where they were maintained with aeration for one night prior to the experiment being setup. Temperature was kept constant at the ambient values measured at the collection site (19.5°C). The experiments were conducted at the Experimental Chambers Service of the University of Barcelona.

2.2 Experimental design and setup

Both experiments were conducted using cylindrical transparent aquaria (12 L capacity, 40 cm height x 20 cm diameter) placed randomly in 3 experimental chambers (2.1 m^2). Each aquarium had an independent air pump providing proper aeration. The chambers allowed us to control the water temperature (20°C, 30°C and 35°C) and incident light (270 µmol photons m^{-2} s^{-1}), which was above the saturation irradiance for these plants (Pérez and Romero, 1992) on a 12 h:12 h light:dark photoperiod. To avoid experimental bias and minimize any uncontrolled variability, the aquaria were randomly relocated within the chambers every two days. Moreover, the aquaria were moved from one chamber to another (changing the chamber temperature) so that they spent approximately 1/3 of the experimental period in each chamber. Within 24 hours of collection, twenty shoots (with their corresponding portion of rhizome and roots) were planted in each aquarium, previously filled with 10 cm of sediment and 9 L of filtered seawater. All the aquaria were covered with plastic film to prevent water evaporation. For the TNUT experiment, a total of 27 aquaria were prepared and distributed randomly in groups of 9

in the three experimental chambers; while for the TANOX experiment, a total of 18 aquaria were distributed randomly in groups of 6 (see experimental setting in Fig. 1).

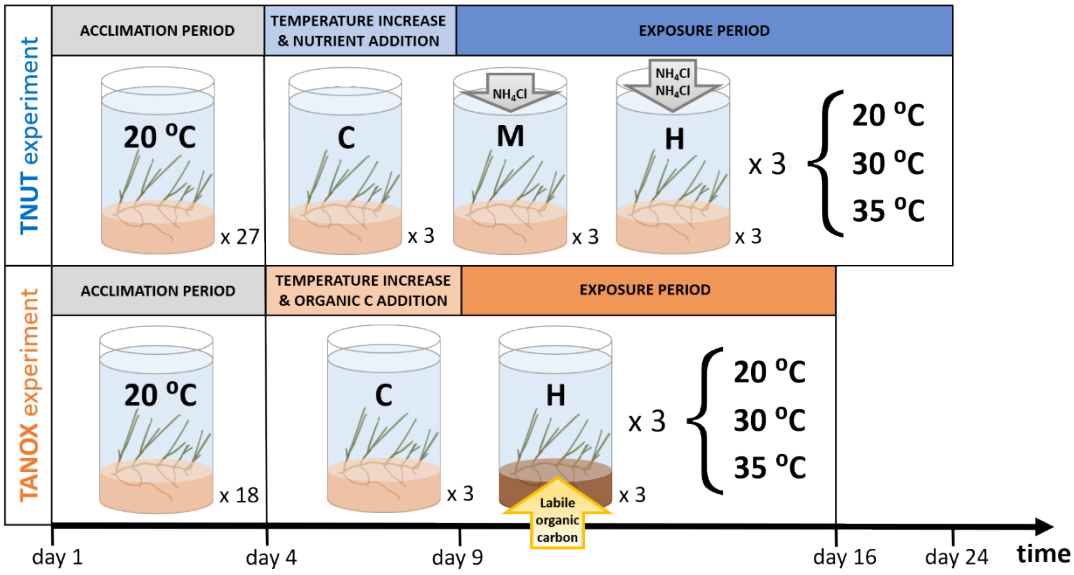


Fig. 1. Experimental setting. Grey arrows indicate ammonium addition in water and yellow arrow indicates labile organic carbon addition to sediment. TREATMENTS: C, control; M, moderate and H, high (see text). Temporal axis indicates: day 1, the beginning of the experimental setup with acclimation at control temperature; day 4, end of acclimation period and progressive increase of temperature; day 9, nutrient or labile organic carbon addition and start of the exposure period; day 16, end of TANOX experiment (7 days exposition); day 24, end of TNUT experiment (15 days exposition).

The aquaria were kept at 20°C for four days (a temperature close to that registered during sampling) to allow for plant acclimation. After the acclimation period, the temperature in two chambers was increased progressively (ca. 3°C per day) until reaching 30°C in one and 35°C in the other. The third chamber was left at 20°C as a control. The choice of the experimental temperatures was based on an unpublished 3-year temperature data series collected by the authors using continuous *in situ* recorders, indicating average summer (July to September) temperatures close to 30ºC, but peaking to 33ºC in the hottest

212 summer. Based on this, we consider that 35ºC during heat waves is a reasonable
213 assumption under a climate change scenario (IPCC, 2013).

214 For the TNUT experiment, once the experimental temperatures were reached, NH_4Cl was
215 added to obtain the following concentrations: control (no NH_4Cl added), moderate
216 concentration (30 μM of NH_4Cl) and high concentration (300 μM of NH_4Cl). The so-
217 called "moderate" value (30 μM) can be observed at eutrophic sites and can trigger
218 responses in some seagrass species (Villazán et al., 2013). The "high" value (300 μM)
219 represents extreme events and would only be reached during some wastewater discharges
220 (Cabaço et al., 2008). To counterbalance plant uptake (roughly estimated from growth
221 requirements, Pérez et al., 1994), NH_4Cl was further added to the experimental aquaria
222 every 5 days: 3.8 mg of NH_4Cl to the moderate ammonium treatment aquaria and 7.6 mg
223 NH_4Cl to the high ammonium treatment aquaria (with a total of two pulses after the initial
224 addition at the beginning of the experiment). Each ammonium treatment was applied to
225 three aquaria, chosen at random, within each temperature chamber, resulting in a
226 complete factorial design with n=3 replicates per experimental condition. The exposure
227 period to both factors lasted 15 days, after which time leaves in the high-temperature
228 treatment (35°C) began to show critical necrosis marks.

229 For the TANOX experiment, once the experimental temperature was reached, the organic
230 matter treatments were applied by adding labile organic C in the form of sucrose to the
231 sediment in the aquaria as follows: control (no sucrose addition, 0.7% DW sediment C
232 content in natural conditions) and high (675 g of sucrose added ≈ 15% DW sediment C
233 content). The labile organic C treatments were applied to three aquaria, chosen at random,
234 within each temperature chamber, resulting in a complete factorial design with n=3
235 replicates per experimental condition. The experiment ended after 7 days of exposure

period, when leaves in the high-temperature treatment (35°C) began to show critical necrosis marks.

2.3 Water and sediment analysis

The concentration of ammonium in the water was analysed in each aquarium at the beginning and end of the TNUT experiment using an FP 2020 Plus fluorometer and following a standard method (Kérouel and Aminot 1997). The redox potential of the sediment was measured at the end of the TANOX experiment using a Thermo Scientific, Orion Star A211 electrode. Measurements were taken in the upper 5 cm of the sediment layer.

2.4 Plant biochemical composition

To verify that the additions of ammonium and labile organic C applied could affect plant conditions, directly (high level of nutrients) and indirectly (anoxic sediment), we determined the N content (TNUT experiment) and S content (TANOX experiment) of different plant parts. To do this, at the end of the experiments, all the remaining shoots were harvested from the aquaria and separated into leaves, rhizomes, and roots. Subsequently, all plant tissues were dried at 60°C and then finely ground and homogenized; finally, they were weighed and packed into tin microcapsules.

For the TNUT experiment, the nitrogen content of leaves, rhizomes and roots was measured using a Carlo-Erba elemental auto-analyser. For the TANOX experiment, vanadium pentoxide was added, and the sulphur content of leaves, rhizomes and roots was determined. Samples were analysed at the Scientific and Technological Centre (CCiT) of the University of Barcelona.

2.5 Measurement of plant traits

259 The plant responses to the different stressors (or their combination) were assessed via
260 measurement of a series of traits, from the physiological to population level.

261 These included maximum quantum yield of PSII (F_v/F_m), incidence of leaf necrosis, leaf
262 growth, rhizome elongation, and shoot demographic balance. All these variables have
263 previously been used in the assessment of seagrass responses to stress and are related to
264 plant health and performance (Beer et al., 1998; Frederiksen et al., 2008; Maxwell et al.,
265 2000; Pagés et al., 2010; Romero et al., 2007). Rhizome elongation was only determined
266 for the TANOX experiment, while all the other traits were measured in both experiments.

267 Maximum quantum yield of PSII (F_v/F_m) was determined using a diving PAM (pulse
268 amplitude modulation) fluorometer (Walz, Germany) after 10 min of plant adaptation to
269 dark conditions. Three shoots were randomly selected from each aquarium (avoiding
270 apical shoots due to their more active growth and photosynthesis) and measurements were
271 obtained from the basal portion of the second youngest leaves, to minimize within-shoot
272 variability (Durako and Kunzelman, 2002; Gera et al., 2012).

273 The incidence of necrosis was assessed at all leaves of five shoots from each experimental
274 condition. Leaves were carefully separated from each shoot and the percentage of necrotic
275 surface, considered as that partially or totally covered by dark brown or black spots, was
276 visually estimated for each leaf and averaged for each aquarium.

277 Leaf growth was measured using a leaf punching method (Zieman, 1974) adapted to the
278 model species (Pérez and Romero, 1994). At the beginning of the experiments, five shoots
279 from each aquarium (avoiding apical shoots) were marked by punching a hole just above
280 the ligule of the outermost leaf with a hypodermic needle. At the end of the experiments,
281 the marked shoots were harvested, epiphytes were removed, and the leaves were carefully
282 separated to measure leaf growth. Shoots were individually sorted into old and newly

283 produced tissues, which were then dried for 48 h at 60°C before obtaining their dry
284 weights. Leaf growth rate was expressed as the new tissue produced per shoot and day
285 (mg DW shoot^{-1} day^{-1}), averaged for each aquarium.

286 To measure rhizome growth, we marked two apical shoots per aquarium with a rubber
287 band. At the end of the experiment, these shoots were harvested, the new portions of
288 rhizome cut, and their weight determined (after drying at 60°C until constant). Rhizome
289 growth was then expressed as weight of new rhizome per day (mg DW rhizome day^{-1}).

290 To estimate the shoot demographic balance (the difference between recruitment, i.e. the
291 number of new shoots, and mortality, i.e. the number of dead shoots), all shoots surviving
292 at the end of the experiments were counted. We computed the instantaneous demographic
293 balance (a) as:

294 $a\ (days^{-1}) = 1/t\ \ln\ (N_t/N_o)$

295 where N_0 is the number of shoots planted in each aquarium at the initial time (20), N_t is
296 the number of shoots alive in each aquarium at the end of each experiment and t is the
297 duration of the experiment (in days).

298 Positive values for *a* occur when shoot recruitment is higher than mortality, indicating a
299 net increase in shoot abundance. Conversely, negative values would indicate a net
300 reduction in shoot abundance, and hence a negative response to the stressor(s) considered.

301 **2.6 Statistical analysis**

302 For all statistical analysis, an aquarium was considered as the experimental unit, with n=3
303 replicates per experimental condition. The significance of the effects of temperature and
304 ammonium, on the one hand, and, temperature and addition of labile organic C, on the
305 other hand, were determined using PERMANOVA analysis based on a similarity matrix

306 created from the Euclidean distances between samples. The analysis was run with two
307 fixed factors: temperature (3 levels: 20°C, 30°C and 35°C) and nutrients (3 levels:
308 Control, Moderate and High, see above) for the TNUT experiment; and temperature (3
309 levels: 20°C, 30°C and 35°C) and addition of labile organic C (2 levels: Control and High,
310 see above) for the TANOX experiment.

311 For each experiment, one multivariate PERMANOVA was carried out for variables
312 related to plant biochemical composition (N and S content of plant tissues, for the TNUT
313 and TANOX experiment, respectively), and a second for the other variables (F_v/F_m,
314 incidence of leaf necrosis, leaf growth, rhizome elongation, and shoot demographic
315 balance), followed by univariate PERMANOVAs performed separately for each
316 individual variable. As in PERMANOVA the test is produced by permutation, the usual
317 normality assumptions of ANOVA (Anderson 2001), that were not met by most of the
318 variables considered, is not necessary. Pairwise comparisons were performed to identify
319 significant differences between individual treatments. In those cases, in which the number
320 of permutations was too low (<999, Anderson et al., 2008), a Monte Carlo test was applied
321 to establish an alternative p-value to validate the analysis. Analysis was carried out using
322 the Primer v6 statistical package (Clarke and Gorley, 2006), in conjunction with the
323 Windows PERMANOVA+ module (Anderson et al., 2008).

324

325 ## 3. RESULTS

326 ### 3.1 Culture conditions and plant biochemical composition

327 The different treatments (additions of nutrient and labile organic C) effectively changed
328 the conditions under which the plants were grown. Thus, on the one hand, in the TNUT
329 experiment, the ammonium concentrations in the water of the moderate and high

treatments were increased (relative to the water in the control aquaria) to the target values at the beginning of the experiment and had decreased at the end of the experiment, despite the repeated additions of ammonium and irrespective of the thermal treatment (Table 1).

Ammonium treatment	Thermal treatment					
	20°C		30°C		35°C	
	$[NH4^+]$ (µM)					
	Initial	Final	Initial	Final	Initial	Final
Control	1.20 ± 0.50	1.54 ± 0.53	0.69 ± 0.18	1.96 ± 1.41	8.64 ± 29.63	17.95 ± 8.29
Moderate	27.76 ± 1.12	0.21 ± 0.09	26.10 ± 0.33	0.62 ± 0.08	45.64 ± 1.30	3.83 ± 2.93
High	288.06 ± 34.01	2.21 ± 0.03	252.33 ± 11.23	4.25 ± 0.79	264.55 ± 7.96	15.82 ± 8.36

Table 1. Ammonium concentrations (in µM, mean ± SEM, *n*=3) in the water at the beginning (just after adding 30 µM and 300 µM to the "Moderate" and "High" treatments respectively) and at the end of the TNUT experiment.

These results show that the plants were subjected at least to one strong initial pulse of ammonium, plus another two pulses during the experiment. On the other hand, in the TANOX experiment, the redox potential of the sediments at the end of the experiment, while maintaining positive values under control conditions, became negative in the mesocosms subjected to large additions of high labile organic C. Temperature affected the redox potential, with lower values at higher temperatures likely due to an enhancement of the bacterial activity (Table 2).

Labile organic C treatment	Thermal treatment		
	20°C	30°C	35°C
	Redox potential (mV)		
Control	180.33 ± 8.31[a]	137.07 ± 16.41[b]	78.59 ± 9.52[c]
High	-24.81 ± 6.90[d]	-230.19 ± 11.38[e]	-281.78 ± 14.84[f]

Table 2. Redox potential values (mean ± SEM, *n*=3) of the sediment in the TANOX experiment for 7 days in three thermal treatments (20°C, 30°C, and 35°C). Lower case letters indicate significant differences (p>0.05) between treatments.

Overall, the biochemical composition of leaves (N and S content) changed in response to the treatments. In the TNUT experiment, addition of ammonium at high concentrations increased the N content of all plant tissues, up to 23% relative to controls (Fig. 2a, b and c; Table 3). In the TANOX experiment, the S content of leaves and roots was significantly higher under conditions with an addition of labile organic C than under control conditions (Fig. 2d and f; Table 3). Temperature had significant effects on biochemical composition in both experiments. The N content of leaves increased with temperature; while the N content of rhizomes decreased at the intermediate temperature. The S content of leaves and rhizomes increased with temperature; and the latter was even higher due to interactive effects between temperature and the addition of labile organic C.

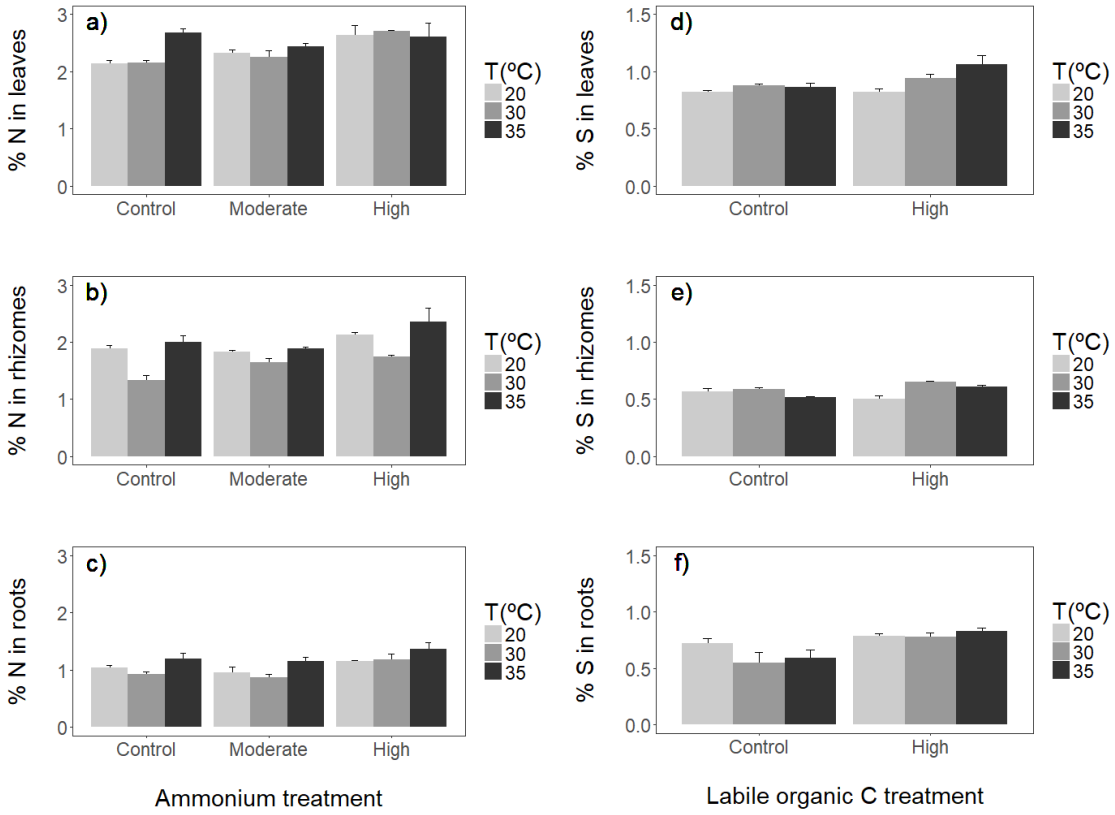


Fig. 2 *Cymodocea nodosa* biochemical composition (N content (mean ± SE, *n*=3) and sulphur content (mean ± SE, *n*=3)) measured in (a & d) roots, (b & e) rhizomes and (c & f) leaves, at 3 thermal treatments (20°C, 30°C, and 35°C, black, light grey and dark grey respectively) in the TNUT (a, b & c) and TANOX (d, e & f) experiments, expressed in percentage (%).

Experiment	Variable	Source	df	SS	MS	Pseudo-F	Unique perms	P	Pair-wise
TNUT	N leaves	***Main test***							
		Temperature	2	1.802	0.901	9.839	9952	**0.0001**	
		Ammonium	2	1.467	0.734	8.011	9947	**0.0002**	
		Temp x Ammonium	4	0.582	0.145	1.588	9931	0.1377	
		Res	18	1.648	0.092				
		Temperature	2	0.254	0.127	3.153	9952	0.0634	
		Ammonium	2	0.605	0.302	7.520	9950	**0.0042**	H>C=M
		Temp x Ammonium	4	0.373	0.093	2.317	9958	0.0955	
		Res	18	0.724	0.04				
	N rhizome	Temperature	2	1.257	0.629	20.145	9949	**0.0001**	35=20>30
		Ammonium	2	0.594	0.297	9.519	9953	**0.0012**	H>C=M
		Temp x Ammonium	4	0.188	0.047	1.506	9956	0.2396	
		Res	18	0.562	0.031				
	N roots	Temperature	2	0.291	0.145	7.219	9943	**0.006**	35>20=30
		Ammonium	2	0.268	0.134	6.655	9945	**0.0059**	H>C=M
		Temp x Ammonium	4	0.021	0.005	0.261	9961	0.9	
		Res	18	0.363	0.02				
TANOX	S leaves	***Main test***							
		Temperature	2	25.162	12.581	9.972	9961	**0.001**	
		Labile organic C	1	8.366	8.366	6.631	9952	**0.0087**	
		Temp x Lab. org. C	2	7.581	3.790	3.004	9940	**0.0443**	
		Res	12	15.140	1.262				
		Temperature	2	0.06	0.03	5.686	9947	**0.0228**	
		Labile organic C	1	0.034	0.034	6.456	9823	**0.0302**	30=35>20
		Temp x Lab. org. C	2	0.032	0.016	3.088	9955	0.0825	C<H
		Res	12	0.063	0.005				
	S rhizome	Temperature	2	0.021	0.011	10.223	9951	**0.0012**	
		Labile organic C	1	0.003	0.003	3.013	9851	0.1117	20=30, 20=35, 30>35
		Temp x Lab. org. C	2	0.021	0.01	9.921	9952	**0.0016**	
		Res	12	0.012	0.001				
	S roots	Temperature	2	0.026	0.013	1.435	9951	0.2638	
		Labile organic C	1	0.146	0.146	16.286	9834	**0.0029**	
		Temp x Lab. org. C	2	0.03	0.015	1.648	9954	0.2393	C<H
		Res	12	0.108	0.009				

Table 3. Results of PERMANOVA testing for the significance of effects of temperature (20°C, 30°C, and 35°C), nutrient level (Control, Moderate, and High additions) and labile organic C addition (Control and High) on plant biochemical composition. Numbers in bold indicate significant effects ($p < 0.05$). The results of the pair-wise tests are indicated in factors with significant influence.

3.2 Effects of temperature on plant traits

Temperature had an overall significant effect on the plant traits measured in both experiments (Table 4). The maximum quantum yield of PSII (F_v/F_m) revealed that the photosynthetic apparatus maintained its integrity at 30°C. However, F_v/F_m was significantly depressed at 35°C (6% and 42% lower than under control conditions, in the TANOX and TNUT experiment, respectively), suggesting that significant damage was caused by warming (Fig. 3; Table 5).

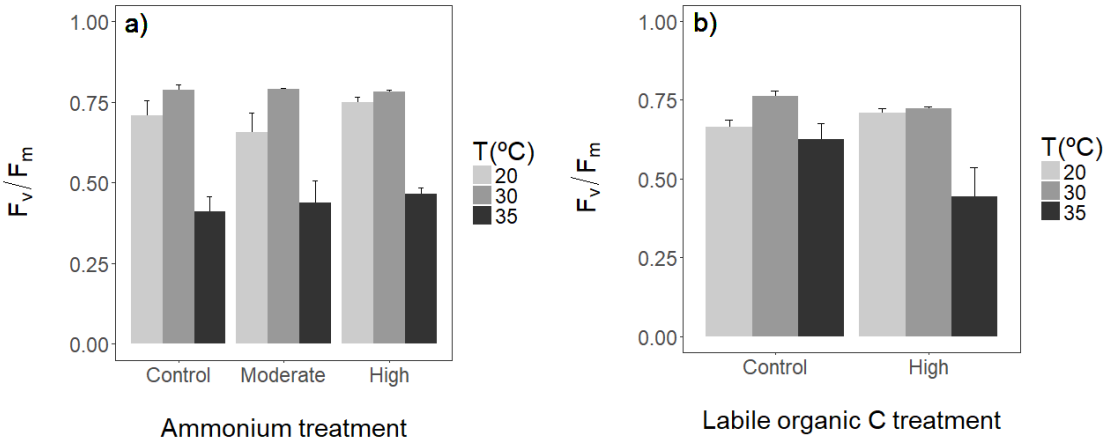


Fig. 3 *Cymodocea nodosa* maximum quantum yield (mean ± SE, *n*=3) at 3 thermal treatments (20°C, 30°C, and 35°C, black, light grey and dark grey respectively) in the (a) TNUT and (b) TANOX experiments.

The incidence of necrosis on the leaves (Fig. 4; Table 5) was low under control conditions (20°C; between 7% and 17% in the TANOX and TNUT experiment, respectively) and at 30°C (<8% in both experiments), but increased significantly to 23% - 33% (depending on the experiment) at 35°C.

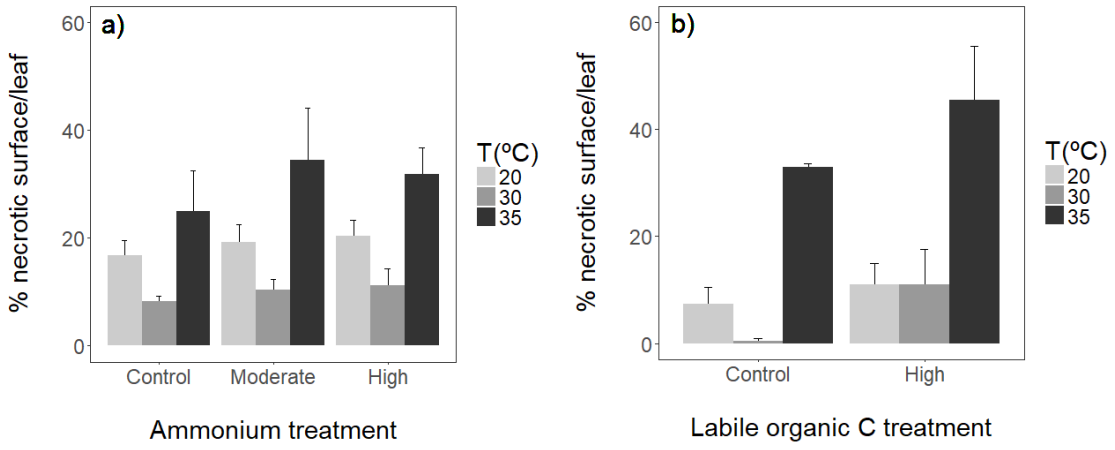
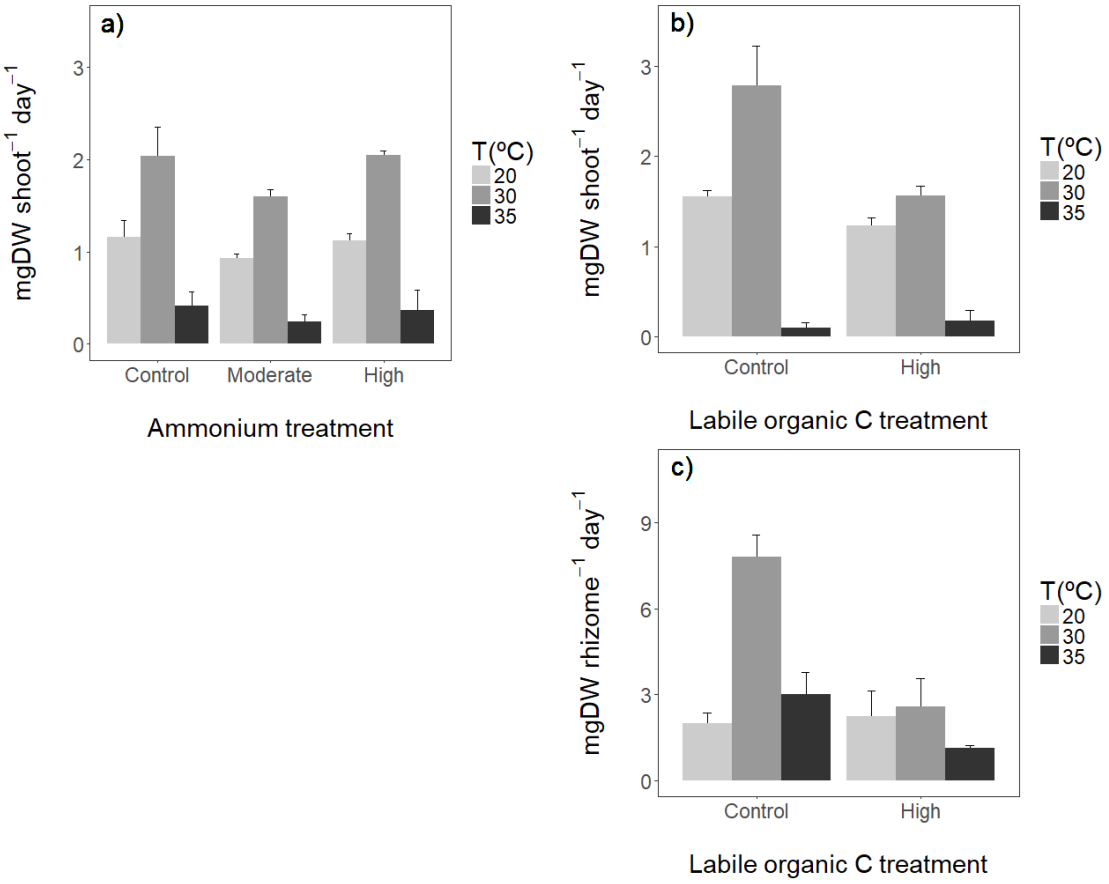


Fig. 4 Leaf necrosis incidence (average necrotic surface per leaf, in %) in *Cymodocea nodosa* (mean ± SE, *n*=3) at 3 thermal treatments (20°C, 30°C, and 35°C, black, light grey and dark grey respectively) in the (a) TNUT and (b) TANOX experiments.

Leaf growth rates (Fig. 5a and b; Table 5) showed higher values at 30°C than under control conditions (45%) and minimum values at 35°C (a decrease of between 63% and 94% relative to control conditions, in the TNUT and TANOX experiment, respectively). Rhizome elongation (only measured in the TANOX experiment) was also significantly higher (74%) at 30°C than at the other two temperatures (Fig. 5c; Table 5).

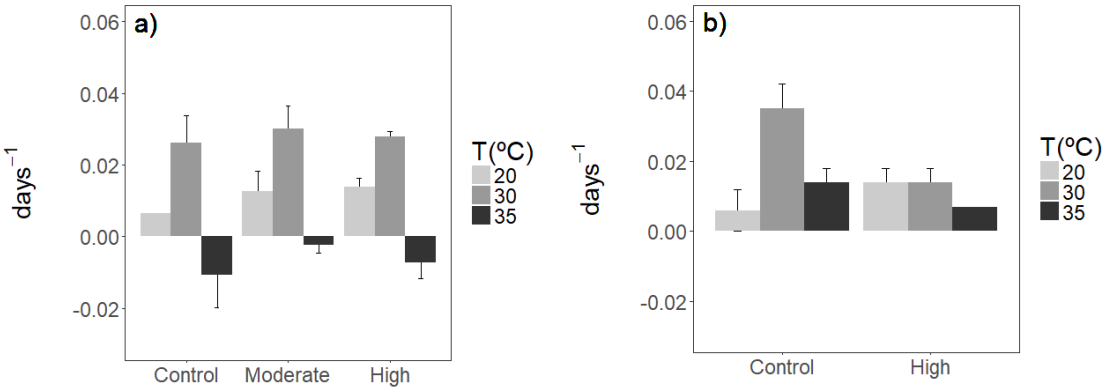


398

399 Fig. 5 *Cymodocea nodosa* growth rate (mean ± SE, *n*=3) at 3 thermal treatments (20°C,
400 30°C, and 35°C, black, light grey and dark grey respectively) in two experiments: (a) Leaf
401 growth rate in the TNUT experiment; (b) Leaf growth rate in the TANOX experiment;
402 (c) Rhizome growth rate in TANOX experiment.

403

404 The shoot demographic balance (i.e. recruitment – mortality) was clearly sensitive to
405 temperature, with a sharp increase (83% on average, relative to the control temperature)
406 under moderate warming (30°C) and a clear decrease under extreme warming (35°C),
407 dropping to negative values in the TNUT experiment (Fig. 6a; Table 5).



408

409 Fig. 6 *Cymodocea nodosa* shoot demographic balance (mean ± SE, *n*=3) at 3 thermal
410 treatments (20°C, 30°C, and 35°C, black, light grey and dark grey respectively) in the (a)
411 TNUT and (b) TANOX experiments.

412

413 **3.3 The effects of additions of ammonium and labile organic C on plant traits**

414 Ammonium addition did not show any effect on any of the plant traits measured (Figs.
415 3a, 4a, 5a and 6a; Tables 4 and 5). The addition of labile organic C did not affect the
416 maximum quantum yield of PSII, the incidence of necrosis or the shoot demographic
417 balance (Figs. 3b, 4b and 6b; Table 5). However, it caused a significant decrease (relative
418 to plants grown in unaltered sediment at 30°C) in leaf and rhizome growth rates (of 44%
419 and 67% respectively; Fig. 5b and c; Table 5).

420

421 **3.4 Interactive effects**

422 Our results did not show any significant interaction between warming and ammonium
423 addition (TNUT experiment) in terms of their effects on plant traits. In contrast, we found
424 interactive effects of warming and the addition of labile organic C to the sediment
425 (TANOX experiment), both overall (Table 4) and in individual traits. Thus, the

426 stimulation of leaf and rhizome growth at intermediate temperatures and the improvement
427 of the shoot demographic balance were cancelled by labile organic C. In addition to this,
428 with the normal organic C content of the sediment, high temperature (35°C) did not alter
429 rhizome growth or the demographic balance, but it did in the sediment with labile organic
430 C added to it.

Experiment	Source	df	SS	MS	Pseudo-F	Unique perms	P
	Main test						
TNUT	Temperature	2	1725.100	862.540	8.612	9950	**0.0030**
	Ammonium	2	50.622	25.311	0.253	9949	0.7830
	Temp x Ammonium	4	144.960	36.241	0.362	9958	0.8326
	Res	18	1802.700	100.150			
TANOX	Temperature	2	105420	52709	105.350	9948	**0.0001**
	Labile organic C	1	435410	435410	870.260	9882	**0.0001**
	Temp x Labile organic C	2	25298	12649	25.282	9948	**0.0001**
	Res	12	6003.800	500.320			

431

432 Table 4. Multivariate PERMANOVA testing for the significance of the general effect of
433 temperature (20°C, 30°C, and 35°C), nutrient level (Control, Moderate, and High
434 additions) and labile organic C addition (Control and High) on plant traits. Numbers in
435 bold indicate significant effects ($p < 0.05$).

436

Variable	Experiment	Source	df	SS	MS	Pseudo-F	Unique perms	P	Pair-wise
Fv/Fm	TNUT	Temperature	2	0.543	0.272	52.937	9952	**0.0001**	20=30>35
		Ammonium	2	0.012	0.006	1.206	9947	0.3277	
		Temp x Ammo.	4	0.012	0.003	0.597	9943	0.6722	
		Res	18	0.092	0.005				
	TANOX	Temperature	2	0.139	0.069	11.488	9956	**0.001**	30>20>35
		Labile organic C	1	0.016	0.016	2.595	9851	0.1297	
		Temp x Lab. org. C	2	0.04	0.02	3.283	9951	0.0555	
		Res	12	0.072	0.006				
Necrosis	TNUT	Temperature	2	1714.000	856.990	8.566	9957	**0.0031**	30<20=35
		Ammonium	2	50.242	25.121	0.251	9954	0.7805	
		Temp x Ammonium	4	144.860	36.215	0.362	9962	0.8436	
		Res	18	1800.900	100.050				
	TANOX	Temperature	2	4060.000	2030.000	23.683	9948	**0.0006**	20=30<35
		Labile organic C	1	354.090	354.090	4.131	9859	0.0655	
		Temp x Lab. org. C	2	62.753	31.376	0.366	9956	0.7057	

		Res	12	1028.600	85.714				
Leaf growth rate	TNUT	Temperature	2	10.538	5.269	53.080	9950	**0.0001**	30>20>35
		Ammonium	2	0.367	0.183	1.848	9950	0.186	
		Temp x Ammonium	4	0.091	0.023	0.228	9951	0.9224	
		Res	18	1.787	0.099				
	TANOX	Temperature	2	12.713	6.357	54.210	9955	**0.0001**	30>20>35
		Labile organic C	1	1.070	1.070	9.125	9851	**0.0058**	C>H
		Temp x Lab. org. C	2	1.327	0.663	5.657	9951	**0.0075**	
		Res	12	1.407	0.117				
Rhizome growth rate	TANOX	Temperature	2	38.329	19.164	15.536	9954	**0.0011**	30>20=35
		Labile organic C	1	23.510	23.510	19.059	9739	**0.0012**	C>H
		Temp x Lab. org. C	2	22.905	11.452	9.284	9948	**0.0035**	
		Res	12	14.803	1.234				
Shoot demographic balance	TNUT	Temperature	2	0.005	0.003	33.785	9964	**0.0001**	30>20>35
		Ammonium	2	0.001	0.001	1.097	9957	0.3624	
		Temp x Ammonium	4	0.001	0.001	0.152	9962	0.9594	
		Res	18	0.001	0.001				
	TANOX	Temperature	2	0.001	0.001	6.767	9952	**0.0119**	30>20=35
		Labile organic C	1	0.001	0.001	3.571	9758	0.0887	
		Temp x Lab. org. C	2	0.001	0.001	5.231	9959	**0.0219**	
		Res	12	0.001	0.001				

Table 5. Results of PERMANOVA testing for the significance of effects of temperature (20°C, 30°C, and 35°C), nutrient level (Control, Moderate, and High additions) and labile organic C addition (Control and High) on each plant trait. Numbers in bold indicate significant effects ($p < 0.05$). The results of the pair-wise tests are indicated in factors with significant influence.

DISCUSSION

Climate change is having an impact on a world that has already been altered by a panoply of local stressors. Our results show how cumulative stress, in this case derived from the joint action of warming and eutrophication, can worsen, either through additive or interactive effects, the negative consequences of each stressor acting in isolation.

As already known, temperature increases can negatively affect different functional mechanisms of seagrasses (Koch et al., 2013). The response patterns and the thresholds involved are largely species specific (Campbell et al., 2006; Collier et al., 2011). In the

452 model species used here (*C. nodosa*), moderate warming seems to be beneficial for the
453 plant, whose performance (photosynthesis, growth and shoot demographic balance)
454 increase at 30°C, relative to those found at the basal spring temperature (control) of 20°C.
455 This pattern is fully consistent in the two experiments we conducted. This is in accordance
456 with previous reports for this species, suggesting an optimum temperature close to 30°C
457 (Olsen et al., 2012; Pérez and Romero 1992; Savva et al., 2018; Terrados and Ros 1995;
458 Tutar et al., 2017). In contrast, plant performance was severely depressed at 35°C; not
459 only relative to the 30°C optimum, but also relative to control conditions. This suggests
460 there is a thermal threshold of clear negative effects on plant activity between 30°C and
461 35°C. This thermal threshold is relatively high (see Lee et al., 2007 for comparisons), and
462 it is in accordance with the subtropical distribution of this species (Green and Short 2003;
463 Reyes et al., 1995) and its facultative habitat in confined environments, where summer
464 temperatures can easily be 5°C above open sea temperatures.

465 Exposure to extreme thermal values damaged the integrity of the photosynthetic
466 apparatus, as shown by a clear drop in F_m/F_v to values below those considered acceptable
467 for healthy plants (0.7-0.8, Campbell et al., 2006; Ralph et al., 1998), as previously found
468 for other seagrass species (e.g. *T. testudinum*; Koch et al., 2007; e.g. *Z. noltii;* Massa et
469 al., 2009). While photosynthesis is depressed, respiration is probably stimulated by
470 thermal stress (not measured in this study; but see Pérez and Romero, 1992), leading to
471 impairment of the C budget (Collier and Waycott, 2014), which could be the cause of the
472 reduced growth and the low to negative shoot demographic balance observed in our
473 experiments. Plants exposed to high temperatures may have to use their energy reserves
474 (stored non-structural carbohydrates) to cope with this stress and the consequent energy
475 requirement (Collier et al., 2011; Massa et al., 2009), probably leading to exhaustion of
476 the internal C reserves (Marín-Guirao et al., 2018). Indeed, thermal stress also affects

other metabolic processes, causing, for instance, oxidative stress (Tutar et al., 2017), and ultimately affecting plant health, which deteriorated in our experiments as shown by the increase in the incidence of necrosis.

Reducing the shoot demographic balance can be critical for *C. nodosa*. This species has a very high shoot turnover, with a yearly shoot mortality reaching 1/2 to 2/3 of the total number of shoots in unaltered meadows. This mortality takes place in late summer to autumn and is balanced by massive recruitment in late spring (Mascaró et al., 2014). Any event altering the shoot demographic balance, such as a heat wave, will cause a drop in seagrass density, eventually leading to meadow extinction. This is relevant for projections of distribution and abundance of this species in future warming scenarios since the frequency and intensity of heat waves are predicted to increase (IPCC, 2013). Those predictions suggest that the threshold temperature (thermal tolerance limit) could be reached during these extreme climate events, mainly in confined areas such as shallow bays or lagoons. However, the threshold is quite unlikely to be reached in the open sea, where warming will be much more moderate and could have beneficial effect on the species which could extend its distribution, maybe at the expenses of the Mediterranean species *P. oceanica*, which is much more sensitive to warming (Marín-Guirao et al., 2016; Olsen et al., 2012).

Regarding eutrophication, *C. nodosa* is affected by an increase in organic matter in the sediment but not by pulses in nutrient concentrations. None of the traits studied were modified by addition of ammonium, despite the high concentrations attained (up to 300 μM) and the fact that ammonium was depleted from the aquaria. A coarse N mass balance, estimating N incorporation in the plant through leaf growth, new shoots and N increase in tissues, suggests that most of this depletion was caused by plant activity, being microbial activity in the sediment the most likely explanation for the rest. Seagrasses

seem to be unable to downregulate N uptake, probably due to a lack of inhibitory feedback mechanisms (Touchette and Burkholder, 2000). This failure in regulation could generate ammonium accumulation in cells, which in turn may have toxic effects (Invers et al., 2004). However, while some species seem to be more vulnerable to this toxicity (e.g. *Z. marina*, Burkholder et al., 1992; van Katwijk et al., 1997; Villazán et al., 2013a; and *Z. noltei*, Moreno-Marín et al., 2016) others show great resistance (*C. nodosa*, Egea et al., 2018 and *Z. marina*, Kaldy et al., 2014). It has been suggested that the key mechanism to endure large ammonium pulses may be an efficient mechanism that is capable of rapidly converting the excess of ammonium into organic forms (Brun et al., 2002; Invers et al., 2004). Second-order (indirect) effects of ammonium pulses, such as an increase in epiphytic load or a decrease in water transparency, were not studied here and cannot be ruled out.

In contrast, the addition of labile organic C had a detrimental effect on plants. The organic additions to the sediment seemed to enhance bacterial respiration and thus oxygen demand, leading to oxygen exhaustion and anoxic conditions (up to -290 mV of redox potential). Under these conditions, sulphate reduction is stimulated, resulting in sulphide accumulation. Consistently with this, we found higher sulphur contents in our exposed plants than in controls. The oxidation of sulphide to sulphur compounds that are further stored in tissues has been shown to be a mechanism that can help cope with sulphide intrusion. However, once the capacity of detoxification of this mechanism is surpassed, the detrimental effects appear (Hasler-Sheetal and Holmer, 2015). Although *C. nodosa* is highly resistant to eutrophication (Oliva et al., 2012), highly negative values (such as those created in our experiment, close to -250 mV) clearly seem to be harmful for plant production and fitness.

526 Beyond the effects of warming and eutrophication highlighted above, and given that both
527 stressors will act jointly in most real-world conditions, the assessment of their potential
528 interactions is of great interest. Our results show that there were no interactive effects
529 between warming and ammonium; but in contrast the effects of warming on key processes
530 (leaf and rhizome growth and the demographic balance) were strongly mediated by the
531 amount of labile organic C in the sediment. The interaction between temperature and
532 organic matter was detected at the individual (leaf and rhizome growth) and population
533 (shoot demographic balance) level, but not at the physiological one (F_v/F_m). Clearly, the
534 processes affected are critical for meadow persistence, which underlines the relevance of
535 such interactions for the prediction of future seagrass meadow dynamics. However, the
536 mechanisms through which these interactions function have not been elucidated by our
537 work. A possible explanation would be a synergistic effect on environmental conditions.
538 This is supported by the fact that the addition of labile organic C and temperature
539 decreased sediment redox potential synergistically, probably through the stimulation of
540 oxygen demand and cascading effects on sulphide production and plant performances.
541 Other mechanisms, including the amplification of sulphide effects by temperature, should
542 not be ruled out.

543 Despite multiple stressors studies have increased in the last decades, our results add
544 evidence to the need to further assess the interactive effects of different stressors, and
545 understanding how the organisms or communities will respond to the impact of multiple
546 co-ocurrent stressors is still a matter of concern (Côté et al., 2016). Seemingly, synergistic
547 effects are quite frequent, as revealed by Crain et al. (2008), which found in a review
548 focused on coastal ecosystems that 36% of the cases examined showed synergy. A
549 thorough literature search on interactive effects on seagrass ecosystems (Table 6)
550 confirms that synergy is more the rule (50%) than the exception (36% additive; only a

551 small part of the studies found antagonistic interaction). There is an urgent need to
552 incorporate those interactive effects to improve predictions of the consequences of
553 climate change in marine ecosystems, which can be seriously underestimated when
554 assessing thermal effects in isolation. In addition, results such as those presented here can
555 support strategies to increase ecosystem resilience to climate change by managing other
556 stressors at a local or regional scale. In this respect, shallow bays and coastal lagoons,
557 which are more vulnerable to both extreme thermal events and eutrophication, may
558 represent a critical scenario for the survival of seagrass species growing close to their
559 upper thermal limit (York et al., 2013, Koch et al., 2007), but also an opportunity to test
560 the above mentioned strategies.

561 Even though our findings, it is important to keep in mind that the results of this work were
562 obtained from a mesocosm experiment focusing only on two factors (warming and
563 eutrophication) without considering any other disturbance that may be found in the
564 environment. In this sense, they should only be extrapolated to natural conditions
565 cautiously. In spite of these limitations, this research highlights the importance of
566 evaluating the impact of global and local stressors jointly; not only to generate more
567 realistic predictions of the impacts that climate change might have, but also to design and
568 implement strategies to improve (or at least not to impair) seagrass resilience to global
569 warming.

Stressor 1	Stressor 2	Stressor 3	Overall interaction type	Species	Source
Temperature	Nitrate and ammonium		Additive	*Zostera marina*	Kaldy 2014
Temperature	Ammonium		Additive	*Cymodocea nodosa*	This study
Temperature	Labile Organic C		Synergy	*Cymodocea nodosa*	This study
Temperature	Acidification		Additive	*Zostera noltii*	Repolho et al., 2017
Temperature	Herbicide		Antagonism	*Halophila ovalis*	Wilkinson et al., 2017
Temperature	Light		Additive	*Zostera muelleri*	York et al., 2013
Temperature	Light		Synergy	*Halodule uninervis* and *Zostera muelleri*	Collier et al., 2011
Temperature	Floods		Synergy	*Amphibolis antarctica*	Fraser et al., 2014
Temperature	Macroalgae		Synergy	*Zostera marina*	Höffle et al., 2011
Temperature	Salinity		Synergy	*Zostera marina*	Salo and Pedersen, 2014
Temperature	Sulfide		Synergy	*Halodule wrightii* and *Thalassia testudinum*	Koch et al., 2007
Nitrate	Acidification		Additive	*Thalassia testudinum*	Campbell and Fourqurean, 2014
Nitrate	Acidification		Additive	*Thalassia hemprichii* and *Halodule uninervis*	Ow et al., 2016
Nitrate and phosphate	Acidification		Additive	*Thalassia testudinum*	Campbell and Fourqurean, 2018
Nitrate, ammonium and phosphate	Waves		Synergy	*Zostera noltii*	La Nafie et al., 2012
Ammonium	Light		Synergy	*Zostera marina*	Villazán et al., 2013b
Ammonium	Macroalgae		Antagonism	*Zostera noltii*	Moreno-Marín et al., 2016
Ammonium	Salinity		Synergy	*Zostera marina*	Villazán et al., 2015
Organic matter	Burial		Synergy	*Posidonia oceanica*	Ceccherelli et al., 2018
Temperature	Ammonium	Light	Synergy	*Zostera marina*	Moreno-Marín et al., 2018
Temperature	Ammonium	Acidification	Additive/Synergy	*Cymodocea nodosa*	Egea et al., 2018
Ammonium	Phosphate	Light	Antagonism	*Zostera noltii*	Brun et al., 2008

570 Table 6. Synthesis of multiple stressors studies on seagrasses and the interaction type effects.

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Author contributions

YO, EG, NS, JMR, JR and MP planned and designed the study. YO, EG and NS conducted fieldwork, performed the experiments, samples processing and lab work. YO and JB analyzed the data. YO, JR and MP led the writing of the paper with contributions from the rest of authors. All Authors reviewed the manuscript.

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