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1 Remote influence of off-shore fish farm waste on Mediterranean seagrass
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Abstract

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

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

1. Introduction

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

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

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

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

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

2. Materials and Methods

2.1. Study area

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

2.2. Sampling design and seagrass variables

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

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

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

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

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

2.3. Laboratory analysis

In the laboratory, epiphytes were scraped and separated from the leaves of each shoot using a razor blade, and the epiphytes were dried to a constant weight (60° C, 24 h) to estimate their biomass (mg DW shoot⁻¹). The length and width of each leaf on a shoot were measured to calculate shoot size as leaf surface area per shoot (cm² shoot⁻¹); epiphyte biomass was normalized to shoot size to estimate epiphyte load (mg cm⁻²). Dried epiphyte and leaf tissues (young leaves, 0-50 days old) were finely grounded in a mortar and, for each replicate, approximately 2 mg of dry weight was encapsulated to determine total (%DW) and isotopic (δ¹⁵N, ‰) nitrogen. POM samples obtained in sediment traps were filtered in laboratory through pre-combusted fibreglass filters (Whatman GF/F), and the filters were dried and ground for analysis of total and isotopic nitrogen. Total N content was measured using a Carlo-Erba CNH elemental autoanalyzer. The nitrogen stable isotope composition was measured using an EA-IRMS (Thermo Finnigan) analyzer in continuous flow configuration, and was compared with a worldwide standard (atmospheric N₂) to estimate the relative amount of ¹⁵N, or δ¹⁵N in plant tissues and POM, as described by the following equation (Peterson and Fry, 1987):

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

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

2.4. Statistical analysis

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

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

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

3. Results

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

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

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

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

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

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

4. Discussion

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

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

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

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

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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 ($\delta^{15}\text{N}$).

13

14 Figure 1

Figures 2:

Figures 3: