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IMPACT OF ENVIRONMENTAL CONDITIONS ON THE COMPOSITION AND PROPERTIES OF ALGAE

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Project .....

Study of Acidification, CO_2, and Fe in the Marine Environment
Main researcher: Juana Magdalena Santana Casiano

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Index

Abstract

The following algae species collected from Las Canteras beach (Gran Canaria) arrivals between June and August in the summer of 2021 were studied in terms of their carbohydrates and malondialdehyde contents, and antioxidant activities (measured through the radical scavenging activity and ferric reducing power assays): *Cymopolia barbata, Caulerpa prolifera, Lobophora variegata*, *Dictyota dichotoma*, *Dictyota fasciola*, *Sargassum vulgare,* and *Hypnea spinella*.

Their content in free carbohydrates (in mg g^{-1} of dry algal biomass) ranged from 0.02 in *Dictyota fasciola* to 7.64 in *Lobophora variegata* and from 3.68 in *Dictyota fasciola* to 10.09 in *Cymopolia barbata* for total carbohydrates. *Caulerpa prolifera, Sargassum vulgare* and *Cymopolia barbata* extracts were the most active in inhibiting 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) (71.84, 75.09 and 79.60% respectively) and showed low levels of malondialdehyde (MDA) (7.02, 5.62 and 4.93 mmol g^{-1} respectively). *Cymopolia barbata* also showed the highest capacity of reducing iron (0.112 mmol of iron (III) g^{-1} of algae). These results confirm the potential possibilities of the arriving algal biomass as a new sustainable source of raw material with nutritional, functional, and/or biomedical properties.

The seasonal variation in composition and antioxidant activities of samples of the two more abundant species in the arriving algae (*Cymopolia barbata* and *Lobophora variegata*) collected in January, April, August, and November were also evaluated. Phenolic compounds gallic acid, epicatechin and syringic acid were found in both algae, but *Cymopolia barbata* showed considerably higher concentrations as well as higher radical scavenging activity with a maximum in August (79.69%).

Samples of seawater enriched with exudates from coccolithophore *Emiliania huxleyi* were studied to improve the understanding of the extracellular release of organic ligands under future marine acidification scenarios. Cultures of *Emiliania huxleyi* were grown under different pH conditions (7.75, 7.9, 8.1 and 8.25). The highest amounts of exuded phenolic compounds (5.04 µg L^{-1} and 3.47 µg L^{-1}) were observed in cultures with lower cell densities (9.15×10^7 at pH 7.75 and 9.98×10^7 at pH 8.25, respectively), indicating that these cells exuded higher amounts of phenolic compounds (55.1 and 34.7 fg $cell^{-1}$respectively).

1.- Introduction

1.1. Algae washed up on Las Canteras beach

Algae is a term that encompasses a large and diverse group of photosynthetic eukaryotic organisms with a great diversity of forms and sizes, ranging from unicellular microalgae to pluricellular macroalgae that can grow up to 50 meters. In certain conditions, algae can wash ashore. Arrivals are large volumes of algae occasionally deposited on the coasts. These events are generally associated with storms or ocean currents that generate massive landslides in the benthic communities of the nearby bottoms, but they can also be a result of the daily tides force that rips the seaweed from the sea floor and drags them to the shore (Kirkman & Kendrick, 1997).

This phenomenon occurs several times a year in Las Canteras beach, which is the main urban beach in the city of Las Palmas de Gran Canaria (Gran Canaria, Canary Islands). These algae arrivals entail an economic cost to the city as budget must be allocated to the collection and disposal of seaweed. However, the biomass is not completely removed to provide nutrients for plant communities and invertebrates. Other negative impacts include decreased tourism, pollution and bad odors derived from the algae decomposition (Darriba, 2022). According to Portillo (2008), about 1.200 tons are deposited each year on the shore of Las Canteras beach, where the maximum biomass was collected in 1994 (approximately 3.127 tons). Portillo (2008) also indicated that 300 tons of brown algae were deposited in only five days in November 2011. The last reported event took place in April 2022, when 173 tons of seaweed were collected from Las Canteras and the nearby beach of Castillo de San Cristobal (Cope, 2022) and transported in trucks to its final destination: the landfill.

The most common algae species washed up on Las Canteras beach are: *Lobophora variegata* and the *Cymoplia barbata*. Other species with less representation are: *Dictyota dycotoma*, *Dictyota fasciola*, *Sargassum vulgare*, *Hypnea spinella* and *Caulerpa prolifera*.

Figure 1. *Algae arrivals in Las Canteras beach. Sources: Darriba (2021) (left) Cope (2022) (right).*

1.2. Impact of environmental conditions

It is known that environment conditions such as temperature, salinity, photoperiod, light intensity, nutrient availability, or water pH strongly affect algae composition and growth (Gani et al., 2019). Nowadays, the environment conditions are not only affected by seasonality as our planet is currently undergoing a global change. Global temperature has risen 1.01°C since 1880 with nineteen of the warmest years occurring since 2000 (NASA, 2022). This phenomenon is due to the high levels of heat-trapping gases such as carbon dioxide, methane, nitrous oxide, water vapor, and fluorinated gases released into the atmosphere (Denchak, 2022). The high CO_2 concentration released into the air directly affects oceans pH, as well as global temperature rise indirectly results in sea-surface warming. Seawater pH and temperature are two major variables that control all chemical and biological cycles. When CO_2 dissolves and reacts with water molecules to form bicarbonate and carbonate, protons are released into the environment acidifying it (Millero, 2005) as expresed in the equilibrium ecuations below.

$$CO_{2\,(g)} \leftrightarrow CO_{2\,(aq)} \quad (1)$$

$$CO_{2\,(aq)} + H_2O \leftrightarrow HCO_3^- + H^+ \quad (2)$$

$$HCO_3^- \leftrightarrow CO_3^{2-} + H^+ \quad (3)$$

$$Ca^{2+} + CO_3^{2-} \leftrightarrow CaCO_{3\,(s)} \quad (4)$$

Pre-industrial seawater pH was 8.25. At the moment, it has already dropped to 8.10. It is expected to reach a seawater pH of 7.85 within this century and a drop of up to 0.7 units more by the year 2300 (Caldeira & Wickett, 2003; Jacobson, 2005). The acidification of seawater is believed to affect deeply the oceanic ecosystems in many levels as it will alter the chemical balances of the ocean (*Understanding Ocean Acidification*, 2022). For example, it will modify the dissolved concentrations of metals such as iron and cooper, both essential for marine life (Hoffmann, 2012). Trace metal complexation in seawater is controlled by the dissolved organic matter, including phytoplankton exudates that contain metal chelators.

These changes could affect the composition and properties of algae reaching the shore of Las Canteras beach, and so the potential benefits and uses of the biomass. In fact, some species of algae live in extreme environmental conditions (salinity, temperature, nutrient limitation, Irradiation, etc.) (Malavasi 2020). Often, their response to these adverse conditions consists in producing a tremendous diversity of biologically active substances that allow adaptation to survive which creates metabolic pathways in algae that produce unique metabolites that cannot be found in any other organisms (Welker et al., 2012).

1.3. Algae as source of biological active compounds

Algae are an interesting natural source of novel compounds with biological activity for food, feed and biomedical applications and uses (Munir et al., 2013). As photosynthetic organisms, algae biosynthesize free radicals and oxidative reagents under normal conditions due to the

exposure to oxygen and light, but the production of these compounds increases under stressful situations. Therefore, in order to protect themselves from oxidation, they carry out the biosynthesis of antioxidant substances. These are compounds that can prevent, or delay cell damage caused by free radicals (NCCIH, 2013).

Oxidative stress also plays an essential role in the development of human diseases such as diabetes, neurodegenerative disorders, and cancer among others (Hayes et al., 2020). Dietary antioxidants are thought to minimize the effects of oxidative stress decreasing the levels of free radicals. Thus, algae are considered an important source of antioxidants of great relevance for the healthy food and pharmaceutical industries (Sathasivam et al., 2019).

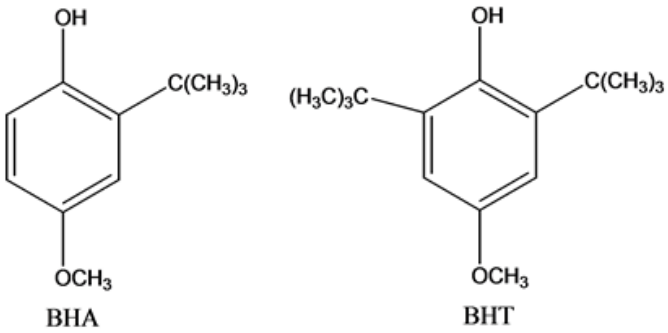

Figure 2. *Chemical structure of synthetic antioxidants BHA and BHT.*

In addition, oxidation is the major cause for the deterioration of food**.** Currently, synthetic antioxidants such as butylhydroxyanisole (BHA, E320) and butylhydroxytoluene (BHT, E321) (Figure 2) are widely used as food additives despite being considered carcinogenic and responsible for multiple damaging side-effects (Saito et al., 2003). Therefore, a large number of studies have focused on finding natural, safe, and effective antioxidants to replace BHA and BHT (Affan et al., 2007; Kumar et al., 2015; Zahid et al., 2019). The following algal metabolites could be a functional alternative to these synthetic compounds with a wide variety of health benefits (Sathasivam et al., 2019):

- **Phenolic compounds.** Their structure consists of a benzene ring substituted by at least one hydroxyl group (Ghani, 2020). When there is more than one hydroxyl group, it is considered a polyphenolic compound (several examples are displayed in Figure 3). They chelate metal ions and prevent radical formation by electron delocalization. The more interconnected rings the polyphenol possesses, the greater the ability to scavange free radicals since the electrons are more delocalized. Polyphenolic substances are commonly used as food additives for their antioxidant properties but can also have pharmacological applications in acute and chronic diseases, neurodegenerative diseases, type 2 diabetes, and cardiovascular diseases (Cory et al., 2018).
- **Polysaccharides.** The basic structure of carbohydrates consists of carbon, hydrogen and, oxygen following the general empirical structure $(CH_2O)_n$. These are organic compounds that organise in the form of aldehydes or ketones with multiple hydroxyl groups coming off a carbon chain and form the building blocks of carbohydrates known as monosaccharides (Aryal, 2022). Complex carbohydrates are formed by various monosaccharides joined by

glycosidic bonds (Holdt & Kraan 2011). The functional properties of the polysaccharide depend on its structure. They are considered a source of dietary fibre (Bauer et al., 2021) with antioxidant, antifungal, antiviral, antibacterial and antitumoral properties, among others (Kashif et al., 2018). Therefore, algae biomass with a high content of polysaccharides is used in many different industries such as cosmetics, pharmacy, nutrition, or feeds.

- **Carotenoids.** These are a group of fat-soluble pigments that are naturally found in plant material and have biological properties. Carotenoid's structure consists of hydrocarbons with 40 carbon atoms and two terminal rings. This is a large group comprising more than 700 compounds divided into two classes: (a) carotenes such as beta-carotene with a linear structure and (b) oxygenated derivatives of carotenes called xanthophylls. Carotenoids are mainly used in the food, pharmaceutical and cosmetic industries (Mezzomo & Ferreira, 2016). A carotenoid of special interest is astaxanthin, which is biosynthesized by microalgae such as *Haematococcus pluvialis* or *Chlorella zofingiensis* (Han et al., 2013). Astaxanthin is useful against oxidative stress as well as having a potential role in medicine. It has anti-inflammatory properties, so its application as a treatment for cardiovascular diseases is being studied (Fassett & Coombes, 2011).

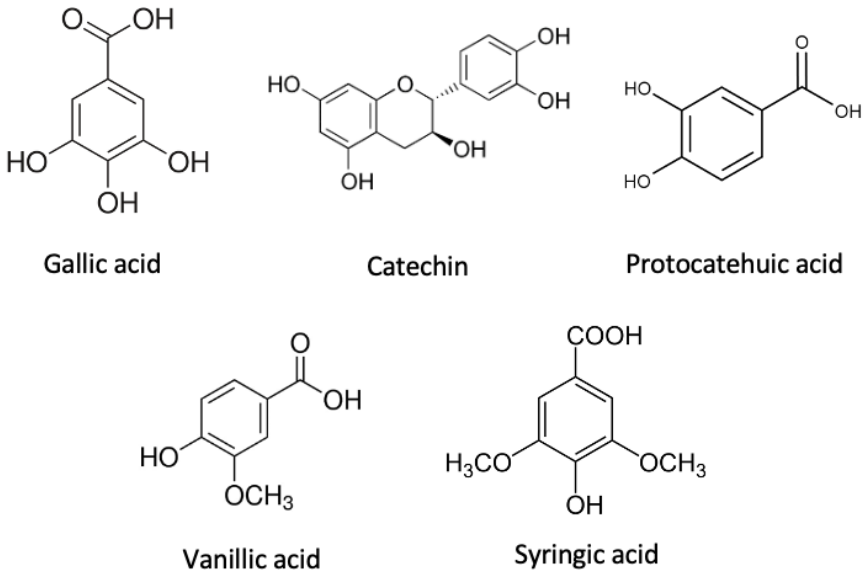

Figure 3. *Polyphenols examples*

The massive influx of algae on the coasts could become a revulsive for the local economy by developing new commercial and industrial activities. These algae could be a sustainable source of natural products with several applications in the food, feed, cosmetic and pharmaceutical industries. Portillo (2008) reported that collected algae from Las Canteras show high content of nitrogen, phosphorus, potassium, and micronutrients which make them a source of organic fertilizers. Globally, the main destination of algal biomass is the extraction of alginates and agar (Rebours et al., 2014). Seaweed collected from arrivals are also used as fertilizer or as food in mariculture (Kirkman & Kendrick 1997; Eyras et al., 2008).

1.4. Objectives

Overall, this project aims to:

- Study the composition and the antioxidant capacity of algae that massively arrive at Las Canteras beach through a series of assays to evaluate their potential use in different industrial sectors (cosmetics, health, food…). To assess the antioxidant activity the following tests were conducted: the free radical inhibition assay, an assessment of the ferric reducing power and an estimation of the lipid peroxidation in the cells. The samples composition was also studied by the determination of total and free carbohydrates.
- Have a more profound understanding of the impact of environment in the algae, the seasonal variability in composition and antioxidant activity of the two most common algae arriving at the shore (*L. variegata* and *C. barbata*) was assessed by carrying out the assays cited above in samples collected in January, April, August, and November 2021. In addition, the identification and quantification of polyphenols was also performed. To evaluate how seasonal changes affect said composition and properties allows both to understand the defense mechanisms that these algae present in response to different environmental conditions and to determine the season in which the use of the algae arrivals would be more profitable.
- Determine how marine acidification may affect the composition of exudates from the marine microalgae *Emiliania huxleyi* (*E. huxleyi*). Oceanic changes of temperature and pH will alter the bioavailability of essential trace metals Fe and Cu. Organic ligands such as the phenolic compounds play a major role in overall metal bioavailability. To evaluate how pH variation affects algae, cultures of microalgae *E. huxleyi* were grown at four different pH: 7.75, 7.9, 8.1 and 8.25 (Samperio-Ramos et al., 2017). Extracellular phenolic compounds released from microalgae were identified and quantified using a Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) equipment.

2.- Material and methods

2.1. Algal material

The following species collected from Las Canteras beach (Gran Canaria) arrivals between June and August in the summer of 2021 were studied: green algae *Cymopolia barbata (C barbata)* and *Caulerpa prolifera (C. prolifera);* brown algae *Lobophora variegate (L. variegate)*, *Dictyota dichotoma (D. dichotoma)*, *Dictyota fasciola (D. fasciola)* and *Sargassum vulgare (S. vulgare),* and red algae *Hypnea spinella (H. spinella)*. Their content in carbohydrates and polyphenols, as well as their antioxidant activities were estimated. The seasonal variation of these same parameters in samples of the two more abundant species *C. barbata* and *L. variegata* collected in January, April, August, and November was also evaluated. These algae samples were collected and pretreated (washed and freeze-dried) by the graduated Fernando Esquíroz Martel as a part

of the project “Development of actions and studies on the Bahía del Confital-Las Canteras” (Ref.: S2020/03) cofinanced by the “Ayuntamiento de Las Palmas de Gran Canaria”.

Besides, samples of seawater enriched with exudates from *E. huxleyi* microalgae cultivated under different pH conditions (7.75, 7.9, 8.1 and 8.25) were also studied to identify and quantify the polyphenols present and their antioxidant activities. These microalgae were provided by the Spanish Bank of Algae.

2.2. Extraction methods

Extracts were made following different methodologies for diverse purposes.

Aqueous solvents were used to extract polar metabolites such as carbohydrates (Ma, 2015):

- Algal biomass (50 mg) were added to 2.5 mL of distilled water. The mixture was kept for one hour (h) in a vortex mixer (Ika Genius 3) to extract free carbohydrates.
- Hydrochloric acid solution (2.5 mL, 3 M) was added to 50 mg of dried biomass, and the mixture was incubated for 1 h at 121ºC in an autoclave to quantify total carbohydrates.

Extractions were also prepared with organic solvents for evaluating polyphenols and determining the antioxidant activity through different tests:

- Methanol (1 mL) was added to 20 mg of algal biomass of each species. These extracts were used to determine the antioxidant activity by inhibiting the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), the ferric reducing power assay (FRAP) and the lipid peroxidation product malondialdehyde with the thiobarbituric acid test (TBA test).
- Methanol (3 mL) was mixed with 100 mg of algal biomass. The purpose of these extracts was to be injected into the HPLC equipment for the identification and quantification of polyphenols.

All these mixtures were sonicated for 30 min at 40-50ºC and subsequently centrifuged for 10 min in the centrifuge (ThermoFisher Scientific ALC 4232) at 3500 revolutions per min (rpm). Supernatant was collected and stored in the fridge for future tests. After sonication, the process carried out for the methanol extracts intended for HPLC analysis was different: The mixture was first shaken in the vortex mixer for 1 h and then, it was concentrated in a rotary evaporator, the residue was dissolved in methanol (300 µL) and filtered through a 0.22 µm micropore filter to be injected into the HPLC equipment for the identification and quantification of polyphenols. Extracts for DPPH testing and the FRAP assay were also filtered through a 0.22 µm micropore filter.

All extracts and assays described herein were performed by triplicate to ensure reproducibility.

2.3. Antioxidant activity

2.3.1. Free radical inhibition assay

The absorbance of a DPPH solution decreases when substances with antioxidant activity are added because this free radical is inhibited (Bondet et al., 1997). A solution (1 mL) of the free radical DPPH (0.1 mM) was mixed with 100 μL of each sample and the absorbance was recorded after 20 min at 515 nm using a UV-visible spectrophotometer (Shimadzu PHARMASPEC 1800). All algae strain extracts were tested, as well as the solutions of two commercial antioxidant products prepared at the maximum concentrations in food allowed by law (200 ppm) (Real Decreto 142/2002, 2002): butylhydroxyanisole (BHA, E320) and butylhydroxytoluene (BHT, E321).

Half-life time (time required for the initial absorbance of DPPH solution to be reduced to one half) was expressed as seconds (s). The percentage of DPPH inhibition was calculated by equation:

$$\left(1 - \frac{Sample\ absorbance\ with\ DPPH}{DPPH\ absorbance\ without\ sample}\right) \times 100$$

2.3.2. Iron reducing power assay

The presence of antioxidants in the samples would result in the reduction of complex 2,4,6-tri(2-pyridyl)-triazine (TPTZ)-Fe (III) to TPTZ-Fe (II) by donating an electron. This reducing ability could be used as an index to evaluate potential antioxidant properties (Benzie & Strain, 1996). Methanol algae extracts (50 μL) were mixed with 1.5 mL of a pre-warmed reagent in a water bath at 37ºC for 10 min. The reagent consisted of a mixture of the following three solutions in volumetric proportion of 10:1:1 respectively: (i) 0.3 M acetate buffer solution at pH 3.6 (prepared by dissolving 3.1 g of sodium acetate and 16 mL of glacial acetic acid in distilled water up to 1 L); (ii) 10 mM TPTZ in HCl (40 mM); (iii) and 2.5 mL of $FeCl_3 \cdot 6H_2O$ solution (20 mM).

Subsequently, the absorbance was measured at 593 nm. Results were expressed as mmol of reduced Fe (III) g^{-1} of freeze-dried algae calculated from a calibration curve ranging from 0.1 to 2 mM (regression line equation y = 0.6018x + 0.0287, R^2 =0.996).

2.3.3. Malondialdehyde determination assay

Malondialdehyde (MDA) is one of the final products of polyunsaturated fatty acids peroxidation in the cells. An increase in free radicals results in an overproduction of MDA, which is used as a biomarker of oxidative stress. MDA reacts with TBA to produce a pinkish-red adduct with an absorbance maximum at 532 nm (Hodges et al., 1999; Gaweł et al., 2004). Following the method established by Fan (2002), 1 mL of aqueous extract was mixed with either: (i) 1 mL of the "-TBA" aqueous solution consisting of 20% trichloroacetic acid with BHT (0.01%); (ii) 1 mL of the "+TBA" aqueous solution, identical to the one above but with 0.5% TBA added. All samples were incubated at 95ºC for 25 min, cooled to approximately 5ºC, and centrifuged at 1300 rpm for 10 min. The absorbance was measured at three wavelengths to correct the interferences of

carbohydrates and pigments (440, 532 and 600 nm). According to Hodges et al. (1999), the equivalents of MDA in nmol mL^{-1} were calculated as follows:

1) $(Abs\ 532_{+TBA}) - (Abs\ 600_{+TBA}) - (Abs\ 532_{-TBA} - Abs\ 600_{-TBA}) = A$
2) $[(Abs\ 440_{+TBA} - Abs\ 600_{+TBA})0.0571] = B$
3) $MDA\ equivalents\ (nmol \cdot ml^{-1}) = (A - B/157\ 000)10^6$

The results were expressed as milimoles (mmol) of MDA equivalents g^{-1} of freeze-dried algae.

2.4. Total and free carbohydrates quantification

The colorimetric method described by Brooks et al. (1986) was used to quantify carbohydrates in algae extracts. Anthrone reacts with carbohydrates forming a green-blue chromophore with an absorption maximum of 625 nm. Samples (1 mL) were mixed with 2 mL of anthrone reagent (100 mg in 50 mL of sulfuric acid (98%)). The mixtures were stirred in a vortex mixer for 30 s, placed in a water bath at 80ºC for 10 min and then in an ice bath for 10 min more. Finally, the absorbance was measured at 625 nm. A calibration curve was prepared with glucose standards ranging from 20 to 200 μg mL^{-1} and the concentrations of carbohydrates in the samples were calculated using the regression line equation y = 0.0103x + 0.1459 (R^2 = 0.9982). The results are expressed as mg of carbohydrates g^{-1} of dry algal biomass.

2.5. Identification and quantification of polyphenols by liquid cromatography

All the solvents used in the HPLC equipment are previously filtered through millipore paper filters with a pore size of 0.22 μm, and are subjected to treatment in the ultrasound equipment for 15 min.

2.5.1. Algae samples analysis

Chromatographic analysis was performed with a Jasco LC-4000 HPLC equipped with a quaternary pump (PU-4180), an autosampler (AS-4150), photodiode array detector (MD-4015), an LC-Net interface II and a Varian C18 column (250 mm × 4.6 mm, 5 μm). The eluents were milliQ water with 0.1% formic acid (A) and methanol (B). The gradient elution method for A was: from 0 to 5 min, 80% isocratic; from 5 to 30 min, linear gradient from 80% to 40%; column was washed with a mixture of A and B (10:90) and the column was conditioned for the next analysis. Simultaneous monitoring for quantification was set at 270 nm (gallic acid, protocatechuic acid, catechin, vanillic acid, rutin, epicatechin, and syringic acid) (Santiago-Díaz et al., 2021). Algae samples were analysed by triplicate. The results were expressed as mg per 100 g of freeze-dried algae.

2.5.2. Water samples analysis

Samples of extracellular phenolic compounds exuded by the marine coccolithophore *E. huxleyi* were analysed using the method described in section 2.5.1. Samperio-Ramos et al. (2017) cultivated *E. huxleyi* coccolithophore (initial cell density of 10^6 cells L^{-1}) under different pH conditions (7.75, 7.9, 8.1, and 8.25) reached by acidification of seawater at initial pH 8.25

bubbling a gas mixture of CO_2-free air and pure CO_2. Seawater samples (700 mL) enriched with exudates were previously subjected to solid phase extraction (SPE) at a flow rate of 2 mL min^{-1} using Macherey-nagel Chromabond Easy cartridges (500 mg). The retained analytes were eluted with 6 mL of methanol, which was subsequently evaporated on a rotary evaporator. The residue was dissolved in 300 µl of methanol and filtered through a 0.22 µm filter to be injected into the HPLC equipment. The results were expressed as µg of polyphenols per liter of seawater as well as femtograms (fg) of polyphenols exuded per cell. These samples were also used to measure their free radical scavenging capacity according to the method described in section 2.3.1, by mixing 50 µl of sample with 1 mL of DPPH solution (0.04 mM). In addition, the total amount of compounds capable of inhibiting DPPH radical per liter of seawater enriched with exudates was calculated from the percentage of DPPH scavenged divided by the number of cells (expressed as picomol (pmol) of DPPH inhibited per cell)

3.- Results

3.1. Antioxidant activity

3.1.1. Free radical inhibition assay

The capacity of the different species of algae to scavenge the DPPH radical (expressed as percentage of inhibition) leads to a comparison of the antioxidant effectiveness, as seen in Figure 4.

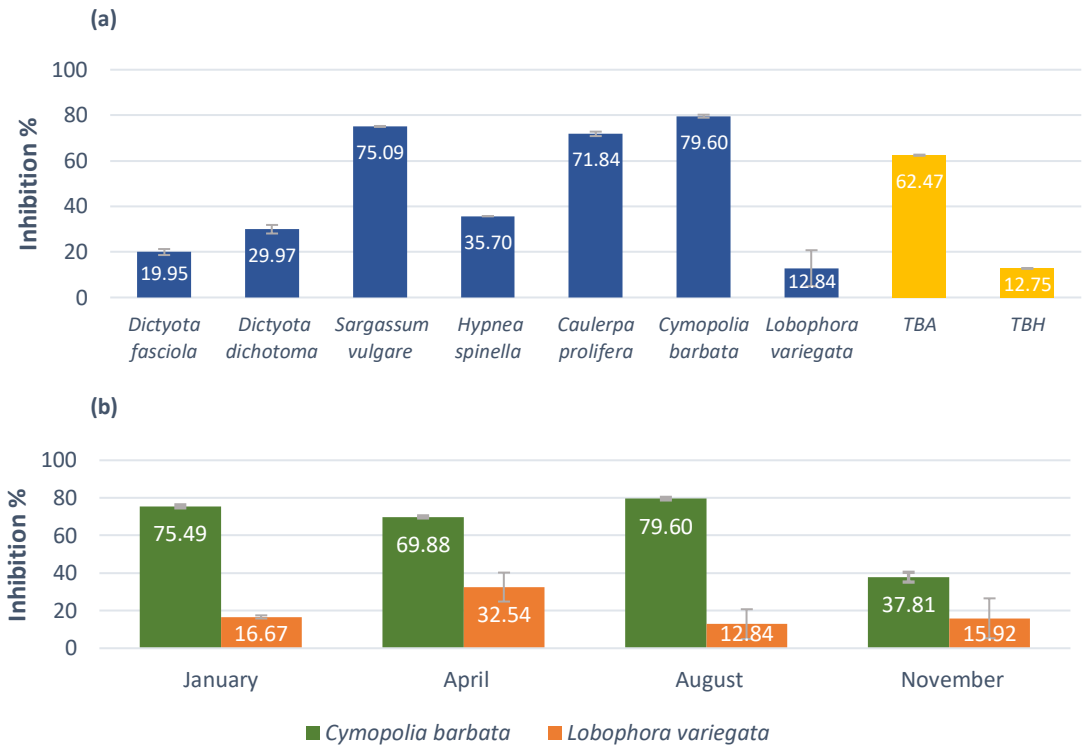

Figure 4*. (a) DPPH inhibition percentages by extracts of algae collected from Las Canteras beach during the summer months of 2021. (b) Seasonal variability of DPPH scavenging capacities of C. barbata and L. variegate (expressed as inhibition percentages).*

The antioxidant activity of the algae samples was compared with those of the commercial antioxidant products BHA (E320) and BHT (E321). All the algae strains showed higher antioxidant activity than BHT (12.75%), and the following three species exceeded BHA activity (62.47%): *C. prolifera* (71.84%), *S. vulgare* (75.09%), and *C. barbata* (79.60%) (except for the samples collected in November) (Figure 4). Among the two most abundant algae species in Las Canteras beach arrivals, *C. barbata* was more efficient in inhibiting free radical DPPH than *L. variegata*. Regarding the seasonal variability, *C. barbata* (Figure 4b) collected in November showed considerably less antioxidant activity (37.81%) than those from January, April, and August (75.49%, 69.88% and 79.69% respectively), being the latter the ones with the highest activity. *L. variegata* exhibited a different pattern as the highest antioxidant activity was found in samples collected in April (32.54%) and the lowest in August (12.84%).

The half-life time expressed in seconds in Table 1 allows the comparison of the inhibition speed of the different species analysed. *C prolifera*, *S. vulgare*, and *C. barbata* species were at least twice as fast in inhibiting 50% of DPPH than BHA (648 s).

Table 1. *Free radical DPPH inhibition effectiveness of algae extracts expressed as half-life time*

Sample	Mean half-life time (s)	Tipical deviation
Dictyota fasciola	*	
Dictyota dichotoma	*	
Sargassum vulgare	**33.6**	0.6
Hypnea spinella	*	
Caulerpa prolifera	**65.45**	12.3
*Cymopolia barbata (*January)	**135**	4.4
*Cymopolia barbata (*April)	**283.8**	33.6
*Cymopolia barbata (*August)	**109**	5.1
Cymopolia barbata (November)	*	
Lobophora variegata	**	
BHA	**648**	13.6
BHT	*	

** Half-life time greater than 20 min*

*** Half-life time greater than 20 min in all studied month*

3.1.2. Iron reducing power assay

Figure 5a displays the FRAP results expressed as mmol of reduced Fe (III) g^{-1} of dry algae. *C. barbata* showed the highest capacity of reducing Fe (0.112 mmol g^{-1}). Meanwhile, *C. prolifera* was the least effective in reducing Fe (0.008 mmol g^{-1}). Between the two most abundant algae, the Fe reducing activity of *C. barbata* (0.112 mmol g^{-1}) highly exceeded that of *L. variegata* (0.023 mmol g^{-1}).

Regarding seasonal variability of these two algae, both exhibited a maximum Fe reducing activity in August, but *C. barbata* presented a minimum in November whilst *L. variegata*'s lowest was found in April (Figure 5b). Even so, excepting the samples collected in August, all of *L. variegata* samples presented similar Fe reducing power (0.010 to 0.013 mmol g^{-1}).

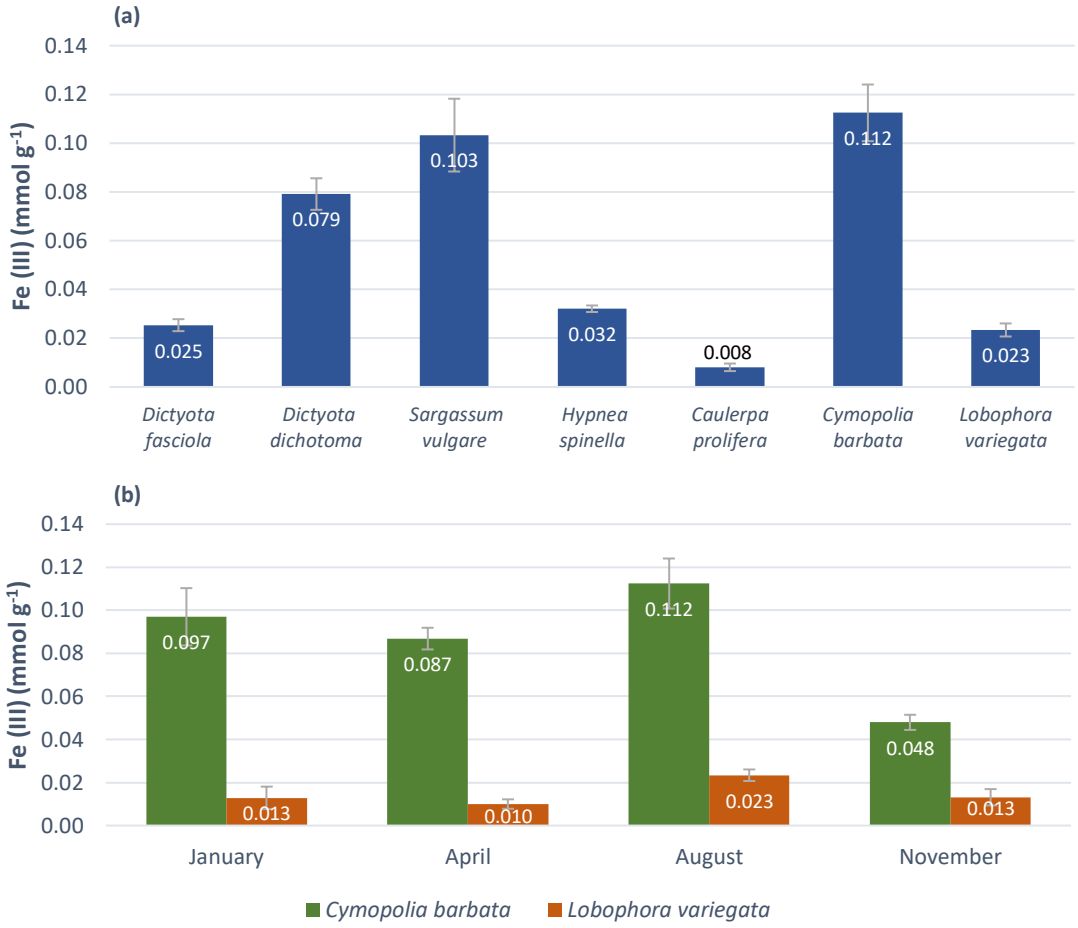

Figure 5. *(a) Fe reducing power of algae collected from Las Canteras beach during the summer months of 2021. (b) Seasonal variability of the capacity of reducing Fe of C. barbata and L. variegate. Both expressed as mmol of reduced Fe (III) g^{-1} of freeze-dried algae.*

3.1.3. Malondialdehyde determination assay

When the level of peroxidation of lipids was evaluated by measuring MDA contents (in mmol of MDA equivalents g^{-1} of dry algal biomass) with TBA, the results shown in Figure 6 were obtained. *D. fasciola* and *D. dichotoma* presented the highest concentrations of MDA (16 and 13.95 mmol

respectively) and, therefore, are believed to have been subjected to the most stressful conditions.

The remaining five species of algae presented MDA concentrations ranging from 8.78 mmol in *H. spinella* to 4.93 mmol in *C. barbata.* Figure 6b shows that *C. barbata* presented higher concentration of MDA equivalents than *L. variegata* all months except for August, when it reached its minimum (4.93 mmol). Meanwhile, the concentration of MDA equivalents of *L. variegata* in August reached its maximum (7.57 mmol).

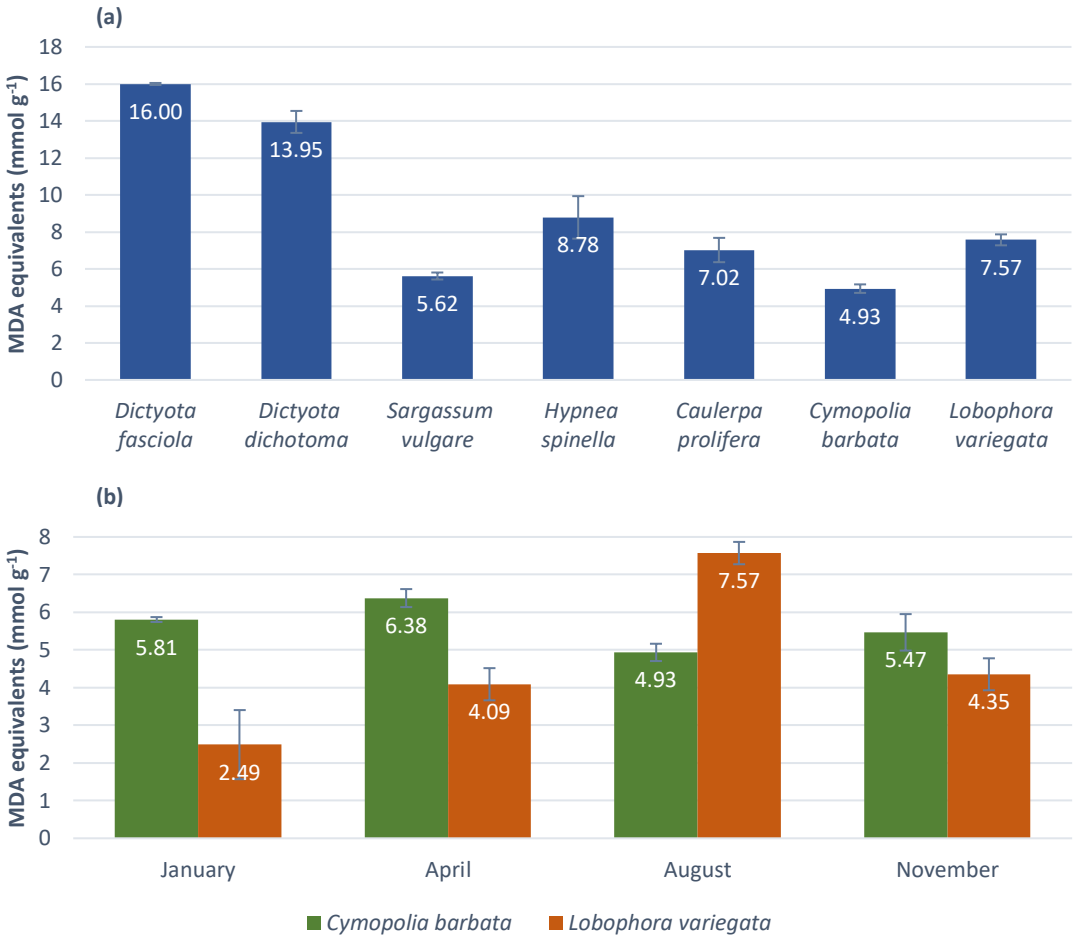

Figure 6. *(a) Concentration of MDA in algae collected from Las Canteras beach during the summer months of 2021. (b) Seasonal variability of the concentration of MDA of C. barbata and L. variegate. Both expressed as mmol g^{-1} of dry algae.*

3.2. Total and free carbohydrates quantification

The carbohydrates contents (expressed as mg of glucose g^{-1} of dry algal biomass) are shown in Figure 7. The concentration of free carbohydrates ranged from 0.02 mg in *D. fasciola* to 7.64 mg in *L. variegata*. Once some of the glycosidic bonds that maintain monosaccharides joined as polysaccharides are broken by the addition of hydrochloric acid, the concentration of

carbohydrates increased and ranged from 3.68 mg in *D. fasciola* to 10.09 mg in *C. barbata*, followed by *L. variegata* with 9.30 mg.

In relation to the seasonal variability of *C. barbata* and *L. variegata* (Figure 7b), the samples collected in November presented the lowest concentration of free and total carbohydrates, except for the free carbohydrates minimum that was found in the January sample of *L. variegata* (0.89 mg). The maximum percentages of free and total carbohydrates of *C. barbata* were found in April (5.96 and 11.45 mg, respectively) and, in *L. variegata* were detected in August (7.64 and 9.30 mg, respectively).

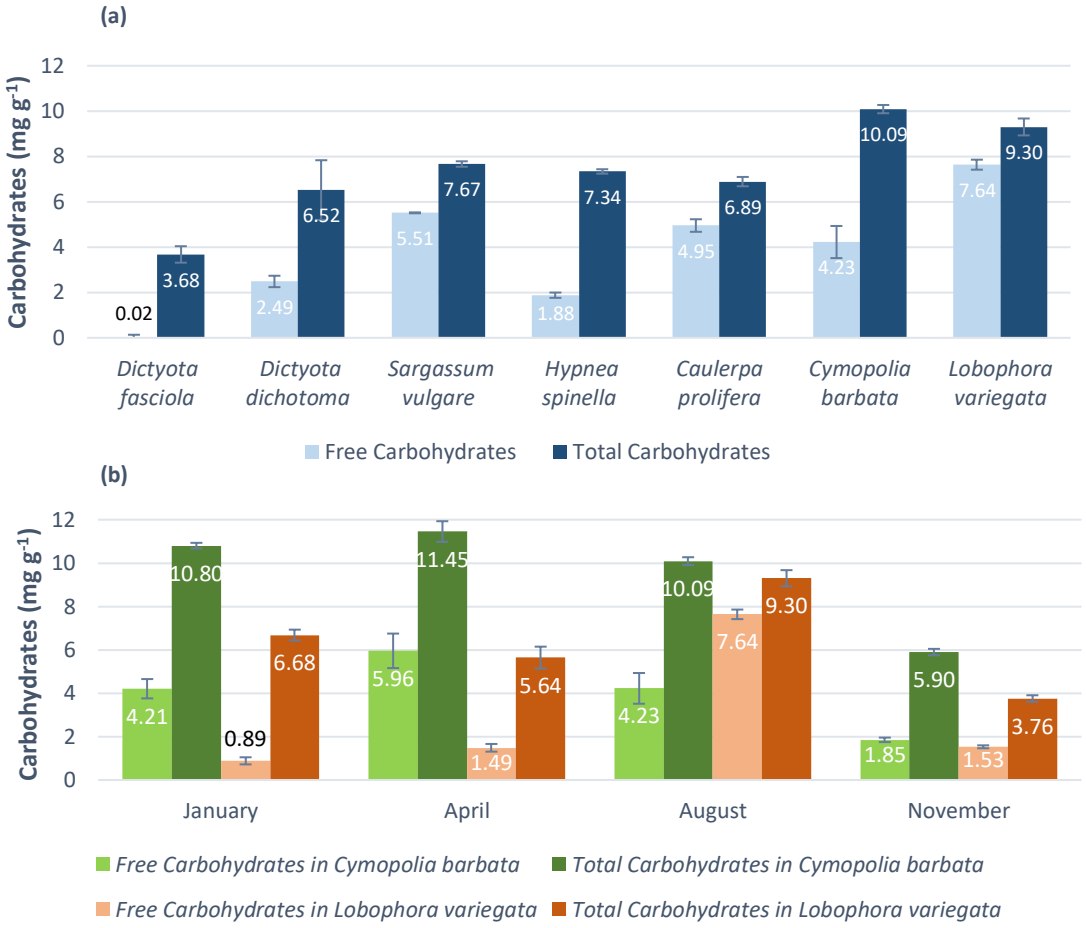

Figure 7. *(a) Concentration of free and total carbohydrates in algae collected from Las Canteras beach during the summer months of 2021. (b) Seasonal variability of the concentration of free and total carbohydrates of C. barbata and L. variegate. Both expressed as mg of glucose g^{-1} of dry biomass.*

3.3. Identification and quantification of polyphenols by RP-HPLC

3.3.1. Algae samples analysis

Polyphenols identified in the methanolic extracts of *C. barbata* and *L. variegata* are displayed in Figure 8 (expressed as mg per 100 g of dry biomass). Gallic acid, epicatechin and syringic acid were found in both algae. Catechin was also identified in *C. barbata*, where the polyphenol

concentration was considerably higher than in *L. variegata*. Gallic acid was detected in all samples ranging from 7.39 to 13.28 mg in *C. barbata* and from 0.75 to 3.11 mg in *L. variegata.* Catechin was spoted in all *C. barbata* samples except for those collected in April and the highest concentration was located in November with 41.86 mg (Figure 8a). *C.* barbata samples collected in January present all four polyphenols studied, being epicatechin the most abundant (35.40 mg) and syringic acid the least one (7.54 mg). *L. variegata*, on the other hand, presents only gallic acid except for the analysed samples collected in January, that also show epicatechin with a concentration of 29.55 mg and syringic acid with 5.66 mg (Figure 8b).

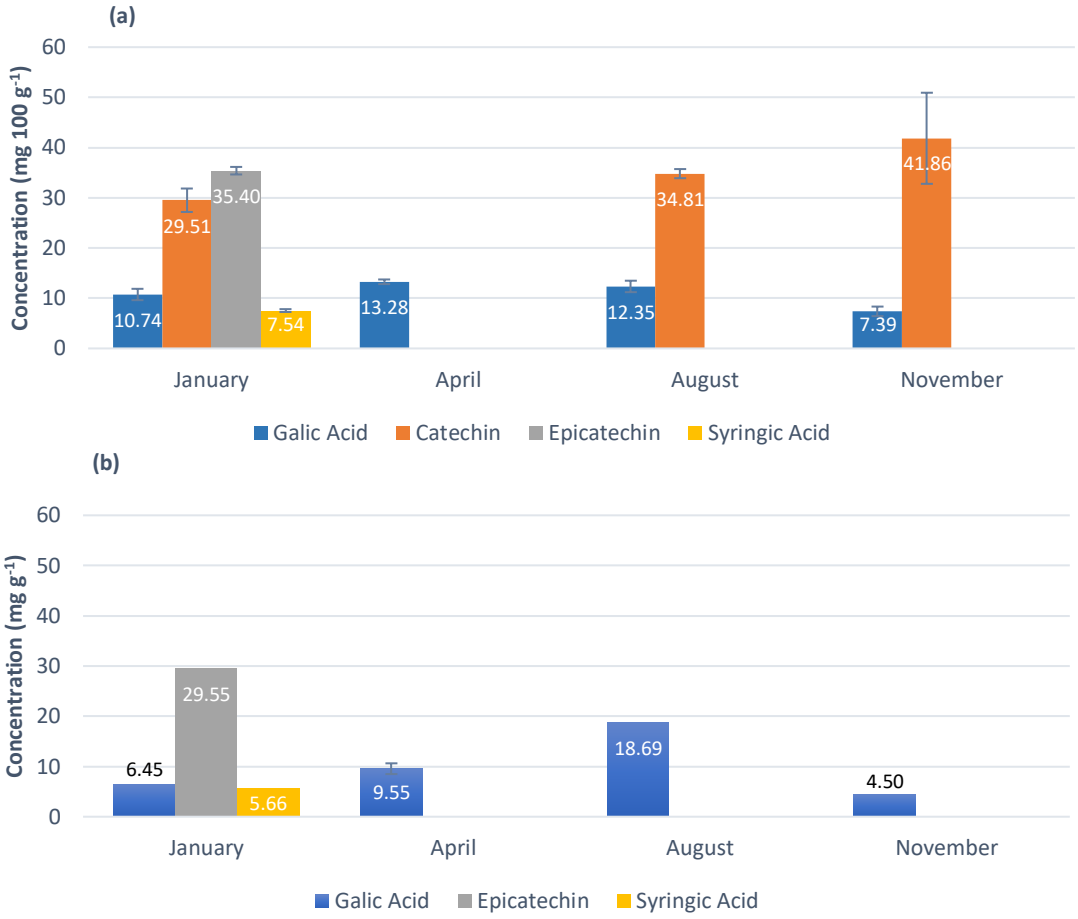

Figure 8. *(a) Seasonal variability of the polyphenolic profile of C. barbata expressed as mg per 100 g of freeze-dried algae. (b) Seasonal variability of the polyphenolic profile of L. variegata expressed as mg per 100 g of freeze-dried algae.*

3.3.2. Water samples analysis

Samperio-Ramos et al. (2017) reported that the cell numbers increased continuously during the 8 days of study to 9.98×10^{7} (pH 8.25), 1.07×10^{8} (pH 8.1), 1.04×10^{8} (pH 7.9) and 9.15×10^{7} (pH 7.75). In this study, the phenolic profile of seawater samples enriched with *E. huxleyi* exudates are summarized in Figure 9. Gallic acid, protocatechuic acid, catechin, vanillic acid and syringic acid were identified, being the last two detected in all pHs studied. The highest concentrations of exuded polyphenols were found at pH 7.75 (5.04 µg L^{-1}) and 8.25 (3.47 µg L^{-1}). In addition,

each cell exuded higher amount of phenolic compounds at pH 7.75 and 8.25 (55.1 and 34.7 fg cell^{-1} respectively) than those exuded at pH 7.9 and 8.1 (25.2 and 12.6 fg cell^{-1} respectively).

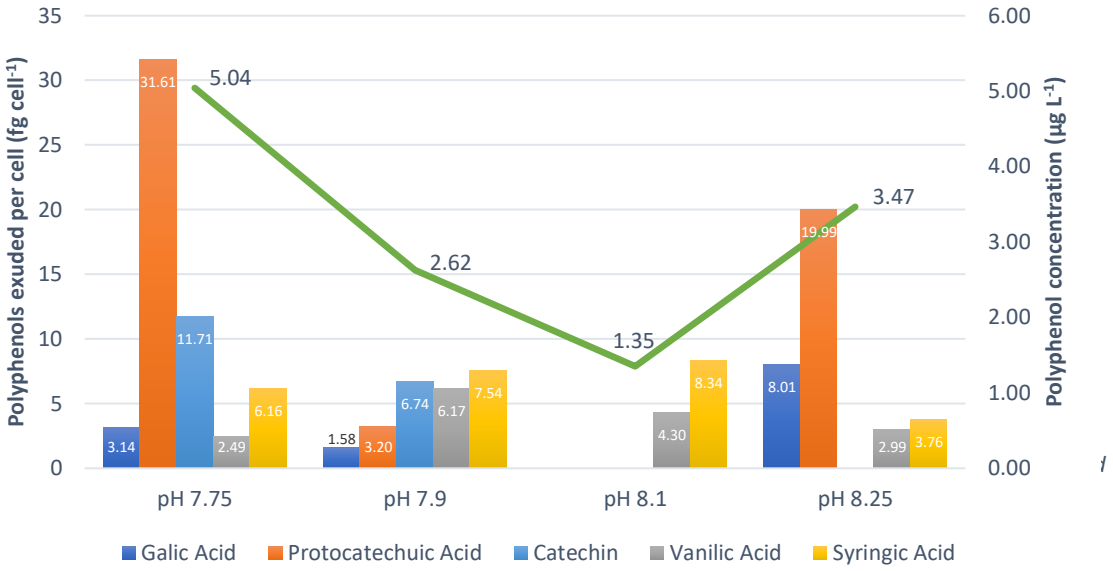

Figure 9. *Poliphenolic profile of E. huxleyi exudates according to the seawater pH of the culture expressed as fg exuded cell^{-1} (bar chart) and as µg of total polyphenols per liter (green line).*

The ability of these samples to inhibit DPPH radical was tested and the results are shown in Figure 10. Samples at pH 7.9 showed the highest radical scavenging activity (40.2%) followed by sample at pH 8.1 (23.2%). Similar activity was found in samples at pH 8.25 and 7.75 (18.6 and 18.2% respectively). Cells growing at pH 7.9 exuded enough antioxidants to inhibit 3.09 pmol of DPPH, followed by cells growing at pH 8.1 (1.74 pmol cell^{-1}). At pH 7.75 and 8.25, the extracellular release of antioxidants was enough to inhibit 1.60 and 1.50 pmol of DPPH respectively.

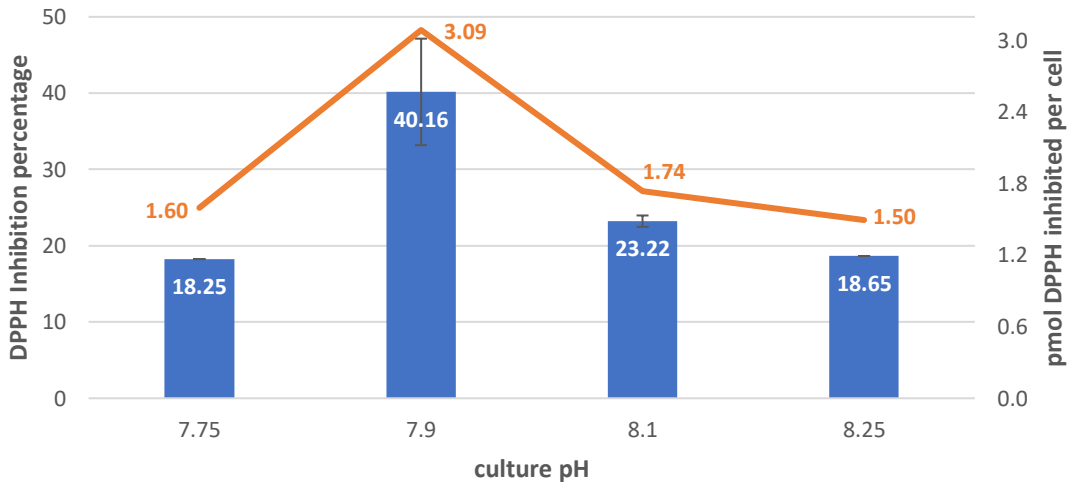

Figure 10. *DPPH Inhibition percentages by exudates from E. huxleyi cultured in different pHs (bar chart) and pmol of inhibited DPPH cell^{-1} (orange line).*

4.- Discussion

4.1. Extraction procedure

It is necessary to correctly collect the material from the beach so the gathering system must seek to minimize the sand content to facilitate the subsequent treatment of the biomass (Portillo, 2008). It is also decisive to choose a suitable extraction method for the successfully recovery of metabolites. The method must be able to break the cell wall without damaging the bioactive constituents that are kept inside (Agboola, 2018). Kröger et al. (2019) and de Farias Neves et al. (2019) concluded that freeze-drying improves the extraction yields. Wang et al. (2008) enhanced the extraction yields of bioactive compounds from plant materials with sonication treatments using methanol as solvent. Therefore, in this study all algae samples were washed, freeze-dried, and crushed. Sonication and mechanical stirring methods were combined with non-mechanical methods such as the addition of chemical agents (Stirk et al., 2020).

4.2. Analysis of algae collected in the summer months

It has been demonstrated that the different species of algae studied present diverse bioactive properties. Through different tests, it has been proven that these algae have great free radical inhibition capacities. Some of them are even higher than those of industrial antioxidant products such as BHA and BHT (Figure 4). These algae also show high FRAP values, which are considered to measure the total antioxidant potential (Figure 5). The results here reveal that *C. barbata* and *S. vulgare* are the algae with the highest free radical inhibition capacity as well as the ones with the highest Fe reducing power (Figures 4 and 5). In addition, these are the species with the lowest concentration of MDA (Figure 6) and, therefore, the ones that seems to show the least oxidative stress. In view of the results, it seems that the high antioxidant activity shown by *C. barbata* and *S. vulgare* confers protection against stress by free radicals.

In the opposite case, *D. dichotoma* and *D. fasciola* present low values in terms of DPPH inhibition and Fe reduction (Figures 4 and 5) but the highest concentration of MDA (Figure 3), which could mean high levels of oxidative stress, maybe caused by the lack of antioxidants in these species. The case of *L. variegata* shows parallelisms with *Dictyota spp.*: it presents the lowest percentage of DPPH inhibition (12.84%), the second lowest concentration of reduced Fe (0.023 mmol g^{-1}) and only 7.57 mol of MDA per gram of freeze-dried algae. In fact, it is approximately three times closer to the MDA content of *C. barbata* (with 4.93 mol g^{-1} of dry biomass) than to *D. fasciola* (16 mol g^{-1}).

L. variegata and the two species of *Dictyota (D. dycotoma and D. fasciola*) showed difference in the level of MDA (7.57, 13.95 and 16 mmol g^{-1} respectively), despite all three exhibited low antioxidant activity. While the two species of *Dictyota* presented the lowest values of free (0.02 and 2.49 mg g^{-1}) and total (3.68 and 6.52 mg g^{-1}) carbohydrate contents, *L. variegata* exhibited the highest levels in both categories (7.64 and 9.30 mg g^{-1}). This significant amount of carbohydrates found in *L. variegata* could be due to the presence of high contents of fucans

(sulfated polysaccharides common in brown algae) according to Paiva et al., (2011). These have proven in laboratory assays to be potent antioxidants capable of reducing and removing reactive oxygen species. Other studies have demonstrated the antioxidant activity of *L. variegata* extracts and suggest their use as nutraceuticals (Sathyaseelan et al., 2015).

Among the studied algae, *C. barbata* and *S. vulgare*, together with *L. variegata*, have the highest carbohydrate content, both free (4.23, 5.51 and 7.64 mg gr^{-1} respectively) and total (10.09, 7.67 and 9.30 mg gr^{-1} respectively), which could enhance their antioxidant properties. According to Hu et al. (2016), the phenolic and protein constituents in complex carbohydrates may play a significant role in the antioxidant activity of complex carbohydrates, which agrees with other literature reports (Moraes et al., 2015; Dlamini et al., 2007).

4.3. Seasonal variability of the phenolic and carbohydrate contents, and antioxidant activities of *C. barbata* and *L. variegata*

Arrivals with the highest biomass usually occur between November and March, although a considerable amount of algae also arrive in late spring and during the summer months due to swell caused by persistent and powerful anticyclones. Depending on the time of the year in which the biomass is collected, its properties may vary. In view of the results, the summer months seem to be better to collect *C. barbata* for applications as an antioxidant, since it presents the highest percentage of free radical inhibition, the greatest Fe reduction capacity (Figures 4 and 5), the lowest MDA concentration (79.60%, 0.112 mmol gr^{-1} and 4.93 mmol gr^{-1}) and the highest content of total carbohydrate (11.45 mg gr^{-1}) (Figure 6). However, the maximum free carbohydrate levels were presented by *L. variegata* in August (7.64 mg gr^{-1}) and a considerable amount of total saccharides (9.30 mg gr^{-1}), the highest recorded for this species. as well as the highest values in ferric reducing power for this species (0.023 mmol g^{-1}). Even so, the highest free radical scavenging activity for *L. variegata* was found in April which agree with those reported by Celis-Plá et al., (2016) who indicated that brown macroalgae *Cystoseira tamariscifolia* produced the highest levels of antioxidants in spring. Moreover, the highest diversity of polyphenols were found in January samples of *L. variegata* possibly a cause for the low levels of MDA produced in that month (2.49 mmol g^{-1}).

4.4. Phenolic content and antioxidant activities of *E. huxleyi* exudates

As a whole, the marine acidification conditions can trigger a physiological response of phytoplankton such as *E. huxleyi*, that can imply an overexertion of the organisms. It has been proven that the decrease in ocean pH can lead to a change in the phenolic profile of exuded organic ligands and, therefore, can have a significant impact on the bioavailability of metals in the future ocean (Samperio-Ramos et al., 2017; Rico et al., 2013). The higher content of polyphenols in the exudates could also improve the ability to acquire essential trace metals

(Santana-Casiano et al., 2014) under the acidification scenario in which the bioavailability of some metals such as Fe seems to decline (Shi et al., 2010).

Protocatechuic acid reached the highest concentration in the exudates at pH acidified to 7.75. This compound is a potent antioxidant by both chelating metal transition ions as well as by scavenging free radicals (Psotová et al., 2003). Catechin is a flavonoid with powerful antioxidant activity and also with cytotoxic properties that could be especially helpful for survival in the face of adverse conditions (Bernatoniene & Kopustinskiene, 2018). It was only detected at pH 7.9 and 7.75. Vanilic and syringic acids are found in the exudates of all cultures and their concentration remains quite stable.

The highest concentrations of total exuded polyphenols were found in cultures at pH 7.75 (5.04 µg L^{-1}) and 8.25 (3.47 µg L^{-1}), where the cell densities were lower (9.15×10^7 and 9.98×10^7 respectively). Therefore, at these pHs each cell exuded higher amount of phenolic compounds (55.1 and 34.7 fg $cell^{-1}$ respectively). At pH 7.9 and 8.1 a greater number of cells (1.04×10^8 and 1.07×10^8 respectively) exuded less polyphenols (2.62 and 1.35 µg L^{-1} respectively) (Figure 9).

However, the exudates with the highest content of phenolic compounds (pH 7.75) gave the lowest radical scavenging activity (18.2%), while exudates from cells cultured at pH 7.9 exhibited the highest activity (40.2%) (Figure 10). Therefore, phenolic compounds could not be the only substances responsible for the antioxidant activity. In fact, Samperio-Ramos et al. (2017) found that extracellular release of dissolved combined carbohydrates (expressed as fmol C $cell^{-1}$ day^{-1}) increased from 44.43 (at pH 8.25) to 47.39 at pH 7.9, decreasing up to 46.95 at pH 7.75. This maximum reached at pH 7.9 could be associated to the highest antioxidant activity found at this pH (40.2%). In our study, the compounds capable of directly scavenging DPPH (expressed as pmol of inhibited DPPH $cell^{-1}$) increased from 1.50 pmol at pH 8.25 to the maximum level of 3.09 pmol at pH 7.9, decreasing to 1.60 pmol at pH 7.75 (Figure 10).

As stated above, cells cultured at lower pH exuded higher amounts of phenolic compounds per cell (55.1, 25.2 and 12.6 fg $cell^{-1}$ at pH 7.75, 7.9 and 8.1 respectively) with the exception of cells cultured at pH 8.25 (34.7 fg $cell^{-1}$) (Figure 9). These results partially agree with those of Samperio-Ramos et al. (2017) who reported increasing extracellular release of phenolic compounds (determined using the colorimetric test of Arnow (1937)) as the levels of CO_2 became higher in the culture medium, obtaining the lowest amount of phenolic compounds at pH 8.25.

Moheimani and Borowitzka (2011) studied the effects of changes in CO_2 and pH on biomass productivity and carbon uptake of *Pleurochrysis carterae* and *E. huxleyi*. They observed the highest growth rate and productivities in *E huxleyi* cultured at pH between 8.1 and 8.4 reached under pH initially unregulated conditions. Under regulated addition of CO_2, these authors reported that *E huxleyi* cultures continued to grow well at pH between 7.9 and 7.7, obtaining the highest productivities at pH 7.8. *E. huxleyi* could not grow at a pH less than 7.5. These findings agree with those here, where two pHs of maximum productivity were also detected (pH 7.75 and 8.25).

5.- Conclusions

Based on the initial approach of the study, satisfactory answers have been obtained for the three main objectives.

Firstly, among the analysed algae species collected in summer:

- Three species exhibited higher radical scavenging activity than BHA: *C. prolifera*, *S. vulgare*, and *C barbata* and the lowest MDA concentrations.
- *C. barbata* and *S. vulgare* also showed the highest capacity of reducing iron and, together with *L. variegata*, the highest content of total carbohydrates.

Secondly, we have demonstrated the noticeable effect of the environmental changes on the antioxidant properties of the two most abundant algae in Las Canteras arrivals:

- According to our results *C. barbata* showed higher antioxidant activity than *L. variegata* in every assay as well as higher content of polyphenols.
- Samples of *C. barbata* collected in summer showed the highest radical scavenging activity and FRAP values while *L. variegata*'s maximums were found in spring and summer respectively.
- The maximum percentages of free and total carbohydrates were found in April for *C. barbata* and in August for *L. variegata*.
- Phenolic compounds gallic acid, epicatechin and syringic acid were found in both algae, but *C. barbata* showed higher concentration than *L. variegata*.
- The study of seasonal variability was carried out on samples collected during the year 2021. It would be interesting to repeat the tests for samples gathered during the following years to evaluate the interannual variability and thus be able to determine the possible effects of climate change on the algal community of Las Canteras beach.

Algal biomass analysed in this study has great properties and bioactive metabolites. Our results open the possibility of using extracts from algae such as *L. variegata* for nutraceuticals, compost, feed, or even human nutrition, replacing current synthetic antioxidant products (BHA, BHT). This could become a revulsive for the local economy by developing new commercial and industrial activity using arriving algae as raw material.

Thirdly, changes in the phenolic profile of exudates from the coccolithophorid *E. huxleyi* have been observed when modifying the pH of the culture seawater.

The highest amounts of exuded phenolic compounds were observed in cultures with lower cell densities (at pH 7.75 and 8.25), indicating that these cells exuded higher amounts of phenolic compounds. These pH showed the maximum productivity of phenolic compounds per cell.

In conclusion, the results obtained in this study expand the pre-existing information and open a range of possibilities for future studies in the fields of biotechnology and climate change.

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