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

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Abstract

To advance the methodological standardization of the biomonitoring technique using macroalgae, we comprehensively characterized the intra-thallus and temporal patterns of variation in concentrations of a wide set of elements (Al, As, Cd, Co, Cr, Cu, Fe, Hg, Ni, Pb, Zn, N) and $\delta^{15}N$ signal in 6 consecutive *Fucus vesiculosus* apical dichotomous sections collected monthly over a four-year period (2015–2019) at 3 sites on the NW coast of Spain. The concentrations of Al, Co, Fe, Ni, Pb and Zn increased significantly from the youngest to the oldest dichotomies regardless of the sampling time and collection site; As, Cd, N and $\delta^{15}N$ showed the opposite trend. Time series analysis revealed a significant and consistent seasonal variation of As, Cd, Co, Cu, Fe, Hg, Ni, Zn, N and δ N concentrations, with maximum values in winter and minimum values in summer. We discussed the possible mechanisms driving these two sources of variation and proposed an efficient and effective sampling strategy to minimize their impact in the results of biomonitoring studies, in which the part of the algal thallus selected for chemical analysis and the sampling frequency were carefully considered. This protocol will improve the conclusions and comparability of biomonitoring data from coastal environments.

Keywords

Aquatic pollution; seasonality; algae; metals; $d^{15}N$

1. Introduction

From its inception as useful environmental tools in biomonitoring marine pollution in the early 1950s (e.g. Black and Mitchell, 1952), macroalgae have become one of the most commonly used biomonitors of trace elements worldwide (Bonanno and Orlando-Bonaca, 2018; García-Seoane et al., 2018). However, many basic aspects of this monitoring technique have not been fully addressed so far, despite that they can affect the interpretation of the results obtained in biomonitoring studies (Costa et al., 2020; Al-Homaidan et al., 2021). Two important aspects that are frequently overlooked are the variability in the concentrations of pollutants within the thallus of the algae and the temporal variability within a given site.

Several studies reported differences in metal concentrations between different parts of the algal thalli (intra-thallus variability), especially in fucoid species. These differences were mainly related to the age and physiological condition of the different tissues (Favero and Frigo, 2002; Savage and Elmgren, 2004). Despite this evidence, García-Seoane et al. (2018) recently pointed out that the majority of the studies using macroalgae for biomonitoring purposes do not specify the part of the thallus selected for chemical analysis. Only few of the articles reviewed (10%) specified this information and, in such cases, researchers tended to select the growing tips or apical sections of a fixed length (e.g. 1–3 cm). This demonstrates the lack of standardization of the part of the algae used for chemical analysis, which restricts the comparability of the results across different studies and limits the capacity of researchers to draw reliable conclusions (Burger et al., 2007; Sáez et al., 2012; Chalkley et al., 2019).

Another key aspect of the biomonitoring technique with macroalgae is the temporal variation in the concentrations of elements in the algae. The temporal variation, i.e. intra-annual variability, determines the temporal representativeness of the concentrations of pollutants in algae collected in a given area. If this variation is not well characterized, samples collected within the same sites in different months/seasons will not be comparable, and inter-study comparisons will also be restricted. Sample collection in most biomonitoring surveys is performed once a year, more often in summer due to better sampling conditions (García-Seoane et al., 2018). With this sampling strategy, the existence of intra-annual variation could lead to an over or underestimation of the actual mean annual concentrations at each site. 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, Villares et al. (2013) found coefficients of variation of 25%, 32%, 38% and 133% for N, Fe, Zn and Cu respectively in samples of *Fucus vesiculosus* collected fortnightly throughout a year.

Intra-annual variation has been generally attributed to changes in environmental conditions (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 (Wright and Mason, 1999; Villares et al., 2002; Malea et al., 2015). Regardless of the source, some authors proposed a strategy to minimize the effect of this variability: collection of several samples throughout the year at each site, commonly monthly, and their integration in a composite sample, an approach also known as “Time-bulking” (Phillips and Segar, 1986; Stoeppler et al., 1986; García-Seoane et al., 2019). The main problem of this approach is that the greater the magnitude of the intra-annual variability, the largest the number of samplings needed, leading to huge sampling and economic efforts. Recently, Al-Homaidan et al. (2021) tried to standardize the sampling time for biomonitoring purposes using common brown macroalgae species from the Arabian Gulf, carrying a systematic monthly sampling in 2018. These authors, however, could not give a scientific-based recommendation on optimal sampling frequency, probably due to the restricted period monitored (samples collected for less than one year), which is insufficient to characterize long-term temporal patterns of variation. The lack of a comprehensive long-term characterization of the temporal variability in the concentrations of elements in algae impedes the standardization of the optimal sampling frequency required to yield representative values of the mean annual concentrations (Malea et al., 2015; García-Seoane et al., 2018).

Some authors have adopted a different strategy to study the temporal patterns of concentrations in algae, i.e. exploit the dichotomously branched condition in some brown species like *F. vesiculosus* (e.g. Savage and Elmgren, 2004; Stengel et al., 2005; Carballeira et al., 2014). These authors suggested that, in these species, the thalli are dichotomously divided once a year, which has led to the interpretation that each dichotomy corresponds to an annual growth segment. In the case of *F. vesiculosus*, the species 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). To our knowledge, no previous studies addressed the growth periodicity of *F. vesiculosus* in terms of the number of dichotomies formed each year, although an attempt has been made for a different species in the same genus (Kashutin et al., 2019). Additionally, during past field work campaigns, we observed that the thallus forking frequency is presumably higher than previously described for this species. In this case, the use of dichotomies to study the temporal patterns of concentrations in algae would be misguided.

The present study aims to investigate the existence of intra-thallus and intra-annual variation patterns in the concentrations of trace elements, N and $\delta^{15}N$ in *F. vesiculosus* over a 4-year period in 3 sampling sites. In addition, it evaluates for the first time the relationship between intra-thallus and intra-annual variability of the concentrations of these elements, as both aspects had always been addressed separately. The possible implications that the existence of both types of variability in the tissue contents of these elements would have on the interpretation of results in biomonitoring studies will be discussed. As a result, an appropriate sampling design for routine use in biomonitoring programs, representative of the intra-thallus and intra-annual variability, will be proposed. Finally, the periodicity in the occurrence of new dichotomies in the thallus of this species, and their implications in the interpretation of the results of temporal variation studies of pollutants will also be considered.

2. Material and methods

2.1. Sampling

Samples of the brown seaweed *Fucus vesiculosus* L. (Class Phaeophyceae) were collected monthly from November 2015 to November 2019 at three sampling stations (SS) located in the coast of Galicia, NW Spain (Fig. 1). 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=557,811, Y 4,812,476; UTM 29N ETRS89), considered one of the most contaminated rias by heavy metals in the coast of Galicia (Cobelo-García and Prego, 2004). The second site (SS2) was selected within the Ría de Muros e Noia (X=506,599, Y=4,737,191), an area with scarce industrial activity. The last site (SS3) was located in the Ría de Pontevedra (X=523,837, Y=4,697,147), characterized by the presence of a paper pulp factory and several small cities in its margins.

At each SS, 30 subsamples (consisting of individual algae 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 of the concentrations. A more detailed description of the sampling, washing and sample processing protocols 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 tissues and sections heavily affected by epiphytes. As illustrated in Fig. 2, consecutive dichotomous sections of the algae thallus were separated with a glass spatula at the base of every pair of air bladders underneath the angle formed by each fork, following the method used in previous studies (e.g. Savage and Elmgren, 2004, Stengel et al., 2005; Carballeira et al., 2014). The 3 most apical dichotomies were separated during the first year of the study. One more dichotomy (4th dichotomy) was separated the next year. The same procedure was followed during the third and fourth years, in which the 5th and 6th dichotomies were included respectively. 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 Toledo XP26). Dichotomies (each of ca. 2–8 g dry weight, d.w.) were then stored at room temperature in hermetically sealed vials until chemical analysis.

2.3. *Growth measurements*

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

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 (Ethos-1, Milestone) in 3 successive steps (10 min at 100 °C, 7 min at 150 °C, 25 min at 190 °C), by adding 10 mL of HNO_3 (65%), 2 mL of H_2O_2 (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 (Agilent 7700x) at the Research Support Services Unit from Universidade de Santiago de Compostela.

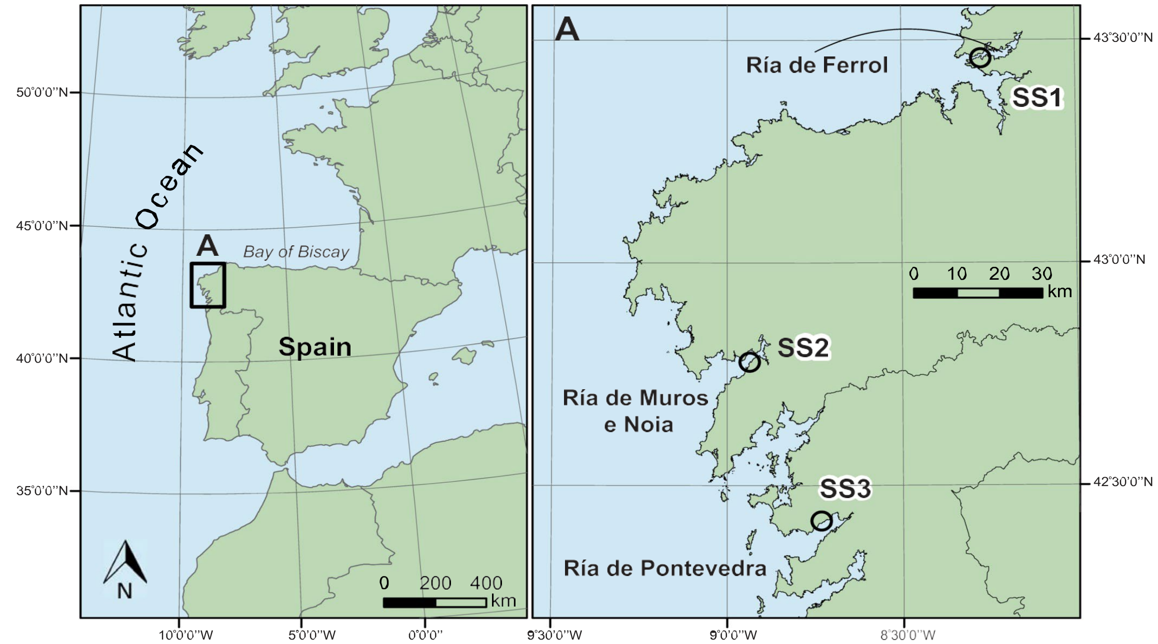

Fig. 1. Map of the coast of Galicia (NW Spain) showing the location of the three sampling sites where *Fucus vesiculosus* samples were collected in all sampling surveys (November 2015– November 2019).

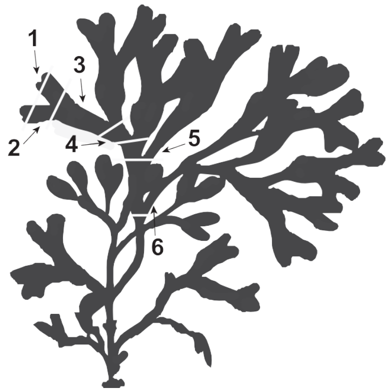

Fig. 2. Diagram showing the different sections (from 1–apical to 6–basal) used throughout the study in the dichotomously divided thallus of *Fucus vesiculosus*.

The concentrations of Hg were determined in an elemental analyzer (DMA 80 Milestone) at the Ecology Unit from the same university. For determination of %N and $\delta^{15}N$ signal, 3 ± 0.1 mg (d.w.) of sample were packaged in tin capsules (EuroVector) and processed in an elemental analyzer (FlashEA1108 Carlo Erba Instruments) coupled to a mass spectrophotometer (MAT253 ThermoFinnigan). Determinations were made at the Instrumental Techniques of Analysis Unit 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 Al (around 13%), Cr and Hg (around 10% for both elements). The percentage of recovery from the reference materials ranged between 70% (Cr) and 97% (Hg), usually around 84% for BCR–279; and between 79% (Pb) and 103% (Hg), usually around 90% for ERM–CD200. De-terminations were above the corresponding limits of quantification (LOQ), with exception of Cu (in 6% of the cases), Ni (10%), Co (14%), Cd (18%), Pb (38%), Al (40%) and Cr (76%). Data from those SS where element concentrations were below the LOQ in more than 30% of the samples, i.e. Al (SS3), Pb (SS2 and SS3), and Cr (all SS), were not included in the statistical analysis.

To simplify the readability throughout the text, although $\delta^{15}N$ signal is not an element per se, but is an isotopic relation of elements, from now on, we will refer to all the elements determined (including $\delta^{15}N$ signal) as “elements”.

2.5. Data analysis

Intra-annual variability in the concentrations at each dichotomy was studied by calculating the coefficients of dispersion (COD) as the ratio between the median absolute deviation (MAD) and the data median. Statistical analysis was performed using R-3.4.0 (R Development Core Team, 2008).

2.5.1. *Intra-thallus variability*

The normality of the concentrations of the different elements in the samples was tested using Lilliefors modifications of the Kolmogorov–Smirnov test (Dallal and Wilkinson, 1986). Elements with non-normal distributions, i.e. all elements except N and $\delta^{15}N$, were successfully normalized using Box–Cox transformations (Box and Cox, 1964): log(x) transformation for Cd, Co, Cu, Fe, Hg, Ni and Pb, sqrt(x) transformation for Al, and 1/sqrt(x) transformation for As and Zn. A Three-way ANOVA was then used to test for differences in the concentrations of the different elements (dependent variables) between the six dichotomous sections of the thallus selected along the 4-year survey. Sampling month (with 12 levels), SS (with 3 levels), and dichotomy (with 6 levels) as well as their interaction were used as predictors in the following model: concentration ~ sampling month * SS * dichotomy. A Two-Way ANOVA test was done only with data from SS1 for Pb because the concentrations of this element were < LOQ in SS2 and SS3. In this case, sampling month, dichotomy and their interaction were regarded as predictors. When the interactions were not significant but the main effect was significant ($p < 0.05$), a Tukey's Post–Hoc test was used to identify homogeneous subgroups. *P*-values were adjusted for multiple testing using the Benjamini and Hochberg (1995) method to obtain false discovery rates with the "p.adjust" function from the base package Stats.

2.5.2. *Analysis of the structure of the temporal series*

The classical analysis of time series assumes that the values of the response variable (concentrations of elements in the algae in this case) are determined by the combined effect of three components, i.e. trend, seasonal and random (Brockwell and Davis, 2002; Chatfield, 2003; Anderson, 2011). Thus, the first two components were isolated from the dataset in two sequential steps for each dichotomy and element separately. Only the first four apical dichotomies were considered in order to increase the robustness of the time series analysis (four complete years for the first 3 dichotomies, and three complete years for the 4th dichotomy). First, the trends in raw data were isolated by linear regression analysis and, applying the so-called series detrending procedure (Chat-field, 2003; Box et al., 2014), were subsequently removed from those significant (p 0.01) non-stationary time series (i.e. series whose mean and/or variance changed systematically over time). Second, to assess the degree of dependency (correlation) between observations, autocorrelograms were calculated from the regression residuals in the detrended series or directly from raw data in the stationary series (constant mean and variance over time). The "acf" function available in the package gstat was used to plot correlograms (Pebesma, 2004; Gräler et al., 2016). Correlations outside the 95% confidence interval (bounds 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 and Davis, 2002).

The seasonal variation was further studied to identify the dominant periods (frequencies) of the time series fitting periodic (cyclic) regression models with a sine and a cosine component (Chatfield, 2003). The "spectrum" and "lm" functions available in the package TSA were used to calculate the spectral density and determine the dominant periods, and to illustrate the model estimated respectively (Cryer and Chan, 2008; Shumway and Stoffer, 2017). 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 models fitting was evaluated by calculating the determination coefficients (r^2), considered significant at p 0.01. A more detailed description of the periodic regression model and its parameters can be found in Table S1 (Supplementary material).

3. Results

The annual range of variation in concentrations of the different elements in the six dichotomies of the thallus at each SS and year is shown in Table 1. Maximum concentration values reported in this study for Al, As, Cd, Co, Fe, Ni, N and $\delta^{15}N$ were observed in SS2. A general comparison between the ranges of variation within each year of study revealed that, for elements such as As, Cd, N and $\delta^{15}N$, the minimum and maximum range values decreased from the youngest to the oldest dichotomy in the SS studied. Whereas in elements like e.g. Al, Co, Fe, Ni, Pb and Zn, the minimum and maximum concentrations values were highest in the oldest dichotomy compared to the youngest. The COD usually ranged between 20% and 70% for Co, Cu, Fe, Hg, Ni and Zn. Low COD values (< 20%) were observed for N and $\delta^{15}N$. Elements such as Al, As, Cd and Pb showed higher variation in concentrations, with COD usually > 100%. The COD differed among SS, with SS3 showing higher COD than SS1 and SS2 for most of the elements. The COD values also varied among dichotomies in SS1, with the apical dichotomy being clearly less variable. No differences were observed among dichotomies in the other two SS.

3.1. *Intra-thallus variation*

The mean concentrations of all elements differed significantly among dichotomies as shown by the significant main effect of this factor in the Three-way ANOVA (p 0.001 in all cases, Table 2; Two-way ANOVA for Pb with F= 15.8 and $p<0.001$). This test showed a significant inter-action between sampling month and SS for Cd, Cu, Fe, Hg, Ni, Zn, N and $\delta^{15}N$ ($p < 0.01$), and between SS and dichotomy for Cd and Zn ($p<0.05$). Interactions were not significant for the remaining factors compared. The concentrations of the elements investigated were not homogenously distributed across the thallus of *F. vesiculosus*. Three patterns of variation were identified depending on the elements considered (Fig. 3): i) mean concentrations of Co, Fe, Pb and Zn increased up to two-fold from the youngest to the oldest dichotomy, even five-fold in those of Ni; Al also increased with the age of the dichotomy but from 2nd dichotomy onwards; ii) mean concentrations of As, Cd, N and $\delta^{15}N$ followed the opposite pattern, as they decreased up to almost half its value from the youngest to the oldest dichotomy; and

iii) mean concentrations of Cu and Hg seem to be u-shaped, with no obvious differences between young and old dichotomies (see homogeneous subgroups in Fig. 3).

3.2. *Temporal variation*

3.2.1. *Trend component*

The results of the linear regression analysis carried out in order to determine whether the time series were stationary or non-stationary are shown in Table 3. In SS1, Al, Co, Cu, Fe, Ni, Pb, N and $\delta^{15}N$ consistently exhibited stationarity (non-significant trend component) in the four dichotomies studied. In SS2 and SS3 however, all the elements showed non-stationarity at least in one dichotomy, with the exception of N in both sites and Cd in SS2. Interestingly, the 4th dichotomy consistently exhibited a stationary behavior across sites, except for Hg at SS3.

Time plots of the variation of the concentrations over time in the four apical most dichotomies of the thallus are shown in Fig. 4 for some representative elements of the different behaviors observed: i) N showing a stationary series, significant seasonal pattern and autocorrelation (Fig. 4A); ii) Zn showing a non-stationary series, significant seasonal pattern, non-significant autocorrelation (Fig. 4B); and iii) Fe showing a non-stationary series, non-significant seasonal pattern or autocorrelation (Fig. 4C). Nitrogen values remained more or less constant throughout the sampling period. By contrast, the concentrations of Hg and Zn significantly decreased from 2015 to 2019 in at least one dichotomy in all the SS (see slope values in Table 3, Fig. 4B for Zn). Likewise, a significant decrease was observed in the concentrations of Co and Ni (1st, 2nd and 3rd dichotomies), and those of Fe (2nd and 3rd dichotomies) in SS2 and SS3 (Fig. 4C for Fe in SS2). On the contrary, the concentrations of As in all SS, and $\delta^{15}N$ in SS2 and SS3, significantly increased with time in nearly every dichotomy (slope values in Table 3). In the case of As, this increment was more notable at the 1st dichotomy,

Table 1

Annual range of variation in the concentrations of elements (μg g^{-1}, except ^{a}: ng g^{-1}, ^{b}: % and ^{c}: ‰) in six dichotomies of *Fucus vesiculosus* thalli collected at each sampling site (SS) throughout the sampling period (four years, between November 2015 and November 2019). Maximum concentrations are shown in boldface for each element. Concentrations below the limit of quantification are not shown. Dic.: dichotomy.

	Year	Dic.	Al	As	Cd^{a}	Co^{a}	Cr	Cu	Fe	Hg	Ni^{a}	Pb^{a}	Zn	N^{b}	δ^{15}N^{c}
SS1	1st	1st	12.6–226	35.5–61.5	642–1027	463–1900	–	2.60–4.06	50.7–187	14.–35.5	591–2826	184 – 517	21.5–157	0.431–0.924	1.97–3.07
		2nd	17.7–281	24.9–35.9	313–779	600–2689	–	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
		3rd	30.7–310	20.3–27.3	291–836	811–3253	–	2.61–5.18	65.2–324	15.4–48.9	1648–6702	249 – 1535	34.5–235	0.286–0.721	1.73–2.58
	2nd	1st	43.3–248	61.0–158	1053–2790	254–640	–	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
		2nd	34.4–174	43.6–102	382–1027	252–888	–	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
		3rd	54.6–198	29.1–63.2	260–814	482–1250	–	1.82–4.67	76.1–186	7.28–28.4	916–2295	330 – 640	38.0–114	0.312–0.806	1.88–2.63
		4th	70.3–362	22.9–54.6	216–786	633–1573	–	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
	3rd	1st	47.8–284	88.5–148	1261–3575	347–1057	–	2.69–7.32	84.5–185	9.80–25.5	513–1243	274 – 663	25.9–77.4	0.455–1.01	2.14–3.06
		2nd	37.7–232	49.3–107	558–1708	355–4209	–	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
		3rd	62.6–327	29.1–61.5	360–1479	491–4716	–	2.09–10.0	113–378	7.92–32.6	843–10,682	414 – 1519	32.3–**288**	0.293–0.885	1.89–2.52
		4th	59.1–352	24.0–50.7	338–1376	568–4592	–	2.41 – 9.58	116–415	9.10–31.5	1061–10,636	441 – 1614	41.4–260	0.266–0.921	1.85–2.51
		5th	74.3–418	21.9–144	380–2506	509–4562	–	3.00–8.29	141–410	12.1–29.6	549–10,319	220 – **1683**	40.4–279	0.234–0.769	1.81–2.37
	4th	1st	33.1–281	109–168	1382–3928	383–1341	–	2.52–**14.5**	49.8–199	11.0–43.0	461–2693	211 – 726	22.9–74.6	0.327–0.946	1.73–3.00
		2nd	33.5–338	72.7–127	471–2339	354–1696	–	2.28–6.03	50.8–229	8.70–17.5	665–3613	982 – 833	20.1–119	0.234–0.925	1.59–2.93
		3rd	17.1–335	50.1–76.8	279–999	614–3323	–	2.68– 6.14	56.2–230	7.05–27.4	1169–3317	102 – 860	34.8–145	0.205–0.983	1.52–2.96
		4th	30.7–311	37.9–55.3	230–993	780–2381	–	2.88–6.23	62.7–245	9.50–24.9	1750–4668	323 – 934	43.9–167	0.194–0.758	1.61–2.55
		5th	39.2–326	35.6–47.1	293–989	1067–2875	–	3.51–5.78	68.3–287	12.3–27.0	2830–6258	341 – 1183	54.0–179	0.182–0.712	1.69–2.34
		6th	36.5–364	35.5–49.1	334–1011	1256–3080	–	3.92–5.88	92.6–278	12.9–28.6	3612–7192	424 – 1350	59.6–186	0.197–0.677	1.63–2.23
SS2	1st	1st	26.9–296	47.0–70.0	809–2100	491–9115	–	2.50–4.49	84.8–218	10.1–27.0	402–7613	–	18.6–139	0.732–1.21	2.53–3.25
		2nd	36.5–556	22.4–42.9	528–2029	771–15,127	–	1.80–4.39	91.9–415	11.2–30.2	710–15,140	–	14.8–219	0.663–1.08	2.41–3.05
		3rd	57.8–**712**	18.6–33.3	514–1970	1290–**16,121**	–	2.04–5.47	142–**591**	10.8–21.8	1427–**17,002**	–	21.1–207	0.608–0.950	2.56–2.82
	2nd	1st	46.3–157	105–181	1481–3269	308–1169	–	2.26–6.45	87.8–145	5.89–41.6	342–859	–	21.1–54.1	0.824–1.11	2.78–3.55
		2nd	31.7–135	60.0–132	596–1378	486–1880	–	1.47–4.09	67.3–126	4.44–24.8	576–1700	–	19.6–56.2	0.504–1.01	2.36–3.16
		3rd	34.0–187	34.3–80.5	390–913	705–3027	–	1.52–4.15	80.0–177	4.67–27.0	1025–7755	–	18.7–76.5	0.689–0.930	2.56–2.99
		4th	63.5–441	27.2–59.5	308–898	997–4165	–	1.66–4.52	111–346	5.89–24.0	1615–5569	–	23.2–92.0	0.617–0.890	2.52–2.81
	3rd	1st	22.5–212	102–162	1407–3936	351–3168	–	2.46–5.88	68.3–147	7.97–20.9	365–1740	–	26.0–71.3	0.800–1.18	3.01–**3.57**
		2nd	2.16–217	36.0–116	343–1754	86.5–4684	–	1.36–3.57	41.0–128	4.61–11.7	441–3462	–	19.5–91.1	0.436–1.09	2.69–3.21
		3rd	12.0–163	32.3–61.5	426–1474	734–7456	–	1.36–3.14	84.3–184	4.35–19.2	1259–6017	–	24.9–122	0.640–1.02	2.68–3.15
		4th	28.1–242	24.2–42.5	360–1422	998–8922	–	1.32–3.21	111–269	6.17–20.4	1757–8865	–	23.8–141	0.563–**1.38**	2.57–3.05
		5th	61.8–421	22.3–38.1	385–1382	1352–9657	–	1.73–3.74	152–403	6.76–12.4	2684–11,508	–	26.6–148	0.537–0.872	2.48–3.05
	4th	1st	40.1–206	102–**207**	1514–**5107**	387–3160	–	2.83–9.93	68.2–212	6.67–43.4	440–1736	–	23.2–88.5	0.733–1.22	2.79–3.41
		2nd	22.1–167	32.7–138	230–1559	295–2502	–	1.60–6.31	59.9–152	6.47–20.0	796–1819	–	18.8–102	0.613–1.04	2.44–3.19
		3rd	27.5–191	47.3–82.9	452–1750	1027–6171	–	1.88–5.38	71.7–169	6.37–16.3	1350–7029	–	21.0–142	0.640–0.928	2.37–3.02
		4th	31.8–403	34.2–60.3	427–1700	1256–8110	–	2.03–5.04	86.7–308	8.28–20.3	1554–9959	–	25.1–159	0.594–0.911	2.31–2.99
		5th	53.4–235	30.4–46.8	445–1462	1623–8284	–	2.48–4.93	106–255	9.53–19.2	2180–11,681	–	28.1–145	0.565–0.838	2.22–2.91
		6th	87.8–350	30.0–45.7	451–1483	1876–8148	–	2.87–5.56	139–351	10.9–19.5	3152–12,196	–	33.4–135	0.519–0.769	2.22–2.64
SS3	1st	1st	–	22.2–47.6	285 – 765	190–2016	–	0.910–3.52	29.1–148	15.8–43.4	346–4341	–	16.0–124	0.511–1.01	1.99–2.94
		2nd	–	16.5–22.7	192–728	292–2178	–	0.872–3.64	39.3–187	12.7–36.5	660–4967	–	21.3–139	0.485–0.810	1.78–2.60
		3rd	–	14.9–26.1	171–817	318–2208	–	1.13–3.66	43.3–245	10.3–31.0	1357–6872	–	29.1–116	0.471–0.728	1.60–2.40
	2nd	1st	–	48.0–132	746–1625	31.4–528	–	1.56–3.80	34.2–158	13.5–42.1	293–1753	–	19.5–50.1	0.501–1.00	2.66–3.47
		2nd	–	28.8–83.5	245–519	21.3–764	–	1.25–2.37	34.8–124	8.98–43.0	503–1221	–	17.6–48.9	0.414–0.996	2.43–3.22
		3rd	–	15.9–48.0	152–394	109–1507	–	0.986–2.31	37.9–165	10.3–30.7	827–1727	–	19.0–73.2	0.385–0.850	2.36–3.02
		4th	–	14.3–34.9	152–398	342–2037	–	1.14–2.84	44.7–246	12.2–37.2	1262–2785	–	24.4–88.9	0.361–0.792	2.14–2.89
	3rd	1st	–	44.2–156	777–2053	55.9–1072	–	1.32–5.14	26.8–135	8.59–29.4	314–1522	–	17.0–69.8	0.426–1.10	2.39–3.55
		2nd	–	31.5–93.0	260–916	84.3–1717	–	1.08–3.42	32.3–128	5.56–41.3	489–3257	–	20.6–101	0.326–1.10	2.27–3.37
		3rd	–	19.0–47.0	167–796	133–2393	–	0.739–3.22	46.1–110	6.78–23.3	914–5671	–	24.7–134	0.293–1.02	2.14–3.13
		4th	–	17.3–29.0	190–687	194–2660	–	0.731–5.58	55.1–126	6.20–22.9	1596–8466	–	28.4–141	0.323–0.900	2.18–2.85
		5th	–	18.6–26.1	232–697	295–3082	–	0.950–3.61	65.7–156	8.68–31.0	2323–12,503	–	34.8–152	0.330–0.767	1.83–2.54
	4th	1st	–	70.1–123	872–1907	113–731	–	2.26–9.44	31.4–108	15.8–**59.8**	291–1379	–	21.5–52.5	0.406–1.06	1.84–3.49
		2nd	–	52.8–84.2	232–679	128–916	–	1.71–4.32	29.9–71.9	8.82–29.2	245–1569	–	19.7–66.6	0.383–0.980	2.23–3.26
		3rd	–	31.5–50.4	142–318	195–1214	–	1.09–2.92	37.3–94.4	10.7–19.0	748–2490	–	23.8–77.0	0.335–0.904	2.20–2.90
		4th	–	24.9–88.8	146–669	273–1573	–	1.15–3.04	41.7–149	9.47–19.4	719–3864	–	22.3–88.3	0.306–0.800	1.97–3.06
		5th	–	23.9–29.4	147–303	256–1841	–	1.63–3.08	57.1–172	12.8–20.7	1872–5330	–	35.3–92.4	0.300–0.706	1.67–2.42
		6th	–	23.5–32.5	185–321	434–1788	–	2.01–3.18	64.6–209	12.9–21.9	2828–6813	–	38.8–86.5	0.306–0.657	1.34–2.40

Table 2

Results of the three-way ANOVA test comparing the concentrations of elements measured in six dichotomous sections of the thallus of *Fucus vesiculosus*, monthly collected in a four-year survey at three sites in the coast of Galicia. df: degrees of freedom; SS: sum of squares; MS: mean square; F: F statistical value.

Source	Variable	df	SS	MS	F
Month	Al	11	760	69.1	4.64***
	As	11	0.028	0.002	2.15*
	Cd	11	6.64	0.603	24.2***
	Co	11	24.6	2.23	26.3***
	Cu	11	2.51	0.228	12.8***
	Fe	11	2.41	0.219	6.62***
	Hg	11	4.28	0.389	13.2***
	Ni	11	10.7	0.971	15.4***
	Zn	11	0.502	0.046	81.3***
	N	11	11.1	1.01	133***
	$\delta^{15}N$	11	7.35	0.668	9.82***
Site	Al	1	87.5	87.5	5.87*
	As	2	0.116	0.058	48.4***
	Cd	2	15.3	7.65	307***
	Co	2	32.5	16.2	191***
	Cu	2	8.16	4.08	229***
	Fe	2	10.7	5.37	162***
	Hg	2	2.32	1.17	39.5***
	Ni	2	1.36	0.681	10.8***
	Zn	2	0.127	0.064	113***
	N	2	5.73	2.87	377***
	$\delta^{15}N$	2	36.4	18.2	268***
Dichotomy	Al	5	531	106	7.12***
	As	5	0.416	0.083	68.9***
	Cd	5	23.6	4.71	189***
	Co	5	21.0	4.19	49.5***
	Cu	5	1.46	0.293	16.4***
	Fe	5	4.66	0.931	28.2***
	Hg	5	1.62	0.325	11.0***
	Ni	5	40.9	8.19	129***
	Zn	5	0.158	0.032	56.4***
	N	5	4.44	0.889	117***
	$\delta^{15}N$	5	20.8	4.16	61.2***
Month X Site	Al	11	248	22.6	1.51
	As	22	0.012	0.000	0.452
	Cd	22	1.86	0.084	3.39***
	Co	22	2.02	0.092	1.08
	Cu	22	1.47	0.067	3.75***
	Fe	22	1.99	0.090	2.73***
	Hg	22	1.29	0.060	2.01***
	Ni	22	2.65	0.121	1.91**
	Zn	22	0.038	0.002	3.06***
	N	22	1.69	0.077	10.1***
	$\delta^{15}N$	22	9.07	0.412	6.06***
Dichotomy X Month	Al	55	393	7.14	0.479
	As	55	0.010	0.000	0.161
	Cd	55	0.458	0.008	0.335
	Co	55	1.10	0.020	0.237
	Cu	55	1.16	0.021	1.18
	Fe	55	0.513	0.009	0.282
	Hg	55	0.660	0.012	0.408
	Ni	55	1.13	0.021	0.325
	Zn	55	0.024	0.000	0.798
	N	55	0.113	0.002	0.270
	$\delta^{15}N$	55	1.98	0.036	0.530
Dichotomy X Site	Al	5	95.8	19.2	1.29
	As	10	0.007	0.000	0.610
	Cd	10	0.534	0.053	2.14*
	Co	10	0.215	0.021	0.254
	Cu	10	0.237	0.024	1.33
	Fe	10	0.242	0.024	0.731
	Hg	10	0.435	0.043	1.48
	Ni	10	0.348	0.035	0.551
	Zn	10	0.012	0.001	2.16*
	N	10	0.038	0.004	0.506
	$\delta^{15}N$	10	0.332	0.033	0.488
Dichotomy X Month X Site	Al	53	418	7.89	0.530
	As	110	0.011	0.000	0.086
	Cd	110	0.696	0.006	0.254
	Co	110	1.32	0.012	0.141
	Cu	110	0.778	0.007	0.396
	Fe	108	1.12	0.010	0.313
	Hg	110	0.865	0.008	0.267
	Ni	110	1.29	0.012	0.185
	Zn	110	0.011	0.000	0.181
	N	110	0.365	0.003	0.437
	$\delta^{15}N$	110	3.21	0.029	0.429
Residuals	Al	284	4230	14.9	
	As	432	0.520	0.001	
	Cd	427	10.6	0.025	
	Co	429	36.3	0.085	
	Cu	429	7.66	0.018	
	Fe	428	14.1	0.033	
	Hg	429	12.6	0.029	
	Ni	427	26.9	0.063	
	Zn	431	0.243	0.001	
	N	432	3.28	0.008	
	$\delta^{15}N$	432	29.4	0.068	

* $p \leq 0.05$.

** $p \leq 0.01$.

*** $p \leq 0.001$.

followed by the 2nd and 3st ones. The concentrations of Cd in SS1, and those of Cd and Cu in SS3, also increased over the period studied in the 1st dichotomy.

3.2.2. Seasonal component

Seasonal variation in tissue concentrations was studied using correlograms (Fig. 4). For most of the elements analyzed, i.e. for N and Zn in all SS (see Fig. 4A and B as examples), As, Cd, Co, Cu and $\delta^{15}N$ in SS1, Cd and Cu in SS2, and Fe in SS3, correlograms were quite similar among dichotomies within the same SS, and showed a clear seasonal pattern repeated every 12 lags (periodicity of 12 months). In the case of Cu and Hg in SS1, $\delta^{15}N$ in SS2, and Cd and Hg in SS3, this pattern was only identified in correlograms of the 1st dichotomy. These cyclic fluctuations in the concentrations were characterized by the alternation of clusters of high positive and negative autocorrelations (approximately 6 in each case), corresponding to those periods of higher and lower bio-concentration in the tissue (end–winter and end–summer months respectively) (Table S2). Specially in the case of N, significant positive autocorrelations (exceeding the significance bounds for the autocorrelation) were observed at lags 0–2, and again at lags 10–13 and 23–25, while significant negative autocorrelations were detected at lags 4–8, and again at lags 16–19 and 28–30 (Fig. 4A, Table S2). It should be noted that for the elements showing seasonal behavior, autocorrelations (both positive and negative) decreased in magnitude with increasing lag, damping down to low and insignificant levels close to zero. This periodic seasonal pattern (alternating and tapering trend) can be described as a quasi-cyclical behavior. Finally, most correlograms for Al, Fe, Hg, Ni and Pb (see Fe in Fig. 4C and Table S2) did not reveal any clear seasonal pattern at the studied scale, with the coefficients of autocorrelation varying at random around the same level.

The time plots including the fitted periodic regression models (Fig. 4, Table 3) also showed a cyclic seasonal oscillation qualitatively similar for all SS and dichotomies within the same SS. For most elements and SS, significant models showed a cyclic pattern 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 N, with maximum concentration peaks at the end of winter (February–April) and minimum at the end of summer (August–October). Concentrations of Fe in the 3rd dichotomy at SS1, and Cu in the 2nd dichotomy at SS3 showed, however, periodic fluctuations of 10 months, while for Cd in SS3, a seasonal cycle was identified in both dichotomies every 11 months. In contrast, for Al and Pb in SS1, Fe in SS2, and $\delta^{15}N$ in SS3, no significant models could be fitted for any of the dichotomies.

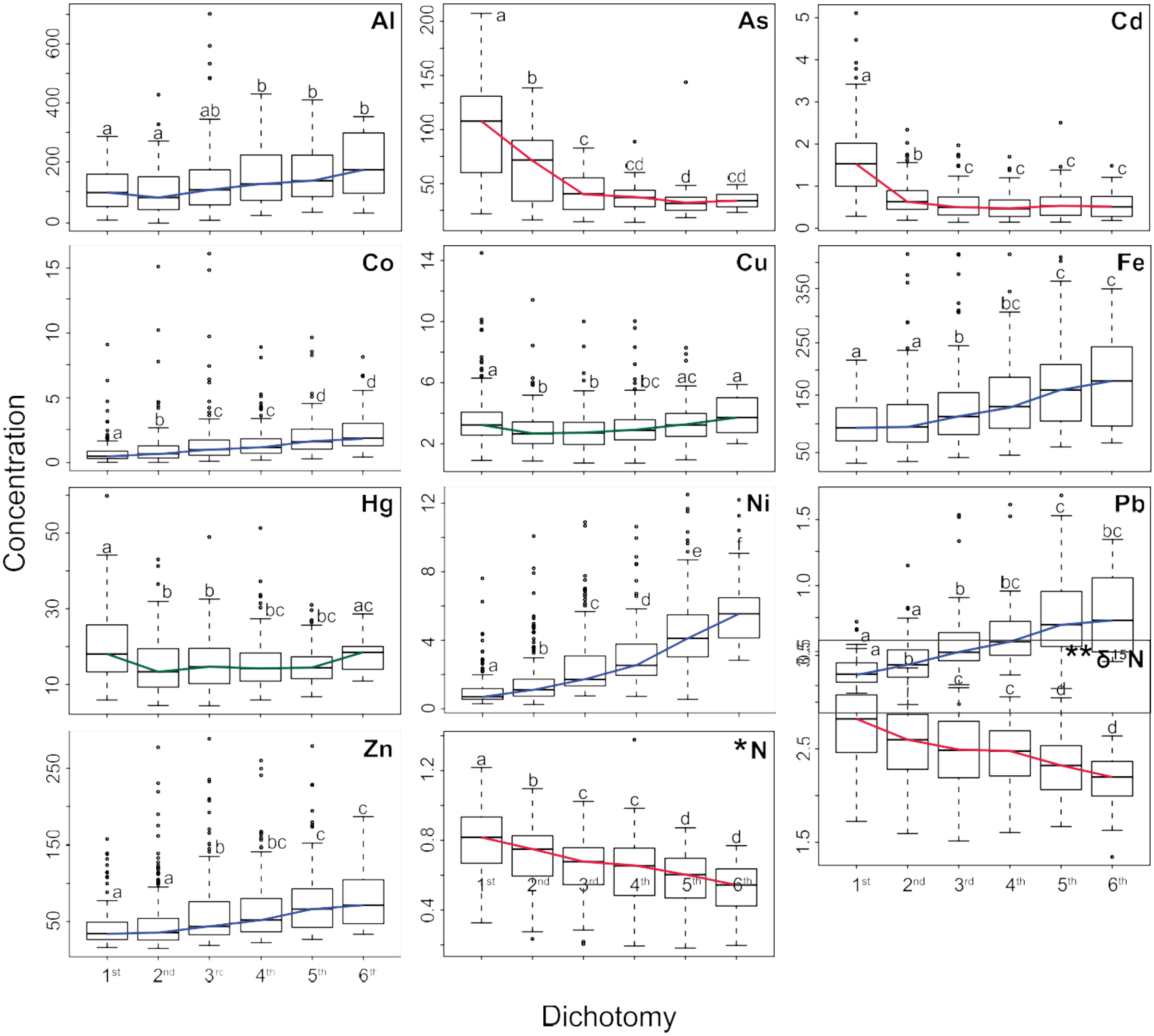

Fig. 3. Boxplots of the concentrations of the different elements in the six dichotomies (1st–apical, 6th–basal) of the *Fucus vesiculosus* thallus from the three study sites. The whiskers extend from 1.5 IQR (interquartile range) of the lower quartile to the 1.5 IQR of the upper quartile. Data exceeding these limits are drawn outside the boxplot. Different letter(s) above the error bars denote significant differences ($p \leq 0.05$) of mean concentrations among dichotomies based on results from Tukey's Post–Hoc test. Concentrations are expressed in μg g^{-1} (except *: % and **: ‰). In each boxplot: n = 144 (1st, 2nd and 3rd dichotomies), n = 108 (4th dichotomy), n = 72 (5th dichotomy), and n = 36 (6th dichotomy). For Al and Pb, the value of n is respectively one-third and two-thirds lower in each dichotomy, as some of the sampling sites were eliminated from data analysis. Lines joining the boxplots represent the three observed patterns: increasing in blue; decreasing in red, and u-shaped in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2.3. Growth measurements

Growth monitoring revealed that healthy thalli formed 2–3 new dichotomies every 3 months in the study sites (up to 5 new dichotomies in some cases), what is ca. 1 dichotomy/month (Fig. S1). The increment in length of the thallus was more variable between measurements. Thalli in SS1 and SS2 grew 7.0 ± 3.5 cm (mean SD) in 3 months after the first measurement, and 5.8 ±3.0 cm in 3 months after the second measurement, which corresponds to a growth rate of between 1.5 and 2.5 cm per month in both sites.

Several circumstances made difficult the study of the growth of *F. vesiculosus* in the field. First, some of the individual thalli died soon after the growth monitoring period started. Second, several individuals gradually disappeared at some point during the checkup. In addition, due to a strong herbivory event in the apical tissues of the algae and the significant loss of individuals, measurements were not considered in SS3. Finally, the strikingly fast growth of many individuals made it impossible to follow the number of dichotomies formed after the first six months. Although these drawbacks led to a significant loss of statistical power for the analysis, this experiment provided important preliminary information about the growth and dichotomous branching patterns of variation in *F. vesiculosus*.

4. Discussion

The main scope of applied biomonitoring studies is to characterize the spatiotemporal patterns of distribution of pollutants in the environment. However, concentrations of pollutants in tissues of bio-monitoring organisms are often subject to a number of sources of

Table 3

Time series analysis of the concentrations of the different elements in the first 4 dichotomies of *Fucus vesiculosus* corresponding to 4 (first three dichotomies) and 3 (fourth dichotomy) years of monthly measurements in three sampling sites (SS). Trend component: significant trends ($p < 0.01$) obtained in the linear regression analysis. Seasonal component: regression model parameters adjusted to the significant seasonal models ($p \le 0.01$). Dic.: dichotomy; r^2: determination coefficient; β_0: mean of the series; β_1 and β_2: regression coefficients; ϵ_t: residuals; *per*: period. The parameters β_0, β_1, β_2 and ϵ_t are expressed in μg g^{-1}, except for N (%) and $\delta^{15}N$ (‰). Further details in Table S1, Supplementary material.

			Component of the time series							
			Trend		**Seasonal**					
		Dic.	r^2	**Slope**	r^2	β_0	β_1	β_2	ϵ_t	*per*
Al	**SS1**	**1st**	–	–	–	–	–	–	–	–
		2nd	–	–	–	–	–	–	–	–
		3rd	–	–	–	–	–	–	–	–
		4th	–	–	–	–	–	–	–	–
	SS2	**1st**	–	–	–	–	–	–	–	–
		2nd	–	–	–	–	–	–	–	–
		3rd	0.26	−5.79	–	–	–	–	–	–
		4th	–	–	0.22	155	−77.4	11.6	−14.6	12
As	**SS1**	**1st**	0.47	1.95	0.31	-3.50E-04	19.4	−13.3	−1.17	12
		2nd	0.50	1.55	0.29	1.75E-04	14.9	−8.43	0.492	12
		3rd	0.46	0.80	0.39	4.52E-04	9.66	−5.03	0.001	12
		4th	–	–	0.33	42.1	5.62	−3.80	0.418	12
	SS2	**1st**	0.31	1.61	0.22	-4.03E-04	20.7	−11.8	−6.70	12
		2nd	0.20	1.09	0.15	-3.49E-04	14.1	−11.7	−1.46	12
		3rd	0.23	0.63	0.17	5.62E-04	9.95	−2.95	−3.91	12
		4th	–	–	0.22	41.4	6.74	−1.48	−0.983	12
	SS3	**1st**	0.28	1.39	0.33	-1.42E-03	15.1	−20.9	−2.05	12
		2nd	0.39	1.05	0.13	-9.22E-04	5.72	−8.78	−3.48	12
		3rd	0.27	0.39	0.16	7.23E-04	2.45	−5.01	−2.36	12
		4th	–	–	–	–	–	–	–	–
Cd	**SS1**	**1st**	0.32	0.04	0.48	1.16E-03	0.733	0.040	0.020	12
		2nd	–	–	0.35	0.816	0.333	−0.031	−0.012	12
		3rd	–	–	0.47	0.613	0.244	−0.068	−0.040	12
		4th	–	–	0.49	0.608	0.257	−0.106	−0.025	12
	SS2	**1st**	–	–	0.18	1.92	0.427	−0.297	−0.069	12
		2nd	–	–	0.52	0.890	0.225	−0.303	−0.030	12
		3rd	–	–	0.36	0.753	0.204	−0.249	−0.041	12
		4th	–	–	0.35	0.672	0.143	−0.213	−0.059	12
	SS3	**1st**	0.40	0.02	0.28	-1.80E-02	0.232	−0.135	−0.017	12
		2nd	–	–	0.25	0.435	−0.034	−0.116	−0.035	11
		3rd	0.21	−0.01	0.17	-7.50E-03	−0.082	−0.040	−0.002	11
		4th	–	–	–	–	–	–	–	–
Co	**SS1**	**1st**	–	–	0.37	0.649	0.264	−0.161	−0.010	12
		2nd	–	–	0.27	0.952	0.422	−0.383	−0.053	12
		3rd	–	–	0.34	1.32	0.503	−0.626	−0.086	12
		4th	–	–	0.34	1.46	0.467	−0.668	−0.078	12
	SS2	**1st**	0.15	−0.05	0.19	-5.99E-04	0.530	−0.894	−0.217	12
		2nd	0.20	−0.09	0.17	-4.65E-04	0.539	−1.45	−0.419	12
		3rd	0.16	−0.09	0.20	-1.03E-03	0.519	−1.94	−0.587	12
		4th	–	–	0.33	2.56	0.584	−1.50	−0.302	12
	SS3	**1st**	0.22	−0.02	0.17	-9.75E-03	0.175	−0.238	−0.002	12
		2nd	0.20	−0.02	0.16	1.14E-03	0.142	−0.309	−0.058	12
		3rd	0.15	−0.02	0.24	-3.82E-04	0.108	−0.393	−0.033	12
		4th	–	–	0.37	0.750	0.255	−0.429	−0.073	12
Cu	**SS1**	**1st**	–	–	0.38	4.55	2.05	−0.582	−0.142	12
		2nd	–	–	0.21	3.69	0.904	−0.780	−0.402	12
		3rd	–	–	0.25	3.75	0.399	−1.09	−0.288	12
		4th	–	–	0.31	4.28	0.494	−1.49	−0.304	12
	SS2	**1st**	–	–	0.25	3.82	0.695	−0.734	−0.113	12
		2nd	–	–	0.31	2.91	0.437	−0.636	−0.037	12
		3rd	0.14	−0.02	–	–	–	–	–	–
		4th	–	–	–	–	–	–	–	–
	SS3	**1st**	0.18	0.04	0.22	7.52E-04	0.674	−0.541	−0.096	12
		2nd	–	–	0.19	2.06	−0.344	0.314	−0.072	10
		3rd	–	–	0.31	1.89	−0.462	−0.156	−0.154	12
		4th	–	–	–	–	–	–	–	–
Fe	**SS1**	**1st**	–	–	–	–	–	–	–	–
		2nd	–	–	–	–	–	–	–	–
		3rd	–	–	0.36	159	38.0	49.9	−3.88	10

(*continued on next page*)

Table 3

			Component of the time series							
			Trend		**Seasonal**					
		Dic.	r^2	**Slope**	r^2	β_0	β_1	β_2	ϵ_t	*per*
		4th	–	–	–	–	–	–	–	–
	SS2	**1st**	–	–	–	–	–	–	–	–
		2nd	0.30	−3.23	–	–	–	–	–	–
		3rd	0.27	−2.91	–	–	–	–	–	–
		4th	–	–	–	–	–	–	–	–
	SS3	**1st**	–	–	0.14	65.2	8.22	17.4	−11.4	12
		2nd	0.34	−1.72	0.17	1.70E-03	2.15	21.0	−2.04	12
		3rd	0.31	−1.62	0.31	6.95E-04	2.84	27.2	−1.61	12
		4th	–	–	0.37	90.3	16.7	31.3	−7.73	12
Hg	**SS1**	**1st**	–	–	0.32	19.6	6.62	−0.176	−1.25	12
		2nd	0.34	−0.27	0.18	-6.87E-05	3.12	−1.35	−0.262	12
		3rd	0.21	−0.26	–	–	–	–	–	–
		4th	–	–	0.23	17.1	4.96	−3.14	−1.62	12
	SS2	**1st**	–	–	–	–	–	–	–	–
		2nd	0.27	−0.23	–	–	–	–	–	–
		3rd	0.33	−0.22	–	–	–	–	–	–
		4th	–	–	0.21	13.1	3.38	1.04	−0.712	12
	SS3	**1st**	–	–	0.24	25.2	5.16	5.65	−1.77	12
		2nd	–	–	0.17	17.7	3.93	4.15	−0.029	12
		3rd	0.31	−0.27	–	–	–	–	–	–
		4th	0.21	−0.32	–	–	–	–	–	–
Ni	**SS1**	**1st**	–	–	0.23	0.940	0.403	−0.155	−0.047	12
		2nd	–	–	0.19	1.67	0.813	−0.558	−0.308	12
		3rd	–	–	0.23	2.48	0.938	−1.01	−0.416	12
		4th	–	–	0.29	2.48	0.938	−1.01	−0.416	12
	SS2	**1st**	0.26	−0.05	–	–	–	–	–	–
		2nd	0.32	−0.08	–	–	–	–	–	–
		3rd	0.24	−0.08	–	–	–	–	–	–
		4th	–	–	0.44	3.74	0.701	−1.77	−0.149	12
	SS3	**1st**	0.25	−0.03	–	–	–	–	–	–
		2nd	0.28	−0.05	–	–	–	–	–	–
		3rd	0.14	−0.04	0.22	-8.19E-05	−0.424	−0.843	−0.160	12
		4th	–	–	0.23	2.78	−0.369	−1.15	−0.153	12
Pb	**SS1**	**1st**	–	–	–	–	–	–	–	–
		2nd	–	–	–	–	–	–	–	–
		3rd	–	–	–	–	–	–	–	–
		4th	–	–	–	–	–	–	–	–
Zn	**SS1**	**1st**	0.15	−0.80	0.43	−0.906	24.3	−7.72	1.46	12
		2nd	–	–	0.38	68.8	42.2	−26.3	−6.32	12
		3rd	–	–	0.45	87.4	47.3	−37.6	−2.78	12
		4th	–	–	0.55	100	44.2	−47.6	−3.13	12
	SS2	**1st**	–	–	0.38	42.5	18.1	−15.5	−4.64	12
		2nd	0.14	−1.02	0.29	-4.28E-04	17.5	−22.7	−9.45	12
		3rd	–	–	0.44	54.5	22.2	−33.1	−1.54	12
		4th	–	–	0.51	55.7	15.1	−31.1	−2.24	12
	SS3	**1st**	0.17	−0.70	0.31	-2.22E-04	13.2	−12.4	0.37	12
		2nd	0.21	−0.97	0.29	1.91E-04	10.1	−18.5	−2.02	12
		3rd	0.15	−0.76	0.34	-4.54E-04	6.79	−20.8	−4.13	12
		4th	–	–	0.46	50.8	8.38	−21.1	−0.75	12
N	**SS1**	**1st**	–	–	0.85	0.713	0.198	−0.071	0.017	12
		2nd	–	–	0.73	0.627	0.215	−0.078	0.019	12
		3rd	–	–	0.73	0.556	0.205	−0.087	0.011	12
		4th	–	–	0.85	0.531	0.231	−0.078	−0.012	12
	SS2	**1st**	–	–	0.66	0.939	0.055	−0.134	−0.004	12
		2nd	–	–	0.52	0.832	0.036	−0.145	0.007	12
		3rd	–	–	0.64	0.783	0.053	−0.107	0.005	12
		4th	–	–	0.51	0.761	0.065	−0.139	−0.003	12
	SS3	**1st**	–	–	0.73	0.750	0.160	−0.148	0.011	12
		2nd	–	–	0.60	0.690	0.146	−0.126	−0.005	12
		3rd	–	–	0.64	0.625	0.119	−0.154	0.002	12
		4th	–	–	0.71	0.584	0.141	−0.150	−0.009	12
δ^{15}N	**SS1**	**1st**	–	–	0.49	2.48	0.305	−0.077	0.010	12
		2nd	–	–	0.57	2.28	0.299	−0.173	−0.001	12
		3rd	–	–	0.66	2.17	0.269	−0.209	0.023	12

Table 3

		Component of the time series							
		Trend		**Seasonal**					
	Dic.	r^2	**Slope**	r^2	β_0	β_1	β_2	ϵ_t	*per*
	4th	–	–	0.65	2.12	0.211	−0.184	0.015	12
SS2	**1st**	0.16	0.01	0.22	3.42E-05	0.059	0.143	0.012	12
	2nd	0.16	0.01	–	–	–	–	–	–
	3rd	0.17	0.01	–	–	–	–	–	–
	4th	–	–	0.23	2.71	0.114	−0.019	0.015	12
SS3	**1st**	–	–	–	–	–	–	–	–
	2nd	0.19	0.01	–	–	–	–	–	–
	3rd	0.21	0.01	–	–	–	–	–	–
	4th	–	–	–	–	–	–	–	–

variation that are independent of these patterns (e.g. seasonality underlying temporal trends), and that could mask them to the point that they are not detectable anymore (Keogh et al., 2011). Uncovering and characterizing such kind of sources is key for the reliability and accuracy of the monitoring data, as well as for the comparability of the results in studies carried out at different time points and sites (Sáez et al., 2012; García-Seoane et al., 2018). This work provides a comprehensive understanding of two of these important sources of variation affecting the elemental concentrations in *F. vesiculosus*, while contributing to the methodological standardization of the biomonitoring technique.

4.1. Intra-thallus variation

Intra-thallus variation in concentrations of different elements has been reported before for different species of algae (reviewed in García-Seoane et al., 2018), and previously observed among dichotomies of *F. vesiculosus* (e.g. Savage and Elmgren, 2004; Carballeira et al., 2014), and among different structural parts of the thallus (e.g. Bryan and Hummerstone, 1973; Stengel et al., 2005); nonetheless, the conclusions of previous studies were based on less complete and robust datasets compared to the presented here (monthly measurements for 4 years up to 6 dichotomies 3 scenarios) and did not use the necessary statistical tools to study in depth the intra-thallus variation as well as the interactions between the concentrations in the different dichotomies and factors as the month of collection or the SS. In this study, the concentrations of all elements differed significantly among the six dichotomies analyzed showing increasing/decreasing trends with the age of the dichotomy, and the differences observed did not depend on the sampling month (dichotomy * month) nor the SS (dichotomy * site), except for Cd and Zn in the dichotomy * site interaction (Table 2); hence, intra-thallus and temporal (e.g. monthly, seasonal) variation must be approached independently.

Several mechanisms, both intrinsic and extrinsic to the algae, would contribute to the differences in the concentrations of elements among dichotomies in an element-specific manner, leading to the patterns observed in this study. Intrinsic mechanisms include: i) differences in growth rates and metabolic activity among tissues (e.g. Rice and Lapointe, 1981; Stengel et al., 2005); and ii) differences in the structural composition of the algal tissues (e.g. tissue-specific variations in cation exchange capacity and alginate concentrations) (e.g. Bryan and Hummerstone, 1973; Barreiro et al., 1993; Sez et al., 2012). Young tissues, i.e. apical most dichotomies, have higher growth rates than old tissues which, according to some authors, would lead to a higher uptake capacity due rapid increases in biomass with a higher proportion of free binding sites to capture elements (i.e. higher cation exchange capacity) (e.g. Higgins and Mackey, 1987; Stengel et al., 2005). This relationship between bioconcentration and growth rate was suggested to be metal-specific (Stengel et al., 2005; Sáez et al., 2012), which would explain why in this study the concentrations of As and Cd decreased with the age of the dichotomy but not those of the other trace elements (Fig. 3). The potential direct relationship between growth rate and uptake capacity would lead to maximum concentration peaks in summer and minimum concentration peaks in winter, as the growth rate of *F. vesiculosus* is higher in summer (Lehvo et al., 2001; Viana et al., 2015b). However, we observed exactly the opposite trend (Fig. 4) which suggests that this mechanism is not the main driver of the concentration patterns observed here.

Both young and old tissues uptake/release metals until they reach a dynamic equilibrium with the concentrations present in the environment (García-Seoane et al., 2020). These processes of element uptake and release are assumed to be faster in new and metabolically more active tissues because the ability to regulate them in macroalgae de-creases with the course of aging (Malinovskaya, 1998; Viana et al., 2015a). According to this, the younger more active growing regions of the thallus (i.e. apical dichotomies in species with predominant tip growth such as *F. vesiculosus*) would have different and likely higher metabolic requirements, leading to higher accumulation of nutrients such as N (e.g. Rice and Lapointe, 1981; Stengel et al., 2005; Viana et al., 2015a). The enrichment of δ^{15}N on the new growing tips, compared to older dichotomies, may be explained by the preferential release of light N isotopes, as suggested by Raimonet et al. (2013) and Viana et al. (2015a). The increasing concentration with age found for these two elements is consistent with other studies reporting differential N and δ^{15}N enrichment along the regions of *F. vesiculosus* thallus (e.g. Carlson, 1991; Dohler et al., 1995; Carballeira et al., 2014; Viana et al., 2015a).

Finally, the variation in the amount and chemical composition of the alginates (polyanionic polysaccharides) present in the cell walls of the algae could contribute to the differences in the concentrations of elements observed along the thallus dichotomies. Alginates are considered the major constituents of the cell walls in brown macroalgae (Rosell and Srivastava, 1984; Rabille et al., 2019) and represent the 40–80% of dry defatted algal biomass (Zvyagintseva et al., 2003). They have a great potential to reversibly bind important amounts of elements, including metal pollutants (García-Seoane et al., 2020) and, although the variation of alginates along the thallus has been scarcely studied in brown algae species, it has been suggested that the changes in the molecular conformation of alginic acid in the different structural components of the thallus can be responsible for differences in their affinity for metals (Saez et al., 2012).

The extrinsic mechanisms contributing to the differences in the concentrations of elements among dichotomies include differences in the amount of epiphyte and fine particle contamination along the thalli (e.g. Försberg et al., 1988; Malinovskaya, 1998; Villares et al., 2005). It is well known that the influence of sediment particles on the concentrations of metals such as Al, Cr, Fe and Pb in F. vesiculosus is mainly related to their abundance in the sediment (Barreiro et al., 2002). These elements, highly correlated in F. vesiculosus thalli (García-Seoane et al., 2019), are naturally bound to fine sediments (Viana et al., 2010), usually indicating contamination by particulate material. Also, older dichotomies generally show greater epiphyte contamination as they have been available for colonization for longer periods. These extrinsic factors would in part explain the higher concentrations of Al, Co, Fe, Ni, Pb

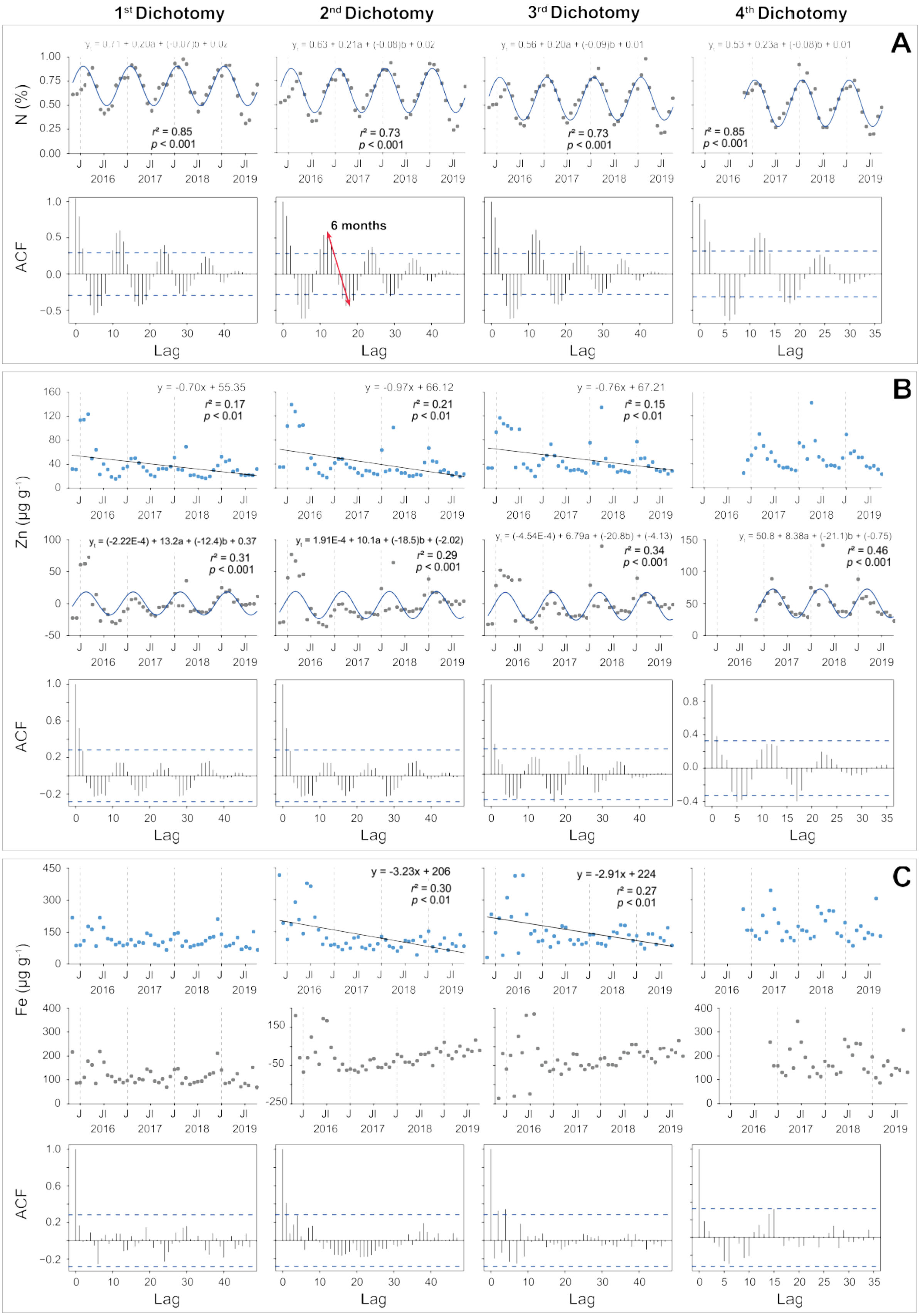
1st Dichotomy
2nd Dichotomy
3rd Dichotomy
4th Dichotomy
A
N (%)
y_t = 0.71 + 0.20a + (-0.07)b + 0.02
r² = 0.85
p < 0.001
y_t = 0.63 + 0.21a + (-0.08)b + 0.02
r² = 0.73
p < 0.001
y_t = 0.56 + 0.20a + (-0.09)b + 0.01
r² = 0.73
p < 0.001
y_t = 0.53 + 0.23a + (-0.08)b + 0.01
r² = 0.85
p < 0.001
J Jl J Jl J Jl J Jl
2016 2017 2018 2019
ACF
6 months
Lag
B
Zn (µg g⁻¹)
y = -0.70x + 55.35
r² = 0.17
p < 0.01
y = -0.97x + 66.12
r² = 0.21
p < 0.01
y = -0.76x + 67.21
r² = 0.15
p < 0.01
y_t = (-2.22E-4) + 13.2a + (-12.4)b + 0.37
r² = 0.31
p < 0.001
y_t = 1.91E-4 + 10.1a + (-18.5)b + (-2.02)
r² = 0.29
p < 0.001
y_t = (-4.54E-4) + 6.79a + (-20.8b) + (-4.13)
r² = 0.34
p < 0.001
y_t = 50.8 + 8.38a + (-21.1)b + (-0.75)
r² = 0.46
p < 0.001
ACF
Lag
C
Fe (µg g⁻¹)
y = -3.23x + 206
r² = 0.30
p < 0.01
y = -2.91x + 224
r² = 0.27
p < 0.01
ACF
Lag

Fig. 4. Results of the time series analysis in *Fucus vesiculosus* dichotomies. A) Seasonal models fitted to the time series of N in sampling site 1 (SS1) (first row), and correlograms for N (second row). The red arrow indicates the time lag between maximum and minimum autocorrelations (6 months). B) Time series of Zn in SS3 and significant linear regression models fitted to the trend component in the non-stationary series (1st, 2nd and 3rd dichotomies) (first row). Seasonal models fitted to the residuals of the series after detrending and directly to raw data in the 4th dichotomy (second row). Correlograms for residuals (1st, 2nd and 3rd dichotomies) and raw data (4th dichotomy) of Zn (third row). C) Time series of Fe in SS2 and significant linear regression models fitted to the trend component in the non-stationary series (2nd and 3rd dichotomies) (first row). Residuals of the series after detrending and directly to raw data in no detrended series (second row). No significant seasonal models could be fitted in Fe series. Correlograms for residuals (2nd and 3rd dichotomies) and raw data (1st and 4th dichotomies) of Fe (third row). Common in-formation 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. J: January, Jl: July. 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. Time lag (months). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and Zn in the older dichotomies observed here (Fig. 3), and are consistent with the results reported in previous studies for *Fucus* species (e.g. Barnett and Ashcroft, 1985; Försberg et al., 1988; Söderlund et al., 1988; Barreiro et al., 1993; Stengel et al., 2005).

Although the reasons for the different patterns observed are not well understood, because they have never been studied in detail, the results of the present study suggest that all these factors are not mutually exclusive, rather the greater accumulation of elements in young or old tissues would be the result of the complex interaction between more than one factor. For example, 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 contribute to the differences observed in the element distribution patterns.

4.2. Temporal variation

All elements displayed important levels of temporal variation, either showing long-term temporal trends (across years), yearly oscillations (within years), or both (Table 3). Co, Fe, Hg, Ni and Zn showed a significant decrease from 2015 to 2019 (annual declines between 10% and 20%), which is also consistent with the decreasing patterns for the same elements reported by Viana et al. (2010), although of a lesser extent (5–10% variation) over a longer period of time (2001–2007). The downward trends continued over time may indicate an outstanding decrease of bioavailable metals in the rias ecosystems of Galicia, suggesting that the measures implemented during the last decades to reduce the release of pollutants in the environment (e.g. The European Water and Marine Framework Directives: WFD–2000/60/EC and MSFD–2008/56/EC, and the Galician Water Law 9/2010 of 4 November) are being effective in the study region. On the other hand, the concentrations of As (in all SS) and δ^{15}N (in SS2 and SS3) increased from 300% to 600% and from 30% to 100% respectively throughout the monitoring period, indicating an increase in pollution with As. The upward trends observed in δ^{15}N contrast with the stationarity in N concentrations, suggesting that the slight changes in δ^{15}N are not a direct consequence of N available concentrations, but are the sources of N which ultimately determine the δ^{15}N in *F. vesiculosus* (Viana and Bode, 2013). These results demonstrate that *F. vesiculosus* is useful for monitoring long-term changes in the concentrations of elements in marine ecosystems, and that can also be suitable to evaluate the effectiveness of environmental management measures.

The intra-annual variation levels in the concentrations of trace elements and nitrogen observed in this study (Table 1) are comparable to the findings for the same elements in multitude studies of temporal trends using macroalgae as biomonitors (i.e. coefficients of variation between 35% and 58%, except for N < 30%; reviewed in García-Seoane et al., 2018). More precisely, similar variability in the concentrations of Al, Cd, Co, Cu, Fe, Hg, Ni, Pb and Zn was reported by Viana et al. (2010) in F. *vesiculosus* in the same study region, with e.g. Al and Pb also showing the highest annual coefficients of variation, usually > 100% (vs. COD usually >100% in our observations). Importantly, according to the correlograms and the cyclic regression models fitted to the time series, the intra-annual variation observed in this study is not random (Fig. 4, Table 3, Table S2), rather it can be reasonably attributed to the existence of seasonality in the bioconcentration patterns of *F. vesiculosus*, consistent across elements, dichotomies and sites. Concentrations of Cu, N and Zn exhibited even more than two-fold changes between both seasons, resulting in very marked seasonal patterns.

Seasonal variation has been 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 elements typically occur during winter and are lowest in summer (García-Seoane et al., 2018). However, none of the previous studies explored seasonal variation using autocorrelation analysis. Most of the studies using the term “seasonality” to describe variations be-tween winter and summer only collected samples for a one-year period or less, which does not rule out the possibility that the variations observed were due to a different cause, for example to an isolated pollution event at the time when the highest concentrations were detected. Proving the existence of seasonality requires a more powerful experimental design in which samples are collected at a high frequency (e.g. one per month) repeatedly over several years, like in the present study. Based on our findings, all the elements except Al, Fe and Pb showed significant cyclical fluctuations every 12 months in at least two of the three SS (Table 3). The lack of significant seasonality in the concentrations of these elements could indicate the presence of particulate material in the surface of the analyzed samples, and the variation observed in their concentrations may be not so influenced by biological factors as might occur with other elements, but largely determined by variations in the sediment present in the immediate environment.

Seasonal variation in tissue element concentrations may be attributed to local and regional seasonal changes in environmental parameters such as temperature, precipitation, salinity, pH or light conditions (e.g. Stoeppler et al., 1986; Haroon et al., 1995; Wright and Mason, 1999; Villares et al., 2013), that could lead to similar variations in the bioavailability of metals in seawater and sediments (e.g. Burdon-Jones et al., 1982; Wright and Mason, 1999), or directly affect important biological processes such as algae metabolism and growth rates (e.g. Raimonet et al., 2013; Malea et al., 2015). For example, the seasonal pattern of N with minimum values in summer could be associated with metabolic processes, as it is the main limiting nutrient for primary production in marine coastal waters, and summer corresponds to the period of maximum productivity in which nutrient limitation by this element might occur (Villares et al., 2013). Moreover, algae accumulate N during the autumn and winter to use it in summer when concentrations in seawater are too low to fulfill their metabolic requirements and the environmental conditions (e.g. light and temperature regime) are favorable for growth (Carlson, 1991; Lehvo et al., 2001). Occasional fluvial and terrestrial inputs during specific seasons could also contribute to seasonal changes in the levels of the elements in solution (e.g. Lacerda et al., 1985; Villares et al., 2002; Fink and Manley, 2011). However, this contribution would only be observed in our study if sea-sonal discharges of all elements studied had occurred annually in all three SS, which is highly unlikely.

We believe that factors intrinsic to the algae undergoing seasonal fluctuations, such as metabolism (photosynthesis and respiration), growth, reproduction and alginates production (e.g. Rao and

Indusekhar, 1989; Wright and Mason, 1999; Malea et al., 2015; Farías et al., 2019), together with the environmental factors affecting the bioavailability of metals in seawater and sediments, contribute the most to those periodic changes observed in the concentrations of elements. Most authors, however, have primarily attributed the seasonal patterns to a diluting effect of the bioconcentrated elements due to fast growth of algae in the warmest periods, causing lower concentrations in summer (e.g. Riget et al., 1995; Martin et al., 1997; Vasconcelos and Leal, 2001; Villares et al., 2002, 2013; Malea et al., 2015). As mentioned in the section above, metals are not irreversibly bound to the algae, and tissues quickly uptake/release them until they are balanced with the ambient concentrations (equilibrium between heavy metals in seawater and *F. vesiculosus* tissues is presumably reached in < 7 days, unpublished data) irrespective of their growth rate (García-Seoane et al., 2020). Moreover, this species is also able to incorporate N-enriched solutions rapidly reaching equilibrium with the solution within ca. 2 weeks (Bailes and Gröcke, 2020). To this point, the effect of rapid growth of *F. vesiculosus* in late spring and summer (Carlson, 1991) on the seasonal dynamics of elemental concentrations might be limited. Therefore, we suggest that seasonal variation in the concentrations of alginates (e.g. Davis et al., 2003; Jothisaraswathi et al., 2006; Khan and Qari, 2009), also displaying maximum winter and minima in summer in *F. vesiculosus* (Fletcher et al., 2017), could be a major contributor to the annual sea-sonal patterns observed for the different elements. Yet, a further examination on the uptake processes of trace elements in *F. vesiculosus*, particularly involving the polysaccharides present in the cell walls, would be helpful to explain the mechanism driving their accumulation in macroalgae.

Most likely none of the mechanisms mentioned in Sections 4.1 and 4.2 fully explain the element-specific intra-thallus variation patterns nor the seasonal variation differences between elements by itself. Rather, bioconcentration of elements in F. vesiculosus is most likely dominated by complex inter-relations among environmental and biological factors (Vasconcelos and Leal, 2001; Villares et al., 2002), together with the physicochemical characteristics of the elements which determine their capacity to compete for binding sites (e.g. Stengel et al., 2005; Ryan et al., 2012). Disentangling the major drivers of both sources of variability would require specific experimental studies.

Finally, since the thallus of *F. vesiculosus* has been found to form several new dichotomies each year, which is comparable to earlier observations for *F. distichus* subsp. *evanescens* from southeastern Kam-chatka (Kashutin et al., 2019), dichotomies in this species and study area should no longer be considered equivalent to annual growth cohorts, and the method for determination of time-trends in trace elements and nutrients (N and $\delta^{15}N$) based on the number of dichotomous branches is no longer suitable for populations of *F. vesiculosus* in the Galician coast, and should be critically evaluated in other regions.

5. Conclusions

In the study region, the concentrations of Al, As, Cd, Co, Cu, Fe, Hg, Ni, Pb, Zn, N, as well as $\delta^{15}N$ signal depend on the tissue age in *F. vesiculosus*. This species preferentially bioconcentrates As, Cd, N and $\delta^{15}N$ in young dichotomies and Al, Co, Fe, Ni, Pb and Zn in old dichotomies. As the concentrations of trace elements and nitrogen 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, according to our findings, the concentrations of trace elements and N in *F. vesiculosus* are subject to high seasonal variation, with the maximum values in an annual cycle being several times greater than the minimum values. 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.

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

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

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Supplementary Material

Figures

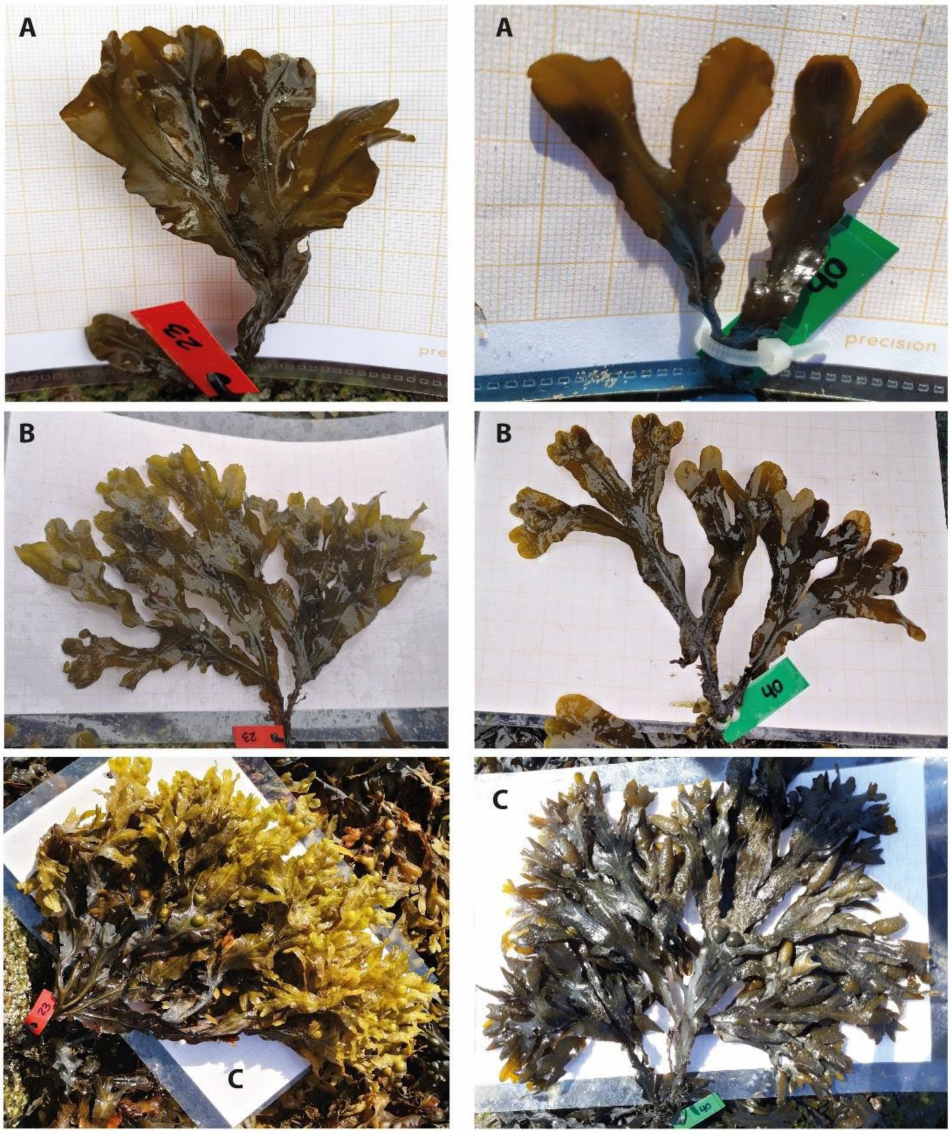
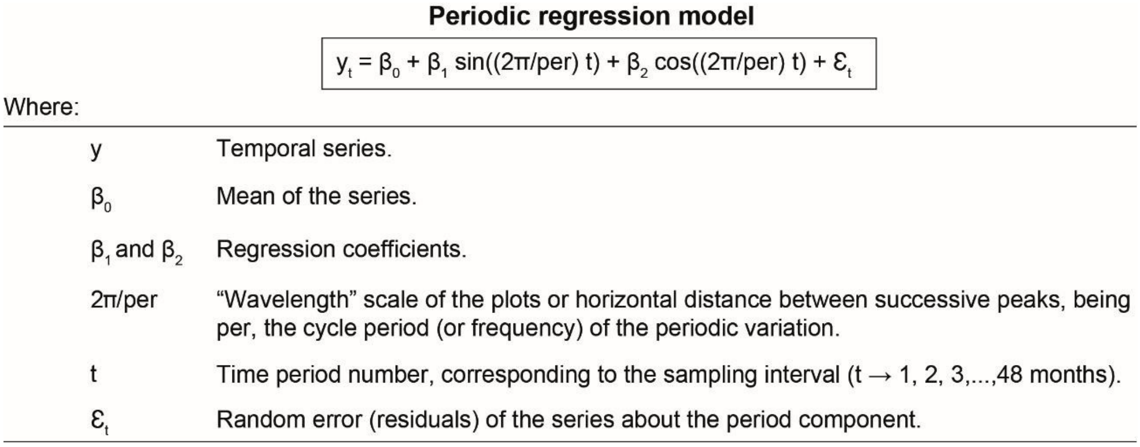


Fig. S1. Growth progression in two *Fucus vesiculosus* thalli from SS1 (red tag) and SS2 (green tag), respectively. A: January; B: April; C: July. Growth monitoring was not possible in October.

Tables

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



Periodic regression model

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

Where:

y	Temporal series.
β_0	Mean of the series.
β_1 and β_2	Regression coefficients.
$2\pi/per$	“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 → 1, 2, 3,...,48 months).
ε_t	Random error (residuals) of the series about the period component.

Table S2. Description of the correlation patterns in time series of the different elements for the 1st, 2nd, 3rd and 4th dichotomies in *Fucus vesiculosus* thalli at the sampling sites (SS). Correlations are showed from lag 1 to lag 47 (1st, 2nd and 3rd dichotomies), and from lag 1 to lag 35 (4th dichotomy). Upward and downward arrows represent positive and negative correlations, respectively. Colored arrows represent significant correlations: green arrows (significant correlations for the 1st dichotomy), yellow arrows (significant correlations for the 2nd dichotomy), red arrows (significant correlations for the 3rd dichotomy), blue arrows (significant correlations for the 4th dichotomy). Background shading has been included to facilitate interpretation of alternating positive and negative correlation patterns. Dic.: dichotomy.

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		4th	**↑**	↑	↑	↑	↓	↓	↓	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↑	↓	↓	↓	↓	↑	↓	↓	↑	↑	↑	↑	↑	↑	↑	↑	↓	↓												
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		2nd	**↑**	**↑**	**↑**	↑	↓	↓	↓	↓	↓	↑	↑	**↑**	**↑**	↑	↑	↓	↓	**↓**	**↓**	**↓**	↓	↓	↓	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↓	↑	↓
		3rd	**↑**	**↑**	**↑**	↑	↓	**↓**	**↓**	**↓**	↓	↑	↑	↑	↑	↑	↑	↓	↓	↓	**↓**	**↓**	**↓**	↓	↓	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↑	↑
		4th	**↑**	**↑**	**↑**	↑	↓	**↓**	**↓**	**↓**	↓	↓	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↑	↑												

	SS2	1st	**↑**	**↑**	↑	↑	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↓	↓	↓	↓	**↓**	↓	↓	↓	↓	↑	↑	↓	↓	↓	↓	↓	↑	↓	↑	↑	↑	↑	↑	↑
		2nd	**↑**	**↑**	↑	↓	**↓**	**↓**	**↓**	**↓**	↓	↓	↑	↑	**↑**	↑	↑	↓	↓	↓	**↓**	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑
		3rd	**↑**	**↑**	**↑**	↑	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	**↓**	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑
		4th	**↑**	**↑**	↑	↑	↓	**↓**	**↓**	↓	↓	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↑	↓	↑	↑												
	SS3	1st	**↑**	**↑**	↑	↑	↓	↓	**↓**	↓	↓	↑	↑	↑	↑	↑	↓	↓	**↓**	**↓**	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↑	↓	↓	↑	↑	↓	↑	↑	↓	↓
		2nd	**↑**	↑	↑	↑	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↑	↑	↑	↑	↓	↓	↑	↓	↓	↓	↓	↑	↑	↑	↑	↓	↓	↓	↑	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓
		3rd	**↑**	**↑**	↑	↓	↓	↑	↓	↓	↓	↓	↑	↓	↑	↑	↓	↓	↓	↓	↑	↑	↑	↑	↓	↑	↑	↑	↓	↓	↓	↓	↑	↑	↑	↑	↓	↓	↓	↑	↑	↓	↓	↑	↑	↑	↑	↑	↓	↓
		4th	**↑**	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↓	↓	↓	↓	↑	↑	↑	↑	↓	↑	↑	↑	↓	↓	↓	↓	↓	↑	↓	↑	↑	↑												
Co	SS1	1st	**↑**	**↑**	**↑**	↑	↑	↓	↓	↓	↓	↑	↑	↑	↑	↓	↓	↓	↓	**↓**	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
		2nd	**↑**	**↑**	↑	↑	↓	↓	↓	↓	↓	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	**↑**	**↑**	↑	↑	↑	↓	↓	↓	↓	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
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		4th	**↑**	**↑**	**↑**	↓	↓	**↓**	**↓**	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑												
	SS2	1st	**↑**	**↑**	**↑**	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↓	↓
		2nd	**↑**	**↑**	**↑**	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↓	↓	↓
		3rd	**↑**	**↑**	**↑**	↑	↓	↓	↓	↓	↓	↓	↓	↓	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↓	↓	↓
		4th	**↑**	**↑**	↑	↓	↓	**↓**	**↓**	↓	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑												
	SS3	1st	**↑**	**↑**	**↑**	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↓	↑	↑	↑	↑	↑	↑	↓	↓
		2nd	**↑**	**↑**	**↑**	**↑**	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↓	↑	↑	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↑	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓
		3rd	**↑**	**↑**	↑	↑	↓	↓	↓	↓	↓	↓	↑	↑	↓	↑	↓	↓	**↓**	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑
		4th	**↑**	**↑**	↑	↑	↓	**↓**	**↓**	↓	↓	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑												
Cu	SS1	1st	**↑**	**↑**	**↑**	↑	↓	**↓**	**↓**	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↑	↑
		2nd	**↑**	**↑**	↑	↓	↓	**↓**	**↓**	↓	↓	↑	↑	↑	↑	↑	↑	↑	↓	↑	↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↓	↓	↑	↑	↓	↓	↓	↑	↑	↑
		3rd	**↑**	**↑**	↑	↓	↓	**↓**	**↓**	**↓**	↓	↑	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↓	↓	↓	↑	↓	↓	↓	↑	↑	↑	↓	↓	↓	↑	↑	↓	↓	↓	↑	↑	↑
		4th	**↑**	**↑**	↑	↓	↓	**↓**	**↓**	↓	↓	↑	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↑	↑	↑	↓	↓	↓	↓	↑	↑	↓	↓	↑	↑	↑												
	SS2	1st	**↑**	**↑**	**↑**	↓	↓	**↓**	**↓**	↓	↑	↑	↑	↑	↑	↑	↓	**↓**	↓	↓	↓	↑	↑	**↑**	↑	↑	↑	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑
		2nd	**↑**	**↑**	**↑**	↑	↓	↓	**↓**	↓	↓	↑	↑	↑	↑	↑	↓	↓	**↓**	**↓**	↓	↓	↓	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↓
		3rd	**↑**	**↑**	**↑**	↑	↑	↑	↓	↑	↓	↓	↑	↑	↓	↓	**↓**	**↓**	**↓**	**↓**	**↓**	↓	↓	↓	↓	↓	↑	↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↑	↑	↓	↓	↑	↑	↑	↓	↓	↓	↑	↑	↑
		4th	**↑**	**↑**	↑	↑	↓	↓	↓	↓	↓	↓	↓	↑	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↑	↑	↓												

	SS3	**1st**	**↑**	**↑**	↑	↓	**↓**	**↓**	**↓**	↓	↓	↑	**↑**	↑	↑	↑	↑	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↓
		2nd	**↑**	**↑**	↑	↑	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↑	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↑	↑	↓	↑	↓	↓	↓	↓	↓
		3rd	**↑**	**↑**	↑	↓	**↓**	**↓**	**↓**	↓	↓	↓	↑	**↑**	↑	↑	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↑	↓	↓	↓	↑	↑	↑	↑	↓	↑	↓	↓	↓	↓	↓	↓	↓	↓
		4th	**↑**	↓	↑	↑	↓	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↑	↑												
Fe	**SS1**	**1st**	**↑**	**↑**	↑	↓	↓	↓	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↑	↓	↓	↑	↓	↓	↓	↓	↑	↓	↓	↓	↓	↑	↑	↑	↑	↓	↓	↑	↑	↑	↑	↓	↓	↓	↓	↑	↑	↑	↓	↓
		2nd	**↑**	**↑**	↑	↓	↓	↓	↓	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↓	↓	↓	↑	↑	↑	↑	↓	↓	↓	↑	↑	↑	↓	↓	↓	↓	↓	↑	↑	↓	↓	↓
		3rd	**↑**	**↑**	↑	↓	↓	↓	↓	↓	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↓	↓	↓	↓	↑	↑	↑	↑	↓	↓	↓	↓	↑	↑	↑	↓	↓	↓	↓	↓	↑	↑	↑	↓	↓
		4th	**↑**	**↑**	**↑**	↑	↓	↓	↓	↑	↑	↓	↓	**↓**	**↓**	**↓**	↓	↑	↑	↑	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓												
	SS2	**1st**	**↑**	↑	↓	↑	↑	↓	↓	↑	↑	↓	↑	↑	↑	↓	↓	↑	↓	↓	↑	↑	↑	↓	↑	↓	↓	↓	↓	↑	↓	↑	↑	↑	↓	↑	↓	↓	↓	↑	↑	↓	↓	↓	↑	↓	↓	↓	↑	↓
		2nd	**↑**	**↑**	↑	↑	↑	↑	↓	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↓	↑	↑	↑	↑	↓	↑	↑	↑	↓	↑	↑	↑
		3rd	**↑**	↓	**↑**	↓	**↑**	↓	↓	↓	↑	↓	↑	↓	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↑	↑	↑	↓	↑	↑	↓	↓	↓	↓	↓	↓	↓
		4th	**↑**	↑	↑	↓	↓	↓	↓	↑	↓	↓	↓	↑	↑	↑	↑	**↑**	↑	↓	↓	↓	↓	↓	↑	↑	↓	↑	↑	↑	↓	↑	↓	↓	↓	↓	↑	↓												
	SS3	**1st**	**↑**	**↑**	**↑**	↑	↓	↓	↓	↓	↓	↓	↓	↓	↑	**↑**	↑	↑	↑	↓	↓	↓	↓	↑	↓	↓	↓	↓	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑
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		3rd	**↑**	**↑**	**↑**	↓	↓	**↓**	**↓**	↓	↓	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↑	↑	↑	↑	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓
		4th	**↑**	**↑**	↑	↓	↓	**↓**	↓	↓	↑	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓												
Hg	**SS1**	**1st**	**↑**	**↑**	↑	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↓	↓	↓	**↓**	↓	↓	↓	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	↑	↑	↓	↓	↓	↓	↑	↑	↑
		2nd	**↑**	**↑**	↑	↑	↑	↑	↓	↓	↑	↑	↑	↑	↑	↑	↓	↓	↓	**↓**	**↓**	**↓**	**↓**	↓	↓	↑	↑	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
		3rd	**↑**	**↑**	↑	↓	↓	↓	↓	↓	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↓	↓	↓	↓	↓	↓	↑	↑	↑	↓	↑	↓	↓	↑	↑	↑	↑	↓	↑	↑	↑	↓

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		3rd	**↑**	**↑**		↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↓	↓	↓	↓	↓	↑
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	SS3	1st	**↑**	**↑**		↑	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓
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Zn	SS1	1st	**↑**	**↑**		↑	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓
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	SS2	1st	**↑**	**↑**		↑	↑	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓
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		4th	**↑**	**↑**	↑	↑	↓	**↓**	**↓**	**↓**	↓	↑	↑	↑	↑	↑	↑	↓	↓	**↓**	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑												
N	**SS1**	1st	**↑**	**↑**	**↑**	↓	**↓**	**↓**	**↓**	**↓**	↓	↓	**↑**	**↑**	**↑**	**↑**	↑	↓	**↓**	**↓**	**↓**	**↓**	↓	↓	↑	**↑**	**↑**	↑	↑	↓	↓	**↓**	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓
		2nd	**↑**	**↑**	**↑**	↓	**↓**	**↓**	**↓**	**↓**	↓	↑	**↑**	**↑**	**↑**	**↑**	↑	↓	**↓**	**↓**	**↓**	**↓**	↓	↓	↑	**↑**	**↑**	↑	↑	↓	↓	**↓**	**↓**	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓
		3rd	**↑**	**↑**	**↑**	↓	**↓**	**↓**	**↓**	**↓**	**↓**	↓	**↑**	**↑**	**↑**	**↑**	↑	↓	**↓**	**↓**	**↓**	**↓**	↓	↓	↑	**↑**	**↑**	**↑**	↑	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓
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	SS2	1st	**↑**	**↑**	**↑**	↓	**↓**	**↓**	**↓**	**↓**	↓	↑	**↑**	**↑**	**↑**	**↑**	↑	↓	**↓**	**↓**	**↓**	↓	↓	↑	↑	**↑**	**↑**	↑	↓	↓	**↓**	**↓**	↓	↓	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑
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		4th	**↑**	**↑**	**↑**	↓	**↓**	**↓**	**↓**	**↓**	↓	↓	↑	**↑**	**↑**	**↑**	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑												
δ¹⁵N	**SS1**	1st	**↑**	**↑**	↑	↓	↓	**↓**	**↓**	**↓**	↓	↓	↑	↑	**↑**	↑	↑	↑	↑	↓	↓	↓	↓	↑	↑	↑	**↑**	↑	↑	↑	↓	↓	↓	↓	**↓**	↓	↓	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↑
		2nd	**↑**	**↑**	↑	↓	↓	**↓**	**↓**	**↓**	↓	↓	↑	**↑**	**↑**	↑	↑	↓	↓	↓	↓	↓	↓	↓	↑	↑	**↑**	↑	↑	↑	↓	↓	**↓**	**↓**	**↓**	↓	↑	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↑	↑	↑
		3rd	**↑**	**↑**	**↑**	↑	**↓**	**↓**	**↓**	**↓**	**↓**	↓	↑	**↑**	**↑**	**↑**	↑	↓	↓	↓	**↓**	**↓**	↓	↓	↑	**↑**	**↑**	**↑**	↑	↑	↓	↓	**↓**	**↓**	**↓**	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↑	↑	↑
		4th	**↑**	**↑**	**↑**	↑	↓	↓	**↓**	**↓**	**↓**	↓	↑	**↑**	**↑**	**↑**	↑	↑	↓	↓	↓	**↓**	↓	↓	↑	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓												
	SS2	1st	**↑**	**↑**	**↑**	↑	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	**↓**	**↓**	**↓**	↓	↓	↓	↑	↑	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↓	↓
		2nd	**↑**	↑	↑	↑	↑	↑	↑	↑	↑	↑	↓	↓	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	**↓**	**↓**	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
		3rd	**↑**	**↑**	↑	↑	↑	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	**↓**	**↓**	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
		4th	**↑**	**↑**	↑	↑	↓	↓	↓	↓	↓	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↓	↑	↓	↑	↑												
	SS3	1st	**↑**	**↑**	**↑**	↑	↑	↑	↑	↓	↓	↑	↓	↓	↑	↓	↓	↓	↓	↓	↓	↑	↓	↓	↓	↓	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
		2nd	**↑**	**↑**	**↑**	↑	↑	↑	↓	↑	↓	↓	↓	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↓	↑	↑	↑	↑	↑	↑	↑	↓	↓
		3rd	**↑**	**↑**	**↑**	↑	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↑	↑	↓	↓	↑	↑	↑	↑	↑	↑	↑	↓
		4th	**↑**	↑	↓	↓	↓	↓	↑	↑	↑	↓	↑	↑	↑	↓	↓	↓	↓	↑	↓	↓	↓	↓	↑	↑	↑	↑	↑	↓	↓	↓	↑	↑	↑	↑	↑	↓												