ECOTOX BRASIL
SOCIEDADE BRASILEIRA DE ECOTOXICOLOGIA

Ecotoxicol. Environ. Contam.,v. 18, n. 2, 2023, 47-72
doi: 10.5132/eec.2023.02.07

EEC

Review

Mechanisms of Tolerance in Metal-exposed Brown Algae and the Importance of Monitoring Studies: A Review

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Received October 08, 2023; Accept January 30, 2024

Abstract

Marine habitats are under threat from the continued release of contaminants into waterways, and monitoring becomes crucial to future management decisions and regulations to protect these environments. A key aspect of this monitoring is the choice of a marine species that is able to cope with this pollution, and the tolerant species exhibit more relevant ecological responses. These species-responses are called biomarkers of tolerance and are tested using a variety of measurements that reflect exposure to chemicals. Marine brown macroalgae can be found in a wide range of marine environments, including those with high metal pollution and they are widely used in monitoring programs. Mechanisms of tolerance are vital to the survival of this group of macroalgae in polluted habitats, but these mechanisms have not yet been reviewed. The aim of this critical review article is to summarize the historical information available regarding metal tolerance in marine brown macroalgae. We review studies about tolerance mechanisms in marine brown macroalgae exposed to metals, conducted both under experimental conditions and in the field. Also, bibliometric analyses are used to identify the most relevant investigations, country contributions and gaps in knowledge on the topic. Results showed that the principal extracellular tolerance mechanisms described for marine brown macroalgae are physical exclusion, exudated ligands, and metal-binding to the cell wall. Epiphytic microorganisms were also found to be capable of accumulating a given metal. Internally, metallothioneins and phytochelatins were important chelators; furthermore, antioxidant responses may increase metal tolerance. Omics analyses were used to reveal the internal mechanisms used by these macroalgae. Biomarkers of tolerance have occasionally been used in monitoring studies, providing additional information regarding tolerance to complex chemical mixtures found in the field. Future studies should make use of these biomarkers to improve our understanding of metal-tolerance strategies.

Keywords: Antioxidants; Chelators; Exudates; Heavy metal; Phaeophyceae; Polysaccharides.

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INTRODUCTION

Coastal areas have been and continue to be used as dumping grounds in which heavy metal contaminants have become a serious issue and one of the main drivers of ecological degradation in marine ecosystems (Yasuhara *et al.*, 2012; Mousavi *et. al.*, 2023). Although efforts have been made globally to improve waterway management and pollution cleanup, there is still a need for better environmental strategies to be developed for the monitoring of marine pollution (Tavakoly Sany *et al.,* 2014). One important consideration as part of monitoring is the degree of tolerance of a chosen species to a chemical pollutant. A tolerant species would be of greater ecological relevance, thus making it more suitable as a biological marker (Phillips and Rainbow, 1993). Amiard-Triquet *et al.* (2011) emphasize that the importance of tolerance lies in its enormous implications for the sustainability of biodiversity and the continuity of ecosystem functions around the world. Tolerance consumes considerable energy and may result in a reduction in the allocation available to ensuring success in reproduction and growth (Amiard-Triquet *et al.*, 2011).

Plants have considerable ability to adapt to metal stress (Labudda *et. al.,* 2022) and metal tolerance mechanisms in plants have been reviewed by several authors (more details in Hall, 2002; Bjerregaard and Andersen, 2007; Gupta *et al.,* 2013; Hasan *et al.,* 2017). Five general mechanisms of metal tolerance are reported: (1) symbiotic association, in which metal is absorbed by the symbiotic organism instead of by the plant; (2) sequestration/immobilization on the cell wall; (3) intracellular chelation by metallothioneins and phytochelatins; (4) interference in transference through the cell membrane; and (5) action of proteins associated with heavy metal-related stress, such as heat shock proteins. Mechanisms of tolerance to copper (Cu) in green and red macroalgae reviewed by Moenne *et al.* (2016; 2020) showed similar strategies to those of plants.

Given the cosmopolitan nature of marine macroalgae, they have been widely used as bioindicators of pollution. For instance, some important monitoring programs around the world have incorporated macroalgae into their surveys. These include the Waste Framework Directive (WFD) in Europe; the ALGAMONY project, which standardized methodologies for monitoring of seaweeds across Nordic countries; and the Intertidal Reef Monitoring Program (IRMP), which conducts censuses of invertebrates and macroalgae in Australia (for more detail, see Farrugia-Drakard *et al.*, 2018; D'Archino and Piazzi, 2021). As such, there is a huge amount of information on macroalgae as bioindicators, and excellent reviews have been conducted by a number of authors (e.g., Burridge and Bidwell, 2002; Prygiel and Haury, 2006; Sánchez-Quiles *et al.,* 2017; García-Seoane *et al.,* 2018; Vázquez-Arias *et al.,* 2023). However, the main focus of attention has been on metal concentrations in macroalgal tissue, and only a few field studies have employed biomarkers associated with tolerance.

Marine brown macroalgae (class Phaeophyceae) are classified into around 300 genera comprising more than 2,000 species (Kawai and Henry, 2016). These multicellular macroalgae almost exclusively inhabit marine environments, primarily along rocky coastlines in temperate to polar regions (Duarte *et al.,* 2022). Some authors have attributed their worldwide distribution to their ability to rapidly change their shape (Tesson and Charrier, 2014). They also play an important ecological role, providing habitats for a wide range of fauna (Sjøtun *et al.,* 2021; Gibbons and Quijon, 2023). Moreover, this group of macroalgae are good accumulators of metals (Ma *et al.*, 2018), possessing a high biosorption capacity and retaining twice as much metal as any of the other macroalgae tested (Romera *et al.,* 2007). These characteristics were a motivation to review and explore the different tolerance mechanisms by which marine brown macroalgae cope with highly metal-polluted environments. The present review article is divided into three sections. The first focuses on experimental published works, and the second presents a review of that literature which tests biomarkers of tolerance in brown macroalgae in the field. The final section consists of a bibliometric analysis exploring the main contributions from each country, the principal mechanisms studied, and gaps remaining on the topic in this group of seaweeds.

Mechanisms of tolerance in brown macroalgae

Mechanisms of tolerance in marine brown macroalgae were reviewed based primarily on the work of Gaur and Rai (2001), but other further studies were also consulted, such as Hurd *et al.* (2014) and Moenne *et al.* (2016; 2020). The mechanisms of tolerance identified were external ones known as exclusion and relating to membrane permeability; exudation and formation of complexing ligands; binding of metal ions to the cell wall; and epibiont organisms able to adsorb metals. When metal ions succeed in entering cells, internal mechanisms come into operation, such as metallothioneins (MTs), phytochelatins (PCs), and other intracellular ligands, all of which have also been studied in marine brown seaweeds. In addition, some authors have suggested that oxidative stress and antioxidant responses are also part of the tolerance mechanism of these macroalgae, highlighting the importance of these responses as mechanisms of defense against metal exposure.

Extracellular mechanisms

Exclusion

Laboratory studies

Exclusion mechanisms have not been evaluated extensively in marine brown macroalgae except by a few studies on Cu. Hall *et al.* (1979) exposed three specimens of *Ectocarpus siliculosus* from two uncontaminated sites (Hilbre Island, England, and Rhosneigr, north Wales), one individual that is tolerant (Salcombe, England) and two individuals that are not tolerant to different concentrations of Cu. They observed that the tolerant macroalgal specimen had significantly less Cu than one of the non-tolerant ones, and a difference in growth was also observed: the tolerant specimens continued growing, while one of the non-tolerant individuals (Hilbre Island) was completely inhibited. The authors suggested that an exclusion mechanism was operating in these tolerant specimens; however, little information was provided by their study. Nielsen *et al.* (2003b) state that Cu accumulation depends, to a certain degree, on metal exclusion mechanisms, but also on the contamination history of the macroalgae's location. The scholars used brown macroalgae *Fucus serratus* from sites in England: a contaminated site (Restronguet Creek, Fal Estuary), and two control (unpolluted) sites. After exposure, macroalgae from the control sites exhibited higher total Cu content ($[Cu^{2+}]_{ext}$) compared with macroalgae from the contaminated site. In fact, metal accumulated in *F. serratus* specimens from the contaminated site was only half as concentrated. Roncarati *et al*. (2015) found evidence of exclusion mechanisms in specimens of *E. siliculosus*. They found that in a tolerant specimen (Es524 from a polluted site in Chile), the intracellular fraction of Cu was around 30% lower than in the LIA individual at the reference site in Scotland, while the extracellular fraction was around 70% higher. Similar to the findings of Hall *et al.* (1979), the tolerant individual (polluted site) continued to grow, while in the specimens from the control site, growth was impeded (Table 1). The authors proposed that this was due to a successful intracellular mechanism of defense in the tolerant specimen, which reduced the toxic effect of Cu at high concentration and allowed the plant to continue to grow. Celis-Plá *et al.* (2018) exposed the seaweed *Cystoseira tamariscifolia* (now *Ericaria selaginoides*) to similar concentrations of Cu (2.0 μM) as Roncarati *et al.* (2015), finding that intracellular concentrations accounted for around half of the total accumulated. Unfortunately, the contamination history of the macroalgae used was not provided.

Exudation: complexing ligands

The combination of metal ion and a ligand is referred to as coordination or complex formation (Pankow, 1991; Stumm and Morgan, 1995; Mason, 2013). Chelation refers to the attachment of cations at more than one site on the ligands, and the strength of these complexes formed is indicated by conditional stability constants or log *K* (Mason, 2013). In solution, metal ions are complexed as free metal ions with inorganic and organic ligands. The importance of complexation by organic ligands is its effect of reducing the concentration of the free ion of a certain metal in solution, which thus reduces the toxicity or bioavailability of this metal (Donat and Dryden, 2009).

In order to comprehend the role of metal ions as essential or toxic elements, we must first understand their speciation (chemical forms) in solution, as not all metal forms can be absorbed by organisms (Mason, 2013). Organic speciation of Cu, zinc (Zn), and iron (Fe) dominates dissolved fraction speciation in oceanic surface waters and, as such, is crucially important to regulating the free metal ion concentrations of these metals (Donat and Dryden, 2009). Furthermore, these metals are essential to the living processes of organisms. The above may go some way towards explaining why the organic Cu complexation capacity of micro and macroalgae has been a primary focus of study (e.g., do Nascimento Júnior, *et al.,* 2019; Shi *et al*., 2021). The importance of speciation and toxicity of Cu in marine macroalgae has been reviewed by Gledhill *et al.* (1997). Marine brown macroalgae are known to release extracellular organic matter, such as humic material, polyphenolic material, and sulfonated polysaccharides (Dogs *et al*., 2017; Lozada *et al*., 2021).

Laboratory studies

Schramm (1993) found that organic substances exuded by *Fucus vesiculosus* and *Laminaria saccharina* decreased Cu toxicity, thus exhibiting the potential to alter the toxicity of Cu or its bioavailability. Likewise, Sueur *et al.* (1982) detected strong chelating abilities (log K = 10.2) of organic material exuded by *E. siliculosus* under exposure to Cu, suggesting its important role in altering the availability of metals for other organisms as well as themselves. In addition, Gledhill *et al.* (1999) observed one class of ligands in *F. vesiculosus* with a similar strength to that found by Sueur *et al.* (1982), proposing that exudation of the Cu complexing ligands may be due to detoxification mechanisms, as these increased at higher Cu concentrations (0.5 μM). Andrade *et al.* (2010b) explored the nature of the brown macroalgal exudates by measuring them quantitatively and qualitatively. The authors found that *Lessonia nigrescens* responds rapidly, exuding ligands and reducing the toxicity of Cu in only hours. Furthermore, they found weak ligands (log K = 8.6) compared with the previous studies mentioned. This was supported by Fellous *et al.* (2017), who demonstrated that exudates of another species of kelp, *Lessonia spicata*, were able to protect not only the algae itself but also crustacean larva to which the algae provides habitat. Murray *et al*. (2014) tested the impact of exudate ligands in three brown macroalgae under experimental exposure to Cu and Zn and in terms of metal bioavailability. They found that exuded ligands substantially decreased the concentration of labile metals. Their findings supported the hypothesis that ligands exuded by these macroalgae constituted a metal tolerance defense mechanism rather than being the result of nutritional deficiencies. This is consistent with another study in which two species of early-life kelps (*L. nigrescens and U. pinnatifida*) were exposed to higher concentrations of Cu: more Cu-binding ligands were reported (Table 1), suggesting that these ligands may work as a mechanism of defense against this metal toxicity (Leal *et al.,* 2018). An important point, suggested by some authors (Andrade *et al*., 2010b; Murray *et al*., 2014), is the need to identify these ligands, such as the polyphenols.

Table 1 Studies of brown algae using biomarkers of tolerance in experiments.

Order	Species	Metal/Metalloid	Concentration/Time	Biomarkers	Reference
Dictyotales	*Dictyota kunthii*	$CuCl_2$	0.74 µM / 4 d	CAT, GPx, PRx, DHAR, GR, APx, LPx	Sordet *et al.* (2014)
	Padina gymnospora	$CdCl_2*H_2O$, $Zn_2SO_4*7H_2O$	Cd: 5 µM, Zn: 3.5 µM / 7 d	Polysaccharides	Andrade *et al.* (2002)
Ectocarpales	*Ectocarpus siliculosus*	$CuSO_4$	0, 1.5, 3.13 µM / 14 d	Exclusion	Hall *et al.* (1979)
	E. siliculosus	$CuSO_4*5H_2O$	0, 0.4, 0.8, 1.6, 2.4 µM / 10 d	Exclusion, PCs, GSH	Roncarati *et al.* (2015)
	E. siliculosus	$CuSO_4$	0.3–5.5 µM / 30 d	Exudates ligands	Sueur *et al.* (1982)
	E. siliculosus	$FeCl_3$	30 µM / 24–168 h	Polysaccharides	Miller *et al.* (2014)
	E. siliculosus	$CuCl_2$	2.5 µM / 24 h	Genes PCs, MTs	González *et al.* (2018a)
	E. siliculosus	$CuCl_2$	0.37, 1.1, 1.86, 3.7 µM / 4, 8 h, 6–10 d	vBPO	Ritter *et al.* (2010)
	E. siliculosus	$CuCl_2$	3.7 µM / 4, 8 h	vBPO, Oxylipins	Ritter *et al.* (2014)
	E. siliculosus	$CuSO_4*5H_2O$	0, 0.4, 0.8, 1.6, 2.4 µM / 10 d	SOD, CAT, AP_X, ASC, DHA, GSH, GSSG, Phenolic compounds	Sáez *et al.* (2015c)
	E. siliculosus	$CuSO_4*5H_2O$	0, 2.4 µM / 10 d	Genes CAT, AP_X, Fe-SOD	Sáez *et al.* (2015b)
	E. siliculosus	$CuCl_2$	2.5 µM / 24 h	Genes associated with antioxidant enzymes	González *et al.* (2018b)
	Scytosiphon lomentaria, *S. gracilis* (now *Planosiphon gracilis)*	$CuCl_2$	0.74 µM / 96 h	CAT, MDHAR, APx, GPx, DHAR, GR	Contreras *et al.* (2007)
	S. lomentaria	$CuCl_2$	0.15, 0.3, 0.74 µM / 2, 6, 12, 24, 48, 96 h	CAT, GR, APx, GPx, DHAR, MDHAR, LP_X, ROS	Contreras *et al.* (2009)

	S. gracilis (now *P. gracilis)*	$CuCl_2$	0.74 µM / 4 d	Proteins associated with ROS scavenging	Contreras *et al.* (2010)
	S. gracilis (now *P. gracilis)*	$CuCl_2$	0.04, 0.074, 0.15, 0.3, 0.74, 2.2 µM / 4, 12, 24, 72, 96 h	PR_X, LP_X	Lovazzano *et al.* (2013)
Fucales	*A. nodosum*, *Fucus vesiculosus*	Cu, Zn	Cu: 0, 7.86, 15.70 nM, Zn: 0, 15.20, 91.70 nM / 7 d	Exudates ligands	Murray *et al.* (2014)
	Ascophyllum nodosum	$CuCl_2$	0.074–0.37 µM / 30 d	Phenolic compounds	Toth and Pavia (2000)
	A. nodosum, *F. vesiculosus*	$ZnCl_2$	3.67, 14.7, 36.7 µM / 2–4 d	Polyphenols	Ragan *et al.* (1980)
	A. nodosum	^{85}Sr ^{65}Zn	10 µCi/L in the seawater / 4, 40 d	Polysaccharides	Skipnes *et al.* (1975)
	A. nodosum	$CuSO_4*2H_2O$, $FeCl_2$	3, 10 ppm of metals dissolved in filtered seawater from Portaferry, County Down, Ireland / 10 d	Epiphytic organisms	Stengel and Dring (2000)
	Cystoseira tamariscifolia (now *Ericaria selaginoides*)	$CuSO_4*5H_2O$	0, 0.5, 2.0 µM / 14 d	Phenolic compounds, Exclusion	Celis-Plá *et al.* (2018)
	Fucus ceranoides	$AgNO_3$	0, 0.29, 0.59, 0.88 µM / 14 d	H_2O_2, LPO	Ramesh *et al.* (2015)
	F. serratus	$CuSO_4*H_2O$	0, 42.2, 211, 422, 844 nM / 12, 23 d	Exclusion	Nielsen *et al.* (2003b)
	F. serratus	$CuSO_4*5H_2O$	0, 0.1, 0.5, 2.0 µM / 12 d	Phenolic compounds	Nielsen and Nielsen (2010)
	F. serratus, *F. vesiculosus*	$Cd(NO_3)_2$	4.2 µM / 2, 4, 7, 11 d	PCs, GSH	Pawlik-Skowrońska *et al.* (2007)
	F. serratus	$CuSO_4*H_2O$	2110 nM / 160 s	ROS	Nielsen *et al.* (2003a)
	F. spiralis, *Hizikia fusiformis* (now *Sargassum fusiforme*)	As^V	0, 13.3 µM, 0.133 mM / 24 h	PCs, GSH	Wood *et al.* (2011)

F. vesiculosus	$CuCl_2$	0.04–0.74 µM / 3–6 wk	Exudates ligands	Schramm (1993)
F. vesiculosus	Cu	0.02, 0.1, 0.5, 1 µM / 19 d	Exudates ligands	Gledhill *et al.* (1999)
F. vesiculosus, *A. nodosum*	$CuSO_4*5H_2O$	0, 0.4, 4, 20 µM / 1, 2, 7, 15 d	Phenolic compounds	Connan and Stengel (2011)
F. vesiculosus, A. nodosum	Pb, Cd, Cu, Hg, Ni, Zn	Pb: 3 µg/L, Cd: 1.0 µg/L, Cu: 10 µg/L, Hg: 2.5 µg/L, Ni: 4 µg/L, Zn: 250 µg/L / 14 d	Phenolic compounds	Pedersen (1984)
F. vesiculosus	$CdCl_2$	10 µM, 0.1 mM, 2.5 mM / 5 d	Polysaccharides	Lignell *et al.* (1982)
F. vesiculosus	$CuCl_2$	18.6 µM / 72 h	MTs	Morris *et al.* (1999)
F. vesiculosus	As_2O_3, $CdSO_4$	As: 5 mM, Cd: 50–150 µM / 4 h	MTs	Merrifield *et al.* (2004)
F. vesiculosus	$CdSO_4$, $Zn(NO_3)_2$	Cd: 50–150 µM / 4 h	MTs	Merrifield *et al.* (2006)
F. vesiculosus	$As^{(III)}$, $As^{(V)}$	10 µM / 20 min	MTs	Singh *et al.* (2008)
F. vesiculosus	$CuSO_4$	0, 0.02, 0.19, 0.94, 1.88, 3.76 µM / 0, 24, 48, 72, 96 120, 144 h	MTs	Owen *et al.* (2012)
H. fusiformis (*Sargassum fusiforme*)	$CuSO_4*5H_2O$, $CdCl_2*2.5H_2O$	Cu: 0, 0.1, 0.2, 0.4, 2.0 µM, Cd: 0, 0.88, 17.52, 26.27, 35.03, 43,79 µM / 7 d	MDA, CAT, SOD, NR	Zhu *et al.* (2011)
H. fusiformis (*Sargassum fusiforme*)	$HgCl_2$	0, 0.07, 0.15, 0.37, 0.74, 1.47 µM / 7 d	MDA, CAT, SOD, NR	Zhu *et al.* (2015)
Sargassum cymosum	$CuCl_2$, $PbCl_2$	10, 25, 50 µM / 7 d	Phenolic compounds	Costa *et al.* (2016)

	Sargassum cinereum	$NiSO_4$, $MnCl_2$, $ZnCl_2$, $CuSO_4$, $CdCl_2$, $K_2Cr_2O_7$, $Pb(NO_3)_2$	10 mM of each metal, with the exception of Pb at 1, 2, 3, 5 mM / 30 d	Epiphytic organisms	Damare (2015)
	S. cymosum	$CdCl_2$	0, 0.55, 1.09, 2.18, 4.36 µM / 7, 14 d	Phenolic compounds	Costa *et al.* (2017)
	S. cymosum	Trace metals from mining tailings	Mud dissolved in seawater / 5, 15 d	Phenolic compounds	Costa *et al.* (2019a)
	S. cymosum	Trace metals from mining tailings	Mud dissolved in seawater / 7 d	CAT, GP_X, SOD, Phenolic compounds	Costa *et al.* (2019b)
	S. fusiforme	$CuCl_2$	8 µM, 47 µM / 1, 7 d	MDA, SOD, CAT, POD, NR, ASC	Zou *et al.* (2014)
	S. fusiforme	$CuCl_2$	0, 4, 8, 24, 47 µM / 1, 3, 5, 7 d	Proteins associated with ROS scavenging, carbohydrates metabolism	Zou *et al.* (2015)
	S. fusiforme	$CuSO_4$	0, 0.16, 0.47, 0.93 µM / 7 d	SOD	Wen and Zou (2021)
	S. muticum	$Cd(NO_3)_2$	20 µM / 2 d	PCs	Gekeler *et al.* (1988)
Laminariales	*Laminaria digitata*	$N_4O_{10}Ru$	20 mL of Ru^{106} in seawater / 20 d	Polysaccharides	Jones (1980)
	L. digitata	$CuCl_2$	2.23 µM / 6, 24, 48, 72 h	Oxylipins, LPO, Genes SOD, PRx, GST	Ritter *et al.* (2008)
	Laminaria hyperborea	Cu, Zn	Cu: 0, 7.86, 15.70 nM, Zn: 0, 15.20, 91.70 nM / 7 d	Exudates ligands	Murray *et al.* (2014)
	Laminaria saccharina (now *Saccharina latissima*)	$CuCl_2$	0–0.74 µM / 3–6 wk	Exudates ligands	Schramm (1993)
	Lessonia nigrescens	$CuCl_2$	42, 117, 157 nM / 48 h	Exudates ligands	Andrade *et al.* (2010b)
	L. nigrescens, *Undaria pinnatifida*	$CuCl_2$	2.47, 3.63 µM / 9 d	Ligands	Leal *et al.* (2018)

L. nigrescens	$CuCl_2$	0.15, 0.3, 0.74 µM / 2, 6, 12, 24, 48, 96 h	CAT, GR, APx, GPx, DHAR, MDHAR, LP_X, ROS	Contreras *et al*. (2009)
L. nigrescens	$CuCl_2$	0.04, 0.074, 0.15, 0.3, 0.74, 2.2 µM / 4, 12, 24, 72, 96 h	PR_X, LP_X	Lovazzano *et al.* (2013)
Lessonia spicata	$CuCl_2$	0.74–6.32 µM / 48 h	Exudates ligands	Fellous *et al.* (2017)
Macrocystis pyrifera	$FeCl_3$	3 µM / 4, 8, 24 h, 19 d	Polysaccharides	Miller *et al.* (2016)
M. pyrifera	$CuSO_4$*5H_2O	IC50: 100–250 ppm isolated bacterial / 18–20 h	Epiphytic organisms	Busch *et al.* (2015)
M. pyrifera	$K_2Cr_2O_7$	0, 1.7, 6.8, 34, 68 µM / 24 h	Protein expression of GP_X, GST, GR, vBPO	Wang *et al.* (2021)
M. pyrifera	$CuSO_4$	15 µM / 3, 6, 12, 24 h	Genes GST	Gu *et al.* (2023)
Saccharina japonica	$CuSO_4$	0.06, 0.6, 1.2 µM / 3 d	Genes APx, GST, CAT, GPx, SOD, POD, vBPO	Zhang *et al.* (2019)

Abbreviations: ASC = Ascorbate; APx = Ascorbate peroxidase; CAT = Catalase; DHA = Dehydroascorbate; DHAR = Dehydroascorbate reductase; MDHAR = Monodehydroascorbate reductase; GPx = Glutathione peroxidase; GR = Glutathione reductase; GSH = Reduced glutathione; GSSG = Oxidized glutathione; GST = Glutathione transferase; LPO = Lipid peroxidation; MTs = Metallothioneins; PCs = Phytochelatins; ROS = Reactive oxygen species; SOD = Superoxide dismutase; PRx = Peroxiredoxins; LPx = Lipoperoxides; NR = Nitrate Reductase; POD = Peroxidase, vBPO vanadium-dependent bromoperoxidase; MDA = Malondialdehyde contents.

Phenolic compounds (phlorotannins) have been found in marine brown macroalgae in insoluble form extracellularly on the cell wall and in soluble form both intracellularly in the physodes and exuded in seawater (Koivikko *et al.*, 2005; Imbs and Zvyagintseva, 2018; Mannino and Micheli, 2020). In the present study I will begin by reviewing exuded and extracellular phenol content as external mechanisms of tolerance. Intracellular phenolic compounds will be reviewed in section 1.2.3.

Polyphenols can be exuded by marine brown seaweeds as a result of several factors, including UV radiation (Amsler and Fairhead, 2005; Lomartire *et al*., 2021; Cruces *et al.,* 2012; Creis *et al.*, 2015), increased exudation of polyphenols as a defense mechanism against herbivores (Pavia and Toth, 2000; Koivikko *et al.* 2005; Lemesheva and Tarakhovskaya, 2018; Mannino and Micheli, 2020), and the presence of heavy metals (Ragan *et. al.*, 1979; Amsler and Fairhead, 2005). Ragan *et al.* (1979) investigated the chelation capacity of various divalent metal ions in the macroalgae *A. nodosum* and *F. vesiculosus*, finding similar chelation pattern in both species, especially with Cu^{2+} and Pb^{2+}, and consequently proposing that marine brown macroalgal polyphenols may be a significant natural chelator.

Laboratory studies

In the case of metals, Ragan *et al.* (1980) exposed phytoplankton to different concentrations of Zn^{2+}, supplementing it with polyphenols from *Ascophyllum nodosum* and *F. vesiculosus*. They found increased growth rate effects in phytoplankton at lower concentrations of Zn (3.67 µM), supporting the direct ability of polyphenols to detoxify heavy metal ions. Increased total phenolic content and physodes displaced toward cortical cells were suggested as a defense mechanism in the brown algae *Sargassum cymosum* following exposure to Cu (10–50 µM) and lead (Pb) (Costa *et al*., 2016). Likewise, other authors observed increased total phenolic compounds in brown macroalgae under exposure to Cu even at lower concentrations (0.4–2.4 µM) (Sáez *et al*., 2015c; Celis-Plá *et al*., 2018). Costa *et al.* (2019a), after exposing *S. cymosum* to different metals from mining waste, also found an increase of phenolic content (4.95 mg g^{-1} dry weight; about two times higher from the control), acknowledging the greater ability of this species to detoxify metals in comparison to a red algae. Additionally, polyphenol content has been evaluated in order to identify associations with metals and other internal or natural stressors by scholars such as Pedersen (1984), who found no connection between various heavy metals and phenol compounds; rather, the latter were associated with age and salinity in two fucoids. Nielsen and Nielsen (2010) exposed two populations of the *F. serratus*—tolerant, from a polluted site (Restronguet Creek, England), and non-tolerant, from an unpolluted site (Bantham Quay, England)—to different concentrations of Cu^{2+} and a light acclimation (high and low), finding that total polyphenol content was the principal mechanism of defense in the non-tolerant algae group, with values decreasing at both low and high light irradiation levels under the highest Cu exposure (2.0 µM). For the tolerant group, polyphenol content also decreased at low light irradiation but not at high light irradiation under the highest Cu exposure. This suggests that polyphenol content is not the main mechanism of tolerance, and that other mechanisms must be working to keep Cu^{2+} away or to remove it from the algae. By contrast, Connan and Stengel (2011) evaluated the phenolic pool: content and composition (intracellular and extracellular) and phenolic exudation in the presence of Cu^{2+} and salinity in the brown algae *A. nodosum* and *F. vesiculosus*. When Cu exposure was increased, they observed a decrease in total phenolic content, accompanied by an increase in the quantity of phenols on the cell wall and an increase in phenolic exudation in both algae. The authors explained that the total phenolic compound decreased in the presence of Cu, probably due to higher phenolic exudation or photosynthesis inhibition. Intracellular phenolic and exuded phenolic compound in the water were found to be directly associated with salinity stress. By contrast, phenolic compounds bound on the cell wall were associated only with the increase in Cu, suggesting it as a protection mechanism in both algae.

Binding: polysaccharides

Marine brown macroalgae have a cell wall comprising an external surface of cellulose fibers that constitute the skeleton of the walls. In addition, there is a matrix formed primarily of two polysaccharides: alginic acid or alginate, and fucoidan (fucans) (Mazur *et al.,* 2018; Mazéas *et al*., 2023). The alginates are linear polysaccharides comprised of two uronic acids: mannuronic acid and guluronic acid. Alginates are located in the cell wall and in the intercellular region (Mazur *et al.*, 2018; Rabillé *et al*., 2019). Fucoidans are also found in the cell wall but are sulphated polysaccharides. Fucoidans provide the structure of the wall, allow the cross connection of cellulose and alginates (Kloareg *et al.,* 1986; Li *et al*., 2021)/肿, and permit the anchoring of zygotes and germlings so that these are not dislodged by waves (Graham and Wilcox, 2000).

Alginates and fucoidans play a role in preventing desiccation (Ponce and Stortz, 2020), preventing hydrophilic colloids (Mariani *et al.,* 1985), and exchanging ions (Mariani *et al*., 1985; Kloareg *et al*., 1986; Mariani *et al*., 1990). In this cation exchange role, they are negatively charged, adsorbing cations and partially excluding anions; as such, they act as an ionic barrier (Mariani *et al.*, 1985). Thus, they are able to adsorb toxic metals from the environment and impede the passage of these into the cells (Foday Jr., 2021). For fucoidans, the interaction is totally electrostatic for both univalent and divalent ions, as it is not selective between the principal cations (Na^+, K^+, Mg^{2+}, Ca^{2+}) of seawater (Kloareg *et al.*, 1986). Haug (1961) found that affinity for divalent metals would depend on the chemical composition of the alginates under study. For instance, they found that alginates rich in guluronic acid have greater affinity for divalent metals. Haug and Smidsrød (1967) confirmed that ion exchange affinity was associated with composition of uronic acid and with different divalent metals such as strontium (Sr), calcium (Ca), and magnesium (Mg) in marine brown macroalgae.

Laboratory studies

Skipnes *et al.* (1975) tested uptake of Sr and Zn in the *A. nodosum* and the role of anionic polysaccharides, finding that polysaccharides act as an ion buffer. For instance, Jones (1960) evaluated uptake of ruthenium-106 in three phylogenetically distinct macroalgae, finding that the laminarian species accumulated more of this metal and suggesting that this phenomenon was attributed to extracellular polysaccharides on the macroalgal surface. Andrade *et al.* (2002) exposed the brown algae *Padina gymnospora* to cadmium (Cd) and Zn and confirmed previous field findings by Mariani *et al.* (1985) (for more detail, see section 2), namely a major abundance of polysaccharides in the thallus. In addition, crystalline deposits discovered by Andrade *et al.* (2002) indicated that sulphated polysaccharides were associated with the immobilization of metals, confirming that the cell wall is an important site of mineralization of different cations, and thus plays a key role in accumulation of and tolerance to metals in the algae.

Additionally, Miller and colleagues found that carboxylate groups from alginates are the principal ligand in the cell wall in *E. siliculosus* and *Macrocystis pyrifera* exposed to Fe (Miller *et al.*, 2014; Miller *et al.*, 2016). Proteomic analyses in two species of marine brown macroalgae exposed to Cu also revealed protein expression of cytosolic phosphomannomutase (PMM) and GDP-mannose associated with biosynthesis of polysaccharides in the cell wall (Contreras *et al.* 2010; Zou *et al.* 2015).

Sequestration by microfouling

According to Hurd *et al.* (2014), another mechanism of metal tolerance in marine macroalgae involves the ability of microorganisms that inhabit the algal surface to sequester metals by adsorption or detoxification. Several complex mechanisms are involved in macroalgae-bacteria interactions (Martin *et al.*, 2014; Kouzuma and Watanabe, 2015; Ramanan *et al.*, 2016; You *et al.,* 2021;Wang *et al.,* 2022). These interactions have received little attention in relation to metal pollution (see Paix *et al*., 2021; You *et al*., 2021).

Laboratory studies

Busch *et al.* (2015) isolated bacteria from the surface of *M. pyrifera* and from the water that this algae was inhabiting in order to evaluate the influence of Cu. Busch and colleagues found that some isolated bacteria from the most polluted site tolerated high levels of the metal, proposing that, due to high levels of Cu pollution in the habitat, they acquired the ability to tolerate the metal. Other epibionts have been reported in marine brown seaweeds. For instance, Stengel and Dring (2000) exposed the fucoid *A. nodosum* to Fe and Cu for 10 days, and they found that the most Fe was located in pennate diatoms from the algal surface, and no Fe was found in algae without diatoms. They found no difference in Cu between epiphytic diatoms and macroalgae. Damare (2015) used thraustochytrid protists from the surface of the brown algae *Sargassum cinereum* to evaluate the effects of several metals, finding that Mg, nickel (Ni), chromium (Cr), and Zn were the best tolerated by two of the protists studied: *Aurantiochytrium* spp. and *Ulkenia* sp. Although holes on the protists' surface indicated high levels of metal accumulation and resulting damage, the authors suggested that thraustochytrids from the algae may contribute to its potential to accumulate metals in polluted waters.

Intracellular mechanisms

Metallothioneins

Some protective mechanisms that decrease the concentration of free pollutants in cells involve binding them to another molecule prior to excretion or storage (Walker *et al.*, 2012). Metallothioneins (MTs) are proteins rich in cysteine and with low molecular weight. They bind metal ions and are typical biochemical indicators of metal pollution (Fabrin *et al.*, 2018). MTs and phytochelatins (PCs) are used to chelate metals in the cytosol and subsequently sequester these metals in particular subcellular compartments (Jan and Parray, 2016). These two classes of chelators are important ligands and probably the main mechanism of detoxification and tolerance of heavy metals (Hall, 2002). Metallothionein proteins and genes have been detected in plants and animals, as well as in prokaryotes (Verkleij *et al*., 2003). Different types of MT have been identified, and they can vary in sequence and function; for instance, four types have been found in plants (Leszczyszyn *et al.,* 2013). Robinson *et al.* (1993) assert that these various metal-binding MTs have the potential to play different roles in the metabolism of metal ions.

Morris *et al.* (1999) isolated and characterized the metallothionein gene from *F. vesiculosus*. Leszczyszyn *et al.* (2013) discovered that metallothionein sequences of *Sargassum binderi* (now *Sargassum aquifolium*) and also showed similarities to MT type I of the model plant *Arabidopsis thaliana.*

Laboratory studies

The majority of information available in the literature concerning marine brown macroalgae is related to Cu and arsenic (As). In a study of the algae *F. vesiculosus*, Morris *et al.* (1999) observed an induction of MTs under Cu exposure, and found that, in anaerobic conditions, Cd was replaced by Cu. This pointed to the affinity of this algae for the latter metal. In an extension of this work, Owen *et al*. (2012) tested the response of the same species of macroalgae to acute concentrations of Cu, finding that *MT* gene expression was associated with time and dose responses (0.02–0.19 µM). Similar results were observed by González *et al.* (2018a) regarding the expression of *MT* and phytochelatin synthase (*PS*), which were up-regulated following exposure to Cu. They also found participation of some calcium-sensor proteins such as calcineurin B-like proteins (CBLs), calcium-dependent protein kinases (CDPKs) and calmodulins (CaMs) mediated in the increased expression levels of MT and PC (Figure 1). In the case of As, Merrifield *et al.* (2004) described the bond between arsenite (As^{3+}) and metallothioneins in *F. vesiculosus*. Singh *et al.* (2008) reported that recombinant MT from the same species of algae had a higher affinity for As^{3+} and lower affinity for arsenate (As^{5+}). Ngu *et al.* (2009) complemented the previous study by describing the complete reaction when As^{3+} is bound to recombinant MT from the same algae species. Furthermore, Merrifield *et al.* (2006) showed that in the same algae species, MT is bound to Cd and Zn.

Phytochelatins

Similar to MTs, phytochelatins (PCs) are cysteine-rich peptides that participate in binding metals. They have been identified in several species of plant and microorganisms

(Cobbett and Goldsbrough, 2002), and fungi, yeasts, some algae, and some aquatic plants (Gekeler *et al.*, 1988; Inouhe, 2005; Shine *et al.,* 2015). It has been suggested that PCs play a significant role in detoxification of metals and in the physiology of plants (Cobbett and Goldsbrough, 2002; Hasan *et al.*, 2017). Studies in plants, for example, have shown that PCs play an essential role in detoxification mechanism, mainly of Cd and As, as both have strong affinity for PCs (Cobbett, 2000; Cobbett and Goldsbrough, 2002; Hall, 2002; Verkleij *et al.,* 2003; Inouhe, 2005; Uraguchi *et al.*, 2017).

Laboratory studies

The presence of PCs in marine brown macroalgae was first reported in 1988 in the species *Sargassum muticum* (Gekeler *et al.,* 1988). These authors discovered them following exposure to Cd. PC_2 was the most abundant in the species, followed by PC_3 and PC_4. Pawlik-Skowrońska *et al.* (2007) evaluated PCs under Cd and Zn exposure in the algae *F. serratus*, observing an increase of $PC_{2\text{-}4}$ concentrations in both metals. Moreover, Roncarati *et al*. (2015) detected dimer PC_2, trimer PC_3, and tetramer PC_4 under exposure to Cd in a specimen of *E. siliculosus*. In relation to Cu, Roncarati *et al.* (2015) observed intra-specific differences in phytochelatins; PC values were higher in the Es524 specimen from a polluted site (Chañaral, Chile) and two types of PC were detected: PC_2 and PC_3. In the control specimen LIA (Arisaig, Scotland), only PC_3 was detected. A similar trend was also observed in the expression of genes; *PS*, *γ*-glutamylcysteine synthetase (*γ-GCS1*, *γ-GCS2*), and, glutathione synthase (*GS*), which were up-regulated in the polluted individual (Es524) at the highest exposure concentration (2.4 µM). Because of the contamination history of this individual, the authors suggest that it was prepared to produce more reduced glutathione (GSH) and, thus, able to synthetize more GSH-PCs than the specimen from the control site. In addition, the authors detected PCs before adding Cu into the experiment, proposing the potential role of PCs in homeostasis and in the storage of essential elements. In this context, but with As, Wood *et al.* (2011) investigated the synthesis of PCs in two species: *Fucus spiralis* and *Hizikia fusiformis* (now *Sargassum fusiforme*). Both species presented with As^{3+}, dimer PC_2, and GSH, but *H. fusiformis* showed higher levels of the latter (about forty times). The authors concluded that arsenite-phytochelatins complexes appeared not to be the main storage strategy in short-term exposure in both macroalgae. Despite the fact that As^{3+} and As^{5+} are strong inducers of PCs, no more PCs were produced under exposure to As.

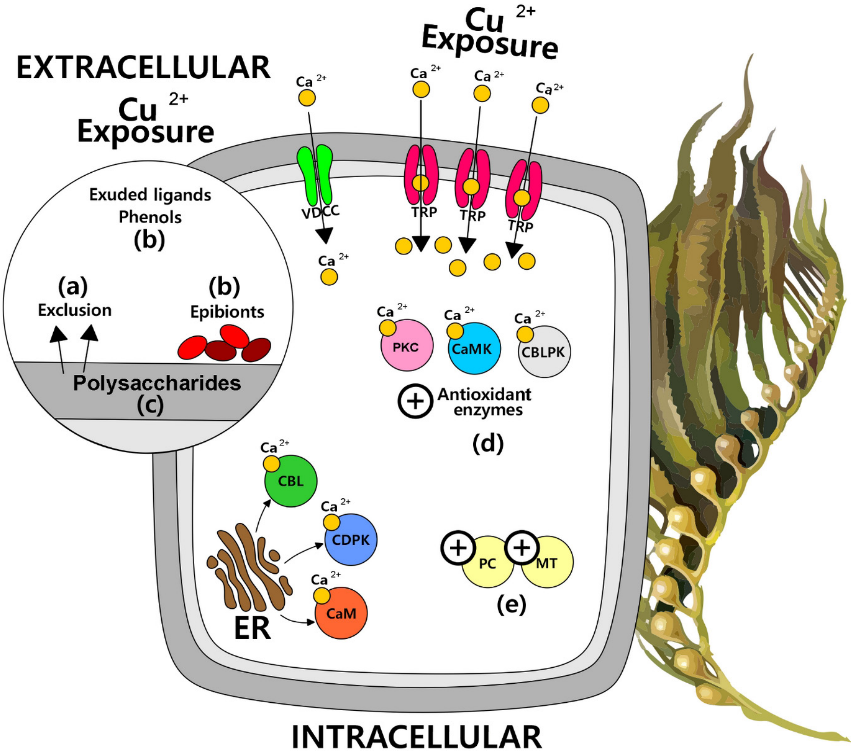

Fig. 1 The different mechanisms of metal tolerance in brown macroalgae. When metal ions—for instance, under exposure to Cu—are present in the environment of a macroalgae, four possible external mechanisms become relevant: (a) physical exclusion; (b) extracellular complexation by exudation of ligands, if present; (b) possible epibiont organisms that may be able to absorb metal ions; and (c) polysaccharide formation in the cell wall. If metal ions manage to enter the cell, (d) antioxidant enzymes trigger different responses to balance the oxidative stress that metals cause. In addition, intracellular chelation occurs by (e) chelators: PC and/or MT. This illustration is a compilation of previous diagrams by Gaur and Rai (2001), Hurd *et al.* (2014), González *et al*. (2018a; b), and Geddie and Hall (2019).

Abbreviations: CaM = Calmodulin; CaMK = Calmodulin-dependent kinase; CBL = Calcineurin B-like protein; CBLPK = Calcineurin B-like (CBL)-dependent protein kinase; CDPK = Calcium-dependent protein kinase; ER = Endoplasmic reticulum; MT = Metallothionein; PKC = Protein kinase C; PC = Phytochelatin; TRP = Transient receptor potential channel; VDCC = Voltage-dependent calcium channel; Yellow circles = calcium ions.

Intracellular phenolic compounds

Soluble polyphenols (phlorotannins) are a type of phenolic compound located in the physodes found solely in marine brown macroalgae. These chemicals contain groups of hydroxyl that are able to bind to divalent metals (Amsler and Fairhead, 2005; Imbs and Zvyagintseva, 2018). Some authors have found metals in the physodes (Lignell *et al.*, 1982; Smith *et al.*, 1986; Salgado *et al.,* 2005). However, Salgado *et al.* (2005) also found sulfated fucans in the physodes, suggesting that accumulation of metals in physodes can be associated with sulfated fucans rather than directly with polyphenol contents.

Laboratory studies

Connan and Stengel (2011) exposed isolated phlorotannins under Cu (4–20 μM), and no differences were seen in Cr, but Zn and Cd concentrations of the phenolic portion decreased at 20 µM, demonstrating the strong affinity intracellular phenolic content for Cu. However, Toth and Pavia (2000) did not find any difference in Cu concentrations in phlorotannins, although the concentrations used were much lower (0.074–0.37 μM). Long-term exposure to metals was associated with an increase in polyphenols in seaweeds. After 14 days of Cd exposure, Costa *et al*. (2017) reported migration of a high number of physodes close to the cell wall in *S. cymosum*. Chemical analysis supported their findings.

Reactive oxygen species

Reactive oxygen species (ROS) are produced in cells due to an imbalance between radical species and antioxidants (Potin, 2008). Low levels of ROS function as signaling pathways to regulate a number of physiological processes and are crucial to life. However, high concentrations of ROS can overwhelm antioxidant production, damaging macromolecules such as nucleic acids, lipids, and proteins (Holmström and Finkel, 2014; Schieber and Chandel, 2014; Halliwell and Gutteridge, 2015; Reczek and Chandel, 2015; Rezayian *et al.*, 2019). ROS is produced by several stressors, both natural (e.g., waves, desiccation, osmotic shock) and anthropogenic (e.g., metals) (for more details, see Coelho *et al.*, 2002; Abele *et al.*, 2011; Pikula *et al.,* 2019; Rezayian *et al.*, 2019; Yang *et al.*, 2019; Nowicka, 2022).

Previous studies have investigated the role of ROS in stress tolerance in marine brown macroalgae. Collén and Davison (1999a; b) confirmed the hypothesis that metabolism of reactive oxygen is factor in stress tolerance, finding that the tolerant species *Fucus* spp. presented higher antioxidant activity in comparison with the less tolerant species *F. distichus*, with the latter showing a considerable increase in ROS under abiotic stressors. Pinto and colleagues indicated in their review that tolerance of algae to heavy metal contamination is likely to rely strongly on defense mechanisms to prevent oxidative damage, suggesting that antioxidant responses are crucial to providing tolerance in species (Pinto *et al.*, 2003). Subsequently, other authors also highlighted the importance of antioxidant defense in the tolerance of marine macroalgae to metal pollution (for examples, see Torres *et al.,* 2008; Nowicka, 2022).

Laboratory studies

An early study reported increased production of reactive oxygen species in embryos of *F. serratus* under exposure to Cu (Nielsen *et al.*, 2003a). Other researchers studying exposure of the same metal, detected generation of lipid peroxidation (LPO) (Ritter *et al.*, 2008; Lovazzano *et al.*, 2013; Contreras *et al*., 2009), presence of free polyunsaturated fatty acids (PUFAs), and presence of oxylipins (Ritter *et al.,* 2008; 2014). Ritter *et al.* (2008) suggested that the oxylipins pathway in marine seaweeds is involved as a protection mechanism under Cu exposure.

Other researchers have also evaluated the activity of enzymatic antioxidants, such as glutathione peroxidase (GPx), catalase (CAT), glutathione s-transferase (GST), superoxide dismutase (SOD), ascorbate peroxidase (APx), glutathione reductase (GR) and peroxiredoxin (PRx); and of nonenzymatic antioxidants such as reduced glutathione (GSH), and ascorbate (ASC) in marine brown macroalgae exposed to metals. For example, Gu *et al.* (2023) tested the ability of *M. pyrifera* to tolerate Cu. They obtained six GST genes from the kelp and evaluated their expression in a cyanobacteria, showing that the modified individuals had the ability to increase their tolerance, while the wild individuals almost died under Cu exposure.

Some authors have supported the hypothesis of intra-specific variations, showing that more tolerant macroalgal specimens, namely those collected from contaminated habitat, responded with higher antioxidant activities than non-tolerant specimens (Sáez *et al.*, 2015b; Sáez *et al.*, 2015c). Other researchers obtained different results in other marine brown macroalgae species, supporting the hypothesis of physiological plasticity or a constitutive trait (Contreras *et al.*, 2007; Sordet *et al.*, 2014). Although results are not conclusive in terms of the evolution of metal tolerant populations, there are some general similarities. Increases in antioxidant activities but an inhibition of GR under Cu excess in different species of brown macroalgae led authors to propose that Cu directly affects cysteine in the catalytic site of the GR enzyme (Contreras *et al*., 2007; Contreras *et al.*, 2009; Sordet *et al.*, 2014).

Interactive experiments analyzing the effects of Cu exposure and atmospheric CO_2 in young sporophytes (*S. fusiforme*) demonstrated that with increased CO_2, the algae would resist Cu stress more efficiently by increasing SOD activity (Wen and Zou, 2021). Experiments with the same economically important brown macroalgae *H. fusiformis* (*S. fusiforme*) exposed to Cd, mercury (Hg), and Cu revealed an increase in malondialdehyde contents (MDA), as well as SOD and CAT; however, at higher concentrations of these metals, a decrease in the enzymatic antioxidants was observed (Zhu *et al*., 2011; Zhu *et al*., 2015). In the case of mercury, Zhu *et* *al.* (2015) suggested that this reduction was due to the metal's property as a pro-oxidant able to inhibit the thiol contained in antioxidants. Different phylogenetic group responses were proven in an *in vitro* study evaluating the interactions between mud with different degrees of metal enrichment and pH. They observed that both a red macroalgae and the brown species *S. cymosum* presented a decrease by 50% in SOD activity under highly enriched treatment and under acidic treatment when compared to the control. In addition, it was observed that *S. cymosum* alone was able to respond better to all treatments, showing increased synthesis of phenolic compounds, greater chlorophyll content, and higher enzymatic activities, assuming that the red macroalgae may compete with *S. cymosum* for nutritional resources (Costa *et al.,* 2019b).

More recently, authors have undertaken molecular and 'omics' analyses to reveal the internal workings of tolerance mechanisms in which antioxidants play an important role under exposure to Cu (Ritter *et al.*, 2008; Contreras *et al.,* 2010; Ritter *et al.*, 2010; 2014; Zou *et al.,* 2014; Roncarati *et al*., 2015; Sáez *et al*., 2015b; Zou *et al*., 2015; González *et al.,* 2018b; Zhang *et al*., 2019). For instance, some studies have focused on the importance of calcium signaling in tolerance mechanisms in marine green macroalgae under Cu exposure (e.g., see Laporte *et al*., 2016; Moenne *et al*., 2020), and their findings show similarities with brown seaweeds, for example, in terms of receptor types.

Calcium ion is a universal second messenger found in all eukaryotes. Second messengers play the role of amplifying signal perception and pathway-essential processes where a series of biological events are involved in modulating a number of enzymes (Hopkins and Hüner, 2008; Taiz *et al.*, 2015; Bhatla 2018). Each specific signal must be received by a receptor which is a physical component that transduces this message to amplify it or induce a cellular response (Hopkins and Hüner, 2008; Taiz *et al.*, 2015; Bhatla 2018). González and colleagues studied *E. siliculosus* under acute Cu exposure, revealing the activation of Voltage-Dependent Calcium Channels (VDCCs) and Transient Receptor Potential Channels (TRPs)—ion channels that are interconnected. When the macroalgae was exposed to excess Cu, there was an activation of TRPs that allowed the entry of extracellular calcium, which activates sensor proteins—calcineurin B-like (CBL)-dependent protein kinases (CBLPK), cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), calmodulin-dependent kinases (CaMK), and protein kinase C (PKC)—promoting the upregulation genes related to antioxidant enzymes (González et al., 2018b). Simultaneously, the action of these last two sensor proteins triggers the induction of VDCCs, allowing the entry of extracellular calcium and the release of intracellular calcium from the endoplasmic reticulum (ER). The entry of intracellular calcium actives the CDPKs, CBLs, and CaMs that were associated with the expression of two chelators (González *et al.*, 2018a). Thus, molecular analyses confirm the involvement of the important above-mentioned chelators, as well as the crucial role of antioxidant defenses as strategies deployed by this group of algae in order to tolerate metals (Figure 1).

Molecular analysis has also helped to highlight the importance of vanadium-dependent bromoperoxidase (vBPO), found only in marine brown and red macroalgae (Leblanc *et al.*, 2006). It has been suggested that these peroxidases are key elements in processes that drive ROS detoxification (Küpper *et al*., 2008; Roeder *et al.*, 2005), and especially under chronic exposure to Cu stress (Ritter *et al.*, 2014). Additionally, under metal exposure, similar chaperones and transporters found in plants such as ABC transporters and metal-transporting ATPases have been reported in marine brown macroalgae (Contreras *et al*., 2010; Ritter *et al.*, 2010; 2014; Zhang *et al.,* 2019, Wang *et al*, 2021), but in-depth investigation of their specific role is still needed.

Table 2 Studies of brown macroalgae using biomarkers of tolerance in field conditions.

Order	Species	Location	Biomarkers	Reference
Desmarestiales	*Himantothallus grandifolius*	South Shetland Islands, Antarctica	LR, CAT, SOD, GST	González *et al.* (2017)
Dictyotales	*Dictyota dichotoma* *Dictyopteris polypodioides* *Padina pavonica*	The Aegean Sea	Polysaccharides and extracellular organic materials	Karavoltsos *et al.* (2013)
	Padina gymnospora	Ribeira Bay and Sepetiba Bay, Brazil	Polyphenol content	Karez and Pereira (1995)
	P. gymnospora	Ribeira Bay and Sepetiba Bay, Brazil	Polysaccharides	Andrade *et al.* (2004)
	Padina tetrastromatica	Western coast of India, Arabian Sea	H_2O_2, LPO, CAT, GST, ASC	Maharana *et al.* (2010)

	P. gymnospora	Cat Island and Ribeira Bay, Brazil	Polysaccharides	Andrade *et al*. (2010a)
Ectocarpales	*Colpomenia sinuosa*	The Aegean Sea	Polysaccharides and extracellular organic materials	Karavoltsos *et al*. (2013)
	Ectocarpus siliculosus	Central Chilean coast	H_2O_2, LPO, GSH, CAT, GSSG, GR, ASC, DHA, APx, SOD, Total phenolic compounds	Sáez *et al.* (2015a)
	Scytosiphon lomentaria	North-central Chilean coast	LPO, TRAP, CAT, SOD	Gaete-Olivares *et al.* (2016)
	S. lomentaria	Northern Chilean coast	ROS, LPO, CAT, APx, ASC, DHA, DHAR, GPx, GR, GSH, GSSG, MDHAR, Phenolic compounds	Contreras *et al*. (2005)
Fucales	*Ascophyllum nodosum*	Swedish West Coast	Phlorotannin	Toth and Pavia (2000)
	Cystoseira barbata (now Treptacantha barbata)	Chioggia, Venice, Italy	Polysaccharides	Mariani *et al.* (1990)
	C. barbata (now T. barbata)	Romanian Black Sea coast	TRAP	Negreanu-Pirjol *et al.* (2012)
	C. barbata (now T. barbata) *C. foeniculacea* *C. compressa* *Cystoseira corniculata*	The Aegean Sea	Polysaccharides and extracellular organic materials	Karavoltsos *et al*. (2013)
	C. crinita	Izmir Bay, Turkey	LPO, CAT, SOD, APx	Akakçe *et al*. (2023)
	C. indica (now P. indica)	Northern coast of Gulf of Oman, Arabian Sea	MTs, PCs	Sinaei *et al.* (2018)
	C. tamariscifolia (now E. selaginoides)	Atlantic coast of Morocco	Total phenolic compounds	Boundir *et al.* (2019)
	Fucus serratus *Fucus vesiculosus*	South West England, UK	PCs, GSH	Pawlik-Skowrońska *et al.* (2007)
	Fucus spiralis	Poole Harbour, UK	MTs	Oaten *et al.* (2017)

	F. vesiculosus, A. nodosum	Western coast of Norway	Phenolic content	Pedersen (1984)
	F. vesiculosus	The Severn Estuary and Fal Estuary, UK	MTs	Owen *et al*. (2012)
	F. virsoides	Chioggia, Venice, Italy	Polysaccharides	Mariani *et al.* (1985)
Laminariales	*Laminaria digitata* *L. hyperborea* *Saccharina latissima* *Alaria esculenta*	Clachan Sound, Isle of Seil, Scotland	Polyphenol content	Schiener *et al.* (2015)
	Lessonia nigrescens	Northern Chilean coast	ROS	Andrade *et al.* (2006)
Sphacelariales	*Halopteris scoparia*	Izmir Bay, Turkey	LPO, CAT, SOD, APx	Akakçe *et al.* (2023)

Abbreviations: ASC = Ascorbate; APx = Ascorbate peroxidase; CAT = Catalase; DHA = Dehydroascorbate; DHAR = Dehydroascorbate reductase; MDHAR = Monodehydroascorbate reductase; GPx = Glutathione peroxidase; GR = Glutathione reductase; GSH = Reduced glutathione; GSSG = Oxidized glutathione; GST = Glutathione transferase; LPO = Lipid peroxidation; LR = Lipid radical; MTs = Metallothioneins; PCs = Phytochelatins; ROS = Reactive oxygen species; SOD = Superoxide dismutase; TRAP = Total antioxidant capacity; H_2O_2 = Hydrogen peroxide.

BIOLOGICAL RESPONSES ASSOCIATED WITH TOLERANCE AND MONITORING

Field studies

In this section, I review results obtained in aquatic habitats in order to understand the different strategies utilized by marine seaweeds in complex chemical mixtures commonly found in the field (Table 2). Karez and Pereira (1995) evaluated concentrations of metals in polyphenol fraction in the algae *P. gymnospora* along the Brazilian coast. At these sites, high metal contents, especially of Cu, Pb, and Zn (alongside lower levels of Cd and Cr), were more strongly concentrated in polyphenols compared to the macroalgae as a whole, supporting their acute affinity for these metals. In addition, Amado Filho *et al.* (1999) used the same species—*P. gymnospora*—along with the species *Sargassum stenophyllum* for long-term monitoring of Zn and Cd between 1990 and 1997 along the Brazilian coast. They detected Zn in the cell wall of algae *P. gymnospora* in the same polluted bay, supporting the assertion that the cell walls play an important role in the accumulation of metals. Boundir *et al.* (2019) identified certain trends, as the most metal-polluted sites they studied presented the highest levels of total phenolic compounds (1.54 mg g-1 DW) detected in the seaweed *C. tamariscifolia* (*Ericaria selaginoides*) from the Atlantic coast. However, Toth and Pavia (2000) did not find differences in phenolic contents in the fucoid *A. nodosum* at different contaminated sites studied, proposing other mechanisms of internal detoxification. Connan and Stengel (2011) evaluated Cu, Zn, Cd, and Cr concentrations in isolated polyphenols from the same species of fucoid collected from an unpolluted location. They reported that intracellular phenolics presented higher concentrations of Cu and Zn than Cd and Cr, pointing to affinity for the former.

The use of polysaccharides as biomarkers has received little attention in terms of response to metal contamination. Eide *et al.* (1980) suggested that uptake of Pb by *A. nodosum* along the Norwegian coast follows the ion exchange process, but no polysaccharides were evaluated. Early studies by Mariani *et al.* (1985; 1990) expanded on findings reported in field about the thallus, namely that this was the main tissue location of polysaccharides. Other field investigations have been conducted by Andrade and colleagues. They first characterized polysaccharide morphology (alginic acid and sulfated fucans) in the brown algae *P. gymnospora* and found that Zn was present in polysaccharides from algae at a polluted site (Andrade *et al.*, 2004). Further study, let them to support the hypothesis that sequestration of metals by extracellular polysaccharides is the main mechanism of tolerance in this brown algae. They detected an overproduction of polysaccharides on the cell wall in this algae inhabiting a contaminated site (Andrade *et al.*, 2010a).

Gledhill *et al*. (1998) acknowledged the importance of monitoring heavy metals using marine macroalgae, they tested the best methods for removal of material on algal surface following the discovery that bacteria enriched the metal concentration there by comparison with the algae tissue itself. In turn, it has also been reported that the surface of macroalgae is covered by other organisms, such as cyanobacteria and pennate diatoms (Cundell *et al*., 1977).

MTs and PCs were assessed in the marine brown seaweeds *C. indica* along the northern coast of the Gulf of Oman in Arabian Sea. MT levels were most strongly associated with Zn, followed by Cd, Ni, Pb, Cr, and Cu. By contrast, PC levels were related only with Zn and Cd, highlighting the restricted use of PCs in metal monitoring studies (Sinaei *et al*., 2018). According to Pawlik-Skowrońska *et al.* (2007), phytochelatins differed not only between phylogenetic groups (i.e., Phaeophyceae, Chlorophyceae, and Rhodophyceae) but also within the same genera. PCs were detected only in the two species (*F. serratus* and *F. vesiculosus*), in a red macroalgae (*Solieria chordalis*), and in a much lower concentration in a green macroalgae (*Rhizoclonium tortuosum*). The two brown macroalgae presented the highest total PC concentration at a highly contaminated site (Restronguet Creek, England). Phytochelatins detected in *F. serratus* had the longest chain lengths ($PC_{2\text{-}4}$) by comparison with $PC_{2\text{-}3}$ found in *F. vesiculosus*. The authors also measured GSH concentration, finding that these two species of *Fucus* spp. had higher concentrations compared with the red algae *S. chordalis*.

Levels of ROS and antioxidant defenses have been used in the field to monitor pollution. Some studies have focused on responses in transplanted macroalgae and others have compared oxidative defenses between habitats with differing degrees of metal pollution. Andrade *et al.* (2006) transplanted juveniles of *L. nigrescens* to a Cu-enriched habitat and observed that after exposure was completed, high levels of ROS continued to indicate the macroalgae's limited ability to block Cu. Ecotypes versus physiological plasticity hypotheses were also tested in the field. Authors transplanted two specimens of *E. siliculosus* into a polluted and reference site. They found that the tolerant specimen had a strong antioxidant capability, thus supporting the ecotype hypothesis (Sáez *et al.,* 2015a). Another study involved the transplanting of the algae *Scytosiphon lomentaria* from a reference site to a Cu-polluted habitat and vice versa. Both groups showed adaptation in their antioxidant responses, exhibiting responses that were very similar to the local group, and supporting the alternative hypothesis (Contreras *et al*., 2005).

Three phylogenetic groups of macroalgae were evaluated in a survey conducted in south-west England, where the highest concentrations of metals and GSH were found in *Fucus* spp. at the most polluted site. For Cu, higher levels of GSH and production of phytochelatins (PCs) were reported in a red macroalgae and in *Fucus* spp. The authors suggested that these help them to grow in highly polluted habitat and that high GSH production was a successful mechanism to combat oxidative stress in metal mixtures (Pawlik-Skowrońska *et al*., 2007).

Other studies have also compared the efficiency of different groups of macroalgae in coping with metals in the field. González *et al*. (2017) compared iron content, lipid radicals, enzymatic antioxidants, and nonenzymatic compounds in the red macroalgae *Gigartina skottsbergii* and the brown macroalgae *Himantothallus grandifolius*, showing the latter species to be more adapted to different physicochemical variations, including iron exposure, because of higher antioxidant ability. A similar trend was observed by Negreanu-Pirjol *et al.* (2012), who collected samples on the Black Sea coast in Romania and found that the brown macroalgae *Cystoseira barbata* (now *Treptacantha barbata*) had a higher antioxidant capacity than a red and a green macroalgae.

Gaete-Olivares *et al*. (2016) surveyed two species of macroalgae: the green *Ulva rigida* and the brown *S. lomentaria* from the northern and central Chilean coast. Results showed that both species exhibited higher antioxidant activities in polluted sites, where *S. lomentaria* showed high SOD, CAT, and LPO activities. Results were correlated with concentrations of Cu and As. Authors recommended use of both types of algae to assess these oxidative stress biomarkers on the Chilean coast, although they presented inter-specific differences. In a bay in Turkey, other researchers found that one type of brown algae activates the activities of SOD and CAT under concentrations of manganese (Mn) and radionuclide ^{210}Po better that other brown algae, suggesting that the former species could be used to understand the antioxidant mechanisms in both metals and radionuclides (Akakçe *et al.,* 2023). Maharana *et al.* (2010) collected samples of *Padina tetrastromatica* on the central-western coast of India and found elevated levels of LPO and hydrogen peroxide, along with high ASC, CAT, and GST activities. The authors also suggested antioxidant responses as useful biomarkers for pollution monitoring.

CITATION NETWORK ANALYSIS

Bibliometric analyses were performed using VOSviewer 1.6.19 software, which helped to visualize trends and patterns, cooperation between countries, and gaps in knowledge. Web of Science/Dimensions were used to extract the information for the theme explored, along with references cited in other articles of interest. A total of 1,357 papers published in English between 1960 and March 2023 were found. Metals and metalloids were included in the review. Keywords used in the search included tolerance AND pollutants, OR macroalgae, brown algae. In addition, more specific words such as metallothioneins, phytochelatins, polyphenols, polysaccharides, epiphytic, oxygen reactive species, and ligands were used in combination with other words. The Scopus database was also used to create a network visualization map of the co-occurrence of keyworks, and the Dimensions database was used to create a network visualization map of collaborations to the topic by country, due to configuration settings.

From the first visualization map (Figure 2), four clusters were observed according to size (keywords most frequently used in the articles). Cluster 1 (brown) covered heavy metals associated with biosorption in marine brown seaweeds, and was closely connected with cluster 2 (pink), featuring studies focused on Cu and phenolic compounds. Another separate but important cluster consisted of studies on oxidative stress responses and macroalgae (cluster 3, green-yellow). The last prominent cluster (light green-yellow) focused on marine brown macroalgae and it was more closely related to a small cluster (red), consisting of studies of external biomarkers of tolerance such as polysaccharides and phenolic content. In addition, a smaller number of studies were represented by four small clusters: one (blue) was mainly associated with seaweed toxicity, where As was the most studied metal. This was also linked to studies on biomonitoring and bioindicators (sky blue). Lastly, two closely connected clusters were focused on nonenzymatic antioxidants such as GSH and ASC (purple) and on other biomarkers such as MT and antioxidant enzymes with emphasis on Cu (orange). Interesting new technologies such as proteomics were also mentioned in this cluster.

Based on the literature data, an overlay visualization map was also created to analyze the evolution of investigations of metal tolerance on brown seaweed over the years. It was observed that, between 2005 and 2010, the main focus of attention was on bioaccumulation of heavy metals and external mechanisms of tolerance in this type of macroalgae. Later, around 2015, the focus shifted to biomonitoring studies of enzymatic and nonenzymatic antioxidants under Cu exposure. Since then, investigations have continued to focus on the fields of antioxidants, toxicity, and MT, with Cu remaining the metal most studied (Figure S1).

Collaborative research analysis reveals the thirty most important countries engaged in cooperation (Figure 3). China, the USA, and India are leaders in scientific productivity, but studies from the USA are the most cited. The Chinese networking group has the most recent piece of research on the topic, and they collaborate primarily with other Asian countries, some Middle Eastern countries, and Australia. The USA's collaboration networks include some European countries, Canada, Brazil, and Chile. The third network observed involved Russia, Iran, Czechia, Poland, and Austria.

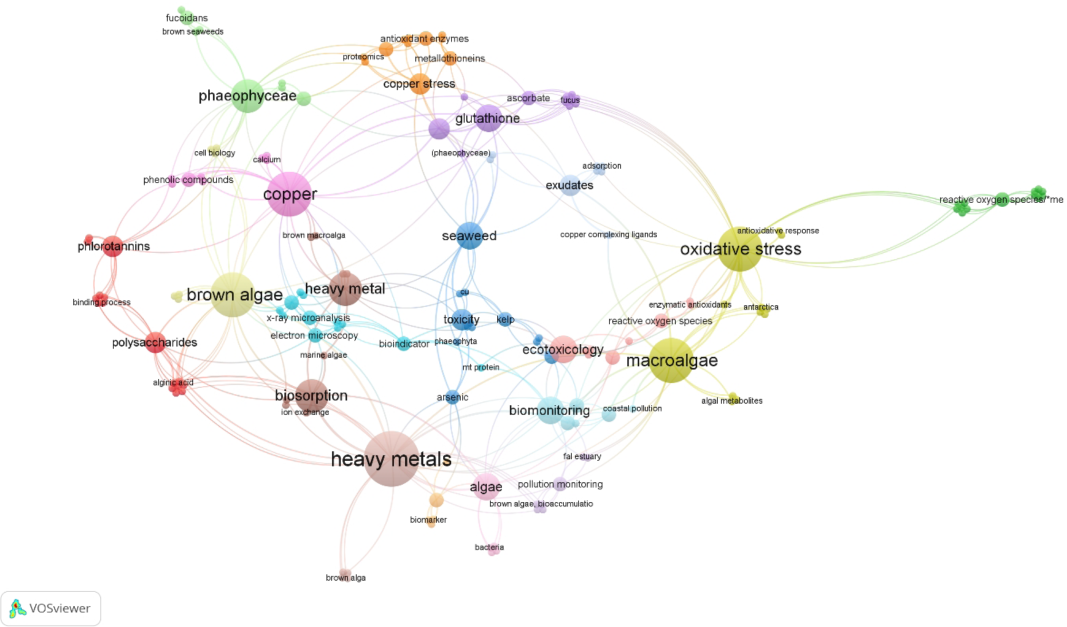

Fig. 2 Network visualization map of the co-occurrence of the main keywords used in studies related to tolerance in marine brown macroalgae, based on a "total link strength" study (software: VOS viewer version 1.6.19). The size of the clusters indicates the number of keywords used in the articles, the colors indicate the association between them, and the distance between clusters indicates similarities in the use of the keywords.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The ability of marine brown seaweeds to live in a variety of marine habitats, including those with high levels of metal pollution, make this group a particular target of research not only in order to understand the mechanisms behind this tolerance, but also for their potential use in monitoring programs. The main points to highlight regarding the different mechanisms of metal tolerance found in marine brown macroalgae were:

Initial physical exclusion and exudation of a variety of ligands—one of the most relevant being phenolic compounds. Phenolics on the cell wall and extracellular polysaccharides also constitute external metal tolerance strategies. In addition, studies on fouling microorganisms have hinted at additional "reinforcement" to cope with high metal concentrations. When the first barriers are crossed and metal ions enter the cell of macroalgae, MTs and PCs are important internal chelators for coping with metals. Marine brown macroalgae, in general, have better antioxidant defenses in comparison with other macroalgal groups, a characteristic that may, in part, enhance tolerance to metal stress (Figure 1). 'Omics' analyses have highlighted certain pathways involved in the activation of some genes associated with MTs, PCs, and antioxidant defense as important mechanisms of tolerance in marine brown macroalgae.

Bibliometric analyses have shown that Cu has received considerable attention in the exploration of the different mechanisms of metal tolerance in marine brown seaweeds, and confirm that extensive investigations have been undertaken on all types of antioxidants and on MT. China, the USA and India have contributed the most to the topic, and the former has produced the most recent investigations in conjunction with other Middle Eastern countries, some Asian countries, and Australia.

Although new technologies have permitted a more in-depth understanding of the internal mechanisms of tolerance, in which antioxidants and MT play an important role in the survival not only of marine brown macroalgae but of all organisms, there are still few studies that cover the internal workings of these mechanisms, or the use of other metals with this type of macroalgae. One possible reason is that these technologies are mainly applied to other good bioindicators such as plants, where mechanisms under metal exposure are investigated extensively. Biomarkers of tolerance in marine seaweeds have been underutilized in monitoring studies but have provided additional information regarding the tolerance of these organisms to complex mixtures in the field. As such, further research is required.

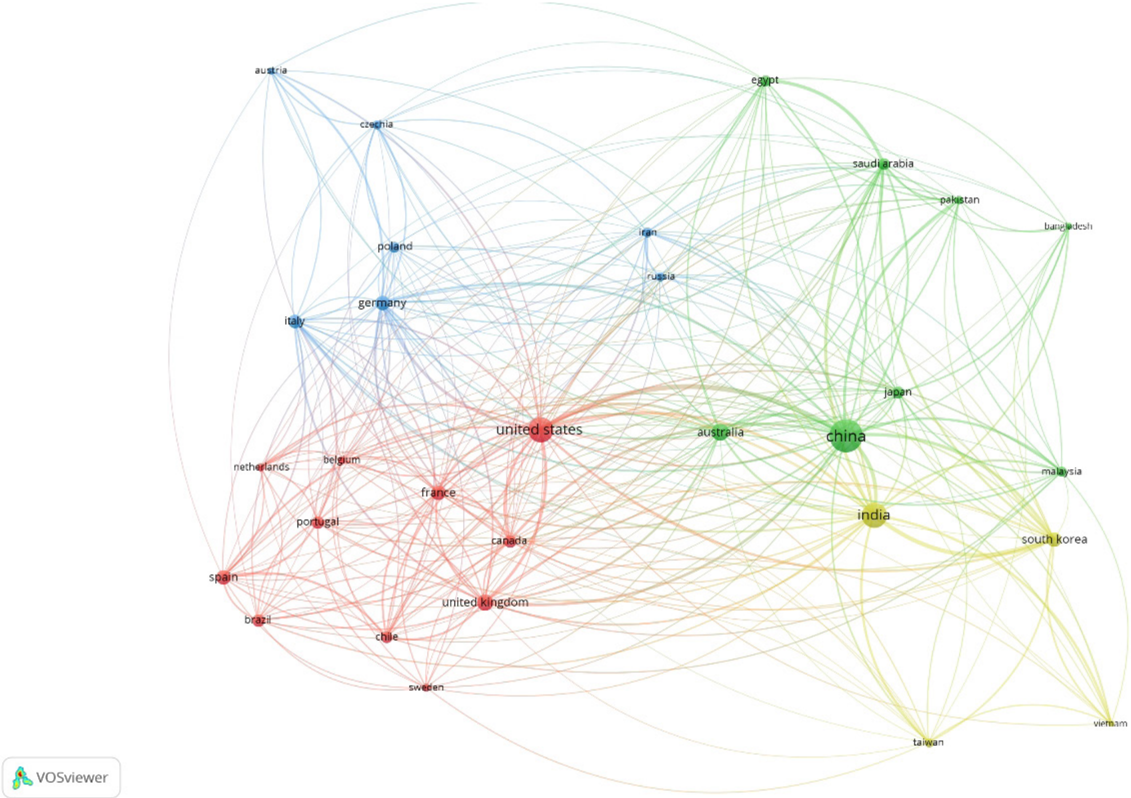

Fig. 3 Network visualization map of collaboration among the 30 most active countries related to tolerance in brown algae, based on "total link strength" study (software: VOS viewer version 1.6.19). The size of the clusters indicates the number of articles; the colors indicate the association between them, and the distance between clusters indicates the strength of their connection to the topic.

ACKNOWLEDGMENTS

The author would like to thank to Dr. C. Sáez for his suggestion of the topic and for providing database access; Aaron Estrada for helping to design the macroalgae drawing; and Betza Jara for her contribution to the mechanisms of tolerance figure. The National Fund for Scientific and Technological Development (Fondecyt 3200799), Chile for their economic support. The author is sincerely grateful to the anonymous reviewers for their helpful suggestions for the improvement of this manuscript.

AUTHOR CONTRIBUTION

Diana Montenegro (DM): Conceptualization, Methodology, Data curation, Writing Draft and Original, Visualization, Reviewing and Editing.

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SUPPLEMENTARY MATERIAL

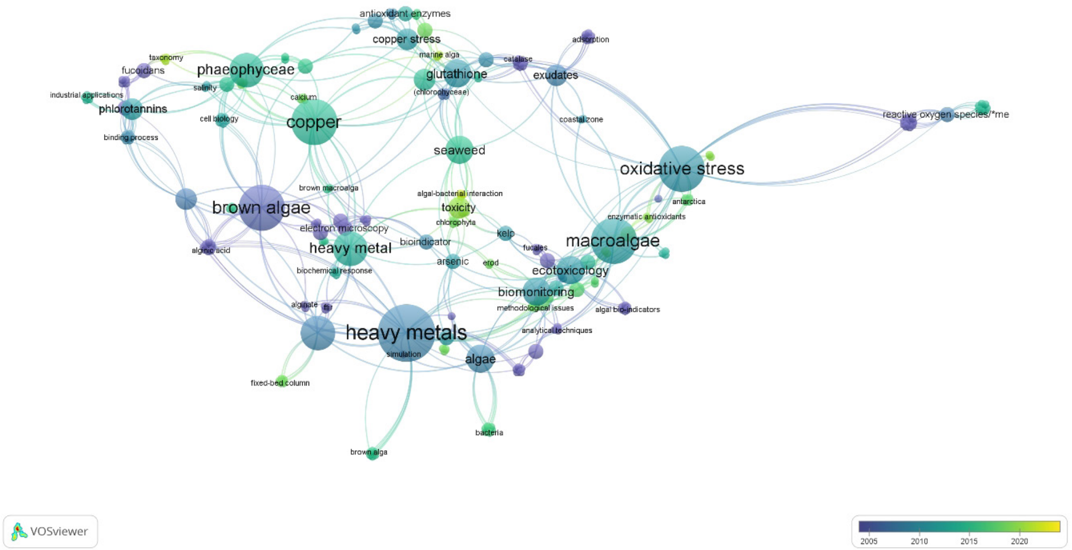

Fig. S1 The overlay visualization map of the main keywords used in studies related to tolerance in brown algae over a time based on "total link strength" investigation (Software VOS viewer version 1.6.19). The side of the clusters indicated the numbers of articles contributions; colors indicated the years of publications by average. and the distance between clusters indicated the how connected are them in relation to the topic.