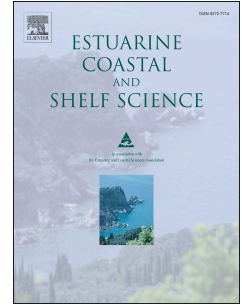


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Epiphytes and nutrient contents influence *Sarpa salpa* herbivory on *Caulerpa* spp vs. seagrass species in Mediterranean meadows.

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15 **Abstract:** Mediterranean seagrass ecosystems are endangered by increased
16 colonization of *Caulerpa* species, which may replace them, affecting key ecosystem
17 processes. The fish *Sarpa salpa* (L.) is one of the main macroherbivores in the western
18 Mediterranean seagrass meadows which is known to feed on a wide range of macroalgae such
19 as *Caulerpa* species. In order to elucidate if this consumption could minimize the spread of
20 invasive species, during summer-autumn 2012, we investigate the importance of *S. salpa*
21 herbivory pressure on *C. prolifera* and *C. cylindracea* compared to *Posidonia oceanica* and
22 *Cymodocea nodosa* in a mixed meadow. A combination of field experiments and dietary
23 analyses were used to investigate consumption rates, dietary contributions, and feeding
24 preferences for the different macrophytes, including the role of epiphytes and nutrient
25 contents in mediating fish herbivory. In summer, *C. nodosa* was the most consumed
26 macrophyte (12.75 ± 3.43 mg WW·d⁻¹), probably influenced by higher fish densities, higher
27 nutritional quality of leaves and epiphytes, and by differences in epiphyte composition.
28 Feeding observations suggest that fish may have a variable diet, although with a consistent
29 selection of mixed patches with *C. nodosa* and *C. prolifera*. Indeed, food choice experiments
30 suggest that when seagrass leaves are not epiphytized, fish prefer feeding on *C. prolifera*. Gut
31 content and stable isotopic analyses supported the dietary importance of epiphytes and *C.*
32 *prolifera* but also suggested that *C. cylindracea* could occasionally be an important food item
33 for *S. salpa*. Our results highlight the role of epiphytes in *S. salpa* feeding decisions but also
34 suggest that *C. nodosa* and *C. prolifera* may have an important contribution to fish diet. The
35 variability in *S. salpa* diet confirm the need to carry out multiple approach studies for a better
36 understanding of its potential influence over different macrophytes species.

37 **Key words:** Food choice, *Sarpa salpa*, *Cymodocea nodosa*, *Posidonia oceanica*,
38 *Caulerpa prolifera*, *Caulerpa cylindracea*, epiphytes, nutrients

39 **INTRODUCTION**

40 Mediterranean seagrass meadows are dominated by *Posidonia oceanica* (L.) Delile
41 (Den Hartog 1970, Thayer et al. 1984), while *Cymodocea nodosa* (Ucria) Ascherson is
42 commonly found in small patches within these meadows (Pérès & Picard 1964). Herbivory
43 rates on these seagrass species are extremely variable according to the available literature (2–
44 57 % of *P. oceanica* leaf productivity, Cebrián et al. 1996a, Prado et al. 2007; 1–50 % of *C.*
45 *nodosa* leaf productivity, Cebrián et al. 1996b). This variability in estimated herbivory has
46 been suggested to be partly a consequence of the different methods employed for
47 quantification (Tomas et al. 2005a). Previous estimates of leaf consumption rates were
48 assessed using indirect methods, such as quantifying herbivore bite marks, which are now
49 known to underestimate seagrass consumption (e.g. Cebrián et al. 1996a) compared to the less
50 frequently-used estimates provided by tethering experiments (e.g. Tomas et al. 2005a, Prado
51 et al. 2007). Direct methods have shown that, in some instances, grazing can be heavy and
52 determine the structure and distribution of temperate macrophyte assemblages (e.g. Tomas et
53 al. 2005a, b, Taylor & Schiel 2010). In addition, these works also provide evidence that
54 herbivory can be highly variable through space and time, displaying different patterns of
55 defoliation between meadows and/or seasons (Prado et al. 2007, 2010, Steele et al. 2014).
56 Nevertheless, further studies are required to compare herbivore impacts on different
57 macrophyte species, including the two main seagrass species and abundant macroalgae, and to
58 determine the role of food preferences in the ecological functioning of mixed Mediterranean
59 seagrass meadows.

60 Mediterranean seagrass ecosystems are endangered by increased colonization by
61 *Caulerpa* species, which may replace them; consequently affecting key ecosystem functions
62 and services (Hendriks et al. 2010). Among the main *Caulerpa* living in the Mediterranean,
63 only the chlorophyte *Caulerpa prolifera* (Forsskål) J.V. Lamouroux, is endemic. It develops

64 in shallow subtidal waters, co-occurring with the seagrasses *C. nodosa* and *P. oceanica*
65 (Vergara et al. 2012, Marco-Méndez et al. 2015). The green alga *Caulerpa cylindracea*
66 (Sonder) [formerly *Caulerpa racemosa* (Forsskål) J. Agardh var. *cylindracea* (Sonder)
67 Verlaque, Huisman et Boudouresque; (hereinafter, *C. cylindracea*, according to Belton et al.
68 2014, Marín-Guirao et al. 2015)], originally described from southwestern Australia, has
69 rapidly spread throughout the western Mediterranean during the last 20 years (Verlaque et al.
70 2000, 2003). The alga has successfully colonized a wide variety of soft and hard substrata,
71 including dead *Posidonia oceanica* rhizomes or “matte” (tough, lignified roots and rhizomes
72 admixed with sediment; Boudouresque & Meisnez 1982) and *C. nodosa* meadows (Vazquez-
73 Luis et al. 2008).

74 Common chemical components such as phenolics (Mariani & Alcoverro 1999; Verges
75 et al. 2007, 2011) are known to deter feeding in algae and terrestrial plants mediating plant–
76 herbivore interactions (Orians et al. 2002; Taylor et al. 2002; Vergés et al. 2007; 2011). In
77 particular, *Caulerpa* species contain different levels of caulerpenyne (Jung et al. 2002), a
78 secondary metabolite that acts as a feeding deterrent that inhibits the growth of
79 microorganisms and is toxic to larvae and adults of potential herbivores (Lemée et al. 1996,
80 Ricci et al. 1999). This chemical deterrence and the consequential lower palability has been
81 often considered one of the main causes for *Caulerpa* species invasion success (Sant et al.
82 1996). However, recent studies suggest that Mediterranean herbivores have evolved the
83 capability to tolerate this secondary metabolite (Cornell & Hawkins, 2003), allowing them to
84 consume large quantities of *Caulerpa* spp (Cebrian et al. 2011; Tomas et al. 2011a,b, Marco-
85 Méndez et al. 2015). Since fish generally have higher mobility and greater consumption rates
86 than invertebrate herbivores, they have been hypothesized to be able to limit the spread of
87 introduced algae (e.g. Weijerman et al. 2008, Vermeij et al. 2009). According to this, fish
88 herbivory pressure on *Caulerpa* species could eventually benefit seagrass species by reducing

89 the proliferation of these species and their negative impact on the dynamics of Mediterranean
90 seagrass meadows (Ruitton et al. 2005).

91 The fish *Sarpa salpa* (L.) is one of the main macroherbivores in the western
92 Mediterranean, and is commonly observed in shallow seagrass meadows and rocky bottoms
93 (Verlaque 1990) feeding on a wide range of macroalgae and seagrasses (Havelange et al.
94 1997). This species has been reported to account for 70 % of the total leaf consumption of *P.*
95 *oceanica* (Prado et al. 2007) and is known to ingest large quantities of *Caulerpa* species such
96 as *C. prolifera* (Marco-Méndez et al. 2015) and *C. cylindracea*, providing at least some
97 resistance to invasion of native assemblages (Tomas et al. 2011b). In general, studies point to
98 higher feeding activity of *S. salpa* in summer to accumulate reserves for the winter period,
99 when fish eat less and adults prepare for reproduction (Peirano et al. 2001). However, *S. salpa*
100 herbivory pressure seems to vary greatly over space and time (Prado et al. 2007, 2010, Steele
101 et al. 2014). It is also influenced by other factors such as macrophyte availability and
102 accessibility, habitat heterogeneity, nutritional quality, human pressure on herbivore
103 populations, herbivore recruitment, predation and patterns of movement (Prado et al. 2008 a,
104 b, 2011).

105 Preferences and feeding rates of marine herbivores may be driven by enhanced
106 nitrogen and protein content, epibiotic load, or lower amounts of chemical and structural
107 components (Mariani & Alcoverro, 1999, Vergés et al. 2007, 2011). Varying levels of
108 structural carbohydrates in seagrass leaves (cellulose), may affect food digestibility and
109 absorption (e.g. Klumpp & Nichols, 1983) and differences in nutritional quality among
110 seagrass species or between seagrasses and epiphytes could result in different levels of
111 herbivory (Alcoverro et al. 1997b, Cebrián & Duarte, 1998, Prado et al. 2010). Furthermore,
112 it has been shown that secondary metabolites of both macroalgae and seagrasses chemically
113 deter herbivores, although inhibition varied between consumers (Vergés et al. 2007, 2011).

114 Given that several factors could be involved in the complex seagrass–herbivore
115 interactions, studies require combined experimental approaches and dietary analyses
116 integrating temporal variability in resource acquisition. Among methods used to quantify
117 dietary contributions, stomach content analysis is the most accurate, although it applies to
118 very short time periods and requires extensive sampling (Legagneux et al. 2007). In contrast,
119 more recent techniques, such as stable isotopes (reviewed in Kelly 2000), provide useful
120 complementary and time-integrative methods in dietary studies (Marco-Méndez et al. 2012),
121 based on the premise that consumers’ tissues will resemble the long-term isotopic
122 composition of the diet (Fry & Sherr 1984, Minagawa & Wada 1984).

123 The aims of this study were to compare the importance of *S. salpa* herbivory on
124 *Caulerpa* species vs. seagrasses in a mixed meadow, and to elucidate if this consumption
125 could eventually control the spread of invasive species. With these aims we investigated
126 summer and autumn abundances and consumption rates of *S. salpa* on *P. oceanica*, *C.*
127 *nodosa*, *C. prolifera* and *C. cylindracea*, as well as their potential relationship with temporal
128 changes in the abundances of those macrophytes in a western Mediterranean mixed meadow.
129 In addition, we investigated whether *S. salpa* feeding preferences, epiphytes presence and
130 nutrient content in macrophytes could explain herbivory pattern observed in the field. To this
131 end, a combination of field experiments and dietary analyses were used to investigate
132 consumption rates, dietary contributions, and feeding preferences for the different macrophyte
133 species, including the role of epiphytes and nutrient contents in mediating herbivory by *S.*
134 *salpa*. We hypothesize that: 1) *S. salpa* abundances and consumption rates will be higher in
135 summer than in autumn influenced by higher fish abundances and macrophytes availability;
136 2) Epiphytes and macroalgae will have lower C:N ratios than seagrasses which will trigger
137 higher herbivory pressure and 3) *Caulerpa* species abundance could be responding not
138 only to seasonal changes but to *S. salpa* herbivory pressure.

139 **MATERIAL AND METHODS**

140

141 **Study site**

142 The study site was located at Cabo de Santa Pola (38° 12'34.56"N, 0° 30'31.55"W,
143 western Mediterranean) in a mixed habitat (depth range: 2-4 m; study area: ~0.75 km²) formed
144 by intertwined patches of variable size of *Posidonia oceanica*, *Cymodocea nodosa*, *Caulerpa*
145 *prolifera*, unvegetated sandy substrate, and rocky substrate covered by *Caulerpa cylindracea*
146 and, other macrophyte species to a lesser extent (e.g. *Cystoseira compressa*; *Dylophus sp*;
147 *Enteromorpha compressa*; *Jania rubens*, *Padina pavonica* and *Halopteris scoparia*). *C.*
148 *cylindracea* was first recorded in 2002 at a site located around ten km north of the study area,
149 where it colonized soft sediments and dead matte of *P. oceanica*. Two months later, it was
150 detected on the rocky platform of our study area (Pena-Martín et al. 2003). Currently, this
151 nonindigenous alga occurs in extensive areas of ecologically important rocky bottoms, as well
152 as on sandy and muddy substrates, and on dead matte of *P. oceanica* (Marín-Guirao et al.
153 2015). It also occurs intermixed with *C. nodosa* in seagrass meadows, with a patchy
154 distribution (Vazquez-Luis et al. 2008).

155 The main aims of this study were to compare the importance of *S. salpa* herbivory on
156 *Caulerpa* species vs. seagrasses in a mixed meadow, to identify mediating factors influencing
157 herbivory and to elucidate if this consumption could eventually control the spread of invasive
158 species. To achieve these goals, we investigated *S. salpa* abundances and consumption rates
159 on *P. oceanica*, *C. nodosa*, *C. prolifera* and *C. cylindracea* and their potential relationship
160 with temporal changes in those macrophytes abundances in a western Mediterranean mixed
161 meadow. The study was carried out in two randomly selected locations (A and B) 2-3 km
162 apart (Fig. 1) and in two different times during 2012, summer (July-August) and autumn
163 (September-October). In summer macrophytes biomasses are expected to be higher in

164 response to temperature, nutrients and light conditions (Alcoverro et al. 1997b) and the
165 feeding activity of *S. salpa* to be more intense in order to accumulate reserves for the winter
166 period (Peirano et al. 2001). In autumn (September-October), both macrophytes biomasses
167 (Alcoverro et al. 1997b) and *S. salpa* feeding activity are expected to decrease before reaching
168 their minimum in winter, when fish eat less and adults prepare for reproduction (Peirano et al.
169 2001).

170

171 **Bottom characterization**

172 At each location (A and B) and study time (hereafter, T1: summer; T2: autumn)
173 seagrasses shoot density (number per m²) was measured by counting shoots in a 40 x 40 cm
174 quadrant placed in three haphazardly selected patches within the mixed habitat. Percentage of
175 bottom covered was estimated visually by scuba divers using a 25 meters tape measure (n = 3)
176 and recording the length covered by the seagrasses and other substrates or macrophytes
177 species (rock, sand, *Caulerpa prolifera*, etc.). Subsequently the data were expressed as
178 percentages of bottom coverage (Sánchez-Lizaso 1993). Because of the heterogeneity of this
179 mixed habitat three additional 20 x 20 cm quadrats were haphazardly selected at each study
180 location and all macroalgae and seagrass within them carefully removed and placed in plastic
181 bags. In the laboratory, all macrophytes were sorted into species, dried for 24 h at 80 °C, and
182 weighed. Accordingly to standardized methods (Romero 1985, Alcoverro et al. 1995, Ruitton
183 et al. 2005), we estimated the percent cover by each macrophyte species relative to the total
184 weight of sample scraped off the rocky substrate. . When during visual characterization two
185 species were highly mixed in the same patch, we recorded it as: e.g. *C. nodosa* and *C.*
186 *prolifera*.

187

188 **Fish abundances and feeding observations**

189 Individuals of *S. salpa* were counted by scuba divers using the visual transect census
190 method (Harmelin-Vivien et al. 1985, Francour 1997). At each time and at each study
191 location, visual censuses were carried out in two different days (T1: one day in July and
192 another in August; T2: one day in September and another October) recording 16 censuses
193 each day ($n = 32$ total censuses per time and location). All *S. salpa* encountered along
194 haphazardly located 50 m² line transects were counted (ind. m⁻²), and their average size (total
195 length, TL) estimated. All fish counts were performed at the same time of the day
196 (approximately between 10:00 and 13:00 h) to minimize possible variability due to
197 differences in fish behaviour (Spyker & Van Der Berghe 1995).

198 The feeding activity of *S. salpa* was also recorded through visual observations. In this
199 case, scuba divers recorded a total of 18 schools of fish feeding at each time and study
200 location (each ca. 7 min in duration). On each occasion, we followed a school of fish and
201 recorded the number of individuals within the school, their average size, and if they were
202 swimming or feeding, in which case the food items consumed were recorded. The percentage
203 of individuals swimming or feeding on the different items was estimated relative to the total
204 of individuals observed.

205

206 **Tethering experiments**

207 Consumption rates of *C. prolifera*, *C. cylindracea*, *C. nodosa* and *P. oceanica* by *S.*
208 *salpa* were estimated with tethering experiments deployed within monospecific patches at the
209 two different times and locations of study (one tethering line per species, time, and location;
210 i.e., a total of 4 tethering lines per macrophyte during the study). Each tethering line consisted
211 of 18 replicates, with similar amounts of freshly collected macrophyte biomass (collected the
212 morning of the experiment). Tethering lines consisted of floating replicates (by using small
213 buoys) to avoid benthic invertebrate herbivores (e.g. sea urchins). Floating replicates were

214 tied to a thin cord and deployed in the field for a week; the lines were elevated a few
215 centimeters from the bottom but integrated at the height of the surrounding vegetation, mixing
216 them within the monospecific macrophyte patches. Each end of the line was secured to the
217 bottom with rebar stakes. Controls for changes in wet weight unrelated to herbivory were
218 simultaneously made to each of the tethering experiment performed during the study. For
219 each species, 18 control replicates of identical portions of macrophyte (individually protected
220 from herbivores by 0.5 cm² mesh cages) were deployed in the field during the same period
221 (see Tomas et al. 2011b). To avoid any interference that fouling organisms could cause in the
222 light entering the mesh, cages were checked every day and cleaned when needed. All
223 replicates were cut down to remove previous herbivore marks and blotted dry of excess water
224 before measuring initial and final wet weight (3 g wet weight per replicate). After a week,
225 tethering and control replicates were collected and biomass consumption by *S. salpa*, whose
226 bite marks are easily distinguishable (e.g. Tomas et al. 2005b), was estimated as $[(H_i \times C_f / C_i)$
227 $- H_f]$, where H_i and H_f were initial and final wet weights of tissue exposed to herbivores, and
228 C_i and C_f were initial and final weights in controls (Cronin & Hay 1996; Parker & Hay 2005;
229 Tomas et al. 2011a, b). Macrophytes consumption was expressed as mg of wet weight
230 consumed per day.

231

232 **Food choice experiments**

233 Given the high consumption of *Caulerpa* spp by *S. salpa* shown in previous studies
234 (Tomas et al. 2011b, Marco-Méndez et al. 2015), and the reported importance that *C. nodosa*
235 and *P. oceanica* can have in its diet (Prado et al. 2007a; Marco-Méndez et al. 2015), we
236 conducted paired feeding experiments to examine the relative palatability of *C. prolifera*, *C.*
237 *cylindracea*, *C. nodosa* and *P. oceanica*. In addition, since epiphytes can have a large
238 influence on herbivore feeding choices (Marco-Méndez et al. 2012), paired experiments were

239 carried out with epiphytized and non-epiphytized seagrass leaves (*Caulerpa* species were not
240 epiphytized). These experiments did not only test fish selectivity for seagrass or *Caulerpa*
241 species but also helped to elucidate whether the manifested selectivity is consistent in
242 presence and absence of epiphytes in seagrass leaves. Despite seagrass are naturally
243 epiphytized, these experiments will help to understand whether *S. salpa* consumption rates
244 and feeding behavior observed in the field could be related to food preferences and epiphytes
245 presence. Food choice experiments were conducted in summer, when macroalgae and
246 epiphyte biomasses have maximum values and they undergo the highest pressure from *S.*
247 *salpa* (Alcoverro et al. 1997a). Experiments were deployed in large sandy patches (ca. 2-4 m
248 depth; at least 5 m away from rocks, seagrasses or macroalgae) to ensure that no other
249 macrophytes could interfere with fish feeding choices and that invertebrate herbivores did not
250 have access to experimental setups. A total of 9 paired floating tethering experiments were
251 carried out with the following paired combinations: 1) *C. nodosa* epiphytized vs. *C. prolifera*
252 ; 2) *C. nodosa* epiphytized vs. *C. cylindracea*; 3) *P. oceanica* epiphytized vs *C. prolifera* ; 4)
253 *P. oceanica* epiphytized vs *C. cylindracea* ; 5) *C. nodosa* non-epiphytized vs. *C. prolifera* ; 6)
254 *C. nodosa* non-epiphytized vs. *C. cylindracea* ; 7) *P. oceanica* non-epiphytized vs. *C.*
255 *prolifera*; 8) *P. oceanica* non-epiphytized vs. *C. cylindracea* and 9) *C. prolifera* vs. *C.*
256 *cylindracea*. For each experiment, similar amounts of freshly collected algal and seagrass
257 biomass were offered in pairs (ca. 3 g wet weight). Replicate pairs (n = 18) and their
258 respective controls (individually protected from herbivores by 0.5 cm² mesh cages) were
259 deployed at least 1 m apart and collected after four days. Consumption was estimated as for
260 tethering experiments and expressed as mg wet weight lost by *S. salpa* bite marks. Despite the
261 amount of epiphytes was not quantified before and after experiments, we used control leaves
262 to make some calculation in order to estimate the amount of epiphytes potentially growing in
263 *C. nodosa* and *P. oceanica* leaves offered in food choice experiments. Estimates of epiphytic

264 loads growing on *P. oceanica* leaves offered with *C. prolifera* were 219.02 ± 8.22 mg DW
265 per shoot and with *C. cylindracea* was 279.51 ± 8.17 mg DW per shoot. For *C. nodosa* leaves
266 offered with *C. prolifera* estimated loads were 100.46 ± 3.46 mg DW per shoot and with *C.*
267 *cylindracea* 138.36 ± 5.57 mg DW per shoot. Differences in the amount initially offered with
268 leaves possibly respond to natural differences between shoots collected. We considered that
269 the most important changes in the epiphytic community occurring during four days would be
270 in terms of biomass, which is also related with epiphytic coverage, so using control leaves for
271 weight correction would resolve this issue. Despite we did not analyzed the epiphytic
272 community before and after the experiments, we think that giving the short duration of the
273 experiments, changes in the community composition in terms of taxa composition would
274 possibly be small.

275

276 **Gut contents, stable isotope analyses and nutrient contents**

277 A total of 26 individuals of *S. salpa* (average length: 23.12 ± 0.62 cm) were
278 haphazardly collected within the area for a dietary study. Since individuals were caught at two
279 different moments, we studied them separately ($n = 13$ individuals per group or school). We
280 used all individuals for gut content analyses and 10 individuals ($n = 5$ from each school) for
281 nutrient content and stable isotope analyses (SIA). In the laboratory, fish muscle was isolated
282 for SIA and nutrient content analysis. Gut contents were extracted and food items separated
283 under the microscope (eg., *P. oceanica* leaves, *C. prolifera*, *C. cylindracea* and epiphytic
284 macroalgae). Each fraction was dried to constant weight at 60°C .

285 Samples of *Caulerpa* and seagrass species were haphazardly collected from the study
286 area for SIA and nutrient content analyses. These samples included: *C. prolifera*, *C.*
287 *cylindracea*, *P. oceanica* and *C. nodosa* epiphytized leaves with and without epiphytes ($n = 5$
288 of each type), as well as epiphytes themselves ($n = 5$ of each type). The latter included both

289 epifauna (heterotrophic metazoans) and epiflora (macroalgae). Samples from schools and
290 food resources were dried to constant weight at 60° C and ground to fine powder for
291 determination of nutrient contents (C:N) and isotopic signatures ($\delta^{15}\text{N}$ and $\delta^{13}\text{C}$). Analyses
292 were carried out with an EA-IRMS (Thermo Finnigan) analyzer in continuous flow
293 configuration at the Technical Unit of Instrumental Analyses (University of La Coruña,
294 Spain). The average difference in isotopic composition between the sample and reference
295 material ($\delta_{\text{sample-standard}}$, expressed in ‰) corresponds to:

$$296 \quad [(R \text{ sample} - R \text{ standard}) / R \text{ standard}] \times 1000 = \delta_{\text{sample-standard}}$$

297 where R sample is the $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$ ratio in the sample; R standard is the $^{13}\text{C}/^{12}\text{C}$ or
298 $^{15}\text{N}/^{14}\text{N}$ ratio for the reference material (i.e. CaCO_3 from belemnite (PBD) for $\delta^{13}\text{C}$ and
299 atmospheric nitrogen for $\delta^{15}\text{N}$ measurements), calibrated against an internal standard (i.e.
300 atropine, IAEA and/or UGS).

301

302 **Epiphytic community**

303 The epiphytic community of both *P. oceanica* and *C. nodosa* was investigated in
304 shoots ($n = 10$) collected at the two study times (summer and autumn 2012). For each shoot
305 the oldest leaf was selected as representative of the epiphyte community during the entire life
306 span of the shoot (Prado et al. 2008a). Epiphytic cover (%) on the leaf surface was estimated
307 visually, and then organisms were scraped off gently for identification to genus level under
308 the microscope. Finally, epiphytes were dried to a constant weight at 60° C for biomass
309 determination ($\text{mg DW}\cdot\text{cm}^{-2}$)

310

311 **Data analyses**

312 The significance of differences in the cover of each macrophyte species (percentage of
313 bottom covered and rocky substrate covered) between “Time” (fixed factor with two levels)

314 and “Location” (random factor with two levels) was investigated using the PERMANOVA+
315 β 20 software package (Anderson and Gorley, 2007).

316 Differences in consumption rates by *S. salpa* among macrophyte species, times (T1:
317 summer; T2: autumn) and locations (A and B) were investigated with a three-way ANOVA
318 design with two fixed factors (“Macrophyte” and “Time”) and a random orthogonal factor
319 (“Location”). The factor “Macrophyte” had four levels (*C. prolifera*, *C. cylindracea*, *C.*
320 *nodosa*, *P. oceanica*). Factors “Time” and “Location” both had two levels in all analyses.

321 Differences in the abundances of *S. salpa* during the study were analyzed with a two-
322 way ANOVA with “Time” and “Location” as fixed and random factors, respectively.

323 A two-way ANOVA was also used to analyze differences in the number of epiphyte
324 taxa, cover and biomass between *P. oceanica* and *C. nodosa* leaves during the two times of
325 study. Factors “Macrophytes” and “Time” were both fixed and had two levels.

326 Differences in isotopic signatures ($\delta^{15}\text{N}$ and $\delta^{13}\text{C}$) and nutrient content (C: N molar
327 ratio) among food resources were tested through a one-way ANOVA with 8 levels (*C.*
328 *prolifera*, *C. cylindracea*, *P. oceanica* epiphytized, *P. oceanica* non-epiphytized, *C. nodosa*
329 epiphytized, *C. nodosa* non-epiphytized, epiphytes of *C. nodosa* and epiphytes of *P.*
330 *oceanica*). Differences in the mean isotopic signatures between the two different schools of
331 fish were subjected to standard t-tests. The IsoSource (Phillips & Gregg 2003) isotope mixing
332 model was used to identify the contributions of each food source to the diets of school 1 and 2
333 separately. Since results by Prado et al. (2012) concluded that there is a strong dietary effect
334 on fractionation (i.e. seagrass, macroalgae, and omnivorous diet fractionations were different)
335 and both schools of *S. salpa* were collected where all those diets were available, the model
336 was run with the means of the fractionation values found for seagrass and macroalgae diets
337 (0.63 ± 0.29 ‰ for $\delta^{15}\text{N}$ and 2.49 ± 0.25 ‰ for $\delta^{13}\text{C}$, means \pm SE). Since seagrasses have very
338 low digestibility, those mean values were considered more accurate than assuming the

339 theoretical 3.4‰ enrichment between trophic levels. The input parameters for the model were
340 the isotopic values of the consumer and trophic resources (measured in this study) and the
341 overall fractionation rates (Mean \pm SE). Since no significant differences were found between
342 epiphytes of *C. nodosa* and epiphytes of *P. oceanic* and this prevents the detection of
343 differences by the model (Phillips & Gregg 2003), $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values were averaged to run
344 the model. We also used averaged $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values for *C. cylindracea* and for *C.*
345 *prolifera*, since no significant differences were found between their $\delta^{15}\text{N}$ values, despite $\delta^{13}\text{C}$
346 values differed.

347 ANOVA assumptions of normality and homogeneity of variance were assessed with
348 the Kolmogorov-Smirnov and Cochran's C- tests, respectively. When necessary, an
349 appropriate transformation was performed before further analysis. When assumptions were
350 not met, the level of significance was set at 0.01 to reduce the possibility of committing Type
351 I errors (Underwood 1997). Student-Newman-Keuls post-hoc tests were used to single out
352 significant groupings. The statistical tests were done using PASW software and GMAV 5
353 software (University of Sydney, Australia).

354 The n-MDS ordination (Bray-Curtis similarity index), ANOSIM and SIMPER
355 (available in the PRIMER-E v.6 software package, Clarke & Warwick 1994) were applied to
356 stomach contents (percentage) and epiphytic assemblages (presence-absence transformation).

357 Wilcoxon signed-ranks paired test was applied to food-choice experiments, due to lack
358 of normality and homoscedasticity of data.

359 Pearson's product-moment correlation coefficient (r) was used to test how fish
360 densities correlate with feeding rates and whether these two variables correlate negatively with
361 macrophytes cover.

362

363 RESULTS

364

365 **Bottom characterization**

366 No significant differences were found in the shoot density of *Posidonia oceanica* and
367 *Cymodocea nodosa* either between times or between locations (Summer, *C. nodosa*: $1029.2 \pm$
368 217.4 shoots·m⁻²; *P. oceanica*: 478.1 ± 66.6 shoots·m⁻²; Autumn, *C. nodosa*: 875.0 ± 219.7
369 shoots·m⁻²; *P. oceanica*: 367.7 ± 45.1 shoots·m⁻²).

370 PERMANOVA analyses for bottom coverage did not showed significant effects
371 between “Times” ($P = 0.5296$) or “Location” ($P = 0.317$). The highest percentages of cover
372 were recorded for *P. oceanica* (Summer: 46.2 ± 6.6 %; Autumn: 34.6 ± 8.4 %; average of A
373 and B) and *Caulerpa prolifera* (T1: 19.7 ± 6.8 %; T2: 27.5 ± 5.3 %; average of A and B). On
374 rocky substrates, no significant effects were found between “Times” ($P = 0.992$) or
375 “Location” ($P = 0.988$). The highest percentage of rocky substrate covered was recorded in
376 summer for *C. prolifera* (48 ± 22.2 %). *Caulerpa cylindracea* and *P. oceanica* recorded
377 similar percentages during the study, with values slightly higher in summer (8.65 ± 1.74 %
378 and 8.98 ± 5.28 % respectively) than in autumn (5.95 ± 3.54 % and 4.6 ± 4.6 %). The
379 remaining percentages corresponded to other macroalgae species identified (*Cystoseira*
380 *compressa*; *Dylophus sp*; *Enteromorpha compressa*; *Jania rubens*, *Padina pavonica* and
381 *Halopteris scoparia*).

382

383 **Herbivore densities and feeding observations**

384 There was a significant “Time x Location” interaction in the abundance of *S. salpa*.
385 The highest abundances reported during the study were those recorded in summer and
386 location A (0.56 ± 0.15 ind·m⁻²) (Two way ANOVA; $p < 0.01$; Fig. 2A).

387 Feeding observations showed that individuals fed on a variety of species during
388 summer (*P. oceanica*, *C. nodosa*, *C. prolifera*, and other algae) but mainly on *C. prolifera* and

389 *C. nodosa* during autumn (Fig. 2B). The average fish sizes recorded for the schools observed
390 during the study were in summer: 15.72 ± 1.14 cm in location A and 17.78 ± 1.22 cm in
391 location B; while in autumn: 13.05 ± 2.25 cm in location A and 12.5 ± 2.58 cm in location B.
392 These sizes were considered small-medium size individuals (small < 17cm and medium 17-29
393 cm), based on Francour (1997),

394

395 **Tethering experiments**

396 There were significant differences for the interaction “Macrophyte x Time” (Fig. 2C;
397 Table 1). Consumption rates of *C. nodosa* were only significantly higher than the
398 consumption recorded for the other macrophyte species in summer. In addition, consumption
399 rates of *C. nodosa* in summer were also significantly higher than in autumn (0.51 ± 0.13 % of
400 wet plant biomass per day). Despite SNK analyses did not detect further significant
401 differences between the rest of macrophyte species or times, consumption of *C. prolifera*
402 tended to be higher in summer; *P. oceanica* displayed low but consistent consumption rates,
403 and *C. cylindracea* showed no herbivory (Fig. 2C; Table 1).

404

405 **Correlations between bottom coverage, herbivore abundances and consumption** 406 **rates**

407 No significant correlations were found between any of the variables analyzed. Bottom
408 coverage of *C. nodosa* and *P. oceanica* displayed a tendency to correlate negatively with fish
409 abundances ($r = -0.70$; -0.550 respectively) and *C. prolifera* and *P. oceanica* with
410 consumption rates ($r = -0.350$; -0.068 respectively). Consumption of *C. nodosa* showed a
411 tendency to correlate positively with fish abundances ($r = 0.070$).

412

413 **Food choice experiments**

414 *Sarpa salpa* displayed higher consumption rates of *C. prolifera* relative to *C. nodosa*
415 non-epiphytized (NE) (Fig. 3A) but not of *C. cylindracea* vs. *C. nodosa* non-epiphytized (NE)
416 (Fig. 3B). Regarding *P. oceanica*, a significant higher consumption of *C. prolifera* was
417 recorded vs. *P. oceanica* non-epiphytized (NE) (Fig. 3C) but no consumption of *C.*
418 *cylindracea* or *P. oceanica* (NE) was detected (Fig. 3D). In presence of epiphytes, no
419 significant differences were found either in the consumption of *C. nodosa* (E) vs. *C. prolifera*
420 or in that of *C. nodosa* vs. *C. cylindracea* (Fig. 3E, F). Similarly, no significant differences
421 were found either in the consumption of *P. oceanica* epiphytized (E) vs. *C. prolifera* or in *P.*
422 *oceanica* (E) vs. *C. cylindracea* (Fig. 3G, H). Finally, the consumption of *C. prolifera* was
423 significantly higher than *C. cylindracea* (Fig. 3I). The highest consumption rates for *C.*
424 *prolifera* were observed vs. *C. nodosa* non-epiphytized (2.58 ± 0.91 mg WW·d⁻¹), followed
425 by those observed vs. *C. cylindracea* and *P. oceanica* non-epiphytized (1.07 ± 0.49 mg WW·
426 d⁻¹; 0.34 ± 0.17 mg WW· d⁻¹ respectively).

427

428 **Gut contents**

429 Gut contents of *S. salpa* individuals from school 1 comprised epiphytes (6.7 %), *P.*
430 *oceanica* (39.7 %) and *C. prolifera* (53.5 %), while school 2 samples showed a diet of *P.*
431 *oceanica* (0.5 %), *C. prolifera* (31.8 %) and *C. cylindracea* (67.7 %).

432 *n*-MDS ordination of the gut items showed different groupings between individuals
433 from schools 1 and 2. ANOSIM results confirmed that gut contents of these two schools were
434 significantly different (Global R: 0.48; $p = 0.001$). The average similarity among school 1 gut
435 contents was 42.87% and school 2 was 59.85 %. The average dissimilarity between the two
436 schools of fish was 80.81 %, mostly due to *C. cylindracea* (41.89 %), *C. prolifera* (29.44 %)
437 and *P. oceanica* (24.52 %).

438

439 **Stable isotope analyses**

440 Both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures showed significant differences among food items (one-
441 way ANOVA, $p < 0.001$; Fig. 4A; Table 2). The highest $\delta^{15}\text{N}$ values were recorded for *C.*
442 *prolifera* and *C. cylindracea* (7.25 ± 0.27 ‰ and 7.59 ± 0.07 ‰ respectively) and the lowest
443 for *P. oceanica* epiphytized and non-epiphytized (4.49 ± 0.07 ‰ and 4.36 ± 0.09 ‰
444 respectively). For $\delta^{13}\text{C}$, the highest values were recorded for *C. nodosa* non-epiphytized (-
445 9.58 ± 0.01 ‰) and the lowest for *C. cylindracea* (-16.67 ± 0.11 ‰), and the epiphytes from
446 *P. oceanica* and *C. nodosa* leaves (-17.06 ± 0.17 ‰; -15.93 ± 0.23 ‰ respectively).
447 Regarding consumers, significant differences were found in the $\delta^{15}\text{N}$ values between the two
448 *S. salpa* schools ($t = 5.004$; $df = 7.527$; $p = 0.001$) and these values (school 1: 12.85 ± 0.46
449 ‰; school 2: 9.95 ± 0.35 ‰) were closer to *C. cylindracea* and *C. prolifera* values. In
450 contrast, no significant differences were found for $\delta^{13}\text{C}$ signals between the two schools ($t =$
451 2.620 ; $df = 7.824$; $p = 0.31$) and values (school 1: -16.23 ± 0.42 ‰; school 2: -17.90 ± 0.35
452 ‰) lay much closer to *C. cylindracea*, *C. prolifera* and epiphytes (including both
453 ‘macroalgae’ and metazoans), than to seagrass values (Fig. 4A; Table 2).

454 Results from the IsoSource model indicated that, in the long term, the diet of both
455 schools of *S. salpa* consisted of *Caulerpa* spp, epiphytes and seagrasses (school 1, *P.*
456 *oceanica*: 20 %; *C. nodosa*: 12 %; epiphytes: 28 %; *Caulerpa* spp: 32 %; school 2, *P.*
457 *oceanica*: 10 %; *C. nodosa*: 16%; epiphytes: 38 %; *Caulerpa* spp: 30 %; both at the percentile
458 50%).

459

460 **Nutrient contents in seagrass leaves and epiphytes**

461 There were significant differences among C:N molar ratios of food items, with the
462 highest values found for *P. oceanica* non-epiphytized and epiphytized (34.06 ± 0.45 ; $29.93 \pm$
463 1.48 respectively) and the lowest for both types of epiphytes (epiphytes of *C. nodosa*: $14.66 \pm$

464 0.33; epiphytes of *P. oceanica*: 10.06 ± 0.98 ; one-way ANOVA, $p < 0.001$; Fig. 4B; Table 2).
465 For *S. salpa*, no differences were found in the C:N molar ratios between the two schools (3.47
466 ± 0.01 for both; $t = -0.188$; $df = 7.957$; $p = 0.855$).

467

468 **Epiphytic community**

469 Significant differences were found in the epiphytic biomass due to the interaction
470 “Macrophyte x Time” (Two way ANOVA; Table 3). *C. nodosa* supported the highest
471 epiphytic biomass in autumn (6.233 ± 0.284 mg DW·cm⁻²; Table 3) but no differences were
472 detected between the epiphytic biomass of *C. nodosa* in summer and the recorded in *P.*
473 *oceanica* leaves, which epiphytic biomass was similar between the two seasons (two-way
474 ANOVA; Table 3). We found significant differences in leaf epiphytic cover with respect to
475 “Macrophyte” and “Time” (two-way ANOVA; Table 3). During the study, the recorded
476 values were consistently higher for *C. nodosa* vs. *P. oceanica* leaves and in autumn vs.
477 summer (Table 3). Concerning the number of epiphytic taxa, significant differences were
478 found for the interaction “Macrophytes x Time” (two-way ANOVA, $p < 0.01$; Table 3). The
479 highest number of epiphytic taxa was found on *C. nodosa* leaves at both times of study and
480 the lowest was recorded on *P. oceanica* leaves in autumn.

481 n-MDS ordination of epiphytic taxa displayed four distinctive groupings considering
482 times (T1: summer; T2: autumn) and seagrass species (*C. nodosa*; *P. oceanica*) (one-way
483 ANOSIM, four levels: Global $R = 0.749$, $p = 0.001$). SIMPER analyses indicated that the
484 epiphytic community on *C. nodosa* leaves displayed an average similarity of 70.19 % at time
485 and 82.97 % at time 2. The epiphytes on *P. oceanica* had an average similarity of 63.14 % at
486 time 1 and 65.33 % at time 2. The average dissimilarity between *C. nodosa* and *P. oceanica*
487 was 64.50 % at time 1 ($R = 0.85$; $p = 0.001$) and 62.75 % in time 2 ($R = 0.99$; $p = 0.001$),
488 mainly due to *Myrionema magnusii*, *Ceramium* sp, *Lyngbya* sp and *Sphacelaria cirrhosa*. The

489 epiphytic community on *C. nodosa* leaves showed an average of dissimilarity between time 1
490 and time 2 of 53.79 % mainly due to *Sphacelaria cirrhosa*, *Myriactula gracilis* and
491 *Cladophora* sp. ($R = 0.90$; $p = 0.001$) while *P. oceanica* epiphytic community did not display
492 significant dissimilarity between times (32.55 %; $R = -0.092$; $p = 0.99$). The average
493 dissimilarity between T1-Sum-CE and T2-Aut-PE was 63.91 % and between T2-Aut-CE and
494 T1-Sum-PE was 63.54 %, due in both cases mainly to *Myrionema magnusii* ($R = 0.85$; $p =$
495 0.002 and $R = 0.92$; $p = 0.001$ respectively).

496 **DISCUSSION**

497 This study points to seagrass *Cymodocea nodosa* and green alga *Caulerpa prolifera* as
498 the “most consumed” and the “most preferred” food species, respectively, by the
499 Mediterranean fish *Sarpa salpa*. In summer, *C. nodosa* recorded the highest consumption
500 rates in the mixed meadow, which seems to be related to higher fish abundances. *C. prolifera*
501 was the most preferred macrophytes in food choice experiments but different nutritional
502 content and epiphyte presence likely explains why the preference of *S. salpa* for *C. prolifera*
503 was not sustained vs. epiphytized leaves and therefore did not deflect herbivory pressure on
504 the most epiphytized and nutritious seagrass *C. nodosa*, the “most consumed” macrophyte in
505 the mixed meadow. Our results highlight the possible mediating role of epiphytes and nutrient
506 contents in *S. salpa* selectivity. However, results also show the high variability in *S. salpa* diet
507 and herbivory pattern as a consequence of the multiple factors potentially involved.

508 Tethering experiments showed that *C. nodosa* was the most consumed macrophyte,
509 recording in summer consumption rates significantly higher than the reported for the other
510 macrophytes species during the whole study. Despite analyses did not detect further
511 significant differences among the rest of the species, the consumption of *C. prolifera* in
512 summer tended to be higher than in autumn (~2.5 times), while consumption of *P. oceanica*
513 was consistently low and no consumption of *C. cylindracea* was detected during the study.
514 Our results evidence that herbivory on *C. nodosa* can even exceed some previous estimates on
515 *P. oceanica* (ca. 2 times higher in our study than in Prado et al. 2007). The high variability
516 observed during the study concurs with the high temporal and spatial variability in the
517 previous estimates of *S. salpa* herbivory on *P. oceanica* (Prado et al. 2007, Tomas et al.
518 2005a). In addition, results also suggest that *C. prolifera* and *C. nodosa* bottom coverage tend
519 to decrease with higher consumption and fish abundances, reinforcing the idea that herbivory
520 on these macrophytes species can also be important (see also Marco-Méndez et al. 2015,

521 Tomas et al. 2011b) and should be considered when studying herbivory in Mediterranean
522 seagrass meadows.

523 The high variance in herbivory has been partially attributed to changes in herbivore
524 abundance and distribution, which can be a consequence of the interaction among recruitment
525 rates (Camp et al. 1973), predation effects (McClanahan et al. 1994) or fishing pressure
526 (Klumpp et al. 1993; Prado et al. 2008). In addition, the fish *S. salpa* displays seasonal
527 mobility patterns according to nutritional and life cycle needs. This accounts for massive
528 schools of fish feeding actively in summer on seagrass meadows in order to accumulate
529 reserves for the winter period, when fish eat less, migrate to greater depths and prepare for
530 reproduction (Peirano et al. 2001). This seasonal migration explains the high temporal
531 variability in the abundances of *S. salpa* individuals detected in our study, with the highest
532 fish densities during summer (up to $0.56 \pm 0.15 \text{ ind}\cdot\text{m}^{-2}$ in location A) but decreasing during
533 autumn (Tomas et al. 2005a, Prado et al. 2007). Since the significantly higher fish abundance
534 in location A was recorded in summer, it was probably related to variability in the mobility
535 pattern within the home range of the species (ca. 4.3 ha; Jadot et al. 2002, 2006), rather than
536 to spatial differences in recruitment rates, predation or overfishing. Accordingly, temporal
537 variability in fish abundance strongly influenced the more intense herbivory in summer,
538 especially on *C. nodosa*, and the low consumption rates of all macrophyte species during the
539 autumn (Ruitton et al. 2006, Tomas et al. 2011b). In fact, our results suggest that *C. nodosa*
540 consumption tend to increase and its coverage to decrease with higher fish densities.
541 Nevertheless, these results contrast with a previous study carried out in a differently located
542 mixed meadow (Marco-Méndez et al. 2015), where herbivory by *S. salpa* on *C. nodosa* in late
543 summer was not detected despite similar fish densities and habitat features. Furthermore, the
544 lack of consumption of *C. cylindracea* detected by the tethering experiment, feeding
545 observations, and food choice trials contrasts with its presence in gut contents and with the

546 findings of Tomas et al. (2011b), where *S. salpa* consumed large quantities of that invasive
547 alga. This apparent contradiction between studies reinforces the idea that herbivory varies
548 strongly both spatially and temporally (Tomas et al. 2005a, Prado et al. 2008b). It is not only
549 influenced by temporal changes in fish abundances but probably also by their home-range
550 size, habitat selection or variability in individual behavior (Jadot et al. 2002, 2006). Plant
551 availability and accessibility or feeding preferences for some macrophyte species could also
552 be mediating herbivory on Mediterranean seagrasses meadows (Prado et al. 2008b, 2009,
553 2010).

554 Food choice experiments recorded the highest consumption on *C. prolifera* vs. *C.*
555 *nodosa* non-epiphytized leaves (2.58 ± 0.92 mg WW·shoot⁻¹·d⁻¹) and showed that *S. salpa*
556 individuals only preferred to feed on *C. prolifera* vs. *P. oceanica* and *C. nodosa* when
557 epiphytes were removed, pointing to the mediating role of epiphytes in herbivore selectivity
558 (Tomas et al. 2005b, Marco-Méndez et al. 2012, 2015). In addition, their consistent
559 preference for *C. prolifera* vs. *C. cylindracea* suggests other factors inherent to macrophyte
560 features could also be involved. Even though some experiments showed no preferences for *C.*
561 *cylindracea* vs. seagrasses (epiphytized or non-epiphytized leaves), the strong preference for
562 *C. prolifera* and the lack of consumption detected by tethers suggest that *S. salpa* may prefer
563 feeding on native species. In fact, a large number of studies have evidenced that preferences
564 and feeding rates of marine herbivores may respond to a combination of high nitrogen and
565 protein content, enhanced epibiotic loads, or with low amounts of chemical and structural
566 components (Cebrián & Duarte 1998, Mariani & Alcoverro, 1999, Verges et al. 2007).

567 In our study, differences in C:N ratios among *Caulerpa* species, seagrass species and
568 epiphytes are likely to have influenced the observed patterns of herbivory and selectivity.
569 However, although both *Caulerpa* species recorded lower C:N ratios than seagrasses,
570 preferences were only manifested for *C. prolifera*. On the one hand, lower C:N ratio values

571 are consistent with *S. salpa*'s preference for *C. prolifera* vs. *C. cylindracea*. On the other, the
572 preference for *C. prolifera* vs. seagrasses, which was dissipated in the presence of epiphytes
573 suggest that epiphytes and macroalgae sustain a comparatively higher herbivore pressure than
574 seagrass leaves (Duarte & Cebrián 1994), due to their typically lower C:N ratios (Duarte
575 1992). In fact, C:N ratios values were ca. 2 times higher in non-epiphytized leaves of both
576 seagrasses than in *C. prolifera*. These nutritional differences were slightly reduced when
577 seagrasses were epiphytized. Together with the significantly lower C:N ratios and higher
578 nutritional content of epiphytes compared to *C. prolifera* (% N was ca. 3 times higher and %
579 C ca. 4 times higher), such differences could explain why preference for *C. prolifera* vs.
580 seagrasses is dissipated in the presence of epiphytes and also the higher herbivory on *C.*
581 *nodosa* recorded in the mixed meadow (i.e. tethering results). It also confirms that epiphytes
582 and their higher nutritional value (e.g. Alcoverro et al. 1997a, 2000) can mediate herbivore
583 preferences and consumption rates (Marco-Méndez et al. 2012). Furthermore, it seems
584 plausible that higher consumption of *C. nodosa* compared to *P. oceanica* in mixed meadows
585 (Marco-Méndez et al. 2015) is explained by the lower C:N ratio of its leaves, plus the
586 increased nutritional value resulting from the presence of epiphytes.

587 Variability in epiphyte composition has also been reported to influence herbivore
588 consumption and preferences (Marco-Méndez et al. 2012, 2015). In the present study, the
589 epiphytic community structure revealed important differences between seagrass species and
590 times. Such differences were probably influenced by differences in light shading (Carruthers
591 1994), and the effects of shoot morphology and leaf age on the surface area and timing of
592 epiphytic colonization (Lavery & Vanderklift 2002). *C. nodosa* leaves were found to support
593 the highest epiphytic biomass, cover and taxa during the study, which may account for the
594 undergoing of more intense grazing rates than in *P. oceanica*.

595 Although they were not measured in this study, we cannot rule out the influence of
596 other macrophytes features in plant–herbivore interactions and feeding preferences (Orians
597 2002, Taylor et al. 2002; Vergés et al. 2011). For instance, high levels of structural
598 carbohydrates in seagrass leaves, which make their digestion less effective (Thayer et al.
599 1984, Cebrián & Duarte 1998), could have also influenced selectivity for *C. prolifera* vs. non-
600 epiphytized seagrass leaves. Despite *Caulerpa* species can synthesize caulerpenyne, a
601 secondary metabolite that plays a major role in their chemical defense (Pohnert & Jung 2003)
602 against epiphytes and herbivores (Erickson et al. 2006), the observed preference of *S. salpa*
603 for *C. prolifera* relative to seagrasses (without epiphytes) suggests that this fish could have
604 evolved some tolerance to this compound. Yet, since lower levels of caulerpenyne have been
605 reported (Jung et al. 2002) for the invasive *C. cylindracea* compared to the non-invasive *C.*
606 *prolifera* (which was the preferred *Caulerpa* species in this study), chemical deterrence was
607 unlikely to be a factor determining the patterns of *S. salpa* herbivory observed in the mixed
608 meadow. From such evidence, it seems that the more intense herbivory on *C. nodosa* and the
609 selectivity for *C. prolifera* must have been mostly influenced by differences in nutritional
610 content rather than in chemical compounds, which seem not to inhibit *S. salpa* herbivory.
611 Although this could theoretically also trigger higher selectivity for *C. cylindracea* vs.
612 seagrasses, our results evidence that *S. salpa* prefers feeding on native species.

613 During the whole study period, feeding observations revealed that although *S. salpa*
614 individuals were feeding on a mix of species, feeding activity on mixed patches of *C.*
615 *prolifera* and *C. nodosa* was reiterative, which concur with tethering results and food choice
616 experiments. For *C. prolifera*, gut content analyses were consistent with previous results,
617 confirming it as a “preferred food item” in the diet of the two schools of fish sampled. In
618 contrast, the absence of *C. nodosa* coupled with the presence of *C. cylindracea* in gut samples
619 did not agree with tethering results. Since coverage of *P. oceanica*, *C. prolifera*, *C.*

620 *cylindracea* and mixed patches of *C. nodosa*-*C. prolifera* were consistently present
621 throughout the study without temporal variation in the mixed meadow, different availability
622 could not explain gut content analyses. We hypothesize that spatial variability may be
623 involved and that *S. salpa* mobility across other sites with lower abundance of *C. nodosa*, or
624 higher abundance of *C. cylindracea* within its home range (ca. 4.3 ha according to Jadot et al.
625 2002, 2006), could account for the absence or enhanced presence of these species within gut
626 contents, also explaining the dietary differences between the two schools. IsoSource mixing
627 model results showed that both seagrasses as well as *Caulerpa* species and epiphytes all
628 contribute to the long-term diet of *S. salpa*, and highlights the importance of *Caulerpa* species
629 (which seems to be mainly attributed to the high consumption of *C. prolifera*) and epiphytes
630 in their diet. This analytical contribution ultimately reflects preferences and consumption
631 patterns observed during the study and supports the previously reported importance of
632 epiphytes (Marco-Méndez et al. 2012, 2015) and *Caulerpa* species in *S. salpa* herbivory
633 (Ruitton et al. 2006, Tomas et al. 2011b).

634 In conclusion, our study highlights the importance of *C. nodosa* and *C. prolifera* in the
635 diet of *S. salpa*, and also that herbivory in Mediterranean meadows can be highly variable and
636 mediated by multiple factors. In summer, when densities of *S. salpa* are higher, *C. nodosa*
637 was the “most consumed” macrophyte, likely influenced by the higher nutritional quality of
638 its leaves and epiphytes, as well as by differences in the epiphytic community composition
639 (Marco-Méndez et al. 2015). Food choice, feeding observations and gut content analyses
640 pointed to *C. prolifera* as a food consistently selected by *S. salpa*. In contrast, preference of *S.*
641 *salpa* for *C. prolifera* was not sustained vs. epiphytized leaves, which suggests that epiphyte
642 presence and nutritional contents explain the herbivory patterns in the mixed meadow. In fact,
643 the IsoSource mixing model confirms the importance of *Caulerpa* species, which from our
644 results, seems to be mostly attributable to the high consumption of *C. prolifera* and the role of

645 epiphytes in the long-term diet of *S. salpa*. Although *C. cylindracea* consumption was not
646 observed, the fact that it was found within stomach contents suggests that they may eventually
647 adapt to feeding on this new resource. Our results may suggest that *S. salpa* selectivity for *C.*
648 *nodosa* and *C. prolifera* could eventually influence their abundances in the mixed meadows.
649 However, macrophyte–herbivore interactions are complex and final consumption rates and
650 dietary differences are not only determined by food preferences, but also by home-range
651 mobility, as well as by temporal and spatial differences in the availability of food resources.
652 This study confirm the need to carry out a multiple methodological approach for a better
653 understanding of herbivory patterns on heterogeneous habitats subjected to seasonal variation.

654

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658 providing *S. salpa* individuals and teaching us dissection and field measurement techniques.

659

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865 **Figure 1.** Map of the study area, Cabo de Santa Pola (Spain), showing the two study locations
 866 (A and B).

867

868 **Figure 2.** **A.** *S. salpa* census (ind·m⁻²); **B.** Feeding observations (%): swimming (SW);
 869 feeding on mixed *C. prolifera* and *P. oceanica* (; mixed *C. prolifera* and *C. nodosa*; *C.*
 870 *prolifera* and other algae and **C.** Macrophyte consumption by *S. salpa* (mg WW·d⁻¹) at both
 871 locations (A and B) and both times of sampling (T1: summer 2012; T2: autumn 2012). Mean
 872 ± SE (in SNK, a and b indicate significant groupings).

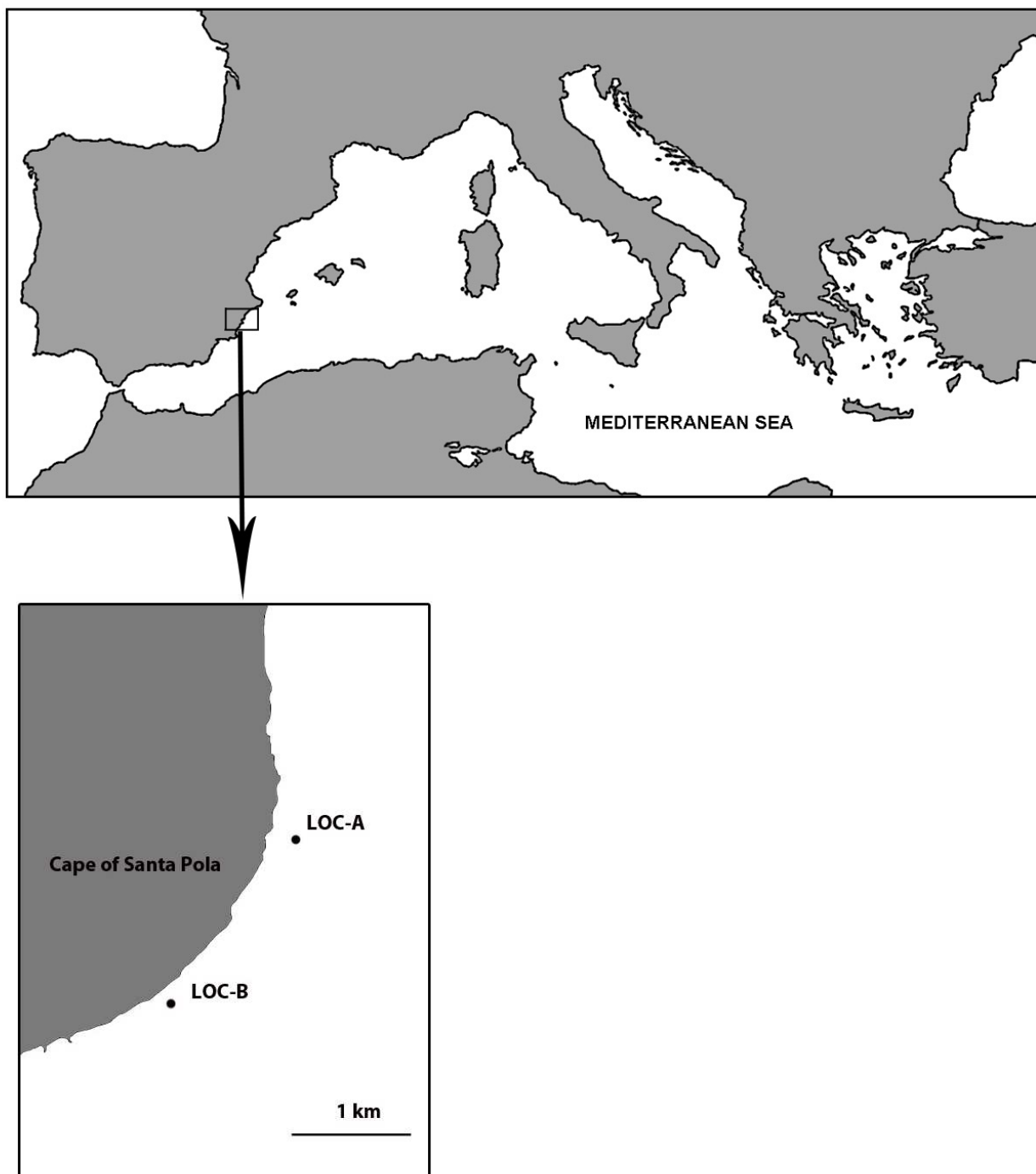
873

874 **Figure 3.** Consumption by *S. salpa* during paired food preference experiments (mg WW·d⁻¹):
 875 **A.** *C. nodosa* non-epiphytized (NE) vs. *C. prolifera*; **B.** *C. nodosa* non-epiphytized (NE) vs.
 876 *C. cylindracea*; **C.** *P. oceanica* non-epiphytized (NE) vs. *C. prolifera*; **D.** *P. oceanica* non-
 877 epiphytized (NE) vs. *C. cylindracea*; **E.** *C. nodosa* epiphytized (E) vs. *C. prolifera*; **F.** *C.*
 878 *nodosa* epiphytized (E) vs. *C. cylindracea*; **G.** *P. oceanica* epiphytized (E) vs. *C. prolifera* ;
 879 **H.** *P. oceanica* epiphytized (E) vs. *C. cylindracea* and **I.** *C. prolifera* vs *C. cylindracea*.
 880 Percentage of the different food items found in the gut contents of *P. lividus* and *S. salpa* (%).
 881 Mean ± SE. *p < 0.05; **p < 0.01; ***p < 0.001; NS = non-significant results.

882

883 **Figure 4.** **A.** δ¹⁵N and δ¹³C signatures of *S. salpa* individuals from the two schools and
 884 collected food items, including epiphytized and non-epiphytized seagrass leaves and their
 885 respective epiphytes (*C. cylindracea*, *C. prolifera*, *C. nodosa* epiphytized (E), *C. nodosa* non-
 886 epiphytized (NE), *P. oceanica* epiphytized (E), *P. oceanica* non-epiphytized (NE), epiphytes
 887 from *C. nodosa* leaves (Epif-*C. nodosa*) and from *P. oceanica* leaves (Epif-*P. oceanica*); **B.**
 888 C:N molar ratios in consumers and food items.. Mean ± SE. *p < 0.05; **p < 0.01; ***p <
 889 0.001; NS = not significant results.

890

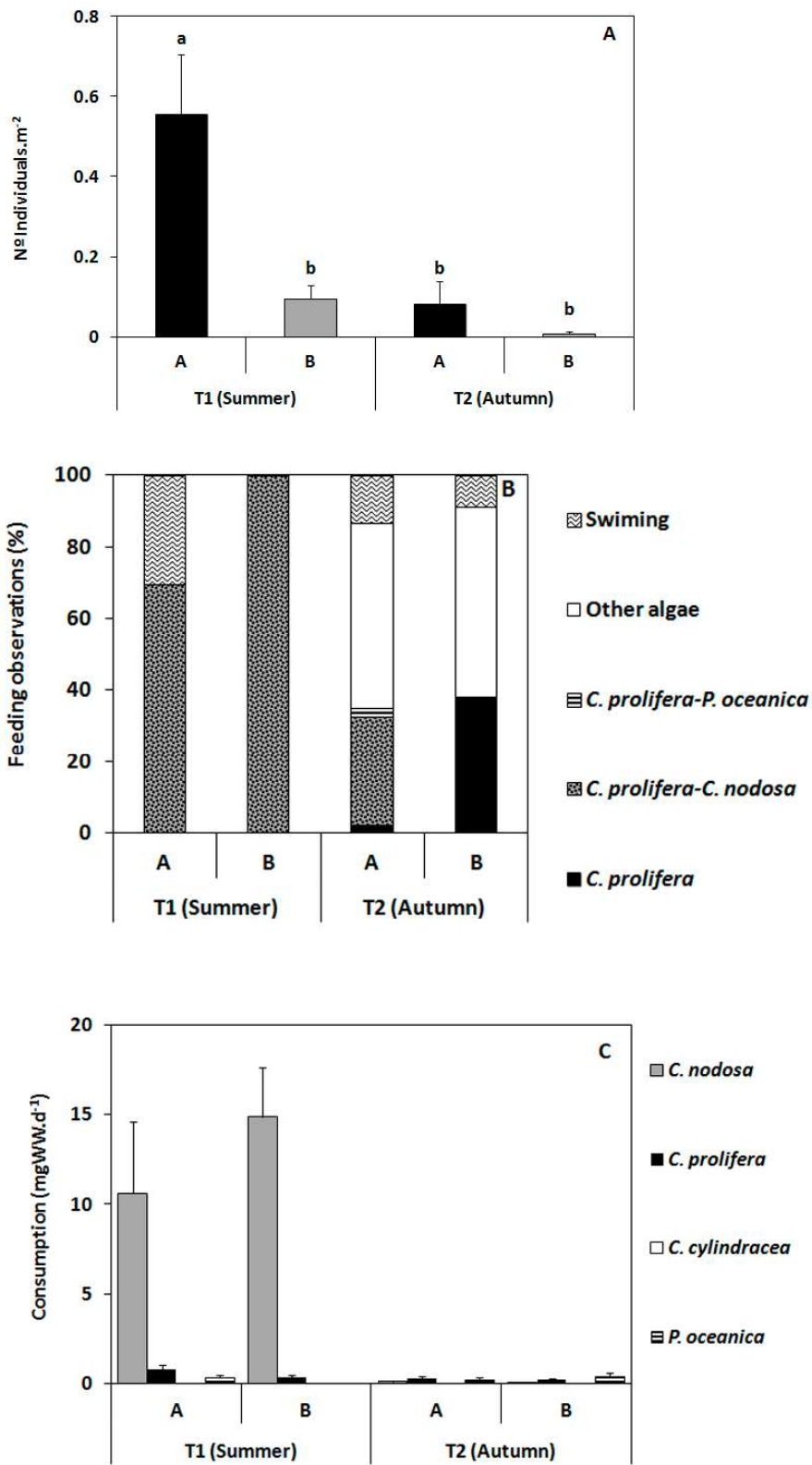
891 **Figure 1.**

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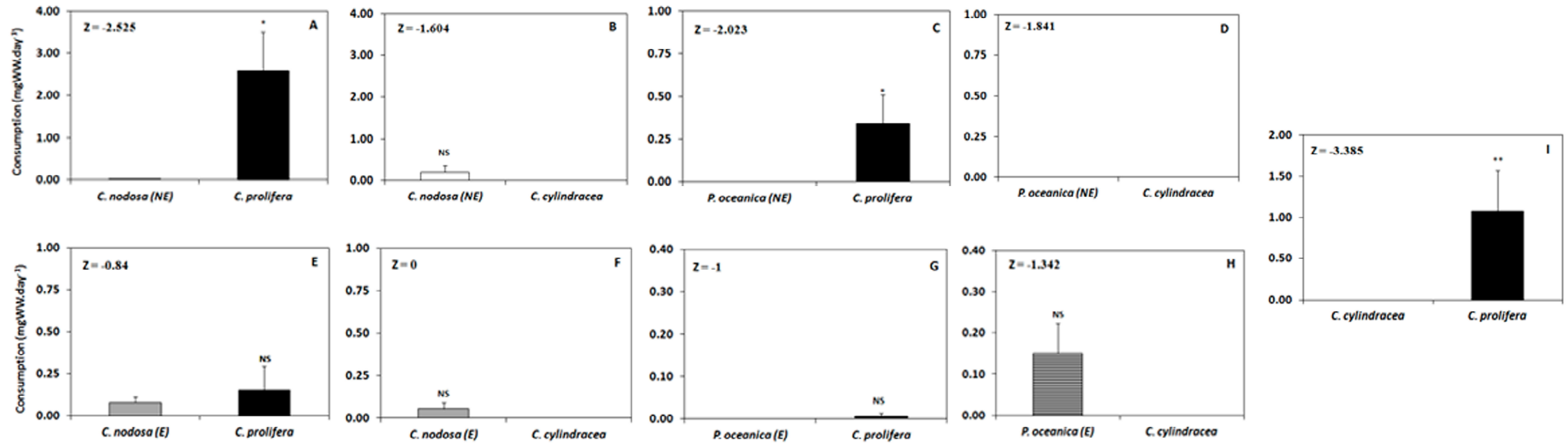
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896 **Figure 2.**

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898 **Figure 3.**

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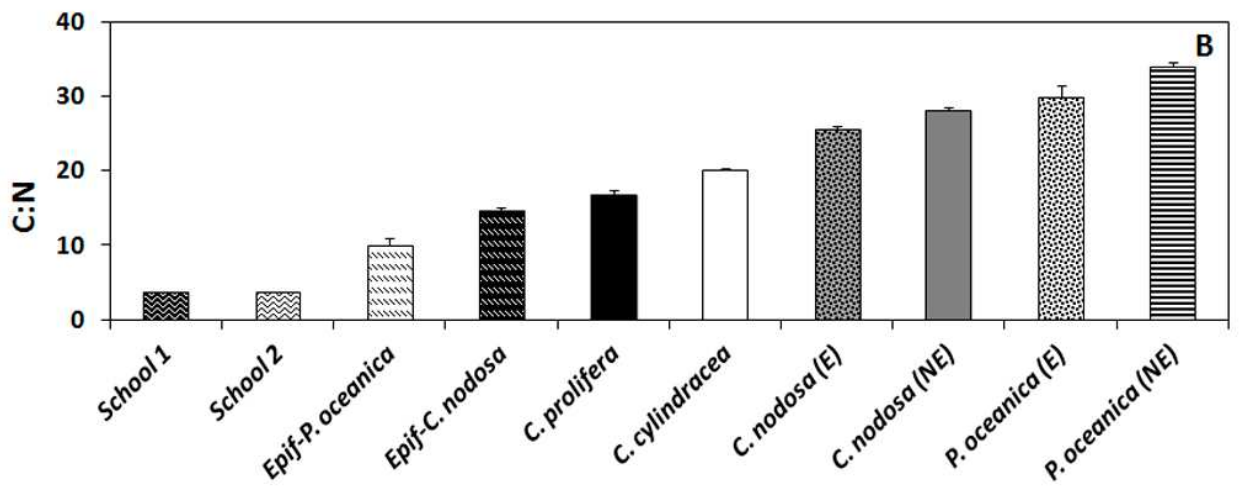
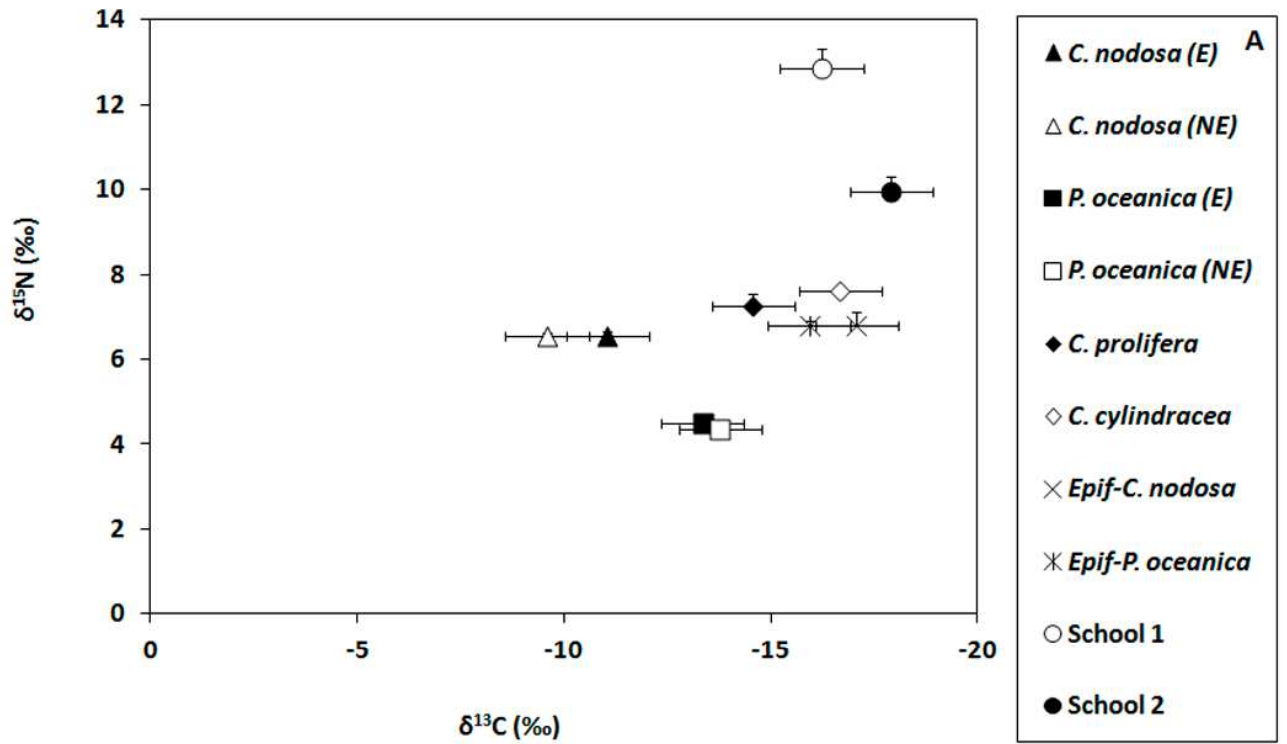


900

901

902 **Figure 4.**

903



905 **Table 1.** Three-way ANOVA showing differences in macrophyte consumption by *S. salpa* (mg WW · d⁻¹) between macrophyte species (CP; CC;
 906 CE; PE), times (T1: Summer; T2: Autumn) and locations (A; B). Labels: *C. prolifera* (CP); *C. cylindracea* (CC); *C. nodosa* (CE) and *P.*
 907 *oceanica* (PE). Significant differences are indicated: * p < 0.05, ** p < 0.01, *** p < 0.001, NS: not significant, NT: no transformation was
 908 carried out. In SNK, significant differences between investigated groups are indicated.

Source of variation	Consumption (mg WW·d ⁻¹)			
	df	MS	F	p
Macrophyte (M)	3.00	694.95	31.35	**
Time (T)	1.00	749.47	56.05	NS
Location (L)	1.00	13.79	0.51	NS
M x T	3.00	718.19	29.67	**
M x L	3.00	22.17	0.83	NS
T x L	1.00	13.37	0.50	NS
M x T x L	3.00	24.20	0.90	NS
RES	272.00	26.85		
TOT	287.00			
SNK	CESum>CEAut=CPSum=CPAut=CCSum=CCAut=PESum=PEAut			
Transformation	NT			

909

Table 2. Differences in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ signatures and nutrient contents (C:N ratios) among food items: *C. cylindracea* (CC); *C. prolifera* (CP); *C. nodosa* epiphytized (CE); *C. nodosa* non-epiphytized (CNE); *P. oceanica* epiphytized (PE); *P. oceanica* non-epiphytized (PNE) and epiphytes of *C. nodosa* (EC) and *P. oceanica* (EP). Significant differences are indicated: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS: not significant, NT: no transformation was carried out. In SNK, significant differences between investigated groups are indicated.

Source of variation	$^{15}\delta\text{N}$				$^{13}\delta\text{C}$				C:N			
	df	MS	F	p	df	MS	F	p	df	MS	F	p
Food item	7	7.2754	48.01	***	7	35.1677	87.69	***	7	345.571	133.12	***
Residual	32	0.1515			32	0.401			32	2.596		
Total	39				39				39			
SNK		CC=CP>EC=EP=CE=CNE>PE=PNE				CNE>CE>PE=PNE=>=CP>EP=CC=EC				PNE>PE=CNE>CE>CC>CP>EC>EP		
Transformation		NT				NT				NT		

910

Table 3. Differences in biomass (mg DW·cm⁻²), coverage (%) and number of taxa (taxa·cm⁻²) between epiphytes of two macrophyte species, *C. nodosa* (EC) and *P. oceanica* (EC) and times (T1: Summer; T2: Autumn). Significant differences are indicated: * p < 0.05, ** p < 0.01, *** p < 0.001, NS: not significant, NT: no transformation was carried out. In SNK, significant differences between investigated groups are indicated.

911

912

Source of variation	Biomass (mgWW.cm ⁻²)				Coverage (%)				Taxa.cm ⁻²			
	df	MS	F	p	df	MS	F	p	df	MS	F	p
Macrophyte (Ma)	1.000	1.901	12.400	**	1.000	8293.030	40.500	***	1.000	19.250	138.110	***
Time (Ti)	1.000	1.856	12.110	**	1.000	2609.269	12.740	**	1.000	0.109	0.780	NS
MacXTi	1.000	3.381	22.060	***	1.000	41.103	0.200	NS	1.000	1.371	9.830	**
Residual	36.000	0.153			36.000	204.750			36.000	0.139		
Total	39.000				39.000				39.000			
SNK	ECAut>ECSum=EPSum=EPAut				Aut>Sum; EC>EP				ECSum=ECAut>EPSum>EPAut			
Transformation	Ln(X+1)				NT				Ln(X)			