Stable nitrogen isotopes in coastal macroalgae: geographic and anthropogenic variability

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Abstract

Growing human population add to the natural nitrogen loads to coastal waters. As the excess nitrogen is readily incorporated in new biomass anthropogenic and natural nitrogen sources may be traced by the measurement of stable nitrogen isotopes ($\delta^{15}$N). In this study $\delta^{15}$N was determined in two species of macroalgae (Ascophyllum nodosum and Fucus vesiculosus), and in nitrate and ammonium to determine the relative importance of anthropogenic versus natural sources of nitrogen along the coast of NW Spain. Both algal species and nitrogen sources showed similar isotopic enrichment for a given site, but algal $\delta^{15}$N was not related to either inorganic nitrogen concentrations or $\delta^{15}$N in the water samples. The latter suggests that inorganic nitrogen inputs are variable and do not always leave an isotopic trace in macroalgae. However, a significant linear decrease in macroagal
δ¹⁵N along the coast is consistent with the differential effect of upwelling. Besides this
geographic variability, the influence of anthropogenic nitrogen sources is evidenced by
higher δ¹⁵N in macroalgae from rias and estuaries compared to those from open coastal
areas and in areas with more than 15x10³ inhabitants in the watershed. These results
indicate that, in contrast with other studies, macroalgal δ¹⁵N is not simply related to either
inorganic nitrogen concentrations or human population size but depends on other factors as
the upwelling or the efficiency of local waste treatment systems.

**Keywords:** upwelling, wastewater, urban populations, biomonitors, *Fucus, Ascophyllum*

1. **Introduction**

Coastal areas, particularly estuaries, have been subjected to increasing nitrogen loads due to
the growing human population and its associated anthropogenic activities (e.g. agriculture,
sewage). As a consequence of these activities, coastal ecosystems are under increasing
pressures of pollution and eutrophication (Paerl et al., 2006; Vidal et al., 1999). The latter, a
problem first limited to enclosed or semi enclosed water bodies, is now being observed in
most coastal areas (Cloern, 2001; Druon et al., 2004; Gilbert et al., 2009; Valiela et al.,
2000). Determining the origin of the dissolved nitrogen in estuarine environments can be an
effective means of evaluating nutrient management policies, and may ultimately lead to more
successful environmental regulation of anthropogenic nitrogen (Ahad et al., 2006).

The adverse effects of anthropogenic nitrogen inputs have led to the development of suitable
indicators to assess water quality of aquatic ecosystems, both for management or biological
issues. Direct quantification of dissolved inorganic nitrogen in water has been frequently
used (e.g. Hickel et al., 1993; Paerl et al., 2006; Rabalais et al., 1996). However, nutrient
concentrations in the water column alone seem not to be adequate to quantify anthropogenic loads as they are highly variable in time because of rapid consumption by primary producers (Fry et al., 2003). Moreover, changes in nitrogen concentrations may be due to anthropogenic inputs but also to natural processes, as coastal upwelling (e.g. Arístegui et al., 2006).

As an alternative to nutrient measurement, the ratio of nitrogen stable isotopes ($\delta^{15}N$) in macroalgae has been increasingly used to quantify the importance of different nitrogen sources for primary producers (Constanzo et al., 2005; Gartner et al., 2002; Lapointe and Bedford, 2007; McClelland and Valiela, 1998; McClelland et al., 1997; Piñón-Gimate et al., 2009; Riera et al., 2000; Savage and Elmgren, 2004; Tucker et al., 1999). Nitrogen has two stable isotopes, and its proportion might vary according to the different metabolic routes that a molecule follows, as light isotopes ($^{14}N$) are mobilized faster by some processes than the heavy ones (isotopic fractionation). For some biological reactions, the reactants are progressively enriched in heavy isotopes while the products are relatively depleted at a rate characteristic of each reaction (Mariotti et al., 1981). Anthropogenic nitrogen sources, as sewage, manure, terrestrial runoff, fish farm waste and groundwater, are often more enriched in $^{15}N$ than seawater (Heaton, 1986; Jordan et al., 1997; McClelland and Valiela, 1998; Vizzini and Mazzola, 2004; Voßland Struck, 1997) because of isotopic fractionation during nitrification and volatilization in the case of $\text{NH}_4^+$, or denitrification in the case of $\text{NO}_3^-$ (Montoya, 2008). In contrast, nitrogen pools from most agricultural facilities are characterized by depleted isotopic values, as they are synthesized from atmospheric $\text{N}_2$ (Heaton, 1986). Furthermore, $\delta^{15}N$ in macroalgae can also be used to detect the intensity and variability of the anthropogenic nitrogen loading (Cole et al., 2004;
Costanzo et al., 2005; Savage and Elmgren 2004) often related to the degree of urbanization in the watershed (Cole et al., 2004, 2005; McClelland and Valiela 1998; McClelland et al., 1997).

Besides nutrients from anthropogenic origin, different natural processes also affect inorganic nitrogen concentrations and in consequence macroalgal isotopic values. For instance, algae from mangrove habitats that were exposed to nitrogen derived from N\textsubscript{2} fixation were depleted in \textsuperscript{15}N while those in habitats with frequent coastal upwelling were relatively enriched (Lamb et al., 2012). In addition, \textit{δ}\textsuperscript{15}N in estuarine waters vary as a consequence of freshwater inputs and local biogeochemical processes (Ahad et al., 2006). Because different combinations of sources may produce similar \textit{δ}\textsuperscript{15}N values, additional information on factors affecting local nitrogen dynamics is required to obtain unequivocal evidence that significant amounts of anthropogenic nitrogen are affecting the coastal zone.

The regions of Galicia and Asturias (NW Spain, Fig. 1) are characterized by the presence of estuaries and rias sustaining high levels of biological production due to seasonal upwelling fertilization (Arístegui et al., 2006). Each of these rias has also an independent river basin, but the nutrient inputs from these rivers are lower than those from the upwelling (Bode et al., 2011b). The upwelling has a larger impact in the production of western and southern rias (Galicia) because the initial nutrient inputs are amplified by remineralization of organic matter in the shelf and subsequent import with estuarine circulation (Álvarez-Salgado et al., 1997). In contrast, upwelling in the northern coast (Asturias) is generally weaker than in the western coast and limited to the vicinity of major capes (Botas et al., 1990). Upwelling nutrients support a larger fraction of primary production in Galicia than in Asturias (Álvarez-Salgado et al., 2002; Bode et al., 2011a). In consequence, geographic variability in the
nitrogen sources, and correspondingly in their isotopic signature, can be expected in NW Spain. Besides, most of the human population concentrates in the coastal zone, which showed large urbanization development in recent years (Viña, 2008). Previous studies of macroalgal $\delta^{15}$N in this region reported high enrichment near large urban areas and inside the rias, suggesting the influence of nitrogen from wastewater (Bode et al., 2006; Bode et al., 2011b; Carballeira et al., 2012; Viana et al., 2011).

In this study the variability in the isotopic composition of two intertidal macroalgae in relation to concurrent measurements of dissolved inorganic nitrogen concentrations and isotopic composition in the NW coast of Spain was analyzed to determine the relative importance of anthropogenic versus natural nitrogen sources. The effect of the coastal upwelling, as the main natural source of nitrogen, was represented by the geographical distribution of sampling sites along the coast, while the main anthropogenic input was represented by the size of the human population in the watershed as a proxy for wastewater production.

2. Material and methods

2.1. Sampling

Samples were collected in the intertidal along the coast of NW Spain at sites representative of environments with variable influence of the upwelling and in a large range of urban influence (Fig. 1). As upwelling in the northern coast is generally weaker than in the western coast (Botas et al., 1990), an arbitrary reference point located at the sea discharge point of the River Miño (Fig. 1) was used to compute the distance along the coast between each sampling site and this reference point. This distance was intended to indicate the lower input of new
nitrogen by the upwelling in the northern coast (Mar Cantábrico, zone I in Fig. 1) compared to those in the western coast (Galicia). In the latter, two zones were considered to investigate potential differences between Rias Baixas (zone III) and other rias (zone II). Sampling sites covered a large range of urban population influence in the watershed (from ~240 to ~246,000 inhabitants) according to Spanish Official Population Census (http://www.ine.es/inebase).

Sampling surveys were carried out mostly during spring and summer 2010 and 2011, but some samples from 2006 were added to complete the range of geographic or urban population values (Table 1).

Two species of Phaeophyceae (brown algae) were selected: Ascophyllum nodosum and Fucus vesiculosus. The species were present at 12 and at 26 sites respectively, and they were cohabiting at 11 sites. Three individuals of each macrophyte species fixed to the substrate were collected from the mesolittoral zone when emerged. Apical parts of the specimens (1 cm) were used for analysis of the stable nitrogen composition. Samples were rinsed with Milli Q water to remove sediments and other material and frozen (-20 °C) before processing. Samples were defrosted and dried (50 °C) until constant weight, before grinding into a homogeneous powder.

Samples of surface water were collected concurrently with macroalgae. Salinity was measured in situ with a portable conductivity meter (YSI Model 30). Water samples were poisoned with HgCl₂ (0.05% final concentration) to prevent microbial alteration and stored in tightly caped Pyrex flasks.

2.2. Chemical analysis
Nitrate, nitrite and ammonium were determined in the laboratory using segmented flow analysis (Braun-Luebbe AAII) following the procedures of Grasshoff et al. (1983). Sensitivity was 0.05, 0.01 and 0.04 µM for nitrate, nitrite and ammonium, respectively. Precision (se of 3 replicates) was better than 14% of the mean value for any of the nitrogen species. Ammonium values >10 µM were excluded from further analysis because of suspect contamination of samples during processing, as values reported for coastal waters in the study region do not exceed 10 µM (e.g. Bode et al., 2011b).

The isotopic composition of total nitrate (NO$_3^-$+NO$_2^-$) was determined by previous conversion into ammonium and later recovery of ammonium on a solid phase. The procedure is an adaptation of the diffusion method (Sigman et al., 1997) involving the incubation of samples in two steps. In this case the resulting ammonium was collected on a small disk of glass-fiber filter placed in the gas headspace of the diffusion flask (Slawyk and Raimbault, 1995). First, aliquots of the samples were incubated (50 °C, 1 week) in the same collecting flask without cap to reduce the volume and concentrate nitrate. Ashed MgO was added to raise pH above 9.7 to remove ammonia by volatilization. In the second step (50 °C, 2 weeks), ashed Devarda’s alloy was added to the reduced volume sample to convert nitrate and nitrite into ammonium. The high pH (>11) of the mixture ensured also the conversion of ammonium into ammonia gas that was collected on a sterilized glass-fiber disk (Whatman GF/F), acidified with 0.5 ml of 0.25N H$_2$SO$_4$ and hooked on a needle fixed to the inner side of the flask cap. Care was taken to ensure that the filter disk did not contact the liquid sample. This extraction procedure does not allow separation between NO$_3^-$ and NO$_2^-$ therefore the values reported are the combined isotopic signatures of total nitrate (Ahad et al., 2006). After the second incubation step the disk filters were dried and
prepared for isotopic analysis. The stable isotope composition of ammonium was
determined in another aliquot of the water samples by an adaptation of the diffusion method
(Holmes et al., 1998). This method involves gas-phase diffusion as described for the second
step of the total nitrate extraction. In all cases corrections for isotopic fractionation during
the whole incubation and diffusion steps were made (Holmes et al., 1998). The measured
values of natural abundance of dissolved inorganic nitrogen were retained for further
analysis when the ammonium recovery after the diffusion procedure exceeded 45% and
isotopic fractionation of internal standards was within 1‰ of values estimated from the
empirical equation in Holmes et al. (1998).

2.3. Stable isotopes

The natural abundance of stable nitrogen isotopes was determined in macroalgae and water
samples (total nitrate and ammonium). For macroalgae, 2.5 mg of dry sample was analyzed
to ensure a minimum of 10 µg of N. For water samples, 1 ml of 4 mM-N (NH₄)₂SO₄ was
added to each sample during the diffusion phase to ensure the detection limit was achieved.
Samples were placed in tin capsules and introduced into an isotope-ratio mass spectrometer
(Thermo Finnigan Mat Delta Plus) via an element analyzer (Carlo Erba CHNSO 1108).
Isotopic results are expressed in delta notation:

\[ \delta^{15}N = \left( \frac{^{15}N_{\text{sample}}}{^{14}N_{\text{sample}}} / \frac{^{15}N_{\text{std}}}{^{14}N_{\text{std}}} \right) - 1 \times 1000 \]

where the standard (std) for \( \delta^{15}N \) is atmospheric N₂. Precision (se of 5 replicates) was better
than 0.05‰ for either IAEA-N-2, IAEA-N-1 or IAEA-NO-3 standards. The coefficient of
variation of triplicate sample aliquots was always <2%.

2.4. Statistical procedures
Relationships between variables were first analyzed using non-parametric correlation (Spearman $\rho$). Further analyses were made using linear regression after excluding outliers exceeding 1.5 times the interquartile range. In the case of salinity vs. dissolved nitrogen concentrations and macroalgal $\delta^{15}$N vs. geographical distance product-moment regression was used because either the error in estimating the salinity was much lower than the error for dissolved nitrogen or because the resulting slope was further employed to account for systematic variability in $\delta^{15}$N with geographical distance (Sokal and Rohlf, 1981). In the case of the comparison of $\delta^{15}$N between the two macroalgal species standard major axis was used because both variables were measured with the same type of error (Sokal and Rohlf, 1981). In this later case, the obtained regression parameters were compared with the line of slope 1 and zero intercept by a $t$-test (Warton and Ormerod, 2007).

The relative contribution of geographical distance and population size to $\delta^{15}$N was estimated as the sums of squares (Type I) obtained with an ANOVA design including two population size classes (larger and smaller than $15 \times 10^3$ inhabitants, respectively) with distance as covariable. Differences between sampling zones or classes of population size were further analyzed by non-parametric Kruskal-Wallis test (Sokal and Rohlf, 1981).

3. Results

3.1. Dissolved inorganic nitrogen

Total nitrate concentration in the samples ranged from 1.40 to 39.38 µM, while ammonium (excluding $>10$ µM values) ranged from 2.28 to 7.47 µM (Table 1). Total nitrate was negatively correlated with salinity in most samples ($\rho = -0.682$, $P<0.001$, $n=24$) except at O Burgo, where nitrate reached ca. 40 µM (Figure 2). In contrast, ammonium was not
correlated with salinity (P>0.05). These relationships with salinity suggest large potential contributions of nitrate from freshwater in most of the studied area but variable inputs of ammonium unrelated to freshwater discharges.

Because of rapid contamination with ambient ammonia during the analytical preparation steps stable isotope composition of dissolved nitrogen was determined with confidence in a subset of samples only (Table 1). Total nitrate δ¹⁵N varied between 2.5 and 19.6‰ while δ¹⁵N ammonium ranged from -1.6 to 2.6‰ (Table 1). When measured concurrently δ¹⁵N of ammonium and δ¹⁵N of total nitrate were correlated (ρ=0.943, P<0.01, n=6). The highest nitrate value corresponded to the sample from O Latón (Code 26), collected at the discharge outlet of a Water Treatment Plant, but a large value was also observed in Figueras (Code 9), in this case not obviously related to residual water discharges. Values of nitrate δ¹⁵N for marine waters (salinity >35) were near 5‰.

3.2. δ¹⁵N in macroalgae

Stable isotope composition of *F. vesiculosus* and *A. nodosum* were significantly correlated (ρ=0.806, P<0.010, n=10). The resulting regression line did not differ from a line with slope 1 and intercept 0 (P<0.05) indicating that the isotopic composition of these species was equivalent for a given site (Fig. 3).

In contrast, macroalgal δ¹⁵N was not correlated with either dissolved inorganic nitrogen concentrations, salinity or isotopic composition (Fig. 4).

3.3. Geographic variability in δ¹⁵N
Macroalgal δ\(^{15}\)N varied according to the geographical location of samples (Fig. 5). Both species showed a linear decrease in δ\(^{15}\)N with the distance from the reference point in the River Miño (Fig. 5a). The slope of the regression lines indicated a change of δ\(^{15}\)N of 0.3 and 0.4‰ per 100 km of coastline for *F. vesiculosus* and *A. nodosum*, respectively (Table 2). In contrast a significant relationship was found between neither dissolved nitrogen concentrations nor δ\(^{15}\)N of total nitrate with distance, as exemplified by total nitrate concentration (Fig. 5b). No significant differences resulted either when considering the sampling zones (I, II and III) in a Kruskal-Wallis test (P>0.05).

Samples of *F. vesiculosus* collected inside the rias and estuaries (as shown in Fig. 1) had higher δ\(^{15}\)N values than samples collected in open coastal sites (Kruskal-Wallis test, P<0.01). Mean (±se) values for rias and coastal sites, after correction for the geographic variability using the slope in Table 2, were 9.1±1.1‰ (n=17) and 7.6±1.1‰ (n=7), respectively.

### 3.4. Variability of δ\(^{15}\)N with human population

The geographic variability accounted for more than half of total variance in δ\(^{15}\)N for both species (Fig. 6). However, the size of the human population in the watershed was also an important factor for δ\(^{15}\)N, particularly for *A. nodosum*. The isotopic values of both macroalgae, after removal of the geographic trend using the equation in Table 2, increased non-linearly with the size of the human population in the watershed (Fig. 7). Variability in δ\(^{15}\)N was largest at small population sizes (<50x10^3 inhabitants) with clear outliers with unusually large or small values. At the three sites influenced by large populations (>100x10^3 inhabitants) δ\(^{15}\)N values in *F. vesiculosus* (as *A. nodosum* was not found at these
sites) did not follow the increase observed at lower populations. In turn, the distribution of
the human population has no relationship with the geographical gradient found for
macroalgal $\delta^{15}$N (no significant correlation between population size and distance). In any
case, and excluding the outliers, both species showed significantly higher $\delta^{15}$N values at
population sizes larger than $15 \times 10^3$ inhabitants (Fig. 8, Kruskal-Wallis test, $P<0.05$).

4. Discussion

4.1. Natural variability of nitrogen sources

Differences in both concentration and $\delta^{15}$N values of nitrate were expected in the NW
Spanish coast because of the varying influence of the upwelling, as nitrate from the Eastern
North Atlantic Central waters is the main natural source of nitrogen for primary production
in shelf waters of NW Spain (Álvarez-Salgado et al., 2002; Botas et al., 1990; Casas et al.,
1997). Instead, our results indicated no significant spatial variability pattern of nitrate
concentrations or $\delta^{15}$N. Nitrate was the main form of dissolved inorganic nitrogen and its
highest concentrations were found in estuarine waters, suggesting a significant input from
freshwater. However, given the low flow of rivers in this region (Rio Barja and Rodríguez
Lestegás, 1996) the influence of riverine nitrate can be considered only of local importance,
as reported in other studies (Bode et al., 2011b; Gago et al., 2005). This is supported by our
$\delta^{15}$N measurements in nitrate, the first reported for this region, with values close to $5\%e$ in
most cases and particularly in seawater. These values agree with the range reported for
subsurface nitrate in the N Atlantic (Liu and Kaplan, 1989), while the largest values
($>10\%e$) suggest local influence of nitrate from nitrification of ammonium (Mariotti et al.,
Systematic observations of coastal waters revealed the importance of local, short-term upwelling for nutrient inputs in the study area (Álvarez-Salgado et al., 1997; Casas et al., 1997; Nogueira et al., 1998). Because of this nutrient variability, instantaneous nitrogen concentrations and isotopic composition of water samples are not directly reflected in macroalgae collected in the field, in contrast to the findings in laboratory experiments allowing for isotopic equilibration between water nitrogen and algal tissues (Cohen and Fong 2005). Temporal variability in the isotopic composition of inorganic nitrogen is expected to be high, as reported for two northeastern English estuaries (Ahad et al., 2006) and related to changes in either nitrogen sources or in the biogeochemical processing of nitrogen. Such variability and the rapid turnover of surface waters in the region would prevent isotopic equilibration and therefore a close correspondence between the isotopic composition of single water samples and those of macroalgal tissues that integrate isotopic composition over time would not be expected. Both A. nodosum and F. vesiculosus are long lived and perennial macroalgae. Individual fronds can become up to 15 (A. nodosum) and 3 years old (F. vesiculosus) before breakage (Keser and Larson, 1984; Niell, 1979). Both species have apical growth (Moss, 1965; Strömgren and Nielsen, 1986), so the sampled apical tips integrate nutrient concentration and isotopic values from the water nutrients during their growing period. This period can be calculated from their growth rates. F. vesiculosus growth show pronounced latitudinal differences (Mathieson et al., 1976), but at latitudes similar to the study area it ranges between 0.6 and 2.8 cm month$^{-1}$ (Fuentes, 1986; Knight and Parke, 1950). A. nodosum growth rates average 10 cm year$^{-1}$ (Niell, 1979) thus implying that the observed $\delta^{15}$N values are the result of the integration of nitrogen inputs during one month period approximately. In our study macroalgae showed a general $^{15}$N depletion along the coast (Fig. 5), following the higher prevalence of upwelling in the
southern areas compared to those in the northern coast. Therefore, the integration at monthly time scales reflects nitrogen sources more appropriately than water samples. Similar isotopic gradients were observed in intertidal species in other upwelling regions (Hill and McQuaid, 2008).

4.2. Anthropogenic nitrogen inputs and macroalgal $\delta^{15}N$

Notwithstanding the frequent use of macroalgal $\delta^{15}N$ as a tracer for anthropogenic nitrogen in coastal ecosystems in the last decades, only few studies showed experimental evidence of isotopic enrichment in algal tissues after exposure to enriched dissolved nitrogen (Cohen and Fong, 2005; Gartner et al., 2002; Naldi and Wheeler, 2002). Instead, many studies report the progressive change in $\delta^{15}N$ of macroalgae with distance of a clearly identified wastewater discharge point (e.g. Carballeira et al., 2012; Constanzo et al., 2005; Gartner et al., 2002; Riera et al., 2000; Savage and Elmgren, 2004). When anthropogenic nitrogen was provided by diffuse or pulse inputs (e.g. from groundwater) over a relative large area, other studies showed a direct relationship between the size of the anthropogenic load (estimated from computation in the watershed) and macroalgal $\delta^{15}N$ (Cole et al., 2004, 2005; McClelland et al., 1997; McClelland and Valiela, 1998), as the degree of urbanization affects $\delta^{15}N$ of groundwater nitrate (Cole et al., 2006; McClelland and Valiela, 1998). In the latter case, the use of direct measurements of concentration or $\delta^{15}N$ in the water would not reveal clear anthropogenic influence because of the relatively low loading rates. The lack of direct correspondence between water concentrations and isotopic composition and macroalgal $\delta^{15}N$ in our study suggest that the inputs of isotopically enriched nitrogen are from diffuse sources. While the influence of other natural sources of nitrogen, as runoff or precipitation with different isotopic signatures cannot be discarded, in the absence of
specific data on concentrations and isotopic composition of dissolved nitrogen in freshwater of the study region, the relatively high salinity found in most samples (Table 1) would support a minor role of freshwater nitrogen in coastal food webs.

Dissolved nitrogen from urban wastewater generally shows $\delta^{15}\text{N}$ values exceeding 10‰ (e.g. Gartner et al., 2002; Savage and Elmgren, 2004; Tucker et al., 1999). Similarly high values were reported for manure and other organic fertilizers used in agriculture (Kendall, 1998). In our study the sampled nitrate from a water treatment facility (19.6‰) can be considered representative of wastewater nitrogen, and it was considerably enriched when compared to macroalgal samples (Table 1). Therefore it can be interpreted that the sampled macroalgae reflect the assimilation of variable fractions of nitrogen from anthropogenic and marine sources. The amount of nitrogen derived from each source could be estimated using a mixing model to compare the measured macroalgal $\delta^{15}\text{N}$ with that of marine or wastewater nitrogen, as done in other studies (e.g. Bode et al., 2011b; Gartner et al., 2002; Savage and Elmgren, 2004). However, we showed that there was a significant geographic trend of macroalgal $\delta^{15}\text{N}$ (but not in other variables) that must be taken into account when performing further estimations in this region (Table 2).

The influence of anthropogenic sources is evidenced by the higher $\delta^{15}\text{N}$ in macroalgae from rias compared to those in open waters, when the effect of geographical variability is identified. This result agrees with the increasing nitrogen load from anthropogenic sources found in other estuaries (Cole et al., 2004; McClelland and Valiela, 1998; McClelland et al., 1997) and confirms the results from previous studies in the Galician rias (Bode et al., 2006, 2011b). As most of the population concentrates near the rias (Viña, 2008) is not surprising that there was a relationship between the number of inhabitants and macroalgal
δ^{15}N. This relationship, however, is not a simple function of the size of the population, and thus on the potential load of wastewater nitrogen, as found in other studies (McClelland et al., 1997) and a large range of δ^{15}N values was observed below 15,000 inhabitants. Highly 15N enriched isotope values close to small populations (e.g. S. Juan de la Arena, Cedeira, Ramallosa; Table 1) might be due to inefficient or lacking treatment of wastewater before disposal, regardless of the population size, as reported in other studies (Costanzo et al., 2005; Savage and Elmgren, 2004).

Depleted δ^{15}N values (e.g. Soutomaiaor δ^{15}N= -2‰ in A. nodosum and +2‰ in F. vesiculosus, Table 1) may indicate other sources of nitrogen. One possible source would be synthetic fertilizers (δ^{15}N= 1 to 2.6‰, Heaton 1986) but they are much less used in the study area than manure (Nuñez Delgado, 2002). Another depleted source would be atmospheric nitrogen, as macroalgae found in oligotrophic ecosystems supported by diazotrophy (e.g. mangroves) have characteristically low δ^{15}N because of the assimilation of nitrate remineralized from mangrove litter (Lamb et al., 2012). While there are no reports of high atmospheric nitrogen fixation in the study area, most likely depleted δ^{15}N may result from high isotopic fractionation during assimilation of a large pool of dissolved nitrogen. Experimental studies have shown that the assimilation of nitrate caused a decrease in algal δ^{15}N between 0 and 20‰ both in phytoplankton (Needoba et al., 2004; Waser et al., 1998) and macroalgae (e.g. Naldi and Wheeler, 2002) with the highest values associated to high nitrogen concentrations. High isotopic fractionation is expected at Soutomaiaor, located at the innermost zone of the Ria de Vigo, and characterized by high dissolved nitrate concentrations likely resulting from organic matter remineralization in the sediments (Gago et al., 2005). Isotopic fractionation is not generally considered in estimations of source
contributions to macroalgal nitrogen (e.g. Gartner et al., 2002; Savage and Elmgren, 2004) but it can largely affect the estimates, as illustrated by our measurements at Soutomaior. Our wide scale survey of macroalgal $\delta^{15}N$ further supports a dominant role of marine nitrogen in coastal ecosystems of NW Spain, as found in previous studies (Bode et al., 2006, 2011b). Large inputs of anthropogenic nitrogen from wastewater appear limited to local scales, likely related to failures in disposal or treatment procedures. As an example, nitrogen waste for fish farms in Galicia has been traced at scales of a few kilometers with $\delta^{15}N$ in macroalgae (Carballeira et al., 2012) while most macroalgae collected far from dumping sites displayed values similar to marine nitrate (Viana et al., 2011). Because of growing urban pressures wastewater treatment in NW Spain is constantly improving with treatment facilities available not only for large cities but including urban aggregations of 2,000 inhabitants and less (Augas de Galicia, internet). An indirect evidence of this improvement is the correspondence between macroalgal $\delta^{15}N$ and the number of inhabitants in the watershed when the population exceeds $10^5$ inhabitants found in our study. In addition, Viana et al. (2011) noted a general decrease of macroalgal $\delta^{15}N$ in the rias between surveys carried out in 1990 and those in 2007, suggesting a general decrease in the impact of wastewater in this region.

5. Conclusions

Macroalgal $\delta^{15}N$ integrate nitrogen assimilated at time scales of months, thus better reflecting changes in the available nitrogen from different sources than occasional measurements in the water. However, the interpretation of $\delta^{15}N$ values requires a good knowledge of local and regional factors affecting isotopic signatures. Our study showed
that large spatial changes can be due to changes in natural sources, such as the influence of upwelling, while the input of anthropogenic nitrogen is not always related to the size of the human population. These factors are not taken into account in most studies using macroalgal $\delta^{15}N$ to estimate anthropogenic nitrogen impacts in coastal ecosystems. Isotopic fractionation and identification of the main nitrogen processes operating at local spatial scales are also key factors for the interpretation of macroalgal $\delta^{15}N$ because, as pointed out for other systems (e.g. Lamb et al., 2012), $\delta^{15}N$ values alone do not provide unequivocal evidence that large amounts of anthropogenic nitrogen are affecting the coastal zone.

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Needoba JA, Sigman DM, Harrison PJ. The mechanism of isotope fractionation during
algal nitrate assimilation as illuminated by the $^{15}$N/$^{14}$N of intracellular nitrate. J Phycol
Niell FX. Sobre la biologia de Ascophyllum nodosum (L.) Le Jol. en Galicia. III. Biometría,


Figure 1. Location of sampling sites along NW Spain. Three environment types representing coastal sites in large rias (I), sites in or near middle rias (II) and mostly open sea sites at the northern coast (III) were considered. The arrow indicate the River Miño discharge point used as the southernmost reference point to compute intersite distances in this study.

Figure 2. Linear relationships between ammonium (NH$_4^+$, black squares) or total nitrate (NO$_3^-$+NO$_2^-$ gray circles) and salinity in water from the sampling sites. The point encircled was an outlier (>1.5 times the interquartile range) not used in the estimation of the regression line (Spearman $\rho$ = -0.666, P<0.01).

Figure 3. Relationship between stable isotope composition of *Ascophyllum nodosum* and *Fucus vesiculosus* sampled at the same locations. The regression line computed without the outlier (open circle, >1.5 times the interquartile range) is significant and with zero intercept (Spearman $\rho$ = 0.806, P<0.01) while the slope is non-significantly different from 1.

Figure 4. Biplots of macroalgal $\delta^{15}$N and concentrations of total nitrate (a) and ammonium (b) or $\delta^{15}$N in total nitrate (c) and ammonium (d). None of the relationships is significant (Spearman $\rho$, P>0.05).

Figure 5. Variability of $\delta^{15}$N in macroalgae (a) or total nitrate (b, $\mu$M) with the relative distance of sampling locations to the River Miño discharge point (see Fig. 1). The regression lines for *Ascophyllum nodosum* (Spearman $\rho$ = -0.855, P<0.01) and *Fucus vesiculosus* (Spearman $\rho$ = -0.590, P<0.01) are indicated. Outliers of $\delta^{15}$N (>1.5 times the
interquartile range and not used in the estimation of regression lines) are enclosed in circles (a) while the corresponding inorganic nitrogen concentrations are shown as open dots (b).

Figure 6. Contribution of distance to the reference point (as covariable) and human population (as fixed factor with two levels: larger and smaller than \(15 \times 10^3\) inhabitants, respectively) to the variance of \(\delta^{15}\)N in \(Fucus vesiculosus\) and \(Asphorallum nodosum\). The error term includes the remaining variability not accounted for by all other components. The outliers in Fig. 5 were not included in the analysis (ANOVA, \(P<0.05\) for all components).

Figure 7. Variability of \(\delta^{15}\)N in \(Fucus vesiculosus\) (a) and \(Asphorallum nodosum\) (b) with the size of the human population in the watershed. The curves are polynomial (a) or lineal (b) fits and 95% confidence limits only intended for descriptive purposes. Isotopic values were corrected for the geographic variability using the equations in Table 2. Open symbols indicate outliers (>1.5 times the interquartile range) not used to fit the curves.

Figure 8. Box and whisker plots of \(\delta^{15}\)N in \(Fucus vesiculosus\) (a) and \(Asphorallum nodosum\) (b) grouped according to the size of the human population in the watershed. The differences between classes are significant for both species (Kruskal-Wallis test, \(P<0.05\)).
Table 1. Mean (±se) values of total nitrate (NO$_3^-$+NO$_2^-$) and ammonium (NH$_4^+$) concentrations and δ$^{15}$N in water and macrophyte samples at the sampling sites. Salinity (S) and the number of inhabitants in the watershed (population) are also indicated. Code is the number of each site in Fig. 1.

<table>
<thead>
<tr>
<th>Code</th>
<th>Site</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Date</th>
<th>Population</th>
<th>S</th>
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<th>NH$_4^+$</th>
<th>NO$_3^-$ + NO$_2^-$</th>
<th>NH$_4^+$</th>
<th>A. nodosum</th>
<th>F. vesiculosus</th>
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Table 2. Linear regression parameters ($\delta^{15}N = a + b \text{ distance}$) of the variation of $\delta^{15}N$ in *Fucus vesiculosus* and *Ascophyllum nodosum* with the distance in km to the River Miño. P: significance, n: number of data points, se: standard error. The outliers in Fig. 5 were excluded from the estimation.

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<th>species</th>
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<th>$b \pm se$</th>
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<th>P</th>
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Figure 1. Location of sampling sites along NW Spain. Three environment types representing coastal sites in large rias (I), sites in or near middle rias (II) and mostly open sea sites in the northern coast (III) were considered. The arrow indicates the River Miño discharge point used as the southernmost reference point to compute intersite distances in this study.
Figure 2. Linear relationships between ammonium (NH$_4^+$, black squares) or total nitrate (NO$_3^-$+NO$_2^-$, gray circles) and salinity in water from the sampling sites. The point encircled was an outlier not used in the estimation of the regression line (Spearman $\rho = -0.666$, $P<0.01$).
Figure 3. Relationship between stable isotope composition of *A. nodosum* and *F. vesiculosus* sampled at the same locations. The regression line computed without the outlier (open circle) is significant and with zero intercept (Spearman $\rho = 0.806$, $P<0.01$) but the slope is non significantly different from 1.
Figure 4. Biplots of macroalgal $\delta^{15}$N and concentrations of total nitrate (a) and ammonium (b) or $\delta^{15}$N in total nitrate (c) and ammonium (d). None of the relationships is significant (Spearman $\rho$, $P>0.05$).
Figure 5 Variability of $\delta^{15}$N in macroalgae (a) or total nitrate (b, µM) with the relative distance of sampling locations to the River Miño discharge point (see Fig. 1). The regression lines for *A. nodosum* (Spearman $\rho = -0.855$, $P<0.01$) and *F. vesiculosus* ($\rho = -0.590$, $P<0.01$) are indicated. Outliers of (not used in the estimation of regression lines) are enclosed in circles (a) or shown as open dots (b).
Figure 6. Contribution of distance to the reference point (as covariable) and human population (as fixed factor with two levels: larger and smaller than $15 \times 10^3$ inhabitants, respectively) to the variance of $\delta^{15}\text{N}$ in \textit{F. vesiculosus} and \textit{A. nodosum}. The error term includes the remaining variability not accounted for by all other components. The outliers in Fig. 5 were not included in the analysis (ANOVA, P<0.05 for all components).
Figure 7. Variability of $\delta^{15}$N in *F. vesiculosus* (a) and *A. nodosum* (b) with the size of the human population in the watershed. The curves are polynomial (a) or lineal (b) fits and 95% confidence limits only intended for descriptive purposes. Isotopic values were corrected for the geographic variability using the equations in Table 2. Open symbols indicate outliers (>1.5 times the interquartile range) not used to fit the curves.
Figure 8. Box and whisker plots of δ¹⁵N in *F. vesiculosus* (a) and *A. nodosum* (b) grouped according the size of the human population in the watershed. The differences between classes are significant for both species (Kruskal-Wallis test, P<0.05).