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J.M. Ruiz, C. Marco-Méndez, J.L. Sánchez-Lizaso. Remote influence of off-shore fish farm waste on Mediterranean seagrass () meadows. *Marine Environmental Research*, 2010, 69 (3), pp.118. 10.1016/j.marenvres.2009.09.002 . hal-00565102

HAL Id: hal-00565102

<https://hal.science/hal-00565102>

Submitted on 11 Feb 2011

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Accepted Manuscript

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PII: S0141-1136(09)00119-6

DOI: [10.1016/j.marenvres.2009.09.002](https://doi.org/10.1016/j.marenvres.2009.09.002)

Reference: MERE 3368

To appear in: *Marine Environmental Research*

Received Date: 10 June 2009

Revised Date: 1 September 2009

Accepted Date: 2 September 2009

Please cite this article as: Ruiz, J.M., Marco-Méndez, C., Sánchez-Lizaso, J.L., Remote influence of off-shore fish farm waste on Mediterranean seagrass (*Posidonia oceanica*) meadows, *Marine Environmental Research* (2009), doi: [10.1016/j.marenvres.2009.09.002](https://doi.org/10.1016/j.marenvres.2009.09.002)

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1 **Remote influence of off-shore fish farm waste on Mediterranean seagrass**
2 **(*Posidonia oceanica*) meadows.**

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19

20 **Abstract**

21

22 The aim of this study was estimating the remote influence of waste dispersed
23 from a large offshore fish farm complex (6,197 ton year⁻¹) on the nearshore *Posidonia*
24 *oceanica* meadow (26-27 m deep) located at a distance of 3 km. Measurements of
25 isotopic nitrogen content in epiphytes and seagrass leaf tissues, epiphyte biomass, shoot
26 size, herbivory pressure, shoot density and seagrass meadow cover, performed in this
27 meadow (FA area) were compared with those obtained in an undisturbed control
28 meadow (CA area) to evaluate: (1) the remote influence of waste and (2) the impact of
29 such influence on seagrass condition. In addition, $\delta^{15}\text{N}$ measurements in particulate
30 organic matter of natural and anthropogenic origin were used in a single isotope mixing
31 model to elucidate the relative contributions of these sources to the isotopic N signal
32 measured in epiphytes and leaf tissues. Total tissue N content was similar between
33 meadow areas, but $\delta^{15}\text{N}$ signatures were significantly higher in the FA area than in the
34 CA area both in epiphytes and seagrass leaf tissues. Results from the mixing model,
35 together with available information on local currents and previous studies, support the
36 conclusion that the dispersion of farm wastes over large areas (spanning kilometres) are
37 responsible for the elevated $\delta^{15}\text{N}$ signatures found in the FA meadow area. Despite this,
38 no changes in meadow structure were detected and only some changes at the level of
39 seagrass community (epiphytes abundances and herbivores activity) could be
40 interpreted at the light of nutrient-induced effects in the FA area. Results from this study
41 indicate that concentrating aquaculture facilities in offshore areas is a strategy not
42 totally exempt of environmental risk on nearshore sensitive habitats such as seagrass
43 meadows.

44

45 *Keywords:* Fish farms waste; *Posidonia oceanica*; Epiphytes; Mediterranean Sea; Stable
46 isotopes.

47 1. Introduction

48

49 The increasing use of floating net cages in the Mediterranean Sea is a subject of
50 major concern, due to the impact of farm waste on marine ecosystems (FAO, 2006).
51 Floating net cages lead to considerable amounts of dissolved and particulate carbon,
52 nitrogen and phosphorus contained in uneaten feed components, excreta and faeces
53 being released into the water column (Hall et al., 1991, 1992; Holby and Hall 1991;
54 Dosdat et al., 1995). By altering water quality and sediment characteristics (Holmer et
55 al., 2007; Holmer and Kristensen 1992; Iwama 1991; Karakassis et al., 2001; Wu et al.,
56 1994), these aquaculture inputs have been shown to affect both the functioning and
57 structure of microbial (Vezzuli et al., 2002), planktonic (Pitta et al., 1999), infaunal
58 (Karakassis et al., 2000; Mirto et al., 2002;) and seagrass (Apostolaki et al., 2009;
59 Cancemi et al., 2003; Delgado et al., 1997;1999; Ruiz et al., 2001) communities in the
60 Mediterranean coastal ecosystem.

61 Seagrass meadows are dominant habitats of recognized ecological relevance in
62 temperate and tropical coastal areas (Green and Short, 2003), but they are experiencing
63 a worldwide decline mainly associated to human activity (Orth et al., 2006). Net cage
64 farming developed throughout the 1990s on the Spanish Mediterranean coast, with
65 small fish farms (i.e. with an annual fish production of several hundred tons) installed in
66 shallow, sheltered and semi-exposed environments where the endemic seagrass
67 *Posidonia oceanica* forms dense and continuous meadows. In these low dispersive
68 environments, high concentrations of dissolved nutrients and particulate (POM) and
69 sedimentary (SOM) organic matter have led to the loss and deterioration of *Posidonia*
70 *oceanica* meadows (Apostolaki et al., 2009; Cancemi et al., 2003; Delgado et al., 1997;
71 Pergent et al., 1999; Pergent-Martini et al., 2006; Ruiz et al., 2001). This seagrass
72 decline has been related to the effects of nutrient excess on plant physiology (Pérez et
73 al., 2008), the toxic effects of sediment anoxia and high sulphide concentrations on
74 plant tissues (Frederiksen et al., 2008; Pérez et al., 2007), and with nutrient-induced
75 interactions between plants and other components of the seagrass community, such as
76 macroalgae, epiphytes and herbivores (Delgado et al., 1997; Leoni et al., 2006; Ruiz et
77 al., 2001; Ruiz et al., in press).

78 As a result of the limited availability of un-vegetated sheltered sites in nearshore
79 environments and the continuous expansion of this aquaculture industry, it has been
80 necessary to place farms in deeper (>30 m) offshore waters with mainly un-vegetated

81 soft beds (Basurco and Larrazabal, 2000) in order to avoid further degradation of *P.*
82 *oceanica* meadows. In these offshore environments, higher current speeds and greater
83 depths should lead to a greater dispersion rate of farm waste and a reduction of SOM,
84 with benthic communities underneath fish cages being affected less as a result (Aguado-
85 Giménez et al., 2007; Maldonado et al., 2005). However, this contention ignores the
86 possible effects of dispersed farm waste on nearby seagrass communities. The influence
87 of aquaculture waste over a large area has tended to be underestimated by previous
88 studies based on traditional approaches involving only a physicochemical and biological
89 characterization of seawater and sediments, which determine that the extent of the
90 impact of fish farms is very local (no more than a few hundred metres), or even non-
91 existent (Aguado-Giménez et al., 2007; Cromey et al., 2002; Karakassis, 2001; Pearson
92 and Black, 2000; Pitta et al., 1999; Pitta et al., 2006; Telfer and Beveridge, 2001).
93 However, stable isotope techniques applied recently to trace the spatial extent of farm
94 waste in water, sediment, benthic organisms and bioassays revealed that aquaculture
95 products disperse to distances ranging between hundreds of metres and several
96 kilometres (Dolenec et al., 2007; Jones et al., 2001; Lin and Fong, 2008; Sará et al.,
97 2004; Sanz et al., in press; Vizzini and Mazzola, 2004; Ye et al., 1991). Therefore, the
98 suitability of the “offshore strategy” to preserve nearshore *Posidonia oceanica* meadows
99 against aquaculture waste has yet to be properly evaluated.

100 In this study, we combined the use of seagrass bioindicators, stable isotope
101 techniques and spatial analysis to evaluate: (1) the remote influence on the *Posidonia*
102 *oceanica* meadow of waste originated by an offshore fish farm complex, and (2) the
103 impact of such an influence on the condition of the seagrass. To this end, we selected a
104 set of *Posidonia oceanica* variables that have previously shown to effectively reflect
105 both the influence and impact of farm waste at different levels of seagrass organization
106 (Pérez et al., 2007; Ruiz et al., 2001): total and isotopic nitrogen content in epiphytes
107 and seagrass leaf tissues, epiphyte biomass, shoot size, herbivory pressure, shoot density
108 and meadow cover. We examined the null hypothesis that there are no differences in
109 these variables between a meadow area in front of the fish farm facilities and an,
110 unpolluted control area of meadow. A mixing model approach was also applied, using
111 isotopic nitrogen signals obtained in epiphytes and seagrass leaves, to determine the
112 specific contribution of farm waste with regard to other natural and anthropogenic POM
113 sources.

114

115 2. Materials and Methods

116

117 2.1. Study area

118 The study was conducted in a town on the southeast coast of Spain (San Pedro
119 del Pinatar; Murcia; 37° 50' 12.88'' N, 0° 47' 34.26'' W. Fig. 1). A fish farm complex has
120 operated in the waters of this town since 1998, with a current total annual production of
121 6.197 tons of blue fin tuna (*Thunnus thynnus*), sea bream (*Sparus aurata*), meagre
122 (*Argyrosomus regius*) and sea bass (*Dicentrarchus labrax*). Available data on current
123 speed and direction in the farming site taken over a six-month period (Sánchez-Lizaso,
124 unpubl. data) indicate that surface currents (7 m depth) most frequently run parallel to
125 the coastline in NW (34%) and SW (25%) directions, with an average speed of 10 cm s⁻¹
126 and a maximum speed of 68 cm s⁻¹. Deeper currents (25 m depth) run mainly in SE
127 (35%) and NW (25%) directions, with an average speed of 7 cm s⁻¹, and a maximum
128 measured speed of 27 cm s⁻¹. In this coastal area, the *Posidonia oceanica* meadow
129 forms a large and continuous band 25 km long (a total surface area of ca. 5.000 ha),
130 between 1 m and a mean depth limit of 27 m (Ruiz 2007; Fig. 1). Hydrodynamic
131 exposure and substrate type are similar throughout the whole meadow area.
132 Oceanographic characteristics (salinity, nutrients, light attenuation coefficient and Chl
133 a) correspond to typical high oligotrophic waters of this geographical area (Vargas-
134 Yáñez et al., 2007), except in the central area, due to the influence of hypersaline waters
135 of the Mar Menor coastal lagoon. A database of 90 sampling points available for this
136 meadow (Ruiz, unpubl. data) shows that the meadow structure is highly homogeneous
137 throughout this area, and corresponds to a healthy meadow in a good state of
138 conservation.

139

140 2.2. Sampling design and seagrass variables

141 Seagrass sampling was carried out in August 2005 at the deepest point of the
142 *Posidonia oceanica* meadow (26-27 m) in front of the fish farming area (hereinafter
143 'FA', Fig. 1). The nearest distance between the edge of the meadow and the fish cages
144 was 2.8 km. At the time of the sampling (summer), *Posidonia oceanica* shoot and
145 epiphyte biomasses (Ruiz et al., 2001; Sánchez Lizaso 1993) were at their maximum, as
146 was fish production, with annual maximums of light and typical temperatures for the
147 time of year in the Mediterranean (Klein and Roether 2001). The FA meadow was
148 compared with a control meadow area (CA) 12.2 km in a southerly direction at the same

149 depth, very close to the Marine Reserve of Cabo Palos-Islas Hormigas (Fig. 1). This
150 control meadow area was selected due to its similarity with the FA meadow, and to rule
151 out any possible influence of fish farm effluents, hypersaline waters from the coastal
152 lagoon or any other anthropogenic influence. No alternative control sites could be found
153 either northward, due to the influence of continental inputs, or to the south, due to the
154 intense alteration of deep *Posidonia oceanica* meadows caused by illegal trawling over
155 recent decades (Martín et al., 1997).

156 A nested sampling design (Quinn and Keough, 2002) was used to incorporate
157 and partition spatial variability of the selected seagrass variables at the different spatial
158 scales (from metres to kilometres) included in each meadow area. Two replicated
159 locations, separated by approximately 1.5 kilometres, were selected in each meadow
160 area (L1- L2 in FA and L3-L4 in CA, Fig. 1). Two sites separated by 100 metres were
161 selected within each location. Meadow structure showed high patchiness in all sites, so
162 three patches separated by a few metres were selected at random. For seagrass variables
163 measured at the level of individual shoots (i.e. shoot size, epiphyte load, total N content
164 and isotopic N signatures), six vertical shoots of *Posidonia oceanica* were randomly
165 collected within each patch and transported to the laboratory for further measurement
166 and analysis. At the patch level, the selected variables were shoot density and herbivore
167 pressure. Shoot density (number of shoots per m⁻²) was measured by counting shoot
168 numbers in 400 cm² quadrants placed in six randomly selected patches within each site.
169 In each site, herbivory pressure was determined in three randomly selected patches as
170 the frequency (percentage) of leaves with recognizable marks of herbivores bites
171 (Boudouresque and Meinesz, 1982), relative to the total number of leaves contained in
172 40 shoots collected within each patch. At the meadow level (i.e. sites), meadow cover
173 (as a percentage) was measured by visually estimating the percentage of substrate
174 occupied by shoot patches within twelve 1600 cm² quadrants placed at regular intervals
175 along a 25 m line transect.

176 In order to determine the specific contributions of external, natural and
177 anthropogenic organic matter sources to the isotopic N signature measured in plant and
178 epiphytes tissues (see mixing model analysis below), POM was collected in six
179 replicated sediment traps deployed close to the net cages and in one site of the control
180 area (CA) over the seagrass canopy. POM samples at the first site were mainly
181 composed of fish faeces and hence we assume that they represent the external
182 anthropogenic source to be mainly farm waste (FFW); at the second site we assume that

183 POM samples represent the external natural source (NAT) that is composed of a
184 mixture of both autochthonous and allochthonous materials of diverse origin (Dolenec
185 2006, Sarà 2006). Sediment traps consisted of PVC cylinders of 11 cm diameter and 55
186 cm height, as recommended by many authors in similar studies (Cromeey et al., 2002).
187 Trapped particles were concentrated in a small glass tube placed at the bottom end of
188 the cylinder, which was recovered after 24 hours and immediately transported to the
189 laboratory for analysis of stable isotopes (see below).

190 **2.3. Laboratory analysis**

191 In the laboratory, epiphytes were scraped and separated from the leaves of each
192 shoot using a razor blade, and the epiphytes were dried to a constant weight (60° C, 24
193 h) to estimate their biomass (mg DW shoot⁻¹). The length and width of each leaf on a
194 shoot were measured to calculate shoot size as leaf surface area per shoot (cm² shoot⁻¹);
195 epiphyte biomass was normalized to shoot size to estimate epiphyte load (mg cm⁻²).
196 Dried epiphyte and leaf tissues (young leaves, 0-50 days old) were finely grounded in a
197 mortar and, for each replicate, approximately 2 mg of dry weight was encapsulated to
198 determine total (%DW) and isotopic ($\delta^{15}\text{N}$, ‰) nitrogen. POM samples obtained in
199 sediment traps were filtered in laboratory through pre-combusted fibreglass filters
200 (Whatman GF/F), and the filters were dried and ground for analysis of total and isotopic
201 nitrogen. Total N content was measured using a Carlo-Erba CNH elemental
202 autoanalyzer. The nitrogen stable isotope composition was measured using an EA-
203 IRMS (Thermo Finnigan) analyzer in continuous flow configuration, and was compared
204 with a worldwide standard (atmospheric N₂) to estimate the relative amount of ¹⁵N, or
205 $\delta^{15}\text{N}$ in plant tissues and POM, as described by the following equation (Peterson and
206 Fry, 1987):

$$207 \quad \delta^{15}\text{N} (\text{‰}) = (R_{\text{sample}} / R_{\text{standard}} - 1) \times 10^3$$

208 where R is defined as the atomic ¹⁵N/¹⁴N ratio. Acetanilide was used as the internal
209 laboratory standard.

210 **2.4. Statistical analysis**

211 A nested ANOVA model was used to test the null hypothesis that seagrass
212 variables were similar across meadow areas, locations, sites and seagrass patches. For

213 seagrass variables measured at the individual shoot level, a four-factor model was
214 considered: (1) “areas” (fixed factor with 2 levels: FA and CA), (2) “locations” (random
215 factor nested in “areas” with 2 levels: L1 and L2 in area FA and L3 and L4 in area CA),
216 (3) “sites” (random factor nested within each “location” with 2 levels) and (4) “patches”
217 (random factor nested within each “site” with 3 levels). For seagrass variables replicated
218 at site level (meadow cover, shoot density and herbivory pressure), a model with the
219 first three factors considered was used. Prior to the analysis, Cochran’s C-test was used
220 to test the homogeneity of variances (Underwood, 1997), and data were properly
221 transformed when necessary. When no homogeneous variances were rendered with any
222 type of transformation, the significance level was set at 0.01 instead of 0.05, as
223 ANOVA can withstand variance heterogeneity, particularly for large balanced
224 experiments, reducing the possibility of a Type I error (Underwood, 1997).

225 A single-isotope three-source mixing model (Phillips and Gregg, 2003) was used
226 to determine the relative contribution of farm sources in the isotopic N signature of
227 epiphytes in relation to other potential external sources present in the study area.
228 Epiphyte N signatures measured in FA and CA meadow areas were designated as the
229 targets in the model. Seagrass leaf N signature was not included in this analysis, as it is
230 governed not only by external water sources but also by internal physiological
231 mechanisms and sediment pools (Romero et al., 2006; Touchette and Burkholder, 2000)
232 that were not measured in this study. The isotopic signal of three potential sources were
233 considered as the end-members of the model: fish farm waste (FFW), external natural
234 sources (NAT), both of which were measured in this study (see previous section), and
235 urban waste (UW), reported from literature ($\delta^{15}\text{N} = 9.7\text{‰}$, Dolenc et al., 2007). This
236 last external anthropogenic source was introduced into the model due to the presence of
237 an urban sewage outfall 6 km north of the FA area.

238

239 **3. Results**

240

241 Table 1 shows the results of the nested ANOVA test on seagrass variables.
242 Meadow cover varied between $14.5 \pm 3.4\%$ and $27.0 \pm 2.4\%$ (Fig. 2A), but no
243 significant differences were detected between areas or at any other spatial scale within
244 them. Shoot density was also similar ($116\text{-}200$ shoots m^{-2}) between areas and sites, with
245 the exception of the significant higher mean values founded in location L4 in the CA
246 meadow (295.2 ± 12.5 shoots m^{-2}) (Fig. 2B; Table 1). Herbivore pressure on seagrass

247 leaves was generally very low (0-7%), but showed highly significant variation between
248 locations due to the extremely high values measured in location L2 of the FA meadow
249 area (8.3-28%; Fig. 2C and Table 1). This herbivore pressure was accounted for by the
250 herbivorous sparid fish *Sarpa salpa* in L2, but by other unidentified herbivore species
251 (possibly isopods) in the CA meadow locations.

252 Shoot size was slightly (but not significant) lower in the FA meadow ($421.4 \pm$
253 $17.8 \text{ cm}^2 \text{ shoot}^{-1}$) than in the CA meadow ($472.2 \pm 22.0 \text{ cm}^2 \text{ shoot}^{-1}$); spatial variability
254 of this variable was only significant at the smaller scale i.e. between patches (Fig. 3A,
255 Table 1). Epiphyte load showed lower mean values in the FA area ($1.11 \pm 0.23 \text{ mg DW}$
256 cm^{-2}) than in the CA area ($1.77 \pm 0.14 \text{ mg DW cm}^{-2}$) (Fig. 3B), but differences were not
257 significant at this and any other spatial scale (Table 1). Subsequent calculations of the
258 observed power of the test for 'Area' effects (13.8%) indicated a low capability to
259 detect differences observed in epiphyte load (i.e. high probability of Type II error); the
260 non-significant term 'Location' (Table 1) was then pooled to increase the power of the
261 test after considering a conservative significance level of $\alpha = 0.25$ for the pooled term
262 (Sokal and Rohlf, 1995; Quinn and Keough, 2002). After pooling, the power of the test
263 for the 'Area' effect on epiphyte load increased to 70%, and significant differences in
264 epiphyte load between areas were then detected ($df = 1,6$; $F = 10.29$; $P = 0.0184$).

265 Mean epiphyte N content varied between 0.7 and 1.0% throughout the area
266 studied with maximum values concentrated in the FA meadow, but no significant
267 differences were detected at any spatial scale considered in the analysis (Fig. 3C, Table
268 1). Mean leaf N content varied between 1.4 and 1.9% and was also similar between
269 areas, but very significant variability was detected between locations and seagrass
270 patches (Fig. 3E, Table 1). The isotopic signal of nitrogen ($\delta^{15}\text{N}$) in epiphyte tissues was
271 consistently and significantly higher in the FA meadow ($5.36 \pm 0.013 \text{ ‰}$) than the CA
272 meadow ($3.75 \pm 0.013 \text{ ‰}$) (Fig. 3D, Table 1). Variability between localities within each
273 meadow area was also significant for this variable (SNK, Table 1). Isotopic nitrogen
274 measured in seagrass leaves also showed significantly higher mean values in the FA
275 meadow ($3.69 \pm 0.02 \text{ ‰}$) than at CA ($2.98 \pm 0.08 \text{ ‰}$) (Fig. 3F), although the variability
276 between seagrass patches within sites was also significant (Table 1).

277 Mean values of isotopic N measured in POM captured by sediment traps showed
278 significant differences between FFW and NAT sources, with higher values for the
279 former ($6.67 \pm 0.32 \text{ ‰}$) than for the latter ($3.69 \pm 0.17 \text{ ‰}$) ($n = 6$; t-test, $p < 0.05$).
280 Table 2 shows the results of the mixing model applied to determine the relative

281 contributions of distinct isotopic N signatures measured in various sources to the mixed
282 N signature measured in epiphytes. For each meadow area, the table indicates the range
283 (1- 99th percentile) and the mean and standard deviation of the distribution of all
284 feasible contributions from each source to the epiphyte N signature. In the FA area, fish
285 farm waste (FFW = 12-56%) and natural sources (NAT = 44-66%) both provided the
286 most significant contributions to the epiphytes N signature. The contribution of urban
287 waste was very low and ambiguous, since it included zero contributions (UW = 0-22%).
288 In the CA area, epiphyte N signal was unambiguously accounted by external natural
289 sources (NAT = 98-99%) while external anthropogenic contributions (FFW and UW)
290 were almost negligible (0-2%).

291

292 **4. Discussion**

293

294 Both epiphytes and seagrass leaf tissues were particularly and significantly ^{15}N -
295 enriched in *Posidonia oceanica* plants located 3 km from the fish farm complex (i.e. the
296 FA meadow area) in comparison with the control meadow, which seems to reflect the
297 nature of external N sources influencing each meadow area. In the control meadow area,
298 POM (NAT source) had an isotopic N signal similar to that measured in water samples
299 from unpolluted natural sites in near-shore Mediterranean environments (2-4‰, e.g.
300 Dolenc et al., 2007; Sará et al., 2004). It may therefore represent a natural or
301 “background” signal composed by a mixture of detritus material of marine and
302 terrestrial origin, phyto- and zooplankton (Dolenc et al., 2007), and, in this case,
303 seagrass detritus. By contrast, POM collected close to the fish cages (FFW) consisted
304 mostly of uneaten food pellets and fish faeces, and exhibited higher positive $\delta^{15}\text{N}$
305 values as those typically measured in these anthropogenic sources (6-11‰, e.g. Dolenc
306 et al., 2007; Sará et al., 2004). In the water column, rapid mineralization of this initially
307 N-enriched POM results in an impoverishment of its isotopic N signature and an
308 enrichment in the dissolved N pool, due to isotopic fractionation by physical and
309 biological process (Macko and Ostrom, 1994; Sará et al., 2004). Excreta from farmed
310 fish (Dosdat et al., 1995) and wild fish around the cages (Fernández-Jover et al., 2008),
311 as well as re-suspension of enriched SOM (Sará et al., 2006), contribute significantly to
312 this dissolved ^{15}N -enriched pool, which currents immediately disperse toward
313 surrounding areas (Sará et al., 2004, 2006).

314 The isotopic N composition of benthic primary producers reflected that of
315 external N sources (Fry, 2006). High $\delta^{15}\text{N}$ values, such as those observed in epiphytes
316 and seagrass tissues in the FA meadow, are associated with the influence of external
317 anthropogenic sources such as aquaculture loads and land-derived wastewater (Cole et
318 al., 2004; Cole et al., 2005; Constanzo et al., 2001; Constanzo et al., 2004; Dolenc et
319 al., 2007; Jones et al., 2001; Lin and Fong, 2008; Pérez et al., 2008; Vizzini and
320 Mazzola, 2004). Several findings support this hypothesis in our case study: (1) fish farm
321 production in our study was approximately 4.2 times higher than a case study that
322 recorded elevated $\delta^{15}\text{N}$ signatures in *Posidonia oceanica* leaves over an area located up
323 to 3 km from the fish farm (Dolenc et al. 2007); (2) the dominant current direction was
324 NW (34%), but the frequency of currents directed toward the FA meadow area (i.e. SW)
325 was also important (25%); (3) the mean velocity of SW currents was 10 cm s^{-1} and
326 reached maximum values of 58 cm s^{-1} , which has shown to be enough to disperse farm
327 waste over a distance covering several kilometres (Sarà et al., 2006); (4) a spatial
328 gradient of $\delta^{15}\text{N}$ between the fish farm and the FA meadow area was obtained in a
329 previous study by measuring isotopic N in benthic macrophytes incubated in pelagic
330 bioassays deployed at increasing distances from fish cages (García-Sanz et al., in press);
331 and finally, (5) the mixing model used in this study provided consistent evidence of the
332 significant contribution of farm waste (i.e. FFW source) to the isotopic N signature of
333 epiphyte tissues in the FA meadow area. Therefore we can consider that farm waste is
334 responsible for the high $\delta^{15}\text{N}$ signatures measured in the seagrass and epiphyte tissues
335 of the FA meadow located 2.8 km from the aquaculture facilities.

336 With a high availability of nutrients, seagrass and its epiphytes can assimilate
337 and store excess nutrients in their tissues, increasing their total N content (Lepoint et al.,
338 2007; Invers et al., 2004; Touchette and Burkholder, 2000). However, no significant
339 increase in nitrogen was observed in either *Posidonia oceanica* leaves or epiphytes in
340 the FA meadow, despite the influence of farm waste. A possible explanation is that the
341 nutrients supplied are too low to merit long-term N storage, and are probably used to
342 sustain growth, as suggested for macroalgae species exposed to moderate nutrient
343 increase in fertilization experiments (McGlathery, 1995) and from shrimp farm loads
344 (Lin and Fong, 2008). This argument can be particularly valid for epiphytic algae,
345 which quickly exploit any increase in water column nutrient availability (Lepoint et al.,
346 2007; Short et al., 1995). Furthermore, nutrients could favour opportunistic epiphytic
347 algae over seagrass leaves (Duarte, 1995; McGlathery et al., 1995; Prado et al., 2008;

348 Wear et al., 1999), the rapid growth of which prevents N accumulation in their tissue.
349 Nonetheless, epiphytes showed greater variability of N content between seagrass
350 patches within some of the FA meadow sites, as a consequence of the maximum values
351 obtained only in this meadow area (Fig. 3C), which can be interpreted as a
352 heterogeneous influence of farm waste on epiphyte N content at a small spatial scale. In
353 seagrass leaves, the lack of N response to water-dissolved nutrients has a more complex
354 interpretation, as it can also be governed by other limiting factors (e.g. light),
355 competition for nutrients by epiphytes, internal N and C metabolism and sediment
356 nutrient pools (Invers et al., 2004; Romero et al., 1998; Romero et al., 2006; Touchette
357 and Burkholder, 2000).

358 In response to fish farm load, epiphyte abundance in *Posidonia oceanica* leaves
359 can either rise (Cancemi et al., 2003; Delgado et al., 1997, 1999) or decrease due to
360 enhanced consumption of epiphyted leaf tips by seagrass macro-herbivores (Ruiz et al.,
361 2001; Ruiz, 2000; Ruiz et al., in press). Previous experimental studies support the
362 existence of this nutrient-macrogazers interaction in *P. oceanica* meadows (Prado
363 2007; Ruiz, 2000) and in tropical seagrass communities (McGlathery, 1995; Goecker et
364 al., 2005). However, our data did not support the existence of such interaction in the FA
365 meadow since the spatial pattern showed by the herbivorous fish *S. salpa* activity was
366 not coupled with that of epiphyte abundance (Figs. 2C and 3B, respectively).
367 Alternatively, we suggest that nutrient induced changes in the composition of the
368 epiphytic assemblage and/or enhanced mesoherbivores (mainly gastropods) activity
369 (Lin et al., 1996, see Borowitzka et al., 2006, Valentine and Duffy, 2006 Burkholder et
370 al 2007 for a recent review of this topic) might also account for the reduction in
371 epiphyte abundance reported in the FA meadow. Nonetheless, since no measurements
372 of these variables are available this possibility is speculative and other factors must be
373 considered including those responsible for natural spatial patterns of *P. oceanica*
374 epiphyte assemblages (e.g. Balata et al., 2007; Pardi et al., 2006). Apart from these
375 possible changes in seagrass community, no other evidence of any nutrient-related
376 effect was obtained at the level of meadow structure. Smaller shoot size and altered
377 meadow structure (shoot density and meadow cover) are commonly reported responses
378 for *Posidonia oceanica* meadows directly exposed to aquaculture loads (e.g. Delgado et
379 al., 1997; Ruiz et al., 2001) or experimental nutrient enrichment (Leoni et al., 2006),
380 though this kind of alteration was not detected in our study.

381 In conclusion, results based on stable isotope analysis, together with available
382 information on hydrodynamics and from previous studies in the study site, provide
383 consistent evidence that waste delivered by offshore farms has a large area of influence
384 (spanning kilometres) that affects *Posidonia oceanica* meadows located in remote near-
385 shore environments. Results obtained also indicate that such influence was not
386 sufficient to produce significant meadow alterations, unlike those described in near-
387 shore case studies under a more severe influence of nutrients. Only changes in the
388 abundance of epiphytes (and possibly in the activity of grazers) could be interpreted in
389 the light of nutrient-induced effects at the community level, but further evidence would
390 be required to confirm it. Finally, based on this study case, the idea that concentrating
391 aquaculture facilities in offshore areas is a strategy totally exempt of environmental risk
392 cannot be supported and hence site-selection processes for new aquaculture facilities
393 must be careful with regard to the distribution of this and other sensitive habitats, even
394 when these are located at distances greater than one kilometre.

395

396 *Acknowledgments*

397 This study is a part of a project funded by the *Plan Nacional de Cultivos Marinos* of the
398 *Junta Nacional Asesora de Cultivos Marinos (JACUMAR)* to JMR during the period
399 2004-2007. We would like to thank IEO technician Rocío García Muñoz for her
400 valuable help in laboratory work and field sampling. JMR would like to thank *TAXON*
401 *Estudios Ambientales S.L.* and the students Teresa Castro and Eva Vázquez for field
402 assistance. We acknowledge to the *Servizos de Apoio à Investigación* of the *University*
403 *da Coruña* for their support in the analysis of stable isotopes.

404

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647 **Figure Captions**

648

649 Figure 1. Map of the study area indicating the location of the fish farms (empty
650 polygons) and sampled meadow areas (see the Materials and Methods section).

651

652 Figure 2. Mean \pm SE of **A** the percentage of meadow cover; **B** the shoot density (shoots
653 m^{-2}); and **C** herbivore pressure (% of leaves eaten) at sites sampled within each location.

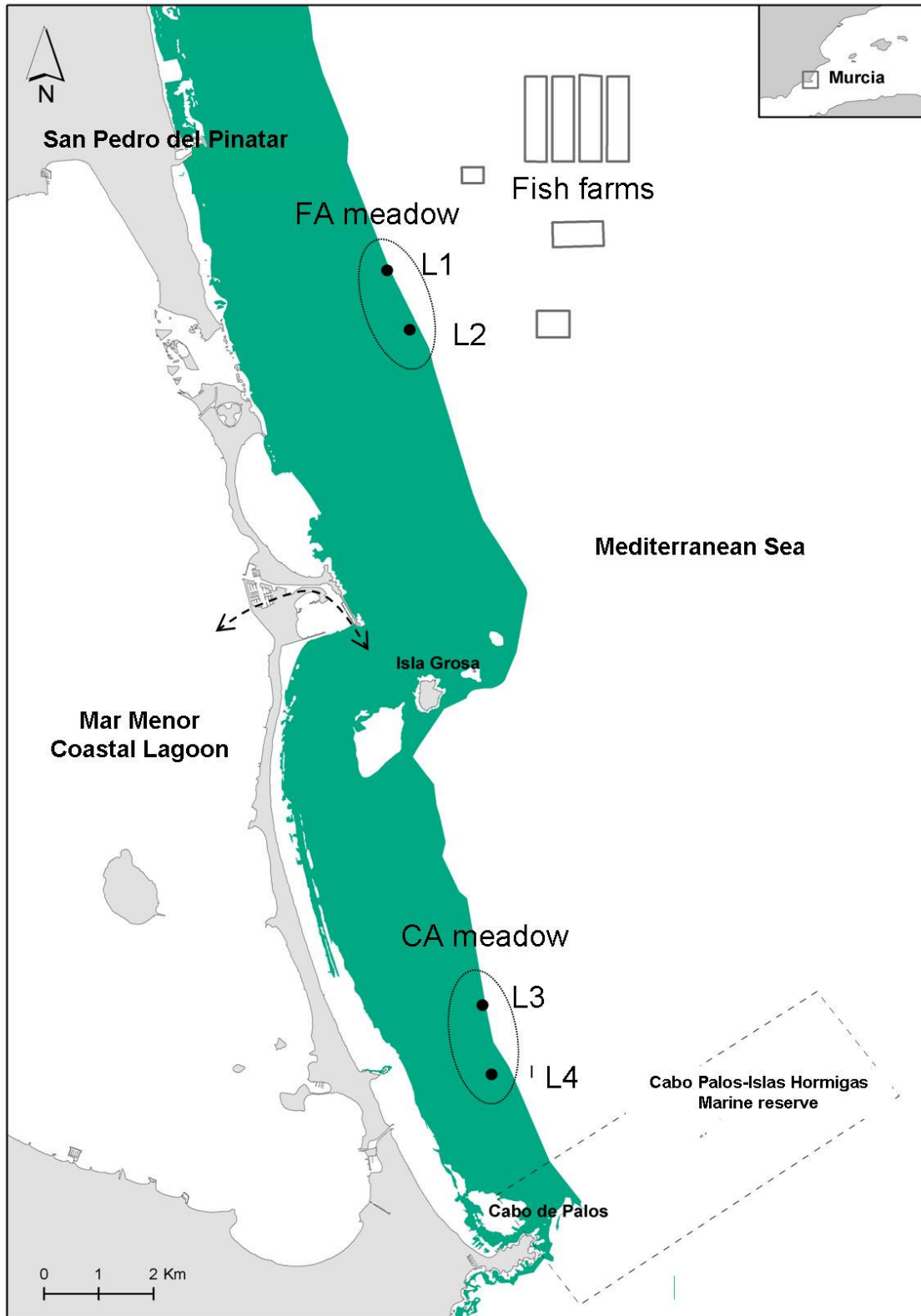
654

655 Figure 3. Mean \pm SE of **A** the shoot size (as leaf surface area, $\text{cm}^2 \text{shoot}^{-1}$); **B** epiphyte
656 load (mg DW cm^{-2}); **C** epiphyte N content (% DW); **D** epiphyte isotopic nitrogen
657 ($\delta^{15}\text{N}$); **E** leaf N content (% DW); and **F** leaf isotopic nitrogen composition ($\delta^{15}\text{N}$).

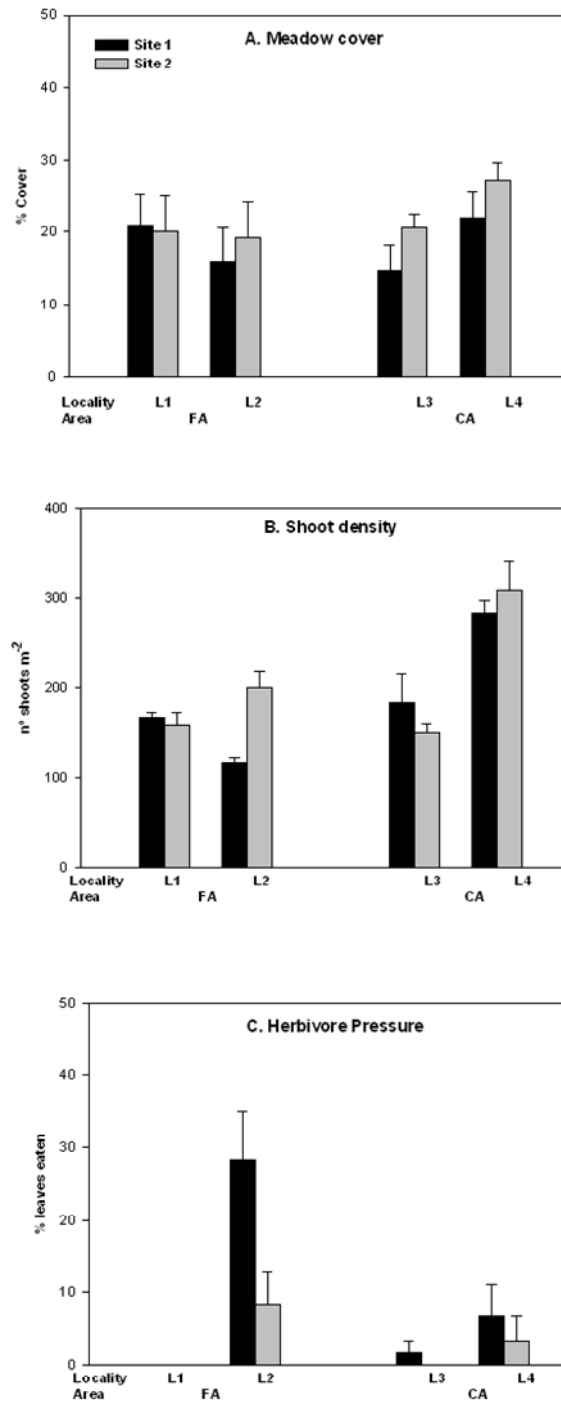
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13

14 Figure 1



Figures 2:



Figures 3:

