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δ15N values of macroalgae as an indicator of the potential presence of waste disposal from land-based marine fish farms

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**Abstract**

The nitrogen isotope ratio (δ15N) in tissues of native macroalgae was evaluated as a means of indicating the intensity and spatial extent of organic contamination due to disposal of waste from land-based marine fish farms (LBMFFs). Three species of macroalgae from the genus *Fucus* and the green macroalgae *Codium tomentosum* were selected for study. The study was carried out at seven flat marine fish farms located in Galicia (NW Spain). Tests were carried out to determine the intra-annual variation in δ15N values and any differences between selected macroalgae. The δ15N values enrichment was observed close to the disposal point, and δ15N values varied more widely throughout the year (±5.57 ‰) at sites affected by the marine fish farm effluent compared to natural conditions (±2 ‰). No significant differences in the isotopic signals were observed in the different species studied (standard major axis). The δ15N values of macroalgae may be an ideal means of detecting the presence of LBMFFs effluents.

Keywords

*Fucus* *Codium tomentosum* Pollution Monitoring Bioconcentration Aquaculture Eutrophication

**Introduction**

Aquaculture activity has increased greatly in coastal areas throughout the world in recent years. This has been possible due to the introduction of new technologies, the increased availability of suitable sites, improvements in food technology, improved understanding of the biology of the species farmed, increased water quality within farming systems and the increased demand for fish products (Read and Fernandes[2003](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html" \l "CR44)). Worldwide, the sector has grown since 1970 and in Europe represents almost 4 % of aquaculture production worldwide (FAO [2010](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR16)). In Galicia (NW Spain), the region under consideration in the present study, the sector represents 86.95 % of the Spanish aquaculture production (APROMAR [2011](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR1)). The Galician Aquaculture Plan contemplates, with extensions and new installations, occupying some 3.106 m2 in land-based marine fish farms (LBMFFs), with an envisaged intensive annual production of flatfish of 30,000 t year−1.

This intensive development has been accompanied by an increase in the environmental impact of these activities (Ervik et al. [1997](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR15); Fernandes et al. [2001](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR17)). Eutrophication is one of the most important of these impacts because of the high levels of ammonium and nitrates that are released as a result of aquaculture activity to coastal communities, which are often limited by nitrogen (Cloern [2001](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR6)). A variety of indices have been developed to quantify the extent of eutrophication brought about by nitrogen loading, as taxonomic shifts and changes in abundance of producers and consumers take place (McClelland et al. [1997](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR34)). However, the disadvantage of these indices is that the effects are only detected when environmental disturbance has already occurred. On the other hand, the water quality parameters traditionally analyzed, i.e. dissolved nutrients, appear to fluctuate significantly over short periods of time (Wolanski et al. [2000](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR70)). This implies that quantification is difficult and is associated with a very costly intensity of sampling (Dalsgaard and Krause-Jensen [2006](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR12); Sarà et al. [2004](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR50)).

Analysis of the ratio of stable isotopes of nitrogen (δ15N) appears to be an ideal alternative, as variations in the relative abundance of the isotopes can be detected before coastal communities undergo alterations in structure and function. The method is based on the fact that nitrogen has two stable isotopes, a light isotope, 14N, and a heavier isotope, 15N, which occur at a constant proportion in the atmosphere, respectively, 99.635 % and 0.365 % (Nier [1950](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR39)). Isotopic abundance is reported on a delta scale (*δ*), which indicates the deviation (expressed in per mille) of the isotopic composition of a sample from an internationally accepted standard, the air (e.g. Robinson [2001](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR47)).

However, this proportion varies according to the different metabolic routes that a molecule follows, as the diverse reactions produce different fractioning of the heavy isotope (15N) (Struck [2012](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR53)). Significant variations in the natural abundance of 15N in marine organisms were first documented in the 1950s (Hoering [1955](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR23)), but the earliest specific studies of nitrogen isotopic abundance in marine systems were carried out in the mid 1960s (Minagawa and Wada [1984](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR36); Miyake and Wada [1967](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR37); Wada et al. [1975](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR68); Wada and Hattori [1976](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR67)). Tracking these variations in the environment was found to have many novel applications in ecological studies, e.g. migration studies, trophic chains and paleoecological studies (Michener and Lajtha [2007](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR35); Struck [2012](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR53)). Such applications included analysis of δ15N to indicate sources of nitrogen in marine systems, although this was not applied until the 1990s (Costanzo et al. [2001](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR8); Dailer et al. [2012](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR11); Lamb et al. [2012](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR25); Mattern et al. [2011](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR33); Rogers [1999](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR48); Savage [2005](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR51); Umezawa et al. [2002](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR57)). The use of stable isotope analysis to trace organic contamination is based on the fact that different anthropogenic sources of nitrogen alter the baseline levels of δ15N values of marine systems (Heaton [1986](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR22)), so that the method can be used to trace and quantify the nitrogen inputs (Filgueira and Castro [2011](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR18); Struck [2012](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR53)).

In marine systems, different matrices (biotic and abiotic samples) can be used for isotope analysis (Lamb et al. [2012](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html" \l "CR25)). However, the advantage of using biotic samples rather than environmental samples (i.e. water and sediment) is that information about the bioavailable fraction, i.e. that associated with the potential risk of eutrophication, can be obtained (Lapointe et al. [2007](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR27); Yang et al. [2008](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR71)). Amongst the biotic samples used, macroalgae have all the main characteristics required for biomonitoring metals (Carballeira et al. [2000](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR3); Viana et al. [2010](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR60)) and nutrients (Villares and Carballeira [2003](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR62), [2004](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR63), [2006](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR64)). Macroalgae are very sensitive to changes in water quality induced by human activities and have been used as biomonitors of δ15N values of many environmental studies (Dailer et al. [2012](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR11); Deutsch and Voss [2006](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR13); Lamb et al. [2012](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR25); Lapointe [2010](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR28); Riera et al. [2000](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR46); Savage [2005](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR51); Savage and Elmgren [2004](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR52); Viana et al. [2011](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR61)). Moreover, macroalgae absorb dissolved inorganic nitrogen in the water (Lobban and Harrison [1994](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR30)) and accumulate it in their tissues without mobilising it, and are therefore not affected by sporadic episodes. In this way, the δ15N values of macroalgae accurately reflect the mean concentration of nitrogen in water sources (McClelland et al. [1997](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR34); Vosz and Struck [1997](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR66)), and the long turnover time allows temporal integration of the 15N source signal (Costanzo et al. [2001](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR8)).

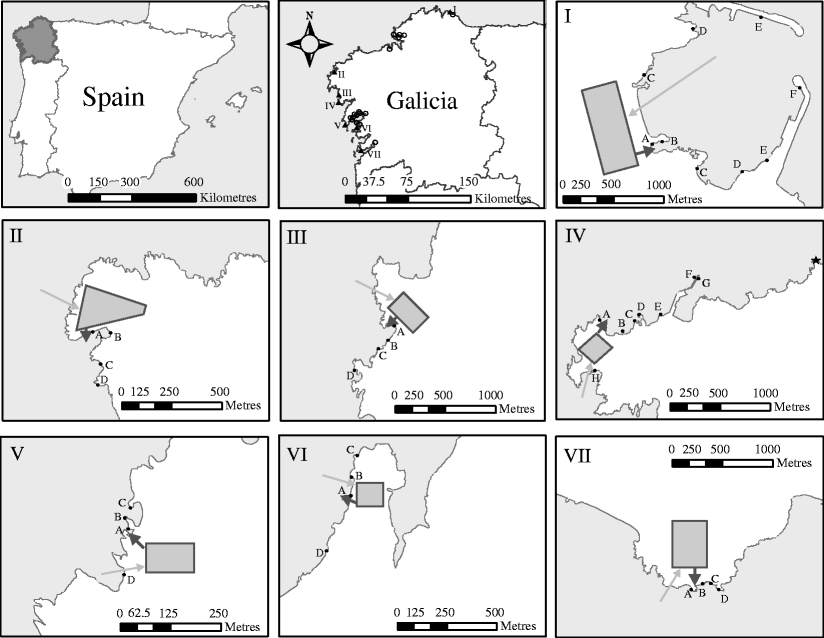
For all of these reasons, using the δ15N values of macroalgae and other autotrophic organisms to detect the presence of effluents from land-based aquaculture (Costanzo et al. [2001](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR8); Jones et al. [2001](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR24); Lin and Fong[2008](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR29); Vizzini and Mazzola [2004](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR65)), and of aquaculture settlements in general (Dolenec et al. [2006](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR14); García-Sanz et al. [2010](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR19); Lojen et al. [2005](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR31)), has become more popular in the last few years. Fish farm waste generally has significantly higher δ15N values because the heavier isotope 15N remains in the effluent source while the lighter isotope 14N is volatilized through microbial processes (Heaton [1986](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR22); Macko and Ostrom[1994](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR32); Van Dover et al. [1992](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR59)). However, further basic information is required in order to establish standardized protocols.

There is a lack of information about aspects such as interspecific differences or the natural seasonal variation of δ15N values of macroalgae (Lapointe et al. [2007](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR27)). Furthermore, nitrogen naturally occurs in the marine environment, so it is important to evaluate the differences between the natural (background) δ15N values of macroalgae and those affected by waste disposal. Different species of native or transplanted macroalgae have been used in marine fish farm studies, but always on offshore fish farms (cages) or shrimp ponds, with very scarce reference to any studies of the effects of LBMFFs (Vizzini and Mazzola [2004](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR65)).

The aim of the present study was to test the effectiveness of the δ15N values of macroalgae for assessing the detection of LBMFFs effluents and the magnitude of nutrient enrichment. In addition, in order to design better protocols for biomonitoring these industries, the following aspects related to δ15N values of macroalgae were considered: (a) interspecific differences of δ15N values between species, (b) annual variability in marine fish farm loads, and (c) comparison between the δ15N of background and those observed near farms.

**Materials and methods**

Macroalgal surveys were conducted in Galicia (NW Spain), in July 2008. Seven LBMFFs, situated in coastal areas with different hydrodynamic conditions and in absence of other nearby sources of organic contamination, were selected (Fig. [1](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html" \l "Fig1)). All of these were dedicated to the cultivation of flatfish, basically turbot (*Psetta maxima*). At each of the seven marine fish farms, between three and eight locations were located on an exponential gradient (approximately 0 to 1,500 m) from the emission point and in the direction of the predominating current (Fig. [1](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html" \l "Fig1)). The mean production on the farms fluctuated between 44 and 2,250 t year−1 (Table [1](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Tab1)). The mean concentration (*n* = 22) of total nitrogen in the input and output water from the LBMFFs under study was 1.34 ± 0.17 and 1.78 ± 0.24 mg L−1, respectively, whereas the concentration of ammoniacal nitrogen was 2.33 ± 1.35 and 2.42 ± 1.56 mg L−1, respectively.



**Fig. 1**

Map showing the location of Galicia in NW Spain, the location of the study areas in Galicia (*solid triangles*) and the location where more than one species coexist are represented by *opene circles*. *Enlargements* show the surroundings of the land-based marine fish farms studied (different scales). In the enlarged figures of the marine fish farms, the sampling locations are shown in *letters* along a down current gradient from the point of discharge to 1,500 m (Table [1](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Tab1)). The *solid star* (fish farm IV) indicates the clean site sampled monthly. The*arrows* indicate the input (*grey*) and output (*black*) of water from the land-based marine fish farms, which are shown as *grey areas*

**Table 1**

δ15N values of (A) *Fucus vesiculosus* and (B) *Codium tomentosum* from the 7 seven land -based marine fish farms at the respective sampling locations (A-–H) arranged in increasing distance from the point of effluent discharge and annual production at the time of sampling. The bold and underlined values in (A) represent locations where *F. spiralis* and *F. serratus* were sampled, respectively. The shaded areas represent locations < 50 m from the point of effluent discharge

| **Farm** | **Production (t year-−1)** | **A** | **B** | **C** | **D** | **E** | **F** | **G** | **H** |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| (A) *Fucus vesiculosus* | | | | | | | | | |
| Fish farm I west | 2,250 | *5.24* | *5.62* | 11.17 | 9.77 | 9.57 | -– | -– | -– |
| Fish farm I east |  |  |  | **9.25** | -– | 10.41 | 8.14 | -– | -– |
| Fish farm II | 292 | *6.37* | 8.44 | 7.89 | 6.22 | -– | -– | -– | -– |
| Fish farm III | 307 | *5.11* | 8.1 | -– | 5.98 | -– | -– | -– | -– |
| Fish farm IV | 1,194 | -– | -– | -– | -– | -– | -– | 5.7 | 5.72 |
| Fish farm V | 347 | -– | -– | -– | -– | -– | -– | -– | -– |
| Fish farm VI | 44 | *4.41* | 8.29 |  | 5.67 | -– | -– | -– | -– |
| Fish farm VII | 285 | *1.27* | 8.6 | -– | -– | -– | -– | -– | -– |
| (B) *Codium tomentosum* | | | | | | | | | |
| Farm |  | A | B | C | D | E | F | G | H |
| Fish farm I west | 2,250 | -– | -– | -– |  | 8.84 | -– | -– | -– |
| Fish farm I east |  | -– | -– | -– | 10.28 | 9.11 | 9.35 | -– | -– |
| Fish farm II | 292 | -– | -– | -– | -– | -– | -– | -– | -– |
| Fish farm III | 307 | *2.85* | 7.87 | 6.58 | -– | -– | -– | -– | -– |
| Fish farm IV | 1,194 | *5.1* | 9.93 | 9.84 | 9.3 | 8.14 | 5.92 | 5.57 | 5.57 |
| Fish farm V | 347 | *1.7* | *4.9* | 5.6 | 6 | -– | -– | -– | -– |
| Fish farm VI | 44 | *4.77* | 8.46 | 6.41 | -– | -– | -– | -– | -– |
| Fish farm VII | 285 | *2.87* | 9.24 | 9.44 | 8.33 | -– | -– | -– | -– |

The *bold and underlined* values in (A) represent locations where *F. spiralis* and *F. serratus* were sampled, respectively. The values in *italics* represent locations <50 m from the point of effluent discharge

The surveys were carried out at low tide in the mesolittoral zone. Each location included 20 m of coastline. At each location, more than 30 specimens of macroalgae attached to the substrate were collected systematically, following a zigzag line, with the aim of covering the degree of variability in the inter-individual concentrations. The specimens were combined to make a composite sample for each location, washed in situ with seawater and transported at 4°C to the laboratory where they were stored at −30°C (for less than 1 month).

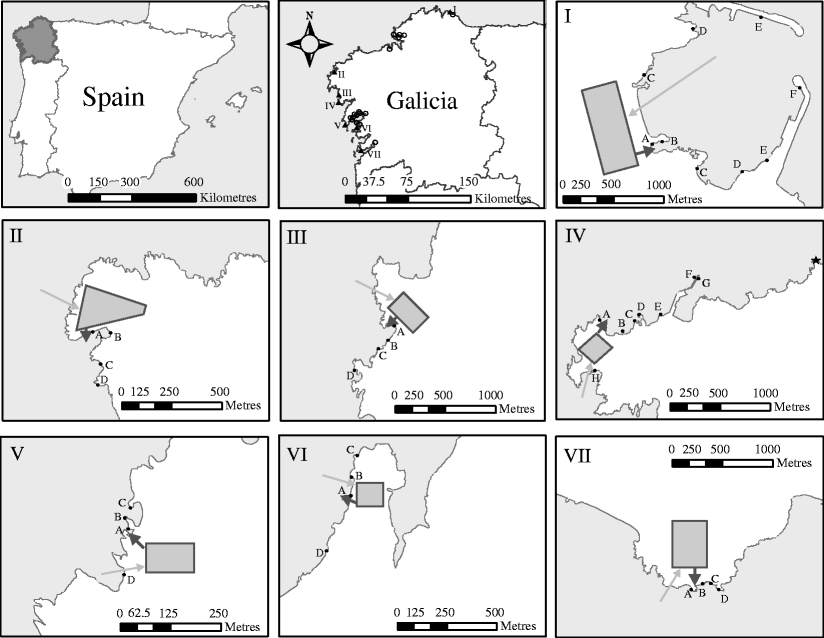
The material was defrosted at room temperature before processing. It was then washed carefully with abundant filtered seawater in successive stages, to remove, as efficiently as possible, any sediment and epiphytes; old and damaged parts of the plants were discarded. The distal 3 cm of the shoots were used to determine the concentrations; these portions were separated from the rest of the plant with a glass spatula, and the samples were homogenized in a laboratory blender. All of the material was dried at 45°C in a forced air oven and homogenized in an ultracentrifugal mill (Retsch ZM 100). Dried samples were stored at room temperature in glass vessels.

**Biomonitors**

For this study, the macroalgae *Fucus vesiculosus* was selected as the main biomonitor, as it is very abundant in the study area. However, in the relatively small area of influenced by the farms, it is not always possible to find *F. vesiculosus*, so other species that are ubiquitous on the coast of Galicia (*Fucus spiralis*and *Fucus serratus*) were chosen as secondary biomonitors. In those cases in which no members of the Phaeophyceae were found, the chlorophyte *Codium tomentosum* was used, as it is often present in the area of influence of marine fish farms.

Stable nitrogen isotope (δ15N) analysis

Aliquots (3 mg) of the dried samples were weighed and packed into tin capsules (EuroVector). The capsules were stored in a desiccator until δ15N analysis (carried out in the Unidad de Técnicas Instrumentales de Análisis (UTIA), Servicios de Apoyo a la Investigación, University of A Coruña). The samples were combusted in an elemental analyzer (FlashEA1112: ThermoFinnigan) coupled to an isotopic ratio mass spectrometer (Deltaplus: ThermoFinnigan). Acetanilide was used as the reference standard for quantifying the nitrogen content. Calibration of the reference gas for atmospheric 15N was carried out with IAEA-N-1 ((NH4)2SO4), IAEA-N-2 ((NH4)2SO4) and IAEA-NO-3 (KNO3) as standards.

The isotopic ratios (15N/14N) of the samples were compared with the standard (atmospheric N2), so that comparable proportions were obtained. The relative abundance of 15N in the sample (δ15N) was calculated from the formula:  , where *R* is the 15N/14N ratio. The overall error was determined by use of analytical replicates and constitutes a measure of the precision of the technique, as it is the coefficient between the standard deviation of the replicates and the number of replicates. The overall error of the replicates of 30 samples was 2 %.

Interspecific differences in δ15N values of macroalgae

The difference between δ15N values of the four species of macroalgae was evaluated by the following comparisons of pair of species: *F. vesiculosus* and *F. spiralis*, *F. vesiculosus* and *F. serratus*, *F. spiralis* and*F. serratus*, and *F. vesiculosus* and *C. tomentosum*. For this, 12, 8, 6 and 11 locations were used in 2008 and 2009 where each pair of species coexisted (Fig. [1](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Fig1)). To study interspecific differences, the model parameters (slopes and elevations) for interspecific bioconcentration regression lines [standard major axis (SMA)] for each pair of species were compared with the line of slope 1 and elevation 0, by use of the *t*statistic. The difference between estimated and hypothesised slope and elevation to the standard error of elevation was thus compared (Warton and Ormerod [2007](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR69)). The calculations were carried out with the “smatr” package (Warton and Ormerod [2007](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR69)), under *R* (R Development Core Team [2008](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR43)).

Establishment of the regional reference levels of δ15N values of macroalgae

Data from a prior survey conducted for different purposes were used in the present study. The samples were collected in a regional coastal survey carried out in July 2007 in Galicia, and deposited in the Environmental Specimen Bank of Galicia (ESBG). The sampling locations in the study were located at distances of more than 300 m from drains, ports or industrial facilities, and more than 150 m from the mouths of first- or second-degree rivers. The data from this survey, as well as the methods of sampling and processing the macroalgae deposited in the ESBG, are described by Viana et al. ([2011](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR61)).

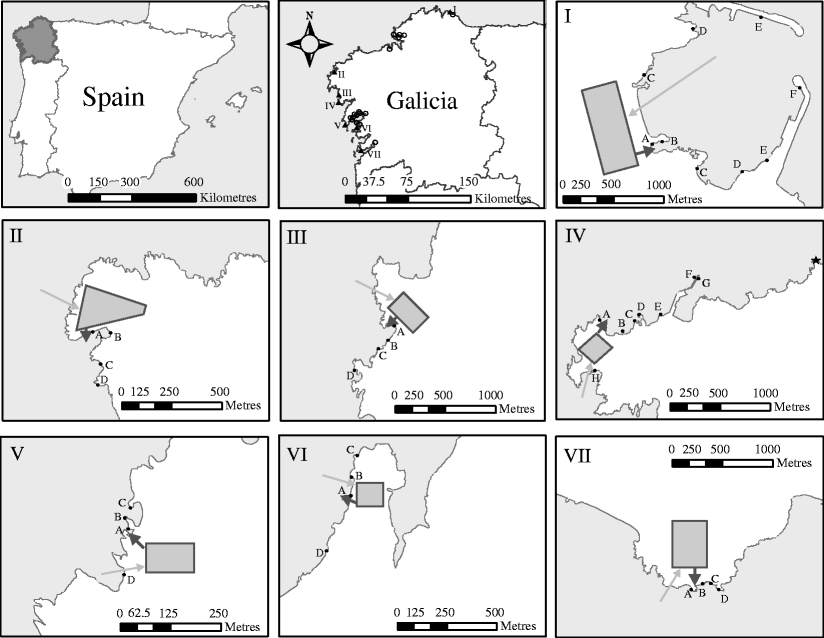
Seasonal variability in δ15N values of macroalgae

The study was conducted at three locations, one of which was located at a supposedly clean site (Fig. [1](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html" \l "Fig1)), and two were located in the surroundings of two different marine fish farms (site C at fish farms II and III, Fig. [1](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html" \l "Fig1)). Samples of *F. vesiculosus* were collected at each of the three locations, and at fish farm III, *F. spiralis* was also sampled. Each location was sampled every month (ten replicates at each location) so that there were a total of 12 mean samples from each location. The experiment began in February 2009 and ended in January 2010.

Results

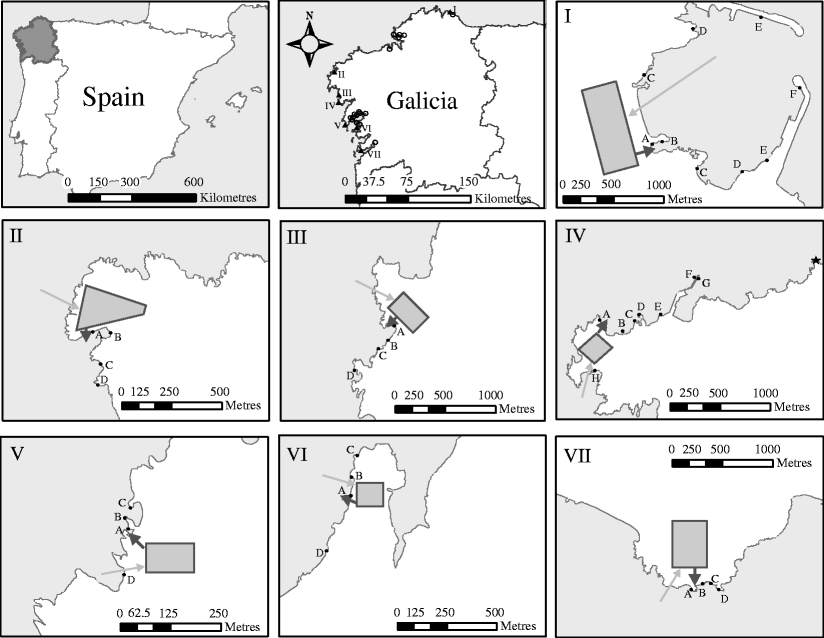
The individual δ15N values of macroalgae collected at the different locations are shown in Table [1](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Tab1). The locations are ordered from A to H, being A, the closest, and H the furthest from the waste disposal point. Those locations at which a species other than *F. vesiculosus* was sampled are shown in Table [1](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Tab1). Similar δ15N values were obtained in different macroalgae sampled at the same S.S [see e.g. fish farm VI (Table [1](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Tab1))]. In all species, there was enrichment of δ15N values of the macroalgae collected from between 100 and 500 m from the marine fish farms (sites B to D). The δ15N values decreased gradually with increasing distance from the dumping point, except in some cases in which similar values were maintained at all locations, for both high values (fish farm I) and low values (fish farm V).

The δ15N values of the different species studied are compared in Fig. [2](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html" \l "Fig2). There generally were no significant differences between the slopes and elevations of the pairs of data studied and the line of slope 1 (*p* > 0.05, null hypothesis accepted); however, the comparison between *F. vesiculosus* and *F. serratus* revealed significant differences between elevations (Fig. [2](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html" \l "Fig2)). The kernel smoothing distribution of the values of δ15N values of *F. vesiculosus* in the 2007 BEAG survey are shown in Fig. [3](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Fig3); this corresponds to a normal distribution (Shapiro–Wilk and Kolmogorov–Smirnov). The distribution of the combination of all of the macroalgae located nearby the marine fish farms (Table [1](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Tab1)) was slightly skewed to the right with respect to the control distribution (Fig. [3](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Fig3)).



**Fig. 2**

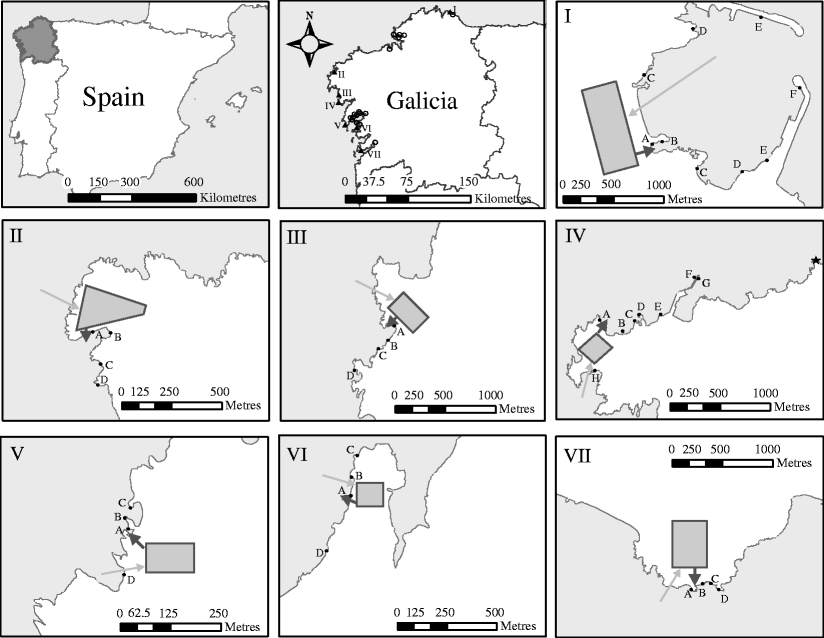
Standard major axis lines of the δ15N values (‰) (per mille) from the pairs of species studied are represented by *fine lines*,; *dashed lines* represent a line with a slope equal to 1. The value of the significance of the comparison of the slopes and elevations are shown (in *bold*) in each case



**Fig. 3**

Distribution of the regional reference values of δ15N in *Fucus vesiculosus* (BEAG, 2007 survey) and distribution of the macroalgae (genus *Fucus* and *Codium tomentosum*) collected around the 7 seven land -based marine fish farms, estimated by kernel smoothing

With regard to the seasonal variation in the δ15N values of macroalgae, the results showed that δ15N values did not vary more than 2 ‰ during the year at the control site (shaded areas, Fig. [4](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Fig4)). By contrast, the range of variation in δ15N values of macroalgae affected by waste discharging from the marine fish farms varied by about 5.57 ‰ throughout the year. The highest value of δ15N was observed in August in both farms and in both species sampled (*F. vesiculosus* and *F. spiralis*). This maximum was not synchronous with that of the control location, which occurred in November (Fig. [4](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Fig4)). The variation of δ15N values throughout the year in the two marine fish farms under study is likely not to enable the detection of farm effluents to be deduced from a single annual sample. Because of this, controls with macroalgae may be carried out during the period July to September (Fig. [4](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Fig4)), with the aim of selecting the maximum annual value of δ15N.



**Fig. 4**

Monthly variations in δ15N (‰) (per mill) in *Fucus vesiculosus* (site C at fish farm II and fish farm III and at a control site, *solid star* at fish farm IV, Fig. [1](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Fig1)) and *Fucus spiralis* (site C at fish farm II, Fig. [1](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html" \l "Fig1)) for the period February 2009 to January 2010

Discussion

The concentrations of dissolved elements and the deposition of particulate residues in the surroundings of a marine fish farm largely depend on the relationship between the production rate of the farm and the dispersive capacity of the environment (Carballeira et al. [2012](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR5)). This relationship varies temporally and spatially and can give rise to multiple environmental interactions that may generate different types of perturbations and responses from the biota (Tello et al. [2010](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR54)). Bacteria preferentially use 14N; thus, marine fish farm effluents are enriched in 15N (Heaton [1986](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR22)), which will be uptaken by primary producers. For this reason, the usefulness of δ15N measured in macroalgae as monitoring biotool of intensive LBMFFs was assessed (Table [1](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Tab1)).

To date, the analysis of δ15N values has been used to evaluate the effects of disposal of aquaculture activities with red macroalgae and one brown macroalgal species (Jones et al. [2001](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR24); Lin and Fong [2008](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR29); Vizzini and Mazzola [2004](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR65)). However, the results obtained in the present work confirmed that macroalgae of the genera *Fucus* and *C. tomentosum* successfully detected the extent of the waste. The isotopic signal was enriched in the macroalgae close to the disposal point, and the maximum values were even higher than in previous studies (Table [2](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Tab2)). The highest macroalgal δ15N values of samples affected by the effluents from the turbot farms ranged from 7.8 ‰ to 11.17 ‰ (Table [1](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Tab1)), whereas values reported in the literature vary between 4.2 ‰ and 7.1 ‰ in red macroalgae from the surroundings of shrimp farms (Costanzo et al. [2004](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR9); Jones et al. [2001](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR24); Lin and Fong [2008](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR29)). In LBMFFs, Vizzini and Mazzola ([2004](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR65)) observed maximum macroalgal δ15N values of 6.3 ± 1.2 ‰. Isotopic enrichment in macroalgae in the surroundings of LBMFFs may be related to isotopic differences in the fish food used. The mean value of δ15N values of the pelleted food provided to the turbot (Nutreco Aquaculture, Skretting) was 7.43 ± 0.45 ‰, while the value of the pelleted food used on shrimp farms was 6.3 ± 0.6 ‰ (Landrum and Montoya [2009](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR26)), and that in the food used on the farms studied by Vizzini and Mazzola ([2004](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR65)) varied between 6.2 ‰ and 11 ‰, according to Sarà et al. ([2004](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR50)).

**Table 2**

Reported δ15N values of marine macroalgae influenced by fish and shrimp farm effluents

| **Species cultivated** | **Production ( t year-−1)** | **Macroalgae species** | **Type of test (duration)** | **δ15N control (‰)** | **δ15N source (‰) (distance, m)** | **Location** | **Reference** |
| --- | --- | --- | --- | --- | --- | --- | --- |
| *Penaeus merguiensis* a | -– | *Catenella nipae* | Transplant (4 days) | 3.3 ± 0.7 | 4.8 ± 0.6 (0-–2000) | North Queensland, Australia | Burford et al. ([2003](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR2)) |
| *Penaeus merguiensis* a | -– | *Catenella nipae* | Transplant (4 days) | ≈3 | 4.2-–4.3 (0) | Australia | Costanzo et al. ([2004](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR9)) |
| *Sparus aurata*,*Dicentrarchus labrax* b | 375 | *Asparagopsis taxiformis* | Transplant (4 days) | 3.9 ± 0.2 | 4-–5 (100) | Canary Islands, Spain | García-Sanz et al. ([2010](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR19))in press |
| *Sparus aurata*,*Dicentrarchus labrax* b | 375 | *Stypopodium zonale* | Transplant (4 days) | 2.5 ± 0.2 | 3-–4 (0) | Canary Islands, Spain | García-Sanz et al. ([2010](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR19))in press |
| *Sparus aurata* b | 800 | *Cystoseira mediterranea* | Transplant (4 days) | 6.1 ± 0.3 | 6-–7 (0) | Catalonia, Spain | García-Sanz et al. ([2010](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR19))in press |
| *Thunnus thynnus* b | 350 | *Dictyopteris polypodioides* | Transplant (4 days) | 1.7 ± 0.1 | ≈4 (0) | Murcia, Spain | García-Sanz et al. ([2010](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR19))in press |
| *Penaeus japonicus* a | -– | *Catenella nipae* | Native | 2.9 | 8.6 (mid creek) | Moreton Bay, Australia | Jones et al. ([2001](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR24)) |
| *Penaeus stylirostris* a | 12 | *Acanthophora spicifera* | Transplant (3 days) | 4.89 | 5.63-–5.96 (10-–495) | Moorea, French Polynesia | Lin and Fong ([2008](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR29)) |
| Shrimp farm | -– | *Gracilaria vermiculophylla* | Native | -– | 7.8-–8.0 (-–)†d | Gulf of California, Mexico | Piñón-Gimate et al. ([2009](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR42)) |
| Shrimp farm | -– | *Hypnea spinella* | Native | -– | 8.5-–11.4 (-–)†d | Gulf of California, Mexico | Piñón-Gimate et al. ([2009](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR42)) |
| Shrimp farm | -– | *Spyridia filamentosa* | Native | -– | 7.7 (-–)†d | Gulf of California, Mexico | Piñón-Gimate et al. ([2009](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR42)) |
| *Dicentrarchus labrax*,*Sparus aurata*,*Diplodus puntazzo* c | 300 | *Sphaerococcus coronopifolius* | Native | -– | 5.8 ± 1.7 (-)(–) | Sicily, Italy | Vizzini and Mazzola ([2004](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR65)) |
| *Dicentrarchus labrax*,*Sparus aurata*,*Diplodus puntazzo* c | 300 | *Padina pavonica* | Native | -– | 6.3 ± 1.2 (-)(–) | Sicily, Italy | Vizzini and Mazzola ([2004](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR65)) |

– unknown

aShrimp farm

bFish cages

cLand -based marine fish farms

†dThe locations are influenced by various types of effluent, - unknown

The degree of effluent detection varied between the farms under study, as a result of differences in production (which determined the amount of dumping) and location (since sites presented different dispersive capacity). The waste from farm I is dumped in a harbour (Fig. [1](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Fig1)) where there was a relatively low rate of hydric renovation, so the δ15N was not less than 8 ‰ in any of the locations within the harbour. In regard to the farms located in zones where there was a high degree of hydrodynamism, the higher the production, the larger the area of influence (Table [1](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Tab1)). Thus, the area of influence of farm IV with an annual production of 2,250 t (Table [1](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Tab1)) was 800 m (site F), whereas for farm III (308 t year−1) and farm VI (43 t year−1), which dumped their waste in similar environments, the area of influence was less than 200 m in the predominating direction of the current.

Interestingly, the location most enriched in δ15N did not correspond to those located closest to the focal point of dumping from these LBMFFs (site A, discharge point, Table [1](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html" \l "Tab1)), contrary to the results from similar studies of macroalgal δ15N values in the vicinity of aquaculture farms (Costanzo et al. [2004](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html" \l "CR9); Jones et al. [2001](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR24); Lin and Fong [2008](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR29); Vizzini and Mazzola [2004](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR65)). In these location, the δ15N was impoverished, and it was even below the control site value in all of the farms under study (0.08–5.1 ‰ in *C. tomentosum* and 1.27–6.37 ‰ in *Fucus* sp., Table [1](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html" \l "Tab1)). These results agree with those of Rogers ([1999](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html" \l "CR48)) where impoverishment of δ15N values in *Ulva lactuca* was observed adjacent to an urban waste dump, where the δ15N decreased from 7.8 ‰ at the reference site to 3.4 ‰ in the location closest to the dumping point. This was associated with the particulate organic matter (POM), for which the δ15N was similar (3.2 ‰), but which did not represent the fraction of N available to macroalgae. Another hypothesis can be that fractionation (preferential use of 14N over 15N) may occur during assimilation of nitrogen in primary producers when excess nitrogen is available for uptake. However, this seemed to be only true for phytoplankton (see e.g. Pennock et al. [1996](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html" \l "CR41)), where lower δ15N values were observed in producers relative to their source. Contrarily, different laboratory-based studies demonstrated that the δ15N values of macroalgae accurately reflected nitrogen from water sources, even at high concentrations (Cohen and Fong [2005](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html" \l "CR7); Naldi and Wheeler [2002](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html" \l "CR38)). Finally, the exceptionally low δ15N values from the sites closest to the output may be related to pH. The mean pH of the input water was 8.03 ± 0.03 and that of the output water was 7.81 ± 0.06 (average value of 68 samples from the marine fish farms studied provided by the water quality monitoring agency, Aguas de Galicia; Carballeira et al. [2012](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html" \l "CR5)). Another possible explanation for the lower δ15N values at the point of discharge and subsequently higher values along the coast is that the majority of the N in the effluent is ammonium. Macroalgae incorporate seven times more NH 4 + than NO 3 − (Deutsch and Voss [2006](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html" \l "CR13)) through passive diffusion (Vallyathan et al.[2002](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html" \l "CR58)). The oxidation of NH 4 + to NO 3 − increases δ15N values (Hadas et al. [2009](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR21)). Therefore, as the ammonium-rich effluent moves away from the point of discharge, more and more ammonium is oxidized to NO 3 − , and the δ15N values subsequently increase.

It must therefore be taken into account that the determination of δ15N values of macroalgae sampled at only one location located close to the dumping point may not adequately reflect the influence of the marine fish farm, and may lead to erroneous conclusions. In this way, the δ15N for detecting LBMFFs effluents should be studied following a non-linear gradient, starting at the discharge point of the LBMFF effluent and following the direction of the prevailing current (Carballeira et al. [2012](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR5); Lapointe et al. [2007](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR27)). Reference sites may be located at the opposite direction of the prevailing current, where most LBMFFs pump the input water, which must be clean. However, when cost is a limiting factor, a single location can be used to standardize the area of exposure. This measure requires the establishment of a maximum intra-annual δ15N threshold value that should not be surpassed at a standard distance. For this type of LBMFFs and results, the standard distance must be more than 200 m from the waste disposal point, to ensure oxidation of the ammonium and re-equilibrium of the pH of the outgoing water with the environment so that it does not inhibit absorption of the N emitted.

The results from this study showed that the δ15N values of macroalgae acted as descriptor of exposure, an indicator of the interaction between the loading estimate and the dispersive capacity of the environment, providing accurate information about the degree of contamination and the area of influence of a farm (Carballeira et al. [2011](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR4)). However, despite the suitability of using native macroalgal δ15N values to trace fish farming activities, certain aspects must be addressed before any plan for monitoring this type of industry can be implemented. These include (1) macroalga interspecific differences, (2) determination of the regional reference ranges of δ15N and (3) intra-annual variability of δ15N.

**Macroalgal interspecific differences**

As different biomonitors are sometimes used at different sites, it was essential to determine whether there are any interspecific differences in the δ15N values amongst species. Moreover, this may be necessary because of the tendency for shifts from perennial, native populations in areas enriched with nutrients, to opportunist macroalgae (Tett et al. [2007](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html" \l "CR55)), which may lead to the need to substitute the species selected as a primary biomonitor. Umezawa et al. ([2002](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR57)) suggested that macroalgae that grow with the same inputs of nutrients (at least between taxonomically related species) have similar values of δ15N. However, Deutsch and Voss ([2006](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html" \l "CR13)) observed large differences in the δ15N values of *Enteromorpha* sp. and *Ulva* sp. even at the same stations. Although several authors have used different macroalgae as biomonitors of δ15N (Gartner et al. [2002](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html" \l "CR20); Piñón-Gimate et al. [2009](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR42); Riera [1998](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR45); Tucker et al. [1999](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR56)), only one quantitative comparison between the different types of macroalgae has been carried out until now (Dailer et al. [2010](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR10)). In the present study, no significant differences in δ15N were observed between the species that co-occur at the same location. For comparison between *F. vesiculosus* and *F. serratus*, the occurrence of significant differences between slopes (Fig. [2](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html" \l "Fig2)) was attributed to the small range of δ15N values, so the slope could not be estimated accurately. This reflected a problem in the distribution of the data rather than real differences between the two species.

**Determination of regional δ15N values**

Knowledge of the regional reference levels of δ15N values of macroalgae may help to provide a more objective interpretation of the results obtained in local studies. The δ15N average value from marine macroalgae worldwide is 7 ‰ (±4 ‰; Owens et al. [1988](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR40); Wada et al. [1975](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR68)). Natural values from different species of macroalgae in different estuarine and marine environments range between 0.01 ‰ and 7.3 ‰ (Burford et al. [2003](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR2); Dailer et al. [2010](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR10); Gartner et al. [2002](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR20); Jones et al. [2001](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR24); Lin and Fong [2008](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR29); Rogers[2003](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR49)).

Because of the large natural variation in δ15N values of macroalgae, it appears that the reference range must be established for each species in a particular region. The regional reference range in the study area was 5.48 ± 1.18 ‰ (calculated from the ESBG 2007 survey data, Fig. [3](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Fig3)). Taking into account the distribution of values, it was found that the location furthest from the dumping point (sites D to H, Table [1](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Tab1)) were within the established regional reference range, and therefore outside the area of influence of the farms. These locations defined the first modal value observed in the distribution of all the locations sampled in the surroundings of marine fish farms (Fig [3](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Fig3)), and which were almost consistent with the modal value of the distribution of the regional reference values. The second modal value, which was higher than the reference range, corresponded to sites that were apparently more influenced by dumping from the marine fish farms. The left tail of the distribution, with values of δ15N lower than reference values, was formed by the sites closest to the waste emission points (site A, Table [1](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Tab1)). The wide fluctuation in the δ15N signal in macroalgae, from 5.48 ‰ (control) to 11.7 ‰ (maximum value observed), enabled better identification of the degree of exposure to the contaminants relative to that in other organisms showing narrower ranges of variations (Lamb et al. [2012](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#CR25)).

**Intra-annual variability**

Macroalgal δ15N values obtained at one particular time of year may not be representative of the entire year because of the potential annual variability in the δ15N values of the source effluent. Although at the control site, the mean δ15N value was approximately 6.29 ± 1.19 ‰ throughout the year (Fig. [4](http://link.springer.com/article/10.1007/s10811-012-9843-z/fulltext.html#Fig4)), the δ15N of macroalgae sampled at the sites affected by disposal from the marine fish farms varied by 5.57 ‰ (4.67 ‰ to 10.24 ‰), the highest values being observed during summer, when the highest temperatures were recorded, and in consequence, the greatest rates of fish production were expected. However, the maximum annual value for the control was not synchronous with the maximum value for any of the locations affected by the marine fish farms.

In conclusion, the present study has shown that macroalgal δ15N values may be an excellent way of monitoring exposure to organic wastes from marine fish farm effluents. This parameter integrated environmental conditions and provided information about the bioavailable fraction of nitrogen. It was easy to interpret and highly replicable. Furthermore, macroalgal δ15N values determined the area of influence and the variation of influence over time. The results obtained also showed that there were no significant differences in δ15N values measured in the species *Fucus* spp. and *C. tomentosum*; therefore, these macroalgae can be used indistinctly as biomonitors.

However, for future studies, correlations should be established between the changes in the descriptor of exposure (δ15N), and direct or indirect changes in the composition or functioning of the affected coastal ecosystems in order to evaluate whether δ15N values can be used to predict environmental deterioration. This way, environmental monitoring could be performed by using this simple, cheap and rapid tool in replacement of more complex measurements.

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