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4 **Seagrass epiphytic assemblages are strong indicators of**

5 **agricultural discharge but weak indicators of host features**

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Patricia Prado^{1*}

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10 ¹IRTA-Aquatic Ecosystems. Ctra. Poble Nou Km 5.5. 43540 Sant Carles de la Ràpita

11 (Tarragona), Spain

12

13

14 *P. Prado

15 e-mail: patricia.prado@irta.cat

16 Telephone: (+34) 977745427

17 Fax: (+34) 977744138

18

19 ABSTRACT

20 Wastewater pulses from rice agriculture are persistently discharged into the northern
21 shore of the Alfacs Bay (Ebro Delta, NW Mediterranean) from April to November. The bay
22 also receives water from coastal lagoons which are subjected to freshwater inputs from
23 the Ebro River mixed to an unknown extent with agricultural wastewater during the same
24 period. This paper compares epiphyte assemblages growing on leaves of *Cymodocea*
25 *nodosa* in sites exposed to agricultural drainage channels, lagoon connection channels,
26 and control sites in the Ebro Delta Natural Park (southern shore of the bay). Leaf epiphytic
27 assemblages of *Zostera noltii* patches in the northern shore of the bay were also compared
28 with those of adjacent beds of *C. nodosa*. Drainage channel sites had consistently
29 distinctive assemblages (higher species richness, biomass load, and taxa composition) than
30 control sites. Assemblages from lagoon channel sites were more variable, with three sites
31 showing particularly high covers of epiphytic algae and two sites more similar to controls.
32 Epiphyte patterns clearly matched in situ measures of nutrient availability, and were
33 consistent with decreased shoot densities in discharge sites. In contrast, differences in
34 epiphyte assemblages between seagrass species were minor, and mostly a result of higher
35 epiphytic loads on *C. nodosa* than on *Z. noltii*, which features thinner leaves. Further
36 research is needed to investigate the consequences of these plant and epiphyte
37 alterations in important ecosystem processes such as decomposition and export rates, as
38 well as overall effects of nutrients and salinity in secondary producers such as associated
39 macroinvertebrate assemblages supporting locally important marine fisheries.

40 *Keywords:* eutrophication; plant architecture; *Cymodocea nodosa*; *Zostera noltii*;
41 Mediterranean; coastal lagoons

42 **1. Introduction**

43 Seagrasses usually colonize soft bottom areas, and their leaves provide the main hard
44 substrate available for the growth of epiphytic organisms, which are continuously
45 renewing and constitute a central component of the ecosystem (Borowitzka et al., 2006).
46 Epiphyte communities may account for over the 50% of the standing stock of seagrass
47 meadows and reach contributions of up to ca. 40-60% of the primary production of these
48 ecosystems (Wear et al., 1999; Borowitzka et al., 2006). They have short generation times,
49 and are able to remove large quantities of nutrients from the water column (Cornelisen
50 and Thomas 2002), often leading to seagrass shading (Dalla Via et al., 1998). Certain
51 groups such as incrusting coralline algae also have an important role in the formation of
52 calcareous sediments (Frankovich and Zieman, 1994), whereas cyanobacterial epiphytes
53 can supply a significant proportion of the nitrogen required for primary production (review
54 by Welsh 2000). In addition, they represent a significant part of the biodiversity within
55 seagrass meadows, and make a significant contribution to food webs and habitat supply
56 for animal communities (Mazzella et al., 1992; Jernakoff et al., 1996).

57 The relative importance of their ecological roles depends on the pattern of epiphyte
58 accrual along the leaf-life span, because of the dynamic succession of species and the
59 increase in diversity and biomass from young to old leaves (Casola et al., 1987; Reyes et
60 al., 1998). Additionally, variations in the number of epiphytic species within seagrass
61 leaves are mostly constrained by the local availability of propagules for algal settlement
62 (Borum et al., 1984). For instance, Reyes and Sansón (1997) studied the epiphytic flora of
63 the leaves of *Cymodocea nodosa* across the annual cycle and found three distinctive
64 groups of species based on their patterns of permanent, seasonal, or occasional presence.
65 In *Posidonia oceanica*, Ballesteros (1987) reported assemblages dominated by incrusting
66 coralline algae and bryozoans during the fall and winter, the typical seasonal community of

67 brown and red algae in spring, and fast-growing opportunistic species during the summer.
68 Hence, coexisting seagrass species exposed to the same sources of propagules should
69 present a similar pool of species, but might differ in total richness and abundances due to
70 variable leaf life-span accrual and possible effects of plant architecture. Although these
71 hypotheses have been seldom tested, Lavery and Vanderklift (2002) evaluated the
72 influence of influence of co-occurring host seagrass type on the patterns of epiphytic
73 assemblages and confirmed that persistent stems of *Amphibolis griffithii* allowed this
74 seagrass to support higher species richness than *Posidonia coriacea*.

75 Among environmental stressors influencing aquatic communities, eutrophication is the
76 most common factor associated with the global decline of seagrass communities during
77 the last decades (see reviews by Romero et al., 2006; Burkholder et al., 2007). Human
78 activities such as terrestrial runoff due to overuse of land or deforestation, domestic
79 sewage, or fish farms have greatly increased nutrient inputs to coastal waters (Peierls et
80 al., 1991; Turner and Rabalais, 1991). The negative effects of eutrophication include
81 nuisance algal blooms, including phytoplankton, free-floating macroalgae, and algal
82 epiphytes, which reduce light availability to seagrass leaves and depress primary
83 production (e.g., Cambridge et al., 1986; Sfriso et al., 2003). Such changes in epiphyte
84 biomass are thought to be directly related to the importance of anthropogenic
85 eutrophication, but often reflect the development of few species (Balata et al., 2008;
86 Prado et al., 2008). In contrast, other community descriptors such as diversity and richness
87 of small species might be initially increased by nutrient availability, because the effect of
88 natural limitation is offset and the epiphytic faunal composition may be also favored
89 (Prado et al., 2007; 2008). Yet, according to experimental experience, the strong seasonal
90 dynamics of epiphyte communities cannot be easily disrupted by nutrient availability and

91 negative effects are mostly restricted to spring and summer, when fast growing
92 filamentous and fleshy macroalgae typically develop (Prado et al., 2008).

93 In the Ebro Delta (Southern Catalonia, NW Mediterranean) ca. 70 % of the total land
94 area is devoted to rice cultivation and agricultural wastewater pulses (ca. $275 \cdot 10^6 \text{ m}^3 \text{ yr}^{-1}$)
95 are discharged directly into the northern shore of the Alfacs Bay between April and
96 October (Garcés et al., 1999). In addition, the Alfacs Bay is connected to coastal lagoons
97 through natural and human made channels that receive seasonal freshwater inputs from
98 the Ebro River (with lower organic matter and nutrients) mixed to an unknown extent with
99 wastewater inputs from rice fields during the same period. The semi-enclosed estuarine
100 water body of the Alfacs Bay plays, however, a very important role in the economy of the
101 region due to its fish and shellfish aquaculture, particularly oysters and mussels (Solé et al.,
102 2009). The shallow submerged bottom (<2 m) is dominated by dense patches of the
103 seagrass *C. nodosa* (Marbà and Duarte, 1995) with some patches of the seagrass *Zostera*
104 *noltii* also occurring along the northern shore (Procaccini et al., 2003) adjacent to points of
105 agricultural discharge. In contrast, the southern shore (the Banyà Sandspit) was included in
106 the Ebro Delta Natural Park in 1986 and is also part of the Natura 2000 network of the
107 European Union because of its importance for both *C. nodosa* and the presence of a very
108 large population of *Pinna nobilis* (Ibáñez 1997; Prado et al., 2014).

109 Since both *C. nodosa* and *Z. noltii* grow together in some northern sites of the Alfacs
110 Bay and seagrass epiphytes have been shown to be an effective indicator of the overall
111 ecosystem integrity (e.g., Martínez-Crego et al., 2010), this scenario provided the
112 opportunity to test for the effects of host type in epiphytic assemblages and compare
113 these effects with those of enrichment. I tested the hypothesis that changes in community
114 structure (species richness, diversity, biomass, and the species composition) could reflect
115 seagrass host features and be a good indicator of environmental impact by seasonal pulses

116 of agricultural wastewater, both directly through discharge canals that are intermittently
117 opened according to agricultural needs, and indirectly through connection canals with
118 coastal lagoons that are permanently opened. Besides, patterns of epiphytic assemblages
119 were compared with measures of seagrass meadow density, as an integrative variable of
120 seagrass vitality (Romero et al., 2007).

121

122 **2. Materials and Methods**

123 *2.1. Study site and sampling design*

124 The study was conducted in the Alfacs Bay (Ebro Delta, Catalonia, NW Mediterranean),
125 a semi-confined estuarine area of ca. 49 km² with an average depth of 3.13 m. It is
126 bordered by rice fields to the north and connected to the Banyà Sandspit through a long
127 sand bar known as the Trabucador that stretches along the eastern side of the bay (Fig. 1).
128 During rice growth and some post-harvest period (April to November), the northern shore
129 of the Alfacs Bay receives pulses of agricultural wastewater through multiple drainage
130 channels that are not permanently opened but bring in significant amounts of nutrients
131 and organic matter (Delgado, 1987). In addition, the northern shore is permanently
132 connected at various points with the Encanyissada and Tancada lagoons (see Fig. 1)
133 allowing the entrance of brackish water. Although these lagoons should be naturally
134 hypersaline in summer, their current seasonal salinity ranges between 12.5 and 27.1 in the
135 Encanyissada and between 18.6 and 28.1 in the Tancada, with the lowest values also being
136 attained during the rice growth period, due to freshwater additions from the River and rice
137 fields by the Ebro Delta Natural Park (see Prado et al., 2013 for further details). As a result,
138 the overall salinity of the north shore may decrease to ranges between 30 and 35 (Garcés
139 et al., 1999). *Cymodocea nodosa* is the dominant seagrass in the area, but in summer is
140 often overgrown by fast growing macroalgae (*Chaetomorpha linum* and *Ulva* spp. P. Prado,

141 personal observation), and some areas with *Zostera nolti* do also occur. The salinity along
142 the southern shore of the bay is more similar to the open sea (Table 1) and dominant
143 macrophytes are *C. nodosa* with sparse patches of the benthic macroalgae, *Caulerpa*
144 *prolifera*.

145 Study sites along the northern shore were selected in front of a drainage channel (N=
146 5), or in front of a connection channel with a lagoon (N= 5), within the *C. nodosa* seagrass
147 bed, at a distance of about 50 m from the discharge point. Along the southern shore,
148 control sites with no water input (N= 5) were randomly selected at similar depths within
149 the *C. nodosa* seagrass bed for comparative purposes. Where the seagrass *Z. nolti* was
150 available (growing in patches adjacent to those of *C. nodosa* (N= 5)), samples were
151 collected for comparison between epiphyte communities growing on both species. This
152 occurred in one drainage channel site (D4) and in four lagoon sites (L1, L2, L4, and L5; see
153 Table. 1). At each site, 20 shoots of *C. nodosa* (and *Z. nolti* where available) were collected
154 in late May 2015 (period when seagrass feature its characteristic leaf epiphytic
155 community; Ballesteros 1987) at 0.5-1 m depth and preserved in a 4% formaldehyde-
156 seawater solution. Later in the lab, three shoots featuring an oldest leaf with a similar
157 length were selected among available shoots in order to ensure standardization of the
158 epiphytic community in relation to leaf age and size (Reyes et al. 1998; Prado et al. 2008).
159 Prior to the detachment of epiphytes from the surface of the oldest leaf, the abundance of
160 encrusting and more abundant taxa (e.g. *Hydrolithon* spp. and bryozoans) were
161 determined qualitatively through visual estimation under the microscope. Then, epiphytes
162 were carefully removed with a razor blade, and the determination of covers conducted as
163 in Prado et al. (2008) in which rare and small taxa found only once per leaf were assigned
164 0.1%, and the remaining taxa were given values from 0.2% by comparison with previous
165 abundances (i.e., taxa present only once). Macroalgae and most groups of sessile epifauna

166 (ascidians, bryozoans, hydrozoans, and actinians) were identified to species level, except
167 for polychaeta which were determined at the family level and foraminifera at the phylum
168 level.

169 Species richness was estimated as the total number of taxa recorded growing on the
170 oldest leaf of each shoot, and the alpha diversity was calculated with the Shannon index:

$$171 \quad H' = - \sum_{i=1}^s p_i \log p_i$$

172 where s is the total number of species and p_i is the proportion of species i observed in
173 the sample.

174 The three selected shoots per sample were also used to determine the number of
175 leaves per shoot, leaf length and width, and the presence of bite marks (fish or sea urchin)
176 or broken apices. After identification, epiphytes on the oldest leaf of each shoot were
177 dried at 60°C for 48 h, and weighed for biomass estimation (mg DW).

178

179 *2.2. Environmental and seagrass variables*

180 To evaluate differences in water quality characteristics among study sites water
181 samples and abiotic measures were taken directly in front of each drainage channel and
182 lagoon connection, next to the area of collection of seagrass samples. At each study site,
183 one sample of dissolved nutrients (PO_4 , NH_4 , NO_2 , and NO_x (NO_3 and NO_2)) was collected
184 with 100 ml water bottles and kept frozen at -20 °C until determination following the
185 Koroleff method (Koroleff, 1977). Water pH and salinity were measured within a two-hour
186 range with an YSI 6660 multiparametric probe (equipped with a 650 MDS data logger).
187 Alkalinity (TA) was also quantified once per site with a Hanna Checker alkalinity meter HI
188 755 (± 5 ppm accuracy at 25 °C). Finally, seagrass shoot density was measured in each site

189 and for each seagrass species as an indicator of plant vitality and ecological status (Romero
190 et al., 2007) using a 20 x 20 quadrat (N= 3) and results expressed as numbers per m².

191

192 2.3. Data analysis

193 Patterns of nutrient concentrations, pH, salinity, and alkalinity among water discharge
194 treatments (fixed factor: drainage channels, lagoon channels, and controls without
195 channels) were investigated with a one-way ANOVA.

196 Differences in shoot density of *C. nodosa*, and epiphyte species richness, alpha
197 diversity, and biomass across water discharge treatments (fixed factor, 3 levels), and sites
198 (fixed factor) were investigated with a 2-way nested ANOVA. Differences in species
199 richness, alpha diversity, and epiphyte biomass between seagrass species (fixed factor, 2
200 levels: *C. nodosa* and *Z. nolti*) and among sites (fixed factor) were investigated with a 2-
201 way factorial ANOVA.

202 ANOVA assumptions of homogeneity of variances and normality were tested by
203 Cochran's test and Kolmogorov-Smirnov distribution-fitting test of the residuals,
204 respectively. NO_x and NO₂ data were double square root transformed and epiphytic
205 biomass Asen x transformed for statistical analysis, because homoscedasticity and
206 normality were clearly improved and balanced ANOVA is robust against violations of these
207 assumptions (Sokal and Rohlf, 1995). For all ANOVA analyses, the critical level of
208 significance was fixed at p < 0.05. Student-Newman-Keuls (SNK) post hoc comparisons
209 were used to identify significant differences between treatments at each month of
210 sampling.

211 Patterns of spatial variation in epiphytic assemblages were investigated using the
212 PRIMER software package from Plymouth Marine Laboratory (UK). Leaf cover values for
213 each species were log (x +1) transformed prior to analysis and then used to build a Bray-

214 Curtis (BC) dissimilarity matrix. Non-metric multidimensional scaling (n-MDS) was applied
215 to examine the influence of discharge type and site, and that of seagrass species and site
216 in the structure of epiphyte assemblages. Analysis of similarities (ANOSIM) was also
217 conducted to test whether differences in species composition and abundance observed in
218 n-MDS results were significant. A 2-way nested design was used for discharge type
219 comparisons and a 2-way factorial design for seagrass species comparisons. In addition,
220 SIMPER analyses were used to investigate which taxa had the highest contributions to
221 both similarities and dissimilarities within and among factor groups, respectively.

222

223 **3. Results**

224 *3.1. Environmental variables*

225 Nitrate and nitrite (NO_x) concentrations in the water column were significantly higher
226 in drainage channel sites (D: $933.5 \pm 542.3 \mu\text{g} \cdot \text{L}^{-1}$) than in lagoon and control sites which
227 were not significantly different (L: 490.8 ± 247.6 ; and C: $48.4 \pm 9.4 \mu\text{g} \cdot \text{L}^{-1}$; $F_{2, 12} = 4.67$, $p =$
228 0.031). Yet, lagoon sites L3 to L5 also showed very high nutrient levels within the same
229 range as drainage sites (see Table 1). NO₂ alone also showed significant differences across
230 all levels of discharge treatments ($F_{2, 12} = 6.84$, $p = 0.010$), with higher values in lagoon
231 channels (L: $28.1 \pm 17 \mu\text{g} \cdot \text{L}^{-1}$), intermediate in drainage channels (D: $15.2 \pm 4.1 \mu\text{g} \cdot \text{L}^{-1}$) and
232 lowest in controls (C: $1.55 \pm 0.5 \mu\text{g} \cdot \text{L}^{-1}$). In contrast, the levels of NH₄⁺ and PO₄⁻³ tended to
233 attain higher values at drainage and lagoons channels (Table 1) but showed non-significant
234 effects (NH₄⁺: $F_{2, 12} = 1.87$, $p = 0.195$; PO₄⁻³: $F_{2, 12} = 2.74$, $p = 0.105$). Control sites showed
235 higher salinities (32.8 ± 1.9) than drainage and lagoon channel sites (20.52 ± 1.9 , and 23.7
236 ± 7 , respectively), although differences were non-significant ($F_{2, 12} = 2.13$, $p = 0.161$) as were
237 those for pH ($F_{2, 12} = 0.78$, $p = 0.476$). In contrast, alkalinity was found to be significantly

238 higher in control and drainage sites (C: 104.4 ± 6.3 ; D: 85 ± 10.7) than in lagoon sites (L: 74
239 ± 9.2 ; $F_{2, 12}$: 4.067, $p= 0.044$; Table 1).

240 Shoot density of *C. nodosa* was significantly higher in control sites, than in lagoon and
241 drainage channels (1483.3 ± 140 , 1155 ± 53 , and 833 ± 33 shoots $\cdot m^{-2}$, respectively; $F_{2, 38}$ =
242 8.54, $p= 0.0008$) with no differences among sites nested with drainage type. Compared to
243 *C. nodosa*, shoot density of *Z. noltii* was ca. 3.8 times higher (3578 ± 178 shoots $\cdot m^{-2}$; $F_{1, 24}$ =
244 188.93, $p= 0.0000$).

245

246 3.2. Seagrass leaf features and epiphytic taxa

247 The oldest leaf used for assessment of the epiphytic community (specifically selected
248 for similar size) featured an average length of 15.3 ± 0.2 cm by 0.31 ± 0.006 cm width in *C.*
249 *nodosa*, and an average length of 22 ± 0.5 cm by 0.15 ± 0.0006 cm width in *Z. nolti*. This
250 resulted on a higher leaf area indexes (LAI) in *C. nodosa* (4.8 ± 0.1 cm²) and compared to *Z.*
251 *nolti* (3.3 ± 0.1 cm²).

252 A total of 45 taxa were found across all types of discharge sites and seagrass species
253 (see percent cover values in supplementary Annex 1). The following taxa were identified:
254 Bacillariophyceae (1 taxa), Rodophyta (11 taxa), Chlorophyta (8 taxa), Hydrozoans (6 taxa),
255 Actiinidae (1 taxa), Bryozoans (2 taxa), Polychaeta (3 taxa), Ascidians (1 taxa),
256 Foraminifera, and invertebrate eggs. Dominant taxa, which varied among sites, were: the
257 Corallinaceae *Hydrolithon farinosum* (0 to 40%) and *Titanoderma pustulatum* (0 to 25%),
258 the Rodophyta *Chondria marei* (0 to 7%), *Ceramium diaphanum* (0 to 60%), and
259 *Polysiphonia* sp. (4 periaxial cells; 0 to 15%), the Chlorophyta *Chaetomorpha mediterranea*
260 (0 to 5%), the Hydrozoans *Agaophenia harpago* (0 to 12%) and *Kirchenpaueria pinnata* (0
261 to 12%), the anemone *Paranemonia cinerea* (0 to 8%), the three Polychaeta families
262 (Spirorbidae, Nereididae, and Ampharetidae; 0 to 20%), the Bryozoan *Mymosella gracillis*

263 (0 to 10%), and the Ascidian *Botryllus schlosseri* (0 to 15%). The rest, constituted a long tail
264 of small and rare species which were of lesser importance to differences among discharge
265 conditions and between species (Annex 1).

266

267 3.3. Univariate analyses

268 3.3.1. Discharge conditions

269 Results from ANOVA showed that both discharge type and site had significant effects
270 on the number of epiphytic species growing on the oldest leaf of *C. nodosa* (Table 2a).

271 Drainage channels sites showed more than double the species richness than control sites
272 (8.5 ± 0.5 , and 4 ± 0.3 species leaf⁻¹, respectively), and were also significantly higher than
273 lagoon channel sites (4.8 ± 0.2 species leaf⁻¹) (Fig. 2a).

274 Patterns for the Shannon index were similar to those of species richness, with
275 significant effects in both investigated factors (Table 2a). Drainage channels showed higher
276 alpha diversity (1.7 ± 0.1) than control and lagoon channel sites which featured similar
277 values (1.2 ± 0.1) (Fig. 2b).

278 There were significant differences in the values of epiphyte biomass across discharge
279 types and sites (Table 2a). The highest biomasses were attained at some lagoon channels
280 sites followed by drainage channels sites and control sites (54.4 ± 13 , 21.9 ± 3.2 , and $3.16 \pm$
281 0.7 mg DW leaf⁻¹, respectively; Fig. 2c).

282

283 3.3.2. Species comparisons

284 There were significant effects of site and a Species x Site interaction, but not species
285 (5.6 ± 0.5 , and 5.3 ± 0.4 species leaf⁻¹, respectively for *C. nodosa* and *Z. noltii*). Significant
286 differences were exclusively due to the D4 site, which showed higher number of epiphytic
287 species in *C. nodosa* than in *Z. noltii* (Table 2b, Fig. 2d).

288 For the Shannon index, both factors (species and site) and their interaction displayed
289 significant effects (Table 2a). Values were slightly higher in *Z. noltii* than in *C. nodosa* ($1.5 \pm$
290 0.1 , and 1.3 ± 0.1 , respectively), which more mostly due to higher values in the L2 and L4
291 sites (Fig. 2e).

292 Epiphyte biomass showed significant differences in between species, sites, and a
293 marginally significant interaction (Table 2b). *C. nodosa* showed overall higher values than
294 *Z. noltii* (48.4 ± 13.5 , and 22.3 ± 6 mg DW leaf⁻¹, respectively), and highest values were
295 recorded in L4 and L5 sites (Fig. 2f).

296

297 3.4. Multivariate analyses

298 For the discharge type design, nMDS analyses evidenced distinctive groupings among
299 discharge treatments. Samples of drainage channel sites were clustered together on the
300 middle of the plot, a second cluster was formed by lagoon sites 3 to 5, and the remaining
301 lagoon sites (L1 and L2) were mixed with control sites without freshwater inputs (Fig. 3a).
302 Further ANOSIM results confirmed the significance of observed differences among
303 discharge types and, to a lesser extent, by variability among sites (2-way nested ANOSIM,
304 $R= 0.889$, $p= 0.001$; and $R= 0.519$, $p= 0.001$ for discharge and site effects, respectively).

305 SIMPER analyses also indicated important differences among discharge treatments,
306 ranging from ca. 79 to 83% (see Table 3a). Drainage channel sites showed higher cover of
307 coralline algae (30% of dissimilarities), and polychaetes of the families Nereididae (17.3%)
308 and Spirorbidae (5.1%), but lower amounts of invertebrate egg masses (6.7%) and of the
309 ascidian *Botryllus schlosseri* (5.2%) than control sites. Lagoon channel sites L3 to L5
310 showed lower cover of coralline algae (13.7% of dissimilarities) than control sites (and also
311 lower amounts of invertebrate egg masses and the bryozoans *Aglaophenia harpago*; 9.5
312 and 5.1% of dissimilarities, respectively), and higher amounts of filamentous algae (the

313 Rhodophyta *Ceramium diaphanum* (15.4%), *Chondria marei* (11.9%), *Polisiphonia* sp.
314 (6.8%), and the Chlorophyta *Cladophora dalmatica* (9.3%)), and of the anemone
315 *Paranemonia cinerea* (5.6%). Yet, two of the lagoon sites (L1 and L2) were more similar to
316 control sites and showed higher covers of coralline algae and the absence of *C.*
317 *diaphanum*.

318 For species comparisons, nMDS analyses showed similar assemblages in both
319 seagrasses, with no clear grouping of samples (Fig. 3b). ANOSIM results showed significant
320 R ratios for both factors, although comparatively lower for Species (2-way crossed
321 ANOSIM, $R= 0.419$, $p= 0.001$ and $R= 0.985$, $p= 0.001$, respectively for Species and Site).
322 SIMPER results also evidenced low percentage of variability between species compared to
323 dissimilarities among sites (Table 3b). *C. nodosa* showed higher covers of coralline algae
324 (*Hydrolithon farinosum*) (18.5% of dissimilarities), filamentous algae (the Rhodophyta
325 *Chondria marei* (10.6%) and *Polisiphonia* sp. (5%), and the Chlorophyta *Cladophora*
326 *dalmatica* (13.2%)), and invertebrate eggs masses (10.1%). In contrast, *Z. noltii* showed
327 higher abundance of the polychaete family Nereididae (10.4%), and of the filamentous red
328 algae *Anotrichium furcellatum* (7.6%) and *C. diaphanum* (6.8%).

329

330 **4. Discussion**

331 Epiphyte assemblages of *Cymodocea nodosa* mirrored local conditions of water
332 discharge, whereas differences with assemblages growing on adjacent shoots of *Zostera*
333 *noltii* were very weak. Sites subjected to pulses of agricultural wastewater discharge
334 consistently displayed distinctive epiphyte assemblages (higher species richness, biomass
335 load, and taxa composition) compared to control sites (see also Balata et al., 2008; Prado
336 et al., 2008) and showed the highest availability of nutrients, particularly nitrate, and
337 nitrite. In contrast, assemblages close to lagoon connection channels were more variable,

338 with two sites featuring especially high covers of fast growing epiphytic algae and two
339 more comparable to control sites, concurring with lower nutrient conditions. Differences
340 between coexisting seagrass species were restricted to higher epiphytic loads and total
341 number of taxa growing on *C. nodosa* vs. *Z. noltii*, as expected by higher leaf area indexes
342 (Vermaat et al., 1993). Overall, the results show that both direct (drainage channels) and
343 indirect (connection channels with coastal lagoons) wastewater discharges from rice field
344 agriculture into the Alfacs Bay are driving alterations in the abundance and composition of
345 epiphytic assemblages. Given the central role of epiphytic assemblages in the functioning
346 of seagrass beds (Borowitzka et al., 2006), the combined effects of nutrients and epiphytic
347 loads may trigger a sequence of flow-on effects that range from altered plant growth and
348 herbivory pressure (Prado et al., 2010) to changes in the mobile epifauna and secondary
349 production of the system (Bologna and Heck, 1999). Evidence of such undergoing changes
350 is also provided by considerably lower shoot densities in drainage and lagoon sites than in
351 control sites (by ca. 22.1 to 40.5%), suggesting long-term eutrophication of the northern
352 shore of the Alfacs Bay.

353 Seagrass epiphyte assemblages are regarded as sensitive to eutrophication and positive
354 relationships between epiphyte biomass and nutrient concentrations are commonly
355 reported (Borum et al., 1984; Wear et al., 1999). During the study, the highest covers of
356 epiphytic algae (by ca. 30 times) were evidenced in lagoon sites L4 and L5 connecting the
357 Western basin of the Tancada lagoon with the Alfacs Bay (mostly the Rodophytes
358 *Ceramium diaphanum*, and *Chondria marei*, and the Chlorophyta *Cladophora dalmatica*).
359 Although strong variability in nutrient conditions resulted on non-significant effects for
360 lagoon channels, those two sites had ca. 22 times higher availability of NO_x than controls.
361 This region of the Tancada lagoon has been indicated to remain mostly unvegetated, and
362 to feature high turbidity due to destabilized sediments and historical nutrient loading

363 (Prado et al., 2013), which are steadily discharged into the Alfacs Bay. In addition, given
364 the particular location of the sites, opposite from the mouth of the bay, more reduced
365 water exchange rates might have additionally favored the development of epiphytic algae.
366 Drainage water sites, also displayed 7 times higher epiphytic loads than control sites,
367 suggestive of discharge activity from rice fields during the study period (Comín et al.,
368 1991). Mean nitrate and nitrite concentrations in drainage sites were ca. 10 to 20 times
369 higher than control sites, although with strong peak at the D3 site (also for PO_4^{3-}),
370 evidencing a pulse of agricultural discharge at the moment of sampling. Patterns of
371 nutrient availability may have also triggered growth responses of encrusting corallines (see
372 Burkepile and Hay, 2009; Smith et al., 2001) and partly account for observed differences in
373 leaf cover across discharge treatments (>20% in drainage channels, 0 to > 20% in lagoon
374 channels, and < 3% in control sites). Alternatively, the abundance of coralline algae could
375 be also connected to local inputs of freshwater from the Ebro River with higher
376 concentration of calcium carbonate than marine waters (Bouza-Deaño et al., 2008),
377 although only lagoon sites displayed significantly higher alkalinities than control sites (by
378 ca. 30%). Unexpectedly, none of the study treatments displayed species of Phaeophyta,
379 including those typically found growing on seagrass leaves during the spring period (e.g.,
380 *Castagnea* spp., *Giraudia sphacelarioides*, or *Myrionema magnusii*, among others;
381 Ballesteros 1987), the reasons for this result being unclear.

382 The number of epiphyte species (Annex 1) per leaf was two-fold higher in drainage
383 water sites, whereas values in lagoon sites were only slightly (but significantly) higher than
384 controls. These results suggest that under enhanced spring light conditions, nutrient
385 availability first acts triggering the development of a multiplicity of macroalgae that have
386 positive effects on the overall diversity. Later in the summer, the community usually
387 becomes dominated by few species of fast-growing algae, and diversity decreases despite

388 higher number of small and occasional species can be observed (see Prado et al., (2008)
389 for similar results). In addition, higher cover of epiphytic fauna in drainage channel sites
390 may have contributed to diversity patterns. Higher ratios of epifauna vs. epiphytic algae
391 have been related to degraded seagrass meadow conditions (Prado et al., 2007; Martínez-
392 Crego et al., 2010) since enhanced turbidity and reduced light availability can interfere
393 with the photosynthetic activity of primary producers. For instance, polychaetes (families
394 Nereididae and Spirorbidae) were more abundant in drainage channels sites and could be
395 indicative of enhanced inputs of organic matter and lower size of sediment particles
396 (Giangrande et al., 2005; Dauvin and Ruellet, 2007).

397 Recurrent runoff conditions in the northern shore of the bay also resulted in decreased
398 shoot densities of *C. nodosa* in drainage and lagoon channel sites (1155 and 883 shoots ·
399 m⁻², respectively) compared to control sites (1483 shoots · m⁻²), an effect typically
400 attributed to enduring eutrophication in seagrass beds (Romero et al., 2007). Pérez et al.
401 (1994) found that high nutrients concentrations may result in enhanced shoot mortality
402 due to limitation in light availability and/ or oxygen supply to belowground tissues. In
403 addition, leaf shading by large loads of fleshy epiphytes during the summer period (P.
404 Prado, personal observation) causes light limitation and may result in lower plant growth
405 and declined meadow density (Silberstein et al., 1986). Enhanced tolerance to low
406 salinities in *Z. noltii* (see Fernández-Torquemada and Sánchez-Lizaso, 2006) may also
407 account for its distribution along the northern shore of the bay, particularly in lagoon
408 channels sites subjected to longer historical discharge. Low salinity may also be
409 responsible for alterations in the local abundance of macrograzers such as the sea urchin
410 *Paracentrotus lividus*, which is totally absent from sites subjected to agricultural discharge
411 (P Prado, personal observation) thus favoring the accumulation of epiphyte loads.

412 The influence of seagrass host type in epiphyte assemblages was lower than that
413 caused by water discharge conditions. Both seagrass species featured similar mean
414 numbers of epiphyte species (5.6 and 5.3 species per leaf, respectively for *C. nodosa* and *Z.*
415 *noltii*, respectively), and alpha diversity (1.3 and 1.5, also respectively for *C. nodosa* and *Z.*
416 *noltii*), and evidenced stronger compositional differences among sites than between
417 species. According to Pérez and Romero (1994), the longevity of *C. nodosa* leaves during
418 the month of May is around 90 days, and that reported for the *Zostera* genus also has a
419 similar mean (see Hemminga et al., 1999). In addition, leaf and plant architecture is
420 comparable in the two species, which altogether may account for similar patterns of
421 epiphytic accrual compared to other species with more contrasting structure and patterns
422 of leaf development (e.g., *Amphibolis griffithii* vs. *Posidonia coriacea*; Lavery and
423 Vanderklift, 2002). Although results of this study were limited to a single month, epiphyte
424 assemblages usually feature higher number of taxa in early spring than in summer, when
425 few species of fast-growing algae tend to dominate the communities (e.g., Reyes and
426 Sanson, 1997; Prado et al., 2008). Yet, the total number of species found on leaves of *C.*
427 *nodosa* was higher than that found on *Z. noltii* (25 vs. 15, respectively) mostly due to
428 higher numbers of occasional macroalgae. Also, epiphytic biomass on *C. nodosa* attained
429 ca. double values than those found growing on *Z. noltii*, accordingly to 1.5 higher values of
430 leaf area index as also found in other studies (Vermaat et al., 1993). Differences in
431 epiphyte biomass between species may increase in summer when few fast growing species
432 become dominant, particularly in sites subjected to recurrent nutrient discharge (Prado et
433 al., 2008).

434

435 **5. Conclusions**

436 Agricultural run-off, rather than host plant features was found to be an effective driver
437 of epiphyte community structure. Seasonal agricultural discharges through drainage
438 channels and persistent arrival of nutrients from areas featuring historical eutrophication
439 (Western basin of the Tancada lagoon) could be evaluated through measures of epiphyte
440 species richness, biomass loads, and community structure as well as seagrass shoot
441 density and species composition (see also Prado et al., 2008). Some lagoon channels
442 investigated, although permanently open, are connected with restored areas of the
443 Encanyissada lagoon or subjected to lower historical discharge (Forés et al., 2002; Prado et
444 al., 2013) and present communities that are more similar to control sites. For species
445 comparisons, differences in epiphyte community structure were mostly restricted to
446 enhanced biomass loads in *C. nodosa* than *Z. noltii*, possibly associated with higher leaf
447 area index. Given that *Z. noltii* tolerates hyposalinity conditions much better than *C.*
448 *nodosa* (Fernández-Torquemada and Sánchez-Lizaso, 2006), and that enhanced nutrient
449 availability appear to cause a decline in the shoot density of *C. nodosa*, agricultural
450 discharges are not only altering epiphytic communities (both directly by promoting algal
451 growth and indirectly by possibly affecting macrograzer assemblages), but also the
452 seagrass species composition and meadow density of Alfacs bay seagrass habitats. These
453 changes in plant-epiphyte composition and productivity, coupled with alterations in
454 salinity may further alter central ecosystem processes such as decomposition, and export
455 rates (Mateo et al., 2006), impacting associated macroinvertebrate assemblages (Bologna
456 and Heck, 1999) that support locally important marine fisheries.

457

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464

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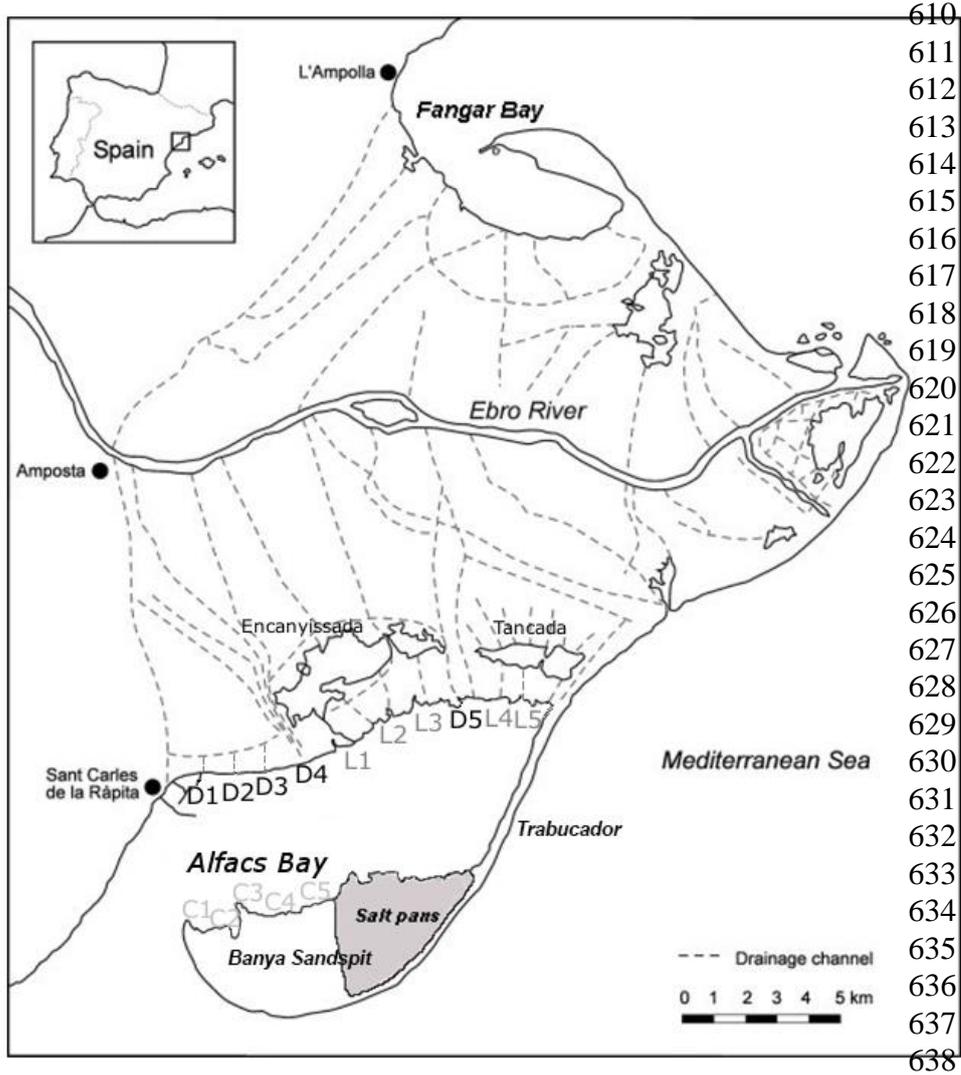
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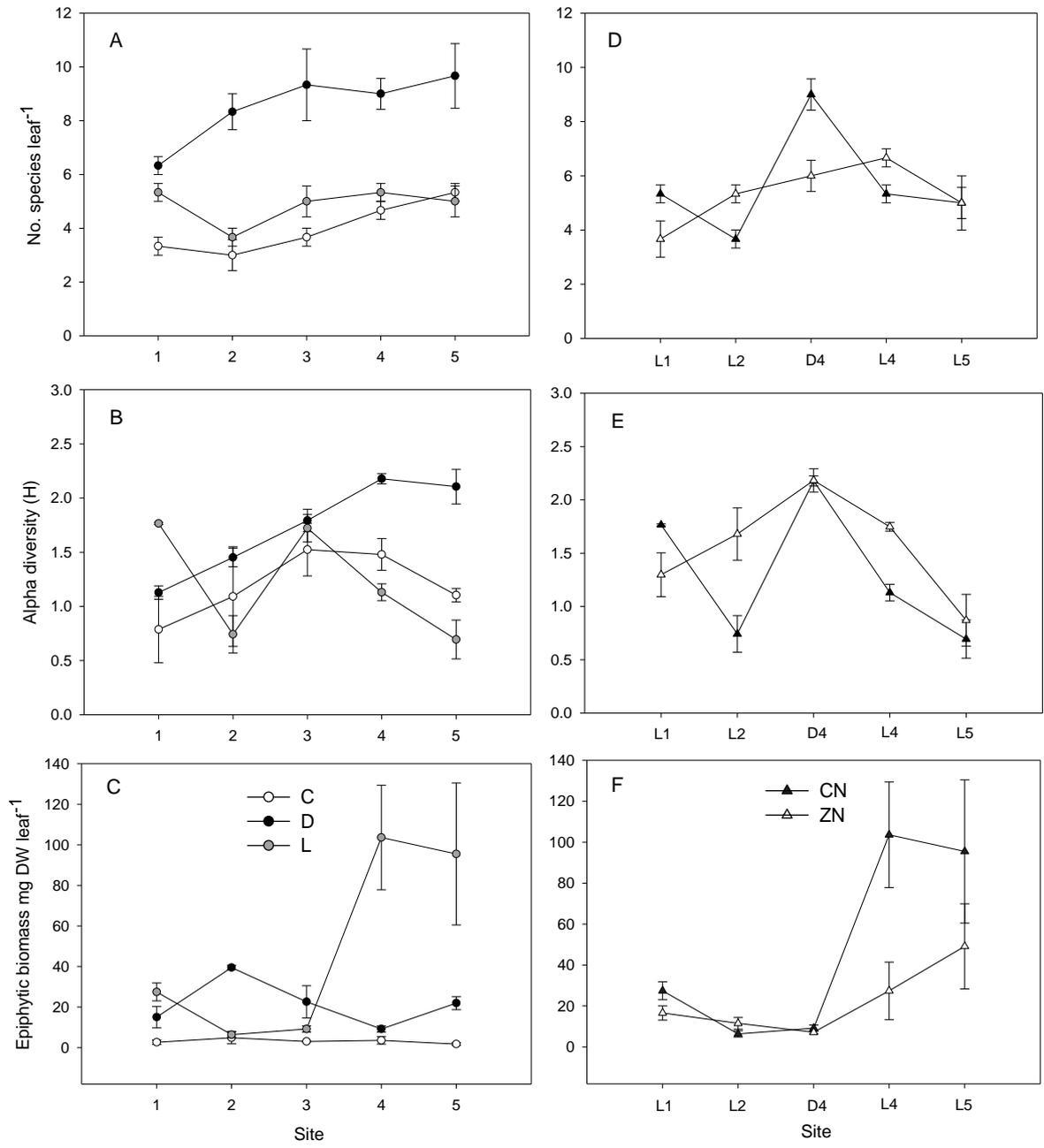
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639
640 **Fig. 1.** Map of the Ebro Delta showing the network of channels draining water to the Alfacs
641 Bay. The situation of study sites is showed with stars. Control sites (C) along the Banyà
642 Sandspit are indicated in light grey, lagoon sites (L) in dark grey, and drainage (D) channels
643 in black.
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645
 646 **Fig. 2.** Univariate measures (species richness, alpha diversity, and epiphytic biomass)
 647 recorded in the oldest leaf in: (1) sites featuring water discharge from canals and lagoons
 648 and in control sites with no impact (A, B, and C); and (2) sites including both *C. nodosa*
 649 and *Z. noltii* (D, E, and F). C: Control, D: Drainage, L: Lagoon channel sites.
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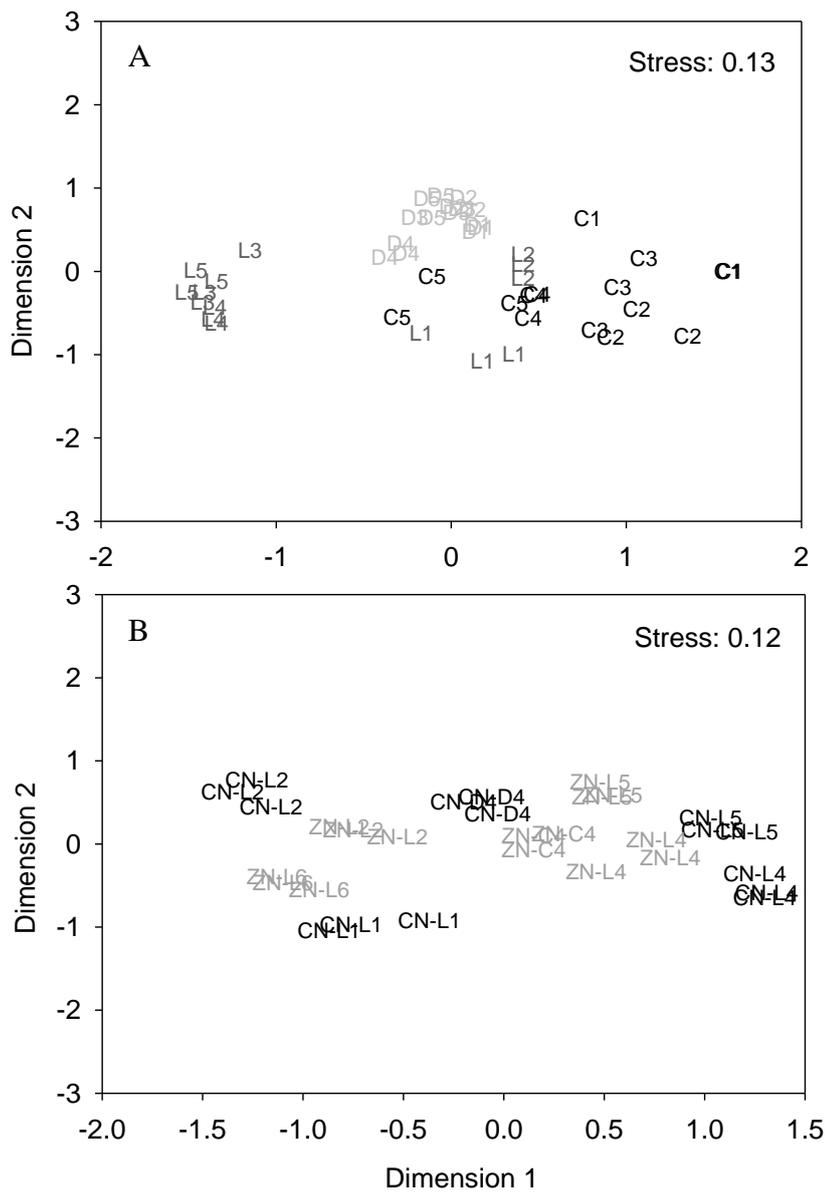


Fig. 3. nMDS showing patterns of epiphytic assemblages: A) Among sites featuring different types of water discharge (D= drainage channel, L= lagoon, C= control sites with no water input); and B) Among sites with *Cymodocea nodosa* (CN) and *Zostera noltii* (ZN). Data were Log (x + 1) transformed.

Site	Shoot density CN No. m ⁻²	Shoot density ZN No. m ⁻²	pH	Alkalinity (ppm)	Salinity	NH ₄ ⁺ (μg L ⁻¹)	NO ₃ ⁻ + NO ₂ ⁻ (μg L ⁻¹)	NO ₂ ⁻ (μg L ⁻¹)	PO ₄ ⁻³ (μg L ⁻¹)
D1	1458 ± 139		7.44	107	33.79	468.54	220.39	9.59	23.65
D2	1633 ± 68		7.5	107	36.56	619.21	320.43	7.86	25.86
D3	792 ± 88		7.45	70	6.71	359.35	3089.45	29.84	110.94
D4	933 ± 8.3	3692 ± 365	8	52	6.26	493.07	463.93	18.86	33.56
D5	958 ± 30		7.33	89	35.14	718.54	573.12	10.08	154.12
L1	933 ± 60	3908 ± 566	8.01	59	17.01	375.27	42.74	5.65	<20.65
L2	817 ± 36	3408 ± 248	7.45	78	27.31	261.4	38.2	3.02	<20.65
L3	1317 ± 36		8.01	82	19.89	472.8	186.23	20.06	27.16
L4	683 ± 60	3008 ± 271	7.88	81	21.59	619.33	1123.35	95.09	52.46
L5	667 ± 44	3875 ± 485	7.82	70	16.82	218.73	1063.74	16.71	27.32
C1	1867 ± 44		6.75	127	33.25	308.69	36.6	2.02	<20.65
C2	2008 ± 162		8.2	104	35.86	308.1	67.31	0.91	<20.65
C3	883 ± 8		8.15	91	25.32	684.27	69.33	3.33	<20.65
C4	1808 ± 8		7.93	107	34.81	191.96	19.62	0.95	<20.65
C5	850 ± 66		7.98	93	34.69	69.49	49.36	0.52	<20.65

Table 1. Plant and environmental variables measured during the study (N= 3). D= Drainage channel, L= Lagoon channel, and C= Control site. CN= *Cymodocea nodosa* and ZN= *Zostera nolti*. Errors are SE.

A) Among discharge types				B) Between <i>C. nodosa</i> and <i>Z. noltii</i>			
	<i>R</i>	<i>p</i> (%)	Sim./ Diss (%)		<i>R</i>	<i>p</i> (%)	Sim./ Diss (%)
Discharge	0.889	0.1		Species	0.419	0.1	
D-L	0.468	1.6	81.34	CN-ZN			46.77
D-C	0.82	0.8	79.27	CN			79.10
L-C	0.38	5.6	83.51	ZN			78.83
D			59.01				
L			29.04				
C			33.19				
Site	0.519	0.1		Site	0.985	0.1	
				L1-L2	1	1	64.65
				L1-D4	1	1	72.68
				L1-L4	1	1	86.64
				L1-L5	1	1	84.60
				L2-D4	1	1	56.13
				L2-L4	1	1	88.13
				L2-L5	1	1	81.82
				D4-L4	0.852	1	55.20
				D4-L5	1	1	55.45
				L4-L5	0.926	1	49.85
				D4			79.36
				L1			76.24
				L2			78.08
				L4			77.55
				L5			83.61

Table 2. Results of 2-way ANOSIM (global effects and pair-wise comparisons) and SIMPER analyses (Similarities/ dissimilarities) based on dissimilarity matrices derived from abundance of epiphyte species ($\log(x + 1)$ transformation). A) Effects among discharge types (only one factor indicated for the SIMPER analysis due to constrictions imposed by the nested design), and B) Effects between species and among sites (factorial design). *R*: ANOSIM statistic; *p*: significance level of sample statistic (%). In SIMPER results, pair-wise comparisons indicate dissimilarities and single groups similarities.

ANOVAs	Species richness				Alpha diversity			Biomass (Asen x)		
A) Discharge	df	MS	F	p	MS	F	p	MS	F	p
Discharge= D	2	86.87	73.75	0.0000	1.392	13.29	0.0000	25.96	70.88	0.0000
Site= Si(D)	12	3.18	2.70	0.014	0.557	5.32	0.0000	1.63	4.45	0.0000
Error	30	1.179			0.1047			0.366		
B) Species	df	MS	F	p	MS	F	p	MS	F	p
Species= S	1	0.833	0.93	0.347	0.482	6.57	0.019	1.779	5.81	0.026
Site= Si	4	9.75	10.83	0.0000	1.562	21.27	0.0000	16.70	16.70	0.0000
S x Si	4	5.917	6.57	0.002	0.447	0.447	0.002	2.91	2.91	0.047
Error	20	0.900			0.073			0.306		

Table 3. 2-way ANOVA results for testing for overall variability in species richness, alpha diversity, and biomass of epiphytes A) among sites subjected to distinctive water discharges, and B) between *Cymodocea nodosa* and *Zostera noltii*. Epiphytic biomasses were Asen (x) transformed to meet ANOVA assumptions. Statistically significant results are indicated in **Bold**.