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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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 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).
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).

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-glycidic acid) is represented by the formula depicted in Figure 1d. D. marginata also biosynthesize specific water-soluble structural polysaccharides: sulfated xylomannans and neutral xylans (Usow 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).

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, this 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 elathratus (Vimala & Poonghuzhalai 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 (23°50’15”S / 40°17’40”W), during low tides. A voucher specimen (SP 428.540) was deposited at the Maria Eneyda P. Kauflman 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 °C and the oven temperature was initially set at 60 °C and increased at 3 °C per min, until reaching 260 °C, 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 °C, 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).
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)

<table>
<thead>
<tr>
<th>Nº</th>
<th>Material analyzed</th>
<th>Rt (min)</th>
<th>S</th>
<th></th>
<th>KI</th>
<th>Substance</th>
<th>Other names</th>
<th>Molecular formula</th>
<th>Molecular mass (g mol⁻¹)</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>A; B; C</td>
<td>48.850</td>
<td>97</td>
<td></td>
<td></td>
<td>Phytol</td>
<td>3,7,11,15-tetramethyl-2-hexadecen-1-ol</td>
<td>C₂₉H₄₄O</td>
<td>296.539</td>
<td>Souza &amp; Nes 1969</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td>54.200</td>
<td>96</td>
<td></td>
<td></td>
<td>Triacantane</td>
<td>N-triacantane</td>
<td>C₂₀H₃₂</td>
<td>422.826</td>
<td>Moustafa et al. 2008</td>
</tr>
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<td>3</td>
<td>A; C; D; E</td>
<td>78.117</td>
<td>91</td>
<td></td>
<td></td>
<td>Demosterol</td>
<td>Cholesta-5,24-dien-3-ol</td>
<td>C₂₇H₄₄O</td>
<td>384.648</td>
<td>Burns et al. 2015</td>
</tr>
<tr>
<td>4</td>
<td>C; D</td>
<td>74.775</td>
<td>94</td>
<td></td>
<td></td>
<td>Cholesta-5,22-dien-3-β-ol</td>
<td>22-dehydrocholesterol</td>
<td>C₂₇H₄₄O</td>
<td>384.648</td>
<td>Burns et al. 2015</td>
</tr>
<tr>
<td>5</td>
<td>A; C; D</td>
<td>76.317</td>
<td>87</td>
<td></td>
<td></td>
<td>Epicholesterol</td>
<td>Cholesta-5-en-3-ol</td>
<td>C₂₇H₄₄O</td>
<td>386.664</td>
<td>Govindan et al. 1993, Plouguerné et al. 2006</td>
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</table>

**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)

<table>
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<tr>
<th>Nº</th>
<th>Material analyzed</th>
<th>Rt (min)</th>
<th>Si</th>
<th>Kl</th>
<th>Substance</th>
<th>Other names</th>
<th>Molecular formula</th>
<th>Molecular mass (g mol$^{-1}$)</th>
<th>Substance information</th>
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<tr>
<td>1</td>
<td>A</td>
<td>15.183</td>
<td>85</td>
<td>1200.1</td>
<td>Dodecane</td>
<td>Diethyl</td>
<td>C$<em>{12}$H$</em>{25}$</td>
<td>170.340</td>
<td>II;</td>
<td>EFSFA 2008, EP0002349 B1</td>
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<tr>
<td>2</td>
<td>A</td>
<td>23.650</td>
<td>92</td>
<td>1400.9</td>
<td>Tetradecane</td>
<td>N-tetradecane</td>
<td>C$<em>{14}$H$</em>{30}$</td>
<td>198.394</td>
<td>II; VIII</td>
<td>Fujimura et al. 1990, Kalbtor et al. 2017, EP0002349 B1</td>
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<tr>
<td>3</td>
<td>A; B; C; G; H</td>
<td>28.817</td>
<td>93</td>
<td>1529.6</td>
<td>Dihydroactinidiolide</td>
<td>5,6,7,7a-tetrahydro-4,4,4-trimethyl-2(4H)-benzo[4]3</td>
<td>C$<em>{17}$H$</em>{30}$O$_{3}$</td>
<td>180.247</td>
<td>V</td>
<td>Yao et al. 1998, Xian et al. 2006, Bork 2014, US20130014771 A1</td>
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<td>4</td>
<td>A; C; G; H</td>
<td>31.583</td>
<td>97</td>
<td>1601.0</td>
<td>N-hexadecane</td>
<td>C$<em>{16}$H$</em>{32}$</td>
<td>226.448</td>
<td>I</td>
<td></td>
<td>Islam et al. 2013</td>
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<tr>
<td>5</td>
<td>A; G</td>
<td>35.033</td>
<td>97</td>
<td>1694.4</td>
<td>Heptadecane</td>
<td>1-heptadecane</td>
<td>C$<em>{17}$H$</em>{36}$</td>
<td>238.459</td>
<td>I; V; VIII</td>
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<td>6</td>
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<td>N-heptadecane</td>
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<td>240.475</td>
<td>V</td>
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<td>7</td>
<td>C</td>
<td>35.088</td>
<td>94</td>
<td>1716.1</td>
<td>Tetradecene</td>
<td>1-tetradecene</td>
<td>C$<em>{14}$H$</em>{28}$</td>
<td>196.378</td>
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<td>8</td>
<td>A</td>
<td>37.575</td>
<td>94</td>
<td>1766.2</td>
<td>Myristic acid</td>
<td>Tetradecanoic acid</td>
<td>C$<em>{14}$H$</em>{28}$O$_{2}$</td>
<td>228.376</td>
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<td>9</td>
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<td>38.783</td>
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<td>Pentadecyl acid</td>
<td>C$<em>{15}$H$</em>{30}$O$_{2}$</td>
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<td>11</td>
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<td>40.317</td>
<td>94</td>
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<td>Phytone</td>
<td>6,10,14-trimethyl-2-pentadecanone</td>
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<td>268.485</td>
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<td>13</td>
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<td>92</td>
<td>1926.7</td>
<td>Methyl palmitate</td>
<td>Hexadecanoic acid, methyl ester</td>
<td>C$<em>{16}$H$</em>{32}$O$_{2}$</td>
<td>270.457</td>
<td>I; III; IV; V; VI</td>
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<td>14</td>
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<td>95</td>
<td>1952.3</td>
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<td>9-hexadecenoic acid</td>
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<td>254.414</td>
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<td>C$<em>{17}$H$</em>{34}$O$_{2}$</td>
<td>284.484</td>
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<td>94</td>
<td>2100.2</td>
<td>Heneicosane</td>
<td>Methyl-eicosano</td>
<td>C$<em>{22}$H$</em>{44}$</td>
<td>296.583</td>
<td>I</td>
<td>Abbas-Ell-Wafa et al. 2011, US8859098 B2</td>
</tr>
<tr>
<td>20</td>
<td>A; G; H</td>
<td>50.021</td>
<td>89</td>
<td>2154.4</td>
<td>Neophytadiene</td>
<td>2-(4,8,12-trimethyltridecylo) buta-1,3-diene</td>
<td>C$<em>{22}$H$</em>{42}$</td>
<td>278.524</td>
<td>V</td>
<td>Plaza et al. 2010, Oliveira et al. 2012, Peres et al. 2012, US2012211015 A1</td>
</tr>
<tr>
<td>21</td>
<td>G; H</td>
<td>50.417</td>
<td>86</td>
<td>2167.9</td>
<td>Ethyl oleate</td>
<td>Oleic acid ethyl ester</td>
<td>C$<em>{18}$H$</em>{32}$O$_{2}$</td>
<td>310.522</td>
<td>II; III; V; VI</td>
<td>Bookstaff et al. 2003, Phaichumrul &amp; Svedkvaip 2012, Castillo et al. 2012, Abdel-Aal et al. 2015</td>
</tr>
<tr>
<td>22</td>
<td>A; G; H</td>
<td>51.383</td>
<td>95</td>
<td>2200.8</td>
<td>Docosane</td>
<td>N-docosane</td>
<td>C$<em>{22}$H$</em>{44}$</td>
<td>310.610</td>
<td>II</td>
<td>Karabuy-Yavasoglu et al. 2007, US8980346 B2</td>
</tr>
<tr>
<td>23</td>
<td>A; F; G; H</td>
<td>59.967</td>
<td>89</td>
<td>2518.1</td>
<td>Oleic acid</td>
<td>C$_{18}$-9-docosenoic acid</td>
<td>C$<em>{18}$H$</em>{32}$O$_{2}$</td>
<td>282.468</td>
<td>I; VI</td>
<td>Khotimchenko et al. 2002, Huang et al. 2010, Sabu et al. 2013</td>
</tr>
<tr>
<td>Nº</td>
<td>Material analyzed</td>
<td>Rt (min)</td>
<td>SI</td>
<td>KJ</td>
<td>Substance</td>
<td>Other name</td>
<td>Molecular formula</td>
<td>Molecular mass (g mol⁻¹)</td>
<td>Substance information</td>
<td>Reference/ Patent</td>
</tr>
<tr>
<td>-----</td>
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<td>---------------------------------</td>
<td>-------------------</td>
<td>------------------------</td>
<td>------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>1</td>
<td>C; D; E; H</td>
<td>5.267</td>
<td>97</td>
<td>913.8</td>
<td>Tetrachloroethane</td>
<td>1,1,2,2-tetrachloroethane</td>
<td>C₄H₈Cl₄</td>
<td>167.838</td>
<td></td>
<td>ATSDR 2008</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td>10.933</td>
<td>73</td>
<td>1095.3</td>
<td>2-hydroxy-3-methyl-2-cyclopenten-1-one</td>
<td>Cyclotene</td>
<td>C₇H₁₀O₂</td>
<td>112.128</td>
<td></td>
<td>EP0008600 B1, EP0413368 B1</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
<td>16.175</td>
<td>76</td>
<td>1223.5</td>
<td>Cyclohexyl/piperidine</td>
<td>1-piperidinocyclohexane</td>
<td>C₁₁H₁₈N</td>
<td>167.296</td>
<td></td>
<td>EP0392317 B1, EP0406111 B1</td>
</tr>
<tr>
<td>4</td>
<td>A</td>
<td>19.442</td>
<td>95</td>
<td>1300.5</td>
<td>Tridecane</td>
<td>N-tridecane</td>
<td>C₁₃H₂₆</td>
<td>184.367</td>
<td></td>
<td>EP0002002 B1</td>
</tr>
<tr>
<td>5</td>
<td>A; C; D; E; F; H</td>
<td>20.825</td>
<td>90</td>
<td>1333.5</td>
<td>Dodecane</td>
<td>1-octadecanol</td>
<td>C₁₈H₃₈O</td>
<td>270.501</td>
<td>I; VI</td>
<td>Horl &amp; Kannan 2008, EP025364 B1, EP0246007</td>
</tr>
<tr>
<td>7</td>
<td>A; B</td>
<td>27.150</td>
<td>88</td>
<td>1487.2</td>
<td>4-bromo-octanol</td>
<td>4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one</td>
<td>C₂₀H₂₄BrO₂</td>
<td>362.350</td>
<td>II; I</td>
<td>Laloo et al. 2007, EP0012246 B1</td>
</tr>
<tr>
<td>8</td>
<td>A; G; H</td>
<td>27.708</td>
<td>95</td>
<td>1501.0</td>
<td>Pentadecane</td>
<td>N-pentadecane</td>
<td>C₁₅H₂₅</td>
<td>212.421</td>
<td>I</td>
<td>El-Boujdadaini et al. 2010, EP0008819 B1</td>
</tr>
<tr>
<td>11</td>
<td>G; H</td>
<td>37.139</td>
<td>92</td>
<td>1753.9</td>
<td>Ocitoxinate</td>
<td>2-ethylhexyl 4-methylcinnamate</td>
<td>C₁₆H₂₆O₂</td>
<td>290.403</td>
<td>V</td>
<td>Schlumpf et al. 2004, Axelstad et al. 2011, US20160089317 A1</td>
</tr>
<tr>
<td>12</td>
<td>A; B; C; D; G; H</td>
<td>41.092</td>
<td>97</td>
<td>1869.2</td>
<td>Diisobutyl phthalate</td>
<td>1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester</td>
<td>C₂₀H₂₂O₂</td>
<td>278.348</td>
<td>VI</td>
<td>Poon et al. 1997, Koch et al. 2006, Gao &amp; Wen 2016</td>
</tr>
<tr>
<td>13</td>
<td>C</td>
<td>41.433</td>
<td>96</td>
<td>1879.4</td>
<td>N-hexadecanol-1-ol</td>
<td>Cetilic alcohol</td>
<td>C₁₈H₃₈O</td>
<td>242.447</td>
<td>II; III; V</td>
<td>US8865144 A1</td>
</tr>
<tr>
<td>15</td>
<td>A</td>
<td>44.150</td>
<td>91</td>
<td>1963.0</td>
<td>Methyl glycol phthalate</td>
<td>1,2-benzenedicarboxylic acid, bis(2-methoxyethyl) ester</td>
<td>C₂₀H₂₄O₂</td>
<td>282.292</td>
<td>I; VII</td>
<td>Poon et al. 1997, Koch et al. 2006, Gao &amp; Wen 2016</td>
</tr>
<tr>
<td>16</td>
<td>A</td>
<td>51.033</td>
<td>93</td>
<td>2188.8</td>
<td>Butyl hexadecanoate</td>
<td>Hexadecanoic acid, butyl ester</td>
<td>C₂₁H₴₂O₂</td>
<td>312.538</td>
<td>II; V</td>
<td>Khan et al. 2016</td>
</tr>
<tr>
<td>17</td>
<td>B; G; H</td>
<td>56.883</td>
<td>95</td>
<td>2399.3</td>
<td>Diethylhexyl adipate</td>
<td>Hexadecanoic acid, bis(2-ethoxy) ester</td>
<td>C₂₀H₃₈O₂</td>
<td>370.574</td>
<td>I; VI</td>
<td>Jobling et al. 1995, Fauman et al. 2012, US20160089321 A1</td>
</tr>
<tr>
<td>18</td>
<td>G; H; I</td>
<td>60.717</td>
<td>95</td>
<td>2548.0</td>
<td>Bis(2-ethylhexyl)phthalate</td>
<td>1,2-benzenedicarboxylic acid, 1,2-bis(2-ethoxy) ester</td>
<td>C₂₁H₴₀O₂</td>
<td>390.564</td>
<td>I; VII</td>
<td>Poon et al. 1997, Koch et al. 2006, Gao &amp; Wen 2016, US20160087367 A1</td>
</tr>
<tr>
<td>19</td>
<td>A; C; D; F; G</td>
<td>60.775</td>
<td>97</td>
<td>2550.3</td>
<td>Monoethylphthalate</td>
<td>1,2-benzenedicarboxylic acid, mono(2-ethoxy) ester</td>
<td>C₂₁H₴₀O₂</td>
<td>278.344</td>
<td>I; VI</td>
<td>Koch et al. 2006, Poon et al. 1997, Gao et al. 2016</td>
</tr>
<tr>
<td>21</td>
<td>C; D</td>
<td>64.650</td>
<td>75</td>
<td>2708.1</td>
<td>Pregnenolone methyl ether</td>
<td>Pregnenolone 3-methyl ether</td>
<td>C₂₅H₴₀O₂</td>
<td>330.512</td>
<td>III</td>
<td>Marx et al. 2011</td>
</tr>
<tr>
<td>22</td>
<td>C; D; E; G</td>
<td>66.267</td>
<td>91</td>
<td>2777.0</td>
<td>Eucalyamide</td>
<td>13-decenoyl amide</td>
<td>C₂₅H₄₀NO</td>
<td>337.592</td>
<td>III</td>
<td>US20160083586 A1, US2013017703 A1</td>
</tr>
</tbody>
</table>

Rt: Retention time; SI: Similarity index; KJ: Kovats index; A: total hexane extract; B: C-1 (Fr 10-15); C: C-1 (Fr 16-27); D: C-1 (Fr 28-33); E: C-1 (Fr 34-37); F: C-1 (Fr 38); G: C-1 (Fr 26-29); H: C-1 (Fr 30-33) e I: C-1 (Fr 34-38).
I: industrial inputs; II: food additives; III: drugs; IV: household products; V: products for personal use; VI: plasticizers; VII: endocrine disruptors.

**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)**
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.

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

<table>
<thead>
<tr>
<th>No</th>
<th>Substance (common and IUPAC name)</th>
<th>Molecular formula</th>
<th>Molecular mass (g mol⁻¹)</th>
<th>[M+H]⁺</th>
<th>Theoretical m/z</th>
<th>Observed m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3-methylcyclopenta-2-enone</td>
<td>C₉H₁₀O</td>
<td>96.0675</td>
<td>97.0647</td>
<td>97.0644</td>
<td>I; II; V</td>
</tr>
<tr>
<td>2</td>
<td>α-ionone</td>
<td>C₁₀H₁₄O₂</td>
<td>192.202</td>
<td>193.1567</td>
<td>193.1562</td>
<td>V</td>
</tr>
<tr>
<td>3</td>
<td>Dihydroxyacridinedisulfone</td>
<td>C₁₈H₁₈O₄</td>
<td>180.247</td>
<td>181.1223</td>
<td>181.1228</td>
<td>V</td>
</tr>
<tr>
<td>4</td>
<td>Diisobutyl Phthalate</td>
<td>C₁₂H₁₄O₂</td>
<td>278.348</td>
<td>279.1590</td>
<td>279.1590</td>
<td>VI</td>
</tr>
<tr>
<td>5</td>
<td>n-butyl-9-octadecanamide</td>
<td>C₂₅H₄₂NO</td>
<td>337.592</td>
<td>338.3417</td>
<td>338.3416</td>
<td>I</td>
</tr>
<tr>
<td>6</td>
<td>Palmitoleic acid</td>
<td>C₁₇H₃₃O₂</td>
<td>284.414</td>
<td>285.2240</td>
<td>285.2276</td>
<td>V</td>
</tr>
<tr>
<td>7</td>
<td>Homomethyl salicylate</td>
<td>C₁₇H₃₃O₂</td>
<td>262.349</td>
<td>263.1641</td>
<td>263.1645</td>
<td>V</td>
</tr>
<tr>
<td>8</td>
<td>Oxybenzone</td>
<td>C₁₆H₃₀O₂</td>
<td>229.247</td>
<td>230.0859</td>
<td>229.0844</td>
<td>V</td>
</tr>
<tr>
<td>9</td>
<td>2-propenoic acid 3- (4-methoxyphenyl)-2-ethylhexyl ester (octinoxate)</td>
<td>C₁₅H₂₀O₡</td>
<td>290.403</td>
<td>291.1954</td>
<td>291.1954</td>
<td>V</td>
</tr>
<tr>
<td>10</td>
<td>Bis(2-ethylhexyl) phthalate</td>
<td>C₁₅H₂₁O₄</td>
<td>390.564</td>
<td>391.2842</td>
<td>391.2853</td>
<td>VI</td>
</tr>
<tr>
<td>11</td>
<td>β-ionone</td>
<td>C₁₀H₁₄O₂</td>
<td>192.202</td>
<td>193.1568</td>
<td>193.1555</td>
<td>I; II</td>
</tr>
<tr>
<td>12</td>
<td>7,9-di-tert-butyl-1-oxaspiro (4,5) dec-a-6-9-diene-1,8-dione</td>
<td>C₁₅H₂₂O₂</td>
<td>276.376</td>
<td>277.1798</td>
<td>277.1787</td>
<td>I</td>
</tr>
<tr>
<td>13</td>
<td>Tributyl acetyl citrate</td>
<td>C₁₅H₃₃O₉</td>
<td>402.484</td>
<td>403.3226</td>
<td>403.3244</td>
<td>VI</td>
</tr>
<tr>
<td>14</td>
<td>Nonylphenol</td>
<td>C₁₃H₂₅O</td>
<td>296.403</td>
<td>297.1849</td>
<td>297.1832</td>
<td>III</td>
</tr>
<tr>
<td>15</td>
<td>Octinoxate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Ethinylestradiol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

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

![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)](image-url)
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\textsubscript{12}, C\textsubscript{14} and C\textsubscript{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). Phthalates 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 molecules 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 (Paolini 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, homomethyl salicylate, methyl glycol phthalate, pregnenolone 3-methyl ether, erucylamide, n-buty1-9-octadecenamide, oxybenzone, tributyl acetyl citrate, dipyrone, and 2-hydroxy-3-methyl-2-cyclopenten-1-one have a caboxyl group. Ethinylestradiol, oxybenzone, nonylphenol, and homomethyl salicylate are phenolic substances; erucylamide and n-buty1-9-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, Martin 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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