

Research Article

First approach of characterization of bioactive compound in *Pyropia orbicularis* during the daily tidal cycle

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ABSTRACT. The red seaweed *Pyropia orbicularis* is an economic important species that occurs predominantly in the high intertidal zone along the Chilean coast where it experiences extreme conditions under daily turning tides. Its gametophytic phase has been demonstrated to have a high desiccation tolerance, both at the genomic and proteomic levels, but studies at the metabolomic level are still lacking. This research aimed at characterizing compounds related to desiccation stress tolerance by performing several phase solid extractions with different solvents. Bioactivity-guided fractionation (antioxidant and antibacterial activities) was made for a more specific characterization. Compounds identification was done using LC-MS/MS. Results showed that *P. orbicularis* produces different compounds depending on the state of hydration during the tidal cycle. For example, minoxidil was only found under hydration, while vincamine only during desiccation. It was also found that the main antioxidant activity was most likely due to lutein and the antibacterial activity could be mainly attributed to compounds of lipid nature such as phosphatidylethanolamine (PE), phosphatidylserine (PS) and monogalactosyldiacylglycerol (MGDG). These results help to get a better understanding of the stress tolerance mechanisms in *P. orbicularis* and place it like a potential source of bioactive compounds.

Keywords: *Pyropia orbicularis*; oxidative stress; bioactive compounds; stress tolerance; antibacterial activity; antioxidant activity

INTRODUCTION

The abiotic and biotic gradients factors along the rocky intertidal zone, such as temperature, light intensity, desiccation, competition and predation among others, make it a highly heterogeneous environment (Stephenson & Stephenson, 1949; Davison & Pearson, 1996). The organisms inhabiting the intertidal present diverse mechanisms of stress tolerance depending of the distribution zone (high, middle, low or rocky walls)

or small scale microenvironments (Davison & Pearson, 1996; Flores-Molina *et al.*, 2014; Meynard *et al.*, 2019; Zapata *et al.*, 2019). Organisms, under constant environmental pressures, generate an overproduction of reactive oxygen species (ROS), such as superoxide ion (O_2^-), hydrogen peroxide (H_2O_2) or hydroperoxides (ROOH) among others (Ray *et al.*, 2012), which in turn can cause oxidative damage to important biomolecules (membrane lipids, nucleic acids and proteins) (Mittler, 2002). Tolerance to this oxidative condition triggered

by environmental factors, such as temperature, salinity, air exposure and pH, is mainly related to antioxidant enzymatic activity (Foyer *et al.*, 1997; Schafer & Buettner, 2001; Lovazzano *et al.*, 2013; Hancock, 2017), and compounds (*e.g.*, pyridoxic acid, ascorbic acid, carotenoids, polysaccharides, phycobiliproteins, and peptides) which are involved in lowering the overall levels of cellular ROS (Zhao *et al.*, 2006; Zhang *et al.*, 2009; Yabuta *et al.*, 2010; Isaka *et al.*, 2015; Cian *et al.*, 2016). Some of these compounds are also important in scavenging ROS during stress induced by salinity (Kumar *et al.*, 2010), temperature (Strimbeck *et al.*, 2015) and UV radiation (Zhang *et al.*, 2018). In addition, seaweeds are subjected to a constant biotic pressure, mainly during high tide, with a complex interaction with its diverse epibiota community guided by metabolite and chemical communication (Dobretsov *et al.*, 2009; Egan *et al.*, 2013).

Among representatives of the order Bangiales (Rhodophyta) along the South Pacific coast, Guillemín *et al.* (2016) established that the red alga *Pyropia orbicularis* (Ramírez *et al.*, 2014) is distributed between 32°S and 53°S in the upper rocky intertidal with seasonal variations of its abundance (Betancourt *et al.*, 2018). Under air exposure during low tide, the gametophytic phase (*i.e.*, macroscopic laminar phase) of *P. orbicularis* can lose up to 96% of its relative water content and remains dried for up to six hours being able to return to its morphological and metabolic basal condition in less than two hours after being rehydrated (Contreras-Porcía *et al.*, 2011). Desiccation tolerance of *P. orbicularis* has been described at genomic and proteomic levels. It has been determined, that during the first four hours of natural air exposure this species presents changes in the expression of genes involved in cellular respiration, cell wall metabolism, antioxidant activity, synthesis and degradation of proteins and dismantling of photosystems, showing a rapid recovery to the basal states during rehydration (Contreras-Porcía *et al.*, 2013; Fierro *et al.*, 2017). Additionally, it has been documented that under desiccation an increase in the synthesis of proteins linked to energy production occur such as the enzyme triose phosphate isomerase and aconitines; along with down regulation of actin and an upregulation of cofilin/actin depolymerizing factor 3 and phycobiliproteins, all related to cell wall structure preservation or chemical tolerance to oxidative stress triggered by air exposure (López-Cristoffanini *et al.*, 2015). The activation of these tolerance mechanisms would be modulated by the accumulation of the phytohormone abscisic acid (ABA), which act by promoting changes in the metabolism of carbohydrates and in the synthesis and degradation of proteins related to photosynthesis (Guajardo *et al.*, 2016), such as has

been determined in vascular plants (Larkindale & Kinght, 2002; Agarwal *et al.*, 2005). Therefore, the presence of this compound is essential within the mechanisms of tolerance to desiccation, as demonstrated in sensitive species when adding exogenous ABA (Contreras-Porcía *et al.*, 2012).

Tolerance to desiccation can be modulated by both primary and secondary metabolites. This has been documented both in vascular plants and algae (Kumar *et al.*, 2011; Suguiyama *et al.*, 2014; Guajardo *et al.*, 2016). Indeed, the roles of secondary metabolites overlap with definitions of bioactive compounds; for example, “secondary metabolites, which modulate one or more metabolic process, eliciting pharmacological or toxicological effects in humans and animals” (Guaadaoui *et al.*, 2014). In the case of stress due to desiccation, it has been shown that sugars are a fundamental part of tolerance mechanisms in plants (Peters *et al.*, 2007; Toldi *et al.*, 2009), as are also amino acids, organic acids, carbohydrates and fatty acids (Suguiyama *et al.*, 2014). These compounds act physiologically and morphologically, as protective metabolites of the cell membrane (Peters *et al.*, 2007), in the regulation of gas exchange (Nunes-Nesi *et al.*, 2007), as well as antioxidant compounds (Jaiswal *et al.*, 2011). In the red seaweed *Pyropia haitanensis* the accumulation of floridosides and other volatile compounds such as 3-octanone, 1-octanol was observed under desiccation which would be fulfilling the function of stabilizers of the cell wall (Qian *et al.*, 2015).

Some compounds which would not only play an important role in environmental stress tolerance, but also may act as bioactive compounds and be beneficial for human consumption. For example, fucoxanthin, dimethylsultonipropionate have been described in *Fucus vesiculosus* as antibacterials (Saha *et al.*, 2011; Svensson *et al.*, 2013); and porphyrans and other polysaccharides have been characterized as antioxidant compounds in species of the genera *Porphyra* and *Pyropia* (Zhang *et al.*, 2003; Zhao *et al.*, 2006; Isaka *et al.*, 2015). Recently, the presence of polyphenols, flavonoids and anthraquinones has also been demonstrated, which would have an antioxidant and anti-cancer activity in the seaweeds *Desmarestia ligulata*, *Dictyota kunthii*, *Chondracanthus chamosoi* and *Laurencia chilensis* (Miranda *et al.*, 2018). In this context, our purpose was to identify compounds associated to desiccation stress tolerance mechanisms, but also those having a potential role as bioactive compounds under desiccation. Therefore, the aims of the present study were i) to identify compounds related to desiccation stress tolerance in the gametophytic phase of *P. orbicularis* and given its economic

importance ii) to evaluate its potential source of bioactive compounds having antibacterial and antioxidant activities.

MATERIALS AND METHODS

Algal samples

Pyropia orbicularis gametophytes were collected randomly along a 200 m long transect perpendicular to the coast from the intertidal zone of Maitencillo, Chile (31°29'S, 71°26'W). Algae were collected in two tide conditions: at high tide, referred as naturally hydrated (NH), and at low tide during four hours, referred as naturally desiccated (ND). The algae were washed either with filtered seawater (0.22 µm) or de-ionized water (18.2 MΩ-cm) for the metabolite and bioassay extraction respectively, and a random mix from different individuals for each condition was separated in 1 g (for the metabolomic analysis) and 25 g packages (for the bioactivity-guided fractionation). Finally, samples were frozen *in situ* with liquid nitrogen. For the *in vitro* experiments, a part of naturally hydrated algae was transported in seawater at 4-7°C, acclimated overnight at 12-14°C in filtered seawater (H) and washed accordingly to the different extraction procedures. Half of these samples were frozen with liquid nitrogen and the other half were subjected to desiccation stress (D), which was done during 4 hours as described by Contreras-Porcia *et al.* (2011). All the biomass was maintained at -20°C until its extraction.

Reagents

HPLC grade acetone, acetonitrile, dichloromethane, ethanol, n-hexane, methanol and ammonium acetate were purchased from Merck (Merck KGaA, Darmstadt, Germany). Ammonium acetate, ammonium formate, kanamycin and ascorbic acid were purchased from Sigma Aldrich (Merck KGaA, Darmstadt, Germany). The n-heptane, chloro-form, ammonium hydroxide, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Merck (Merck KGaA, Darmstadt, Germany).

Metabolomic characterization

Metabolites extraction procedure

Samples from *in situ* experiment was grounded in a mortar with liquid nitrogen and 1 g of powder was extracted with 5 mL of either de-ionized water, acetonitrile, ethanol or n-hexane. The mixture was agitated using an orbital shaker at 4°C for 4 h, and then centrifuged at 1790 g and 4°C for 40 min. The extracts were filtered with a syringe filter of 0.22 µm and kept in an amber vial at 4°C until chromatographic analysis.

Metabolites chromatographic analysis

The samples were analyzed by liquid chromatography/tandem mass spectrometry (LC-MS/MS) with electrospray (ESI) as ionization method in an Agilent 1100 HPLC system (Agilent Technologies Inc., CA-USA) coupled to tandem mass spectrometry system with electrospray-triple quadrupole (ESI-QqQ) Esquire 4000 ESI-IT model (Bruker Daltonik GmbH, Germany) both in negative and positive ion mode by spray voltage of 3 kV; heated capillary temperature was maintained at 365°C with nitrogen as drying gas at 45 psi and 10 L min⁻¹. Chromatographic separation was carried out in a C-18 column Inertsil ODS-3 250×4.6 mm with 5 µm size particle (GL Sciences Inc., USA) at 30°C; previously, samples were filtered with a syringe filter at 0.22 µm and 20 µL were injected in a LC-MS/MS. The mobile phase used consisted in a gradient of 0.1% m/v ammonium acetate in de-ionized water (A) and 0.1% m/v ammonium acetate in acetonitrile (B) at a constant flow of 0.6 mL min⁻¹ (Table 1a), and running time of 45 min. Compounds were identified using the softwares Mass++ (Tanaka *et al.*, 2014) and the databases MassBank (Horai *et al.*, 2010), MassBase (<http://webs2.kazusa.or.jp/massbase/>), MetaboSearch (Zhou *et al.*, 2012), and BinBase (<http://finehnlab.undavis.edu/db>).

Bioactivity characterization

Bioassay extraction procedure

Samples of *in situ* and *in vitro* experiments were grounded, separately, in a mortar with liquid nitrogen and 20 g was treated under solid phase extraction with a Soxhlet extraction system (Isolab Laborgeräte GmbH, Germany) during three hours with 200 mL of either methanol, acetone, dichloromethane or n-hexane, in different batches to establish the greatest bioactivity. The extracts were concentrated in a rotary evaporator (RE-300, Stuart) at 30°C, and kept at -20°C.

Fractionation of bioactive extract with the greatest activity

The n-hexane extract, showing the greatest bioactivity (see next section), was fractionated in a preparative silica gel (Silica G-60, Dynamic Adsorbent) open

Table 1. HPLC gradient elution programs.

a) Gradient elution for metabolomic analysis			b) Gradient elution for bioactivities analysis		
Time (Min)	Phase A (% V/V)	Phase B (% V/V)	Time (Min)	Phase A (% V/V)	Phase B (% V/V)
0	100	0	0	5	95
10	50	50	5	3	97
20	25	75	10	3	97
30	25	75	10.01	0	100
42	0	100	58	0	100
45	100	0	60	5	95

column chromatography as described by Vieira *et al.* (2017), with some modifications. Briefly, 1 mL of extract was transferred to the n-hexane equilibrated column, and eluted with 2, 10, 20, 30 and 100% acetone to obtain the fractions 1, 2, 3, 4 and 5, respectively. The fractions were evaporated at 30°C in a rotary evaporator, resuspended in 500 µL methanol and kept at -20°C.

Antioxidant assay

The antioxidant activity was measured with DPPH assay by thin layer chromatography (TLC). 50 µg of each extract was spotted onto 20×20 cm TLC plates (Merck KGaA, Darmstadt, Germany), and were developed with the mobile phase water:ethanol:chloroform:heptane:ammonium hydroxide (2:7:1:1:1). 50 µg of the fractions of n-hexane extracts (showing the greatest bioactivity) were spotted onto 6×4 cm TLC plates (Dynamic Adsorbent, silica Gel 60 F254) and were developed with the mobile phase hexane:acetone (7:3). The plates were sprayed with a methanolic solution of 0.2% of DPPH and the antioxidant activity was quantified by Sorbfil TLC densitometry (v.2.5 JSC SorbPolymer). To compare antioxidant activity (TLC-DPPH) of each extract ascorbic acid was used as positive control.

Antibacterial assay

The antibacterial activity was measured by agar disk-diffusion assay. 100 µL of *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus* cultures were inoculated ($OD_{600} = 0.5$) in 10 mm Petri dishes with Mueller-Hinton agar and incubated overnight at 37°C. For each bacterial culture, sterile sensidisks were impregnated with 50 µg of each extract or fraction which were placed onto the surface of the agar, using 30 µg of Kanamycin (Sigma Aldrich, Merck KGaA, Darmstadt, Germany) as a positive control (Boyle *et al.*, 1973).

Compounds identification

The bioactive fractions were separated using LC-MS/MS (see Chromatographic analysis section) in a C-18 column Inertsil ODS-3 250×4.6 mm with 5 µm size particle at 30°C. Previously, samples were filtered with a syringe filter at 0.22 µm and 20 µL were injected in a LC-MS/MS. The mobile phase used consisted in a gradient of 10 mmol L⁻¹ ammonium formate in de-ionized water (A) and 10 mmol L⁻¹ ammonium formate in methanol (B) at a constant flow of 1.5 mL min⁻¹ (Table 1b), and running time of 60 min. Compounds were identified using the software Mass++, OpenMS (Pfeuffer *et al.*, 2017), MS-Dial (Tsugawa *et al.*, 2015) and LipidXplorer (Herzog *et al.*, 2013) and the

databases MassBank, and LipidBlast (Kind *et al.*, 2013).

Statistical analysis

For statistical comparisons between compounds identified under NH and ND we performed the following analyses. To describe in multivariate space and search for differences of the metabolomic profile between extraction capacity of the several solvents, we performed a principal coordinate analysis (PCoA) with a similarity index of Bray-Curtis, without differentiating between NH and ND. Since PCoA showed that metabolomic profiles were different between solvents, we performed a permutational ANOVA (Permanova) with a similarity index of Bray-Curtis and 9999 permutations, for each solvent to detect differences between NH and ND. To compare the relative concentration of each compound between NH and ND, an ANOVA was applied. It is worth mentioning that since endogenous acetyl-CoA signal intensity did not show statistical differences between treatments or solvents, it was used as reference parameter to determine the other compounds relative concentrations. Then we considered that the relative concentration is the signal area of the compound obtained with the MS in relation with the signal area obtained by acetyl-CoA. The overexpression and inhibition percentage of relative concentration for each compound under ND was categorized in comparison to NH treatment. After the compound identification, these were categorized into different groups depending on their biological function. For all compounds clustered under each group, significant differences in the relative concentration of compounds under ND (using NH as reference) were assessed applying an ANOVA followed by the *post-hoc* Tukey multiple comparison test. For the statistical comparison between treatments and solvents in relation to their antioxidant (or antibacterial) bioactivities, we performed a one-way ANOVA, followed by a *post-hoc* Tukey HSD tests to determine the solvent or treatment presenting the highest bioactivities. Once we defined that n-hexane (and the hydration treatments, H or NH) presented the highest (antioxidant/antibacterial) bioactivities, we performed a one-way ANOVA (fraction as factor), followed by a *post-hoc* Tukey HSD test to determine the fraction showing the highest bioactivity (for each antioxidant, or antibacterial bioactivity assay, considered independently). All differences were considered statistically significant at a $P < 0.05$. Prior to the statistical analyses, data were checked for normality and homogeneity of variances using Shapiro-Wilk and Levene's tests, respectively. We carried out all the extraction, fractionation and bioactivities experiments

considering three replicates for all the treatments. All statistical analyses were done using PAST software v.3.01 (Hammer *et al.*, 2001), R Core Team (2017).

RESULTS

General metabolome analysis

Principal coordinate analysis (PCoA) showed that metabolomic profiles of *Pyropia orbicularis* were different between the solvents utilized for the compound's separation (Fig. 1). The first coordinate, which corresponds to the used solvents, explains 27% of data variation, and the second coordinate, which corresponds to NH (naturally hydration) and ND (naturally desiccation) treatments, explains 25% of the data variation (52% in total). The PERMANOVA revealed a significant difference between NH and ND for all solvents, except n-hexane [de-ionized water ($P = 0.013$), acetonitrile ($P = 0.0362$), methanol ($P = 0.0213$), ethanol ($P = 0.024$) an n-hexane ($P = 0.0597$)] (Fig. 2). Throughout the LC-MS/MS analysis, a total of 30 signals were detected among all the extracts, 15

being observed in both treatments. 4 out of 19 and 10 out of 25 compounds were exclusively observed in NH and ND, respectively. From the total of the signals, we were able to identify 11 compounds according the

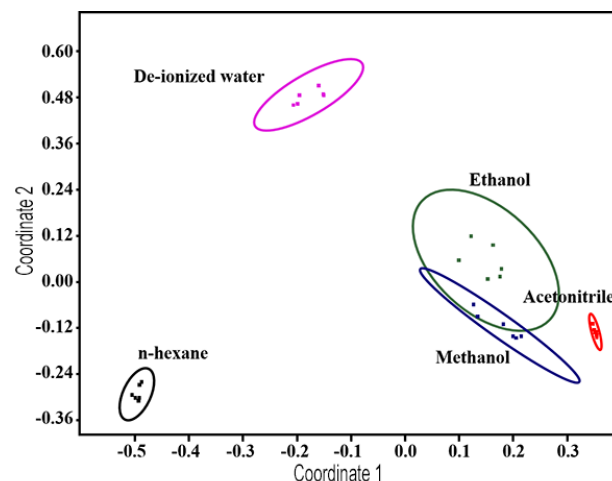


Figure 1. Principal coordinate analysis (PCoA) showed that metabolomic profiles of *Pyropia orbicularis* were different between solvents utilized.

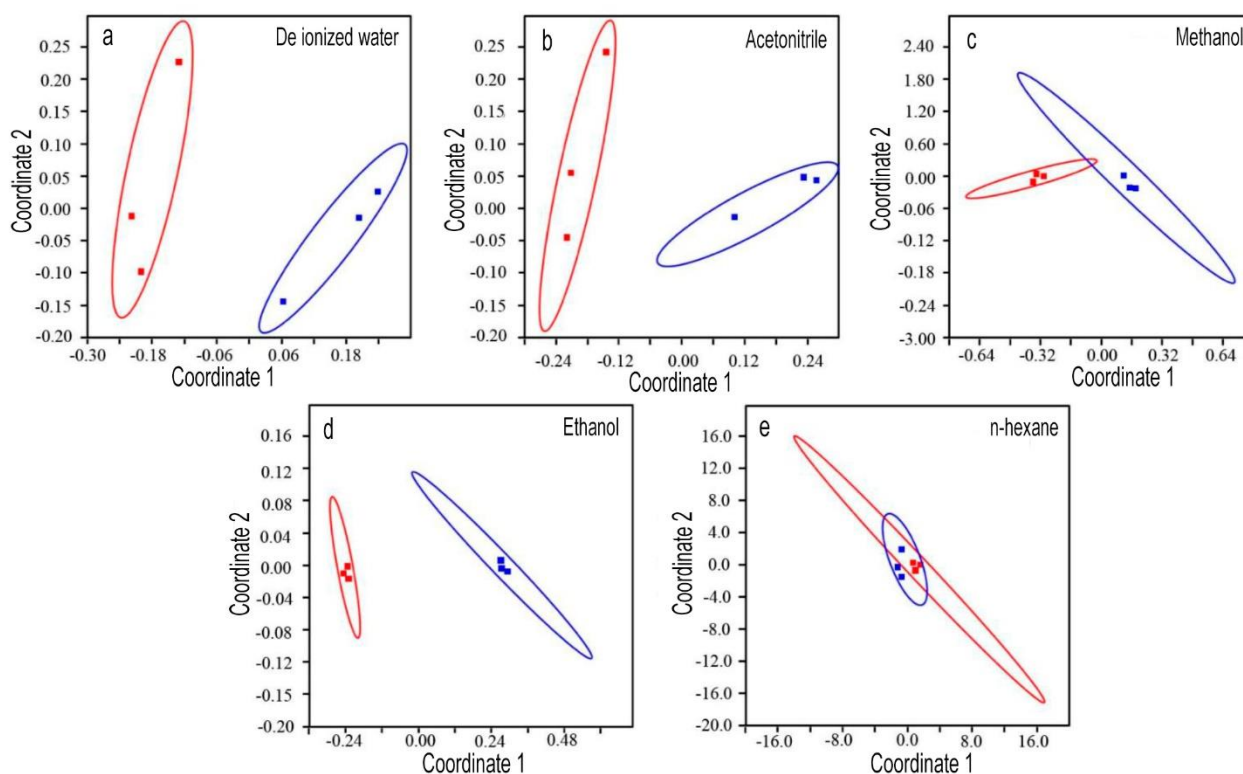


Figure 2. Principal coordinate analyses between naturally hydrated (blue) and naturally desiccated (red) for each extraction solvent: a) de-ionized water, b) acetonitrile, c) methanol, d) ethanol or e) n-hexane.

Table 2. List of compounds identified with LC-ESI/MS-MS extracted with all solvents. The overexpression (mean increase rate of relative concentration \pm standard deviation) and inhibition percentage of relative concentration for each compound under ND was categorized in comparison to NH treatment. The significant differences of the relative concentrations between ND and NH were established with $P < 0.05$ (ANOVA). *Represents the absence of significance differences. The numbers above the name of the compound represent de solvent used in the extraction: deionized water¹, methanol², acetonitrile³. References make mention of previous descriptions of the main function of each compound in other organisms. NH: natural hydration, ND: natural desiccation.

Compound	Function	Presence	Overexpression	Inhibition (%)	Reference
4-Piridoxic acid ³	Energy generation	ND	-	-	Fitzpatrick <i>et al.</i> (2012)
Acetil-CoA ³	Energy generation	NH/ND	*	*	Szutowicz <i>et al.</i> (1996)
Adenosine ¹	Energy generation	ND	-	-	Ramkumar <i>et al.</i> (1995)
Chrysoiriol ³	Cell defense factor	NH/ND	1.2 \pm 0.1	-	Kim <i>et al.</i> (2010); Rose <i>et al.</i> (2012)
Colchicine ³	Cell defense factor	ND	-	-	Korkmaz <i>et al.</i> (2011)
Dexametasone ¹	Cell defense factor	NH/ND	0.22 \pm 0.04	-	Smits & Williams (1999)
Emetine ³	Cell defense factor	NH	-	~100	Lee & Wurster (1995)
Minoxidil ¹	Membrane transport	NH	-	~100	Leonhardt <i>et al.</i> (1997)
Phenolic compound ¹	Cell defense factor	NH/ND	*	*	Pinelo <i>et al.</i> (2004)
Possible glyceride		NH/ND	0.35 \pm 0.05	-	
Quercetin ^{1,2}	Cell defense factor	NH/ND	1.3 \pm 0.09	-	Wang & Zheng (2001)
Vincamine ¹	Cell defense factor	ND	-	-	Hasa <i>et al.</i> (2013); Woods <i>et al.</i> (2013)

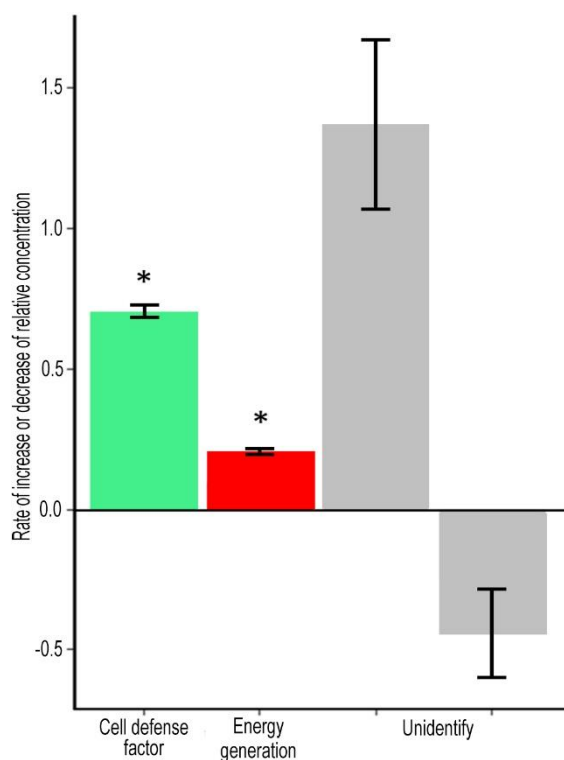


Figure 3. Rate of overexpression (or inhibition) in the concentration (mean \pm SD) of compounds belonging to each biological function group (cell defense factor, energy generation or unidentified) under naturally desiccated (ND) in relation to naturally hydrated (NH). Asterisks (*) correspond to statistically significant differences ($P < 0.05$) between ND and NH within biological function group (CDF or EG), unidentified compounds not being included in the statistical analysis. Error bars represent (plus/minus) one standard deviation from the mean.

databases using MS spectrum data: 4-piridoxic acid, acetil-CoA, chrysoiriol, colchicine, dexametasone, emetine, minoxidil, phenolic compound, a glyceride, quercetin and vincamine. In some cases, a decrease or increase in the relative concentration of these compounds was observed (Table 2). Several signals could not be interpreted since they lacked good resolution in terms of the second ionization from mass spectrum and some of those unidentified compounds could be new since no correspondences were found in the databases.

The identified compounds were separated into three groups depending on their biological function: cell defense factor (CDF), energy generation (EG) and membrane transport (MT) (Table 2). The group with the highest number of compounds identified was the cell defense factor, 62.5% in NH and 70% in ND. Then comes the group of energy generation, with 25% in NH and 30% in ND. The MT group was only observed in the NH treatment and was only composed by minoxidil. The average relative concentration of the CDF group was 0.7 times higher in ND than in NH, and the EG group was 0.2 times higher in ND than in NH. It should be noted that for the unidentified compounds groups, most compounds increased (1.3 times) under ND (Fig. 3).

Bioactive analysis

The antioxidant activity of crude extracts from *Pyropia orbicularis* was maintained among all the conditions studied. An increased and statistically significant activity was found in n-hexane extracts in comparison to extracts using other solvents (Fig. 4), but no differen-

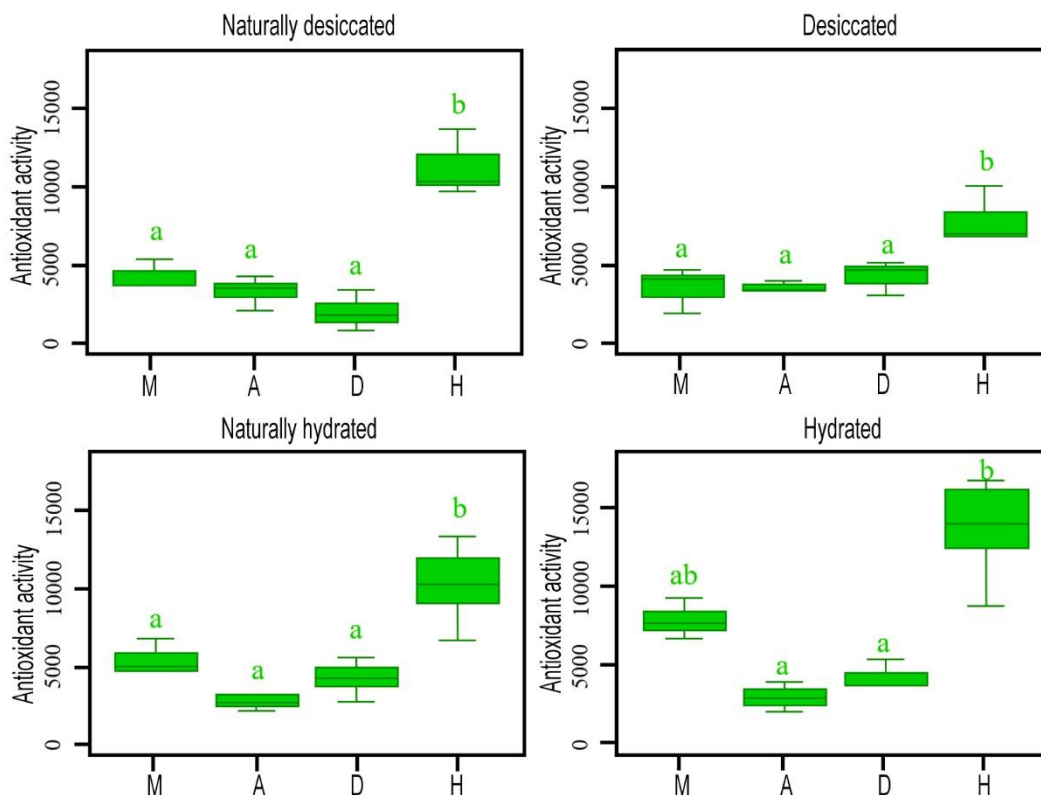


Figure 4. Boxplots of antioxidant activity (median, and lower and upper whiskers) of crude extracts of *Pyropia orbicularis* with different solvents [M: methanol, A: acetone, D: dichloromethane and H: n-hexane] under the conditions of natural desiccation, in vitro desiccation, natural hydration and in vitro hydration. The antioxidant activity was measured by the direct assay TLC-DPPH with ascorbic acid used as a positive control. For each treatment considered independently, levels not connected by the same letter (a or b) correspond to solvents showing significantly different bioactivities (antioxidant activity) based on *post-hoc* Tukey HSD analysis with $P < 0.05$.

ces was observed under hydration (H or NH) and desiccation (D or ND) treatments. This was also observed in the antibacterial assay, where the highest bacterial inhibition was obtained from n-hexane extract against *Bacillus cereus* (Fig. 5), particularly under the natural treatments (ND and NH) in comparison to the *in vitro* conditions (H and D) (Fig. 5).

Since both antibacterial and antioxidant capacities showed a higher activity in n-hexane extracts, these extracts were chosen for fractioning and identification (see methodology). The highest inhibition against *Bacillus cereus* and *Escherichia coli* was observed in the fraction 4 from n-hexane extract, which was comparable to the activity of kanamycin (Fig. 6). Low antibacterial activities (inhibition halos) for all fractions were evidenced in *Staphylococcus aureus*.

Since the main antioxidant fraction from *P. orbicularis* carried a bright yellow color, it was most likely a carotenoid compound. Then, a HPLC-UV method was developed to further separate the compounds of each fraction. In fact, with a purified fraction of

carotenoids from *Haematococcus pluvialis* using as control (Sun *et al.*, 2015), we were able to determine the presence of lutein at retention time 9.42 min in the fraction 4 of *P. orbicularis* (Table 3).

To identify the compounds responsible for the antibacterial activity, each bioactive fraction (3, 4 and 5) was submitted to LC-ESI/MS-MS and analyzed using the software MS-DIAL. The signals that appeared on all the chromatograms were identified using the databases available in MassBank and LipidBlast. These analyses allowed determining the presence of monogalactosyldiacylglycerol (MGDG), lysophosphatidylcholine (LPC), phosphatidylserine (PS) and diacylglycerols (DAG). In order to quantify the abundance of different lipid classes within each fraction, and to identify the possible source of antibacterial activity, the LipidXplorer software was subsequently used. The lipid classes PC-O (phosphatidylcholineether), PE (phosphatidylethanolamine), PS (phosphatidylserine), PI (phosphatidylinositol), GICer (glucosylceramide), TAG (triacylglycerols) and SM

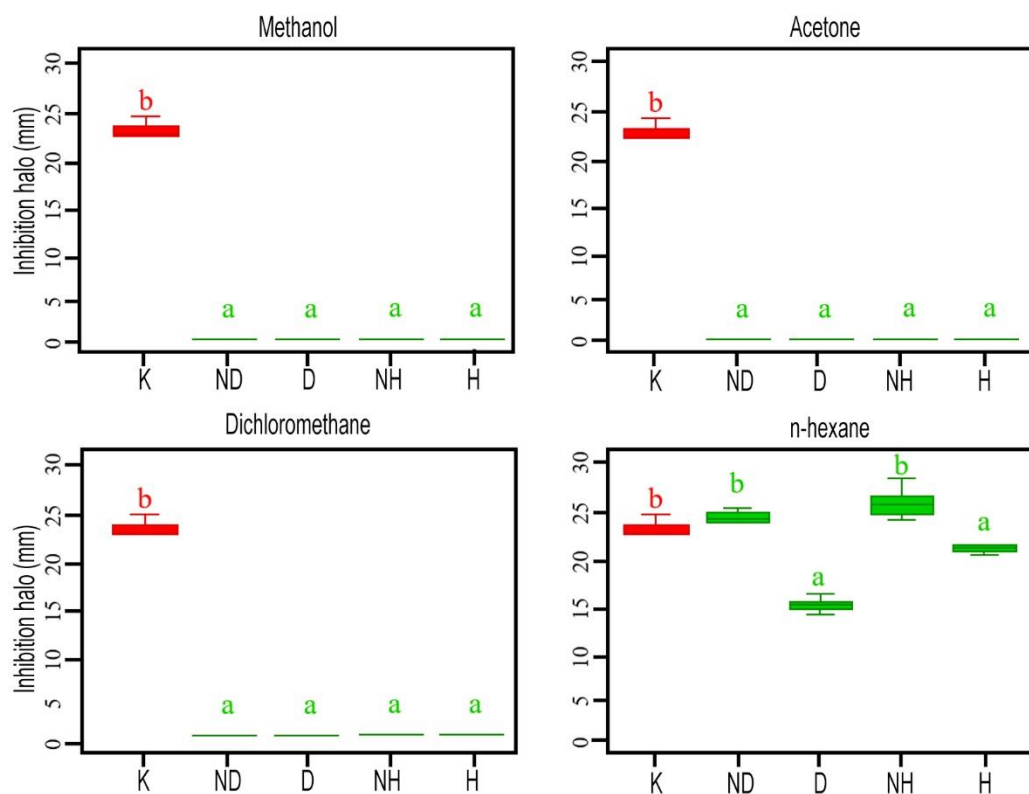


Figure 5. Boxplots of antibacterial activity (median, and lower and upper whiskers) against *Bacillus cereus* of crude extracts of *Pyropia orbicularis* with different solvents (methanol, acetone, dichloromethane and n-hexane) under the conditions of natural desiccation (ND), desiccation (D), natural hydration (NH) and hydration (H) with kanamycin (K) as a positive control. For each solvent considered independently, levels not connected by the same letter a or b correspond to treatments showing significantly different bioactivities (inhibition halos) based on *post-hoc* Tukey HSD analysis with $P < 0.05$.

(sphingomyelin) were more abundant in all the fractions that showed high antibacterial activity; while PE-O (phosphatidylethanolamineether), DAG (diacylglycerols), LPC (lysophosphatidylcholine) and LPE (lysophosphatidylethanolamine) presented higher concentrations in fractions with low bioactivity (Fig. 7).

DISCUSSION

General metabolome analysis

In accordance with previous studies of Contreras-Porcía *et al.* (2013); López-Cristoffanini *et al.* (2015) and Fierro *et al.* (2017) at the genomic and proteomics levels in *Pyropia orbicularis*, our results show a differential metabolomic response in natural desiccation (ND) in comparison to natural hydration (NH). This differential response has been extensively studied in resurrection plants, for which several compounds have been identified (Moore *et al.*, 2009; Yobi *et al.*, 2012; Gechev *et al.*, 2013; Suguiyama *et al.*, 2014). Few studies have been published on this subject in the

Table 3. Carotenoid retention time. Carotenoid extraction from different species, this data was used to prove the presence of lutein in *Pyropia orbicularis*. *Porphyra* CHF corresponds to a Chilean genetic species identified by Guillemín *et al.* (2016).

Species	Carotenoid	Retention time (min)
<i>Heamatococcus pluvialis</i>	Lutein	9.4
	Astaxanthin	6.1
	Astaxanthin monoester	29.4
	Astaxanthin monoester	30.2
<i>Pyropia orbicularis</i>	Lutein	9.4
<i>Agarophyton chilensis</i>	Lutein	9.4
<i>Porphyra</i> CHF	Lutein	9.4

Bangiales, for example Qian *et al.* (2014) showed that in *P. haitanensis*, a rapid accumulation of volatile compounds and floridosides is observed under ND. The compounds identified in our study, during NH and ND, fulfill diverse functions. The 4-pyridoxic acid is a participant in the biosynthesis of vitamin B6, having an active role as a cellular defense factor against oxidative

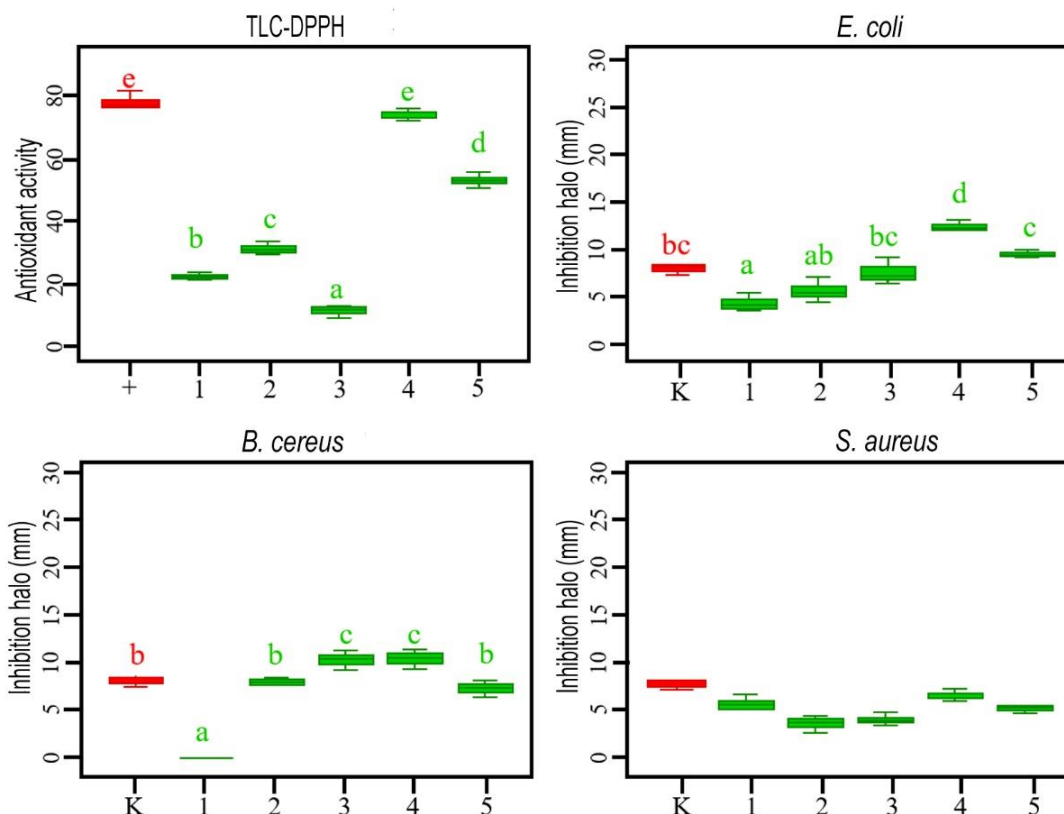


Figure 6. Boxplots of bioactivities (median, and upper and lower whiskers) of the fractions (1-5) from the hexane extract of natural hydration *Pyropia orbicularis*. Antioxidant activity was measured by TLC-DPPH and represented as DPPH inhibition (%) with ascorbic acid (+) as a positive control. Antibacterial activity against *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus* with Kanamycin (K) as a positive control. For each (antioxidant, or antibacterial) bioactivity assay, considered independently, levels not connected by the same letter correspond to fractions showing significantly different activities based on post-hoc Tukey HSD analysis with $P < 0.05$.

stress (Havaux *et al.*, 2009). It is also used as a cofactor for enzymes involved in metabolic pathways of fatty acids and glucose (Fitzpatrick *et al.*, 2012). We suggest that 4-pyridoxic acid was identified only in ND because in this condition *P. orbicularis* uses more energy than in NH in order to induce the synthesis of desiccation stress tolerance compounds and proteins as described by López-Cristoffanini *et al.* (2015). Like 4-pyridoxic acid, adenosine is involved in biochemical processes such as energy transfer and has been shown to act as an endogenous promoter of the antioxidant cellular defense system (Maggirwar *et al.*, 1994; Ramkumar *et al.*, 1995). Chrysoeriol has multiples functions such as UV cell protection in plants, antioxidant activity and induction of the transcription of nod genes in *Rhizobium meliloti*, a symbiotic bacterium of legume plants (Hartwig *et al.*, 1990; Kim *et al.*, 2010). The overexpression of this compound in ND may be related to its antioxidant activity and its UV protection capacity, since *P. orbicularis* is exposed directly to

sunlight at low tide. Colchicine inhibits cellular division with reported antioxidant and anti-inflammatory activity (Korkmaz *et al.*, 2011) and it was detected only in ND. Quercetin, which has antioxidant properties (Jullian *et al.*, 2007) but most importantly modulates growth, development and cell death in plants (Wang & Zheng, 2001), showed a greater relative concentration in ND than in NH. Then, both colchicine and quercetin could be regulating the growth of *P. orbicularis* directly or indirectly and acting as a ROS scavenger in the ND condition. The alkaloid, emetine, has antiprotozoal and antioxidant activity (Lee & Wurster, 1995). This compound was only identified in NH, so it could be acting like antiprotozoal in high tide, since in high tide *P. orbicularis* may be exposed to a higher microbial load, as previously mentioned. Emetine is also part of the release of mRNA, from stress granules, involved in the synthesis of proteins playing a pivotal role in environmental stress tolerance in human cell lines (HeLa) (Kedersha *et al.*, 2000, 2005).

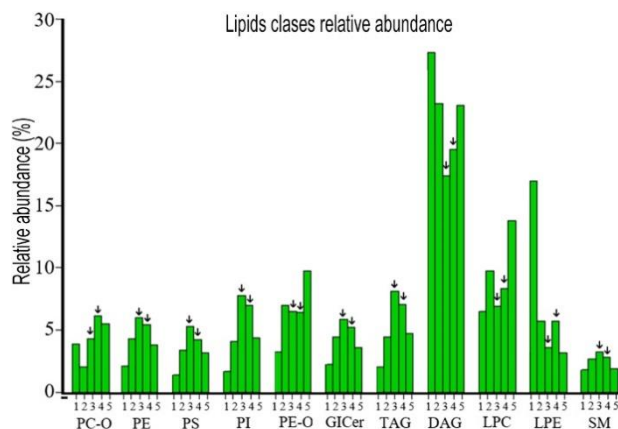


Figure 7. Lipid classes relative abundance identified in the fractions (1-5) from the hexane extract of natural hydration *Pyropia orbicularis*. Lipid classes Phosphatidylcholineether (PC-O), Phosphatidylethanolamine (PE), Phosphatidylserine (PS), Phosphatidylinositol (PI), Phosphatidylethanolamineether (PE-O), Glucosylceramide (GICer), Triacylglycerols (TAG), Diacylglycerols (DAG), Lysophosphatidylcholine (LPC), Lysophosphatidylethanolamine (LPE) and Sphingomyelin (SM) were identified by LipidXplorer. Arrows indicate fractions 3 and 4 which showed the highest antibacterial activities.

Nonetheless, the presence of stress granules in seaweeds has not been proven, which is why we propose more studies in this area. Vincamine is an alkaloid with antioxidant properties (Woods *et al.*, 2013) which may be part of the ROSs scavenger mechanisms in the ND condition. Dexamethasone has anti-inflammatory functions and is an immunosuppressor in birds (Smits & Williams, 1999), but there are no reported activity in plants or algae related with oxidative stress tolerance. The other compounds in the list are not included in this description because they do not have a relevant function related to oxidative stress tolerance or an exact identification of them is not still available. For those unidentified compounds, more exhaustive and specific extractions, and other characterization analyses such as gas chromatography-MS (GC-MS) and nuclear magnetic resonance (NMR) are needed to identify them. Nevertheless, unidentified compounds showing higher average concentration under ND than NH could play an important role in environmental stress tolerance. This statement is based on previous studies showing that, in both plants and algae, most compounds increasing their concentration under stress conditions are related to tolerance mechanisms (Whittaker *et al.*, 2001; Suguiyama *et al.*, 2014; Chen *et al.*, 2016). Then, we found compounds in NH and ND probably corresponding to constitutive responses; whereas, other compounds were exclusively presents in ND may indicate an induced response.

On the other hand, some of these compounds could be classified as antioxidant compounds while others may be classified as bioactive compounds, *i.e.*, eliciting pharmacological or toxicological effects.

Bioactive analysis

The (antioxidant and antibacterial) bioactivities observed in this study showed no major differences under desiccation in comparison to hydration in *P. orbicularis*, suggesting that there is no induction of antibacterial or antioxidant compounds through the daily tidal cycle. This alga would then show a constitutive response. Since antibacterial activity was highest under the natural desiccation than the *in vitro* treatments, other abiotic conditions should be considered, such as temperature or light intensity to fully understand the trigger of this activity. For example, the synthesis of antibacterial compound zinc oxide by the angiosperm *Hibiscus subdariffa* (Malvales order) has been shown to be dependent on temperature (Bala *et al.*, 2015)

In relation to the main antioxidant capacities, within Rhodophyta, Bangiophyceae can produce both lutein and zeaxanthin (Takaichi *et al.*, 2016) and there are reports of their presence in *Porphyra* and *Pyropia* spp. (Schubert *et al.*, 2006; Oh *et al.*, 2013). In agreement with that, in our study, we identified lutein in *P. orbicularis*. However, there were no significant differences in the antioxidant activities observed from the n-hexane extracts under the ND and NH treatments suggesting that the lutein content would not be affected by this factor. This is in accordance with previous studies (Wu, 2016) where, for example, there was no differences in the modulation of the carotenoid levels in *Pyropia haitanensis* cultivated under different light intensities, despite observing differences in the concentration of other photosynthetic pigments such as chlorophyll-*a*, phycoerythrin and phycocyanin.

PE and PS lipids extracted from seaweeds have been previously linked to antibacterial activity against gram positive and negative bacteria (Abd & Baky, 2014). It has been documented that under natural desiccation *Laurencia snackeyi* would not be degraded by microorganisms, most likely due to MGDG antibacterial activity, unlike during *in vitro* desiccation (Vairappan, 2003). Our results showed that desiccation alone does not seem to induce exclusively the antibacterial activity found in *Pyropia orbicularis*, suggesting that it should remain throughout the tidal cycle. The antibacterial activity found in this study was not attributed to a single compound, and it is most likely due to lipids and its derivatives, or at least to nonpolar compounds. Indeed, it has been previously reported that different seaweed present a highest antibacterial

activity from the nonpolar fraction in comparison to the polar one, suggesting that lipid classes might be involved (Kamenarska *et al.*, 2009; Abd & Baky, 2014). In fact, lipids may decay on derivative compounds such as the biologically active oxylipins (Mosblech *et al.*, 2009) and there are reports of prostaglandins obtained from *Gracilaria* spp. which may cause food-poisoning (Fusetani & Hashimoto, 1984; Imbs *et al.*, 2012) and serve as chemical defense against epiphytes (Weinberger *et al.*, 2011).

CONCLUSIONS

Our study demonstrates that stress tolerance during the tidal cycle in *Pyropia orbicularis*, at the metabolomic level, is not only modulated by antioxidant compounds, but rather by a variety of other compounds. Among these other compounds, we found some showing pharmacological effects (beneficial effects in human health) and other eliciting toxicological (antibacterial) effects. Our results suggest that the lipid fraction fulfills an important antioxidant and antibacterial role. It would be necessary to further characterize the lipid fraction involved in the oxidative stress defense mechanisms. This result helps to understand the environmental stress tolerance in seaweed in the intertidal zone and to propose *P. orbicularis* as a potential source of bioactive compounds.

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