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► To cite this version:

T. García-Sanz, J.M. Ruiz-Fernández, M. Ruiz, R. García, M.N. González, et al.. An evaluation of a macroalgal bioassay tool for assessing the spatial extent of nutrient release from offshore fish farms. *Marine Environmental Research*, 2010, 70 (2), pp.189. 10.1016/j.marenvres.2010.05.001 . hal-00602601

HAL Id: hal-00602601

<https://hal.science/hal-00602601>

Submitted on 23 Jun 2011

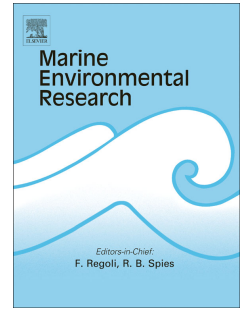
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Accepted Manuscript

Title: An evaluation of a macroalgal bioassay tool for assessing the spatial extent of nutrient release from offshore fish farms

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PII: S0141-1136(10)00068-1

DOI: [10.1016/j.marenvres.2010.05.001](https://doi.org/10.1016/j.marenvres.2010.05.001)

Reference: MERE 3448

To appear in: *Marine Environmental Research*

Received Date: 26 June 2009

Revised Date: 26 April 2010

Accepted Date: 3 May 2010

Please cite this article as: García-Sanz, T., Ruiz-Fernández, J.M., Ruiz, M., García, R., González, M.N., Pérez, M. An evaluation of a macroalgal bioassay tool for assessing the spatial extent of nutrient release from offshore fish farms, *Marine Environmental Research* (2010), doi: 10.1016/j.marenvres.2010.05.001

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An evaluation of a macroalgal bioassay tool for assessing the spatial extent of nutrient release from offshore fish farms

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1 **An evaluation of a macroalgal bioassay tool for**
2 **assessing the spatial extent of nutrient release from**
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1 Abstract

2 In this study we develop and evaluate a macroalgal bioassay tool for monitoring the
3 spatial extent of dissolved wastes loaded from offshore fish farms into the marine
4 coastal ecosystem. This tool is based on the analysis of the nitrogen stable isotope ratio
5 ($\delta^{15}\text{N}$) in tissues of several benthic primary producers (macroalgae and *P. oceanica*
6 epiphytes) incubated, by means of incubation devices, in the water column at increasing
7 distances (from 0 m to 1000 m) from the fish cages. The bioassays were performed in
8 three fish farms situated in different geographical locations (the Canary Islands, Murcia
9 and Catalonia) and we test: the suitability of the different macroalgae species used in
10 relation with their resistance to incubation and their sensitivity to fish-farm wastes and
11 the most appropriate incubation depth (5m or ~20m) and incubation time (2, 4 or 6
12 days) to detect the spatial distribution of $\delta^{15}\text{N}$ around fish farms. In general terms, the
13 results showed a significant increment of $\delta^{15}\text{N}$ values towards the fish cages with
14 respect to the reference (initial) and control values for all the species of macroalgae
15 tested except for the red algae *Asparagopsis taxiformis* from Canary Islands. The
16 magnitude and shape of the reported spatial responses varied as a function of the
17 experimental settings analyzed as well as a function of the nutrient regime
18 characteristics of each coastal area. The spatial gradient was more consistent in the
19 shallow part of the water column (5m depth), than in the deeper part (~20m) and was
20 statistically significant after an incubation period of four days. These results confirm the
21 effectiveness and reliability of the method proposed, enabling the spatial extent of
22 nutrients derived from fish farms to be assessed in an effective and simple manner,
23 suitable for use in monitoring programs around offshore fish farms.

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27 **Keywords:** Nitrogen Stable isotopes ($\delta^{15}\text{N}$), Fish farms, Macroalgae, Bioassays, Indicators.

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1 Introduction**2**

3 The use of floating net cages in marine aquaculture has grown steadily over recent years
4 in the Mediterranean (FAO, 2007). This development has sparked much concern over
5 the threats posed by fish-farm wastes entering marine ecosystems. Fish farm wastes
6 increases the concentration of particulate organic matter (POM) and dissolved nutrients
7 in the surrounding waters (Pitta et al., 1999; Karakassis et al., 2001; Norvdarg and
8 Johansson, 2002) which has lead to significant alterations of benthic marine
9 communities, mainly in soft-bottom macro- and meio-benthos (Mazzola et al., 2000;
10 Mirto et al., 2002) but also in more vulnerable and valuable habitats, particularly
11 seagrasses (Pergent et al., 1999; Ruiz et al., 2001; Holmer et al., 2003; Pergent-Martini
12 et al., 2006; Holmer et al., 2008; Ruiz et al, in press). Most of these negative effects
13 have been reported in shallow and sheltered nearshore environments, and as a
14 consequence, most of fish farm facilities are presently placed in deeper, offshore waters,
15 where aquaculture waste is presumed to have a lower resident time thus minimizing the
16 impact on benthic communities (Wu et al., 1994; McGhie et al., 2000; Alongi et al.,
17 2003).

18 Despite the reported evidence of environmental degradation caused by aquaculture
19 activities, there are currently no specific and reliable tools to provide ecologically
20 meaningful information about the impact of fish-farm wastes on marine ecosystems.
21 Classic descriptors based on water-column and sediment physicochemical variables (i.e.
22 DIN, POM, among others) have been systematically used in monitoring programs of
23 aquaculture impacts to assess the spread of fish-farm wastes in marine environments
24 (Dosdat et al., 1995; Roque d'Orbcastel et al., 2008). However, these methods have
25 usually failed to detect nutrient enrichment in receiving waters surrounding fish farms,
26 thus underestimating the spatial extent of the dissolved fraction of aquaculture wastes
27 (Jones et al., 2001 and references therein). This lack of response is attributed to the fast
28 dispersal of dissolved nutrients, its assimilation by organisms or loss to the atmosphere
29 through volatilization and denitrification (Pitta et al., 1999; Wolanski et al., 2000;
30 Karakassis et al., 2001; Sarà, 2007). This is especially true in offshore environments
31 where the dilution and spread of released nutrients is faster than at more confined sites.

1 Biological indicators (bioindicators) represent an alternative to physical and chemical
2 analyses to assess water quality since they can integrate both persistent and pulsed
3 changes in external nutrient availability over time (Lyngby 1990; Costanzo et al., 2000;
4 Jones et al 2001). Benthic primary producers (macroalgae, seagrasses and epiphytes) are
5 considered excellent potential bioindicators of nutrient availability for several reasons:
6 they are widely distributed and abundant, their biomasses persist over relatively long
7 periods, and they show well-characterized physiological responses to nutrient exposure
8 (Udy and Dennison, 1997; Lyngby et al., 1999). Indeed, the number of studies that use
9 these organisms to assess the influence of external sources of nutrients on coastal
10 ecosystems has recently increased (Fong et al., 1998; Savage and Elmgren, 2004; Lee et
11 al., 2004; Dalsgaard and Krause-Jensen, 2006; Pérez et al., 2008). On the other hand, in
12 the last decade, the analysis of the isotopic nitrogen ratios ($\delta^{15}\text{N}$) in benthic primary
13 producers has been revealed as a technique that is particularly effective in tracing
14 external nutrient inputs from certain human activities (see Lepoint et al., 2004 for a
15 review, Sarà et al., 2004, 2006). Organic loads from urban wastes and aquaculture are
16 enriched in the heavy nitrogen isotope (N^{15}) relative to natural sources (Jones et al.,
17 2001; Costanzo et al., 2003, 2004; Cole et al., 2004, 2005). This enrichment in N^{15} can
18 be detected in macrophyte tissues exposed to wastes since they show only small or no
19 fractionation during nitrogen uptake and assimilation (Gartner et al., 2002; Cohen and
20 Fong, 2005; Deutsch and Voss, 2006).

21 The analysis of the nitrogen isotopic signal in benthic vegetation has been applied
22 successfully to determine the spatial scale of the dispersal of aquaculture effluents
23 derived from land-based farms (Jones et al., 2001; Vizzini and Mazzola, 2004).
24 However, the distribution of benthic macrophytic communities along the coast is not
25 continuous and it is depth limited so these communities are usually absent from deeper
26 areas where offshore aquaculture is expanding. This fact restricts the use of this kind of
27 biological indicators to quantify the impact of fish farm effluents in open waters. To
28 overcome this problem some researchers have analyzed variations of $\delta^{15}\text{N}$ in samples of
29 macroalgae collected from natural stands and placed in mesh bags staked to sediment at
30 unvegetated bottoms to detect the spatial extent of shrimp-farm (land-based) effluents
31 (Lin and Fong 2008). However, to our knowledge its potential has not still been
32 evaluated in marine offshore fish farms. So, we present here a study that uses pelagic
33 macroalgal bioassays in combination with isotopic analysis to determine the spatial

1 extent of offshore fish-farm wastes in open waters.

2 This study represents an assessment of a method based on the analysis of $\delta^{15}\text{N}$
3 signatures in tissues of benthic primary producers (macroalgae and *P. oceanica*
4 epiphytes) used as active bioindicators (i.e. bioassays) to determine the spatial extent of
5 dissolved wastes derived from offshore aquaculture facilities. Three case studies (fish
6 farms) were selected in three geographically separated sites along the Spanish coast to
7 perform this study. In each case, bioassays were deployed by means of incubation
8 devices in the water column at increasing distances from the fish farm facilities to
9 analyse the effects of several key experimental conditions (species of macroalgae, depth
10 of incubation and duration of incubation) on the variations of $\delta^{15}\text{N}$ signatures. In
11 addition, spatial variations of $\delta^{15}\text{N}$ were compared with those indicated by other
12 measures such as total nitrogen in plant tissues.

13

14 **Materials and methods**

15

16 *Study sites*

17

18 Three offshore fish farms were selected to perform the study. One was located in waters
19 of the Atlantic coast (Tenerife, Canary Islands), and two in the Mediterranean: one in
20 San Pedro del Pinatar (Murcia Region) and the other in L'Ametlla de Mar (Catalonia
21 Region), on the south-eastern and north-eastern coast of the Spanish Peninsula,
22 respectively (Fig. 1). In the Canary Islands, the annual production of the fish farm
23 during the study period was about 375 tonnes. The species farmed here were sea bream
24 (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*). In Murcia, the annual production
25 of the fish farm was about 350 tonnes. Atlantic blue fin tuna (*Thunnus thynnus*) was
26 farmed in Murcia. However, this facility was at the southern end of a larger complex of
27 seven fish farms. These farms were concentrated in an area of 1.47 km² and farmed blue
28 fin tuna, sea bream and sea bass with an annual production of 6,760 tonnes. On an
29 annual basis, the fish farm in Catalonia produced about 800 tonnes of sea bream. All
30 fish farms selected were located in areas with soft, unvegetated bottoms between about
31 20 and 40 m deep, and located more than 1 km from shore. The study was carried out
32 during July and August in 2005.

1 *Experimental design***2**

3 At each study site, macroalgal samples were collected from unpolluted sites one day
4 before the bioassay deployment. Macroalgae collected varied in availability at each
5 study site. As a result of this variation, the red algae *Asparagopsis taxiformis* (Delile)
6 Trevisande Saint-Léon and the brown algae *Styopodium zonale* (Lamouroux)
7 Papenfuss were used in the Canary Islands study site; the brown algae *Dictyopteris*
8 *polypodioides* (De Candolle) J.V. Lamouroux and the epiphytic community of the
9 seagrass *Posidonia oceanica* (L.) Delile were used in the Murcia fish farm, and the
10 brown algae *Cystoseira mediterranea* (Sauvageau) in the study case of Catalonia. After
11 collection, all macroalgae were maintained until the next day in coolers with well-
12 aerated seawater under low light conditions to avoid further physiological stress by light
13 and temperature. Ten sub-samples were separated from the bulk of collected plant
14 material and kept frozen until the time of analysis to obtain reference values for the
15 variables analyzed.

16 The day after sampling, macroalgae pieces (between 25 and 50 g of plant biomass) were
17 placed into incubation devices (comprising plastic 1 cm mesh cylinders measuring 9 cm
18 in diameter and 15 cm in height) and deployed in the water column at sites increasingly
19 further from the fish cages along a 1 km transect in the main current direction (Fig. 2).
20 All the plant material introduced in the devices was alive and in good condition. The
21 direction of the currents during the course of our study was established at each site from
22 data provided by environmental studies available in the respective monitoring programs.

23 The incubation devices were hung along vertical rope lines deployed at 0, 25, 50, 100,
24 200, 500 and 1000 meters from the fish cages, being the largest distance considered as a
25 control site representative of a natural, unpolluted condition. Rope lines were attached
26 to a concrete cube weighing 30 kg on the seabed and were maintained in an erect
27 position with a buoy. Along each rope line, three groups of four cylindrical devices (n =
28 4) were hung at two incubation depth (5 m (shallow) and between 15 and 20 m (deep))
29 to assess the effect of the incubation depth on the spatial variation of the nitrogen
30 isotopic signal measured in plant tissues. Bioassays hanging at ~20m depth were always
31 separated from the bottom by at least 5 m.

32

1 To evaluate the most adequate incubation time, a group of 4 devices at each depth and
2 each distance (from a total of three groups at each sampling point) was collected at 2, 4
3 and 6 days after the initial deployment (T2, T4 and T6, respectively). The plant material
4 contained in each device was cleaned with deionized water, stored in plastic bags and
5 kept frozen until analysis.

6
7 Light (PAR Irradiance, $\mu\text{mol quanta m}^{-2}\text{s}^{-1}$) profiles were obtained at each incubation
8 distance (and depth) using a LICOR light sensor. No differences in turbidity (k) or in
9 light availability (% SI) existed between incubation distances. Only differences between
10 incubation depths were apparent (Table 1). Thus, like other previous studies have
11 demonstrated (Beveridge et al., 1994; Wu et al., 1994; Mantzavrakos et al., 2007), light
12 availability did not represent an important factor explaining the reported variability of
13 response variables with cage distance. Furthermore, care was taken to avoid the shadow
14 of the cage in the 0 m distance, which was a possible cause of light reduction in this
15 experiment.

16
17

18 *Analysis of plant material*

19
20 Macroalgae samples were dried at 60°C to constant weight for 24 hours, ground using
21 an agate mortar and pestle and preserved in a desiccator at room temperature. From
22 each sample, between 1.8 and 2 mg of dry weight was encapsulated to determine total
23 nitrogen content and the stable isotope ratio ($\delta^{15}\text{N}$).

24 The isotopic analysis technique was based on the two naturally occurring atomic forms
25 of nitrogen ^{14}N and ^{15}N (Mariotti, 1983). By measuring the ratio of ^{14}N and ^{15}N in dried
26 plant tissue, the relative amount of ^{15}N or $\delta^{15}\text{N}$ in the plant can be determined as the
27 relative difference between the sample and a worldwide standard (atmospheric N_2)
28 using the following equation (Peterson and Fry, 1987):

$$29 \quad \delta^{15}\text{N} = \left(\frac{{}^{15}\text{N}/{}^{14}\text{N}_{\text{sample}}}{{}^{15}\text{N}/{}^{14}\text{N}_{\text{standard}}} - 1 \right) \times 1000$$

30 The nitrogen stable isotope ratio ($\delta^{15}\text{N}$) and total N content (%) were determined using a
31 continuous flow isotope ratio mass spectrometer (Thermo Finnigan Delta Plus IRMS).

1 This was carried out after combustion in an elemental analyzer Flash EA from
2 TermoFinnigan coupled via a ConFlo II from Finnigan MAT with the elemental
3 analyzer. The ratio $\delta^{15}\text{N}$ value was expressed in per mil (‰). The internal laboratory
4 standard employed was acetanilide.

5

6 *Statistical procedures*

7

8 The effect of distance from fish cages on the dependent variables ($\delta^{15}\text{N}$ and % N in
9 plant tissues) was analyzed using a one-way ANOVA for each combination of depth
10 and incubation time and for each specie (30 one-way ANOVA for each dependent
11 variable). Before analysis, Cochran's test was used to assess the homogeneity of
12 variances. Differences were considered significant if $p < 0.05$. When significant
13 differences were detected, a *post hoc* pair-wise comparison of means using Student-
14 Newman-Keuls (SNK) test was performed (Zar, 1999). When variances were
15 homogeneous, results were interpreted at the probability level of 0.01 to reduce the
16 possibility of a Type I error (Underwood, 1997). Statgraphics Plus 5.1 data analysis
17 software package was used for the statistical analysis.

18

19

20

21 **Results**

22

23

24 *Nitrogen Stable Isotope Ratio ($\delta^{15}\text{N}$)*

25 Mean $\delta^{15}\text{N}$ values obtained at the incubation time T2 for each transects distance were
26 variable and close to the reference values (Figs. 3, 4 and 5 and Table 2). Such variability
27 was significant in most cases but was not influenced by the presence of fish cages
28 (Table 3). As the incubation time increased, differences between bioassays and the
29 reference values increased, the maximum being in the fish cage (0 m) and minimum at
30 the control site (i.e. 1000 m). However, the significance and strength of this spatial
31 trend depended on the incubation time, the macroalgae species used and the depth of the
32 bioassay, as is described below.

1 With regard to the time of incubation, significant spatial trends were detected only in
2 the macroalgae incubated for four and six days (T4 and T6, respectively) (Table 3). In
3 the Canary Islands a spatial gradient was only detected by *S. zonale* at T4 (Fig. 3). In
4 the case of *A. taxiformis* variations of $\delta^{15}\text{N}$ along the bioassays transect in all times of
5 incubations were not related to the spatial pattern described above, probably due to the
6 deteriorated state of the tissues of this alga at the end of the experimental period
7 (pigment loss, pers. Obs.) (Fig. 3). In Catalonia the spatial gradient defined by *C.*
8 *mediterranea* was evident at T4, while it appeared later (T6) in Murcia for the two
9 species analyzed (Figs. 4 and 5).

10 On the other hand, macroalgae incubated at 5m depth showed a similar response to
11 those incubated at ~20m depth (Table 3) although the spatial gradient between the fish
12 farm and control sites was more clearly defined in the upper layer of the water column
13 than in the deeper part (e.g. *S. zonale*, *P. oceanica* epiphytes and *C. mediterranea*, Figs.
14 3-5). All the macroalgae species tested, except for *A. taxiformis*, showed significant
15 spatial trends at 5m depth. At ~20m depth, significant spatial gradients were also found
16 in all macroalgae species, except for *S. zonale* and *P. oceanica* epiphytes (Table 3).
17 Differences between the fish cage (0 m) and control (1000 m) sites were highest for *D.*
18 *polypodioides* (1.9-fold) and lower for the other species (1.1 to 1.5-fold) (Figs. 3-5).
19 Furthermore, mean $\delta^{15}\text{N}$ values in *D. polypodioides* were highest in comparison with
20 initial samples (i.e. references values), even at the control site, with maximum
21 differences in T6 at the 5m depth (Fig. 4).

22

23 ***Total nitrogen (% N) content***

24 The mean N content values obtained at T2 (2 days) were similar to the reference values
25 (Figs. 6, 7 and 8 and Table 2) and there were no significant differences between transect
26 distances, with two exceptions: *P. oceanica* epiphytes at the 5m depth and *C.*
27 *mediterranea* at both 5m and ~20m depth (Table 4). However, in these cases no clear
28 spatial trend associated with fish cages (Figs. 6, 7 and 8 and Table 4) was detected. In
29 T4 (four days) differences between transect distances were found, such as in *A.*
30 *taxiformis* at the 5m depth and in *D. polypodioides* and *C. mediterranea* at ~20m depth.
31 From these cases, *D. polypodioides* at ~20m depth was the only species to show a

1 perceptible spatial gradation. However, at T6 (6 days) significant differences were
2 found between transect distances in almost every species except *S. zonale* and *P.*
3 *oceanica* epiphytes (at 5m and ~20m depth); *D. polypodioides* and *P. oceanica*
4 epiphytes followed significant trends at the 5m depth, while the same was true for *C.*
5 *mediterranea* at ~20m depth (Figs. 6, 7 and 8 and Table 4). Differences between the
6 fish cage (0 m) and control (1000 m) sites were greater in Murcia than in Catalonia (1.6-
7 fold and 1.2-fold, respectively).

8
9

10 Discussion

11

12 In general terms, the results obtained in this study show a significant increase in the
13 $\delta^{15}\text{N}$ values towards the fish cages with respect to the reference (initial) and control
14 values, in almost all the incubated macroalgae (except in *Asparagopsis taxiformis*),
15 which confirms the validity of the bioassay method employed to detect and characterize
16 spatial gradients of nutrients associated to offshore aquaculture facilities.

17 This response was similar to the increase in $\delta^{15}\text{N}$ in benthic primary producers of
18 natural communities under the influence of organic effluents delivered from sewage
19 outfalls (Costanzo et al., 2001) or from land-based aquaculture facilities (Jones et al.,
20 2001, Vizzini and Mazzola, 2004). Contamination from animal wastes is a common
21 cause of ^{15}N enrichment in aquatic environments due to isotopic fractionation during the
22 ammonification and nitrification processes of urea, which is one of the main forms of
23 excreted nitrogen (Macko and Ostrom, 1994). Thus, higher $\delta^{15}\text{N}$ values in incubated
24 macroalgae indicate a significant assimilation of dissolved inorganic nitrogen forms
25 (ammonia and nitrates) coming from fish farm wastes. Therefore, decreasing $\delta^{15}\text{N}$
26 signature in incubated macrophytes with increasing distance from the fish cages is
27 likely to reflect the dilution of nutrients from the source. This agrees with the high
28 significant negative correlation that we have found between the isotopic signature and
29 the distance from the fish cage for most of the case studies (Table 3).

30 The spatial pattern found for $\delta^{15}\text{N}$ was well-defined and significant after four days of
31 incubation (T4) (Table 3). After two days of bioassay deployment (T2), differences in
32 $\delta^{15}\text{N}$ were not significant and did not show any consistent spatial pattern. In contrast, in

1 T4 (four days) and T6 (six days) well-defined gradients were found. Then, although
2 nutrients released from fish farms usually show large temporal variability (e.g.
3 Karakassis et al., 2001), an incubation time of 4-6 days was sufficient to integrate
4 temporal, short-term changes in the nutrient regime at increasing distances from fish
5 farms. These findings are consistent with results from other studies where macroalgae
6 assimilated sewage-derived DIN and displayed higher $\delta^{15}\text{N}$ values in their tissues at
7 similar times of incubations (Gartner et al., 2002; Costanzo et al., 2004; Deutsch and
8 Voss, 2006).

9 Not all of the macroalgal species tested showed the same tolerance and ability to
10 survive the experimental bioassay procedures. The red algae *A. taxiformis* used in the
11 bioassays at the Canary Islands site was the most obvious case (Fig. 3). In this specie,
12 values of both $\delta^{15}\text{N}$ and total N content along transects did not show any relationship
13 with the fish farm effluent. Indeed, $\delta^{15}\text{N}$ and total N content strongly decreased in
14 relation to references values (Tables 3 and 4). This is explained by tissue decomposition
15 during the bioassay incubation and indicates the low resistance of this macrophyte to
16 experimental manipulation. The other macroalgae species and *P. oceanica* epiphytes
17 were resistant to manipulation as no signs of deterioration were apparent during
18 incubation and we assumed that they were assimilating nitrogen derived from fish-farm,
19 so they could be considered as good indicators of nitrogen derived from fish farms
20 wastes.

21 Spatial gradients between the fish farm and control sites were more clearly defined in
22 the upper layer of the water column (i.e. 5 m incubation depth) than in the deeper part
23 (between 15 and 20 m incubation depth), where this gradient was not well-defined (e.g.
24 *P. oceanica* epiphytes; Fig. 4) or non-existent (e.g. *S. zonale*; Fig. 3). This can be
25 attributed to differences in environmental factors as light (Dudley, 2007) or differences
26 in the dispersion patterns of wastes. Macroalgae incubated at ~20m depth are subjected
27 to lower light levels than macroalgae incubated at the 5m depth. This can affect the
28 photosynthetic rates and hence nitrogen assimilation (Lobban and Harrison 1997). On
29 the other hand, our results indicated a higher dispersion of the dissolved inorganic
30 nitrogen forms derived from fish-farm wastes in the superficial layer than in the deeper
31 one. This can be attributed to a high fish density and activity in the upper layer during
32 feeding operations. Also, in summer, a thermocline was established between the two

1 incubation depths (unpublished data, pers. obs), which prevented effective diffusion and
2 transport of dissolved nutrients towards the deeper layer, so fish farm wastes are more
3 easily dispersed in the superficial layer, and thus detected more easily by the bioassays
4 at the 5m depth than by the 20m depth.

5 The magnitude of $\delta^{15}\text{N}$ enrichment of macrophyte tissues (in relation to reference,
6 initial and control mean values) appeared higher also at 5m than at ~20m depth at all
7 study sites, except in Murcia for *D. polypodioides*. In Murcia study, results show that
8 the dispersion of the fish farm effluent at 5m and ~20m depth reached further than the
9 spatial scale measured in this study (1000 m), especially at the shallower depth, since
10 control bioassays had an isotopic signal higher than those found in reference algae. This
11 is consistent with the larger size of the aquaculture facilities selected in Murcia (see
12 methods), where the annual fish production is 9- and 20-fold higher than in the
13 Catalonia and Canary Islands fish farms, respectively.

14 The sensitivity of the bioassay and its spatial variability may be also influenced by other
15 factors such as the nutrient regime characteristics of each coastal area and the presence
16 of nutrient sources that differ from those derived from offshore fish farms (Ye et al.,
17 1991; Wu, 1995; Cromey et al., 2002; Vizzini and Mazzola, 2004; Sarà et al., 2006).
18 These factors varied between the three fish farms studied here, which is probably
19 reflected in the results. The variation of $\delta^{15}\text{N}$ along the transect was higher in the fish
20 farms of Murcia and the Canary Islands (2.5 -5 ‰), probably due to the oligotrophic
21 character of their waters. This condition, together with the absence of any substantial
22 source of nitrogen in the studied areas, besides fish farms, meant that $\delta^{15}\text{N}$ could be
23 applied as a single tracer to follow the dispersion of the fish farm wastes from source
24 point (Peterson, 1999). In contrast, the variations of $\delta^{15}\text{N}$ in Catalonia were lower than
25 those in Murcia and the Canary Islands, mainly because of higher initial $\delta^{15}\text{N}$ values in
26 natural stands of *C. mediterranea* (6-7 ‰) used as reference values. The $\delta^{15}\text{N}$ values
27 found in *C. mediterranea* in our study site are clearly higher than the background values
28 (3.4 ‰, Pantoja et al., 2002) for dissolved N in the Western Mediterranean and that
29 those found in the genus *Cystoseira* from unpolluted environments (Pinnegar and
30 Polunim, 2000). This could be explained by the high continental influence of the
31 Catalonia fish farm. Coastal waters near continents are enriched in the heavier N isotope
32 transported by runoff from watersheds larger than in islands, and with important urban

1 and agricultural development (McClelland and Valiela, 1998; Bowen and Valiela,
2 2008). Consequently, the contribution of fish farm waste to the enhancement of $\delta^{15}\text{N}$ in
3 incubated algae could have been masked by ambient levels. In these cases the use of the
4 nitrogen stable isotope ratios would not have been as sensitive as in oligotrophic areas
5 to detect dissolved nutrients derived from fish farms (Costanzo et al., 2003).

6 Total N content in marine plants is commonly considered a potential indicator of the
7 concentration of biologically available nutrients in the environment (Duarte, 1990; Fong
8 et al., 2004). Despite this, it was much less sensitive to detect fish farm waste than $\delta^{15}\text{N}$
9 probably due to high dispersion rates in offshore fish farms. In our study, total N
10 content was a poor indicator of nutrient release from fish farms; in only three cases % N
11 content increased towards the cages with respect to the reference and control values (*D.*
12 *polypodioides* and *P. oceanica* epiphytes at 5m depth and *C. mediterranea* at ~20m
13 depth; Table 4). This trend was also reported by Lin and Fong (2008), who reported a
14 much more localized response to nutrient availability in tissue N content than in
15 nitrogen isotope stable ratios in relation to shrimp farm effluents. These authors
16 attributed this response to the fact that nutrient supply was not high enough to induce
17 tissue nitrogen storage (Lin and Fong, 2008), which only occurs when nutrient supply
18 exceeds growth rate (Fong et al., 2004). In our study, the lack of response in tissue
19 nitrogen content could be explained by the same argument; it is likely that the high
20 hydrodynamic conditions in offshore fish farms dilute the nitrogen rapidly. Moreover,
21 the lack of nitrogen response may also be influenced by other aspects related to species-
22 specific physiological traits (Fong et al., 2001, 2003).

23 In summary, our results confirm the potential of $\delta^{15}\text{N}$ in macrophytes (more than the
24 classic measures of total N) to track the dispersion of dissolved nutrients derived from
25 aquaculture discharges in offshore sites without benthic vegetation, and especially in
26 oligotrophic waters. Changes in $\delta^{15}\text{N}$ values measured in incubated macrophytes along
27 transects has provided clear and reliable characterizations of nutrient gradients
28 associated with the fish farm wastes in 4-6 days (except for *A. taxiformis*), and the
29 wastes are going farther in the upper layer of the water column than in the deeper.
30 Furthermore, the technique is simple and low cost; experimental deployment is very
31 easy to perform and requires very little training making this method a strong candidate
32 for application in monitoring programs.

1 Finally, our results have also highlighted the necessity of an assessment study to
2 determine the appropriate experimental conditions (species of macroalgae, distances
3 from fish farms, incubation depth, etc...) that will ensure the bioassay is successful in
4 indicating the area of the spatial extent of fish farm wastes. The results obtained in this
5 study now make it possible to extensively apply the bioassay to other case studies. A
6 proper spatial replication is able to gather most of the spatial variability caused by
7 currents and other local factors of interest (i.e. more than one site per distance and at
8 least two transects either side of the fish farms). In conclusion, the application of this
9 macroalgal bioassays method has a great potential in determining the spatial scale of the
10 influence of aquaculture wastes on the marine environment, which represents a key
11 aspect for monitoring and management this activity in coastal marine ecosystems.

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1 Acknowledgements**2****3****4** This study was financed by the “National Plan of Cage Impact of JACUMAR”**5** (Secretaría General de Pesca Marítima del Ministerio de Agricultura, Pesca y**6** Alimentación), and by the “Instituto Español de Oceanografía” (IEO).**7** The authors would like to thank Oriol Mascaró for his assistance in field work and**8** Natalia Cortazar for her help in laboratory analyses. Moreover we thank the staff of the**9** three fish farms for their collaboration and support during the experiments. We also**10** thank Maria Lema, from the “Unidad de Técnicas Instrumentales de Análisis” (UTIA)**11** of the “Servizos de Apoio á Investigación” (SAI) (Universidade da Coruña), for support**12** in isotopical analysis.**13****14****15****16****17****18****19****20****21****22****23****24****25****26****27****28****29****30****31****32****33****34****35****36****37****38****39****40****41****42****43****44****45****46**

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1 Caption of figures**2****3****4 Figure 1:** Sample sites map.**5****6 Figure 2:** Diagram of the experimental design developed in this study.**7**

8 Figure 3: Nitrogen isotopic ratio ($\delta^{15}\text{N}$) in the two macroalgae analyzed in Canary Islands, *Asparagopsis*
9 *taxiformis* and *Styopodium zonale*. For each species of macroalgae the results for two depths (5m and
10 ~20m) and three times of incubations (T2= 2 days; T4= 4 days; T6= 6 days) at different distances from
11 fish farm are shown. Vertical bars correspond to the standards error of the mean.

12
13 Figure 4: Nitrogen isotopic ratio ($\delta^{15}\text{N}$) in the two macroalgae analyzed in Murcia, *Dictyopteris*
14 *polypodioides* and *Posidonia oceanica* epiphytes. For each specie of macroalgae the results for two
15 depths (5m and ~20m) and three times of incubations (T2= 2 days; T4= 4 days; T6= 6 days) at different
16 distances from fish farm are shown. Vertical bars correspond to the standards error of the mean.

17
18 Figure 5: Nitrogen isotopic ratio ($\delta^{15}\text{N}$) in the macroalgae analyzed in Catalonia, *Cystoseira*
19 *mediterranea*. The results for two depths (5m and ~20m) and three times of incubations (T2= 2 days; T4=
20 4 days; T6= 6 days) at different distances from fish farm are shown. Vertical bars correspond to the
21 standards error of the mean.

22
23 Figure 6: Nitrogen content (% N) in the two macroalgae analyzed in Canary Islands, *Asparagopsis*
24 *taxiformis* and *Styopodium zonale*. For each species of macroalgae the results for two depths (5m and
25 ~20m) and three times of incubations (T2= 2 days; T4= 4 days; T6= 6 days) at different distances from
26 fish farm are shown. Vertical bars correspond to the standards error of the mean.

27
28 Figure 7: Nitrogen content (%N) in the two macroalgae analyzed in Murcia, *Dictyopteris polypodioides*
29 and *Posidonia oceanica* epiphytes. For each species of macroalgae the results for two depths (5m and
30 ~20m) and three times of incubations (T2= 2 days; T4= 4 days; T6= 6 days) at different distances from
31 fish farm are shown. Vertical bars correspond to the standards error of the mean.

32
33 Figure 8: Nitrogen content (% N) in the macroalgae analyzed in Catalonia, *Cystoseira mediterranea*. The
34 results for two depths (5m and ~20m) and three times of incubations (T2= 2 days; T4= 4 days; T6= 6
35 days) at different distances from fish farm are shown. Vertical bars correspond to the standards error of
36 the mean.

37**38**

Table 1 Vertical attenuation coefficient (k) and superficial irradiance (% SI) values obtained at each incubation distance and depth for the Murcia study case during the incubation experiment.

Distance from fish cages (m)	K (m ⁻¹)		% Superficial Irradiance (μmol quanta m ⁻² s ⁻¹)			
			5 m-bioassays		~20 m-bioassays	
	Mean	ES	Mean	ES	Mean	ES
0	0,054	0,003	77,2	7,7	34,7	4,0
25	0,061	0,001	65,0	2,7	28,3	1,1
50	0,052	0,001	70,9	3,2	31,0	1,6
100	0,055	0,0005	84,2	3,7	34,6	0,3
200	0,057	0,001	62,8	2,1	28,3	1,1
500	0,048	0,001	78,3	3,8	37,6	2,6
1000	0,048	0,0009	79,2	0,5	37,7	0,6

Table 2 Mean \pm SE values of $\delta^{15}\text{N}$ and % N in macroalgae collected from unpolluted sites at each location (reference values).

Site	Specie	Reference values (n=10)	
		$\delta^{15}\text{N}$ (‰)	% N
Canary Islands	<i>Asparagopsis taxiformis</i>	3.9 ± 0.2	1.7 ± 0.1
	<i>Styopodium Zonale</i>	2.5 ± 0.2	0.9 ± 0.1
Murcia	<i>Dictyopteris polypodioides</i>	1.7 ± 0.1	1.8 ± 0.1
	<i>P. oceanica</i> epiphytes	3.5 ± 0.1	1.1 ± 0.04
Catalonia	<i>Cystoseira mediterranea</i>	6.1 ± 0.3	1.4 ± 0.2

Table 3 Summary of ANOVA one-way results for $\delta^{15}\text{N}$ values of the macroalgae analyzed. *Post hoc* associations are shown in lower-case letters. For the sake of simplicity, only SNK test results for significant differences are shown. H_0 : there are no significant effects between distances. NS = not significant, * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Site	Specie	Samples		ANOVA one-way (p-value)	df	F-value	SNK -test (distance)						
		Depth	Time				0	25	50	100	200	500	1000
Canary Islands	<i>Asparagopsis taxiformis</i>	5m	T2	ns	27	1.2	-	-	-	-	-	-	-
			T4	***	27	9.6	a	a	a	a	b	a	a
			T6	ns ⁽¹⁾	27	2.2	-	-	-	-	-	-	-
		~20m	T2	**	27	4.1	ab	a	a	a	b	ab	ab
			T4	***	26	14.8	b	ab	a	ab	b	b	c
			T6	ns	25	2.4	-	-	-	-	-	-	-
	<i>Styopodium zonale</i>	5m	T2	***	24	6.9	a	a	b	a	a	a	a
			T4	**	27	4.1	a	a	ab	a	a	ab	b
			T6	*	27	3.1	a	a	a	a	a	a	a
		~20m	T2	ns	22	0.9	-	-	-	-	-	-	-
			T4	ns	27	0.3	-	-	-	-	-	-	-
			T6	ns	27	2.3	-	-	-	-	-	-	-
Murcia	<i>Dictyopteris polypodioides</i>	5m	T2	ns ⁽¹⁾	27	3.2	-	-	-	-	-	-	
			T4	ns	27	1.4	-	-	-	-	-	-	
			T6	***	27	15.4	a	ab	a	ab	bc	c	d
		~20m	T2	*** ⁽¹⁾	26	9.3	a	b	b	b	a	b	b
			T4	***	27	27.5	a	b	b	b	b	c	c
			T6	***	26	37.8	a	a	b	b	b	c	d
	<i>Posidonia oceanica</i> epiphytes	5m	T2	***	27	4.6	a	b	b	b	b	b	b
			T4	**	21	4.8	a	ab	b	ab	ab	b	b
			T6	***	23	9.5	a	a	a	a	ab	c	bc
		~20m	T2	***	26	6.2	a	b	b	b	b	b	b
			T4	ns ⁽¹⁾	23	1.7	-	-	-	-	-	-	-
			T6	***	27	6.4	a	bc	bc	ab	bc	c	c
Catalonia	<i>Cystoseira mediterranea</i>	5m	T2	***	24	9.0	b	a	b	b	a	b	b
			T4	***	25	8.9	a	a	b	b	b		b
			T6	** ⁽¹⁾	27	4.1	a	ab	ab	b	b	b	b
		~20m	T2	***	27	8.8	b	a	ab	b	b	ab	b
			T4	***	24	16.4	a	a	b	ab	c		bc
			T6	ns	25	0.9	-	-	-	-	-	-	-

(1) Non homogeneous variances. Significant differences if $p \leq 0.01$

Table 4 Summary of ANOVA one-way results for % N values of the macroalgae analyzed. *Post hoc* associations are shown in lower-case letters. For the sake of simplicity, only SNK test results for significant differences are shown. H_0 : there are no significant effects between distances. NS = not significant, * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Site	Specie	Samples		ANOVA one-way (p-value)	df	F-value	SNK -test (distance)							
		Depth	Time				0	25	50	100	200	500	1000	
Canary Islands	<i>Asparagopsis taxiformis</i>	5m	T2	ns	26	1.8	-	-	-	-	-	-	-	
			T4	** ⁽¹⁾	26	5.5	a	b	b	b	b	b	b	
			T6	***	26	20.1	c	bc	bc	bc	a	a	b	
		~20m	T2	ns	27	0.9	-	-	-	-	-	-	-	-
			T4	**	26	4.5	ab	ab	ab	ab	a	b	b	
			T6	**	26	4.6	ab	abc	abc	a	bc	c	abc	
	<i>Styopodium zonale</i>	5m	T2	ns	27	1.1	-	-	-	-	-	-	-	
			T4	ns ⁽¹⁾	26	2.2	-	-	-	-	-	-	-	
			T6	*	27	3.4	a	a	a	a	a	a	a	
		~20m	T2	ns	27	1.2	-	-	-	-	-	-	-	
			T4	ns	27	0.4	-	-	-	-	-	-	-	
			T6	ns	27	2.2	-	-	-	-	-	-	-	
Murcia	<i>Dictyopteris polypodioides</i>	5m	T2	ns	27	2.2	-	-	-	-	-	-		
			T4	ns ⁽¹⁾	27	1.1	-	-	-	-	-	-		
			T6	**	27	4.4	a	ab	ab	a	ab	ab	b	
		~20m	T2	ns	27	0.1	-	-	-	-	-	-	-	
			T4	**	27	3.9	ab	ab	ab	a	a	ab	b	
			T6	** ⁽¹⁾	27	5.2	abc	a	bc	ab	abc	c	c	
	<i>Posidonia oceanica</i> epiphytes	5m	T2	**	27	4.1	ab	ab	ab	a	ab	b	b	
			T4	ns	22	0.5	-	-	-	-	-	-	-	
			T6	***	23	7.5	a	ab	ab	bc	bc	c	c	
		~20m	T2	ns	27	1.3	-	-	-	-	-	-	-	
			T4	ns	23	2.4	-	-	-	-	-	-	-	
			T6	ns	27	2.1	-	-	-	-	-	-	-	
Catalonia	<i>Cystoseira mediterranea</i>	5m	T2	*** ⁽¹⁾	26	9.4	a	b	a	a	a	a		
			T4	* ⁽¹⁾	25	3.0	-	-	-	-	-	-		
			T6	*	27	2.8	a	a	a	a	a	a		
		~20m	T2	**	27	4.2	ab	a	ab	ab	b	ab	b	
			T4	*	22	3.5	a	a	a	a	a	a	a	
			T6	*** ⁽¹⁾	26	10.0	a	abc	ab	abc	c	bc	d	

(1) Non homogeneous variances. Significant differences if $p \leq 0.01$

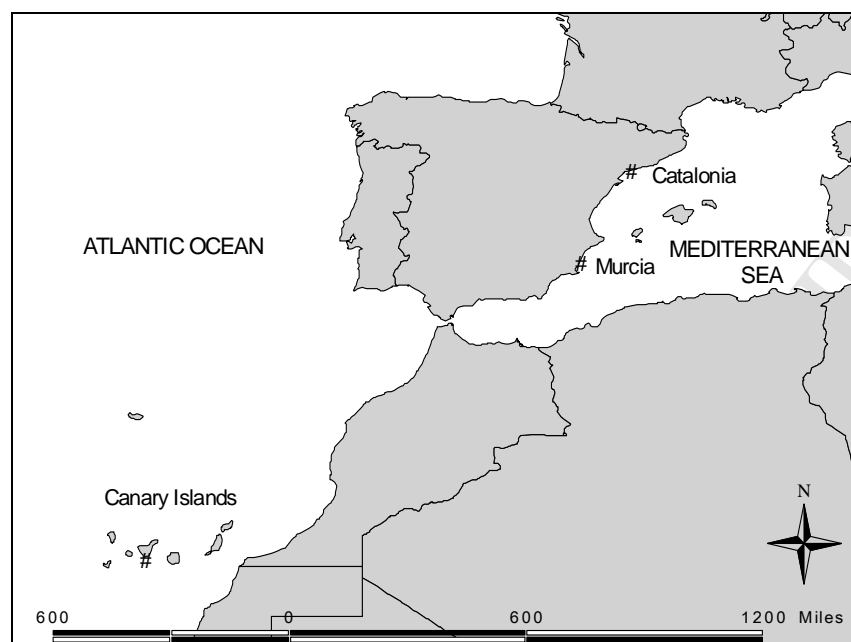
Figure 1

Figure 2

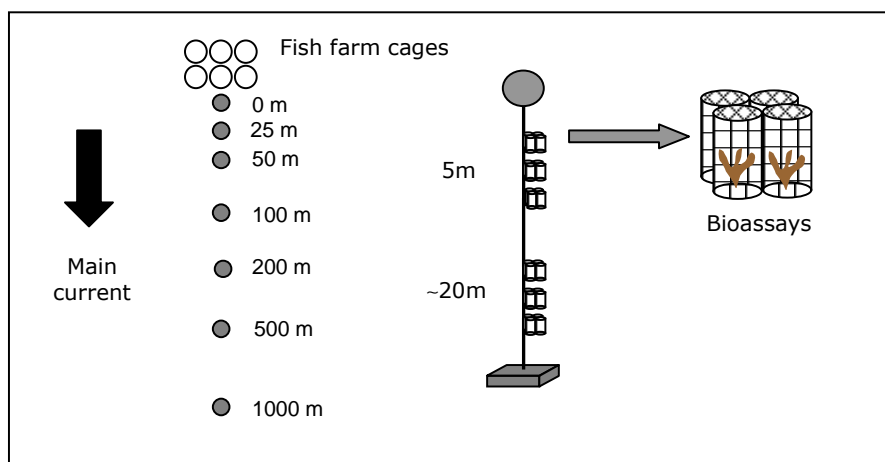


Figure 3

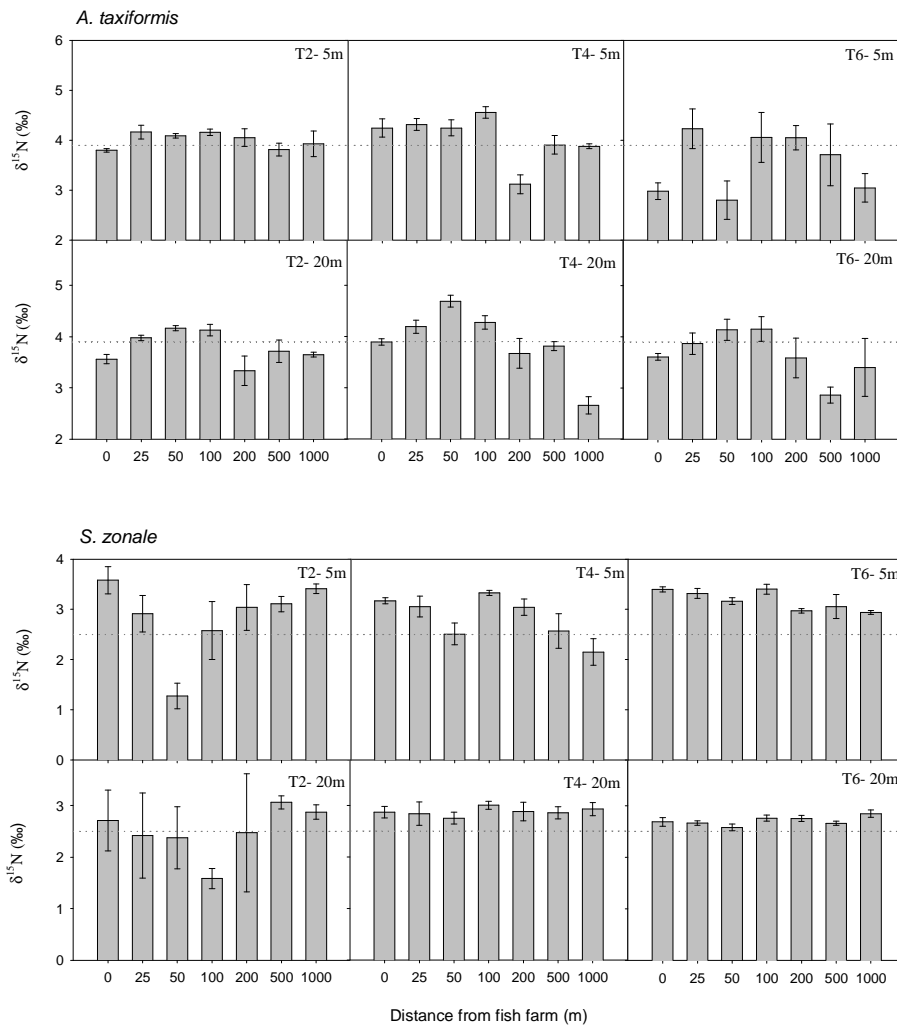


Figure 4

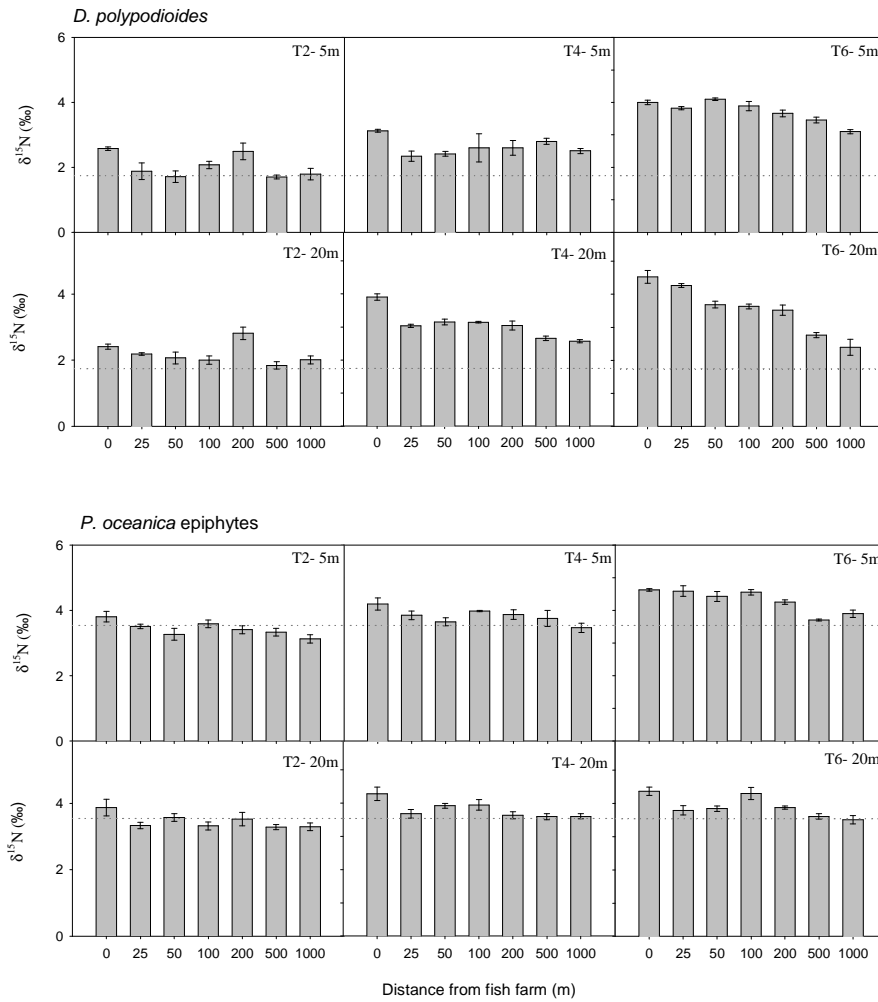


Figure 5

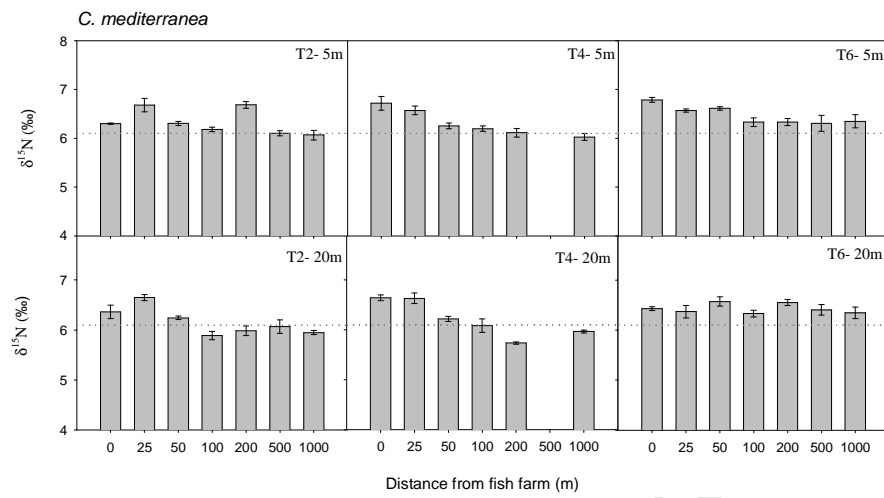


Figure 6

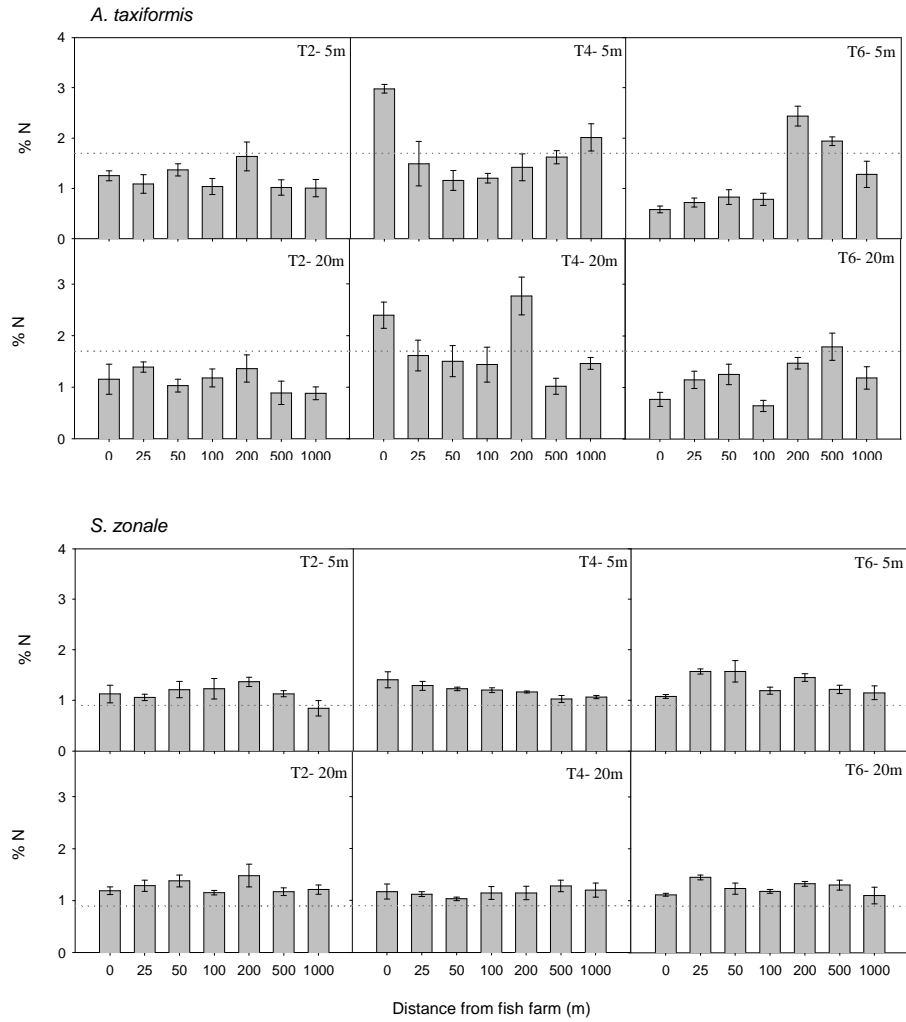


Figure 7

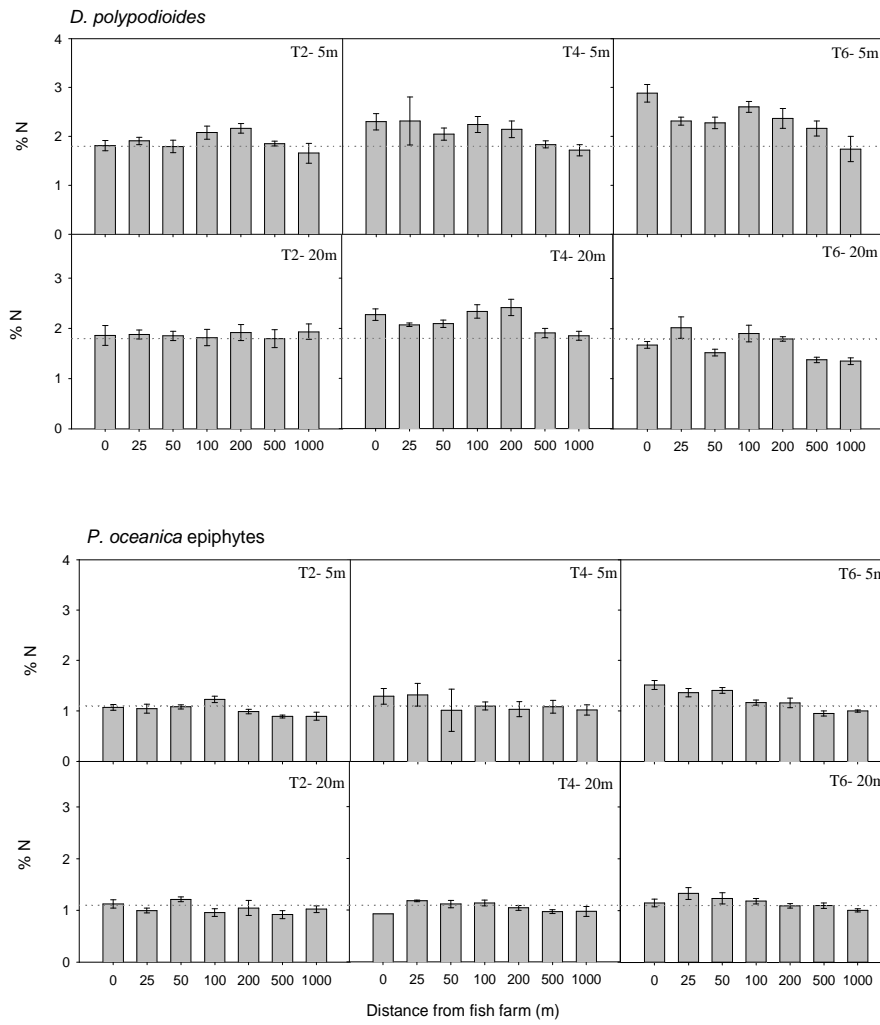


Figure 8

