

TESE DE DOUTORAMENTO

**BROWN ALGAE AS HEAVY METALS
AND NITROGEN BIOMONITORS OF
COASTAL AREAS: A REVIEW OF
PROTOCOLS**

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ESCOLA DE DOUTORAMENTO INTERNACIONAL
PROGRAMA DE DOUTORAMENTO EN MEDIO AMBIENTE E RECURSOS NATURAIS

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Brown algae as heavy metals and nitrogen biomonitors of coastal areas: a review of protocols

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Brown algae as heavy metals and nitrogen biomonitors of coastal areas: a review of protocols

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The doctoral candidate declares no conflicts of interest related to her Thesis.

The articles presented in this Thesis have the signed consent of all authors who have participated in them.





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General Introduction





The 1982 United Nations Convention on the Law of the Sea defined marine pollution as “*the introduction by man, directly or indirectly, of substances or energy into the marine environment, resulting in harm to living resources, hazards to human health, hindrances to marine activities, impairment of the quality of seawater and reduction of amenities*” (UNCLOS, 1982; NEPA, 2008). According to UNESCO (2017), the most important water contaminants originated from human activities are microbial pathogens, nutrients, pesticides, heavy metals, and persistent organic compounds. Over 80% of all these contaminants originates from industrial, agricultural and urban land-based sources (Beiras, 2018; WWF, 2018). Most environmental contamination by metals result from anthropogenic activities such as mining and smelting operations, industrial production and use, and domestic and agricultural use of metals and metal-containing compounds (Goyer & Clarkson, 2001; Bradl, 2005).

As pollution results in the steady degradation of coastal and marine ecosystems, monitoring and assessment of the quality of the marine environment has become a global priority, with legislative measures and strategies to mitigate the impact of pollution and to protect and conserve ecosystems and biological diversity, e.g. The European Water and Marine Framework Directives (WFD, 2000/60/EC; MSFD, 2008/56/EC). United Nations Environment coordinates, since its inception 40 years ago, the Regional Seas Programme, the first global approach to the protection of the coastal and marine environment, including more than 143 countries from all over the world. Today, there are 18 Regional Seas Conventions and Action Plans for the sustainable management and use of the marine and coastal environment. Under the auspices of the European Union, Convention for the Protection of the Marine Environment of the North-East Atlantic, celebrated in 1992, established the OSPAR Commission, who created a coordinated environmental monitoring programme to develop periodic and long-term assessments of hazardous substances in Europe. In addition to hydromorphological controls, the levels of pollutants in these programmes are monitored in seawater, sediments and biota (including fishes, shellfish and seabirds) for an integrated diagnostic

approach. Heavy metals as Cd, Cu, Hg, Pb and Zn, which are persistent, toxic, and liable to bioaccumulate in organisms and transfer to the trophic chains, are also included to provide a more realistic estimate of the impact of these bioavailable elements in the aquatic environment.

Although the analysis of marine macroalgae is not required under the current legislation, these organisms have been widely used as biomonitors of marine pollution (including heavy metals, organic pollutants or radioactive elements among others) since the middle of the 20th century. Brown algae have demonstrated to strongly bind a variety of metal ions, making them one of the most widely used bioabsorbents (Volesky & Holan, 1995; Davis et al., 2003). Species such as *Fucus vesiculosus*, *F. spiralis* or *Ascophyllum nodosum* have been routinely used in metal pollution monitoring programmes around the world (García-Seoane et al., 2018a,b).

The use of macroalgae offers important advantages for effective biomonitoring, when compared to other marine organisms included in some programmes, as e.g. fishes or invertebrates: i) they are sessile, easily identified, collected and transplanted, ii) they are widely distributed and available all year round, iii) they have a long life span, iv) they present wide ranges of environmental tolerance, surviving in highly adverse and polluted environmental conditions, v) the concentrations of pollutants in their tissues are strongly correlated with the concentration in seawater and sediments, etc. (e.g. Haug et al., 1974; Phillips, 1980, 1990).

One of the main reasons for the non-inclusion of macroalgae in environmental monitoring programmes may be the lack of standardized protocols describing how this technique needs to be applied. As it will be pointed out in Chapters I and VI of this dissertation, researchers are currently using a wide variety of protocols differing in aspects such as the species used, or the way in which the algae are collected, processed and analyzed. These variations obey mostly to practical and economic reasons rather than scientific criteria. The absence of protocols based on such criteria limits the interpretation of the results obtained and hampers the comparison of results between studies. Additionally, the scarcity of studies addressing methodological issues also reveals a general lack of concern for

the standardization of the protocol. For this reason, in this dissertation we also addressed some basic aspects of the methodology of the technique to contribute to establish the use of macroalgae in extensive monitoring studies regulated by legislation.

The structure of this Thesis responds to the research gaps identified in the previous paragraph. First of all, we performed a literature review of all the studies using macroalgae as biomonitors of marine pollution, including those using both passive (collection of specimens growing naturally in the area of interest) and active biomonitoring (transplanting individuals from their natural areas to the study sites). Based on these reviews, we proposed a more standardized protocol that served as the basis for the other studies included in this Research Thesis. Some of the most important methodological aspects of the technique for which there was no consensus recommendation, or which have never been studied, were addressed by means of experimental studies. Because the use of native algae has been preferred over the use of transplanted algae, our research efforts focused on optimizing some methodological aspects of the passive biomonitoring techniques: i) number of subsamples and subsample collection strategy to adequately represent the intra-site variability in concentrations (Chapter III), ii) number of samples to significantly differentiate the levels of pollution between sampling sites (Chapter IV), iii) collection period of samples to better represent temporal variation in concentrations (Chapter V), and iv) selection of material for analysis in order to minimize differences in concentrations resulting from tissue variation (Chapter V). In addition, the suitability of macroalgae for assessing natural levels of pollution in the marine environment and detecting sources of pollution was studied in Chapter II. Finally, a study using transplantation techniques was carried out in Chapter VII to assess the possible variation in the uptake capacity of algae under chronic metal exposure.

The species selected for carrying out the experimental studies that make up this Doctoral Thesis is *Fucus vesiculosus* (Linnaeus 1753) because: i) it is the species of brown algae most widely studied in biomonitoring of water quality (García-Seoane et al., 2018b), ii) it meets the characteristics to be a

good biomonitor (Martin et al., 1997), and iii) it is widely spread throughout the Galician coast. The bladder-wrack, as it is commonly known, is a perennial brown macroalgae that belongs to the Phylum Ochrophyta, Class Phaeophyceae, Order Fucales, and Genus *Fucus* (Strasburger et al., 2008). The etymology '*vesiculosus*' derives from the characteristic air bladders on both sides of midrib that confer buoyancy to the thalli (Fig. 1B). Although this is the main distinctive feature of the species, bladderless forms have also been described on more wave exposed shores, even in the Galician coast (Pazó & Romarís, 1979; Bárbara et al., 1995). The species is widely distributed in cold waters at both sides of the Atlantic Ocean, frequent on the Atlantic coasts of Europe (North Sea, Baltic Sea, Greenland, Azores, Canary Islands and Madeira), and North America (from Ellesmere Island and Hudson Bay to North Carolina), but absent in the southern hemisphere (Fig. 1A). *Fucus vesiculosus* is common on semi-exposed or sheltered shores, and dominant in the mid-intertidal on rocky shores, often with *A. nodosum*, and occupying a position in the intertidal zonation below the populations of *F. spiralis*, and in a zone further up from *F. serratus* (Carlson, 1991; Bárbara et al., 1995).

As regards its morphology, this fucoid is characterized by its flattened, and pseudo-dichotomously branched thallus with apical growth crisscrossed by a midrib. The thallus can reach 100 cm in length and it is fixed to the rocky substrate by means of a conical disc or holdfast (van den Hoek et al., 1995) (Fig. 1B). As all members of the genus *Fucus*, the life cycle of *F. vesiculosus* is oogamous diplont with sexual zygotes (South & Whittick, 1987; van den Hoek et al., 1995; Serrão et al., 1999). Unlike other species, like for example *F. spiralis* which is monoecious, *F. vesiculosus* is dioecious, with separate male and female plants. During the fertile season the gametes are produced by meiosis and multiplied by mitosis in the gametangia from male plants (antheridia) and from female plants (oogonia). Antheridia and oogonia are formed in reproductive structures, called receptacles, that develop apically at the tips of the branches (Fig. 1C, 1D). Once mature, haploid gametes (spermatozoids and egg cells) are

released, and diploid zygotes are produced by external fertilization. After fertilization, zygotes attach to the substrate and grow into a new diploid gametophyte (van den Hoek et al., 1995; Serrão et al., 1996; Ladah et al., 2003, 2008). Although sexual reproduction is the most common strategy in fucoids, asexual propagation by clonal reproduction (i.e. detachment of adventitious branches from the parental plant that act as vegetative propagules) has been described in species of the genus *Fucus*, including *F. vesiculosus* (Bergström et al., 2005; Tatarenkov et al., 2005).

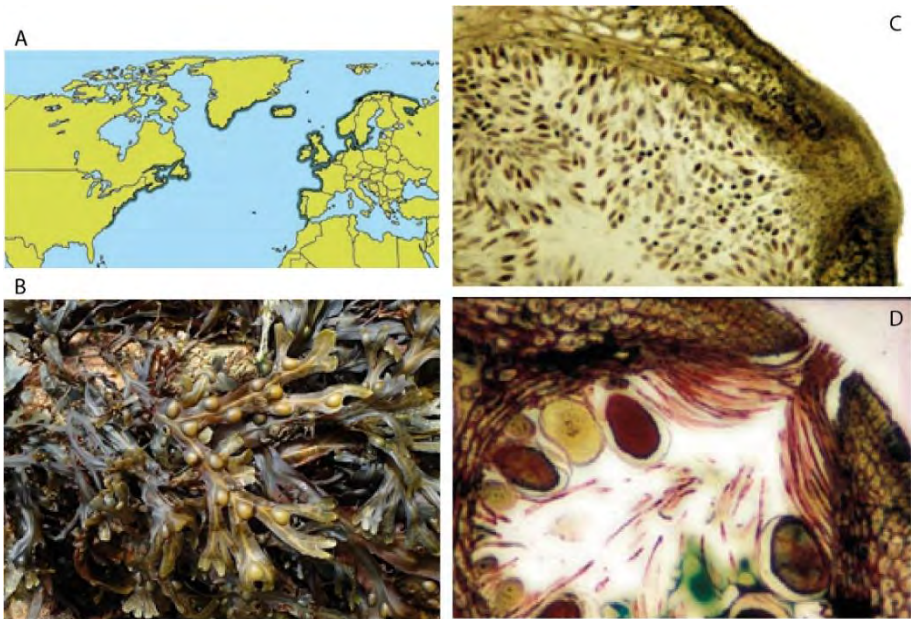


Fig. 1. **A)** Distribution of *Fucus vesiculosus* in the northern hemisphere. **B)** *Fucus vesiculosus* thalli attached to substrate. Detailed images of reproductive structures of *F. vesiculosus*, antheridia (**C**) and oogonia (**D**). Images obtained from www.aphotomarine.com (A), www.bioweb.uwlax.edu (B), and www.seaweed.ie (C, D).



General Objectives





The overall aim of this PhD research was to evaluate the potential of marine macroalgae to monitor heavy metals, metalloids and nitrogen in coastal environments, and to propose a scientifically based protocol for the application of this technique. For this purpose, the information presented in this dissertation is divided into two parts, each consisting of several chapters. Part I (Chapters I to V) encompasses the main body of this work and is dedicated to the use of native macroalgae as biomonitors of pollution (passive monitoring). Part II (Chapters VI and VII) focuses on the use of transplants of macroalgae in biomonitoring studies (active monitoring), as an alternative to the use of native algae. The specific objectives of each chapter are summarized as follows:

1. To carry out a critical review of the methodology used in studies concerning pollution biomonitoring in coastal environments worldwide, and involving the use of native marine macroalgae (passive biomonitoring). The specific aims of this review were: to establish the state of the art of the methodology, to identify its degree of harmonization, and to propose an updated protocol for monitoring pollutants with these organisms (**Chapter I**).

2. To study the potential use of the brown macroalgae *Fucus vesiculosus* to characterize the natural range of variability in the $\delta^{15}\text{N}$ signal, and concentrations of N and Hg in coastal areas not affected by local sources of pollution, and to detect small scale sources of contamination. To achieve this objective, samples of the species were collected in more than 150 sampling sites distributed along the shoreline in 3 study zones affected by different degrees of pollution (**Chapter II**).

3. To propose a sampling strategy for biomonitoring with *F. vesiculosus* that encompasses the intra-site variability (local variability), and accounts for the spatial structure in the concentrations of Al, As, Cd, Co, Cr, Cu, Fe, Hg, Ni, Pb, Zn and N, and in the $\delta^{15}\text{N}$ signal, while optimizing the number of subsamples used to make a composite sample, the sampling effort and costs. For this purpose, 50 subsamples of the algae were collected at random using a regular sampling grid design, in each of 3 sites affected by different levels of pollution (**Chapter III**).

4. To determine the optimal number of samples of *F. vesiculosus* needed to detect statistically significant differences in the mean concentrations of Al, As, Cd, Co, Cr, Cu, Fe, Hg, Ni, Pb, Zn and N, and in the $\delta^{15}\text{N}$ signal between 2 sampling sites affected by different levels of pollution (**Chapter IV**). The experimental set-up is the same as described in the previous chapter.

5. To assess the existence of temporal variation of bioconcentration of heavy metals, metalloids and nitrogen in *F. vesiculosus*, as well as to study the intra-thallus variability in these elements. For this purpose, the concentrations of Al, As, Cd, Co, Cr, Cu, Fe, Hg, Ni, Pb, Zn, N, and the $\delta^{15}\text{N}$ values were determined in samples of the species collected monthly over a period of 3 years at 3 sampling sites. The implications that the temporal (intra-annual/seasonal) and intra-thallus variability in the concentrations may have on the results of biomonitoring studies with algae are discussed. In addition, an appropriate sampling design, representative of the existing variability in the tissue contents of elements, is proposed for application in biomonitoring studies. The growth of the species (in terms of the number of dichotomies formed in the thallus through the year) is also considered, and the implications for interpretation of the results of temporal studies of pollutants are also discussed (**Chapter V**).

6. To evaluate the essential aspects of the methodology and the degree of standardization of the protocols used in studies involving biomonitoring of inorganic pollutants and nutrients in coastal environments worldwide with transplants of marine macroalgae (active biomonitoring). On the basis of the conclusions reached, a scientifically based protocol is proposed for application of the biomonitoring technique with transplanted algae (**Chapter VI**).

7. To compare the accumulation capacity of metals and metalloids in specimens of *F. vesiculosus* growing naturally under different pollution scenarios. Thalli of the species were reciprocally transplanted between 2 polluted and 2 unpolluted sites. The differences in the dynamics of uptake/release rates of Al, As, Cd, Co, Cr, Cu, Fe, Hg, Ni, Pb and Zn were

studied after exposure of the thalli in these environments for 90 days (Chapter VII).





Chapter I





CHAPTER I

Use of macroalgae to biomonitor pollutants in coastal waters: Optimization of the methodology

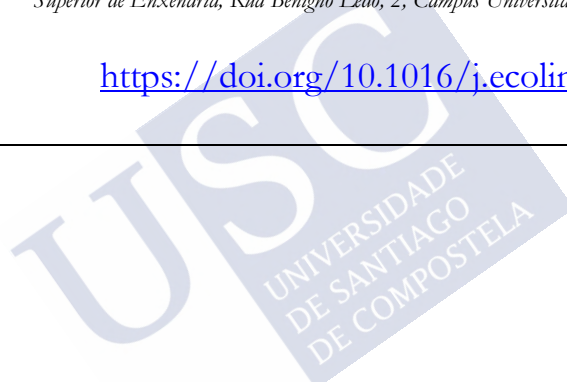
Ecological Indicators, 84 (2018), 710–726

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Chapter II





CHAPTER II

Application of macroalgae analysis to assess the natural variability in selected pollution concentrations (N and Hg), and to detect sources of it in coastal environments

Science of the Total Environment, 650 (2019), 1403–1411

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Chapter III





CHAPTER III

Sampling optimization for biomonitoring metal contamination with marine macroalgae

Environmental Pollution, 255 (2019), 113349

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Chapter IV





CHAPTER IV

Optimal number of *Fucus vesiculosus* subsamples to differentiate between sites affected by distinct levels of heavy metal contamination

Submitted for publication.

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Abstract

The presence of trace elements in marine habitats is a serious environmental problem which increasingly affects ecosystem and human health. The use of macroalgae as contamination biomonitors represents a valuable alternative approach to traditional physicochemical methods. The present study was carried out to determine the optimal number of subsamples of *Fucus vesiculosus* needed to detect statistically significant differences in the mean concentrations of Al, As, Cd, Co Cr, Cu, Fe, Hg, Ni, Pb, Zn, N and $\delta^{15}\text{N}$ between two sampling sites affected by different levels of contamination. For this purpose, we plotted the density distributions of the concentrations of the different elements and examined the local variability at three sites. For sites with mean concentrations differing by more than 30%, 20 subsamples were sufficient to detect significant differences for all of the elements, except Cr. The proposed methodology could be used in other studies in the absence of specific research on each species and region.

Keywords: Contamination monitoring; Heavy metals; Coastal environments; Algae; Local variability; Standardization.

1. Introduction

The use of marine macroalgae to biomonitor contamination levels in aquatic environments was first proposed more than 50 years ago (Phillips, 1977; Bryan et al., 1980; Rainbow & Phillips, 1993). Since then, numerous authors have used the technique to determine the levels of different pollutants in coastal areas around the world: heavy metals (Fuge & James, 1973; Riget et al., 1997; Stengel et al., 2005), non-metallic elements such as N, $\delta^{15}\text{N}$ and P (Fong et al., 2001; Deutsch & Voss, 2006), organic contaminants (Maroli et al., 1993; Net et al., 2015) and even radioactive elements (Wong et al., 1972; Nawakowski et al., 2004).

Many of the biomonitoring studies carried out with macroalgae in coastal environments have involved the use of numerous, widely dispersed sampling sites covering large geographical areas, with the aim of characterizing levels of contamination in the environment (mainly heavy metal contamination). In such studies, small numbers of subsamples of a given species of algae are usually collected at each sampling site and combined to form a composite sample. Fewer than 30 subsamples are generally collected, each consisting of one or several individual thalli: e.g. in previous studies, 8 (Sharp & Bölviken, 1979), 25–30 (Villares et al., 2005) and 30 (Viana et al., 2010) subsamples have been collected per site. Analysis of composite samples enables adequate representation of the intra-site variability in the concentrations of pollutants (García-Seoane et al., 2019), yielding a more accurate picture of the level of contamination in the study area than would be provided by analysis of a single sample per site.

In other studies, small numbers of subsamples of algae (as described previously) are collected and analyzed individually to determine the intra-site variability, information that is lost when bulking a composite sample. In these types of studies the aim is to better characterize each site in order to investigate the differences in metal concentrations between a few locations (e.g. Conti & Cecchetti, 2003; Stengel et al., 2005), or even to

compare the same location at different times (e.g. Miramand & Bentley, 1992; Riget et al., 1995). As already pointed out, the number of subsamples collected is also quite variable, e.g. 5 subsamples of *Ulva* spp. per site (Pereira et al., 2009), 5–6 subsamples of *Sargassum* spp. (Chernova & Sergeeva, 2008), 10 subsamples of *Fucus vesiculosus* (Rainbow et al., 2002), and 15 subsamples of *F. vesiculosus* and *Ascophyllum nodosum* (Rainbow et al., 2011). In this type of study, different statistical tests, often ANOVA, are used to detect differences between two or more sampling sites, based on the concentrations determined in algae growing naturally in the area under study (e.g. Barreiro et al., 2002; Stengel et al., 2004; Rainbow et al., 2011). However, the number of subsamples is usually chosen without any scientific basis, or at least the authors do not provide any justification for their choice (for further details, see García-Seoane et al., 2018). To our knowledge, only one study has investigated the relationship between the number of subsamples collected at a sampling site and the possibility of differentiating it from another study site (Barreiro et al., 1993). Although detecting differences between sites was not the primary objective of the aforementioned study, the authors concluded that 3 or 4 subsamples of *F. ceramoides* per site (each composed by 10 individuals) were sufficient to detect differences of 50% between mean concentrations of Al, Co, Fe, Mn, Ni and Zn.

The number of subsamples collected at each site is an important factor that determines the capacity to differentiate two sites affected by varying degrees of contamination (or the same site over time), in terms of the concentrations of contaminants in the algae. It is possible to estimate the number of subsamples needed to differentiate two sites with the desired statistical power of the test (Zar, 2010), i.e. at a defined probability of rejecting a false null hypothesis (Desu & Raghavarao, 1990; Suresh & Chandrashekhara, 2012). This means that determining the optimal number of subsamples to be collected at each site ensures adequate statistical power to detect any differences. Because the power of the statistical test is positively correlated with the sample size, a larger number of subsamples

guarantees greater power, which ideally should not be less than 80% (Cohen, 1988; Hintze et al., 2008). If the number of subsamples is sufficient to yield good level of statistical power and the test shows no differences between sites, we can reasonably conclude that there are no differences between sites. On the contrary, an inadequate sample size (fewer subsamples than statistically necessary) may lead to unrealistic assumptions being made (Thomas & Juanes, 1996).

As scientific, operational and cost issues go hand-in-hand in biomonitoring studies, determination of the minimum number of subsamples necessary to differentiate between sites is a critical step in achieving scientifically and statistically sound results (Moher et al., 1994). Using an adequate number of subsamples will result in more reliable, valid and generalizable results, and it could also save resources and collection effort. By contrast, if the number of subsamples collected is not sufficient to guarantee a high level of statistical power, the study results will be statistically inconclusive and may lead to failure of the protocol (Suresh & Chandrashekara, 2012).

For all of the above mentioned reasons, we estimated the number of subsamples of the brown algae *F. vesiculosus* required to differentiate, with statistical certainty, between sites on the basis of the concentrations of metal and metalloid pollutants (Al, As, Cd, Co Cr, Cu, Fe, Hg, Ni, Pb and Zn) and also N and $\delta^{15}\text{N}$ levels.

2. Material and methods

The data examined in the present study have been used for other purposes in a previous original publication, and therefore only a brief description of the study area and methods will be presented here (for more details, see García-Seoane et al., 2019).

2.1 Sampling and processing

In July 2016, a study was carried out at three sampling sites (SS) on the NW coast of Spain affected by different levels of contamination. The first two sites (SS1 and SS2) were located in areas affected by low levels of contamination (Cobelo-García & Prego, 2004; Carro et al., 2010), while SS3 was located in an area affected by discharges of urban and industrial origin, resulting in moderate contamination by heavy metals (Prego & Cobelo-García, 2003; Marmolejo-Rodríguez et al., 2007). At each SS, 50 subsamples (subsample = group of 5 thalli collected within a 25 cm radius of a determined point) of the brown macroalgae *Fucus vesiculosus* L. (Class Phaeophyceae) were randomly collected within a regular 1 x 1 m sampling grid. Each subsample was washed in seawater, labelled individually and refrigerated at 5°C until processing (maximum 3 days storage). Growing tips (the youngest and most physiologically active parts) were separated from each thallus with a glass spatula, dried with circulating air at 40°C (72 h) and powdered (Tangential mill, Retsch MM400). Subsamples (each of ca. 7 g dry weight – d.w.) were hermetically stored at room temperature in darkness until chemical analysis.

2.2 Chemical analysis

The tissues were dried again at 40°C for 24 h, and subsamples (1 g d.w.) were digested with HNO₃ (65%) in Teflon vessels in a microwave oven (CEM MDS2100). The concentrations of Al, As, Cd, Co, Cr, Cu, Fe, Ni, Pb and Zn were determined in each subsample by ICP-MS (VARIAN 820-MS ICP quadrupole mass spectrometer). The concentrations of Hg were determined in an elemental analyzer (Milestone DMA80). Each subsample (3±0.1 mg d.w.) was also packed in tin capsules (EuroVector) and processed in an elemental analyser (Carlo Erba Instruments FlashEA1108) coupled to a mass spectrophotometer (ThermoFinnigan MAT253) for determination of the N concentration and δ¹⁵N signature. The analytical procedure was controlled by parallel analysis of analytical

blanks, replicate samples and two certified reference materials (green macroalgae, BCR-279 *Ulva lactuca*, IRMM, Brussels, Belgium; and brown macroalgae, ERM-CD200 *F. vesiculosus*, IRMM, Geel, Belgium), at the same frequency (every 10 samples) for each of the elements determined. The concentrations of all elements were higher than the limit of quantification (LOQ). The mean difference between replicate samples (between 2 and 11%) and the recovery of the reference materials (between 80 and 110%) were generally satisfactory.

2.3 Data analysis

The minimum number of subsamples (n) required to detect significant differences ($p \leq 0.05$) between two SS was estimated by the method recommended by Zar (2010) for normally distributed populations (“Estimation of Required Sample Size for a Two-Sample t test”), where each SS is considered to be a population. For both data sets compared, n can be calculated using the following equation (**Eq. 1**):

$$n \geq \frac{2\sigma_p^2}{\delta^2} (t_{\alpha, \nu} + t_{\beta(1), \nu})^2$$

Where δ is the minimum detectable difference between population means (μ_1 and μ_2); α is the significance level ($\alpha=0.05$); $(1 - \beta)$ is the power of the test; ν is the number of degrees of freedom $((n_1 - 1) + (n_2 - 1))$; and σ_p^2 is the population variance estimated by the pooled variance (**Eq. 2**):

$$\sigma_p^2 = \frac{\sum_{i=1}^n (x_{i1} - \bar{X}_1)^2 + \sum_{i=1}^n (x_{i2} - \bar{X}_2)^2}{\nu_1 + \nu_2}$$

As recommended by Cohen (1988, 1992a, 1992b) for scientific research, a probability of 80% of detecting a genuine difference, i.e. 0.8

statistical power of the test, was considered (hence, a $\beta=0.2$). Thus, when $\delta \geq d$, where d is the difference between population means (μ_1 and μ_2), no significant differences will be detected between the means being compared. By contrast, if $\delta < d$, significant differences will be detected. The calculation is valid for data underlying normal distributions. However, the distributions of the contaminant concentrations (including those of heavy metals) are not usually normally distributed in nature (Olsson & Biegnert, 1997). It is therefore necessary to check the normality of the raw data before carrying out the calculations. The normality of the raw data was then checked by using Lilliefors's modification of the Kolmogorov–Smirnov test. Differences were considered significant at $p \leq 0.05$. When necessary, Box-Cox transformations were applied to normalize those non-normally distributed populations (for all the elements, except N), by choosing a λ value that matched the three SS studied (Box & Cox, 1964). The concentrations of some of the elements (i.e. Fe, Hg and Zn) in the three SS fitted a value of $\lambda=0.5$, and the square root transformation was therefore applied in such cases. For Ni, the best fit value of λ was -0.5 , and an inverted square root transformation was therefore chosen for this element. When the best-fit λ values for a given element did not coincide for the three SS, we applied a square root transformation to maintain uniformity between SS and because this transformation was useful for normalizing the data for most SS. Thus, the mean and variance of each data set for transformed data can be calculated according to each type of transformation. For the square root transformation, $Y = X^{0.5}$, and the mean and variance of X were first calculated as follows (**Eq. 3**):

$$\mu_X = \mu_Y^2 + \sigma_Y^2$$

$$\sigma_X^2 = (2\mu_Y^2 + \sigma_Y^2) * 2\sigma_Y^2$$

Solving these equations yields the mean and variance of Y (μ_Y and σ_Y^2 , respectively) (**Eq. 4**):

$$\mu_Y = \sqrt[4]{\mu_X^2 - \frac{1}{2}\sigma_X^2}$$

$$\sigma_Y^2 = \mu_X - \sqrt{\mu_X^2 - \frac{1}{2}\sigma_X^2}$$

As an explicit expression is not available for the inverted square root transformation, μ_Y and σ_Y^2 were calculated as the mean and variance of the data transformed from the raw variable (then $y_i = x_i^{-0.5}$, **Eqs. 1 and 2**), which although not strictly correct, is more appropriate than using the data from the original variable. All statistical tests and Box-Cox transformations were performed using IBM SPSS Statistics 24 (SPSS Inc., USA).

The density functions for each element were estimated by kernel smoothing, with the Kern-Smooth package (Wand & Ripley, 2006) in R (R development Core Team, 2008). The technique was applied with a Gaussian kernel, the width of which was optimised for each data set by using direct introduction with two levels of functional estimation (Wand & Jones, 1995).

3. Results

The descriptive statistics of the concentrations of the elements determined in the algae collected at the three SS studied are shown in Table 1. In general, contamination levels were higher at SS3 than at SS1 and SS2. As regards the variability of the data, the coefficients of variation and dispersion (median absolute deviation/median) were, in comparison with the other elements, higher for Al, Fe and Pb in all SS, with values of around 40–50%. Additionally, the density distributions for some of the elements and sites studied are shown as an example in Fig. 1. These graphs confirm that the concentrations of most of the elements (other than N) in algae were non-normally distributed at all SS (see Fig. 1 and Table 1). In general, polymodal distributions with positive asymmetry (skewed to the right) were observed (e.g. for $\delta^{15}\text{N}$ in SS1, and Ni in SS2), and in some

cases the skew to the right was so large that there appeared to be some negative asymmetry (e.g. As in SS3). These types of distributions are indicative of the existence of contamination at the SS (Viana et al., 2010). For example, the concentrations of Cr, Fe and Zn include an extreme value 3 times higher than the modal value of the distributions in SS2. Some of these atypical values in the density distributions were identified in the same subsamples for some of the elements determined in the same SS (e.g. Al and Cr in SS1, Fig. 1).

The comparisons between the density distributions of Cu in SS2–SS3 and Zn in SS1–SS2 are shown as examples (see Fig. 1) to illustrate the reasoning behind the calculation of the minimum number of subsamples required to significantly differentiate two sites in terms of metal concentrations (values presented in Table 1). As previously explained, the number of subsamples will be determined by the d value (i.e. the difference between population means μ_1 and μ_2) and by the deviation of each distribution compared (σ). For Cu, the distributions compared are clearly different, with means differing by ca. 50% (high d value), whereas for Zn, the distributions almost overlap and only differ by 5% (low d value). In addition, for Cu the σ values for both distributions (~ 0.8) are lower than those for Zn (4.9 and 8.8). With this information and using the previously defined equations, we found that by collecting a minimum of 3 subsamples from SS2 and another 3 from SS3, we can significantly differentiate ($p \leq 0.05$) between the mean concentrations of Cu at both SS, as the minimum detectable difference between population means, $\delta_3 = 2.22$, is lower than $d = 2.37$. On the contrary, Zn distributions were very variable and the mean values were very similar, so that significant differences would not be detected with 3 subsamples (as $\delta_3 > d$), and a much larger n (119 in each SS) would be required to differentiate between the mean concentrations of Zn at SS1 and SS2 (as $\delta_{119} < d = 1.71$). Thus, the more dissimilar the means ($\mu_2 - \mu_1$) are (i.e. the higher the value of d) and the less variable the data from each distribution are (σ_1 and σ_2) (i.e. the smaller the value of σ), the easier it will be to detect significant differences between

SS (as a small n is required). Likewise, for most of the remaining elements, when pairs of SS with very different means were compared (e.g. Al, As, Fe, Cu, Hg and Pb, for the SS1–SS3 and SS2–SS3 comparisons), the number of subsamples required was small (between 1 and 8), whereas to differentiate between the concentrations for those elements with similar means in the SS compared (e.g. Cr, Cu and Zn in the SS1–SS2 comparison), a larger number of subsamples would be required (more than 400 for Cr and Cu) (Table 1).

Table 1. Descriptive statistics of concentrations ($\mu\text{g g}^{-1}$) of each element determined in 50 subsamples of *Fucus vesiculosus* collected at the sampling sites (SS) under study. Mean values and coefficient of variation (%) are shown in regular typeface for those elements that are normally distributed, and median values and coefficients of dispersion (median absolute deviation/median, %) are shown in italics for those elements and SS that are not normally distributed. The minimum number of subsamples required to differentiate ($p \leq 0.05$) the mean tissue concentrations of the elements studied in *F. vesiculosus* growing at the three SS compared is also shown. Pairs of SS in which the mean concentrations differ by more than 30% are shown in bold type. ^a: ng g^{-1} ; ^b: %; ^c: ‰.

	Sampling sites			Comparisons		
	SS1	SS2	SS3	SS1-SS2	SS1-SS3	SS2-SS3
Al	200 (53)	352 (77)	1437 (50)	24	2	2
As	55.6 (10)	<i>51.3 (11)</i>	28.1 (14)	47	2	1
Cd	0.82 (17)	1.02 (20)	<i>0.47 (9)</i>	32	13	9
Co	0.52 (19)	<i>1.18 (16)</i>	<i>0.99 (10)</i>	3	35	48
Cr	<i>0.31 (24)</i>	<i>0.40 (50)</i>	<i>0.98 (37)</i>	581	29	84
Cu	<i>2.31 (12)</i>	<i>2.53 (17)</i>	4.80 (17)	415	7	3
Fe	<i>191 (35)</i>	299 (58)	1002 (44)	7	1	1
Hg ^a	22.5 (30)	14.5 (20)	59.3 (19)	6	1	1
Ni	1.07 (25)	<i>2.02 (17)</i>	<i>3.75 (6)</i>	9	3	16
Pb	0.40 (41)	<i>0.25 (39)</i>	1.62 (39)	86	8	6
Zn	32.5 (15)	<i>33.1 (10)</i>	<i>46.6 (8)</i>	119	2	4
N ^b	0.40 (11)	0.77 (7)	0.59 (8)	2	4	5
$\delta^{15}\text{N}^c$	1.94 (4)	<i>2.59 (2)</i>	2.81 (4)	2	12	5

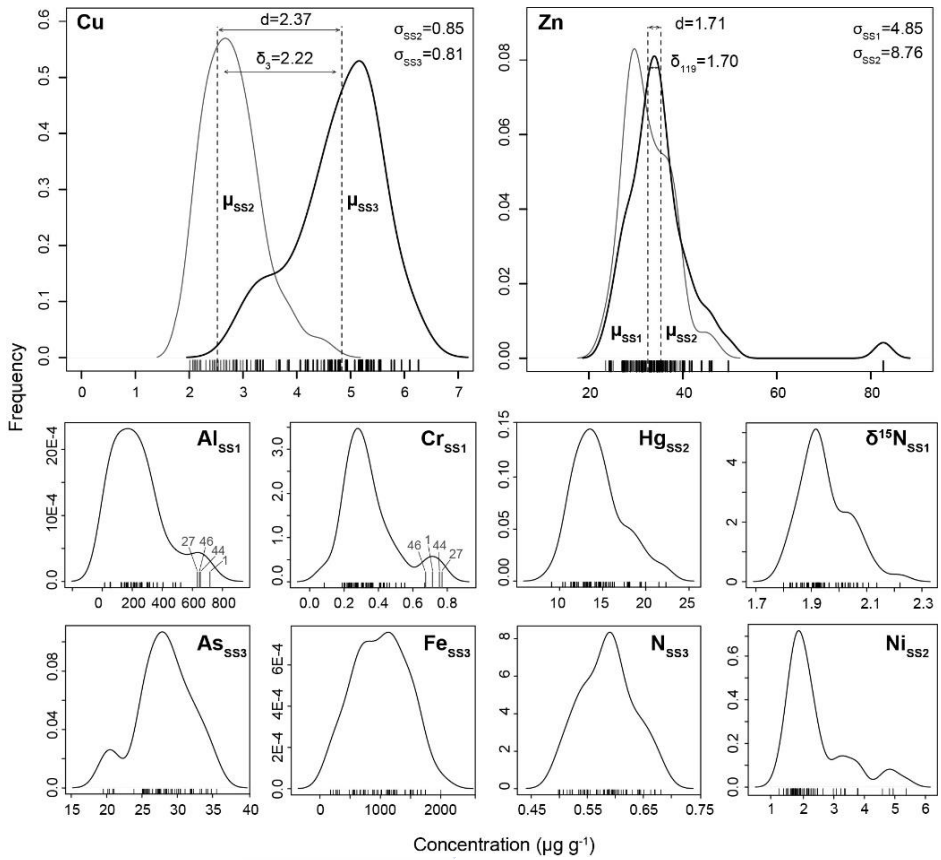


Fig. 1. Density functions for the concentrations of Al, Cr and $\delta^{15}\text{N}$ in sampling site (SS) 1, Hg and Ni in SS2, and As, Fe and N in SS3, determined in the brown algae *Fucus vesiculosus*. Subsamples showing extreme concentrations are highlighted in Al and Cr at SS1 as examples. The upper graphs show the comparisons made between two SS that differ in terms of mean concentrations (μ_1 and μ_2) and deviations (σ_1 and σ_2) for Cu and Zn. The size of each arrow indicates the detectable minimum difference in concentration (δ), and the difference between the mean values (d) of each pair of SS compared, for $n=3$ and $n=119$ for Cu and Zn respectively. Thin and thick lines represent the values of both populations compared. For each kernel, vertical lines along the X-axis correspond to the individual values of the subsamples collected at each SS ($n=50$). The concentrations are expressed in $\mu\text{g g}^{-1}$, except for Hg (ng g^{-1}), N ($\%$) and $\delta^{15}\text{N}$ ($\%$).

4. Discussion

The variability in the concentrations of elements in tissues of algae largely determines the number of subsamples required to differentiate between two SS separated in space, or to differentiate temporal changes in the same SS (e.g. in time trend studies). This variability can be generated by factors that locally affect the accumulation of elements in algae (i.e. factors causing intra-SS variability). Some of these factors have been investigated in detail (e.g. Nickless et al., 1972; Martin et al. 1997; García-Seoane et al., 2019) and are primarily related to the effect of the vertical position of the algae on the shoreline and to variations in environmental conditions (Fuge & James, 1974; Villares et al., 2002). In view of this, and by using the method recommended by Zar (2010), we have verified that in order to detect significant differences between SS in which the mean concentrations of the element under consideration are very different, very few subsamples are needed, e.g. 3 for Cu (mean concentrations differing more than 50% between SS compared, Fig. 1), see Table 1. These results are consistent with those reported by Barreiro et al. (1993) for *F. ceranoides* and *F. vesiculosus* collected in the same region, in which 3–4 subsamples were considered sufficient to detect significant differences between the mean concentrations of Al, Fe, Co, Mn, Ni and Zn, which differed by 50% in the different sites. On the other hand, we observed that the number of subsamples required increases substantially when the differences between the means are small, leading to impractical sample sizes, e.g. 199 subsamples in the case of Zn (Fig. 1 and Table 1). In light of this, we estimated the minimum number of subsamples that would yield the greatest number of significant differences between SS. We found that collection of 20 subsamples at each SS was sufficient to detect significant differences ($p \leq 0.05$) for all the elements whose concentrations differed by more than 30% between the SS compared (except for Cr for SS1–SS3 and SS2–SS3 comparisons, Table 1). For most of these elements (e.g. Al, As, Cu, Fe and Hg), a smaller number (<10 subsamples) would also have been sufficient.

These results (in terms of the number of subsamples required to differentiate SS) are only strictly applicable to studies using *F. vesiculosus* and carried out in the same region, because as mentioned above, the number of subsamples will depend on the existing local variability in the SS and on the similarity or divergence in the level of contamination between the SS compared. Thus, as the vast majority of studies using this technique to compare heavy metal or nutrient levels between SS do not justify the sample size chosen, and most collect a small number of subsamples at each SS (García-Seoane et al., 2018), the results of such studies could be questioned. In particular, in those cases where no significant differences were found on comparing contamination levels between SS, this may be due to the fact that the number of subsamples collected was not sufficiently representative of the high variability existing. For example, Riget et al. (1997) examined differences in element concentrations between different locations by collecting 5 subsamples of each species (*F. vesiculosus*, *F. distichus*, and *A. nodosum*) per site. However, a two-way ANOVA test revealed that the concentrations of the elements displaying the highest levels of natural variability (i.e. Cr and Pb), did not differ significantly between any of the 4 locations compared. According to our estimations, the concentrations of Cr and Pb in *F. vesiculosus* are highly variable (Table 1), and at least for this species, 5 subsamples were not sufficient to differentiate the SS with certainty. We estimated that at least 30 subsamples would be needed to differentiate the sites on the basis of Cr concentrations, and between 6 and 86 subsamples would be required to the same for Pb (Table 1). Likewise, Rainbow et al. (2002) reported no significant differences (Tukey's post-hoc test) between SS with similar mean concentrations of elements such as Pb and Zn, when 10 subsamples of *F. vesiculosus* were collected in each SS. According to our estimations (Table 1), >80 subsamples may have been necessary to detect differences in Pb and Zn concentrations between SS.

5. Conclusions

Although the technique for monitoring contamination with macroalgae is still far from being standardized, one aspect of the sampling protocol has been defined for monitoring heavy metal contamination in coastal waters. Despite the high variability in the concentrations of the different elements (especially Al, Fe and Pb) determined in the subsamples of *F. vesiculosus*, we were able to establish the minimum number of subsamples that should be collected in each SS to allow robust differentiation between SS affected by different levels of metal contamination (ca. 20 subsamples). From a practical point of view, this number of subsamples can easily be collected during sampling surveys.

Although the number of subsamples was estimated using *F. vesiculosus* and applying our definition of a subsample (see Material and methods), our recommendation is valid for studies carried out in other regions and for other algal species, in the absence of new studies under different settings. Finally, in order to improve the results of the studies and increase the reliability and comparability of the data, further research is needed to support and revise the current methodology for using algae to monitor contamination levels.

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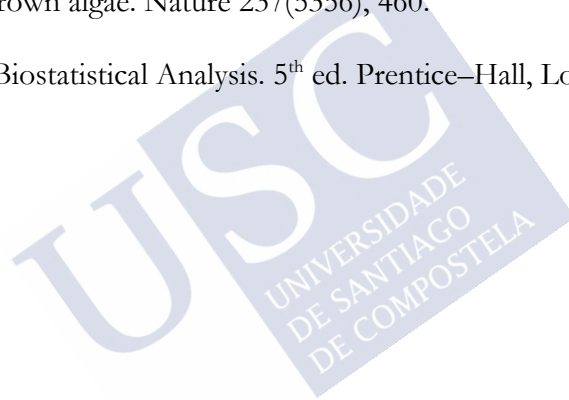
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Chapter V



CHAPTER V

Analysis of temporal and intra-thallus variability of nitrogen and trace elements in *Fucus vesiculosus*: sampling protocol optimization for biomonitoring

In preparation.

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Abstract

Many studies support the use of macroalgae as bioindicators of metal contamination in the marine environment. However, the integration of these organisms in monitoring plans requires for an efficient and effective sampling protocol to avoid misleading conclusions. Factors as the season of collection and the part of the algal thallus selected for chemical analysis should be carefully considered when algae are used for monitoring purposes. The present study first approached the existence of intra-annual/seasonal patterns of bioconcentration of Al, As, Cd, Co, Cr, Cu, Fe, Hg, Ni, Pb, Zn, N and $\delta^{15}\text{N}$ in *Fucus vesiculosus* by means of time series analysis. Samples of this species were collected monthly at 3 sites for 3 years and the concentrations of these elements determined in different dichotomous sections of the thallus. Applying autocorrelations, the same cyclic seasonal pattern in the concentrations was reported every 12 months in all dichotomies over the years, with concentrations increasing at the end of winter and decreasing at the end of summer months. This pattern was especially robust for Cd, Cu, Zn and N. Intra-thallus variation in the concentrations was observed in all sites for most elements: Al, Co, Fe, Ni, Pb and Zn showed an increasing trend with the age of the dichotomy, while As, Cd, Hg, N and $\delta^{15}\text{N}$

exhibited a decrease in concentrations from the youngest to the oldest dichotomies. To minimize the effect of intra-annual variability, a sampling strategy consisting of collecting subsamples 2 times per year separated by 6 months and combined in a composite sample was recommended for biomonitoring purposes with this species. To minimize the effect of intra-thallus variability, it is proposed to make composite samples of the three apical dichotomies of thallus.

Keywords: Aquatic contamination; Biomonitoring; Seasonality; Intra-thallus variability; Algae; Metals.



1. Introduction

From its inception as useful environmental tools in biomonitoring marine pollution in the early 1950s (Wort, 1955; Black & Mitchell, 1952), macroalgae have become one of the most commonly used bioindicators worldwide (Malea & Kevrekidis, 2014; Bonanno & Orlando-Bonaca, 2018; García-Seoane et al., 2018). However, the methods implemented in this type of study have not been properly addressed, and many basic aspects of the technique have not been taken into consideration despite being issues that affect the interpretation of the results obtained.

One such aspect is the temporal representativeness of the concentrations of pollutants in the algae collected in a given area. Temporal variability (intra-annual) in element concentrations in algae must be characterized, otherwise samples collected within the same sites in different months/seasons will not be comparable, and inter-study comparisons will be also restricted. Although sample collection is usually limited to a particular time of the year, typically in summer to promote good sampling conditions (García-Seoane et al., 2018), if the concentrations of elements in algae vary over time, the values obtained making a single sampling survey may not well represent the annual mean value within the site, but an over or underestimation of it. Previous studies in which samplings were carried out over a minimum period of one year covering all seasons, revealed intra-annual variability in more than 75% of the elements considered (García-Seoane et al., 2018). For example, Ferreira & Oliveira (1988) found coefficients of variation (CV) for Hg of 45% in samples of the brown algae *Fucus vesiculosus* collected throughout a year, and similarly, Villares et al. (2013) found annual CV of 17%, 25%, 32%, 38% and 133% for Mn, N, Fe, Zn and Cu respectively for the same species. These variations in the concentrations of elements in algae have been attributed to changes in environmental factors (Stoeppler et al., 1986; Haroon et al., 1995), although most authors think that biological factors, such as metabolism, reproduction and/or growth, constitute the most determinant factors of the temporal variability (Rao & Indusekhar, 1989;

Malea, 1995; Wright & Mason, 1999). Several authors have recommended that to minimize the effect of this variability, various samples should be collected during the year in each site, commonly with monthly samplings, and integrated in a single composite sample, a strategy also known as “Time-bulking” of samples (Phillips & Segar, 1986; Stoepler et al., 1986). The main problem of this approach is that the greater the magnitude of the intra-annual variability (due or not to seasonality), the largest the number of samplings would need to be performed, involving a huge sampling and economic effort. Thus, it is necessary to characterize the temporal variability of the concentrations of elements in algae in order to establish the sampling frequency required to yield representative values of annual concentrations (Malea et al., 2015; García-Seoane et al., 2018).

Along with the intra-annual variability, other aspect of the biomonitoring technique with macroalgae that needs to be closely investigated in order to yield representative and comparable data among studies is the intra-thallus variability (Riget et al., 1997; Burger et al., 2007; Sáez et al., 2012). Several studies have been reported to show differences in metal concentrations between different parts of the algal thalli, especially in fucoid species (García-Seoane et al., 2018), differences mainly related to the age and physiological condition of the tissues (Favero & Frigo, 2002; Savage & Elmgren, 2004). In consequence, selection of the part of the algae used as biomonitor should be previously standardized for the results to be consistent across studies and not draw erroneous conclusions (Sáez et al., 2012). In addition, it is still not known whether there is any relationship between intra-thallus and intra-annual variability.

The present study aims to investigate the existence of temporal variation patterns of intra-annual bioconcentration of nitrogen, $\delta^{15}\text{N}$ and trace elements in *F. vesiculosus* along with the intra-thallus variability in the concentrations of these elements over a three-year period in three sampling sites. The possible implications that the existence of temporal and intra-thallus variability in the tissue contents of these elements would have on the interpretation of results in biomonitoring studies will be discussed, and an appropriate sampling design for routine use in

biomonitoring programmes with macroalgae, representative of the intra-annual and intra-thallus variability, will be proposed. In addition, the periodicity in the occurrence of new dichotomies in the thallus of this species and their implications in the interpretation of the results of temporality studies of pollutants will also be discussed.

2. Material and methods

2.1 Sampling

Samples of the brown seaweed *Fucus vesiculosus* L. (Class Phaeophyceae) were collected monthly from three sampling stations (SS) located in the coast of Galicia (NW Spain) from November 2015 to November 2018. The SS were located far from point sources of pollution, such as port infrastructures or submarine outfalls. The first site (SS1) was located in the Ría de Ferrol (centered in $X = 557811$, $Y = 4812476$; UTM 29N ETRS89), considered one of the most contaminated rias by heavy metals in the coast of Galicia (Cobelo-García & Prego, 2004). The second site (SS2) was selected within the Ría de Muros e Noia ($X = 506599$, $Y = 4737191$), an area with scarce industrial activity. The last site (SS3) was located in the Ría de Pontevedra ($X = 523837$, $Y = 4697147$), characterized by the presence of a chlor-alkali industry and several small cities in its margins. In each SS, 30 subsamples consisted of individual thalli with similar size attached to rocks, were collected within three 50 m bands parallel to the coastline (10 subsamples per band), and combined into a single composite sample to achieve greater representativeness of the intra-SS variability in the concentrations. A more detailed description of the sampling protocol, washing and sample processing can be found in García-Seoane et al. (2019).

2.2 Sample processing

Samples were manually cleaned by removing any adhering material and discarding receptacles, damaged or old tissues and sections heavily

affected by epiphytes (García-Seoane et al., 2018). Consecutive dichotomous sections of the seaweed thallus were separated at the base of every pair of air bladders underneath the angle formed by each fork with a glass spatula, as illustrated in Fig. 1, following the method used in previous studies (e.g. Savage & Elmgren, 2004, Stengel et al., 2005; Carballeira et al., 2014). The three most apical dichotomies were separated during the first year under study. One more consecutive dichotomy (4th dichotomy) was separated the next year. The same procedure was followed over the third year, with the 5th dichotomy being included, making a total of 5 dichotomies at the end of the 3 years of survey. Prior to analysis, each dichotomy was individually dried in a forced air oven at 40°C (72 h), then homogenized in a tangential mixer mill with zirconium oxide grinding vessels (Retsch MM400), and weighed on a precision balance (Mettler ToledoXP26). Dried dichotomies (between ca. 2 and 8 g dry weight, d.w., each) were then stored at room temperature in hermetically sealed vials until chemical analysis.

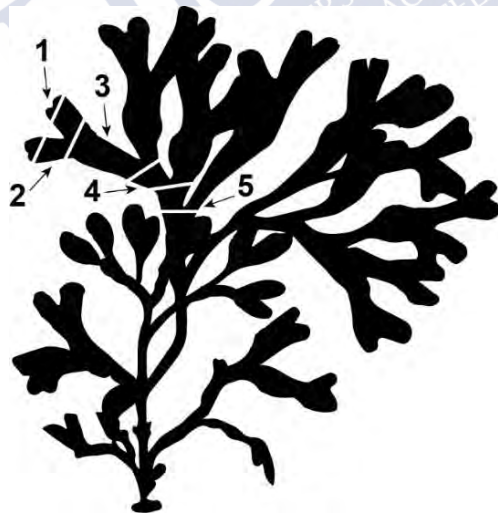


Fig. 1. Diagram showing the different sections (from 1st–apical to 5th–basal) selected over the three-year survey in the dichotomously divided thallus of *Fucus vesiculosus*.

2.3. Growth measurements

Fucus vesiculosus is characterized by apical growth and pseudo-dichotomous branching of the thallus. The thallus grows forming dichotomous ramifications, leading to a parallel although unequal development of the branches (Hoek et al., 1995). Several authors have suggested that the branches in this species are dichotomously divided once a year, which has led to the interpretation that each dichotomy corresponds to an annual growth segment (e.g. Carlson, 1991; Savage & Elmgren, 2004; Stengel et al., 2005; Carballeira et al., 2014). However, during past field work campaigns, the authors have observed that the frequency of the thallus forking is presumably higher than the previously described in these studies. Because we are unaware of any previous studies which addresses the growth periodicity of *F. vesiculosus* (in terms of number of dichotomies formed each year), we believe that it is convenient to assess whether each dichotomy truly corresponds to an age cohort or if several dichotomous divisions occur consecutively during the same year.

To study growth of *F. vesiculosus*, a group of 30 thalli was randomly selected within each SS and individually labeled with coded plastic tags in the basal section of the thallus. The number of new dichotomies formed and the size growth were periodically monitored by taking photographs of each thallus against a background of graph paper. Growth monitoring was carried out every 3 months for a period of 9 months (January 2019 – October 2019). All individuals were measured to the nearest mm from the base of the holdfast to the tip of the longest frond.

2.4 Chemical analysis

Before analysis, samples were dried again at 40°C in a forced air oven. The mineralization of the samples (1 g d.w.) was performed in Teflon vessels in a microwave oven (CEM MDS2100) in three successive steps (10 min at 100°C, 7 min at 150°C, 25 min at 190°C), by adding 10 mL of HNO₃ (65%), 2 mL of H₂O₂ (30%) and 2 mL of MilliQ water. The concentrations

of Al, As, Cd, Co, Cr, Cu, Fe, Ni, Pb and Zn were determined by ICP–MS (VARIAN 820-MS ICP quadrupole mass spectrometer) at the Research Support Services Unit from Universidade de Santiago de Compostela. The concentrations of Hg were determined in an elemental analyzer (DMA 80 Milestone). For determination of %N and $\delta^{15}\text{N}$ signal, 3 ± 0.1 mg (d.w.) of sample were packaged in tin capsules (EuroVector) and processed in an elemental analyser (FlashEA1108 Carlo Erba Instruments) coupled to a mass spectrophotometer (MAT253 ThermoFinnigan). Determinations were made at the Unit of Instrumental Techniques of Analysis from Universidade da Coruña.

To ensure the analytical quality of the process, one analytical blank, one replicate sample and two certified reference materials, Sea lettuce–*Ulva lactuca* [BCR–279] and Bladderwrack–*Fucus vesiculosus* [ERM–CD200] (Institute for Reference Materials and Measurements, IRMM, Belgium) were analyzed once every ten samples. In the case of %N, only the reference material BCR–279 was analyzed. The global error associated with the analytical process was usually lower than 6%, except for Hg (around 10%) and Al (around 17%). The percentage of recovery from the reference materials ranged for BCR–279, between 65% (Pb) and 110% (Hg), usually around 85%, and for ERM–CD200, between 65% (Pb) and 122% (Hg), usually around 90%. Determinations were above the corresponding limits of quantification (LOQ), with exception of Ni (in 5% of the cases), Cu (8%), Co (11%), Cd (15%), Al (33%), Pb (44%) and Cr (69%). Data from those SS where element concentrations were below the LOQ in more than 30% of the samples, i.e. from SS1 for Cr, from SS2 for Cr and Pb, and from SS3 for Al, Cr and Pb, were not included in the data treatment.

2.5 Data analysis

2.5.1 *Intra-thallus variability*

Lilliefors modifications of the Kolmogorov–Smirnov test was used to check the normality of the data. Except for N and $\delta^{15}\text{N}$, which concentrations were normally distributed, those elements with non-normal distributions were successfully normalized using Box–Cox transformations: $\log(x)$ transformation for As, Cd, Co, Cu, Fe, Ni and Pb, \sqrt{x} transformation for Al, and $1/\sqrt{x}$ transformation for Hg and Zn. A Three–way ANOVA test was used to test differences between the five dichotomous sections of the thallus and study the interaction between the factors (categorical variables): “Sampling Month * SS * Dichotomy”. The concentrations of the elements were considered as the dependent variables. When there was no interaction between factors and when significant differences were detected ($p \leq 0.05$), a Tukey’s Post–Hoc test was used to identify differences. Spearman’s rank correlation coefficients were also calculated to determine the relationship between the concentrations of the elements and the weight (d.w.) of the dichotomies. Statistical analysis was performed using R-3.4.0 (R development Core Team, 2008).

2.5.2 *Analysis of the structure of the temporal series*

The classical analysis of time series is based on the assumption that the values taken by the variable are the consequence of three components (trend, seasonal and random components) whose combined actuation results in the measured values (Brockwell & Davis, 2002; Chatfield, 2003; Anderson, 2011). To distinguish between trend and seasonal components, trends in raw data were isolated by lineal regression analysis of the time series. A detrending procedure was then applied to remove the trend component from those non-stationary time series (with trend and/or variability changing systematically over time) with significant trends

($p < 0.01$) (Chatfield, 2003; Box et al., 2014). To assess the degree of dependence in observations of the time series, autocorrelograms were calculated from the regression residuals in those detrended series, or directly from raw data in those stationary series (with constant mean and variance over time), for each dichotomy and for each element separately at all SS. The “acf()” function available in R-3.4.0 (Package gstat) was used to plot correlograms (Pebesma, 2004; Gräler et al., 2016). Correlations outside the 95% confidence interval (bounds $\pm 1.96/\sqrt{n}$, where n is the number of lags and 1.96 is the 0.975 quantile of the standard normal distribution) were deemed significant at the 5% significance level (Brockwell & Davis, 2002). In the absence of data for the three years in the 4th and 5th dichotomies, and to get a more consistent temporal analysis, correlograms and time plots were only studied in the three more apical dichotomies.

In order to study the seasonal variation and to identify the dominant periods (or frequencies) of the series, periodic (cyclic) regression models with a sine and a cosine component were fitted (Chatfield, 2003), using the “spectrum” (to calculate spectral density and determine the periods of the series) and “lm” (to illustrate the model estimated) functions available in R-3.4.0 (Package TSA, R development Core Team, 2008) (Cryer & Chan, 2008; Shumway & Stoffer, 2017). A description of the periodic regression model and its parameters can be found in Table S1 (Supplementary Material). Although the terms inside the sine and cosine functions are known, the regression coefficients are automatically estimated by the regression model fitted of each series. The fitting of the models was done by calculating determination coefficients (r^2), considered significant at $p < 0.01$.

3. Results

Annual range of variation in concentrations of the different elements/ $\delta^{15}\text{N}$ signal in the five dichotomies of the thallus at each SS are shown in Table 1. Although $\delta^{15}\text{N}$ signal is not an element *per se*, but is an

isotopic relation of elements, we will refer from now on to all the elements determined (including $\delta^{15}\text{N}$ signal) as “elements”, to simplify the readability throughout the text. Intra-annual variability in the concentrations at each dichotomy was studied by calculating the coefficients of dispersion (COD), as the ratio between the MAD (median absolute deviation) and the data median. Concentrations varied between 3% for $\delta^{15}\text{N}$ at SS1 (third year, 1st dichotomy) and 747% for Cd at SS3 (third year, 3rd dichotomy). The COD usually ranged between 30% and 60% for most of the elements in all the SS, with the exception of N and $\delta^{15}\text{N}$ (usually <20%), and were especially high (>100%) for Al in SS2, As and Cd in all SS, and Co, Cu and Fe in SS3. A general comparison between dichotomies showed that the apical dichotomy was clearly the least variable in SS1 in the three years studied. No differences were apparently observed between dichotomies in the other SS.

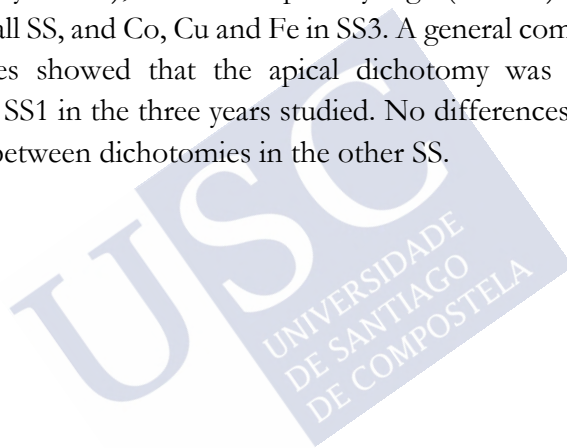


Table 1. Annual range of variation in the concentrations of elements ($\mu\text{g g}^{-1}$, ng g^{-1} , $\%$, $\%$, $\%$) in dichotomies of *Fucus vesiculosus* thalli collected at each sampling site (SS) throughout the sampling period. <LOQ: concentrations below the limits of quantification.

Year	Dichotomy	Al	As	Cd ^a	Co ^a	Cr	Cu	Fe	Hg	Ni ^a	Pb ^a	Zn	Nb	$\delta^{15}\text{N}$, ^c
SS1	1 ^o	12.6–226	35.5–61.5	642–1027	463–1900	<LOQ	2.60–4.06	50.7–187	14.0–35.5	59.1–2826	184–517	21.5–157	0.431–0.924	1.97–3.07
	2 ^o	17.7–281	24.9–35.9	313–779	600–2689	<LOQ	2.28–4.82	52.5–288	15.2–31.9	1080–5401	232–823	27.8–230	0.329–0.826	1.83–2.80
	3 ^o	30.7–310	20.3–27.3	291–836	811–3253	<LOQ	2.61–5.18	65.2–324	35.4–48.9	1648–6702	249–1535	34.5–235	0.286–0.721	1.73–2.58
	2 ^o	43.3–248	61.0–158	1053–2790	254–640	<LOQ	1.91–7.67	66.9–178	7.38–26.8	398–909	226–516	25.7–66.5	0.457–0.952	2.26–3.09
	2 ^o	34.4–174	43.6–102	382–1027	252–888	<LOQ	1.71–8.43	65.9–161	6.39–24.8	522–1542	275–498	30.0–90.1	0.378–0.866	2.02–2.86
	2 ^o	54.6–198	29.1–63.2	260–814	482–1250	<LOQ	1.82–4.67	76.1–186	7.78–28.4	916–2295	330–640	38.0–114	0.312–0.806	1.88–2.63
	2 ^o	70.3–362	22.9–54.6	216–786	633–1573	<LOQ	2.31–10.0	91.1–265	9.39–51.3	1426–4520	428–779	47.9–156	0.268–0.723	1.87–2.63
	3 ^o	47.8–284	88.5–148	1261–3575	347–1057	<LOQ	2.69–7.32	84.5–185	9.89–25.5	513–1243	274–663	25.9–77.4	0.455–1.01	2.14–3.06
	3 ^o	27.7–232	49.3–107	558–1708	355–4209	<LOQ	2.12–11.4	82.5–240	6.08–27.3	567–7923	312–1152	25.4–277	0.364–0.937	1.99–2.82
	3 ^o	62.6–327	29.1–61.5	360–1479	491–4716	<LOQ	2.09–10.0	113–378	7.92–32.6	843–10682	414–1519	32.3–288	0.293–0.885	1.89–2.52
	3 ^o	59.1–352	24.0–50.7	338–1376	568–4592	<LOQ	2.41–9.58	116–415	9.10–31.5	1061–10636	441–1614	41.4–260	0.266–0.921	1.85–2.51
	3 ^o	74.3–418	21.9–144	380–2506	509–4562	<LOQ	3.00–8.29	141–410	12.1–29.63	549–10319	220–1683	40.4–279	0.234–0.769	1.81–2.37
SS2	1 ^o	26.9–296	47.0–70.0	84.8–218	491–9115	<LOQ	2.50–4.49	18.6–139	10.1–27.0	809–2100	<LOQ	10.1–27.0	0.732–1.21	2.53–3.25
	1 ^o	36.5–556	22.4–42.9	91.9–415	771–15127	<LOQ	1.80–4.39	14.8–219	11.2–30.2	528–2029	<LOQ	11.2–30.2	0.663–1.08	2.41–3.05
	3 ^o	57.8–712	18.6–33.3	142–591	1290–16121	<LOQ	2.04–5.47	21.1–207	10.8–21.8	514–1970	<LOQ	10.8–21.8	0.608–0.95	2.56–2.82
	2 ^o	46.3–157	105–181	87.8–145	308–1169	<LOQ	2.26–6.45	21.1–54.1	5.89–41.6	1481–3269	<LOQ	5.89–41.6	0.824–1.11	2.78–3.55
	2 ^o	31.7–135	60.0–132	67.3–126	486–1880	<LOQ	1.47–4.09	19.6–56.2	4.44–24.8	596–1378	<LOQ	4.44–24.8	0.504–1.01	2.36–3.16
	2 ^o	34.0–187	34.3–80.5	80.0–177	705–3027	<LOQ	1.52–4.15	18.7–76.5	4.67–27.0	390–913	<LOQ	4.67–27.0	0.689–0.93	2.56–2.99
	2 ^o	63.5–441	27.2–59.5	308–898	997–4165	<LOQ	1.66–4.52	11.1–34.6	5.89–24.0	1615–5569	<LOQ	23.2–92.0	0.617–0.890	2.52–2.81
	3 ^o	22.5–212	102–162	68.3–147	351–3168	<LOQ	2.46–5.88	26.0–71.3	7.97–20.9	1407–3936	<LOQ	7.97–20.9	0.800–1.18	3.01–3.57
	3 ^o	2.16–217	36.0–116	41.0–128	86.5–4684	<LOQ	1.36–3.57	19.5–91.1	4.61–11.7	343–1754	<LOQ	4.61–11.7	0.456–1.09	2.69–3.21
	3 ^o	12.0–163	32.3–61.5	84.3–184	734–7456	<LOQ	1.36–3.14	24.9–122	4.35–19.2	426–1474	<LOQ	4.35–19.2	0.640–1.02	2.68–3.15
	3 ^o	28.1–242	24.2–42.5	360–1422	998–8922	<LOQ	1.32–3.21	11.1–269	6.17–20.4	1757–8865	<LOQ	23.8–141	0.563–1.38	2.57–3.05
	3 ^o	61.8–421	22.3–38.1	385–1382	1352–9657	<LOQ	1.73–3.74	152–403	6.76–12.4	2684–11508	<LOQ	26.6–148	0.537–0.872	2.48–3.05
SS3	1 ^o	<LOQ	22.2–47.6	29.1–148	190–2016	<LOQ	0.910–3.52	16.0–124	15.8–43.4	285–765	<LOQ	15.8–43.4	0.511–1.01	1.99–2.94
	1 ^o	<LOQ	16.5–22.7	39.3–187	292–2178	<LOQ	0.872–3.64	21.3–139	12.7–36.5	192–728	<LOQ	12.7–36.5	0.485–0.810	1.78–2.60
	1 ^o	<LOQ	14.9–26.1	43.3–245	318–2208	<LOQ	1.13–3.66	29.1–116	10.3–31.0	171–817	<LOQ	10.3–31.0	0.471–0.728	1.60–2.40
	2 ^o	<LOQ	48.0–132	34.2–158	31.4–528	<LOQ	1.56–3.80	19.5–50.1	13.5–42.1	746–1625	<LOQ	13.5–42.1	0.501–1.00	2.66–3.47
	2 ^o	<LOQ	28.8–83.5	34.8–124	21.3–764	<LOQ	1.23–3.27	17.6–48.9	8.98–43.0	245–519	<LOQ	8.98–43.0	0.414–0.996	2.43–3.22
	2 ^o	<LOQ	15.9–48.0	37.9–165	109–1507	<LOQ	0.986–2.31	19.0–73.2	10.3–30.7	152–394	<LOQ	10.3–30.7	0.385–0.850	2.36–3.02
	2 ^o	<LOQ	14.3–34.9	152–398	342–2037	<LOQ	1.14–2.84	44.7–246	12.2–37.2	1262–2785	<LOQ	24.4–88.9	0.361–0.792	2.14–2.89
	3 ^o	<LOQ	44.2–156	26.8–135	55.9–1072	<LOQ	1.32–5.14	17.0–69.8	8.59–29.4	777–2054	<LOQ	8.59–29.4	0.426–1.10	2.39–3.55
	3 ^o	<LOQ	31.5–93.0	32.3–128	84.3–1717	<LOQ	1.08–3.42	20.6–101	5.56–41.3	260–916	<LOQ	5.56–41.3	0.326–1.10	2.27–3.37
	3 ^o	<LOQ	19.0–47.0	46.1–110	133–2393	<LOQ	0.739–3.22	24.7–134	6.78–23.3	167–796	<LOQ	6.78–23.3	0.293–1.02	2.14–3.15
	3 ^o	<LOQ	17.3–29.0	190–687	191–2660	<LOQ	0.731–5.58	55.1–126	6.20–22.9	1596–8466	<LOQ	28.4–141	0.323–0.900	2.18–2.85
	3 ^o	<LOQ	18.6–26.1	232–697	295–3082	<LOQ	0.950–3.61	65.7–156	8.68–31.0	2323–12503	<LOQ	34.8–152	0.330–0.767	1.83–2.54

3.1 Intra-thallus variation

The results show that the concentrations of the different elements investigated were not homogeneously distributed within thalli of *F. vesiculosus*. The Three-way ANOVA test (Table S2, Suppl. Mat.) showed significant interaction ($p \leq 0.05$) between factors only in the case “Sampling Month * SS” for Al, Cd, Cu, Fe, Zn, N and $\delta^{15}\text{N}$. However, significant differences ($p \leq 0.05$) between dichotomies were observed for all the elements studied, regardless of the month and the SS in which the samples were collected. Two patterns of variation in concentrations across dichotomies were identified depending on the elements considered (see boxplots in Fig. S1, Suppl. Mat.). On the one hand, the concentrations of Al, Co, Fe, Ni, Pb and Zn increased with the age of the dichotomy. The mean concentrations increased up to two-fold from the youngest to the oldest dichotomy (even four-fold in those of Ni). The opposite trend was observed for As, Cd, Hg, N and $\delta^{15}\text{N}$. The concentrations decreased up to almost half its value from the youngest to the oldest dichotomy. The pattern seems to be more arbitrary for Cu, with no obvious differences between young and old dichotomies. Although general increasing or decreasing trends were observed across dichotomies, the Tukey’s Post-Hoc test showed that concentrations become stable across the oldest dichotomies, as no significant differences ($p > 0.05$) were found between the 3rd, 4th and 5th dichotomies for most of the elements (see homogeneous subgroups in Fig. S1, Suppl. Mat.).

Significant negative correlations ($p < 0.001$) with dry weight of the dichotomies were observed for As ($\rho = -0.53$), Cd ($\rho = -0.43$), N ($\rho = -0.29$) and $\delta^{15}\text{N}$ ($\rho = -0.48$) in SS1, for Al ($\rho = -0.27$), As ($\rho = -0.56$), Cd ($\rho = -0.60$) and Cu ($\rho = -0.32$) in SS2, and for As ($\rho = -0.49$), Cd ($\rho = -0.34$), and $\delta^{15}\text{N}$ ($\rho = -0.40$) in SS3. Significant positive correlations ($p < 0.001$) were obtained only for Fe ($\rho = 0.30$) and Hg ($\rho = 0.27$) in SS1.

Fig. 2. Results of time series analysis in *Fucus vesiculosus* dichotomies. **A)** Seasonal models fitted to the time series of N in sampling site 1 (SS1) and correlograms for N. **B)** Time series of Zn in SS3 and significant lineal regression models fitted to the trend component in those non-stationary series. Seasonal models fitted to the residuals of the series after detrending (1st and 2nd dichotomies) and directly to raw data in no detrended series (3rd dichotomy). Correlograms for residuals (1st and 2nd dichotomies) and raw data (3rd dichotomy) of Zn. **C)** Time series of Hg in SS2 and significant lineal regression models fitted to the trend component in those non-stationary series. Residuals of the series after detrending (2nd and 3rd dichotomies). No significant seasonal models could be fitted in Hg series. Correlograms for raw data (1st dichotomy) and residuals (2nd and 3rd dichotomies) of Hg. Common information to A, B and C) In time plots: equation of the regression models fitted [$a=\sin((2\pi/per) 12)$; $b=\cos((2\pi/per) 12)$]. r^2 : determination coefficient. p : p -value. per : period. Vertical dashed lines split time plots by years. In correlograms: ACF: autocorrelation function. Vertical lines represent the coefficients of autocorrelation and horizontal dashed lines the 95% confidence interval.

3.2 Temporal variation

3.2.1 Trend component

Lineal regression analysis was carried out in order to determine whether the time series were stationary or non-stationary (Table 2). The coefficient of determination (r^2) calculated for each of the series revealed that, except for Cd, Cu and N, which showed stationarity in most SS (i.e. no significant trend components), time series in the rest of elements exhibited non-stationary behavior (Table 2). In Fig. 2, time plots of the variation in the concentrations over time in the three more apical dichotomies of the thallus are shown for some of the elements as representative examples of the different behaviours observed: i) N in Fig. 2A (stationary series/significant seasonal pattern and autocorrelation); ii) Zn in Fig. 2B (non-stationary series/significant seasonal pattern/no significant autocorrelation); and iii) Hg in Fig. 2C (non-stationary series/no significant seasonal pattern or autocorrelation). The concentrations of Co, Hg, Ni and Zn significantly decreased from 2015 to 2018 in at least one dichotomy in all the SS (see r^2 in Table 2, Fig. 2B-C). A significant decrease in concentrations was also observed in the 2nd and 3rd dichotomies for Al,

Cu and Fe in SS2, and Fe in SS3. The decrease in concentrations appears to be most severe in the oldest dichotomy (3rd dichotomy), followed by 2nd and 1st dichotomies (see slopes of the regressions for Co and Ni at SS2, Table 2). On the contrary, the concentrations of As in all the SS, Cd in SS1, and $\delta^{15}\text{N}$ in SS2 and SS3, significantly increased over time in nearly every dichotomy, but more markedly at 1st dichotomy compared to the others (see slope values for As, Cd and $\delta^{15}\text{N}$, Table 2). The concentrations of N remained, however, more or less constant throughout the sampling period (Fig. 2A, Table 2).

3.2.2 Seasonal component

Seasonal variation in tissue concentrations was studied by constructing correlograms (Fig. 2). For all the elements analyzed, the correlograms corresponding to the three apical dichotomies were quite similar at each of the SS. Correlograms for Cu, Zn and N in all SS (see N in Fig. 2A), as well as for Cd, Fe, Pb and $\delta^{15}\text{N}$ in SS1, Cd in SS2, and Fe in SS3, showed a clear seasonal pattern in the three dichotomies. This pattern usually consisted of a cycle repeated every 12 lags (periodicity of 12 months). Cyclic fluctuations in the concentrations were characterized by the alternation of clusters of high positive and negative autocorrelations (approx. 6 in each case), corresponding to those periods of higher and lower bioconcentration in tissues (i.e. end–winter and end–summer months, respectively) (Table S3, Suppl. Mat.). For elements such as Cd, Cu, N and $\delta^{15}\text{N}$, significant positive autocorrelations (exceeding the significance bounds for the autocorrelation) were observed at lags 1–3, and again at lags 10–14, while significant negative autocorrelations were detected at lags 4–8, and again at lags 16–20. However, from approximately lag number 20, autocorrelations in these elements (both positive and negative) decreased in magnitude with increasing lag, damping down to low and insignificant levels of autocorrelation, approaching zero correlation (see N in Fig. 2A, Table S3 in Suppl. Mat.). This periodic seasonal pattern (alternating and tapering trend) can be

described as a quasi-cyclical behaviour. By contrast, correlograms for Al and Hg (see Hg in Fig. 2C, Table S3 in Suppl. Mat.) did not reveal any clear seasonal pattern at the scale studied, with the coefficients of autocorrelation varying at random around the same level.

In addition to the exposed in the previous paragraphs, time plots (Fig. 2) and models adjusted (Table 2) also showed a cyclic seasonal variation qualitatively similar for all SS and for the 3 apical dichotomies within the same SS. Significant models showed, for most of the elements and SS, a pattern cyclic repeated every 12 observations, with high and low concentration peaks alternated every 6 months. These patterns were especially well defined (high r^2 values) for Cd, Cu, Zn and N, with maximum concentrations peaks at the end of winter (February–April) and minimum at the end of summer (August–October). Concentrations of elements such as Al, Fe and Pb in SS1 showed, however, periodic fluctuations of between 9 and 10 months, while for As in SS2 and $\delta^{15}\text{N}$ in SS3, a seasonal cycle was identified, in the 2nd and 3rd dichotomies, every 36 and 24 months respectively. For Al, Fe, Hg and $\delta^{15}\text{N}$, no significant models could be fitted in SS2 for any of the dichotomies. Comparison of the model parameters between dichotomies revealed that the oldest dichotomy exhibited a better fit of the sinusoidal model than the other two, for all elements in SS1 (except for Cd, Hg and Pb), for Ni and Zn in SS2, and for Cu, Fe, Zn and $\delta^{15}\text{N}$ in SS3.

Table 2. Results from significant lineal regression analysis of time series ($p < 0.01$) in *Fucus vesiculosus* dichotomies from three sampling sites (SS). Determination coefficients (r^2) and the slope of each regression are shown. Significant regression models parameters adjusted to the seasonal component of time series ($p < 0.01$). Sig.: signification level; n.s.: non-significant; β_0 : mean of the series; β_1 and β_2 : regression coefficients; ϵ_t : residuals; per : period (further details in Table S1, Suppl. Mat.).

	Dichotomy	Trend component		Seasonal component								
		r^2	Slope	r^2	Sig.	β_0	β_1	β_2	ϵ_t	per		
Al	SS1	1 st	0.23	3.44	-	n.s.	-	-	-	-	-	
		2 nd	-	-	0.22	<0.01	130	8.86	45.8	2.26	10	
		3 rd	-	-	0.42	<0.001	146	29.7	64.5	4.07	10	
	SS2	1 st	-	-	-	n.s.	-	-	-	-	-	
		2 nd	0.26	-6.56	-	n.s.	-	-	-	-	-	
		3 rd	0.28	-8.99	-	n.s.	-	-	-	-	-	
As	SS1	1 st	0.48	2.63	-	n.s.	-	-	-	-	-	
		2 nd	0.50	1.99	0.24	<0.01	-1.30E-04	12.2	-9.66	-1.99	12	
		3 rd	0.28	0.76	0.33	<0.001	4.56E-05	8.72	-6.37	-1.94	12	
	SS2	1 st	0.33	2.26	-	n.s.	-	-	-	-	-	
		2 nd	0.24	1.63	0.59	<0.001	-2.09E-04	-8.95	-32.54	1.33	36	
		3 rd	-	-	0.44	<0.001	44.6	-12.9	-17.7	0.504	36	
	SS3	1 st	0.23	1.75	0.36	<0.001	2.99E-04	17.0	-24.7	-1.328	12	
		2 nd	0.34	1.39	-	n.s.	-	-	-	-	-	
		3 rd	0.19	0.45	0.20	<0.01	-0.424	1.82	-6.61	-1.56	12	
	Cd	SS1	1 st	0.41	0.05	0.53	<0.001	-1.46E-04	0.664	-0.091	0.040	12
			2 nd	0.21	0.01	0.58	<0.001	7.25E-05	0.293	-0.081	-0.021	12
			3 rd	-	-	0.40	<0.001	0.606	0.223	-0.101	-0.036	12
SS2		1 st	-	-	0.35	<0.001	1.80	0.209	-0.530	-0.103	12	
		2 nd	-	-	0.60	<0.001	0.896	0.193	-0.371	-0.020	12	
		3 rd	-	-	0.44	<0.001	0.766	0.156	-0.330	-0.041	12	
SS3		1 st	-	-	-	n.s.	-	-	-	-	-	
		2 nd	-	-	0.23	<0.01	0.425	-0.053	-0.126	-0.022	11	
		3 rd	-	-	-	n.s.	-	-	-	-	-	
Co	SS1	1 st	0.28	-0.02	-	n.s.	-	-	-	-	-	
		2 nd	-	-	0.25	<0.01	1.01	0.406	-0.467	-0.089	12	
		3 rd	-	-	0.31	<0.001	1.35	0.415	-0.705	-0.076	12	
	SS2	1 st	0.23	-0.10	-	n.s.	-	-	-	-	-	
		2 nd	0.24	-0.14	-	n.s.	-	-	-	-	-	
		3 rd	0.21	-0.16	0.16	<0.01	-6.36E-04	0.177	-2.48	-0.518	12	
	SS3	1 st	0.26	-0.03	-	n.s.	-	-	-	-	-	
		2 nd	0.21	-0.03	-	n.s.	-	-	-	-	-	
		3 rd	-	-	0.26	<0.01	0.752	0.100	-0.498	-0.088	12	
SS1	1 st	-	-	0.32	<0.001	3.99	1.37	-0.486	0.087	12		
	2 nd	-	-	0.20	<0.01	3.72	0.840	-1.01	-0.391	12		
	3 rd	-	-	0.35	<0.001	3.78	0.338	-1.44	-0.340	12		
Cu	SS2	1 st	-	-	0.39	<0.001	3.62	0.373	-0.899	-0.227	12	
		2 nd	0.31	-0.04	0.58	<0.001	-3.10E-04	0.042	-0.748	0.044	12	
		3 rd	0.40	-0.06	-	n.s.	-	-	-	-	-	
	SS3	1 st	-	-	0.35	<0.001	2.38	-0.752	0.310	-0.040	10	
		2 nd	-	-	0.24	<0.01	1.89	-0.339	-0.353	-0.140	12	
		3 rd	-	-	0.47	<0.001	1.84	-0.596	-0.192	-0.115	12	

TABLE 2
(Continued)

	Dichotomy	Trend component		Seasonal component							
		r^2	Slope	r^2	Sig.	β_0	β_1	β_2	ϵ_t	per	
Fe	SS1	1 st	-	-	-	n.s.	-	-	-	-	-
		2 nd	-	-	0.30	<0.001	141	23.7	38.3	-1.21	10
		3 rd	-	-	0.36	<0.001	169	38.0	49.9	-3.88	10
	SS2	1 st	-	-	-	n.s.	-	-	-	-	-
		2 nd	0.41	-5.54	-	n.s.	-	-	-	-	-
		3 rd	0.34	-7.02	-	n.s.	-	-	-	-	-
	SS3	1 st	-	-	0.31	<0.001	68.9	-12.1	-26.1	-6.36	16
		2 nd	0.31	-2.35	-	n.s.	-	-	-	-	-
		3 rd	0.32	-2.46	0.34	<0.001	0.662	0.203	31.2	-1.97	12
Hg	SS1	1 st	0.23	-0.29	-	n.s.	-	-	-	-	-
		2 nd	0.48	-0.48	-	n.s.	-	-	-	-	-
		3 rd	0.28	-0.43	-	n.s.	-	-	-	-	-
	SS2	1 st	-	-	-	n.s.	-	-	-	-	-
		2 nd	0.57	-0.48	-	n.s.	-	-	-	-	-
		3 rd	0.43	-0.36	-	n.s.	-	-	-	-	-
	SS3	1 st	0.23	-0.43	0.27	<0.01	-1.03E-03	-1.21	-6.38	-0.045	10
		2 nd	0.27	-0.47	-	n.s.	-	-	-	-	-
		3 rd	0.38	-0.46	-	n.s.	-	-	-	-	-
Ni	SS1	1 st	0.36	-0.03	-	n.s.	-	-	-	-	-
		2 nd	-	-	0.20	<0.01	1.78	0.858	-0.740	-0.342	12
		3 rd	-	-	0.25	<0.01	2.67	0.993	-1.27	-0.283	12
	SS2	1 st	0.34	-0.10	-	n.s.	-	-	-	-	-
		2 nd	0.37	-0.18	-	n.s.	-	-	-	-	-
		3 rd	0.35	-0.20	0.27	<0.01	-8.04E-04	0.683	-2.20	-0.383	12
	SS3	1 st	0.27	-0.05	-	n.s.	-	-	-	-	-
		2 nd	0.27	-0.07	-	n.s.	-	-	-	-	-
		3 rd	-	-	0.21	<0.01	2.35	-0.480	-1.02	-0.300	12
Pb	SS1	1 st	-	-	0.32	<0.001	0.350	0.006	0.090	-0.009	10
		2 nd	-	-	0.32	<0.001	0.457	0.053	0.145	-0.016	10
		3 rd	-	-	0.23	<0.01	0.604	0.130	0.175	-0.058	10
	SS1	1 st	0.18	-1.26	0.37	<0.001	-0.987	24.3	-8.51	-5.82	12
		2 nd	-	-	0.39	<0.001	74.8	44.3	-33.0	-5.09	12
		3 rd	-	-	0.51	<0.001	95.0	49.3	-48.8	-3.32	12
	SS2	1 st	-	-	0.38	<0.001	43.2	17.7	-19.0	-4.74	12
		2 nd	0.19	-1.77	0.32	<0.001	-2.10E-04	14.7	-28.4	-6.13	12
		3 rd	-	-	0.48	<0.001	56.2	18.9	-39.8	-4.80	12
SS3	1 st	0.22	-1.20	0.30	<0.01	3.46E-04	12.3	-15.1	-1.62	12	
	2 nd	0.24	-1.52	0.32	<0.001	-4.33E-04	7.90	-22.5	-5.57	12	
	3 rd	-	-	0.38	<0.001	51.6	8.17	-26.2	-4.75	12	

TABLE 2
(Continued)

	Dichotomy	Trend component		Seasonal component							
		r ²	Slope	r ²	Sig.	β ₀	β ₁	β ₂	ε _t	per	
N	SS1	1 st	-	-	0.65	<0.001	0.719	0.172	-0.083	0.020	12
		2 nd	-	-	0.65	<0.001	0.626	0.180	-0.089	0.029	12
		3 rd	-	-	0.71	<0.001	0.555	0.178	-0.087	0.021	12
	SS2	1 st	-	-	0.67	<0.001	0.932	0.029	-0.133	-0.004	12
		2 nd	-	-	0.50	<0.001	0.830	0.021	-0.150	0.019	12
		3 rd	-	-	0.67	<0.001	0.778	0.042	-0.116	0.006	12
	SS3	1 st	-	-	0.71	<0.001	0.757	0.147	-0.149	0.013	12
		2 nd	-	-	0.58	<0.001	0.690	0.124	-0.140	-0.003	12
		3 rd	-	-	0.65	<0.001	0.624	0.097	-0.167	0.012	12
δ ¹⁵ N	SS1	1 st	-	-	0.51	<0.001	2.49	0.294	-0.019	0.012	12
		2 nd	-	-	0.50	<0.001	2.29	0.269	-0.132	0.011	12
		3 rd	-	-	0.66	<0.001	2.15	0.244	-0.156	0.016	12
	SS2	1 st	0.36	0.01	-	n.s.	-	-	-	-	-
		2 nd	0.34	0.01	-	n.s.	-	-	-	-	-
		3 rd	0.44	0.01	-	n.s.	-	-	-	-	-
	SS3	1 st	-	-	-	n.s.	-	-	-	-	-
		2 nd	0.31	0.02	0.21	<0.01	4.72E-05	-0.242	-0.076	-0.028	24
		3 rd	0.34	0.02	0.23	<0.01	-1.04E-02	-0.230	-0.044	0.009	24

3.3 Growth measurements

Regarding growth in *F. vesiculosus*, a very short time of experiment revealed the high mortality among the individuals monitored. Several individuals had disappeared after an interval of a few weeks and gradually at some point during the checkup, so these could not be followed for the entire period. Due to a strong herbivory in the apical tissues of algae and the significant loss of individuals in SS3, growth measurements in this site were only possible at 3 months after the initial follow-up. In addition, many individuals experienced significant size growth that it was impossible to follow the number of dichotomies formed from 6 months onwards. Although the loss of individuals not guaranteed data of statistical significance, it allowed a preliminary inquiry into size and dichotomous branching variation in this species. Thus, healthy thalli formed 2–3 new dichotomies every three months (up to 5 new dichotomies in some cases), what is ca. 1 dichotomy/month (Fig. S2, Suppl. Mat.). The growth in thallus length was more variable between measurements. Thalli in SS1 and

SS2 had grown 7 ± 3.5 cm (mean \pm SD) three months after the first measurement and 5 ± 3 cm after the second measurement. The growth after the third measurement was not considered given the significant loss of individuals and the strong herbivory in those who survived.

4. Discussion

4.1 Intra-thallus variation

The concentrations of all elements varied significantly depending on the dichotomy analyzed, but this variation was not dependent on the “Sampling month” neither on “SS” (Table S2, Suppl. Mat.). Regardless the type of trend in concentrations observed along the thallus, the lack of significant differences in concentrations of most elements between the oldest dichotomies suggests that bioconcentration rates in *F. vesiculosus* stabilize with the age of the tissue.

Intra-thallus variation in concentrations of elements was widely reported in algae (García-Seoane et al., 2018), and previously observed in *F. vesiculosus* between dichotomies (e.g. Savage & Elmgren, 2004; Carballeira et al., 2014), and between different structural parts of the thallus (e.g. Bryan & Hummerstone, 1973; Carvalho et al., 1997). However, none of these studies has applied robust statistics to determine whether concentrations in the different dichotomies of the thallus significantly interact with factors as the month of collection or the SS. Furthermore, most of them did not even use statistical tests to determine whether the differences observed between the different parts of the thallus were significant or not, and neither cross-checked the representativeness of their results by comparing among various SS (García-Seoane et al., 2018). In view of this, the gradual increase in concentrations of Al, Co, Fe, Ni, Pb and Zn observed from the growing tips towards the older dichotomies of the thallus (Fig. S1, Suppl. Mat.) is in accordance with the results reported from previous studies for the same elements in *F. vesiculosus*, e.g. for Al, Fe, Pb and Zn (Bryan & Hummerstone, 1973), Fe,

Ni and Zn (Barnett & Ashcroft, 1985), and Al, Co, Fe, Ni and Zn (Försberg et al., 1988; Söderlund et al., 1988). Furthermore, the decreasing trend in concentrations of As, Cd, Hg, N and $\delta^{15}\text{N}$ with the age of dichotomy (Fig. S1, Suppl. Mat.), although is opposed to the $\delta^{15}\text{N}$ values found in the same species by Savage & Elmgren (2004), is also consistent with the results from Carballeira et al. (2014) and Viana et al. (2015), who found highest $\delta^{15}\text{N}$ values in the apical dichotomies compared to the rest of the frond. However, although these authors noted the existence of concentration gradients within the thallus of *F. vesiculosus* and, in most cases, their conclusions agree with those reached in this study, the absence of statistical tests confirming the existence of significant differences between dichotomies, especially in older studies, leads these results to be considered inconclusive.

The presence of a concentration gradient related with the thallus age could be the result of several factors, including: i) the intrinsic uptake characteristics of algae tissues (e.g. variations in cation exchange capacity); ii) the different growth rates and metabolic activity of tissues (e.g. Rice & Lapointe, 1981; Stengel et al., 2005); iii) but also the contamination by epiphytes (e.g. Kangas et al., 1982) and fine particles (e.g. Bryan & Hummerstone, 1973; Försberg et al., 1988; Malinovskaya, 1998); and iv) the presence of pre-existing contaminant load in tissues. However, according to the results of the present study, the preferential concentration of the elements in young or old tissues cannot be explained by a single factor. The physicochemical characteristics of the elements may influence the capacity to compete for binding sites (e.g. Stengel et al., 2005; Ryan et al., 2012), but the variation in the number and/or type of binding sites along the thallus may also explain the differences observed in the distribution patterns of elements. Meanwhile, the different metabolic functions of the elements across different thallus regions can also contribute to the observed differences (e.g. Rice & Lapointe, 1981; Stengel et al., 2005).

As some authors (e.g. Bryan & Hummerstone, 1973; Higgins & Mackey, 1987) suggested, the metal uptake capacity in algae may increase

with growth rate, as algae growth rates determine the quantity of tissue per unit of time that is exposed to the environmental levels of pollutants. Although the relationship between bioconcentration and growth rate is likely to be metal-specific (Stengel et al., 2005). Thus, higher growth rates in young tissues than in old parts would lead to a rapid increase of biomass and, consequently, to a high proportion of free binding sites for capture elements (higher cation exchange capacity in tissues). Furthermore, it is assumed that the active uptake and release processes are faster in new and metabolically more active tissues because the ability to regulate both processes in the macroalgae decrease in the course of aging (Malinovskaya, 1998). All of the above would explain the large differences observed in the concentrations of As, Cd, Hg, N and $\delta^{15}\text{N}$ between young and old dichotomies (Fig. S1, Suppl. Mat.).

Nevertheless, high concentrations of elements such as Al, Co, Fe, Ni, Pb and Zn in the oldest areas of the thallus (Fig. S1, Suppl. Mat.) is probably more related to factors as the appearance of epiphytes (Malinovskaya, 1998) or particulate matter (Bryan et al., 1985; Barreiro et al., 2002). But the possible pre-existing contaminant load compared to youngest tissues or, as suggested by Higgins & Mackey (1987), the increase in metal binding sites in older tissues may also cause those high concentrations. Finally, contrary to the mentioned in the previous paragraph, some authors (e.g. Fuge & James, 1973; Markham et al., 1980; Villares et al., 2002) have pointed out that metal uptake capacity in algae decrease with growth rate, as higher growth rates in apical parts would result in an increase in biomass of tissue, leading to a dilution of concentrations by new growth, and therefore to lower concentrations in young than in old tissues. However, this theory can be discarded based on that, although young tissues grow proportionally more than olds, all tissues will uptake/release metals until they are balanced with the concentrations present in the environment, even while the new tissues is growing, so there could never be a dilution by growth.

To conclude, the strong negative correlations between elements such as As and Cd, and the dry weight of the dichotomies, could indicate that

the greater the amount of tissue, the lower the bioconcentrated concentrations. This could be related to the amount of specific surface available to adsorb these elements which, in proportion, may be less than the rest of the tissue as the algae grow.

4.2 Temporal variation

The concentrations of all elements in all the dichotomies of *F. vesiculosus* varied considerably over years. A significant decreasing pattern in concentrations of elements such as Co, Hg, Ni and Zn was observed in the tissues from 2015 to 2018 in all SS (Table 2), with concentrations decreasing annually between 10% and 20%. Although Viana et al. (2010) studied temporal changes only in apical dichotomies in this species in the same study area, they observed a significant decrease in the concentrations of Al, Cd, Co, Fe, Hg and Zn of ca. between 5% and 10% from 2001 to 2007.

The results of the present study also demonstrate a large degree of intra-annual variation in the concentrations of the elements determined in the *F. vesiculosus* tissue (Table 1), being comparable to the intra-annual variability found for the same elements in a multitude of studies of temporal trends using macroalgae as biomonitors (i.e. coefficients of variation, CV between 35% and 58%, except N, CV <30%) (see García-Seoane et al., 2018). According to the correlograms calculated and to the models fitted to the time series (Fig. 2, Table 2, Table S3 in Suppl. Mat.), this intra-annual variation in concentrations is not random, and can be reasonably attributed to the existence of seasonality in the bioconcentration process of these elements. Except for Al, Hg and Pb, the other elements showed cyclical fluctuations every 12 months in at least one of the SS, consisting on maximum values in winter and minimum values in summer. Concentrations of Cu, Fe, N and Zn exhibited more than two-fold changes between both seasons. Seasonal variation was previously documented in over 80% of publications assessing the effect of temporal variability in macroalgae, and most of these concluded, as in

the present study, that the highest concentrations of metals typically occur during winter and are lower in summer (García-Seoane et al., 2018). Seasonality was observed in *F. vesiculosus* for example for As (Stoeppler et al., 1986), Cd (Riget et al., 1995), Zn (Riget et al., 1995; Villares et al., 2013) and $\delta^{15}\text{N}$ (Lemesle et al., 2015, 2016), but also in other furoid species, e.g. for Cd, Zn (Miramand & Bentley, 1992; Martin et al., 1997) and Ni (Martin et al., 1997). However, although there has been much discussion about the existence of seasonal fluctuations in bioconcentration of elements in algae, until now it has never been proven using autocorrelation analysis. Furthermore, most of those studies using the term seasonality to describe variations between winter and summer, have done so erroneously from our point of view, since in order to establish the existence of seasonality it is necessary to collect samples at a high frequency (e.g. monthly) repeatedly over several years. Those studies comparing concentrations in different seasons collecting samples for a one-year period or less, cannot clearly state that the variations observed correspond to a seasonal pattern, since it cannot be discarded that these differences are due to a different cause, for example, to an isolated pollution event at the time when the highest concentrations were detected. Finally, since there was no significant interaction between “Sampling month” and “Dichotomy” for any of the elements, it can be concluded that the intra-thallus variation occurs independently of the month/season of the year in which the algae are collected.

Seasonal variation in algae concentrations may be attributed to changes in environmental factors such as temperature, precipitation, salinity, pH or light conditions (Stoeppler et al., 1986; Haroon et al., 1995; Wright & Mason, 1999), as the bioavailability of metals in seawater and sediments changes depending on the physico-chemical conditions of the environment (Burdon-Jones et al., 1982; Wright & Mason, 1999). Although seasonal variation could also reflect seasonal changes in the levels of the elements in solution due to occasional fluvial and terrestrial inputs (Lacerda et al., 1985; Fink & Manley, 2011). However, although most authors attributed this pattern to a diluting effect due to growth of

algae in the warmest periods (e.g. Riget et al., 1995; Martin et al., 1997; Vasconcelos & Leal, 2001; Villares et al., 2002, 2013), we do not believe that seasonal fluctuations can be explained by the differences in algae growth rates between summer and winter, since as it was mentioned above, algal tissues uptake/release metals until they are balanced with the ambient concentrations, irrespective of their growth rate. Thus, the possibility that the seasonality of concentrations be due to other factors intrinsic to the algae, such as metabolism (photosynthesis and respiration) and reproduction cannot be discarded (e.g. Rao & Indusekhar, 1989; Wright & Mason, 1999). In the case of N, as it is the main limiting nutrient for primary production in marine coastal waters, the occurrence of minimum values in summer may suggest nutrient limitation by this element, as it corresponds to the period of maximum productivity (Villares et al., 2013).

Regardless, any one of these possibilities fully explain the seasonal variation differences between elements. Bioconcentration of elements in *F. vesiculosus* is most likely due to complex inter-relations among environmental and biological factors (Vasconcelos & Leal, 2001; Villares et al., 2002), and the interaction between these factors can result in seasonal cycles of different period.

Finally, because the thalli of *F. vesiculosus* have been found to form several new dichotomies each year, dichotomies can no longer be considered equivalent to annual growth cohorts, as done in biomonitoring studies studying temporality in this species (e.g. Savage & Elmgren, 2004; Carballeira et al., 2014).

5. Conclusions

The concentrations of Al, As, Cd, Co, Fe, Hg, Ni, Pb, Zn, N, as well as $\delta^{15}\text{N}$ signal depend on the tissue age in *F. vesiculosus*. This species preferentially bioconcentrates As, Cd, Hg, N and $\delta^{15}\text{N}$ in young dichotomies and Al, Co, Fe, Ni, Pb and Zn in old dichotomies. As the concentrations of nitrogen and trace elements will largely depend on the

dichotomy selected for analysis, we assume that the results from biomonitoring studies that use different sections of the thallus of *F. vesiculosus* are not strictly comparable. In addition, accordingly to our findings, the concentrations of nitrogen and trace elements in *F. vesiculosus* are subject to high seasonal variation. Therefore, comparison of samples collected at different times of the year is not acceptable. Thus, the present study proposes the following sampling protocol, that should serve as a guideline for future biomonitoring studies using marine macroalgae on the SS scale:

1. To reduce the effect of variability along *F. vesiculosus* thallus and to ensure inter-comparability between studies, we propose to make composite samples of the three apical dichotomies of thallus (usually available even in the youngest thalli).

2. To minimize the effect of the seasonal variation and provide annual representative data while optimize the sampling effort, subsamples should be collected 2 times per year separated by 6 months (the time lag between the annual maximum and minimum concentrations) and combined in a single composite sample.

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Chapter VI



CHAPTER VI

Biomonitoring coastal environments with transplanted macroalgae: a methodological review

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Chapter VII



CHAPTER VII

Phenotypic differences in heavy metal accumulation in populations of the brown macroalgae *Fucus vesiculosus*: a transplantation experiment

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Abstract

The concentrations of Al, As, Cd, Co, Cr, Cu, Fe, Hg, Ni, Pb and Zn in the thalli of *Fucus vesiculosus* transplanted reciprocally among four sites affected by different degrees of metal pollution (two unpolluted and two industrial environments) were measured with the aim of comparing the capacity of the algae to accumulate these elements under such conditions. At the beginning of the experiment, the concentrations of all elements differed significantly between the individuals from at least one of the unpolluted and one of the polluted sites. After exposure of the algae for 90 days, the concentrations of all of the elements except As, Cd and Cu in individuals transplanted from the unpolluted sites to the most polluted site increased to higher levels than in the algae transplanted within the site of origin. The same was observed for all elements except As and Cd in the thalli transplanted from one of the unpolluted sites to the second most polluted site. By contrast, the concentrations of the elements in transplants from the polluted sites exposed in the unpolluted sites usually decreased to approximately the same levels as in the autotransplants. These results suggest that the heavy metal uptake capacity may be limited in *F. vesiculosus* populations exposed to long-term pollution as an adaptive response to toxicity by metals. We therefore recommend the use of algal transplants to study water quality in highly polluted sites, rather

than of native algae already growing in the sites, to avoid the possible effects of such adaptation.

Keywords: Biomonitoring; Marine pollution; Heavy metals; Fucus; Transplants; Adaptation.



1. Introduction

Biomonitor are organisms that provide quantitative information (measurable responses) about environmental changes, whether natural or human-induced, through chemical analysis of their tissues (Hertz, 1991; Carballeira et al., 2000). In the context of heavy metal pollution in marine environments, an ideal biomonitor should be a net accumulator of metals, i.e. an organism in which a simple correlation between the concentrations of metals in its tissues and the bioavailable concentrations in its surrounding environment is observed (Phillips, 1980; Rainbow, 1995; Markert et al., 1999). The linear relationship between exposure concentrations and body burden should be maintained to provide unequivocal information about the levels of metals available in the environment (Stengel & Dring, 2000).

Marine macroalgae are often used in biomonitoring programmes to estimate metal pollution in a wide range of environments (Phillips, 1980). Some species of brown algae (Class Phaeophyceae) are able to survive in highly polluted environments, such as areas receiving waste from fish farms (Rönnerberg et al., 1992), different types of industries (Marsden et al., 2003), and sewage treatment plants (Oczkowski et al., 2008), and can accumulate high levels of heavy metals in their tissues. Although some metals play important roles in metabolic processes (viz. Cu, Fe and Zn), they may have toxic effects when present at high concentrations and cause physiological stress in algae (Gaur & Rai, 2001; Pearson et al., 2010), affecting growth rates (e.g. Collén et al., 2003), survival rates (e.g. Marsden et al., 2003; Sales et al., 2011) and cover (e.g. Marsden et al., 2003). According to several authors, in algae excess metal can lead to the substitution of essential co-factors and thus to the inactivation of enzymes and proteins and the subsequent inhibition of photosynthesis (Plötz, 1991; Küpper et al., 2002).

Several studies have demonstrated the existence of resistance mechanisms in algal populations continuously exposed to high levels of

metals (Hall et al., 1979; Correa et al., 1996). Defence strategies developed by macroalgae to prevent, or at least reduce, the stress and damage caused by excess metal include the following (see Pinto et al., 2003 and Moenne et al., 2016): cellular exclusion mechanisms (Hall et al., 1979); synthesis of metal-chelating compounds such as metallothioneins and phytochelatins (Morris et al., 1999); activation of reactive oxygen metabolism (antioxidant metabolism) (Rijstenbil et al., 1998; Collén & Davison, 1999; Collén et al., 2003; Contreras et al., 2009); and complexation with ligands such as polyphenols (Smith et al., 1986; Gledhill et al., 1999). Some of these mechanisms may affect the capacity of algae to regulate metal uptake, e.g. by limiting the capture of metals. Although such a strategy would allow algae to survive in environments containing high levels of metals, it would also potentially alter the linearity of the bioaccumulation process, so that the concentration of accumulated metals will not only depend on the exposure concentration but also on the contamination history of their native habitats (Correa et al., 1996; Nielsen et al., 2003). The ability of macroalgae to regulate the metal levels in their tissues would therefore have negative implications for the use of these organisms in biomonitoring studies, as would affect their suitability to act as efficient biomonitors (Phillips, 1990; Nielsen et al., 2003).

In a study involving the simultaneous use of native and transplanted macroalgae to biomonitor metal pollution, Hédouin et al. (2008) looked for any differences in the accumulation capacity of algae from different environments. These researchers observed that specimens of *Lobophora variegata* (Phaeophyceae) transplanted from an unpolluted to a polluted site accumulated more Ag, As, Cd, Co, Cr, Cu, Mn and Ni than native algae from the polluted site after one month of exposure. However, the direct comparison of metal levels in native and transplanted algae is a major drawback of this design. It is known that transplantation may cause additional stress in algae (McCook, 1996; Alquezar et al., 2013). This adds an extra source of variation to the final data set and means that transplanted and native algae are not directly comparable.

In the present study, we aimed to improve the aforementioned design by carrying out a field experiment in which specimens of the brown algae *Fucus vesiculosus* were transplanted both within the same site and among different sites (two polluted and two unpolluted sites) to enable comparison of the capacity of the algae growing under different conditions to accumulate several metals and metalloids. Here we discuss the implications that the possible adaptation mechanisms exhibited by algae growing in polluted environments have on the use of native algae in biomonitoring studies.

2. Material and methods

2.1 Sampling

Four sites in Galicia (NW Spain) were selected for collection and exposure of transplants of the brown seaweed *Fucus vesiculosus* L. (Fucaceae, Phaeophyceae). Two of the sites were located within rías in industrialized environments (polluted sites), i.e. an iron and steel plant (P1, centered in X = 566905, Y = 4816449; UTM 29N ETRS89), and a paper pulp industry/chlor-alkali plant (P2, X = 525918, Y = 4694978), which discharge high levels of metals and metalloids into the seawater, especially As, Cd, Cr, Cu, Hg, Ni, Pb and Zn (E-PRTR, 2017). The other two sites, (U1, X = 500427 Y = 4731363 and U2, X = 507009 Y = 4700994) were located in open sea areas with low pollution levels (unpolluted sites), according to the concentrations found in marine biota and sediments (Carballeira et al., 1997; Viana et al., 2010).

Whole thalli of *F. vesiculosus* (55 in each site) were handpicked using plastic globes and washed with surrounding seawater to remove most of adhering surface material, mainly particulate material. To minimize variability within samples, care was taken to ensure that all the individual thalli collected were of a similar size. Samples were transported to the laboratory in polyethylene plastic bags and stored in refrigerated conditions ($5\pm 1^\circ\text{C}$) until preparation of the transplants. In order to

establish baseline concentrations at the beginning of the experiment (t_0) at each site, a composite sample of 5 of these individuals was made and analysed in the same way as the thalli transplanted.

2.2 Preparation and exposure of the transplants

Transplants were prepared in the laboratory. Each transplant system consisted of 10 thalli, which were attached to a rock (ca. 15 kg weight) by gluing the holdfast with a strong polyurethane adhesive (SIKAFLEX®) and were then covered with a polyethylene mesh net (2 x 2 cm mesh size). A total of 20 transplants were prepared for exposure at each site. The transplants were bound together with ropes in groups of 5, so that 5 replicate transplants (i.e. 5 rocks) with thalli from each of the four original populations sampled (P1, P2, U1 and U2) were exposed in each site. All the transplants were labelled with plastic tags indicating the site of origin (Fig. 1).



Fig. 1. Transplantation systems exposed in the field, each consisting of 10 thalli of *Fucus vesiculosus* attached to a rock and covered with a polyethylene mesh net.

Transplants exposed within the same site in which the thalli were collected (i.e. exposed in their origin environment) will hereafter be referred to as autotransplants, and those exposed in sites other than the original site, will be referred to as crosstransplants. In total, 200 thalli were exposed at each site (i.e. 50 from each of the four populations). Transplants were exposed in the intertidal zone amongst the native populations of *F. vesiculosus*. Experimental exposures were carried out during low tide in April 2017 and lasted 90 days, considered to be long time enough for the element concentrations in the algae to become balanced with those in the surrounding environment.

2.3 Processing and chemical analysis

At the end of the exposure period, the thalli were detached from the rocks, cleaned in the surrounding seawater to eliminate any adhering epiphytes and sediments, placed inside clean PET bags and transported to the laboratory in a refrigerated container ($5\pm 1^\circ\text{C}$). The thalli were processed within 1–2 days after collection. Apical segments (1 cm long) were removed with a glass spatula, and receptacles, damaged or very old tissue, and areas heavily affected by epiphytes were discarded. The apical sections, i.e. the most recent and physiologically active parts of the thallus, were used to standardize the material analysed. These were dried in a forced air oven (72 h, 40°C), to prevent loss of volatile elements (MacNaeidhe, 1995), and homogenized in a tangential mixer mill with zirconium oxide grinding vessels (Retsch ZM400). The material was stored in hermetically sealed vials until chemical analysis.

Before the analytical determinations, samples of algal tissue were dried overnight at 40°C in a forced air oven. Algal samples (0.3 g dry weight - d.w.) were digested with nitric acid (65%) in PTFE (Teflon) containers in a microwave oven (CEM MDS2100). Acidic digestions were carried out at an initial temperature of 100°C (10 min), which was increased up to 150°C for 7 min and then to 190°C for 25 min to complete the mineralization. Each sample volume was then adjusted to 50 mL with Milli-Q water. The

concentrations of Al, As, Cd, Co, Cr, Cu, Fe, Ni, Pb and Zn were determined by inductively coupled plasma mass spectrometry (VARIAN 820-MS ICP quadrupole mass spectrometer) and those of Hg were determined in an elemental analyser (Milestone DMA80). The determinations were carried out at the Research Support Services Unit and the Ecology Unit (Universidade de Santiago de Compostela).

For analytical quality control, one replicate sample and one analytical blank were analysed every 10 samples. The following certified reference materials were analysed in the same way as the samples to test the accuracy of measurements: BCR-279 *Ulva lactuca* (Chlorophyta) (Institute for Reference Materials and Measurements, IRMM, Brussels, Belgium) and ERM-CD200 *F. vesiculosus* (Institute for Reference Materials and Measurements, IRMM, Geel, Belgium). The concentrations of all elements were above the limits of quantification of the analytical technique.

The analytical quality of the process was generally satisfactory, with an overall percentage error ranging between 4 and 12%, except for Al (22%). The recovery of BCR-279 was satisfactory in all cases, ranging between 68% (Cr) and 113% (As), and was usually higher than 85%. The recovery of ERM-CD200 ranged between 71% (Pb) and 107% (Cd), and in most cases it was higher than 90%.

2.4. Data analysis

Prior to the statistical analysis, the normality of the data distribution and homogeneity of variances were assessed using the Kolmogorov–Smirnov and Levene tests respectively. Significant differences among the initial concentrations in the samples from the four sites were explored using a non-parametric Kruskal–Wallis test, as the data were not normally distributed. Elemental concentrations at the end of transplantation period (which were normally distributed) were also compared using a two-way ANOVA (General Linear Model with Type I error), with the exposure site and transplant origin as the grouping variables, and the element concentrations as the dependent variable. To ascertain whether the effect

of the exposure site on the final concentrations differed depending on the origin of the transplant, the interaction between these two variables was further examined by a Simple Effects Test. A Bonferroni post-hoc test was also used to study differences between the crosstransplants and the autotransplants at each site for each element. A significance level of $p \leq 0.05$ was applied in all cases. All statistical analyses were performed with IBM SPSS Statistics 20.

Enrichment of different contaminants in the transplants was expressed in the form of uptake/release rate, calculated for each element as the difference between the final (t_f) and the initial concentrations (t_0), divided by the number of days of exposure.

3. Results

The concentrations of the different elements in the transplants at the beginning and at the end of the experiment for each of the four sites are shown in Figs. 2 and 3. The results of the Kruskal–Wallis test comparing the initial concentrations across sites revealed significantly higher concentrations of Al, As and Fe in algae from P1 than from U1, and significantly higher concentrations of Co, Cr, Ni, Pb and Zn in algae from P1 than from U2. Only the concentrations of Cu and Hg were significantly higher in algae from P2 than in those from U2. The initial concentrations of Cu in algae from U1 were significantly higher than in the algae from P1. No significant differences were found between the initial concentrations of algae from P1 and P2, and those from U1 and U2.

Changes in the mean concentrations of the elements in transplants after exposure in the 4 sites are also shown in Figs. 2 and 3. At the end of the experiment, the concentrations of all the elements in the thalli transplanted to P1 (including crosstransplants and autotransplants) tended to increase, with some exceptions for As and Cd. This increase was more than twofold for Hg, up to threefold for Cu, and more than twentyfold for Al and Fe in the crosstransplants (and for Al and Fe even more than sixtyfold in algae transplanted from U1). The post-hoc tests showed that the

concentrations of metals in the samples transplanted from P2 (for all elements except As, Co and Pb), from U1 (for Co, Fe, Hg and Ni), and from U2 (for Co, Hg and Ni) to P1 were, significantly higher than the levels reached in the autotransplants. A similar pattern of increase in the concentrations was observed in thalli transferred to P2, for fewer elements (i.e. Al, Cr, Cu, Fe, Hg, Pb, and Zn), and with the crosstransplants from U1 (for Al, Cu and Hg) and from P1 (for Pb) reaching significantly higher concentrations than in the autotransplants.

On the other hand, the concentrations of all elements in crosstransplants from P1 exposed in U1 and U2 tended to decrease to approximately the same levels as in the autotransplants. The decrease was of more than half the initial values for Cr, Ni, Pb and Zn, and up to ca. sixfold times for Co and Cu. The concentrations of As, Cu, Hg and Zn (for Zn only in U2) decreased in the algae transplanted from P2 to U1 and U2. For the other elements (i.e. Al, Cd, Co, Cr, Fe, Ni, Pb), the concentrations in the crosstransplants from P2 tended to increase, rather than decrease. By contrast, the final concentrations of Cd were significantly lower in crosstransplants from P1 and U2 exposed in U1, and from P1 exposed in U2 than in the corresponding autotransplants. As an exception, the concentrations of Cd in the crosstransplants from U1 and U2 exposed in P1 and P2 were lower than in the autotransplants, whereas the concentrations in the algae transplanted from P2 to U1 and U2 tended to increase above the concentrations in the autotransplants.

Finally, the two-way ANOVA test applied to the final concentrations (see Table 1) also revealed a significant exposure site*origin site interaction for Al, Cd, Cu, Fe and Hg. The exposure site had a different effect on the final concentration of metals depending on the site of origin of the algae. Although no clear pattern was observed regarding the significant interactions, the Simple Effects Test showed that the effect in the concentrations was associated with exposure sites P1 and P2 (exceptionally also U1 and U2 in the case of Cd).

The uptake/release rates for each element and transplant, along with the variability expressed as the coefficient of variation (CV), were also

calculated (Table 2). Transplants exposed in unpolluted sites (U1 and U2) showed negative rates (release) for all elements, which were especially high in transplants from P1. Release rates were also observed for As and Cd in transplants transferred to P2. On the contrary, the highest positive rates (uptake) were observed in transplants exposed in the polluted sites (mainly for Al, Cu, Fe and Zn). The CV revealed greater variability in the concentrations of some elements in the autotransplants from P1 than in the crosstransplants (for Al, Co, Cr, Cu, Fe, Ni, Pb and Zn). The same was found in P2 for other elements (i.e. As, Cd and Hg). In U1 and U2, auto and crosstransplants were not clearly distinguished in terms of variability.



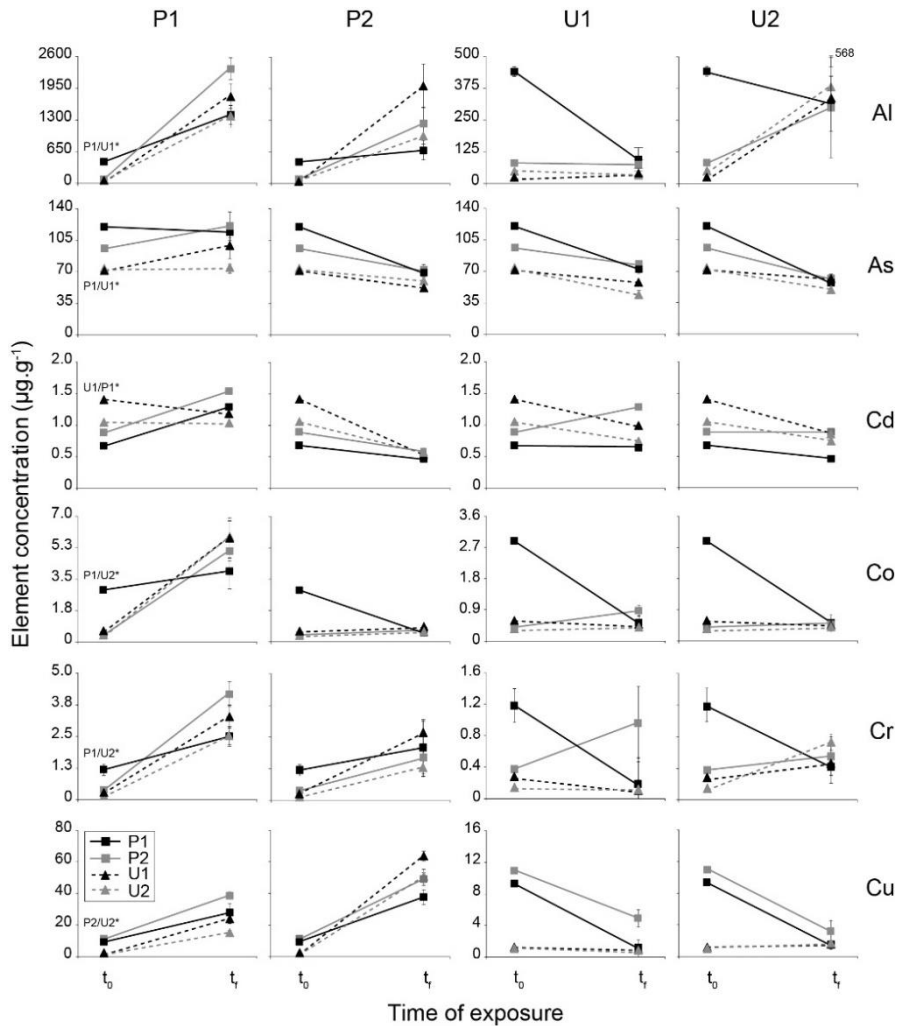


Fig. 1. Changes in the mean concentrations ($\mu\text{g g}^{-1}$ d.w. \pm SE, $n=5$) of Al, As, Cd, Co, Cr and Cu in transplants of *Fucus vesiculosus* exposed in two polluted sites (P1 and P2) and in two unpolluted sites (U1 and U2). Squares and continuous lines: specimens transplanted from the polluted sites (black lines: P1; grey lines: P2). Triangles and discontinuous lines: specimens transplanted from the unpolluted sites (black lines: U1; grey lines: U2). Asterisks indicate the comparisons in which the mean concentrations at initial time were significantly different ($p \leq 0.05$) among sites (results of Kruskal–Wallis test). Note that the scale on the ordinate axis is different for polluted and unpolluted sites. t_0 : initial time; t_f : final time (90 days).

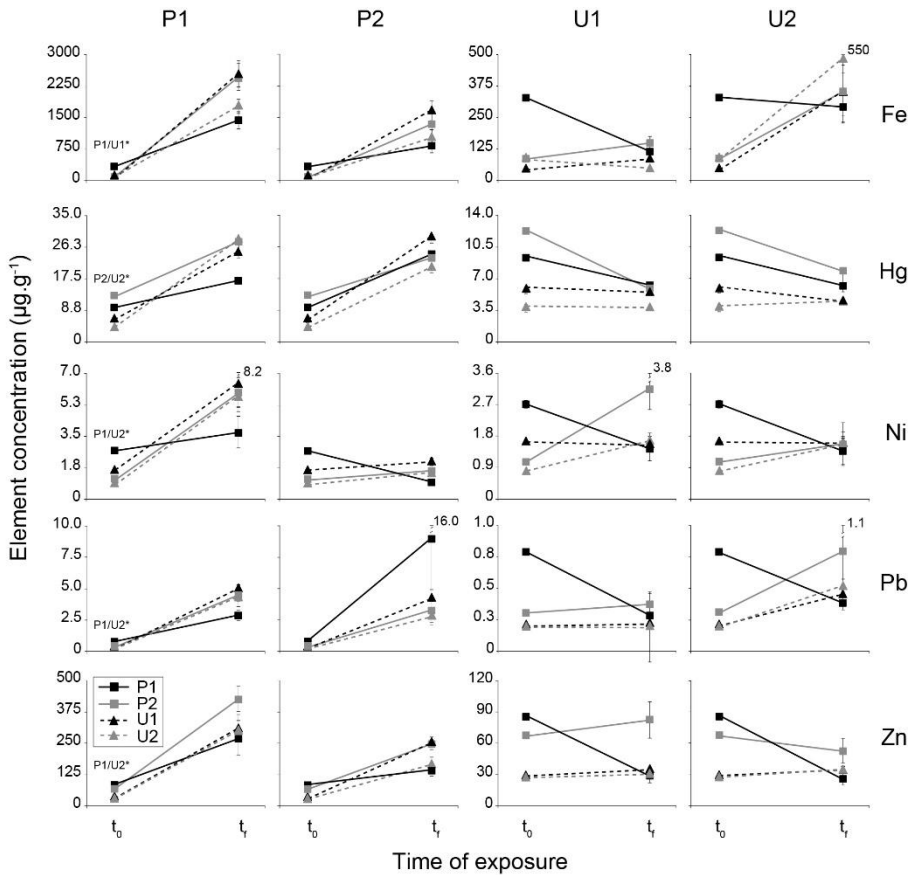


Fig. 2. Changes in the mean concentrations ($\mu\text{g g}^{-1}$ d.w. \pm SE, $n=5$) of Fe, Hg, Ni, Pb and Zn in transplants of *Fucus vesiculosus* exposed in two polluted sites (P1 and P2) and in two unpolluted sites (U1 and U2). Squares and continuous lines: specimens transplanted from the polluted sites (black lines: P1; grey lines: P2). Triangles and discontinuous lines: specimens transplanted from the unpolluted sites (black lines: U1; grey lines: U2). Asterisks indicate the comparisons in which the mean concentrations at initial time were significantly different ($p \leq 0.05$) among sites (results of Kruskal–Wallis test). Note that the scale on the ordinate axis is different for polluted and unpolluted sites. t_0 : initial time; t_r : final time (90 days).

Table 1. Results of the two-way ANOVA comparing final concentrations (t_i) in autotransplants and crosstransplants of *Fucus vesiculosus*, according to the exposure and origin site. F = F statistic; df = degrees of freedom; and $p = p$ value. Significant p values are shown in bold italics ($p < 0.001$), in bold type ($p < 0.01$) and in italics ($p < 0.05$).

Source of variation	Dependent variable	df	Final concentrations		
			Mean squares	F	Sig. (p)
Exposure site	Al	3	1.09E+07	53.43	<0.001
	As	3	7613	30.44	<0.001
	Cd	3	1.708	64.20	<0.001
	Co	3	96.81	87.46	<0.001
	Cr	3	30.31	32.03	<0.001
	Cu	3	9412	216.8	<0.001
	Fe	3	1.42E+07	89.35	<0.001
	Hg	3	1992	192.5	<0.001
	Ni	3	66.02	33.76	<0.001
	Pb	3	99.34	5.38	0.003
	Zn	3	3.31E+05	67.66	<0.001
Origin site	Al	3	7.26E+05	3.569	<i>0.020</i>
	As	3	2218	8.869	<0.001
	Cd	3	0.378	14.22	<0.001
	Co	3	0.809	0.731	0.538
	Cr	3	1.513	1.599	0.200
	Cu	3	218.3	5.028	0.004
	Fe	3	8.72E+05	5.474	0.002
	Hg	3	29.09	2.810	<i>0.048</i>
	Ni	3	4.646	2.376	0.080
	Pb	3	4.612	0.250	0.861
	Zn	3	2.10E+04	4.291	0.009
Exposure site * Origin site	Al	9	5.50E+05	2.703	<i>0.011</i>
	As	9	327.0	1.308	0.254
	Cd	9	0.094	3.546	0.002
	Co	9	1.025	0.926	0.510
	Cr	9	1.170	1.237	0.293
	Cu	9	244.6	5.635	<0.001
	Fe	9	3.45E+05	2.163	<i>0.039</i>
	Hg	9	45.69	4.414	<0.001
	Ni	9	1.853	0.948	0.493
	Pb	9	13.07	0.708	0.699
	Zn	9	4287	0.876	0.552
Error	Al	55	2.04E+05		
	As	55	250.1		
	Cd	55	0.027		
	Co	55	1.107		
	Cr	55	0.947		
	Cu	55	43.42		
	Fe	55	1.59E+05		
	Hg	55	10.35		
	Ni	55	1.955		
	Pb	55	18.47		
	Zn	55	4894		

Table 2. Mean uptake/release rates ($\text{ng g}^{-1} \text{day}^{-1}$) of the elements bioconcentrated in transplants of *Fucus vesiculosus* exposed for 90 days at each study site ($n=5$ replicates). For Al, Fe and Zn, the rates are expressed in $\mu\text{g g}^{-1} \text{day}^{-1}$. Value of the coefficient of variation: CV (%); polluted sites: P1, P2; unpolluted sites: U1, U2.

Exposure Origin site	Al		As		Cd		Co		Cr		Cu		Fe		Hg		Ni		Pb		Zn	
	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV
P1	10.6	45	-64.1	255	6.8	33	11.5	208	14.6	67	202.3	74	12.3	42	82.6	28	11.1	199	23.3	46	2.0	82
	25.2	22	27.61	145	7.2	22	51.7	25	42.3	29	306.8	18	26.5	30	169.9	42	54.1	39	46.4	25	4.0	31
	19.6	32	310.1	118	-2.6	111	58.1	47	33.7	33	253.5	31	27.9	28	210.4	21	54.1	76	53.3	15	3.1	43
	14.8	35	17.5	648	-0.3	732	61.4	33	26.7	30	155.6	20	18.8	19	269.2	34	55.4	27	46.2	36	3.0	29
P2	2.7	190	-563.3	15	-2.4	35	-26.5	10	9.8	286	315.7	37	5.5	74	163.7	16	-19.2	21	91.8	189	0.6	98
	12.7	60	-273.2	56	-3.3	37	2.7	42	14.2	68	422.9	22	13.9	46	119.6	47	6.0	49	33.1	47	2.0	16
	22.1	50	-212.2	25	-9.7	20	2.3	105	26.5	44	692.5	11	18.1	31	253.7	18	5.0	112	44.3	42	2.5	26
	10.3	42	-138.2	36	-5.4	6	2.5	64	13.2	51	549.9	22	10.5	45	184.4	21	8.0	65	28.3	38	1.5	47
U1	-3.8	30	-527.3	24	-0.2	581	-26.3	19	-11.2	14	-91.7	8	-21.48	25	-35.4	50	-14.3	61	-5.6	29	-0.6	26
	-0.1	561	-213.8	43	4.5	32	5.2	72	6.6	173	-68.8	40	0.7	93	-73.0	12	23.5	65	0.8	304	0.2	251
	0.2	1	-156.4	19	-4.8	29	-1.7	42	-1.9	17	-4.3	147	0.5	31	-6.2	115	-1.0	307	0.2	1	0.1	48
	-0.2	1	-315.5	37	-3.3	99	1.0	165	-0.2	574	-6.1	0	-0.4	61	-2.4	116	9.7	58	-0.03	2238	0.04	179
U2	-1.4	151	-712.3	21	-2.3	33	-26.4	18	-8.5	22	-88.0	9	-0.4	308	-35.9	66	-15.1	53	-4.5	36	-0.7	16
	2.4	127	-389.5	4	0.04	6868	1.4	175	1.9	198	-87.5	25	3.0	64	-50.5	73	5.7	165	5.4	92	-0.2	119
	3.6	126	-114.6	91	-6.0	34	-1.4	125	2.2	210	2.3	357	3.5	56	-17.5	58	-0.4	1441	2.8	83	0.1	104
	3.8	47	-247.6	35	-3.3	38	0.9	66	6.8	38	5.1	123	4.5	34	5.5	142	8.6	47	3.7	53	0.1	62

4. Discussion

In the present study we used an experimental design based on a transplantation technique with the objective of studying the differences in heavy metal accumulation capacities among transplants of *F. vesiculosus* exposed in different environments. To accomplish this, we selected a number of sites with high and low levels of contamination and exposed at each of those sites, algal samples from other sites (crosstransplants) and native algae from the exposure site (autotransplants). We found that our approach (in relation to the choice of sites) was adequate for the established objectives, because the differences in the concentrations among sites at the beginning of the experiment confirmed that P1 was the most polluted site, followed by P2, and that U1 and U2 were not polluted, or were affected by much lower levels of pollution than P1 and P2 (Figs. 2 and 3).

Our methodology is an improvement on that used in other studies assessing the response of algae to environmental heavy metal exposure using transplantation techniques (e.g. Eide et al., 1980; Ho, 1984; Amado Filho et al., 1999; Hédouin et al., 2008; Sáez et al., 2015), because these researchers did not use autotransplants, but compared the concentrations in crosstransplants with those in the native algae growing in the site. However, as previously mentioned, this comparison is not strictly valid because the algae collected directly from natural populations and analysed are not subjected to the effect of the transplantation, that may add an extra layer of variation in the final concentrations obtained (Sáez et al., 2015).

After comparing the final concentrations of heavy metals in autotransplants and crosstransplants reached after 3 months of exposure in the different sites, we observed the following: i) the concentrations in the transplanted algae from the unpolluted sites (U1 and U2) and from the polluted site P2 (generally less contaminated than P1) increased and exceeded those in the autotransplants from P1 for all elements studied, in at least one of the autotransplants/crosstransplants comparisons, and ii) algae from P1 and P2 transplanted to the unpolluted sites responded

rapidly to the low metal concentrations, with a large reduction in the metallic content of their tissues to values close to or even lower than the autotransplants for all elements (except Cu and Hg in transplants from P2) (Figs. 2 and 3). These observations confirm, first of all, that metal levels in algae vary dynamically, i.e. algae are able to modify their metal burden in response to the levels of these elements in their surrounding environment. Secondly, the fact that transplants originally growing in less polluted environments are capable of taking up higher levels of metals than native algae when growing under the same conditions, suggests that populations of *F. vesiculosus* differ in their capacity to take up heavy metals.

Although it was initially thought that the accumulation process entailed such a strong bond that the release of metals to seawater was not possible (Munda, 1978), reduction (release) of metal concentrations in algal specimens transferred from polluted to unpolluted sites was previously observed in brown algae (e.g. Eide et al., 1980; Andrade et al., 2006) and described as a metal detoxification mechanism, probably associated with exudation of chelated metal compounds (e.g. Karez & Pereira, 1995; Gledhill et al., 1999; Vasconcelos & Leal, 2001; Pinto et al., 2003). Other authors such as Eide et al. (1980) and Amado Filho et al. (1999) attributed the reduction in metal levels in two species of brown algae to a dilution effect associated with tissue growth during exposure in a metal-free environment. However, our findings are not consistent with the dilution effect hypothesis as we did not observe a reduction in the concentrations of metals in the crosstransplants or autotransplants exposed in the unpolluted sites. On the contrary, concentrations in autotransplants from U1 and U2 remained basically unchanged or increased slightly between t_0 and t_f , the final concentrations were not significantly affected by growth of the algae.

The total metal content in the algae is thus the result of the inputs and outputs from different cellular compartments (i.e. extracellular, intercellular and intracellular) plus the particulate matter adhered to the surface of the thallus and intracellular particles. Metals can also be adsorbed extracellularly to the cell wall or taken up and sequestered in cells

(Morris et al., 1999; Costas & López, 2001; Fink & Manley, 2011). Polyanionic polysaccharides (negatively charged and composed mainly of alginic acids and sulphated fucans) are the main constituents of the cell wall and intercellular matrix and act as ion-exchanger and ionic barrier (Percival, 1979; Veroy et al., 1980; Andrade et al., 2004). These compounds are able to retain metal cations from the surrounding environment by exchanging them with counter ions (e.g. Na^+ , Ca^{+2} and Mg^{+2}) bounded to polyanionic sites, such as amino, carboxyl, phosphate and sulphate functional groups (Percival & McDowell 1967; Mykkestad et al., 1978; Żbikowski et al., 2007). The content of a particular metal in the cellular wall will be therefore determined by the ion selectivity of the polyanions and the competition among metallic ions (Haug & Smidsrød, 1967; Sinnott, 2007; Ryan et al., 2012). When absorbed in the cytoplasm, metal ions are chelated by intracellular macromolecules such as polyphenols, metallothioneins and phytochelatins (Mykkestad et al., 1978). It has been suggested that polyphenols, which have a high capacity to chelate heavy metals and are present in high proportions in brown algae (Ragan, 1976; Targett et al., 1992), may accumulate some metals in brown algal tissues (e.g. Ragan et al., 1979; Pedersén, 1984; Karez & Pereira, 1995; Hédouin et al., 2008).

Based on our knowledge of the mechanisms involved in metal uptake in algae, and on our previous experience, we believe that high proportions of metals in algae are bound to the cell wall polysaccharides and are not localized in the intracellular compartment of the cells, as concluded by Ryan et al. (2012). Our findings in a recent field study with *F. vesiculosus* transplants (unpublished data), showed that the species is able to take up and release Hg in only 5 days; this would probably not be possible if most of the Hg were localized intracellularly, as it would possibly entail a high cost in terms of energy. We therefore hypothesize that the differences we observed in the capacity of the different populations to take up/release heavy metals (intraspecific variability) may be due to the modification of one or more structural components of the cellular compartments involved in metal uptake, and probably to differences in the cell wall polysaccharide

composition. Native algae from the most polluted sites (P1 in the present study), subjected to chronic elevated metal exposure, would have suffered such changes in response to polluted-induced stress, and would have a lower metal uptake capacity than those living in slightly polluted or unpolluted environments (e.g. Morris et al., 1999; Ma et al., 2000; Hédouin et al., 2008). Some authors have suggested that adaptation to this type of environments may imply the genetic differentiation of organisms subjected to the selective pressure generated by contamination (Hédouin et al., 2008; Ritter et al., 2010; Sáez et al., 2015), giving rise to ecotypes of the same species with differential tolerance to heavy metal excess (Moenne et al., 2016). For this reason, algal transplants may therefore be more sensitive biomonitors than native algae, reflecting more accurately the level of metal pollution in the environment, as they will not be affected by the resistance mechanisms that may occur in native populations (e.g. Hédouin et al., 2008; Pereira et al., 2014; García-Seoane et al., 2018), in addition to other advantages over the use of native macroalgae, as e.g. the ability to be exposed in places where resident species do not occur (e.g. Søndergaard et al., 2014; Jona-Lasinio et al., 2015).

5. Conclusions

The linear relationship between metal concentrations in algae and in the surrounding environment is used to justify the use of these organisms as pollution biomonitors. However, our findings suggest that this relationship may not occur in algae continuously exposed to high concentrations of metals. Hence, we recommend the use of transplants (active biomonitoring) rather than native algae (passive biomonitoring) to assess the level of metal contamination, in order to prevent the effect of changes in the metal uptake capacity (tolerance/resistance mechanisms) as a result of chronic exposure to contamination.

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APPENDIX.
SUPPLEMENTARY MATERIAL

APPENDIX. SUPPLEMENTARY MATERIAL (CHAPTER V)

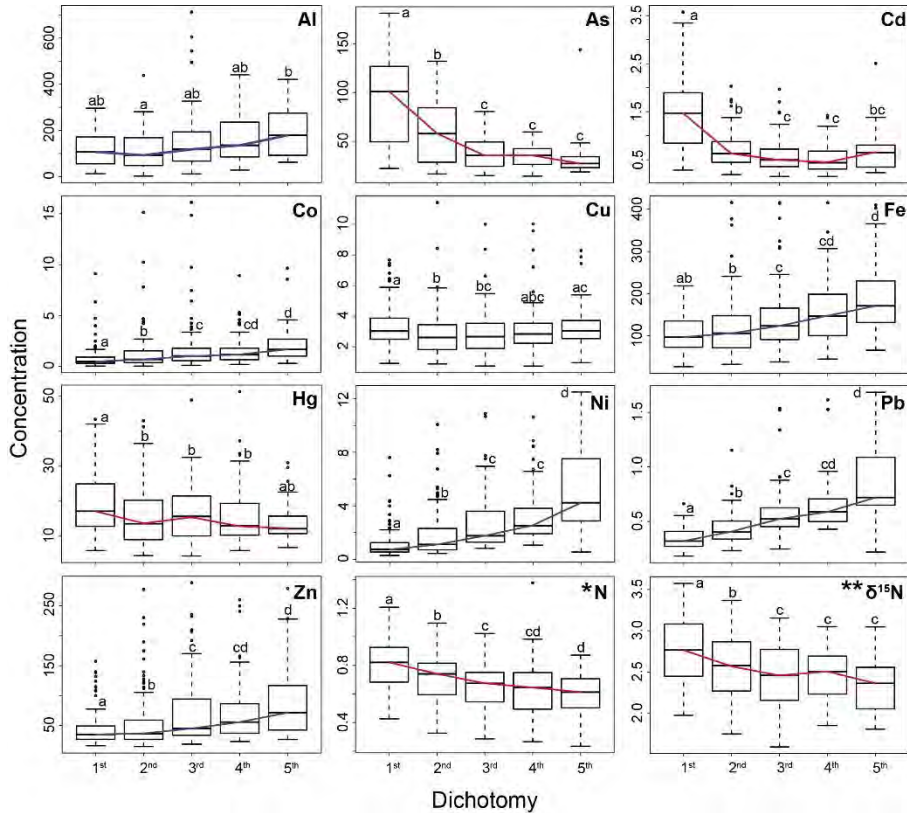


Fig. S1. Boxplots of the element concentrations in the five dichotomies of the thallus of *Fucus vesiculosus* (1st–apical, 5th–basal) from all sites under study. The whiskers extend from 1.5 IQR (interquartile range) of the lower quartile to the 1.5 IQR of the upper quartile. Those data points that exceed these limits are drawn outside the boxplot. Different letter(s) above the error bars denote significant differences of mean concentrations among dichotomies based on results from Tukey's Post-Hoc test ($p \leq 0.05$). The concentrations are expressed in $\mu\text{g g}^{-1}$ (except *: % and **: ‰). In each boxplot: $n=108$ (1st, 2nd and 3rd dichotomies), $n=72$ (4th dichotomy), and $n=36$ (5th dichotomy). For Al and Pb, n value is respectively one-third and two-thirds lower in each dichotomy, as some of the sampling sites were eliminated from data analysis.



Fig. S2. Growth progression in one *Fucus vesiculosus* individual from site 2 (SS2). **A)** January; **B)** April; **C)** July. Growth monitoring was not possible in October.

Table S1. Description of the general periodic regression model and its parameters fitted to the time series.

Periodic regression model

$$y_t = \beta_0 + \beta_1 \sin((2\pi/\text{per}) t) + \beta_2 \cos((2\pi/\text{per}) t) + \varepsilon_t$$

Where:

y	Temporal series.
β_0	Mean of the series.
β_1 and β_2	Regression coefficients.
$2\pi/\text{per}$	"Wavelength" scale of the plots or horizontal distance between successive peaks, being per, the cycle period (or frequency) of the periodic variation.
t	Time period number, corresponding to the sampling interval ($t \rightarrow 1, 2, 3, \dots, 36$ months).
ε_t	Random error (residuals) of the series about the period component.

Table S2. Results of the Three-way ANOVA test comparing the concentrations of elements in the five dichotomous sections of the thallus of *Fucus vesiculosus*. df=degrees of freedom; SS=sum of squares; MS=mean square; F=F statistical value; Sig.=statistical significance level (*p*-value). Significant *p*-values are shown in bold type.

Source	Variable	df	SS	MS	F	Sig.
Month	Al	11	267	24.3	1.41	.166
	As	11	1.16	0.105	1.93	.036
	Cd	11	4.44	0.404	10.0	.000
	Co	11	18.8	1.72	14.2	.000
	Cu	11	2.62	0.238	13.3	.000
	Fe	11	0.992	0.090	2.21	.014
	Hg	11	0.308	0.028	7.35	.000
	Ni	11	9.61	0.874	9.77	.000
	Pb	11	0.676	0.061	1.09	.373
	Zn	11	0.380	0.034	54.2	.000
	N	11	7.05	0.641	79.1	.000
	$\delta^{15}\text{N}$	11	3.57	0.324	3.75	.000
Site	Al	2	3007	1504	87.7	.000
	As	2	2.56	1.28	23.5	.000
	Cd	2	7.32	3.66	90.8	.000
	Co	2	19.4	9.69	80.2	.000
	Cu	2	5.92	2.96	165	.000
	Fe	2	7.41	3.71	90.9	.000
	Hg	2	0.149	0.075	19.6	.000
	Ni	2	0.794	0.397	4.44	.012
	Pb	2	19.9	9.96	176	.000
	Zn	2	0.092	0.046	72.2	.000
	N	2	3.53	1.76	218	.000
	$\delta^{15}\text{N}$	2	24.1	12.0	139	.000
Dichotomy	Al	4	187	46.9	2.73	.029
	As	4	10.9	2.73	50.2	.000
	Cd	4	13.1	3.28	81.3	.000
	Co	4	10.7	2.68	22.2	.000
	Cu	4	0.542	0.135	7.56	.000
	Fe	4	3.11	0.777	19.1	.000
	Hg	4	0.072	0.018	4.70	.001
	Ni	4	20.6	5.16	57.7	.000
	Pb	4	5.55	1.39	24.5	.000
	Zn	4	0.087	0.022	34.3	.000
	N	4	2.17	0.542	66.9	.000
	$\delta^{15}\text{N}$	4	9.26	2.32	26.8	.000

TABLE S2*(Continued)*

Source	Variable	df	SS	MS	F	Sig.
Month * Site	Al	22	711	32.3	1.88	.011
	As	22	0.282	0.013	0.235	1.000
	Cd	22	1.96	0.089	2.21	.002
	Co	22	1.69	0.077	0.635	.897
	Cu	22	1.49	0.068	3.78	.000
	Fe	22	1.76	0.080	1.97	.007
	Hg	22	0.099	0.005	1.19	.255
	Ni	22	2.87	0.130	1.46	.089
	Pb	22	1.66	0.075	1.33	.151
	Zn	22	0.034	0.001	2.41	.000
	N	22	1.23	0.056	6.93	.000
	$\delta^{15}\text{N}$	22	5.81	0.264	3.05	.012
Month * Dichotomy	Al	44	200	4.54	0.265	1.000
	As	44	0.335	0.008	0.140	1.000
	Cd	44	0.663	0.015	0.373	1.000
	Co	44	1.63	0.037	0.307	1.000
	Cu	44	0.693	0.016	0.879	.689
	Fe	44	0.349	0.008	0.195	1.000
	Hg	44	0.075	0.002	0.451	1.000
	Ni	44	1.61	0.037	0.410	1.000
	Pb	44	0.618	0.014	0.248	1.000
	Zn	44	0.030	0.001	1.08	.341
	N	44	0.130	0.003	0.364	1.000
	$\delta^{15}\text{N}$	44	1.51	0.034	0.398	1.000
Site * Dichotomy	Al	8	191	23.9	1.39	.200
	As	8	0.122	0.015	0.280	.972
	Cd	8	0.248	0.031	0.769	.630
	Co	8	0.143	0.018	0.148	.997
	Cu	8	0.186	0.023	1.30	.244
	Fe	8	0.095	0.012	0.293	.968
	Hg	8	0.018	0.002	0.608	.771
	Ni	8	0.305	0.038	0.426	.905
	Pb	8	0.208	0.026	0.460	.884
	Zn	8	0.008	0.001	1.65	.110
	N	8	0.021	0.003	0.328	.954
	$\delta^{15}\text{N}$	8	0.113	0.014	0.163	.995

TABLE S2*(Continued)*

Source	Variable	df	SS	MS	F	Sig.
Month * Site * Dichotomy	Al	88	714	8.11	0.473	1.000
	As	88	0.475	0.005	0.099	1.000
	Cd	88	1.13	0.013	0.318	1.000
	Co	88	1.29	0.015	0.121	1.000
	Cu	88	0.703	0.008	0.446	1.000
	Fe	88	1.12	0.013	0.313	1.000
	Hg	88	0.112	0.001	0.334	1.000
	Ni	88	1.18	0.013	0.150	1.000
	Pb	88	1.77	0.020	0.355	1.000
	Zn	88	0.010	0.000	0.177	1.000
	N	88	0.236	0.003	0.331	1.000
	$\delta^{15}\text{N}$	88	1.39	0.016	0.184	1.000
Residuals	Al	233	3993	17.1		
	As	252	13.7	0.054		
	Cd	251	10.1	0.040		
	Co	250	30.2	0.121		
	Cu	250	4.48	0.018		
	Fe	252	10.3	0.041		
	Hg	250	0.953	0.004		
	Ni	251	22.4	0.089		
	Pb	252	14.3	0.057		
	Zn	251	0.162	0.001		
	N	252	2.04	0.008		
	$\delta^{15}\text{N}$	252	21.8	0.086		

Table S3. Description of the correlation patterns in time series of the different elements, for each dichotomy of the *Fucus vesiculosus* thallus at the sampling sites (SS). Correlations are shown from lag 1 to lag 35. Upward and downward arrows represent positive and negative correlation coefficients, respectively. Significant correlations (whether positive or negative) are showed in bold type. Background shading has been included to facilitate interpretation of correlation patterns.

		Dichotomy																																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	
Al	SS1	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
	SS2	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
	SS3	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
As	SS1	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
	SS2	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
	SS3	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
Cd	SS1	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
	SS2	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
	SS3	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
Co	SS1	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
	SS2	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
	SS3	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
Cu	SS1	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
	SS2	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
	SS3	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
Fe	SS1	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
	SS2	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
	SS3	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑



General Discussion



As primary producers of great ecological relevance that play a key role in the metals transfer cycle in marine food chains (Conti et al., 2007; Conti & Finoia, 2010), macroalgae have been extensively used in monitoring studies of marine pollution worldwide for 50 years. However, these organisms have not been included in environmental monitoring programmes under the current legislation yet (WFD, 2000/60/EC; MSFD, 2008/56/EC), despite their use presents several advantages when compared to other organisms already included (e.g. fish and invertebrates). This fact led us to investigate the reasons why macroalgae are not included in such programmes, and to design several experimental studies that will contribute to improving the methodology that makes use of macroalgae to monitor pollution in coastal environments.

The detailed review of the work carried out on the use of macroalgae in biomonitoring studies worldwide (Chapters I and VI) revealed that there is no well-established methodology for passive nor active biomonitoring with marine macroalgae. This is likely the most important factor preventing macroalgae from being included in current environmental monitoring programmes. Therefore, we focused our efforts in identifying the most relevant methodological aspects influencing the final results of both passive and active biomonitoring studies, and proposed one protocol for each technique. These protocols included recommendations based on scientific criteria, when the aspect had been studied in depth, or on the most commonly used option, when the aspect had not been investigated at all. In Chapter I these aspects were grouped into three main methodological blocks relevant for passive biomonitoring: i) pre-sampling issues; ii) sample collection; and iii) sample processing. In Chapter VI we defined four blocks relevant for active biomonitoring: i) selection and preparation of the macroalgae; ii) preparation of the transplants; iii) exposure of the transplants; and iv) post-exposure treatments. These two protocols will contribute to the standardization of the two techniques, a necessity pointed out before by some authors (e.g. García-Sanz et al., 2010; Malea et al., 2015), although more research is needed in most of these aspects for complete standardization.

Since optimizing each methodological variable is a laborious and time-consuming procedure, we focused our efforts in optimizing some methodological aspects of the passive monitoring technique related to pre-sampling issues (collection period, described in Chapter V), sample collection (Chapters III and IV), and sample processing (selection of material for analysis, Chapter V). One of the main problems when conducting a biomonitoring study with algae is designing the sampling strategy, i.e. defining how many samples, and how they need to be collected within each sampling site (SS) in order to better represent the actual levels of pollutants in each site. In the literature it has been established that collection of several subsamples and their combination in a single composite sample (Phillips & Segar, 1986) provides a better representation of the real concentrations of the SS. However, until now, it had not been studied in depth if the concentrations of pollutants present a spatial structure at the SS level, nor the level of variability of these concentrations in the algae. In addition, the minimum number of samples required to distinguish significantly SS subject to different levels of contamination also remained unclear. If these aspects are not taken into account when designing a sampling plan, the quality of the results obtained will be significantly reduced, compromising the conclusions of the studies. In Chapters III and IV, we investigated these aspects in detail, and observed that there is a high intra-SS variability in the concentrations (local variability), that is dependent on the element studied. Based on that, and in the existence of concentration gradients within the SS, we concluded that to obtain more accurate results, the best sampling strategy is: collection of not less than 30 subsamples evenly distributed within 3 bands parallel to the coastline along the SS (and also at different heights on the rocks if possible) and bulking in a composite sample. Furthermore, we determined that collection of 20 samples in each SS is necessary to statistically differentiate SS with mean concentrations that differ by at least 30%.

Other aspects which have been broadly identified by authors over the years as the main sources of variability in the concentrations of

contaminants in algae are the temporal changes of such concentrations (e.g. Martin et al., 1997; Villares et al., 2013) and the tissue-specific patterns in concentrations (e.g. Malinovskaya, 1998; Stengel et al., 2005). Intra-annual/seasonal variability in the concentrations of elements in algae must be investigated with the aim of establishing the frequency of sampling required to yield representative annual mean values, otherwise samples collected in different months/seasons will not be comparable. Intra-thallus variability has to be studied and taken into account in order to select a homogeneous and representative material that enables comparison of SS and studies. Thus, based on results from Chapter V, we proposed that two samplings separated by an interval of 6 months be carried out each year, and the samples combined in a composite sample to represent the intra-annual variability. On the other hand, regarding the intra-thallus variation, the three apical dichotomies of the thallus should be selected to form a composite sample that integrates this variability.

Brown macroalgae have been frequently used to identify potential sources of pollution, as well as to study the area of influence of a particular source. However, in order to accomplish this, it is necessary to characterize first the natural range of variability in the concentrations of elements in the algae (Carpenter et al., 1991; Riget et al., 1997), given that heavy metals may occur naturally in these environments (Carral et al., 1995). Thus, in Chapter II, we characterized the range of natural variation of certain elements in algae, and used this information to prove that they constitute a valuable tool to identify potential sources of pollution. Chronic exposure to high concentrations of pollutants may lead to phenotypic and even genotypic differentiation of populations to survive in such conditions (Nielsen et al., 2003; Hédouin et al., 2008). The results reported in Chapter VII support this hypothesis demonstrating that prolonged exposure to high levels of pollution lead to differences in the capacity of algal populations to take up heavy metals. The most important requirement for an organism to be considered a good biomonitor is perhaps the maintenance of a linear relationship, throughout the period of exposure, between the concentrations of contaminants in its tissues and

those in the surrounding environment (Philips, 1990). The results reported in Chapter VII indicate that the linearity of the bioconcentration process in algae growing nearby pollution sources has been altered, breaking one of the basic principles of biomonitoring. For this reason, we proposed the use of algae transplants over the use of native algae for the quantitative evaluation of water quality in coastal systems at least when sources of pollution are known in the area.

Finally, despite the advances reported in this dissertation, and as stated in the two literature reviews (Chapters I and VI), some other key methodological aspects of the biomonitoring technique using macroalgae still remain to be standardized. Some aspects related to the use of native algae comprise e.g. issues related with the sample collection (as the size of the SS), and other aspects associated with the sample processing (as the sample cleaning method before chemical analysis to remove particulate material adhered to the thallus surface). Although the use of transplantation techniques has been less frequently implemented in biomonitoring studies (see Chapter VI), it offers certain advantages over the use of native plants as e.g.: i) enables studies to be extended to areas where native populations of the algae species of interest are absent (Fernandes et al., 2012; Jona-Lasinio et al., 2015) and, ii) avoids the influence of some factors that may affect the interpretation of the results, since reduces the effect of the possible spatial variability of concentrations in native algae at the SS (Alquezar et al., 2013), improves the temporal interpretation of the results because the time of exposure is known (Sáez et al., 2015) and, as discussed in Chapter VII, eliminates the possible phenotypic variation of native algae in highly polluted sites (Hédouin et al., 2008). Therefore, future efforts to standardize procedures should be directed towards the optimization of the protocols implemented in monitoring technique using algae transplants rather than native algae.



General Conclusions



It has been demonstrated that *Fucus vesiculosus* can be used to establish the natural range of variability in concentrations of $\delta^{15}\text{N}$, N and Hg in coastal areas. The results also showed that a large part of this natural variability is explained by the presence of a spatial pattern (deterministic component of the variability) that is characteristic of each zone and each contaminant under study. The evaluation of the natural range of variability in concentrations of $\delta^{15}\text{N}$ using this species was useful to detect the presence of sources of small scale contamination by $\delta^{15}\text{N}$, but also enabled to determine the spatial representativeness of the sampling site (SS) (the SS can be moved a distance of up to 900 m without yielding large variations in the $\delta^{15}\text{N}$ signal, at least for the SS studied).

High inter-individual variability was observed between subsamples of *F. vesiculosus* at 3 different SS, with concentrations varying longitudinally and transversally along the SS. This revealed that the position of the subsamples within the SS influences the bioconcentrated concentrations in the tissues. The findings evidenced that, to minimize the spatial intra-SS variability in the concentrations and adequately represent the concentrations within the SS, a minimum of 30 subsamples evenly distributed should be collected within three bands parallel to the coastline along a 30–50 m wide stretch (10 subsamples in each band), and also at different heights on the rocks if possible, and bulked in a single composite sample.

The ability to differentiate significantly the mean concentrations of any element between two SS using *F. vesiculosus* will depend on the number of samples of this species collected and individually analyzed at each SS. The evidences provided in this Thesis allowed to conclude that the number of samples needed to detect such differences will be largely determined by the mean and variance in the concentrations of elements in algae tissues at each SS. Taking into account the results, a minimum of 20 samples should be collected at each SS to detect significant differences for elements whose concentrations differed by more than 30% between the SS compared.

Factors as the time of collection and the part of the algal thallus selected for chemical analysis should be taken into account when standardize the methodology for monitoring pollution levels using algae. Our findings demonstrated the existence of high intra-annual/seasonal, as well as high intra-thallus variation in the concentrations of elements in *F. vesiculosus* tissues. In order to minimize the influence of these sources of variability on the data obtained in biomonitoring studies and to ensure a good representativeness of the results, we suggested that subsamples should be collected 2 times per year separated by 6 months and combined in a composite sample. To minimize the effect of variability along *F. vesiculosus* thallus, it is proposed to make composite samples of the three apical dichotomies of thallus.

The ability of *F. vesiculosus* to take up and/or release elements can be altered when native populations of this algae grow subjected to long-term and high heavy metal pollution levels. We hypothesized that the stress induced by metallic toxicity has altered some of the mechanisms involved in the process of bioconcentration of these elements, restricting their ability to concentrate these compounds. This phenotypic alteration is interpreted as a response of the algae to overcome such extreme conditions. Therefore, we recommend to use algae transplants rather than native algae for biomonitoring studies of water quality in order to prevent the possible effect of such adaptations.

An exhaustive study of the methods used in passive biomonitoring studies with macroalgae revealed that, up to date, there is no standard protocol that enables the widespread implementation of this technique. This lack of standard methodology hampers routine use of these organisms in biomonitoring programmes. Thus, with the objective of normalizing the use of native algae as environmental tools for water quality assessment, we proposed a standard protocol. Although most of the methodological aspects included in this protocol have not yet been thoroughly investigated, our recommendations are based on the conclusions of previous studies involving this technique, especially those who considered methodological issues and, of course, in the results from the experimental studies carried out in the course of this Doctoral Thesis (the latter

are shown in bold type in the Recommendations section). The protocol is described below:

Methodological issues	Recommendations
1. Pre-sampling issues	
1.1 Sampling design	Regular sampling (grids) or transects
1.2 Number of sampling sites (SS)	As many as possible (based on the scope, degree of spatial resolution, economical and operational constraints of the survey)
1.3 Sampling density	1 SS/16 km or 1 SS/32 km ² (1 SS per 16/2 ⁿ km or 1 SS per 32/2 ⁿ km ² , when more resolution is needed)
1.4 Survey frequency	As frequent as possible (economic limitations), but firstly check for seasonality and/or intra-annual variation
1.5 Sampling period	2 samplings per year separated by 6 months (annual composite sample)
1.6 Algae species	1 species (<i>Ulva</i> sp. or <i>Fucus</i> sp.). If more than 1 species is used, qualitative interpretation
2. Sample collection	
2.1 N° of samples/subsamples	To significantly differentiate the levels of pollution between SS: minimum 20 samples (individually analyzed) per SS To represent the intra-site variability in concentrations: composite sample (minimum 30 subsamples)
2.2 Amount of sample collected	Minimum of 5 g dry weight (d.w.)
2.3 Subsamples collection strategy	Evenly distributed in the SS within 3 bands parallel to the coastline (10 subsamples/band), at different heights on the rocks if possible
2.4 Area occupied by the SS	Between 200 and 500 m ²
2.5 Size of the SS	30–50 m wide stretch
2.6 Moment of collection	Low tide (intertidal zone)
2.7 Collection depth	Less than 1 m deep in intertidal zone at low tide
2.8 Spatial representativeness of the SS	SS at 900 m away from the original SS
2.9 Distance between SS and pollution focus	Further research
2.10 Sampling precautions	Use of plastic gloves (pre-cleaned and trace metal free)/sample only material attached to substrate/no smoking Washed thoroughly in seawater in the field
2.11 <i>In situ</i> cleaning	In sealed plastic bags in refrigerated conditions
2.12 Transportation	At 4°C no more than 6 days. Never freeze samples before analysis
2.13 Storage pre-processing	
3. Sample processing	
3.1 Cleaning	Manual cleaning and scraping of epiphytes, without washing
3.2 Selection of material for analysis	Composite sample of 3 apical dichotomies in <i>Fucus</i> sp.
3.3 Drying	Lyophilizing or drying at 40°C + an aliquot at 105°C (d.w.)
3.4 Homogenization	Ground in metal free mills (not sieve)
3.5 Certified reference material for analysis	Certified reference material for macroalgae
4. Additional issues	
4.1. Correction for particulate contamination	Apply at least once per site to rule out the possible contribution of sediment

Since the development of methodology for application of the active biomonitoring technique with algae is practically inexistent, the protocol proposed below is supported by the findings reported in the available literature. In this respect, methodological issues which have never been studied in detail, like the preparation of the algae for transplant (including acclimation, cloning and culture in the laboratory), the vital status of the transplants (whether using living or devitalized material), and exposure of the transplants (e.g. duration of exposure and exposure conditions) should be addressed in the near future.

Methodological issues	Recommendations for species with laminar growth	Recommendations for species with apical growth
1. Selection and preparation of the macroalgae		
1.1 Monitoring species	1 single species (<i>Ulva</i> sp.)	1 single species (<i>Fucus</i> sp.)
1.2 Selection of material for transplant	Discs	Whole thalli
2. Preparation of the transplants		
2.1 Pre-exposure treatments	Acclimation to low nutrient conditions in studies on nutrients Further research	Acclimation to low nutrient conditions in studies on nutrients Further research
2.2 Sample cleaning	Washed in seawater in the field	Washed in seawater in the field
2.3 Cloning and culture in the laboratory	Further research	Further research
2.4 Type of transplant	Cages	Bags
2.5 Amount of algae/transplant	1 g fresh weight (f.w.)/30 cm ³	1 g f.w./100 cm ²
2.6 Vital status	Further research	Further research
3. Exposure of the transplants		
3.1 Support	Buoys	Buoys
3.2 Depth of exposure	ca. 1 m	ca. 1 m
3.3 Duration of exposure	Further research	Further research
3.4 Number of replicates/transplant	Minimum of 3	Minimum of 3
3.5 Initial concentrations and controls	3–5 initial times and 3 controls	3–5 initial times and 3 controls
4. Post-exposure treatments		
4.1 Transportation	Under refrigeration	Under refrigeration
4.2 Storage pre-processing	At 4°C no more than 6 days. Samples never frozen before analysis	At 4°C no more than 6 days. Samples never frozen before analysis
4.3 Cleaning	Washed in seawater in the field and manual cleaning	Washed in seawater in the field and manual cleaning
4.4 Selection of material for analysis	Algal discs	Green parts of the shoot or apical segments (2–3 cm)
4.5 Drying	Lyophilizing or drying at 40°C for 24 h + an aliquot at 105°C (d.w.)	Lyophilizing or drying at 40°C for 24 h + an aliquot at 105°C (d.w.)
4.6 Homogenization	Milling in metal free mills	Milling in metal free mills
4.7 Certified reference material for analysis	Certified reference material for macroalgae	Certified reference material for macroalgae





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1. Introducción

La Convención de las Naciones Unidas sobre el Derecho del Mar, de 1982, definió contaminación marina como: *“la introducción por parte del hombre, directa o indirectamente, de sustancias o energía en el ambiente marino, causando daños a los recursos vivos, siendo un peligro para la salud humana, un impedimento a las actividades marinas, deterioro de la calidad del agua de mar y reducción de los servicios”* (UNCLOS, 1982; NEPA, 2008). Según la UNESCO (2017), entre los contaminantes más importantes del agua, procedentes de actividades humanas, se encuentran patógenos microbianos, nutrientes, pesticidas, metales pesados y compuestos orgánicos persistentes. Más del 80% de todos estos contaminantes provienen de fuentes terrestres industriales, agrícolas y urbanas (Beiras, 2018; WWF, 2018). La mayor parte de la contaminación ambiental por metales se debe a actividades de origen antropogénico, como la minería y la fundición, la producción y el uso industrial, así como el uso de metales y compuestos que contienen metales (Goyer & Clarkson, 2001; Bradl, 2005).

Dado que la contaminación provoca una degradación constante de los ecosistemas costeros y marinos, la vigilancia y la evaluación de la calidad del medio marino se han convertido en una prioridad a nivel mundial, con medidas y estrategias legislativas para mitigar el impacto de la contaminación, y proteger y conservar los ecosistemas y la diversidad biológica, e.g. las Directivas Marco Europeas del Agua y el Medio Marino (WFD, 2000/60/EC; MSFD, 2008/56/EC). Las Naciones Unidas para el Medio Ambiente coordinan, desde su creación hace 40 años, el Programa de Mares Regionales, el primer enfoque mundial para la protección del medio ambiente costero y marino, que engloba a más de 143 países de todo el mundo. En la actualidad, existen 18 Convenciones y Planes de Acción de Mares Regionales para la gestión y el uso sostenible del medio ambiente marino y costero. Bajo el patrocinio de la Unión Europea, el Convenio para la Protección del Medio Marino del Atlántico Nordeste, celebrado en 1992, estableció la Comisión OSPAR, que creó un programa coordinado de vigilancia del medio ambiente para elaborar evaluaciones

periódicas a largo plazo de las sustancias peligrosas en Europa. Además de los controles hidromorfológicos, los niveles de contaminantes en estos programas se vigilan en el agua de mar, los sedimentos y la biota (incluyendo a peces, mariscos y aves marinas) para un enfoque de diagnóstico integrado. Metales pesados como Cd, Cu, Hg, Pb y Zn, que son persistentes, tóxicos y susceptibles de bioacumularse en los organismos y transferirse a las cadenas tróficas, también se incluyen en dichos programas para proporcionar una estimación más realista del impacto de estos elementos biodisponibles en el medio acuático.

Aunque el análisis de macroalgas no es obligatorio en virtud de la legislación vigente, estos organismos se han utilizado ampliamente como biomonitores de la contaminación marina (incluyendo metales pesados, contaminantes orgánicos y elementos radiactivos, entre otros) desde mediados del siglo XX. Las algas pardas han demostrado una alta capacidad para bioconcentrar una amplia variedad de iones metálicos, convirtiéndolas en uno de los bioabsorbentes más utilizados (Volesky & Holan, 1995; Davis et al., 2003). De hecho, especies como *Fucus vesiculosus*, *F. spiralis* o *Ascophyllum nodosum* se utilizan habitualmente en programas de monitorización de la contaminación por metales en todo el mundo (García-Seoane et al., 2018a,b). El uso de macroalgas ofrece ventajas importantes para ser utilizadas en biomonitorización, en comparación con otros organismos marinos incluidos en algunos programas (por ejemplo, peces o invertebrados): i) son sésiles, fácilmente identificables, muestreadas y trasplantadas, ii) tienen una amplia distribución y están disponibles durante todo el año, iii) llegan a ser muy longevas, iv) presentan amplios rangos de tolerancia ambiental, sobreviviendo en condiciones ambientales adversas y de elevada contaminación, v) las concentraciones de contaminantes en sus tejidos están fuertemente correlacionadas con las concentraciones en el agua de mar y los sedimentos, etc. (e.g. Haug et al., 1974; Phillips, 1980, 1990).

Una de las principales razones por las que las macroalgas no están incluidas en la actualidad en los programas de monitorización ambiental quizás sea la falta de protocolos estandarizados que describan cómo debe

aplicarse esta técnica. Como se verá en los Capítulos I y VI de esta Tesis, los investigadores que vienen empleado esta técnica, han aplicado una gran variedad de protocolos que difieren en aspectos como la especie de alga empleada o la forma en la que se recogen, procesan y analizan las muestras. Estas variaciones obedecen más a razones prácticas y económicas que a criterios científicos. La ausencia de protocolos basados en tales criterios limita la interpretación de los resultados obtenidos y dificulta la comparación de los resultados entre diferentes estudios. Además, la carencia de estudios que abordan cuestiones metodológicas también revela una despreocupación y falta de atención general por la estandarización de la técnica. Por este motivo, en esta Tesis también abordamos algunos aspectos básicos de la metodología con el fin de fomentar el uso de las macroalgas en estudios de monitorización extensivos regulados por la legislación vigente.

La estructura de esta Tesis pretende subsanar algunos de los aspectos poco investigados, identificados en el párrafo anterior. En primer lugar, se realizó una revisión bibliográfica de todos los estudios en los que se utilizaron macroalgas como biomonitoras de la contaminación marina, incluyendo los que utilizaron técnicas de biomonitorización pasiva (recogida de individuos que crecen de forma natural en el área de interés) y activa (individuos trasplantados desde sus áreas naturales a otros lugares). Basándonos en estas revisiones, propusimos un protocolo estandarizado que sirvió de base para los otros estudios incluidos en esta Tesis. Identificamos además los aspectos metodológicos más importantes de la técnica para los que no existe una recomendación consensuada, o que nunca han sido estudiados. Dichos aspectos se investigaron mediante estudios experimentales. Debido a que el uso de algas nativas ha prevalecido sobre al uso de algas trasplantadas, nuestros esfuerzos de investigación se centraron en la optimización de algunos de los aspectos metodológicos clave de la técnica de biomonitorización pasiva: i) el número mínimo de submuestras necesario, y la forma en la que éstas deben ser recogidas, para representar adecuadamente la variabilidad de las concentraciones dentro del sitio estudiado (Capítulo III), ii) el número de

muestras que deben ser recogidas para diferenciar significativamente los niveles de contaminación entre dos sitios (Capítulo IV), iii) el período de recogida de las muestras (Capítulo V), y por último iv) la selección de material para el análisis químico del mismo (Capítulo V). Para completar estos estudios metodológicos, en el Capítulo II se evaluó la idoneidad de las macroalgas para estudiar la variabilidad natural de diversos contaminantes en el medio marino y utilizarla como referencia para detectar focos de contaminación a pequeña escala. Finalmente, en el Capítulo VII se llevó a cabo un estudio para investigar posibles cambios en la capacidad de captación de metales en algas expuestas de forma crónica a elevados niveles de estos contaminantes.

La especie seleccionada para la realización de los estudios experimentales que componen esta Tesis Doctoral es *Fucus vesiculosus* (Linnaeus 1753) ya que: i) es la especie de alga parda más estudiada en biomonitorización de la calidad del agua quality (García-Seoane et al., 2018b), ii) reúne las características para ser un buen biomonitor (Martin et al., 1997), y iii) está ampliamente distribuida en toda la costa gallega. El sargazo vesiculoso, como se le denomina habitualmente en castellano, es una macroalga parda perenne que pertenece al Filo Ochrophyta, la Clase Phaeophyceae, el Orden Fucales y el Género *Fucus* (Strasburger et al., 2008). La etimología '*vesiculosus*' deriva de las características vesículas de aire a ambos lados del nervio central que confieren flotabilidad al talo. Aunque este es el principal rasgo distintivo de la especie, también se han descrito formas sin vesículas en costas más expuestas al oleaje, incluso en la costa gallega (Pazó & Romarís, 1979; Bárbara et al., 1995). Esta especie presenta una amplia distribución en aguas frías a ambos lados del Océano Atlántico. Es frecuente en las costas atlánticas de Europa (Mar del Norte, Mar Báltico, Groenlandia, Azores, Islas Canarias y Madeira) y América del Norte (desde la Isla de Ellesmere y la Bahía de Hudson hasta Carolina del Norte), pero se encuentra ausente en el hemisferio sur. *Fucus vesiculosus* es común en costas semiexpuestas o abrigadas, y domina en zonas rocosas del nivel medio del intermareal, a menudo con *A. nodosum*. Ocupa una

posición en la zonificación intermareal por debajo de las poblaciones de *F. spiralis*, y por encima de *F. serratus* (Carlson, 1991; Bárbara et al., 1995).

En cuanto a su morfología, esta alga se caracteriza por presentar un talo aplanado y pseudo-dicotómico ramificado con crecimiento apical, surcado por un nervio central. El talo puede alcanzar los 100 cm de longitud y se fija al sustrato rocoso por medio de un disco cónico (van den Hoek et al., 1995). Como los demás miembros del género *Fucus*, su ciclo reproductor es oogámico, con cigotos sexuales (South & Whittick, 1987; van den Hoek et al., 1995; Serrão et al., 1999). A diferencia de otras especies, como por ejemplo *F. spiralis* que es monoica, *F. vesiculosus* es dioica, presentando tanto plantas masculinas como plantas femeninas. Durante la época fértil, los gametos se producen por meiosis y se multiplican por mitosis en los gametangios de las plantas masculinas (anteridios) y de las plantas femeninas (oogonios). Los anteridios y los oogonios se forman en las estructuras reproductivas que se desarrollan en los receptáculos situados en la parte apical de las ramas. Una vez maduros, los gametos haploides (espermatozoides y óvulos) son liberados, y los cigotos diploides se producen por fertilización externa. Después de la fertilización, los cigotos se adhieren al sustrato y se convierten en un nuevo gametófito diploide (van den Hoek et al., 1995; Serrão et al., 1996; Ladah et al., 2003, 2008). Aunque la reproducción sexual es la estrategia más común en algas de este género, la reproducción asexual por clonalidad (es decir, la separación de ramas adventicias de la planta parental que actúan como propágulos vegetativos) ha sido descrita en especies del género *Fucus*, incluyendo *F. vesiculosus* (Bergström et al., 2005; Tatarenkov et al., 2005).

2. Objetivos generales

Teniendo en cuenta lo anteriormente mencionado, el principal objetivo de esta Tesis Doctoral es la realización de una evaluación exhaustiva de la metodología empleada en los estudios de biomonitorización de contaminantes (principalmente metales pesados y metaloides, pero también nitrógeno) con macroalgas marinas. Para ello, se realizarán dos

revisiones bibliográficas de los artículos disponibles publicados hasta la fecha que empleen las macroalgas marinas (nativas o trasplantadas) como organismos biomonitores. Una vez valorado el estado del arte de esta técnica, el segundo objetivo será evaluar el potencial de las algas para monitorizar la contaminación del medio marino y proponer un protocolo estándar, basado en criterios científicos, que garantice la calidad de los resultados obtenidos y permita la comparación entre estudios.

3. Objetivos, resultados y conclusiones específicos de los diferentes capítulos

3.1 Biomonitorización de contaminantes en zonas costeras mediante el uso de macroalgas: optimización de la metodología (Capítulo I).

Aunque las macroalgas se han utilizado con éxito como biomonitoras de la calidad del agua de mar desde hace décadas, a día de hoy todavía no existe un protocolo estándar que asegure la calidad de los resultados obtenidos en este tipo de estudios. Por este motivo, el objetivo de este capítulo es realizar una evaluación crítica de los distintos aspectos de la metodología empleados en el desarrollo de la técnica, con el fin de proponer un protocolo que sirva de referencia para la aplicación de la biomonitorización pasiva con algas marinas. Tras la revisión de 445 artículos publicados entre 1952 y 2016 que han tratado este tema, concluimos que la mayor parte de la literatura científica existente se centra en el uso aplicado de esta técnica, y no en el desarrollo y mejora de la metodología *per se*. Esto se refleja en que, en general, los autores no describen en detalle ni justifican la metodología elegida, y se limitan a aplicar directamente la técnica basándose en estudios previos, sin cuestionarse si los métodos que utilizan tienen el rigor científico necesario para que los resultados se consideren fiables. Sin embargo, esto no es de extrañar, ya que han sido muy pocos los trabajos que han abordado cuestiones meramente metodológicas. Por este motivo, se ha recopilado toda la información posible a cerca de los diferentes aspectos

metodológicos de la técnica y se ha propuesto un protocolo en el que cada sugerencia ha sido cuidadosa y rigurosamente contrastada con la información disponible en la literatura.

3.2 Aplicación de las macroalgas en la evaluación de la variabilidad natural de las concentraciones de contaminantes (N y Hg), y en la detección de fuentes contaminantes en ambientes costeros (**Capítulo II**).

En este capítulo se describe un nuevo método en el que se utilizan las macroalgas para la detección de fuentes de contaminación por metales y contaminación orgánica en zonas costeras. El procedimiento implica la caracterización del rango de variación natural de las concentraciones de los elementos estudiados en áreas no afectadas por fuentes puntuales de contaminación, y la consiguiente comparación de las concentraciones en el sitio de estudio con esos niveles naturales. El método se aplicó en 3 zonas de estudio, en las que se caracterizó el rango de variabilidad natural de las concentraciones de Hg, N, y la señal isotópica de N ($\delta^{15}\text{N}$) en muestras de *Fucus vesiculosus* (más de 150 sitios muestreados a lo largo de la costa), determinando diferencias en las concentraciones entre pares de muestras separadas a diferentes distancias. El método fue testado en los alrededores de fuentes potenciales de nitrógeno, y resultó ser exitoso para la detección de la contaminación por $\delta^{15}\text{N}$ a pequeña escala, lo que sugiere su utilidad para ser aplicado en otras regiones y con otras fuentes de contaminación.

3.3 Optimización de la etapa de muestreo en la biomonitorización de la contaminación por metales utilizando macroalgas marinas (**Capítulo III**).

Aunque se han aplicado un sinnúmero de estrategias de muestreo en estudios de monitorización de la contaminación con algas marinas, ningún trabajo había estudiado en profundidad la estrategia óptima para representar con exactitud las concentraciones de contaminantes en las algas en el sitio de estudio. Por este motivo, se llevó a cabo un experimento para determinar

el número mínimo de submuestras de *F. vesiculosus* que deberían ser recogidas y combinadas en una muestra compuesta, así como la forma en la que éstas deberían ser recogidas dentro de la parcela de muestreo. Para ello, se estudió la variabilidad de las concentraciones de Al, As, Cd, Co, Cr, Cu, Fe, Hg, Ni, Pb, Zn, N y $\delta^{15}\text{N}$ en 50 submuestras de esta especie, que fueron recogidas al azar dentro de una cuadrícula regular en cada una de las 3 parcelas muestreadas. La estructura espacial de las concentraciones dentro de cada sitio se estudió por medio de semivariogramas, demostrando la existencia de estructura espacial en el 88% de los casos, en los que las concentraciones variaron longitudinal y transversalmente a lo largo del mismo. Simulando 3 tipos de muestreo comunes en biomonitorización mediante técnicas de aleatorización (muestreo homogéneo, muestreo por bandas y muestreo en agregados), y en base a la variabilidad de las concentraciones encontrada, se propuso el siguiente protocolo: en cada sitio, un tramo de entre 30–50 m de ancho debe ser dividido en 3 bandas paralelas a la costa. En cada banda se deben recoger un mínimo de 10 submuestras distribuidas de forma homogénea, que se combinarán para formar una muestra compuesta (30 submuestras) que sea representativa de la variabilidad dentro del lugar. Dada la alta variabilidad de las concentraciones encontrada dentro de los sitios muestreados, y el reducido número de submuestras que se recogen habitualmente en estudios de biomonitorización (sin tener en cuenta la variabilidad dentro de la parcela en la mayoría de los casos) (ver Capítulo I), nos ha permitido concluir que los resultados y conclusiones de muchos de esos estudios se podrían poner en entredicho.

3.4 Número óptimo de submuestras de *Fucus vesiculosus* necesarias para diferenciar entre sitios expuestos a distintos niveles de contaminación por metales pesados (**Capítulo IV**).

Además de para representar la variabilidad de las concentraciones en el sitio muestreado, el número de muestras recogidas en cada sitio también es un factor que va a determinar la capacidad para diferenciar entre sitios

afectados por diferentes niveles de contaminación, o el mismo sitio a lo largo del tiempo. De esta manera, este estudio se llevó a cabo para determinar el número mínimo de muestras de *F. vesiculosus* necesario para detectar diferencias estadísticamente significativas en las concentraciones medias de Al, As, Cd, Co, Cr, Cu, Fe, Hg, Ni, Pb, Zn, N y $\delta^{15}\text{N}$ entre dos sitios. El diseño experimental fue el mismo que el utilizado en el Capítulo III. El estudio de la distribución de las concentraciones en las algas de los 3 sitios muestreados permitió estimar que, para sitios con concentraciones medias que difieren más del 30%, sería suficiente con recoger 20 muestras en cada sitio para detectar diferencias significativas para todos los elementos excepto para Cr, que requeriría un mayor número de muestras.

3.5 Análisis de la variabilidad temporal e intra-talo de nitrógeno y elementos traza en *Fucus vesiculosus*: optimización del protocolo de muestreo para propuestas de biomonitorización (**Capítulo V**).

El objetivo de este capítulo fue evaluar la existencia de variación temporal en la bioconcentración de metales pesados, metaloides y nitrógeno en *F. vesiculosus*. La variación de las concentraciones en las distintas secciones dicotómicas del talo del alga también fue estudiada con el objetivo de proponer la parte del alga que debe ser analizada en estudios de biomonitorización. Para ello, se determinaron las concentraciones de Al, As, Cd, Co, Cr, Cu, Fe, Hg, Ni, Pb, Zn, N y $\delta^{15}\text{N}$ en 5 secciones dicotómicas consecutivas del talo (desde la dicotomía apical), en individuos recogidos mensualmente durante 3 años en 3 sitios con distinto nivel de contaminación. Además, se estudió la periodicidad del crecimiento en esta especie (en relación con el número de dicotomías formadas en el talo a lo largo del año), y se discutieron las implicaciones que dicha periodicidad tendría en la interpretación de los resultados en estudios de temporalidad de contaminantes que emplean esta especie. Los resultados demostraron que las concentraciones de Co, Hg, Ni y Zn decrecieron significativamente, mientras que las de As, Cd y $\delta^{15}\text{N}$ se incrementaron significativamente a lo largo del período estudiado. El

estudio de la variabilidad intra-anual/estacional, realizado por medio de autocorrelogramas, mostró que a pesar de la alta variabilidad intra-anual en las concentraciones, el proceso de bioconcentración siguió el mismo patrón cíclico de variación repetido cada 12 meses, en todas las dicotomías y en todos los sitios estudiados: las concentraciones se incrementaron al final del invierno y decrecieron al final de los meses de verano. Este patrón fue especialmente robusto para Cd, Cu, N and Zn. Además, las concentraciones de Al, Co, Fe, Ni, Pb y Zn tendieron a incrementarse de forma significativa con la edad de la dicotomía, mientras que las de As, Cd, Hg, N y $\delta^{15}\text{N}$ experimentaron un incremento desde las dicotomías más antiguas hacia las más jóvenes. En base a estos resultados, para minimizar el efecto de la variabilidad estacional en las concentraciones, se propuso una estrategia de muestreo que consistió en recoger submuestras 2 veces al año con una separación de 6 meses y combinarlas en una muestra compuesta. Para minimizar el efecto de la variabilidad existente a lo largo de talo de *F. vesiculosus*, se propone hacer muestras compuestas de las tres dicotomías apicales del talo.

3.6 Biomonitorización de ambientes costeros empleando trasplantes de macroalgas: revisión metodológica (**Capítulo VI**).

El uso de trasplantes de macroalgas en la biomonitorización de contaminantes (biomonitorización activa) es una técnica reciente que ha surgido como alternativa al uso de algas nativas (biomonitorización pasiva). Aunque presenta ciertas ventajas con respecto a esta última, su uso no se encuentra tan extendido, la metodología utilizada es demasiado diversa y no ha sido debidamente contrastada. Así, el objetivo de esta revisión, que engloba a un total de 60 artículos publicados entre 1978 y 2017, es determinar el estado de la técnica en base al desarrollo de los aspectos metodológicos. Los resultados muestran que, al igual que ocurre en biomonitorización pasiva (ver Capítulo I), la técnica no está estandarizada, y los investigadores han prestado poca o ninguna atención a las cuestiones metodológicas. De esta manera, para permitir la

comparabilidad entre estudios, se ha propuesto un protocolo estándar que facilite la implementación de esta técnica para biomonitorizar la calidad de las aguas marinas.

3.7 Diferencias fenotípicas en la acumulación de metales pesados en poblaciones del alga parda *Fucus vesiculosus*: un experimento de trasplantes (Capítulo VII).

En este trabajo se pretende estudiar la existencia de diferencias en la capacidad de acumulación de metales pesados y metaloides en el alga *F. vesiculosus* que ha crecido de forma natural en ambientes sometidos a distintos niveles de contaminación. Con esta finalidad, se han determinado las concentraciones de Al, As, Cd, Co, Cr, Cu, Fe, Hg, Ni, Pb y Zn en individuos de esta especie trasplantados recíprocamente (trasplantes cruzados y autotrasplantes) entre 4 sitios (2 contaminados y 2 no contaminados) después de 90 días de exposición a las nuevas condiciones ambientales. Los resultados demuestran que las concentraciones en individuos procedentes de los sitios no contaminados llevados a sitios contaminados se incrementan llegando a superar los niveles en los autotrasplantes. En contraposición, las concentraciones en las algas trasplantadas desde zonas contaminadas a zonas no contaminadas tienden a reducirse hasta aproximarse a los niveles en los autotrasplantes. Estos resultados sugieren que aquellas poblaciones de *F. vesiculosus* que han crecido expuestas a elevados niveles de contaminación de forma continuada, han podido desarrollar estrategias para adaptarse a la contaminación, por ejemplo, mediante la reducción de su capacidad de acumulación de dichos contaminantes. Para evitar la posible influencia de dichos cambios en el proceso de bioconcentración de estos elementos, se recomienda el uso de trasplantes de algas en lugar de algas nativas, al menos cuando se pretenda estudiar la calidad de las aguas en sitios altamente contaminados.

4. Conclusiones generales

El alga *F. vesiculosus* ha demostrado ser útil para establecer el rango de variabilidad natural de las concentraciones de $\delta^{15}\text{N}$, N y Hg en zonas costeras. Los resultados también mostraron que gran parte de esta variabilidad natural se puede explicar por la presencia de un patrón espacial (componente determinístico de la variabilidad) que es característico de cada zona y de cada contaminante estudiado. La evaluación del rango de variabilidad natural en las concentraciones de $\delta^{15}\text{N}$ utilizando esta especie, fue útil para detectar la presencia de fuentes de contaminación de $\delta^{15}\text{N}$, pero también permitió determinar la representatividad espacial del sitio de muestreo (SS) (el SS puede ser movido una distancia de hasta 900 m sin producir grandes variaciones en la señal de $\delta^{15}\text{N}$, al menos en los sitios estudiados).

Entre las submuestras de *F. vesiculosus* recogidas en 3 SS diferentes se observó una alta variabilidad inter-individual. Las concentraciones variaron en las algas, longitudinal y transversalmente a lo largo del SS. Esto demostró que la posición de las submuestras dentro del SS influye en las concentraciones bioconcentradas en los tejidos. Los resultados también mostraron que, para minimizar la variabilidad espacial de las concentraciones en las submuestras dentro del SS, un mínimo de 30 submuestras distribuidas uniformemente, debería recogerse dentro de 3 bandas paralelas a la costa a lo largo de un tramo de 30-50 m (10 submuestras en cada banda) en el SS, y también a diferentes alturas en las rocas si es posible, y analizadas en una única muestra compuesta.

La capacidad para diferenciar significativamente las concentraciones medias de cualquier elemento entre dos SS utilizando *F. vesiculosus* va a depender del número de muestras de esta especie que se recojan y analicen individualmente de cada SS. Los resultados proporcionados en esta Tesis permitieron concluir que el número de muestras necesario para detectar tales diferencias vendrá determinado en gran medida por la media y la varianza de las concentraciones de los elementos en los tejidos de las algas en cada SS. Teniendo en cuenta esto, un mínimo de 20 muestras deberían

recogerse en cada SS para poder detectar diferencias significativas para aquellos elementos cuyas concentraciones difieran más del 30% entre los SS comparados.

El momento en el que se recogen las algas y las partes del talo que se seleccionan para el análisis químico de las mismas, son factores a tener en cuenta a la hora de estandarizar la metodología. Nuestros resultados demostraron la existencia de una elevada variabilidad intra-anual/estacional, además de variación intra-talo, en las concentraciones de los elementos en los tejidos de *F. vesiculosus*. Para minimizar la influencia de estas fuentes de variabilidad en los datos obtenidos en estudios de biomonitorización y asegurar una buena representatividad de los resultados obtenidos anualmente, proponemos que se recojan submuestras 2 veces al año con una separación de 6 meses y combinarlas en una muestra compuesta. Para minimizar el efecto de la variabilidad existente a lo largo de talo de *F. vesiculosus*, se propone hacer muestras compuestas de las tres dicotomías apicales del talo.

La capacidad de *F. vesiculosus* para capturar y/o liberar elementos se puede ver alterada cuando las poblaciones de esta alga crecen expuestas a altos y continuados niveles de contaminación metálica. Creemos que el estrés inducido por la toxicidad de los metales puede haber alterado algunos de los mecanismos involucrados en los procesos de bioconcentración de esos elementos, restringiendo su capacidad para concentrar esos compuestos. Interpretamos esta alteración fenotípica como una respuesta de las algas para poder sobrevivir en tales condiciones extremas. Por lo tanto, recomendamos que en los estudios de biomonitorización de la calidad del agua, se utilicen trasplantes de algas en lugar de algas nativas, para prevenir los posibles efectos de tales adaptaciones.

El estudio exhaustivo de los métodos utilizados en estudios de biomonitorización pasiva con macroalgas ha demostrado que, hasta el momento, no existe ningún protocolo estándar que permita el uso extensivo de esta técnica. Esta falta de metodología estandarizada impide el uso rutinario de estos organismos en programas de biomonitorización.

De esta manera, con el objetivo de normalizar el uso de algas nativas como herramientas ambientales para la evaluación de la calidad del agua, propusimos un protocolo estándar. Aunque la mayor parte de los aspectos metodológicos contemplados en este protocolo no han sido investigados en profundidad, basamos nuestras recomendaciones en las conclusiones de otros trabajos previos que utilizaron esta técnica, o bien en los resultados de los estudios experimentales llevados a cabo en el transcurso de esta Tesis Doctoral (estas últimas se muestran en negrita en la columna de Recomendaciones). Este protocolo se describe a continuación:

Aspectos metodológicos	Recomendaciones
1. Cuestiones pre-muestreo	
1.1 Diseño de muestreo	Muestreo regular (cuadrículas) o transectos
1.2 Número de sitios de muestreo (SS)	Tantos como sea posible (basado en los objetivos, grado de resolución espacial, limitaciones económicas y operativas de los seguimientos)
1.3 Densidad de muestreo	1 SS/16 km ² o 1 SS/32 km ² (1 SS por cada 16/2 ^o km ² o 1 SS por cada 32/2 ^o km ² , cuando se requiera una mayor resolución)
1.4 Frecuencia de los seguimientos	Tan frecuente como sea posible (limitaciones económicas), pero primero estudiar la existencia de estacionalidad y/o variabilidad intra-anual
1.5 Periodo de muestreo	2 muestreos al año con una separación de 6 meses (muestra compuesta anual)
1.6 Especies de algas	1 especie (<i>Ulva</i> sp. o <i>Fucus</i> sp.). Si se emplea más de 1 especie, interpretación cualitativa
2. Recogida de las muestras	
2.1 N° de muestras/submuestras	Para diferenciar significativamente los niveles de contaminación entre SS: mínimo 20 muestras (analizadas individualmente) por SS Para representar la variabilidad intra-SS en las concentraciones: muestra compuesta (mínimo 30 submuestras)
2.2 Cantidad de muestra recogida	Mínimo 5 g peso seco (p.s.)
2.3 Estrategia de recolección de las submuestras	Distribuidas uniformemente en el SS, recogidas dentro de 3 bandas paralelas a la costa (10 submuestras en cada banda), a diferentes alturas en las rocas si fuese posible
2.4 Área ocupada por el SS	Entre 200 y 500 m ²
2.5 Tamaño del SS	un tramo de 30–50 paralelo a la costa
2.6 Momento de la recogida	Marea baja (zona intermareal)
2.7 Profundidad de recogida	Menos de 1 m de profundidad en la zona intermareal con marea baja
2.8 Representatividad espacial del SS	El SS puede ser movido hasta 900 m del SS original
2.9 Distancia entre el SS y el foco de contaminación	Se necesita más investigación
2.10 Precauciones de muestreo	Uso de guantes de plástico (pre-lavados and libres de elementos traza)/muestrear solo material adherido al sustrato/no fumar

(Continued)

Aspectos metodológicos	Recomendaciones
2.11 Limpieza <i>in situ</i>	Lavado a fondo con agua de mar en el campo
2.12 Transporte	En bolsas de plástico selladas en condiciones de refrigeración
2.13 Almacenamiento antes del procesado	A 4°C no más de 6 días. Nunca congelar las muestras antes del análisis
3. Procesado de las muestras	
3.1 Limpieza	Limpieza y raspado manual de epífitas, sin lavado
3.2 Selección del material para el análisis	Muestra compuesta de 3 dicotomías apicales en <i>Fucus sp.</i>
3.3 Secado	Liofilización o secado a 40°C + una alícuota a 105°C (p.s.)
3.4 Homogeneización	Molienda en molinos que no contienen metales (no tamizar)
3.5 Material de referencia certificado para el análisis	Material de referencia certificado para macroalgas
4. Aspectos adicionales	
4.1. Corrección por contaminación particulada	Aplicar al menos una vez en cada sitio para descartar la posible aportación de sedimentos

Dado que el desarrollo de la metodología para la aplicación de la técnica de biomonitorización activa con algas es prácticamente inexistente, el protocolo que se propone a continuación se apoya en los hallazgos recogidos en la bibliografía disponible. A este respecto, deben abordarse en un futuro próximas cuestiones metodológicas que nunca se han estudiado en detalle, como la preparación de las algas para el trasplante (incluida la aclimatación, la clonación y el cultivo en el laboratorio), el estado vital de los trasplantes (ya sea utilizar material vivo o desvitalizado), y la exposición de los trasplantes (e.g. la duración de la exposición y las condiciones de exposición).

Aspectos metodológicos	Recomendaciones para especies con crecimiento laminar	Recomendaciones para especies con crecimiento apical
1. Selección y preparación de las macroalgas		
1.1 Especies monitoras	1 única especie (<i>Ulva</i> sp.)	1 única especie (<i>Fucus</i> sp.)
1.2 Selección del material para el trasplante	Discos	Talos completos
2. Preparación de los trasplantes		
2.1 Tratamientos pre-exposición	Aclimatación a bajos niveles de nutrientes en estudios de nutrientes Se necesita más investigación	Aclimatación a bajos niveles de nutrientes en estudios de nutrientes Se necesita más investigación
2.2 Limpieza de las muestras	Lavado a fondo con agua de mar en el campo	Lavado a fondo con agua de mar en el campo
2.3 Clonación y cultivo en el laboratorio	Se necesita más investigación	Se necesita más investigación
2.4 Tipo de trasplante	Cajas	Bolsas
2.5 Cantidad de alga/trasplante	1 g peso fresco (p.f.)/30 cm ³	1 g p.f./100 cm ²
2.6 Estado vital	Se necesita más investigación	Se necesita más investigación
3. Exposición de los trasplantes		
3.1 Soporte	Boyas	Boyas
3.2 Profundidad de exposición	ca. 1 m	ca. 1 m
3.3 Duración de la exposición	Se necesita más investigación	Se necesita más investigación
3.4 Número de réplicas/trasplante	Mínimo de 3	Mínimo de 3
3.5 Concentraciones iniciales y controles	3–5 tiempos iniciales y 3 controles	3–5 tiempos iniciales y 3 controles
4. Tratamientos tras la exposición		
4.1 Transporte	Bajo refrigeración	Bajo refrigeración
4.2 Almacenamiento antes del procesado	A 4°C no más de 6 días. Nunca congelar las muestras antes del análisis	A 4°C no más de 6 días. Nunca congelar las muestras antes del análisis
4.3 Limpieza	Lavado con agua de mar en el campo y limpieza manual	Lavado con agua de mar en el campo y limpieza manual
4.4 Selección del material para el análisis	Discos de algas	Partes verdes del talo o segmentos apicales (2–3 cm)
4.5 Secado	Liofilización o secado a 40°C durante 24 h+ una alícuota a 105°C (p.s.)	Liofilización o secado a 40°C durante 24 h+ una alícuota a 105°C (p.s.)
4.6 Homogeneización	Molienda en molinos que no contienen metales	Molienda en molinos que no contienen metales
4.7 Material de referencia certificado para el análisis	Material de referencia certificado para macroalgas	Material de referencia certificado para macroalgas





Al finalizar un largo trabajo de investigación, como es el desarrollo de una tesis doctoral, es inevitable sentirse orgulloso de uno mismo, por el gran esfuerzo realizado y los logros conseguidos. Sin embargo, un análisis crítico de cada uno de los momentos que te han llevado a alcanzar tus objetivos te demuestra que gran parte del mérito en tus aportaciones se lo debes a todas esas personas que continuamente te han apoyado y te han ayudado para que ese proyecto llegase a un feliz término. Por ello, aunque hoy sea yo quien firme este trabajo, es para mi un auténtico placer poder dedicar unas líneas para expresarles mi gratitud a todas ellas.

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