American Journal of Plant Sciences, 2023, 14, 323-338
https://www.scirp.org/journal/ajps
ISSN Online: 2158-2750
ISSN Print: 2158-2742

Relationship of Trace Elements Concentration and Growth Rate in *Alsidium triquetrum* (Rhodophyta)

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How to cite this paper: Areces, A.J., Cabrera, R., Díaz-Larrea, J. and Collado-Vides, L. (2023) Relationship of Trace Elements Concentration and Growth Rate in *Alsidium triquetrum* (Rhodophyta). *American Journal of Plant Sciences*, **14**, 323-338.
https://doi.org/10.4236/ajps.2023.143022

Received: January 9, 2023
Accepted: March 28, 2023
Published: March 31, 2023

Abstract

Minerals and trace elements content and concentration in marine algae vary depending on species morphology and physiology; as well as growing environmental conditions. Despite this variability, accumulation of magnesium, and especially iron, seems to be common in Chlorophyta; while Rhodophyta and Heterokontophyta show higher affinity to manganese. The red agarophyte *Alsidium triquetrum* was used to analyze the relationship between metal concentration, environmental conditions and growth rate. Specimens grown *in situ* showed a large variability of Fe, Mn, Mg, and Al, in thallus tissue concentrations. Further, a compelling relationship between the growth rate and the thallus concentration of Mg and Mn, Zn, and Al was detected. Manganese, unlike the other trace elements analyzed showed a positive linear relationship between growth rate and tissue content during the period of greatest vegetative growth.

Keywords

Alsidium, Growth Rate, Manganese, Trace Elements

1. Introduction

Macroalgae have a high affinity and capacity to accumulate metals due to the presence of polysaccharides in their cells [1]. Iron, copper, zinc, and manganese are essential metals in biological metabolic pathways; however, these elements can exhibit toxic effects on macroalgae at high concentrations [2].

Because macroalgae have strong bioaccumulative capacities, their mineral content can be up to 100 times higher than that of land vegetables [3]. Other non-essential metals, such as mercury, lead and cadmium are also accumulated in macroalgae [4]; potentially becoming a health issue to humans even in trace amounts, if they are directly consumed [5].

Nutritional properties and chemical composition of algae are, highly dependent on the species [6], locality [7], seasonality [8] [9] and growing conditions [10] [11]. However, the majority of studies addressing metal content in macroalgae tissue are linked to, among others, commercial important species of the genera *Gracilaria* [12] and *Gelidium* [13], on massive bloom-forming species such as *Sargassum* [14].

Alsidium triquetrum (S.G. Gmelin) Trevisan is very common in Caribbean Reefs. While the exploitation of *Alsidium* gains momentum, fundamental knowledge gaps related to the ecological-physiological relationship with growth precursors such as Mn. One example is the limited information regarding micro elements interactions between farmed macroalgae. *Alsidium* is known to be a source of intermediate quality agar [15], of biogenic compounds [16], shows a wide physiological tolerance to salinity [17], and supported several fastening systems [18], making it an effective candidate for aquaculture [19].

In order to strengthen the knowledge about this species, the levels of tissue accumulation of aluminum, iron, zinc, manganese, copper and magnesium in *Alsidium* are analyzed and their possible relationship with *in situ* growth rates is explored.

2. Materials and Methods

2.1. Study Area and Experimental Period

The research was carried out at two sites with different environmental conditions. One of them in the interior of a shallow bay and other outside the bay in the vicinity of the Institute of Oceanology at Havana Cuba (23˚04'29"N, 82˚28'20"W). The area of the bay was of 15,487m^2 and reaches an average depth of 1.5 m, framed by two breakwaters that limit its entrance, approximately 25 m wide (**Figure 1(a)**).

The bay has a bottom composed of the seagrass *Thalassia testudinum*, rocks and sand in the inner half, and sandy-muddy sediments towards the vicinity of its mouth. Two well-established seasonal periods govern the annual cycle. One is the dry season, from November to April, corresponding to winter, and another is the rainy season from May to October, coinciding with summer.

2.2. General Methodology of the Experiments

Specimens of *Alsidium triquetrum* were collected from a bed in the vicinity of the experimental site. Each thallus was vigorously cleaned with filtered seawater to remove particles and epibionts. With them, a multipurpose experiment was

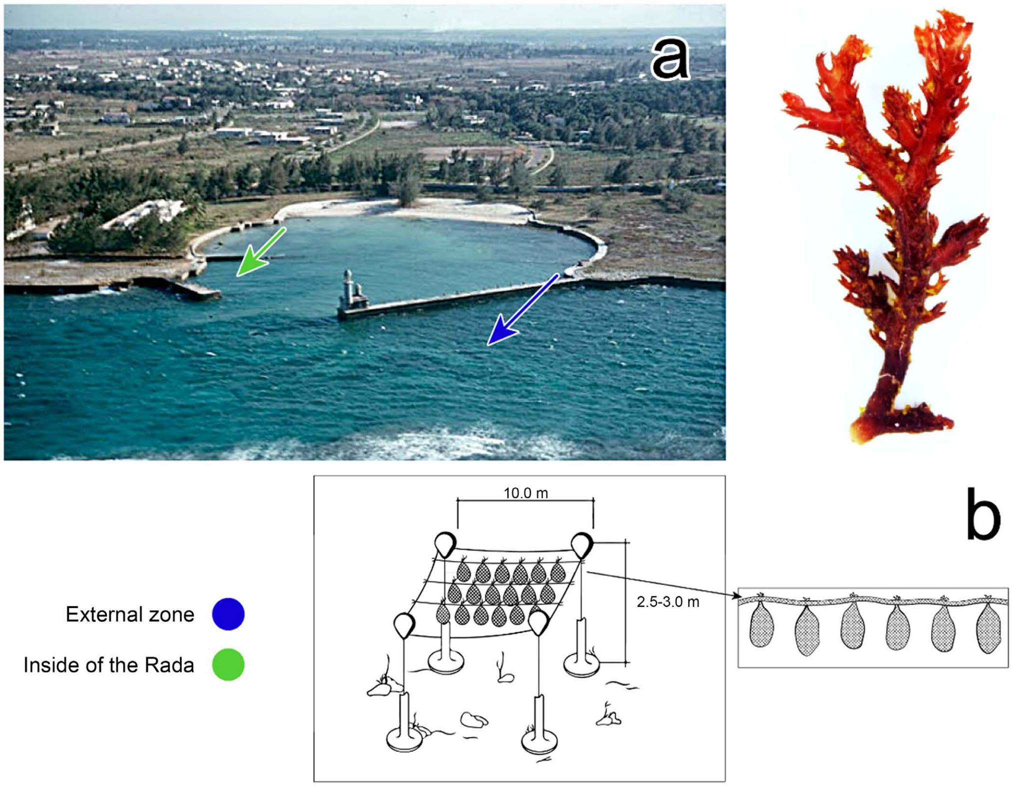

Figure 1. Experimental zone of crop the *Alsidium triquetrum*. (a) study site; (b) Support structure used to hold the ropes with bags with 6 cm holes, the arrow indicates (close up), suspended baskets attached directly to the rope.

designed. Thirty pieces were sectioned into fragments of 25 and 50 g (wet weight = ww) (n = 15 each), each fragment was inserted in a bag to avoid herbivory. The 30 bags were randomly installed onto three 10.5 m polypropylene ropes in such a way that each rope held 10 bags separated one from the next by approximately 90 cm. A squared structure consistent of iron piles inserted into concrete bases was deployed on a sandy-muddy bottom at a depth of 2.5 - 3.0 m to support bags with cultures (**Figure 1(b)**) [for manufacturing methodology consult reference 19].

To determine the content of trace elements, and their relationship with growth rate and tissular concentration of N, P, and K, bags with pieces of 50 g were used. The experiment lasted 60 days both in summer and in winter. The experimental period took place during the months of December-February, (hereafter referred to as winter), and in the wet season (June-August; hereafter referred to as summer). In each period, five bags were extracted every ten days to be analyzed. In addition, a complementary experiment of collection and replenishment of biomass every 30 days were done in the external zone (**Figure 1(b)**; n = 5), from March to October, during the months that best represent the peak of growth of the species [15]. In the same locality, monthly records of the content of Al, Fe, Zn, Mn and Cu were also carried out at apexes and stipe of *A. triquetrum* from the natural mantle. The data obtained were compared to the growing experiment results.

2.3. Samples Processes for Metal Content Estimations

The 50 g biomass from bags, as well as from specimens collected in the external zone was cleaned of epibionts and inorganic residues and then washed in running water and dried in an oven at 60˚C, until reaching constant weight. The apical portions of specimens collected in the external zone were separated from the stipe before drying. After drying, all material was grinded in a rotating mortar mill of agate balls. The obtained material was sieved using a plastic mesh of 0.6 - 0.66 µ. Subsequently, the samples, weighing 4 g, were treated with a concentrated mixture $HCl:HNO_3:H_2O_2$ in a ratio of 1:2:3. All the determinations were made with two, three, or five replicates.

Estimation of Mg, Al, Fe, Zn, Mn, and Cu content were done using atomic absorption spectrophotometer Pye Unicam SP-9-800 with air-acetylene flame.

For weighing, technical balances with an error of ±0.1 g, or when necessary, analytical balances of 0.0001 g precision were used.

Methods Used for the Determination of Nitrogen, Potassium, Phosphorus and Ash Content

The phosphorus content was determined using the Jackson technique [20]. For the estimation of nitrogen, the Kjeldahl method was used [21]. Potassium was determined by flame photometry according to [22]. Ash content was obtained by incineration in an oven at 500˚C. For more details on the procedure see [16].

2.4. Data Sources and Numerical Procedures

The growth of *A. triquetrum* under culture the daily growth rate was calculated as:

$$\textbf{DGR}\ [\text{daily growth rate}] = [(\ln W_t/\text{In}\, W_i)\ 1/t - 1] \times 100$$

where ln = natural logarithm, W_t and W_i are the wet weights of each thallus (g) at time one and time zero, and (t) is the time in days [23]

Inferred Turbulence (IT) was calculated according to the expression:

$$(\text{IT}) = [(f_1 + f_2)/f_t] \times \{[\Sigma(W_{\text{sen}}\Phi)^2]/[\Sigma(W_{\text{sen}}\Phi)^2 + 1]\}$$

where f_t = total number of observations in the period (including calm data), f_1 = number of observations corresponding to marine semicircle according to wind direction, f_2 = number of observations corresponding to terrestrial semicircle according to wind direction, W = wind speed of each observation, Φ = angle formed between the coastal line and heading of wind. As the index was not used to compare water turbulence in different locations, data were not corrected considering fetch.

On-site temperature recordings were carried out with ±0.1˚C precision oceanographic thermometers. The monthly precipitation data (MPD) were referred to the Institute of Meteorology, whose location is close to the working site and were provided by this institution.

Results were expressed as the arithmetic mean bounded by extreme values or by ±1 SD When its variability was considered, it was quantified by mean deviation (MD), standard deviation (SD), or the corrected coefficient of variation for bias [24]. Homogeneity of variance was not reached even after transformation of data; non-parametric test was used. For the contrast of two samples, Mann-Whitney Statistic U and the Fisher Median and Exact Probability tests were used, and for the verification of k independent samples, the analysis of variance of a Kruskal-Wallis range classification was used [25]. Data analysis to assess differences in the Daily growth Rate (DGR) and differences between apices and stipe across seasons was conducted using the Mann-Whitney U test [26] with a level of significance of 0.05.

The effect of growth on trace elements concentration in *A. triquetrum* biomass was explored by means of predictive models using determination coefficient as adjustment criteria [15]. The relationship between trace elements concentration, environmental magnitudes and tissular concentration of biogenic compounds different magnitudes were assessed by linear correlations employing Pearson's linear correlation coefficient.

The numerical calculation and production of the graphs used the systems Statistic 10 [27].

3. Results and Discussion

3.1. Growth Rates per Season and Site

The growth of *A. triquetrum* was shown to be markedly seasonal, a fact demonstrated also under controlled conditions in laboratory experiments, where the highest rate were obtained at 29.05˚C - 29.19˚C, a temperature quite similar to mean water temperature in summer season [15]. When culture was held simultaneously in different environmental conditions; near its natural habitat and sub superficially inside an enclosed area (**Figure 1(a)**), growth rate trends were similar despite the differences in magnitude and showed an abrupt increase from June, maximum magnitudes in July and a rapid decrease in August (**Figure 2**).

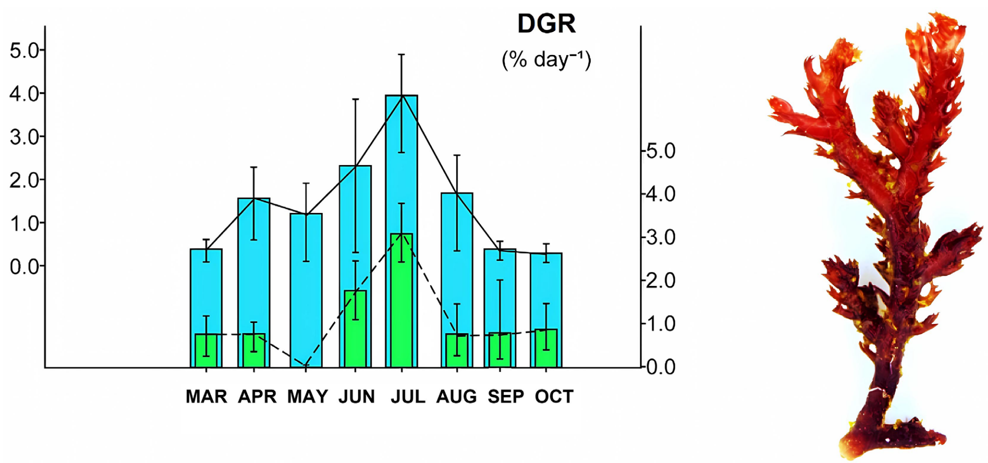

Figure 2. Seasonal trend of *A. triquetrum* DGR in two culture sites (— near the bottom in the vicinity of natural bed; --- superficially, inside an enclosed area). Vertical segments bounded by extreme values (U, p ≤ 0.035).

3.2. Dynamics of Trace Metal Content Considering Growth, Environmental Factors and Biogenic Elements

The bioaccumulation of trace elements by marine autotrophs, is attributed to transport [28]. It is also influenced by availability environment [29], and by dynamics of the speciation of the absorbed metal ion and the type of interaction it has with other bioactive metals [30].

In macroalgae, bioaccumulation has been associated with the reproductive cycle, the stage of development of the species, or the degree of affinity of the species for the metal [31]. It is assumed that it is also related to the prevailing environmental concentration, by the time of exposure, and by the age of the plant [32] but few results related them to growth rate directly.

In spite of the obvious behavior of the seasonal growth of *A. triquetrum*, when his trace metal content is only related to annual sampling time, any relationship is hardly seen in a similar way as the growth rate variations observed. Neither the content behavior of Al, Fe, Zn, Mn and Cu appear to be equivalent. When the tissue concentration of the metal studied are related to the time of observation in the annual cycle only Zn and Mn showed a consistent increase between spring and summer (**Figure 3**).

Manganese is available in dynamic and transition ion pathways, in the form of Mn^{2+} [33], and in the water column through chemical processes linked to oxidative precipitation and adsorption on suspended particles of silt and clay [34]. It is usually deposited on the seabed, where large quantities can accumulate unless extreme events (*i.e.*, storms, dredging and suspension) expose the element to benthic communities [33].

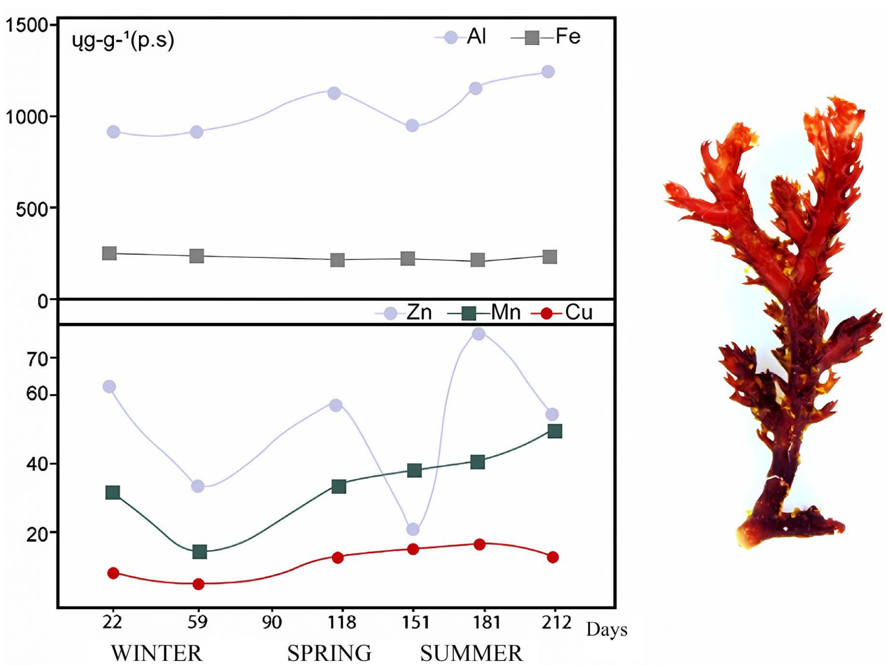

Figure 3. Seasonal dynamics of some trace elements concentration in *A. triquetrum* branchlets analyzed from a natural bed near the experimental site.

Manganese, like other metals of the first transition series, acts as a cofactor or is part of prosthetic group of numerous metallo-enzymes and proteins. It is also well known for its role in the reaction center of the photosystem II, in relation to the conversion of water molecules, as well as in the transport of electrons and oxygen [35] [36]. Concerning intermediate metabolism, also a linear relationship between the rate of absorption of Zn and oxygen production has been demonstrated [37], with the magnitude of this accumulation, being attributed to the availability of organic N ligands in the cell [38].

The importance of some trace metals in plant metabolism and whether they are linked to the DGR can be established considering the content that the plant had in the period of the last sampling, and the DGR that the plant had in the period preceding sampling, that is between the moment of sampling collection for analysis and the date of the previous collection (**Figures 4(c)-(f)**).

When the content of Mg, Al and the trace elements tested is adjusted to predictive models considering DGR as independent variable (**Table 1**), the kinetics of their accumulation in branchlets and young tissue could be seen easily. In the case of Manganese, this ratio is direct and proportional and is represented, with a high coefficient of determination, by a straight line (**Table 1**, **Figure 4(c)**).

Zinc, aluminum and magnesium experience a phenomenon of tissue dilution when the rate of biomass generation is high. Based on the slope value for the first derivative of each polygon (Mg = −1718.54; Al = −168.82; Zn = −30.79), this process appears to be intense with the Zn. Therefore, with regard to DGR, only Mn correlated direct and significantly (**Table 2**). If the absorption rate of

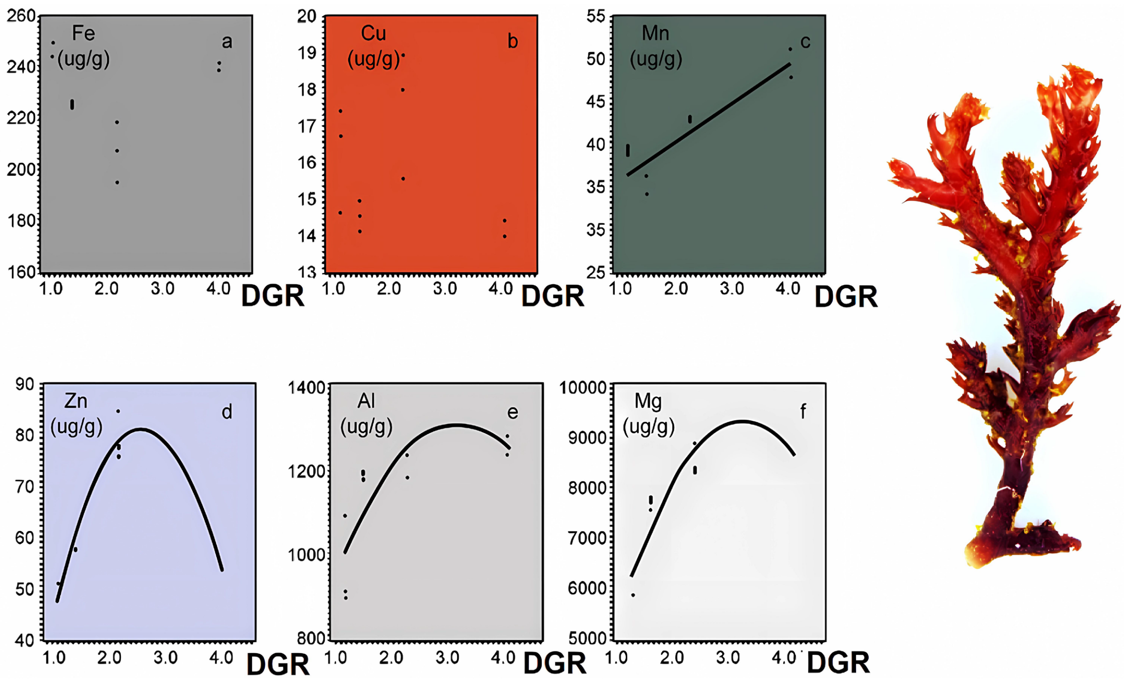

Figure 4. Functional ratio of the magnesium, aluminum, and four trace elements content on the branchlet and apex of *A. triquetrum* in relation to growth rate (DGR) of *A. triquetrum* during the period of greatest vegetative development.

Table 1. Numerical expressions with better-fit and representative coefficients of determination (r^2), used to describe the relationship between the content of some metallic elements present in branchlets and apical portion of *A. triquetrum* and DGR of the plant.

Element	Numerical model	r^2
Mg	$Mg = 864.40 + 527.38 (DGR) - 859.30 (DGR)^2$	0.870
Al	$Al = 516.18 + 498.18 (DGR) - 79.41 (DGR)^2$	0.702
Zn	$Zn = 28.80 + 81.94 (DGR) - 15.40 (DGR)^2$	0.936
Mn	$Mn = 30.56 + 4.79 (DGR)$	

Table 2. Linear correlation coefficient of five trace elements presents in *A. triquetrum*, with respect to the relative growth rate (DGR), its centesimal composition and the magnitudes reached by three environmental variables during the stage of greatest beds development.

Concentration ($ug \cdot g^{-1}$)	Al	Fe	Zn	Mn	Cu
Physical factors					
IT	0.097	0.564*	−0.142	−0.494	−0.490
AST (˚C)	0.127	−0.461	−0.048	0.911***	−0.011
MPA (mm)	0.100	−0.517*	0.535	0.169	0.626**
Macro elements					
Na ($ug \cdot g^{-1}$ d. w.)	−0.134	−0.390	−0.412	0.684**	0.131
Mg	0.576**	−0.160	0.551*	0.490	−0.231
K	−0.206	−0.477	−0.219	0.775***	0.211
N (% d. w.)	−0.034	0.394	0.576**	0.223	−0.745***
P	0.020	0.504*	0.039	−0.837***	−0.132
Metabolic balance					
RGR	0.311	−0.155	−0.028	0.896***	0.490

IT = Inferred Turbulence; AST = Average surface temperature; MPA = Monthly precipitation accumulation; d. w. = Dry weight; *$p \le 0.10$; **$p \le 0.05$; ***$p \le 0.001$.

trace metals is independent of the growth rate of the plant, when DGR increase the tissue concentration of the element usually decreases due to the overall reduction of the exposure time to the medium surrounding the thallus. This effect was observed in *Ulva lactuca* Linn., by [39] for Rb and Cd. The virtual "dilution" of the content in metals such as the Mg, the Al and trace elements such as Zn, is present in the apical portion of *A. triquetrum* when DGR increase.

Between the trace elements quantified in the apical tissue, Mn, Cu, Zn and Fe correlates significantly in one way or other with macro elements linked to intermediate metabolism like magnesium, phosphorus, potassium and nitrogen (**Table 2**).

The significant correlation between the Zn and N contents in *A. triquetrum* (**Table 2**) could also postulate this fact. It is evident when the DGR is high and N availability is limited, competition that can possibly occur between formation and extraction in genesis of enzymes and structural proteins, is evidenced by the research of [40].

Attending to correlations with Inferred Turbulence (IT) and Monthly precipitation accumulation (MPA), tissular Fe content seems to be linked to different sources of this element in sea water during the annual cycle. Due to polar fronts, in winter season the water turbulence enhances, and the suspension of sediments, is higher. In contrast, in summer increase of rain and consequently of terrestrial runoff seems to be the main factor involved. Probably, this phenomenon also determined the strong correlation seen between Cu and MPA due to the presence of riverine pollution in the vicinity of the experimental place.

The increase in the tissue concentration of bioactive metals during the summer reflects their regulation at the metabolic level [41], which also holds for specimens from sites where nutrient availability is greater [42]. According to the seasonal maxima of *A. triquetrum* specimens taken from wild beds, [Al (1204.6 ± 41.7 $\mu g \cdot g^{-1}$ d. w), Fe (200.4 ± 31.7 $\mu g \cdot g^{-1}$ d. w), Zn (67.3 ± 10.8 $\mu g \cdot g^{-1}$ d. w), Mn (52.42 ± 8.7 $\mu g \cdot g^{-1}$ d. w) y Cu (19.9 ± 5.5 $\mu g \cdot g^{-1}$ d. w)], this apparent regularity has been demonstrated.

The hierarchical ordering of these values, according to their magnitude, coincides with the stoichiometric ratio proposed by [26] for the bioactive trace elements registered in the plankton. In order to attain maximum growth, nutrients need to be supplied or acquired by the plant in a stoichiometrically balanced manner [43] [44].

However, as for the ratio between Zn and Mn, at least in tropical and subtropical areas, a significant percentage of the species evaluated by different authors presented a higher content of Mn. Examples: for the Gulf of Mannar, Bay of Bengal, 83.3% [45]; coast of Qatar, 66.7% [46] and for the north coast of Cuba, 41.7% [39] [47].

In some cultivated terrestrial plants, the amount of Fe, Co and occasionally Zn tends to be concentrated in their aerial tissues [48], and is markedly acropetal [49]. Although translocation mechanisms in macroalgae can be quite different from terrestrial plants [50], and their ecophysiological responses very peculiar, there is also a similar relation according to distribution of these compounds and possibly their metabolism in *A. triquetrum.*

The bioaccumulation of these elements in the thallus of agarophytes, although can be associated with the age of the tissue, presented specific peculiarities. In natural beds, the concentration of Al, Fe, and Zn in apical branchlets was somewhat lower than in the oldest parts of the plant, as opposed to what happens with Mn and Cu, the magnitude of which is more than double (**Table 3**).

The effect of this heterogeneous distribution is well seen when, under culture, the plant depresses for instance its growth and increase the proportion of stipes and thick branches, in detriment of the newly formed tissue. That happened in winter, when the rate of grown is lower and the fastening system used, hanging bags, wasn't the best. The differentiated loss of biomass resulting from unfavorable growing conditions will result in an increase in both the ash content of the sample and its trace element concentration (**Figure 5**). This enrichment process

Table 3. Bioaccumulation of Al, Fe, Mn, Zn and Cu in two parts of the thallus of *A. triquetrum* (Mean values ± 1 SD) referred to four replicates.

Element	Concentration (mg·g⁻¹ d. w.)	
	Stipe	Apical portion
Al	1000.1 ± 7.5	972.3 ± 7.6
Fe	252.0 ± 3.2	225.0 ± 4.3
Zn	59.6 ± 0.9	50.5 ± 2.5
Mn	65.1 ± 0.7	31.2 ± 0.6
Cu	30.0 ± 1.6	11.4 ± 0.9

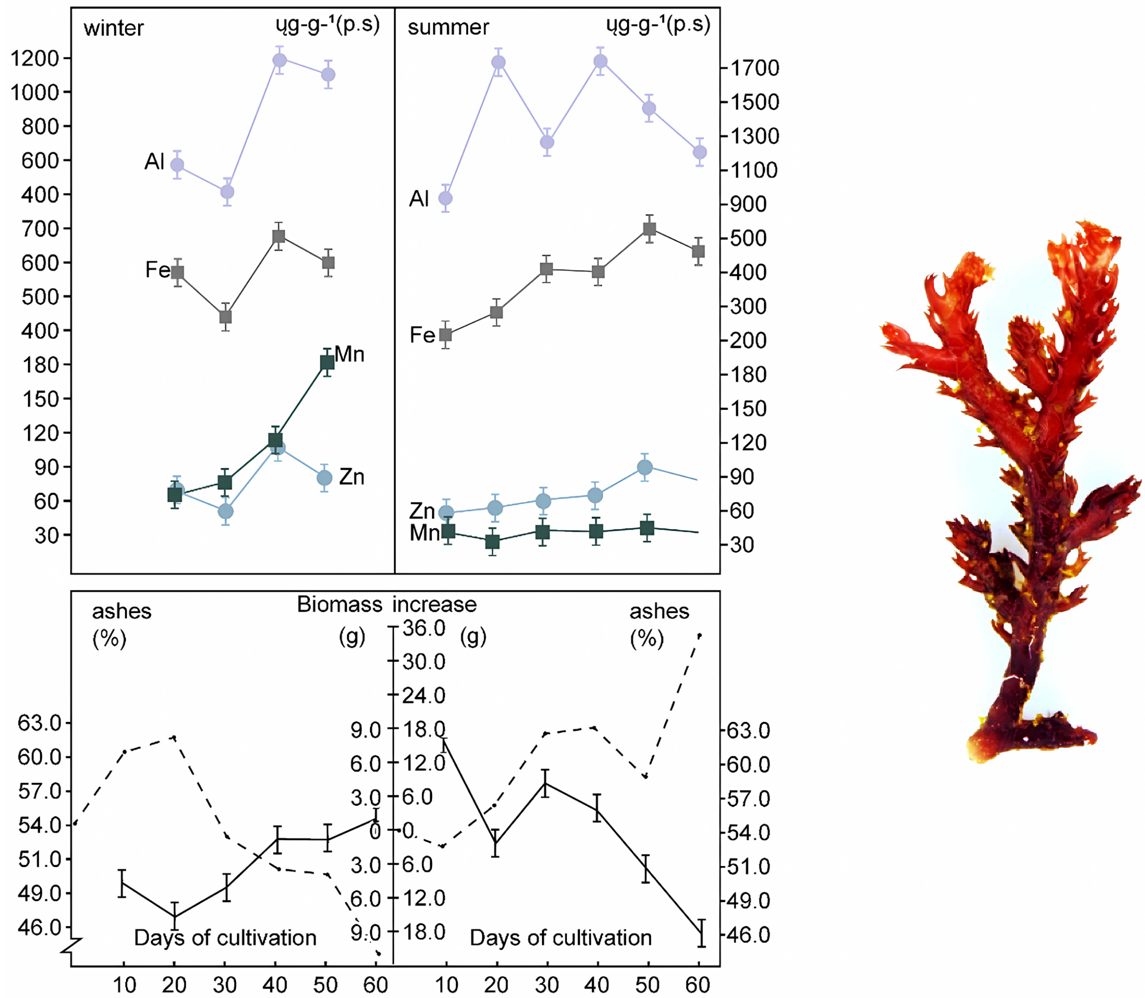

Figure 5. Variation of the concentration of four trace elements in relation to the mean biomass increase (----) and ash content (—). Analyses based on total specimens of *Alsidium triquetrum* grown for 60 days in opposite climatic seasons. The culture technique tested prevented recovery and growth during the winter (Vertical heights equivalent to ±1 DE. Initial weight 50 g).

takes place due to the accumulation of detritus and the presence of abundant epibionts on the affected fronds [51]. When this occurs, trace elements such as Zn and Mn increase their variability concerning tissular content (CV = 31.9 and

50.2% respectively in winter 23.1 *vs* 15.1 in summer). In addition, Mn increase concentration meanwhile the proportion of ancient tissue increase in the thallus (**Figure 5**).

The described process, if sufficiently intense, raises significantly in artificially propagated plants the content of some trace elements with respect to those coming from wild beds. This phenomenon masks the tendency toward an increase in tissue concentrations of these elements during the summer when the analysis was carried out using the entire plant. The decrease in relation to the winter months was significant (U, $p \leq 0.036$) for all the trace metals studied. In samples of apical tissue, it was confined only to Mn and Al (U, $p \leq 0.016$).

In multicellular models such as *Alsidium triquetrum*, the higher accumulation of Mn and Cu in branchlets and apex suggest a greater relationship between them than with other trace elements. If that is factual, the physiological regulation of such interaction would be greater and can mask the differences between the absorption rates of both elements, or their translocation [52]. For phytoplankton a competitive interaction of detoxifying effect has been demonstrated between Mn and Cd [53] and, according to [54], in Rhodophyceae, or maybe just red algae exist also antagonism effects between Fe [55], Zn [56] and Cu [57]. Considering Cu in particular, growth inhibition and deficiency manifestations seem to depend on the ratio between the ionic concentrations of both elements in the medium [30].

The absorption of Mn is physiologically regulated, and is linked to growth rate to a greater degree than other trace elements [58]. Despite the importance of Mn for photosynthesis and other processes, the physiological relevance of Mn uptake and compartmentation in plants has been underrated [59]. This premise may explain very well why, improved Mn uptake capacity will achieve in culture higher growth and yield, even under suboptimal availability, by providing sufficient Mn to PSII and thus increasing their photosynthetic efficiency. Nevertheless, Mn indeed from been a precursor to growth, as also demonstrated by the results of [32], can cause the collapse of cell walls and irreversible metabolic damage, a fact verified in *Sargassum cymosum* C. Agardh. Under experimental conditions, it induced toxic effects at high concentrations, such as reduced growth and diminishing the synthesis of photosynthetic pigments [35] [36].

In correspondence with DeBoer's results [60], it is who suggested that manganese and zinc, could limit growth of macroalgae in nature and demonstrated experimentally in *Ulva lactuca* [61], and in *Sargassum cymosum* [32], our data also suggest that this phenomenon takes place with *A. triquetrum.*

Perhaps it underlies the assertion that macroalgae are among the best biological indicators of the element Mn [62] [63], and because of its practical importance, this relationship should be explored more, specifically in controlled experimental conditions. *Alsidium triquetrum* and other macroalgae are considered as biological models. Hence, the detection of Mn in the apice of *A. triquetrum* can be used to evaluate the physiological status of the plant. Also due to its high concentration in stipe and basal branches, it could be used, together with Cu, to monitor the quality of surrounding environment. This particular allocation is not the same in other macroalgae models. It has been observed that in *Laminaria digitata* (Hudson) JV Lamouroux the concentration of Mn was higher in distal blade sections than in stipes, contrary to what was observed in *Alsidium triquetrum* [64] (**Table 3**).

4. Conclusions

The *on-site* growth data of *A. triquetrum* and its association with the internal concentration of five metals, some of them directly linked to plant metabolism, indicated a differentiation between them in the bioaccumulation and translocation processes in relation to growth.

Apparently, the growth rate regulates the concentration of trace elements directly linked to metabolites such as Mg, Zn and Mn. The kinetics of these three elements as also that of Al, suggests the existence of a tissue dilution process in relation to growth rate. Manganese in particular showed a direct linear relationship with growth.

If proven, this fact could have implications in the definition of physiological criteria of feasibility and fertility on given sites in the the aim to develop culture trials at different scales or for biomonitoring schedules to assess environmental health.

Authors' Contributions

AA, conceived and designed the experiments, analyzed the data, prepared figures, and reviewed all manuscript drafts. RC, prepared figures, and reviewed all manuscript drafts. JDL prepared figures and worked on conceptualization and analysis. LCV contributed to editing and reviewing all drafts of this manuscript. All authors have read and agreed to the version of the manuscript submitted.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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