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Effects of copper and cadmium on physiology and antifouling defense of the marine macroalga *Ulva reticulata*

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Abstract

Heavy metals are major stressors for benthic macroalgal communities in marine ecosystems. In this study, the effects of copper and cadmium on some physiological parameters along with antifouling defense of the marine macroalga *Ulva reticulata* were assessed under laboratory conditions. Macroalgal samples were treated with three concentrations (1 mg l^{-1}, 3 mg l^{-1} and 5 mg l^{-1}) of copper and cadmium for 2 and 7 days. After treatment, algal samples were analyzed for chlorophyll-*a*, carotenoid, total polyphenol and total antioxidant capacity. Also, algal extracts were tested against biofilm-forming bacteria strains to understand differences in antifouling activity. The results indicated that exposure of *U. reticulata* to copper and cadmium, on the one hand, induced protective mechanisms such as total phenol production and antioxidant capacity against metal stress and, on the other hand, reduced photosynthesis. While the extract obtained from control algal samples showed a strong inhibitory effect on the growth of biofilm-forming bacteria, treatment with heavy metals resulted in reduced antibiofilm activity. In general, the results revealed that exposure of macroalgae to heavy metals can affect antifouling defense traits in addition to changes in photosynthetic pigment content.

Key words: marine pollution, heavy metals, antifouling, antioxidant activity, antibiofilm, seaweed, *Ulva*, Red Sea

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1. Introduction

Macroalgal communities play a key role in coastal ecosystem functioning by acting as a primary producer and providing refuge for a number of organisms (Wang et al. 2014; Schiel & Foster 2015). Marine pollution is considered to be one of the factors contributing to the decline of macroalgal populations in coastal ecosystems (Martins et al. 2012; Scherner et al. 2013). Among the pollutants, heavy metals have a significant impact on macroalgal communities. Due to the sensitivity of macroalgae to anthropogenic pressure and environmental factors, these systems are used as a valuable indicator to assess the health of coastal ecosystems (Mannino & Micheli 2020). The effects of metals on algae have been previously studied due to the ability of these organisms to uptake metal ions and accumulate them in their tissues (Flouty & Estephane 2012). Further, heavy metal accumulation in macroalgae has both direct and indirect effects on organisms at higher trophic levels (Contreras-Porcia et al. 2017).

Once accumulated, metals can cause physiological damage depending on the sensitivity of the organisms (Polo et al. 2014; Costa et al. 2016). Higher concentration of metals has severe effects on the growth and metabolism of many algae species (Ismail & Ismail 2017). In brief, exposure of algae to higher concentration of heavy metals resulted in an increase in lipoperoxides and a decrease in antioxidant enzymes, reduction in reactive oxygen species (ROS) production, protein content, free amino acids and carbohydrates (Contreras et al. 2009; Huang et al. 2010; Yadav 2010; Foyer & Noctor 2011). Moreover, heavy metals can also reduce the content of photosynthetic pigments, the growth and cause cell damage (Xia et al. 2004; Saleh 2015; Zhu et al. 2017).

In addition to physiological effects, heavy metals affect the chemical defense properties of marine macroalgal communities (Lurling 2012; Warneke & Long 2015). Macroalgae are characterized by a strong chemical defense mechanism against herbivores and colonizing marine organisms (Paul et al. 2001; Sudatti et al. 2018). Epibiosis or biofouling on surfaces can have detrimental effects on marine macroalgae, mainly reduction in growth and reproduction, biomass loss, depletion of nutrients and tissue damage (Jormalainen & Honkanen, 2008; Da Gama et al. 2014). To reduce fouling, most macroalgae are equipped with antifouling defense mechanisms against both micro- and macrofoulers (da Gama et al. 2008).The antifouling defense is mainly achieved through the production of secondary metabolites. Many bioactive metabolites play an important role in macroalgal defense against fouling organisms and herbivores (Paul et al. 2006; Pereira & Da Gama 2008). In addition, pollutants can interfere with the production and composition of bioactive metabolites in marine algae (Pinto et al. 2011; Gressler et al. 2011). Any change in the biosynthesis of secondary metabolites can reduce the defense properties of macroalgae against fouling organisms and herbivores. Further, algae with fouling organisms on their surface are more attractive to herbivores (Da Gama et al. 2008).

While the physiological effects of metal pollution on macroalgal communities have been studied in detail (Huang et al. 2010; Jiang et al. 2013; Saleh 2015; Costa et al. 2016), the effects of metals on the antifouling defense of marine macroalgae have received little attention. Most of the previous studies have focused on the effects on antioxidant enzymes and total phenolic content (Toth & Pavia 2000; Tzure-Meng et al. 2009; Costa et al. 2016). Therefore, in this study, the effects of two selected metals, copper and cadmium, on macroalgae physiology and antifouling defense were assessed using *Ulva reticulata* as a model green alga. The selection of these two heavy metals was based on previous studies that reported that copper and cadmium can affect gametophyte development and distribution of cellular components in various macroalgae species (Contreras et al. 2007; Leal et al. 2018). The results observed in this study will enhance our knowledge about the effects of metal pollutants on antifouling defense of macroalgae, which is considered one of the important adaptations providing resistance to herbivores and unwanted colonization.

2. Materials and methods

2.1. Collection of macroalga

The green macroalga *Ulva reticulata* Forsskal 1775 was collected from the Obhur Creek (21°42'33.52"N; 39°5'45.71"E), the central Red Sea, Saudi Arabia. Salinity, temperature and pH of the creek water at the time of sampling were 39 PSU, 25.5°C and 8.3, respectively. These parameters were measured *in situ* using a portable multiparameter water quality monitor (Hanna). Many previous studies described hydrographic and environmental properties of the Obhur Creek waters (Alsaafani et al. 2017; Salama et al. 2018). The collected algal samples were brought to the laboratory in a bucket with seawater. In the laboratory, they were initially rinsed with filtered (Millipore, 0.47 µm) seawater to eliminate the epifauna and debris. *Ulva reticulata* was identified based on

morphological characters according to the available identification keys (Dhargalkar & Kavlekar 2004; Brodie et al. 2007). Samples were kept in glass tanks containing filtered seawater. The tanks were kept at 25°C under 80 µmol E m^{-2} s^{-1} illumination (14 h light/ 10 h dark cycle) with gentle aeration. Salinity and pH of seawater in the tank during the experiment were 38 PSU and 8.3, respectively. Salinity and pH of water were measured using a refractometer and pH meter (Hanna), respectively. Samples were acclimatized under laboratory conditions for 5 days prior to experiments.

2.2. Heavy metals and stock toxicant solution preparation

Two heavy metals, copper (Cu) in the form of copper sulfate ($CuSO_4$) and cadmium (Cd) as cadmium chloride ($CdCl_2$), were used as toxicants to study their effects on the alga *U. reticulata*. To prepare a stock solution, 5 g of a metal compound was dissolved in 100 ml distilled water in dark bottles. Fresh stock solution was prepared for each day of the experiment.

2.3. Experimental design

The acclimatized macroalgal samples (100 g) were transferred to small glass tanks (5 l) with filtered seawater. Three different toxicant concentrations, 1 mg l^{-1}, 3 mg l^{-1} and 5 mg l^{-1}, were used to study the effects. The toxicant was added from the stock solution prepared for each metal compound. The experiment was conducted for 7 days in replicates (n = 3, three independent experiments were conducted for each metal). Macroalgae samples kept in the tank without the addition of any of the metal solutions were used as a reference. Seawater in the tanks was changed on each day of the experiment and a fresh metal solution was added. Samples (about 20 g) were collected from each tank after 2 and 7 days of exposure to metals. One portion of the collected samples was processed immediately for pigment analysis (Chlorophyll-*a* and carotenoids). The other portion of an algal sample was dried under shade for one week, powdered and used for the analysis of other parameters. Ethanolic extract of dried algal powder was prepared with 2.5 g of dried algae samples with 10 ml of ethanol. The mixture was kept in a shaker for 3 days and centrifuged at 3000 rpm for 15 min at 4°C. The resulting crude extract was filtered and used for the experiments.

2.4. Chlorophyll-*a* and carotenoid content analysis

Macroalgal samples (500 mg of fresh algal tissue from each sample) were macerated with 10 ml of acetone in a pestle and mortar under dark conditions. The homogenate was centrifuged at 3000 rpm for 15 min at 4°C. The supernatant was collected in test tubes, which were covered with aluminum foil and the absorbance at 470 nm, 647 nm and 664 nm was measured immediately in a UV-Vis spectrophotometer. The content of chlorophyll-*a* (Chl-*a*) and carotenoid was calculated using the formulae provided in the related literature (Jeffrey & Humphrey 1975; Torres et al. 2014).

2.5. Analysis of total phenolic content

The total phenolic content in algal samples was measured by the method described by Singleton & Rossi (1965) with some modifications. Distilled water (1.58 ml) and the Folin–Ciocalteu reagent (100 µl) were added to the macroalgal extract (20 µl). The mixture was then allowed to settle for 5 min at room temperature. Sodium carbonate solution (300 µl) was then added and agitated carefully for 10 min. The mixture was kept for 2 h under dark conditions at 20°C. The optical density of the mixture was measured at 765 nm using a spectrophotometer. The total polyphenolic content in the analyzed samples was determined from the absorbance of the standard (gallic acid) and the values obtained were presented as mg gallic acid equivalents g^{-1} of algal dry weight.

2.6. Total Antioxidant capacity assay

The antioxidant capacity of algal samples was determined by the method described by Prieto et al. (1999). In brief, sulfuric acid (0.6 M), sodium phosphate (28 mM) and ammonium molybdate (4 mM) were mixed to prepare a total antioxidant capacity (TAC) reagent. The TAC (3 ml) was added to 50 µl of algal extracts and kept at 95°C for 90 min in a water bath. After removal from the water bath, the TAC and algal extract mixture was cooled for 10 min at room temperature. The optical density (OD) was then measured at 695 nm in a spectrophotometer using ethanol as blank. The OD of ascorbic acid was used as a standard to calculate the total antioxidant capacity, and the values obtained were presented as equivalents of ascorbic acid (µg ml^{-1}).

2.7. Bacterial growth inhibition assay

This experiment was conducted to understand the variation in antibacterial activity of *U. reticulata* samples after being treated with heavy metals. Two biofilm-forming bacteria strains, *Pseudomonas shioyasakiensis* (NCBI: KY224086) and *Vibrio harveyi*

(NCBI: KY266820), maintained in our laboratory, were used as target organisms for a growth inhibition assay. These bacteria strains were isolated from the artificial material submerged in the Obhur Creek (Balqadi et al. 2018). An overnight grown bacteria culture (marine broth, Difco) was used for the experiment. The bacterial culture (3 ml) was collected into test tubes and 30 µl algal extracts were added. Two types of controls, one with 30 µl of ethanol and another without solvent or algal extracts (only bacteria culture) were maintained. The OD of the culture was measured at 570 nm in a spectrophotometer immediately after the addition of the extracts. The test tubes were cotton plugged and kept in an incubator at 30°C for 24 h. After that, the final OD was measured and the percentage of bacteria growth inhibition by the extracts was calculated using the following formula:

$$Growth\ Rate\ (\%) = \frac{Final\ OD\ value - Initial\ OD\ value}{Initial\ OD\ value} \times 100$$

2.8. Metal accumulation in algal samples

The content of heavy copper and cadmium in algal samples treated with different concentrations of solutions of respective metals was analyzed by the method described in Topcuoglu et al. (2003). The dried algal samples (5 g) were placed in Teflon vessels and digested by adding H_2SO_4 (5 ml). The samples were then heated on a hot plate at 70–80°C for 15 min. The sample was then allowed to cool at room temperature and 2 ml of concentrated HNO_3 was added slowly. This mixture was again heated for 30 min and allowed to cool. After cooling, 15 ml of H_2O_2 was added and heated for 2 h at 150°C. Finally, the solution was diluted to 100 ml with 2% concentrated HNO_3 in a volumetric flask. Blank samples were prepared using the same protocol without algal samples. The content of copper and cadmium in the samples was analyzed (minimum detection limit: 0.001 µg g^{-1}) by inductively coupled plasma mass spectrometry (ICP-MS). The obtained values were presented as µg g^{-1} dry weight of an algal sample.

2.9. Statistical analysis

Data were analyzed for differences in different physiological parameters and bacterial growth inhibitory activity between control and metal-treated algal samples using three-way ANOVA. Concentration, treatment duration and metals were used as factors in ANOVA. Tukey's post-hoc test was used when ANOVA results showed significant differences between algal samples treated with different concentrations of metals. Two-way ANOVA was also used to examine the differences in bioaccumulation of copper and cadmium in algal samples. Treatment concentration and exposure duration were used as factors in two-way ANOVA. Further, simple correlation analysis was performed to determine the relationship between bacterial growth, bioaccumulation of metals and different physiological parameters. All statistical analyses were carried out using STATISTICA and $p < 0.05$ was considered significant.

3. Results

3.1. Chlorophyll content

Chl-*a* content in a control algal sample was 200.31 µg g^{-1} after 2 days and 133.41 µg g^{-1} after 7 days (Fig. 1). Samples treated with copper and cadmium showed a decrease in Chl-*a* after 2 and 7 days of exposure. A concentration-dependent decrease was observed in samples treated with both copper and cadmium (not significant, three-way ANOVA; Table 1). The Chl-*a* content in samples treated with 1 mg l^{-1} of Cu was 165.8 µg g^{-1} after 2 days and 143.72 µg g^{-1} after 7 days. Similarly, the Chl-*a* content in algal samples treated with 3 mg l^{-1} of Cu showed a decrease (159.88 µg g^{-1}) after 2 days and 7 days (99.75 µg g^{-1}). The lowest Chl-*a* content (79.93 µg g^{-1}) was determined in *U. reticulata* samples treated with 5 mg l^{-1} of copper for 7 days (Fig. 1).

3.2. Carotenoid content

The carotenoid content in the control algal sample was 88.05 µg g^{-1} after 2 days and 62.04 µg g^{-1} after 7 days (Fig. 1). Algal samples treated with copper and cadmium varied in carotenoid content. While a gradual decrease in carotenoid content with treatment concentration was observed in samples treated with copper for 2 and 7 days. A slight increase in carotenoid levels was observed in samples treated with 1 mg l^{-1} and 3 mg l^{-1} of cadmium for 7 days. The lowest carotenoid content (27.17 µg g^{-1}) was measured in algal samples treated with 5 mg l^{-1} of cadmium for 7 days (Fig. 1). Three-way ANOVA results indicated that the differences in carotenoid content due to copper and cadmium treatments were not significant (Table 1).

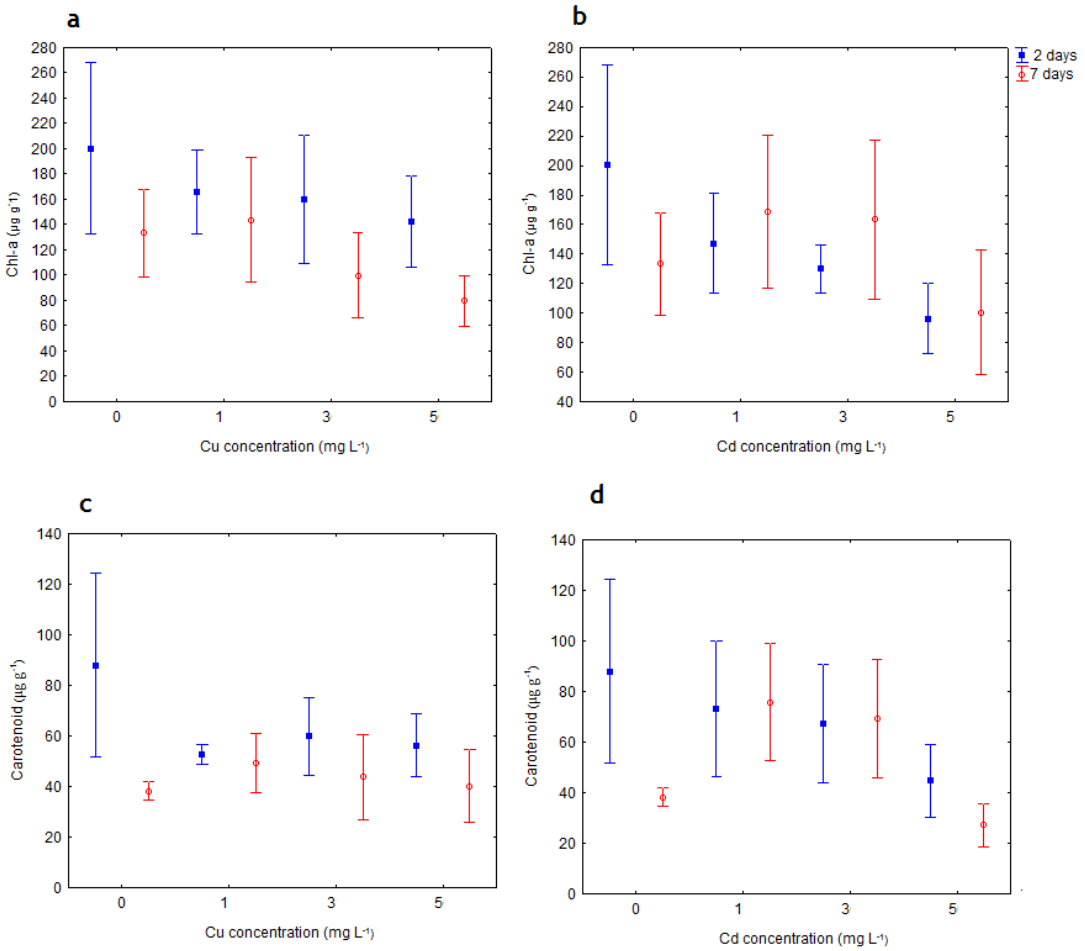

Figure 1

Changes in chlorophyll-*a* and carotenoid content (mean ± SE, n = 3) in the macroalga *U. reticulata* treated with heavy metals for 2 and 7 days. a) Chlorophyll-*a* content in algal samples treated with copper. b) Chlorophyll-*a* content in algal samples treated with cadmium. c) Carotenoid content in algal samples treated with copper. d) Carotenoid content in algal samples treated with cadmium

Table 1

Three-way ANOVA results showing the variations in antifouling defense and physiological parameters of the macroalga *U. reticulata* treated with copper and cadmium. Treatment concentration (0, 1, 3 and 5 mg l^{-1}), duration (2 and 7 days) and metals (copper and cadmium) were used as factors for ANOVA ($p < 0.05$ = significant).

Factors	df	*P. shioyasakiensis*		*V. harveyi*		Chl-*a*		Carotenoids		Phenol		Total antioxidant capacity	
		F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
Concentration	3	18.68	0.000	15.15	0.000	1.57	0.213	1.02	0.395	1.40	0.258	89.65	0.000
Days	1	40.29	0.000	0.91	0.345	1.61	0.213	3.48	0.070	18.08	0.000	133.51	0.000
Metals	1	0.15	0.695	1.98	0.168	0.00	0.934	0.48	0.490	9.10	0.004	16.22	0.000
Concentration x Days	3	8.08	0.000	1.42	0.253	0.44	0.723	1.22	0.317	2.07	0.123	40.36	0.000
Concentration x Metals	3	0.52	0.666	0.77	0.517	0.08	0.970	0.66	0.582	1.99	0.134	34.77	0.000
Days x Metals	1	7.98	0.008	2.28	0.140	1.38	0.248	0.08	0.776	5.68	0.023	120.35	0.000
Concentration x Days x Metals	3	4.98	0.006	3.03	0.043	0.20	0.889	0.04	0.984	1.25	0.306	29.64	0.000
Error	32												
Total	47												

3.3. Total phenolic content

The control algal sample showed a total phenolic content of 3.02 mg GAE g^{-1} after 2 days and 1.86 mg GAE g^{-1} after 7 days. Samples treated with 1 mg l^{-1} of copper showed a decrease in phenolic content after 2 days (2.204 mg GAE g^{-1}) and 7 days (1.103 mg GAE g^{-1}). Samples treated with 3 mg l^{-1} and 5 mg $l^{-1}$of copper also showed a decrease in phenolic content after 2 and 7 days of treatment (Fig. 2). A very low phenolic content of 0.778 mg GAE g^{-1} was observed after 7 days of a copper treatment at a dose of 3 mg l^{-1}. Contrary to the copper treatment, samples treated with cadmium showed an increase in total phenolic content after 2 days of exposure (Fig. 2). In algal samples exposed to cadmium for 7 days, a decrease in total phenolic content was observed in samples treated with 3 mg l^{-1} and 5 mg l^{-1} (1.48 and 0.98 mg GAE g^{-1}, respectively). Further, ANOVA results showed significant differences in the total phenolic content in algal samples depending on the treatment duration (Table 1). Changes in the phenol content in algal samples also showed significant differences between Cu and Cd treatments (Table 1).

3.4. Total antioxidant capacity assay

U. reticulata samples (control) revealed the antioxidant capacity of 330.87 and 262.64 µg ml^{-1} after 2 and 7 days under laboratory conditions. However, samples treated with copper and cadmium showed higher levels (significant variation between the metals; Table 1) compared to the control after both 2 and 7 days of exposure (Fig. 3). The antioxidant capacity was remarkably high in samples observed after 2 days (706.5 µg ml^{-1} for copper-treated samples; 1696.65 µg ml^{-1} for cadmium-treated samples). In general, a concentration-dependent increase in antioxidant capacity was observed in samples treated for 2 days with both copper and cadmium. The highest antioxidant capacity of 1696.65 µg ml^{-1} was observed in the extract of algal samples treated with 5 mg l^{-1} of cadmium for 2 days. Three-way ANOVA results indicated a significant variation in antioxidant capacity as a function of metal concentration and exposure duration (Table 1). Further, significant variations were observed for all interactions between the factors used in ANOVA. While the antioxidant capacity for all concentrations of copper treated samples differed significantly from the control, an algal sample treated with 1 mg l^{-1} of cadmium showed no significant difference from the control (Table 2).

3.5. Bacteria growth inhibition assay

The extract obtained from control algal samples showed a strong growth inhibitory effect on the bacterium *P. shioyasakiensis*. However, algal samples treated with different concentrations of copper and cadmium for 2 days exhibited a relatively weak growth inhibitory effect on *P. shioyasakiensis* (Fig. 4). Further, extracts obtained from algae treated with 1 mg l^{-1} of copper and cadmium for 2 days showed very low growth inhibitory activity. Algal samples treated with

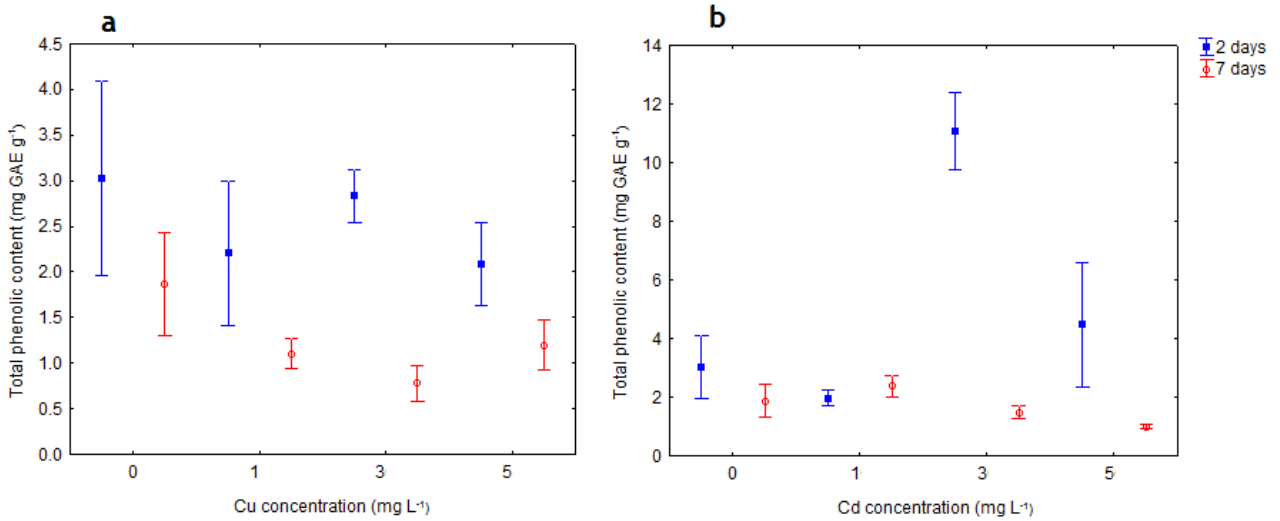

Figure 2

Effects of heavy metal treatment on total phenol content (mean ± SE, n = 3) in the macroalga *U. reticulata*. a) Total phenol content in algal samples treated with copper. b) Total phenol content in algal samples treated with cadmium.

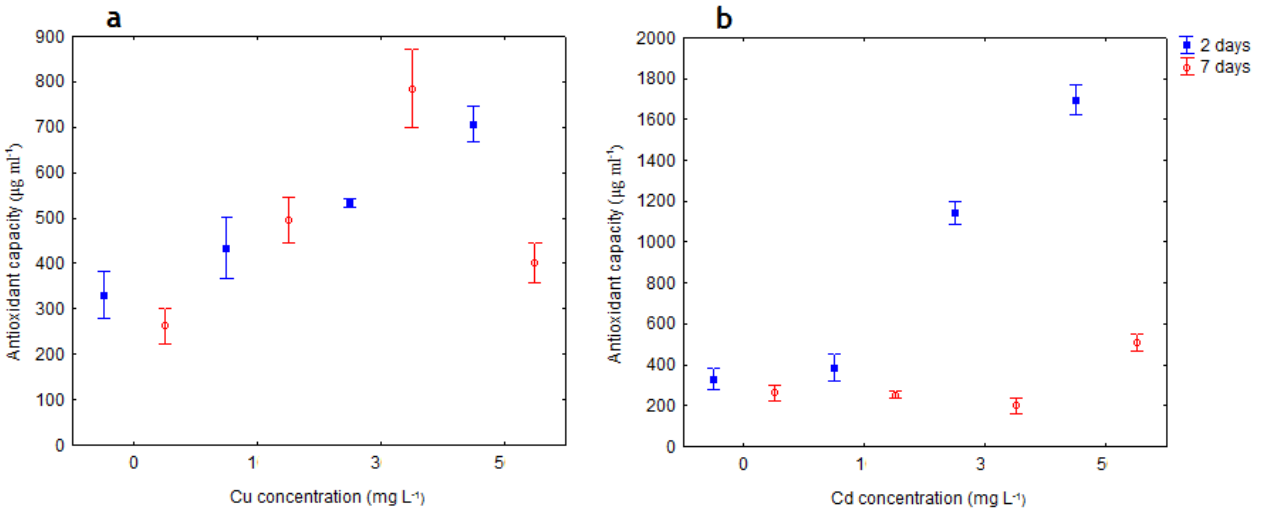

Figure 3

Changes in total antioxidant capacity (mean ± SE, n = 3) in algal samples treated with copper and cadmium for 2 and 7 days. a) Total antioxidant capacity of algal samples treated with copper. b) Total antioxidant capacity of algal samples treated with cadmium.

Table 2

Post-hoc Tukey HSD test results for the effects of different concentrations of copper and cadmium on the macroalga *U. reticulata* ($p < 0.05$ = significant)

Factor 1	Factor 2	Total antioxidant activity		Bacterial growth *P. shioyasakiensis*		Bacterial growth *V. harveyi*	
		Cu-treated samples	Cd-treated samples	Cu-treated algal extracts	Cd-treated algal extracts	Cu-treated algal extracts	Cd-treated algal extracts
Control	1 mg l^{-1}	0.049	0.966	0.028	0.001	0.001	0.000
	3 mg l^{-1}	0.000	0.000	0.020	0.007	0.001	0.005
	5 mg l^{-1}	0.000	0.000	0.000	0.001	0.075	0.001
1 mg l^{-1}	3 mg l^{-1}	0.013	0.000	1.000	0.999	1.000	0.981
	5 mg l^{-1}	0.668	0.000	0.669	1.000	0.811	0.999
3 mg l^{-1}	5 mg l^{-1}	0.473	0.000	0.755	0.999	0.789	0.999

copper and cadmium for 7 days also showed lower growth inhibitory activity (except the extract obtained from samples treated with 5 mg l^{-1} of cadmium). Algal extracts obtained from samples treated with copper and cadmium exhibited a weak growth inhibitory effect on the bacterium *V. harveyi* (Fig. 4). A concentration-dependent reduction in bacterial growth inhibitory activity was observed for extracts obtained from samples treated with cadmium for 2 days. The growth inhibitory activity of algal samples treated with copper and cadmium was very low except for the extract obtained from samples treated with copper at 5 mg l^{-1} for 2 days.

The ANOVA results indicated a significant variation in bacterial growth inhibitory activity of algal extracts depending on the concentration of metals and days of exposure against *P. shioyasakiensis* (Table 1). On the other hand, a significant difference was observed between metal concentration and growth percentage of *V. harveyi* (Table 1). Tukey's post-hoc test revealed significant variation in bacterial growth inhibitory activity between algal samples treated with different concentrations of metals and control samples (Table 2). The growth rate of *P. shioyasakiensis* also showed a significant negative correlation with the bioaccumulation of copper in algal samples (Table 3), but *V. harveyi* showed a significant positive correlation. A positive correlation was determined between the antioxidant activity and the growth rate of *P. shioyasakiensis* (Table 3).

3.6. Metal accumulation in *U. reticulata*

The content of copper and cadmium in algal samples treated with different concentrations of copper sulfate and cadmium chloride is presented

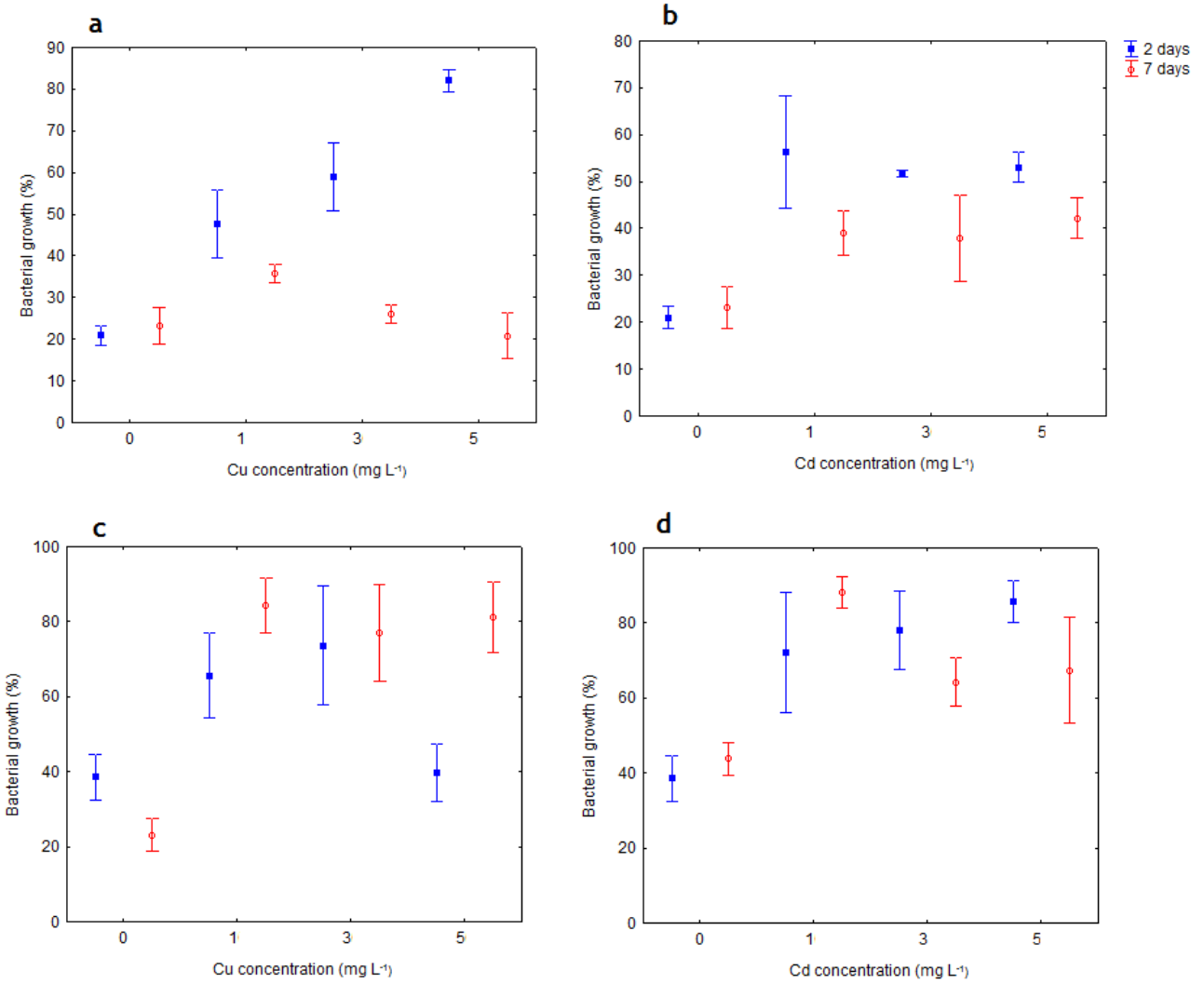

Figure 4

Effects of heavy metal treatment on antifouling defense of the marine macroalga *U. reticulata*. Antifouling activity of algal extracts was tested against two biofilm-forming bacteria. a) Growth inhibitory activity of copper-treated samples against *P. shioyasakiensis*; b) Growth inhibitory activity of cadmium-treated samples against *P. shioyasakiensis*; c) Growth inhibitory activity of the extract from copper-treated algal samples against *V. harveyi*; d) Growth inhibitory activity of the extract from cadmium-treated algal samples against *V. harveyi*. Error bars indicate SE of mean values (n = 3).

Table 3

Correlation between bacterial growth and physiological parameters of the macroalga *U. reticulate* ($p < 0.05$ = significant). Bioaccumulation of copper and cadmium was also used in correlation analysis.

Copper-treated samples	*P. shioyasakiensis*	*V. harveyi*	Cadmium-treated samples	*P. shioyasakiensis*	*V. harveyi*
Antioxidant capacity	0.39	0.31	Antioxidant capacity	0.44*	0.39
Total phenol	0.20	−0.37	Total phenol	0.38	0.29
Chl-*a*	0.16	−0.13	Chl-*a*	−0.05	−0.19
Carotenoid	0.05	−0.08	Carotenoid	0.05	0.02
Cu accumulation	−0.43*	0.43*	Cd accumulation	−0.008	0.047

* = significant

on Figure 5. In control samples, the copper content was 0.057 and 0.049 µg g^{-1} after 2 and 7 days under laboratory conditions. However, copper accumulation in the algal tissue was observed in samples treated with copper sulfate with a maximum of 17.71 µg g^{-1} (samples treated with 5 mg l^{-1} of copper for 7 days). Similarly, cadmium accumulation was observed in algal samples treated with 5 mg l^{-1} of cadmium for 7 days, with a maximum of 0.72 µg g^{-1}. The cadmium content in control algal samples was 0.012 and 0.003 µg g^{-1} after 2 and 7 days. In general, a significant increase in metal content was observed in algal samples with increasing exposure duration and treatment concentrations (Table 4). The bioaccumulation level of copper in algal samples indicated a significant negative correlation with Chl-*a* and the total phenolic content in algal samples (Table 5). However, cadmium concentration in algal samples showed no significant correlation with physiological parameters.

Table 4

Two-way ANOVA of bioaccumulation of copper and cadmium in the macroalga *U. reticulata*. Treatment concentration and duration were used as factors ($p < 0.05$ = significant).

Factors	df	Cu accumulation		Cd accumulation	
		F	*p*	F	*p*
Concentration	3	58.427	0.000	35.030	0.000
Days	1	229.51	0.000	68.458	0.000
Concentration x Days	3	59.790	0.000	37.720	0.000
Error	16				
Total	23				

Table 5

Correlation between metal accumulation and physiological parameters of the macroalga *U. reticulata* ($p < 0.05$ = significant).

Metal accumulation	Chl-*a*	Carotenoid	Total phenol	Antioxidant capacity
Copper	−0.444*	−0.264	−0.481*	0.208
Cadmium	−0.178	−0.277	−0.356	0.158

* = significant

4. Discussion

Heavy metal pollution is one of the major anthropogenic stressors affecting the marine environment throughout the world (Tzafriri-Milo et al. 2019). After entering the coastal waters through various sources, heavy metals accumulate in marine organisms (Saez et al. 2012; Lozano-Bilbao et al. 2019). This study showed that the macroalga *U. reticulata* can accumulate copper and cadmium from water. The accumulation level increases as the concentration of these metals in water increases. Cadmium content in algal samples was lower compared to copper concentrations. This may be due to the characteristic feature of *Ulva* species, which usually have lower cadmium concentrations (Rybak et al. 2012). In general, macroalgal communities can accumulate metal ions dissolved in seawater in proportion to

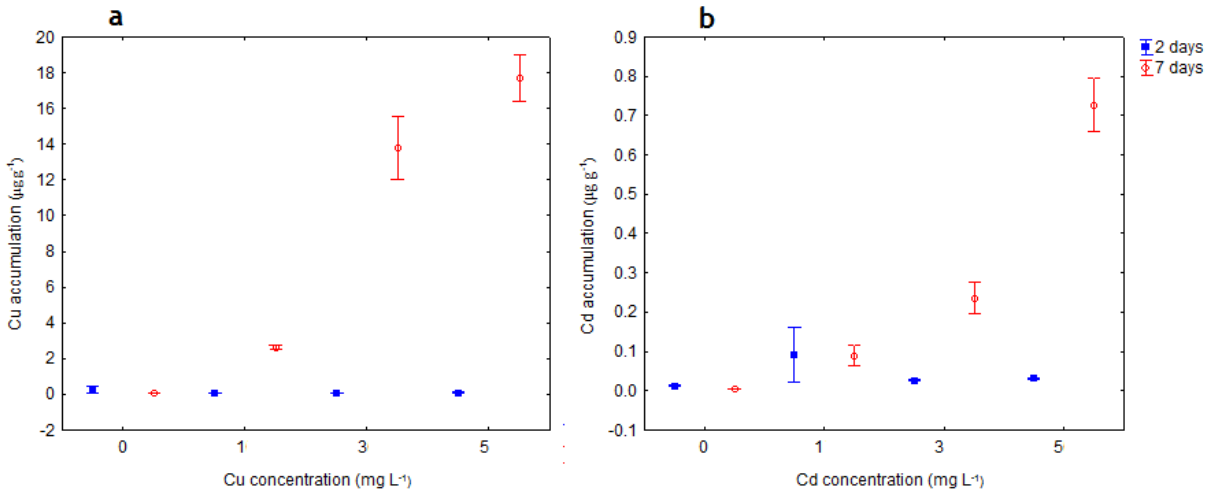

Figure 5

Bioaccumulation of copper (a) and cadmium (b) in the macroalga *U. reticulata*. Algae were exposed to heavy metals for 2 and 7 days at three concentrations. Error bars indicate SE of mean values (n = 3)

their concentration (Wang et al. 2014; Seepersaud et al. 2018). The bioaccumulation capacity of *U. reticulata* indicated that this alga could be used as an indicator species to assess metal pollution in coastal zones. A good bioindicator of metal pollution should show a linear relationship between the concentration of metals in cells and in the environment (Phillips 1990). Previous laboratory studies also showed a strong linear correlation for metal concentration between thalli of *Ulva* species and culture tanks (Tabudravu et al. 2002; Chan et al. 2003). In seawater, however, environmental factors such as salinity, pH and nutrient content will affect the bioaccumulation process in macroalgae (Tabudravu et al. 2002). Therefore, it is important to consider physical and chemical factors of seawater when using *Ulva* species as a bioindicator of metal pollution (Rybak et al. 2012).

The results indicated a decrease in chlorophyll-*a* and carotenoid content in algal samples treated with copper and cadmium for 2 days. While the Chl-*a* and carotenoid levels decreased, the differences between control and metal-treated algal samples were not statistically significant. In a similar study, the Chl-*a* content in the red alga *Sarcodia suiae* was reduced by the cadmium treatment (Han et al. 2020). Another alga, *Gracilaria domingensis*, showed a decrease in Chl-*a* content after exposure to cadmium for 4 days (Dos Santos et al. 2012). Another study by Ji et al. (2018) also indicated that cadmium pollution affects photosynthesis of the alga *Phaeodactylum tricornutum*. On the other hand, a study by Pinto et al. (2011) reported an increase in Chl-*a* and carotenoid content in the marine alga *Gracilaria tenuistipitata* exposed to copper and cadmium for 24 h. Similarly, an increase in carotenoid content was observed in this study in samples treated with cadmium for 7 days. This indicates that physiological effects of heavy metals depend mainly on the exposure duration. In general, higher levels of metals in water may inhibit photosynthesis, reduce concentration of pigments and affect the growth of macroalgae (Xia et al. 2004; Contreras et al. 2007).The increase in photosynthetic pigments in algal samples treated with cadmium may be one of the defense mechanisms against toxicant stress (Ramlov et al. 2014).

Most algae species adapt easily to metal pollution through various physiological mechanisms (Contreras-Porcia et al. 2017). The resistance mechanisms against heavy metal toxicity are mainly involved in the production of polyphenols, which act as chelating agents (Toth & Pavia 2000). In general, phenolics prevent the catalytic functions of metals by acting as metal chelators (Wu & Hansen 2008). The results of the present study indicate that the total phenol content was reduced in algal samples treated with copper, while it showed higher values (after 2 days) in samples treated with cadmium. Further, algal samples treated with copper and cadmium showed an increase in antioxidant capacity. As antioxidant activity is directly related to the total phenolic content (Zhang et al. 2007), the increase in total antioxidant capacity in this study may be a resistance strategy of *U. reticulata* to the metal toxicity.

In this study, antifouling mechanisms of *U. reticulata* were tested against two fouling bacteria strains isolated from artificial substrates. The bacterial growth inhibitory activity in algal samples was reduced after exposure to copper and cadmium (except a slight increase in the algal sample treated with 5 mg l^{-1} of copper for 7 days). The increase in bacterial growth was observed even after an increase in total antioxidant capacity and total phenolic content of algal samples treated with the metals. Phenolic compounds are the major group of secondary metabolites used by algae against pathogens, grazers and biofouling organisms (Da Gama et al. 2014). Therefore, the reduction in bacterial growth inhibitory activity may be due to algae prioritizing the use of available resources to mitigate the toxic effects of metals. On the other hand, chemical defense is an energy consuming process (Nylund et al. 2013) and the reduction in photosynthesis may further reduce the energy reserves of algae. The decrease in energy content affects the production of secondary metabolites that are important for chemical defense (Ramalhosa et al. 2016).

Macroalgae exhibit constitutive (permanent) and induced (temporary) chemical defenses as protective mechanisms against attacks of herbivores and fouling organisms (Cronin & Hay 1996). The induced chemical defense involves increased production of secondary metabolites due to grazing or other competitors. Therefore, the induced defense trait may be more sensitive to abiotic and biotic factors. In a previous study, Warneke & Long (2015) reported that copper contamination reduced the inducible defense in the marine alga *Silvetia compressa*. Moreover, it has been reported that environmental stressors change the defense traits of seaweeds against herbivores (Yates & Peckol 1993; Warneke & Long 2015). The results observed in this study indicate possible indirect effects of metal pollution on macroalgal communities in coastal ecosystems. Specifically, an increase in heavy metal pollutants may change the structure of macroalgal assemblages and thus affect the ecosystem services. In addition to the reduction in photosynthetic pigments, results of this study study revealed that those macroalgae inhabiting the high

metal-contaminated regions may have weak defense traits against epibiosis and herbivores.

Of the two metals used in this study, copper is considered an essential trace element for physiological functions of macroalgae (Leal et al. 2018). However, higher concentrations of copper in seawater are reported to have toxic effects on macroalgal communities and other aquatic organisms (Kramer & Clemens 2006; Gouveia et al. 2013). The significant negative correlation between copper accumulation in algal samples and Chl-*a*, total phenolic content and growth of the bacteria *P. shioyasakiensis* indicate direct effects of this metal on *U. reticulata*. The negative correlation with the growth of *P. shioyasakiensis* may be due to the antifouling activity of copper. Copper-based compounds are commonly used as a booster biocide in antifouling paints (Fay et al. 2019). On the other hand, the positive correlation between the copper content in algae and the growth rate of *V. harveyi* indicates that the effect of copper on microbial communities is species-specific. Unlike copper, cadmium is not an essential element for macroalgae and represents a potential threat to human health due to accumulation in the food chain (Volesky & Holan 1995; Webster et al. 1997). Cadmium can also seriously affect phytoplankton and macroalgae in marine ecosystems (Payne & Price 1999; Kapkow et al. 2011).

In this study, statistically significant differences were observed between the effects of two metals on total antioxidant capacity and total phenolic content of algal samples, with higher phenolic content found in samples treated with cadmium. It has been reported that phenols are secondary metabolites of algae responsible for the antioxidant activity (Ganesan et al. 2008; Pereira et al. 2017). Antioxidant compounds protect algae from the production of reactive oxygen species (ROS) by acting as free radical scavengers (Kokilam & Vasuki 2014). In a previous study, the correlation between stress tolerance and higher levels of antioxidants were observed in brown algae from the genus *Fucus* (Collen & Davidson 1999). Ahmad et al. (2010) reported that phenolic compounds are non-enzymatic antioxidants that usually act as terminators and chelators for free radical chains and redox-active metal ions capable of inducing lipid peroxidation. Although algal samples treated with cadmium showed higher phenolic content after 2 days, further treatment with this metal resulted in reduced total phenolic content. This indicates that the effect of cadmium on the antioxidant defense mechanism of algae may be stronger than that of copper.

In conclusion, the results of the present study indicate that exposure of *U. reticulata* to copper and cadmium induced protective mechanisms against metal stress but reduced photosynthesis. Furthermore, exposure to metals reduced the algal defense against marine biofilm-forming bacteria. Although the results showed bioaccumulation in algal samples exposed to higher concentrations of metals, changes in physiological parameters and antifouling defense were observed even for algae treated with a low concentration of metals for a short period of time (2 days). Further studies using different macroalgae species (instead of a single species) may be helpful in understanding the antifouling response of other algae species to heavy metal pollution. Comparison of defense traits observed in laboratory studies with macroalgal samples collected from presumably polluted sites will also provide more information on physiological and antifouling responses of macroalgal communities to anthropogenic stressors.

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