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Article

Dichotomaria marginata (Rhodophyta) as a bioindicator for marine pollution: An overview about its metabolites and adsorbed pollutants

Dichotomaria marginata (Rhodophyta) como bioindicador para la contaminación marina: Una visión general sobre sus metabolitos y contaminantes adsorbidos

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Resumen.- Las macroalgas se consideran bioindicadores de la contaminación marina, ya que tienen la capacidad de reaccionar rápidamente a los cambios en su entorno. En consecuencia, las poblaciones de macroalgas fluctúan, según las características de las especies y las estrategias de adaptación. Sus polisacáridos de la pared celular contienen grupos sulfato que son capaces de retener y acumular metales pesados. Además de los contaminantes tradicionales, los contaminantes emergentes están siendo reconocidos en ambientes acuáticos. En este trabajo, los contaminantes emergentes se han identificado después de ser desorbidos de la macroalga, *Dichotomaria marginata*, recolectada en la playa de Fortaleza, Ubatuba - SP, Brasil. Basado en el hecho de que las redes de polisacáridos de algas tienen el potencial de formar enlaces de hidrógeno con compuestos polares, se planteó la hipótesis de que estos contaminantes estarían unidos a polímeros de azúcar. Los compuestos presentes en las muestras de *D. marginata* se identificaron mediante cromatografía de gases y líquidos-espectrometría de masas (GC-MS y LC-MS), asistidos por métodos computacionales. Fue posible identificar inequívocamente 22 contaminantes emergentes con GC-MS y 16 sustancias con LC-MS.

Palabras clave: Contaminantes marinos emergentes, polisacáridos de algas, carbonato de calcio, desorbancia, macroalgas, bioindicadores

Abstract.- Macroalgae are considered bioindicators for marine pollution, because they have the ability to quickly react to changes in their environment. In consequence, macroalgae populations fluctuate, according to species characteristics and adaptive strategies. Their cell wall polysaccharides contain sulfate groups that are capable of retaining and accumulating heavy metals. In addition to traditional contaminants, emerging pollutants are being recognized in aquatic environments. Herein, emerging pollutants have been identified after being desorbed from the macroalga *Dichotomaria marginata*, collected from Fortaleza Beach, Ubatuba - SP, Brazil. Based on that algal polysaccharide networks have the potential of forming hydrogen bonds with polar compounds, it was hypothesized that these pollutants would be bound to sugar polymers. Compounds present in the *D. marginata* samples were identified using both gas and liquid chromatography/mass spectrometry (GC/MS and HPLC/MS), assisted by computational methods. It was possible to unequivocally identify 22 emerging contaminants with GC/MS, and 16 substances with HPLC/MS.

Key words: Emerging marine contaminants, algal polysaccharides, calcium carbonate, desorbance, macroalgae, bioindicators

Introduction

Over a century ago it was first proposed that there is a connection between the presence, absence or abundance of algal species and the characteristics of the marine environment (Kolkwitz & Marsson 1908). In fact, many species of algae have adapted to eutrophic conditions by undergoing morphological, physiological and/or ecological changes, which promote the growth of the population (Omar 2010), and the disappearance of others coincides with habitat eutrophication (Sousa & Cocentino 2004, Holt & Miller 2010, Omar 2010).

Previous studies have demonstrated that algae have the ability to incorporate heavy metals and nutrients (Omar 2010, Rajfur 2014, Rajfur & Kłos 2014). This characteristic is attributed to the high adsorption power of the thallus, whose cell walls are made up of polysaccharides, proteins, and lipids.

The polysaccharide composition of the cell walls and the of the intercellular matrix can differ in different groups of red algae, but related to storage carbohydrates, there are basically two types of them (floridosides and digeneaside).

The floridoside [2-O-a-D-galactopyranosylglycerol, (Fig. 1a)] is the prevalent one. Besides that, there are also two diastereoisomeric isofloridosides, {[1-O-a-D-galactopyranosyl-D-glycerol (Fig. 1b)] and [1-O-a-D-galactopyranosyl-L-glycerol (Fig. 1c)]}. The digeneaside (2-O-a-D-mannopyranosyl-D-glyceric acid) is represented by the formula depicted in Figure 1d. *D. marginata* also biosynthesize specific water-soluble structural polysaccharides: sulfated xylomannans and neutral xylans (Usov 2011).

These polysaccharides contain a large amount of hydroxyl groups, which endows these carbohydrates with a hydrophilic character and the ability to form hydrogen bonds with environmental molecules. Hydrogen bonds occur when the hydrogen atom bound covalently to an electronegative atom (hydrogen donor group) electrostatically interacts with electronegative atoms (hydrogen receptor) forming non-covalent bonds. In the hydrogen donor group, usually the electronegative atom is either oxygen, nitrogen or sulfur. Hydrogen receptors are typically O, N, S and also phenols (Schaeffer 2008).

The lipids contain carboxylate, sulfate and phosphate anionic groups, which function as metal-binding sites, as well as positively charged groups. Additionally, environmental pH and the presence of competing ions can impact the adsorption process (Gadd 2009, Chopra & Pathak 2010). Algae also adsorb pollutants through the use of phytochelatins, which are peptides that are capable of chelating heavy metals (Inouhe 2005).

According to Dokulil (2003), the pollutant adsorption process occurs in two steps. In the first step, the pollutant accumulates, by passive adsorption, on the outer surface of the seaweed thallus. In the second step, these compounds/substances are then slowly adsorbed, by metabolic processes. Interestingly, previous studies have demonstrated that these macroalgae can remove alkyl benzene sulfonates, phthalates, and textile dyes from aqueous solutions (Fernandez *et al.* 1995, El-Maghraby 2013), thus emphasizing the adsorptive capabilities of these organisms.

In the marine environment, all organisms are in constant contact with dissolved or suspended substances. For example, in addition to common salts, bases, and organic or inorganic acids, marine organisms are also in contact with halogens, alkali metals, oxygen, nitrogen, silicon, trace elements, organic compounds (produced by aquatic organisms themselves) and pollutants (Bryan 1979, Millero *et al.* 2008).

Among these pollutants are emerging contaminants, which are a group of substances used extensively in everyday life. These contaminants are not currently included in routine water monitoring programs, norms or legislation for environmental control (Calvo-Flores *et al.* 2018). This is primarily because the existing toxicity data are insufficient for establishing the respective reference doses. As a consequence, these unregulated pollutants pose a serious challenge to water quality regulators (Barceló 2003, Farré *et al.* 2008, Sousa *et al.* 2018). Furthermore, these agents have been detected in numerous aquatic organisms (Mearns *et al.* 2015).

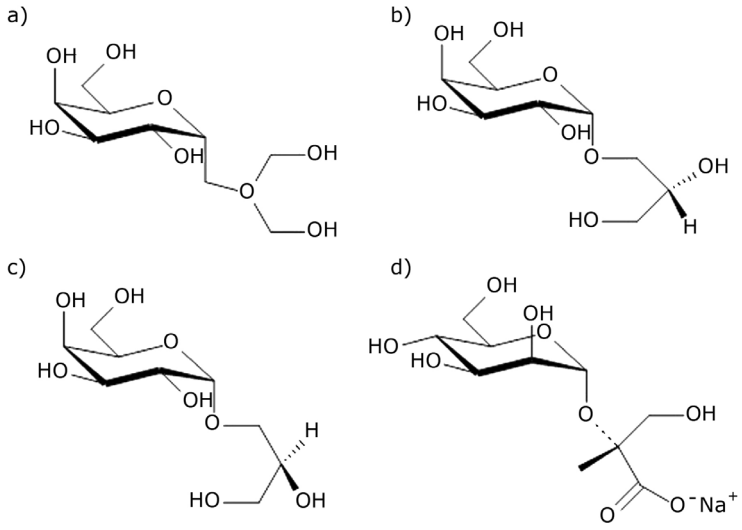

Figure 1. Natural storage carbohydrates biosynthesized by red algae. a-c) Floridoside and d) digeneaside (figure modified from Usov *et al.* 1981) / Carbohidratos de almacenamiento natural biosintetizados por algas rojas. a-c) Floridosido y d) digeneasida (figura modificada de Usov *et al*. 1981)

With regards to macroalgae, compounds such as: surfactants, phthalates and polychlorinated biphenyls have been isolated from their extracts (Mackintosh *et al.* 2004, Gressler *et al.* 2012, Osman *et al.* 2013, Shobier *et al.* 2016, Stranska-Zachariasova *et al.* 2017). These three groups of compounds have antifungal activity (Uyanik *et al*. 2009, Memić *et al*. 2011, Lotfy *et al*. 2018, Paluch *et al.* 2018, Fait *et al*. 2019). In the late 1970s, phthalates were identified as algal components (Noguchi *et al.* 1979), and the bioconcentration, as well as the trophic magnification in the aquatic food web have been evaluated (Mackintosh *et al.* 2004).

In studies with algae, gas chromatography/mass spectrometry (GC/MS) has been utilized for the detection of phthalates, adipates and surfactants, due to the inherent thermal stability, volatility and of low polarity of these compounds (Mackintosh *et al*. 2004, Gressler *et al.* 2012, Osman *et al.* 2013, Shobier *et al.* 2016, Stranska-Zachariasova *et al*. 2017). On the other hand, high performance liquid chromatography coupled with mass spectrometry (HPLC/MS) has been successfully employed in research on non-volatile, thermally unstable and more polar contaminants (Goulitquer *et al.* 2012).

Both analytical platforms (GC/MS and HPLC/MS) become more valuable when assisted by computer tools such as the AMDIS (Automated Mass Spectrometry Deconvolution and Identification System) algorithm, which can separate co-eluting components (deconvolution), provide accurate molecular weights and generate both qualitative and quantitative data for each substance (Du & Zeisel 2013).

The search for biological activities in algae extracts - whether they are polar or nonpolar - is the starting point for the discovery and isolation of substances with interesting biological actions. Regarding antifungal activity, it was detected in the hexane extracts of *Dictyota dichotoma*, *Dictyota dichotoma* var. *implexa* and *Dilophus spiralis* (Moreau *et al.* 1988) and of *Laurencia* species (Stein *et al*. 2011) and *Hydroclathrus clathratus* (Vimala & Poonghuzhali 2017) and in the dichloromethane extracts of *Ceramium rubrum* (Cortés *et al.* 2014).

Thus, the present study was designed to identify metabolites from the algae and potential emerging pollutants, desorbed from the thallus of the benthic marine macroalgae *Dichotomaria marginata* (J. Ellis & Solander) Lamark, 1816, which may have antifungal activity, using a combination of GC/MS and HPLC/MS, followed by spectral deconvolution. The results will demonstrate the utility of *D. marginata* as a bioindicator for emerging contaminants and the interference of some of these contaminants in biological activity assays of crude extracts.

Materials and methods

Sample collection and extraction

Dichotomaria marginata, previously named as *Galaxaura marginata*, is a calcareous alga that belongs to the Rhodophyta (Johnson *et al.* 2014). Samples of this species were manually collected from Fortaleza Beach, in Ubatuba, São Paulo, Brazil (23º50'15"S / 40º17'40"W), during low tides. A voucher specimen (SP 428.540) was deposited at the Maria Eneyda P. Kauffman Fidalgo Herbarium, Instituto de Botânica, São Paulo.

Following collection, samples were washed, frozen and transported inside thermal box with ice to the laboratory. At the laboratory, they were shade dried at room temperature (20-25 °C), and ground into fine particles (Carvalho *et al.* 2003). The powdered biomass (419.63 g) was extracted with hexane (5× 3.45 L) until exhaustion; the pooled extracts were filtered through filter paper (Whatman® No. 5), the solvent was removed under reduced pressure and the final product was weighed (Bhagavathy *et al.* 2011).

Hexane extract fractionation

A portion (130 g) of the extract was subjected to column chromatography (designated as C-I) on silica gel (60, 0.2-0.5 mm, Vetec), and eluted with 400 mL of each of the following solutions: dichloromethane (DCM)/methanol (MeOH) 99.5:0.5 v/v, DCM/MeOH 97.3:2.7 v/v, DCM/MeOH 95:5 v/v and MeOH 100%. Each eluted 10 mL fraction was dried under an air stream, and subjected to Planar Chromatography (PC), using vanillin-sulfuric acid as visualization reagent. Similar fractions were pooled together.

Re-fractionation of pooled fractions from C-I

Combined fractions from C-I (Fractions 4-9) (53.4 mg) were refractionated on a silica gel column (designated as C-II), using DCM/MeOH 99.5:0.5 v/v as the mobile phase, which yielded 55, 1 mL, fractions. After PC analysis, similar fractions were combined. The combined fractions from both columns (C-I and C-II) were then analyzed by GC/MS and HPLC/MS.

Gas chromatography and mass spectrometry

GC/MS analyses of all samples were performed using a mass spectrometer connected to a Shimadzu GCMS-QP2010 plus gas chromatograph (Kyoto, Japan), equipped with a HP-5MS column (5%-phenylmethylpolysiloxane, 30 m × 0.25 mm id, 0.25 µm film thickness). Helium gas was used as the carrier, at a flow rate of 1.0 mL/min. The injector temperature was 250 ºC and the oven temperature was initially set at 60 ºC and increased at 3 ºC per min, until reaching 260 ºC, at which time this temperature was held for 40 min.

The mass spectrometer was operated in full scan mode from 40 to 1000 m/z, at an interface temperature of 240 ºC, and the samples were ionized with an electron beam of 70 eV.

Identification of chemical constituents was performed by comparing the mass spectra with data from the NIST/EPA/NIH Mass Spectral Library (Version 2.0).

The linear retention indices were calculated according to the Kovats method (KI), using a mixture of C6-C28 n-alkanes as external references (Sigma-Aldrich, St. Louis, MO, USA).

Only compounds with retention times and fragmentation patterns that matched reference compounds or spectral data available in literature were considered reliable, and thus included in the text.

HPLC-ESI-EM

These analyses were carried out using a Bruker HPLC instrument (MicroTOFII) coupled to a diode-array detector and a mass spectrometer with electrospray ionization (ESI-MS). The mass spectrometer was operated in the positive ion mode and provided mass spectra data from m/z 50 to 1000.

Twenty microliters of each sample (5 mg mL^{-1}) were injected onto a Kinetex C18 column (150 mm × 3 mm, 2.6 µm; Phenomenex®), at room temperature. For elution, a mixture of methanol/ammonium acetate (1 mM) (70:30) (Eluent A) and acetonitrile/ ammonium acetate (10 mM) in methanol (98:2) (Eluent B) were used, at a flow rate of 0.7 mL min^{-1}. The gradient was as follows: 0% B (0 to 1 min); 0 to 60% B (1-25 min); 60 to 100% B (25 to 27 min); 100% B (27 to 30 min) 100 to 0% B (30-31 min); 0% B (31-32 min). Substances were identified with the aid of the computational deconvolution process.

Thin-layer chromatography/bioautography for detection of antifungal activity

The assay was conducted using the microorganism *Cladosporium cladosporioides* Fresen (SPC 140), which have been maintained at the Instituto de Botânica, São Paulo, Brazil. This fungus was cultured on potato dextrose agar (Difco) for 12 days until sporulation. The spore suspension was then extracted in a solution containing glucose and salt (Homans & Fuchs 1970, Rahalison *et al*. 1994) to a final concentration of 108 spores mL^{-1} and used for qualitative assessment of the antifungal activity of the crude seaweed extract and fractions.

The test was carried out by applying 10 µL of a solution containing 100 µg of crude extract or fractions on silica gel GF 254 TLC plates (Merck, Germany) in DCM/MeOH (98.5:1.5 v/v), which were developed in tanks lined with solvent-saturated Whatman No. 3M. After thorough air drying, the chromatograms were sprayed with fungal spore suspensions and incubated for 72 h at 28 °C. Antifungal compounds on the developed plates appeared as clear zones against the dark backgrounds of the TLC plates. Nystatin was used as positive controls.

Results

In Figure 2A, the inhibitory activity exerted by a *D. marginata* hexane extract against the fungi *Cladosporium cladosporioides* is shown. Likewise, the Figure 2B displays the antifungal bioautographic assay of all fractions of the hexane extract and shows that all of them were active.

The combination of similar fractions from the C-I chromatographic process resulted in 12 fractions, five of which were analyzed by GC/MS. The refractionation (process C-II) of the pooled fractions (Fractions 4-9 from chromatographic process C-I) yielded 16 new fractions, 8 of which were analyzed by GC/MS.

The characteristic algal metabolites identified by the GC/MS analyses are listed in Table 1; among them, there are the diterpene Phytol, which is a product of Chlorophyll degradation (Olofsson *et al.* 2014) and Demosterol, a steroid commonly found in red alga (Sharanagat *et al.* 2019).

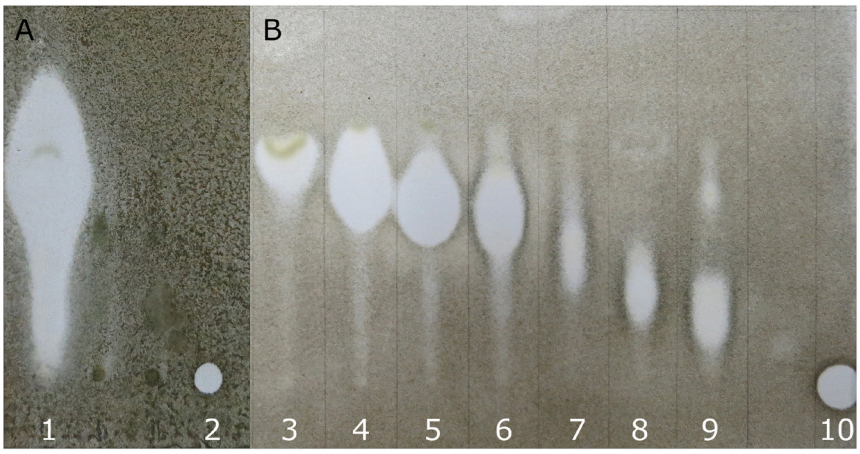

Figure 2. Antifungal TLC (thin layer chromatography) bioautographies of the *D. marginata* against *Cladosporium cladosporioides.* A) Hexane extract (1) and Positive control (nystatin)(2). B) Combined *fractions* from (C-I): 3, (4-9); 4, (10-15); 5, (16-27); 6, (28-33); 7, (34-36); 8, (37-38); 9, (39-40) and 10. Positive control (Nystatin). Mobile phases (A and B) dichloromethane/methanol 98.5:1.5 v/v / Detección por bioautografías de compuestos antifúngicos frente al hongo *Cladosporium cladosporioides* encontrado en *D. marginata*. A**)** Extracto hexánico (1) y Control positivo (nistatina) (2). B**)** Fracciones combinadas de (C-I): 3, (4-9); 4, (10-15); 5, (16-27); 6, (28-33); 7, (34-36); 8, (37-38); 9, (39-40) y 10. Control positivo (nistatina). Fases móviles (A y B) diclorometano/metanol 98,5:1,5 v/v

Table 1. Substances synthesized by algae and identified in *D. marginata* by gas chromatography/mass spectrometry (GC/MS) / Sustancias sintetizadas por algas e identificadas en *D. marginata* por cromatografía de gases/espectrometría de masas (GC/MS)

Nº	Material analyzed	Rt (min)	SI	KI	Substance	Other names	Molecular formula	Molecular mass (g mol^{-1})	Reference
1	A; B; C	48.850	97	2114.6	Phytol	3,7,11,15-tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296.539	Souza & Nes 1969
2	A	54.200	96	2300.5	Triacontane	N-triacontane	$C_{30}H_{62}$	422.826	Moustafa *et al*. 2008
3	A; C; D; E	78.117	91	3134.8	Demosterol	Cholesta-5,24-dien-3-ol	$C_{27}H_{44}O$	384.648	Burn *et al*. 1957
4	C; D	74.775	94	3057.5	Cholesta-5, 22-dien-3-β-ol	22-dehydrocholesterol	$C_{27}H_{44}O$	384.648	Burn *et al*. 1957
5	A; C; D	76.317	87	3097.5	Epicholesterol	Cholest-5-en-3-ol	$C_{27}H_{46}O$	386.664	Govindan *et al*. 1993, Plouguerné *et al*. 2006

Rt: Retention time, SI: Similarity index; KI: Kovats index
A: total hexane extract; B: C-I (Fr 10-15); C: C-I (Fr 16-27); D: C-I (Fr 28-33); E: C-I (Fr 34-37)

Substances that can be used to toiletries and personal care products, which were also herein identified, are in Table 2. They include myristic, oleic and palmitoleic acids, compounds produced by algae but that are also synthesized on an industrial scale to supply cosmetic companies (Gressler *et al*. 2010, Zielinska & Nowak 2014).

The emerging pollutants detected by GC/MS are shown in Table 3. In total, 23 compounds were identified, with industrial contaminants being the most prevalent, followed by personal care products (Octinoxate). Pharmaceuticals (Pregnenolone methyl ether), household cleaning products (Cyclohexasiloxane), food additives (Ciclotene), endocrine disruptors (Nonylphenol) and plasticizers (Diisobutyl phthalate) were also identified.

Table 2. Substances synthesized by algae and produced for various industrial uses, identified in *D. marginata* by gas chromatography/mass spectrometry (GC/MS) / Sustancias sintetizadas por algas y producidas para varios usos industriales, identificadas en *D. marginata* por cromatografía de gases/espectrometría de masas (GC/MS)

Nº	Material analyzed	Rt (min)	SI	KI	Substance	Other names	Molecular formula	Molecular mass (g mol^{-1})	Substance information	Reference/ Patent
1	A	15.183	85	1200.1	Dodecane	Dihexyl	$C_{12}H_{26}$	170.340	I; II	EFSA 2008, EP0002349 B1
2	A	23.650	92	1400.9	Tetradecane	N-tetradecane	$C_{14}H_{30}$	198.394	II; VIII	Fujimura *et al*. 1990, Kalhor *et al*. 2017, EP0002349 B1
3	A; B; C; G; H	28.817	93	1529.6	Dihydroactinidiolide	5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone	$C_{11}H_{16}O_2$	180.247	V	Yao *et al*. 1998, Xian *et al*. 2006, Borik 2014, US20130014771 A1
4	A; C; G; H	31.583	97	1601.0	N-hexadecane	Cetane	$C_{16}H_{34}$	226.448	I	Islam *et al*. 2013
5	A; G	35.033	97	1694.4	Heptadecene	1-heptadecene	$C_{17}H_{34}$	238.459	I; V; VIII	Rezanka *et al*. 1982, Yamamoto *et al*. 2014, US20150376544 A1
6	A; G; H	35.433	97	1705.5	Heptadecane	N-heptadecane	$C_{17}H_{36}$	240.475	V	McInnes *et al*. 1979, Sugisawa *et al*. 1990, Oliveira *et al*. 2012, US8933334 B2
7	C	35.808	94	1716.1	Tetradecene	1-tetradecene	$C_{14}H_{28}$	196.378	I; V	Yamamoto *et al*. 2014
8	A	37.575	94	1766.2	Myristic acid	Tetradecanoic acid	$C_{14}H_{28}O_2$	228.376	I; II; V	Widianingsih *et al*. 2012, Zielińska A & I Nowak 2014, El-Maghraby & Fakhry 2015
9	A; G; H	38.783	96	1800.5	Octadecane	N-octadecane	$C_{18}H_{38}$	254.502	I	Borik 2014, EP0002349 B1, US8961622 B2
10	A	39.675	93	1827.0	Pentadecanoic acid	Pentadecylic acid	$C_{15}H_{30}O_2$	242.403	I	Plaza *et al*. 2010, EP0003032 B1, EP0022871 B1
11	A; B; C; G; H	40.317	94	1846.1	Phytone	6,10,14-trimethyl-2-pentadecanone	$C_{18}H_{36}O$	268.485	I	Eltz *et al*. 2010, Adegoke *et al*. 2015, Hatem *et al*. 2016
12	A; H	42.142	95	1900.5	Nonadecane	N-nonadecane	$C_{19}H_{40}$	268.529	V	Abou-El-Wafa *et al*. 2011, US8859471 B2
13	G; H	42.983	92	1926.7	Methyl palmitate	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.457	I; III; IV; V; VI	Knaggs *et al*. 1965, Parris *et al*. 1973, Lee *et al*. 2010, El-Demerdash 2011, US20160089464 B1, US20160089317 B1
14	A; F; G; H	43.808	95	1952.3	Palmitoleic acid	9-hexadecenoic acid	$C_{16}H_{30}O_2$	254.414	V	Pereira *et al*. 2012
15	A; F; G; H	44.300	92	1967.7	Palmitic acid	N-hexadecanoic acid	$C_{16}H_{32}O_2$	256.430	V	Kamenarska *et al*. 2006, Pereira *et al*. 2012, Sahu *et al*. 2013, US20150191607 A1, US4537782 A
16	G; H	45.175	95	1994.9	Ethyl palmitate	Ethyl hexadecanoate	$C_{18}H_{36}O_2$	284.484	V	Cortés *et al*. 2014, US20160089311 A1, US20160089317 A1
17	A; G; H	45.367	96	2000.9	Eicosane	Icosane	$C_{20}H_{42}$	282.556	I	Karabay-Yavasoglu *et al*. 2007, US8859471 B2
18	G; H	48.158	81	2091.5	Propyl palmitate	Propyl hexadecanoate	$C_{19}H_{38}O_2$	298.511	V	Lui *et al*. 2008, US20160051455 A1
19	A; G	48.425	94	2100.2	Heneicosane	Methyl-eicosane	$C_{21}H_{44}$	296.583	I	Abou-El-Wafa *et al*. 2011, US8598098 B2
20	A; G; H	50.021	89	2154.4	Neophyitadiene	2-(4,8,12-trimethyltrydecyl) buta -1,3-diene	$C_{20}H_{38}$	278.524	V	Plaza *et al*. 2010, Oliveira *et al*. 2012, Peres *et al*. 2012, US2012211015 A1
21	G; H	50.417	86	2167.9	Ethyl oleate	Oleic acid ethyl ester	$C_{20}H_{38}O_2$	310.522	II; III; V; VI	Bookstaff *et al*. 2003, Phaechamud & Savedkairop 2012, Castillo *et al*. 2012, Abdel-Aal *et al*. 2015
22	A; G; H	51.383	95	2200.8	Docosane	N-docosane	$C_{22}H_{46}$	310.610	II	Karabay-Yavasoglu *et al*. 2007, US8980346 B2
23	A; F; G; H	59.967	89	2518.1	Oleic acid	Cis-9-octadecenoic acid	$C_{18}H_{34}O_2$	282.468	I; V; VI	Khotimchenko *et al*. 2002, Huang *et al*. 2010, Sahu *et al*. 2013

Rt: Retention time, SI: Similarity index; KI: Kovats index; A: total hexane extract; B: C-I (Fr 10-15); C: C-I (Fr 16-27); F: C-I (Fr 38); G: C-II (Fr 26-29); H: C-II (Fr30-33).
I: industrial inputs; II: food additives, III: drugs; IV: household products; V: products for personal use; VI: plasticizers; VIII: flame retardants

Table 3. Emergent pollutants identified in *D. marginata* by gas chromatography/mass spectrometry (GC/MS) / Contaminantes emergentes identificados en *D. marginata* por cromatografía de gases/espectrometría de masas (GC/MS)

Nº	Material analyzed	Rt (min)	SI	KI	Substance	Other name	Molecular formula	Molecular mass (g mol^{-1})	Substance information	Reference/ Patent
1	C; D; E; H	5.267	97	913.8	Tetrachloroethane	1,1,2,2-tetrachloroethane	$C_2H_2Cl_4$	167.838	I	ATSDR 2008
2	A	10.933	73	1095.3	2-hydroxy-3-methyl-2-cyclopenten-1-one	Ciclotene	$C_6H_8O_2$	112.128	II	EP0080600 B1, EP0413368 B1
3	A	16.175	76	1223.5	Cyclohexylpiperidine	1-piperidinocyclohexane	$C_{11}H_{21}N$	167.296	III	EP0392317 B1, EP0406111 B1
4	A	19.442	95	1300.5	Tridecane	N-tridecane	$C_{13}H_{28}$	184.367	I	EP0002002 B1
5	A; C; D; E; F; H	20.825	90	1333.5	Dodecamethylcyclohexasiloxane	Cyclohexasiloxane	$C_{12}H_{36}O_6Si_6$	444.924	IV; V	Horii & Kannan 2008, EP0285364 B1, EP0246007
6	A; H	24.792	93	1429.1	α-ionone	Iraldeine	$C_{13}H_{20}O$	192.302	V	Adams *et al*. 1996, Lalko *et al*. 2007, Brechbill 2009, 2012; CA2813334 A1
7	A; B	27.150	88	1487.2	β-ionone	4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one	$C_{13}H_{20}O$	192.302	II; I	Lalko *et al*. 2007, EP0012246 B1
8	A; G; H	27.708	95	1501.0	Pentadecane	N-pentadecane	$C_{15}H_{32}$	212.421	I	El-Boujaddaini *et al*. 2010, EP0000819 B1
9	C	33.840	94	1662.1	1-octadecanol	Stearyl alcohol	$C_{18}H_{38}O$	270.501	I; V; VI	Bingham & Cohrssen 2012, Shore & Shelley 2015, US20160089321 A1, US20160089323 A1
10	F	35.108	89	1696.4	Nonylphenol	N-nonylphenol	$C_{15}H_{24}O$	220.356	IV; VII	Duan *et al*. 2016, He *et al*. 2016, Sieppi *et al*. 2016, EP0004108 B1
11	G; H	37.139	92	1753.9	Octinoxate	2-ethylhexyl 4-methoxycinnamate	$C_{18}H_{26}O_3$	290.403	V	Schlumpf *et al*. 2004, Axelstad *et al*. 2011, US20160089317 A1
12	A; B; C; D; G; H	41.092	97	1869.2	Diisobutyl phthalate	1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester	$C_{16}H_{22}O_4$	278.348	VI	Poon *et al*. 1997, Koch *et al*. 2006, Gao & Wen 2016
13	C	41.433	96	1879.4	N-hexadecanol-1-ol	Cetilic alcohol	$C_{16}H_{34}O$	242.447	II; III; V	US8865144 A1
14	G; H	41.617	86	1884.8	Homomenthyl salicylate	3,3,5-trimethylcyclohexyl salicylate	$C_{16}H_{22}O_3$	262.349	V	Rietschel & Lewis 1978, Sambandan & Ratner 2011, Jiménez-Díaz *et al*. 2013
15	A	44.150	91	1963.0	Methyl glycol phthalate	1,2-benzenedicarboxylic acid, bis (2-methoxyethyl) ester	$C_{14}H_{18}O_6$	282.292	I; VII	Poon *et al*. 1997, Koch *et al*. 2006, Gao & Wen 2016
16	A	51.033	93	2188.8	Butyl hexadecanoate	Hexadecanoic acid, butyl ester	$C_{20}H_{40}O_2$	312.538	II; V	Khan *et al*. 2016
17	B; G; H	56.883	95	2399.3	Diethylhexyl adipate	Hexanedioic acid, bis(2-ethylhexyl) ester	$C_{22}H_{42}O_4$	370.574	I; V; VI	Jobling *et al*. 1995, Fasano *et al*. 2012, US20160089321 A1
18	G; H; I	60.717	95	2548.0	Bis(2-ethylhexyl)phthalate	1,2-benzenedicarboxylic acid, 1,2-bis(2-ethylhexyl) ester	$C_{24}H_{38}O_4$	390.564	I; VII	Poon *et al*. 1997, Koch *et al*. 2006, Gao & Wen 2016, US20160075671 A1
19	A; C; D; F; G	60.775	97	2550.3	Monoetylhexyl phthalate	1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester	$C_{16}H_{22}O_4$	278.344	I; VI	Koch *et al*. 2006, Poon *et al*. 1997, Gao & Wen 2016
20	A; D	64.458	95	2699.9	Tetracontane	N-tetracontane	$C_{40}H_{82}$	563.096	I; III	Mortimer & Luke 1966, Vazquez & Mansoori 2000, US8013091 B2
21	C; D	64.650	75	2708.1	Pregnenolone methyl ether	Pregnenolone 3-methyl ether	$C_{22}H_{34}O_2$	330.512	III	Marx *et al*. 2011
22	C; D; E; G	66.267	91	2777.0	Erucylamide	13-docosenamide	$C_{22}H_{43}NO$	337.592	I	US20160083586 A1, US20130177703 A1

Rt: Retention time, SI: Similarity index; KI: Kovats index; A: total hexane extract; B: C-I (Fr 10-15); C: C-I (Fr 16-27); D: C-I (Fr 28-33); E: C-I (Fr 34-37); F: C-I (Fr 38); G: C-II (Fr 26-29), H: C-II (Fr 30-33) e I: C-II (Fr 34-38).
I: industrial inputs; II: food additives, III: drugs; IV: household products; V: products for personal use; VI: plasticizers; VII: endocrine disruptors

The sixteen pollutants which were identified by HPLC/MS technique compounds are gathered in Table 4. Seven of these compounds are used for personal care, four as food additives, and two are pharmaceuticals. In fact, the drugs dipyrone and ethinylestradiol were only detected by HPLC/MS.

Figure 3 shows the structural formulas of some of these pollutants (Kim *et al*. 2019).

Table 4. Substances identified in *D. marginata* by liquid chromatography/mass spectrometry (HPLC/MS) / Sustancias identificadas en *D. marginata* por cromatografía de líquidos/espectrometria de masas (HPLC/MS)

Nº	Substance	Molecular formula	Molecular mass (g mol^{-1})	$[M+H]^+$		Substance information
				Theoretical m/z	Observed m/z	
1	3-methylcyclopenta-2-enone	C_6H_8O	96.0575	97.0647	97.0644	I; II; V
2	α-ionone	$C_{13}H_{20}O$	192.302	193.1587	193.1562	V
3	Dihydroactinidiolide	$C_{11}H_{16}O_2$	180.247	181.1223	181.1228	V
4	Diisobutyl Phthalate	$C_{18}H_{36}O$	278.348	279.1590	279.1590	VI
5	n-butyl-9-octadecenamide	$C_{22}H_{43}NO$	337.592	338.3417	338.3416	I
6	Palmitoleic acid	$C_{16}H_{30}O_2$	254.414	255.2240	255.2276	V
7	Homomenthyl salicylate	$C_{16}H_{22}O_3$	262.349	263.1641	263.1645	V
8	Oxybenzone	$C_{14}H_{12}O_3$	228.247	229.0859	229.0844	V
9	2-propenoic acid 3- (4-methoxyphenyl) -2-ethylhexyl ester (octinoxate)	$C_{18}H_{26}O_3$	290.403	291.1954	291.1954	V
10	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.564	391.2842	391.2853	VI
11	β-ionone	$C_{13}H_{20}O$	192.302	193.1586	193.1555	I; II
12	7,9-di-tert-butyl-1-oxaspiro (4,5) deca-6-9-diene-1,8-dione	$C_{17}H_{24}O_3$	276.376	277.1798	277.1787	I
13	Tributyl acetyl citrate	$C_{20}H_{34}O_8$	402.484	403.2326	403.2344	VI
15	Dipyrone	$C_{13}H_{16}N_3NaO_4S$	333.076	334.0831	334.0884	III
16	Ethinylestradiol	$C_{20}H_{24}O_2$	296.403	297.1849	297.1832	III

I: industrial inputs; II: food additives, III: drugs; V: products for personal use; VI: plasticizers

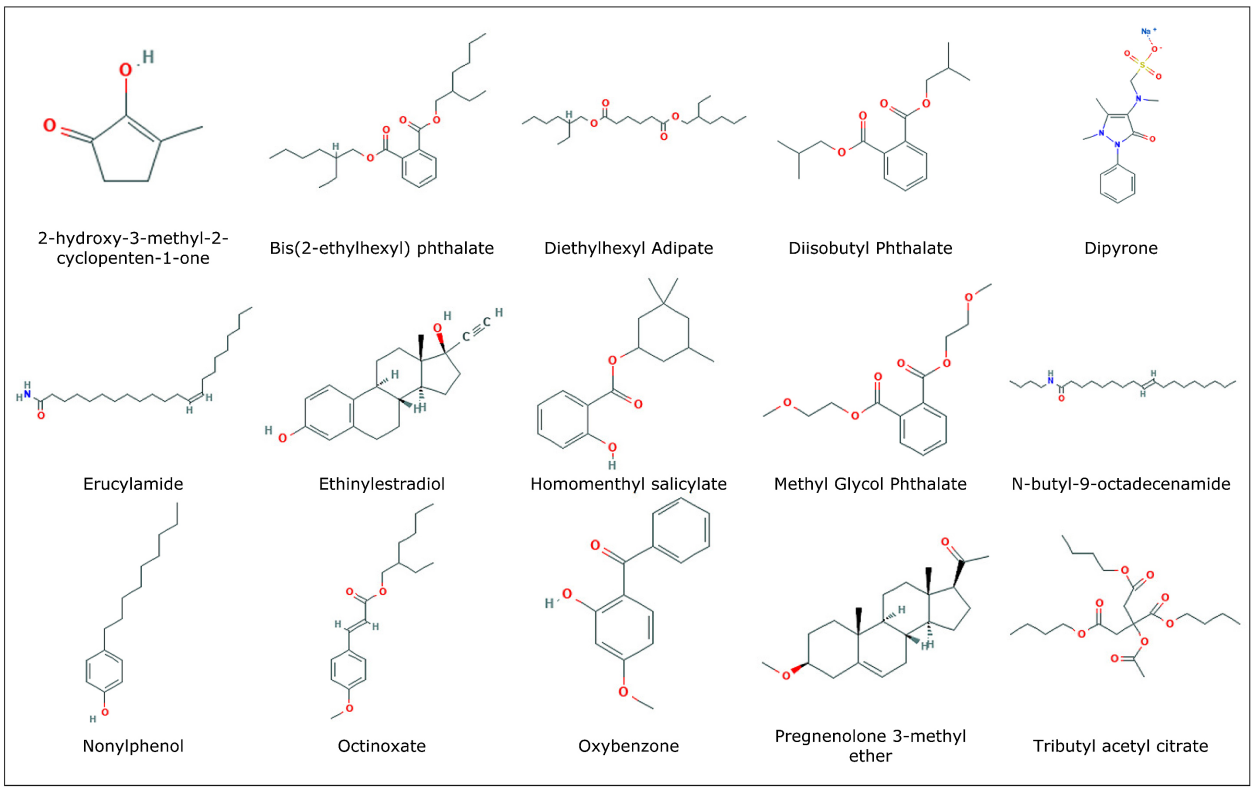

Figure 3. Chemical structure of some emergent pollutants identified in *D. marginata* (figure modified from Kim *et al*. 2019) / Estructura química de algunos contaminantes emergentes identificados en *D. marginata* (figura modificada de Kim *et al*. 2019)

Discussion

Hexane extracts of *D. marginata* presented, in addition to its typical components, compounds with different origins and potential applications, indicating that the species is able to retain them in its thallus.

The main components of the hexane extracts are usually compounds of low molecular weight and low polarity, such as some hydrocarbons, fatty acids, terpenes and aromatics compounds. Antibiotic activities of any kind which were assigned to these extracts (essential oils) oftentimes are due to terpenes and aromatic compounds (Nazzaro *et al*. 2017).

Among the primary metabolites herein identified (Table 1) only one, eicosane, has antifungal activity (Ahsan *et al.* 2017).

Special mention should be made of fatty acids C_{12}, C_{14} and C_{16} (Table 2), since they are valuable components of the human diet, precursors of the eicosanoid biosynthesis and bioregulators of several cellular processes (Gressler *et al*. 2010). Additionally, these compounds are considered to be a useful tool in chemotaxonomy, since they help to distinguish classes, families and genera of macroalgae (Khotimchenko 2005).

Among the compounds listed in Table 3, which are all pollutants, phthalates deserve special attention since they are very often isolated from macroalgae and sometimes mistakenly regarded as the natural components of these organisms (Chan *et al.* 2004, Gressler *et al.* 2012, Avio *et al.* 2016). Phathalates are not only antifungal compounds but also they may have toxic effects on humans and animals (Rowdhwal & Chen 2018). Still in Table 3, various other substances which have adverse effects on living beings are listed (references cited in Table 3).

Apart from four variants of phthalates, the pollutants nonyphenol (Karley *et al*. 1997), and cyclohexasiloxane (Moustafa *et al*. 2013) have antifungal activity as well. The fungitoxic action of these compounds could justify much of the observed fungal inhibition, since only one algal metabolite showed this activity.

The adsorption of these pollutants relied on the ability of the structural polysaccharides of the macroalgae to sequester and accumulate substances, with which these compounds can electrostatically bond.

On the other hand, the molecule*s* of these substances must have oxygen atoms (hydrogen receptors) which are the ones that make up carbonyl (from acids, esters and ketones) and phenolic hydroxyl groups as well, nitrogen atoms which make up amines and amides and sulfur atoms, for sulfate groups (Paoloni *et al*. 1975, Hay *et al*. 2004, Schaeffer 2008, Ouellette & Rawn 2015).

The molecules of the sixteen pollutants herein identified have groups which can act as hydrogen receptors, by bonding with the numerous hydroxyl groups of sugar polymers, forming hydrogen bonds. The pollutants octinoxate, diisobutyl phthalate, homomenthyl salicylate, methyl glycol phthalate, pregnenolone 3-methyl ether, erucylamide, n-butyl-9-octadecenamide, oxybenzone, tributyl acetyl citrate, dipyrone, and 2-hydroxy-3-methyl-2-cyclopenten-1-one have a caboxyl group. Ethinylestradiol, oxybenzone, nonylphenol, and homomenthyl salicylate are phenolic substances; erucylamide and n-butyl-9-octadecenamide are amides and dipyrone is a sulfated substance (Fig. 3).

While the GC/MS method reliably identified emerging pollutants desorbed from the thallus of *D. marginata*, the HPLC/MS procedure, assisted by spectral deconvolution, proved to be a valuable tool for identifying non-metallic, polar, non-volatile and thermally unstable environmental contaminants. These types of compounds are often present in minute quantities in samples with a profuse mixture of compounds (Magi & Di Carro 2016, Martín *et al*. 2017).

In conclusion, the significant number of pollutants detected by both chromatographic methods (GC and HPLC) indicate that macroalgae have the ability to retain a variety of substances dissolved or suspended in the aquatic environment. Due to the fact that some of the identified compounds were from pharmaceuticals residues and industrial debris, the present study has demonstrated that the red alga *Dichotomaria marginata* could be a reliable bioindicator for water pollution. Early detection and subsequent remediation will minimize the harmful effects associated with these pollutants, and benefit the human population, in general.

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