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Effects of new microalgae and macroalgae products on live prey and fish performance

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Effects of new microalgae and macroalgae products on live prey and fish performance

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“Si he visto más, es poniéndome sobre los hombros de Gigantes”

Isaac Newton

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ABBREVIATIONS

% AA: percentage of antioxidant activity

ABTS: 2,20-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)

ALA: α-linolenic acid

ARA: arachidonic acid

BAL: Bile salt-activated lipase

BHT: butylated hydroxytoluene

CAT: catalase

CD: control diet

CD+MOD: control diet with monospecific macroalgal wrack inclusion (*Dictyota* sp.)

CD+MOL: control diet with monospecific macroalgal wrack inclusion (*Lobophora* sp.)

CD+MU: control diet with multispecific macroalgal wrack inclusion

CDNB: 1-chloro-2,4-dinitrobenzene

DAG: diacylglycerol

DHA: docosahexaenoic acid

DGDG: digalactosyl-diacylglycerol

DMSO: dimethyl sulfoxide

DPPH: 1,1-diphenyl-2-picryl-hydrazyl

DW: dry weight

EPA: eicosapentaenoic acid

FA: fatty acid

FAME: fatty acid methyl esters

FFA: free fatty acids

FIFO: fish in fish out ratio

FO: fish oil

GPx: glutathione peroxidase

GR: glutathione reductase

GSH: reduced glutathione

GSSG: oxidized glutathione

GST: glutathione-S-transferase

HCl: hydrochloric acid

H_2O_2: hydrogen peroxide

HSI: hepatosomatic index

IFRE: fresh *Isochrysis galbana*

IFRO: frozen *Isochrysis galbana*

ISD: spray-dried *Isochrysis galbana*

LA: linoleic acid

LC: lipid class

LC-PUFA: long chain polyunsaturated fatty acids

LPC: lysophosphatidylcholine

MAG: monoacylglycerols

MDA: malondialdehyde

MEUR: million euros

MGDG: monogalactosyl-diacylglycerol

MOD: monospecific macroalgal wrack (*Dictyota* sp.)

MOL: monospecific macroalgal wrack (*Lobophora* sp.)

MU: multispecific macroalgal wrack

MUFA: monounsaturated fatty acids

NFRE: fresh *Navicula salinicola*

NFRO: frozen *Navicula salinicola*

NSD: spray-dried *Navicula salinicola*

OA: oleic acid

PBR: photobioreactor

PC: phosphatidylcholine

PE: phosphatidylethanolamine

PI: phosphatidylinositol

PS: phosphatidylserine

PTS: phytosterols

PUFA: polyunsaturated fatty acids

PxI: peroxide index

ROS: reactive oxygen species

SD: standard deviation

SDA: stearidonic acid

SE: sterol esters

SFA: saturated fatty acids

SGR: specific growth rate

SOD: superoxide dismutase

SQDG: sulfoquinovosyl-diacylglycerol

TAC: total antioxidant capacity

TAG: triacylglycerols

TBA: thiobarbituric acid

TBARS: thiobarbituric acid reactive substances

TL: total lipid

TNL: total neutral lipids

TPL: total polar lipids

UKNL: unknown neutral lipids

UKPL: unknown polar lipids

VFI: visceral-fat index

VO: vegetable oil

VSI: viscerosomatic index

SUMMARY

Aquaculture has acquired a worldwide essential importance, growing steadily over the last years and exceeding global capture fisheries. Among the aquaculture products, algae global production is expected to increase to the highest rate in the coming decades. In fact, two algae (*Saccharina japonica* and *Euchema* spp.) are presently at the top of total production statistics. Algae are rich in valuable compounds such as polysaccharides, vitamins, minerals, proteins, phenolic compounds, sterols and other lipids including n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA). They also have high contents of pigments that exert antioxidant and anticancer activities. Thus, algae are considered good candidates to be used in human and animal nutrition because of their biochemical composition. Besides, macroalgal wracks that regularly detach from offshore beds and accumulate in coastal areas are usually removed and discharged, and consequently, new uses for this biological biomass are being currently evaluated.

Live prey are essential tools in aquaculture to feed larvae of marine organisms. However, they are usually poor in some essential nutrients such as LC-PUFA, needing to be enriched prior to larval feeding. In this sense, lipid emulsions and microalgae are commonly used to enrich or feed live preys. While enrichment protocols are usually associated to high oxidative stress condition, which is increased by the addition of a lipid emulsion, the use of microalgae involves high production costs, both leading to a serious bottleneck in hatcheries. Thus, substitutes of live microalgae including pastes, dried formats, microencapsulates or flocculated microalgae are receiving increasing research attention due to its nutritional stability and antioxidant potential, since oxidative stress may be partially compensated by the antioxidant compounds present in microalgae.

On the other hand, algae have been proposed as suitable alternative sources of lipids and proteins for farmed fish due to their high nutritional value and balanced composition, high production rates, and potential availability. In fact, algal inclusion has already demonstrated some beneficial effects when partially replace fish meal and fish oil in aquafeeds.

In our study, new *Isochrysis galbana* and *Navicula salinicola* formats, together or not with lipid emulsions in rotifer and *Artemia* enrichment protocols are assayed. The lipid composition of enriched live preys, and the potential ability of products to palliate oxidative stress is studied. Our results suggest that *I. galbana* was better than *N. salinicola*

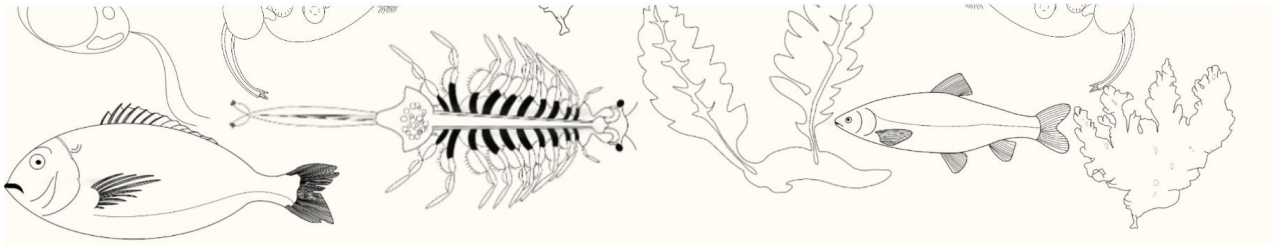
to feed live preys. In particular, spray-dried was the best format to enhance n-3 LC-PUFA content and phospholipids retention, due to a higher nutrient availability in this format. In spite of increasing oxidative stress, the addition of a lipid emulsion for a short enrichment period, together with the spray-dried *I. galbana*, showed the best results regarding lipid composition and oxidative status protection.

On the other hand, lipid characterization of seaweeds regularly present in beach casts from the Macaronesian region is assessed, in order to discuss their potential use for animal feeding and as human food. Thus, a 7% inclusion of a multispecific or two monospecific (*Lobophora* sp. and *Dictyota* sp.) macroalgal wracks in *Ctenopharyngodon idella* and *Sparus aurata* diets is assayed, and the lipid composition, oxidative status, digestive enzymes activities and plasmatic parameters of fish determined.

The present study also provides evidence of the high variability of macroalgal lipid profiles. Their n-6/n-3 ratio, and their content in some benefitial lipids such as phytosterols make the algae analysed interesting from both a nutritional and health perspective.In this sense, *Asparagopsis taxiformis* and *Dictyota dichotoma* can be considered as good sources of n-3 fatty acids for human nutrition because of their EPA+DHA content.

Finally, a 7% of macroalgal wrack dietary inclusion did not display any negative effect in the culture performance of *C. idella* and *S. aurata* juveniles. In fact, the multispecific wrack displayed less fat deposition in *C. idella*, while both multispecific and monospecific (*Lobophora* sp.) wracks caused some protective effect by enhancing the antioxidant capacity in the liver compared to the control. *S. aurata* also showed a higher capacity to mold the glutathione metabolism with the monospecific *Dictyota* sp. wrack feeding. Furthermore, *S. aurata* showed some altered patterns in the digestive enzymes activities with respect to the control, although without any detrimental effects on fish performance.

General introduction



1. GENERAL INTRODUCTION

1.1. Current status of aquaculture

World population was estimated to be about 2.6 billion in 1950. Nowadays, it has raised up to 7.8 billion in 2020, and it is expected to peak at 9.7 billion by 2050, according to the United Nations. This massive expansion of human population has led to a consequent rise in the world food needs and, as a result, productive activities such as agriculture and farming, especially aquaculture, have been intensified in the last decades (Troell et al., 2014).

The anual global consumption of marine origin products has increased in recent times, and, specifically that of fish was 20.5 kg per capita in 2017 (FAO, 2020). Fish and seafood are considered a much healthier food than meat products from terrestrial origin, comprising the third major source of protein consumed by humans after cereals and milk in 2017. That year, marine food products represented a 17.1% of total animal protein supply, higher than that of poultry meat, pig meat, bovine meat, or hen eggs (Tacon et al., 2020). Food from aquatic animals have a higher protein, essential aminoacids, and generally greater mineral (including calcium, magnesium, iron, copper, zinc, iodine, selenium, and trivalent chromium), and vitamin (A, D, E, B12, folic acid, choline, coenzyme Q10) contents than terrestrial meat products. They also have higher content of omega-3 (n-3) fatty acids (FA) than any other animal foodstuff, and lower caloric density than terrestrial meats (Tacon et al., 2020). Moreover, consumption of aquatic food, which includes finfish, crustaceans, cephalopods and other molluscs, aquatic plants, algae, and other aquatic animals such as mammals, insects or sea cucumbers, has demonstrated to benefit human health (Golden et al., 2021; Riediger et al., 2009; Tacon et al., 2020). Thus, its ingestion reduces micronutrient deficiencies (such as vitamin A, calcium or iron), and provides n-3 FA, which may reduce the risk of heart disease and promote brain and eye health (Golden et al., 2021; Zárate et al., 2017). Finally, it decreases the consumption of less-healthy red and processed meat than can cause adverse health outcomes (Golden et al., 2021).

The increasing uptake of marine-origin products has resulted in an over-exploitation of most global fisheries and in a subsequent inability to meet the current nutritional demand through fishing (Tidwell and Allan, 2001). Thus, aquaculture has become a potential activity to cover the nutritional needs of world population (Hixson, 2014). In this context,

annual capture fisheries has kept stable at around 86-93 million tonnes from the 80s, excluding 2018, where it reached the highest level ever recorded at 96.4 million tonnes (FAO, 2020). On the contrary, aquaculture has acquired a global essential importance, growing exponentially over the last years (Figure 1.1), even exceeding total catches by over 18.32 million tonnes in 2017 (Tacon, 2020). It is estimated that the over-exploitation of fisheries, together with the enhancement of technologies will make aquaculture to keep growing over the next decades (Troell et al., 2014).

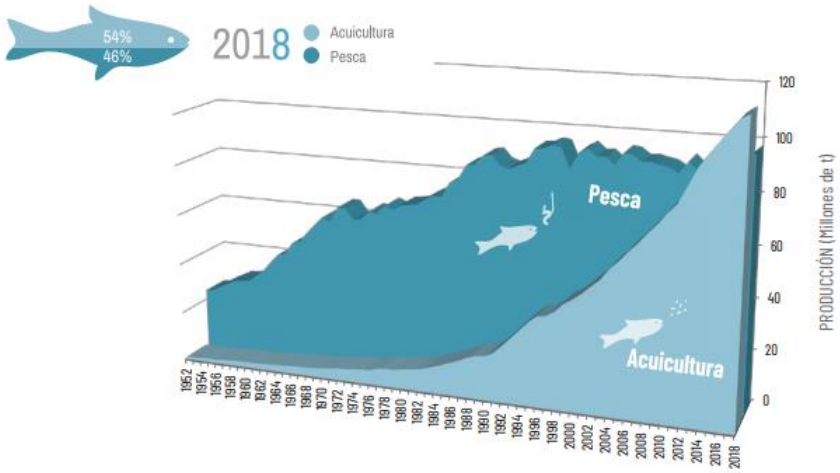


Figure 1.1. World **capture fisheries** and **aquaculture production** from 1950 to 2018 (APROMAR, 2020; FAO, 2020).

Around 580 aquatic species, including both plant and animal species are cultivated all over the world (FAO, 2022). In 2017, fish represented the largest major group produced by aquaculture by weight (47.7% total aquaculture), followed by algae (28.4%), molluscs (15.5%), crustaceans (7.5%), amphibians and reptiles, and miscellaneous invertebrates (Figure 1.2) (Tacon, 2020).

Fish production is growing at an average rate of 5.7% per year since 2000, with over 200 different fish species produced in 2017, dominated by freshwater fish species (83.6% of total fish production) (Tacon, 2020). In particular, the herbivorous freshwater grass carp (*Ctenopharyngodon idella*) has been the major fish species produced in the last years (FAO, 2020). Although algae reached 28.4% of total aquaculture production by weight in 2017, only represented a 4.7% of total production by value. Despite this, over 20 different aquatic plant species cultivated, Japanese kelp (*Saccharina japonica*, previously

known as *Laminaria japonica*) was the top cultured species with 11.17 million tonnes, followed by *Euchema* spp. and *Gracilaria* spp. (8.64 and 4.31 million tonnes, respectively) (Tacon, 2020).

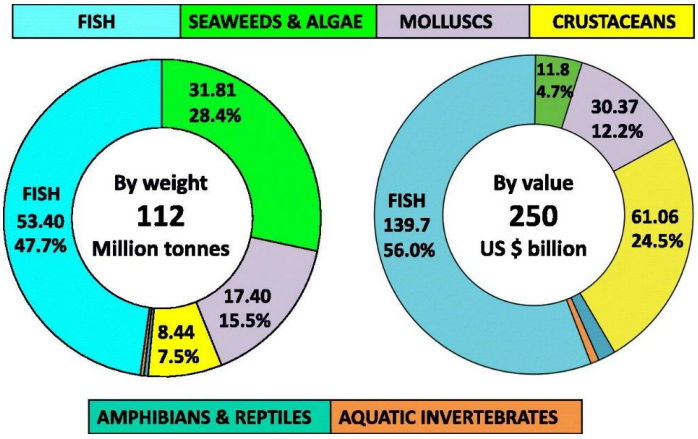


Figure 1.2. Total global **aquaculture production by groups of species** (live weight and value) (Tacon, 2020).

In Europe, aquaculture is an important source of aquatic products accounting for 19.9% of the total production. In addition, aquaculture plays a very significant role in the social and economic development of certain coastal and river areas, as well as in the preservation of their maritime-river and fishing culture (APROMAR, 2020).

Spain produced the largest aquaculture harvest within the European Union in 2018 with 347,825 tonnes, a 25.5% of the total production, followed by the United Kingdom and France with 197,618 tonnes (14.5%) and 185,650 tonnes (13.6%), respectively. However, UK accounts for up to 25% of the total production in value (1,075 million euros, MEUR) followed by France with 16% (678 MEUR), Greece with 12% (509 MEUR) and Spain with 11% (479 MEUR). The main aquaculture products in Europe are finfish and molluscs, while the production of crustaceans, algae and other invertebrates is very limited. The finfish harvest in 2018 was 695,885 tonnes (51% of the total) reaching a value of 3,241 MEUR (74.4% of aquaculture value), while molluscs totalled 667,934 tonnes, 48.9% of the total weight, encompassing 1,108 MEUR (25.4% of the total value). The main species produced in the EU are mussels (*Mytilus* spp.), followed by Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Considering its value,

Atlantic salmon was the first farmed species (1,106 MEUR), followed by rainbow trout (574 MEUR) and seabass (*Dicentrarchus labrax*) (465 MEUR) (APROMAR, 2020).

Marine fish farming started in Spain in the 60s, and it kept growing until 2009. Despite its progressive increase along the 20th century, aquaculture has not been able to overcome the fishing activity. Furthermore, Spanish aquaculture development has stagnated in the last decade about 45,000 tonnes, excluding 2019, in which the production rised to 53,920 tonnes. However, climatic and epidemiological events are believed to affect aquaculture production, expecting a decrease in the next years. The main marine fish producer in Spain in 2019 was the Valencian Community with 16,045 tons, followed by Galicia with 8,337 tonnes, the Canary Islands (8,239 tonnes), Murcia (6,513 tonnes) and Andalusia (5,644 tonnes). The major species produced were mussels (*Mytilus* spp.), followed by seabass (*D. labrax*), rainbow trout (*O. mykiss*) and gilthead seabream (*Sparus aurata*) (APROMAR, 2020). *S. aurata* is the most important finfish aquaculture product in the Mediterranean (Savoca et al., 2021); also being especially important in the Canary Islands together with *D. labrax* (APROMAR, 2020).

1.2. Fish diets

Fish diets should supply both essential nutrients (aminoacids, FA, vitamins and minerals) and macronutrients such as proteins, lipids and carbohydrates (Hixson, 2014). Traditionally, fish diets, mainly those for carnivorous species, have heavily relied on marine ingredients such as fishmeal and FO obtained from wild harvested pelagic fish populations, due to their needs of feeding in similar conditions to wildness. The fish in fish out ratio (FIFO) was created to highlight the specific dependence of the aquaculture industry on wild capture fisheries (Tacon et al., 2022). Aquafeeds reliance on marine ingredients promotes the over-exploitation of fisheries and the loss of diversity of fish species (Naylor et al., 2000; Tacon and Metian, 2008), increasing the FIFO greatly. Hence, carnivorous fish feeding is actually considered as the main environmental impact factor of the aquaculture sector (Ghamkhar and Hicks, 2020; Hixson, 2014). The potential development and social acceptability of aquaculture will depend on both the sustainability of carnivorous fish dietary formulations and the implementation of environmentally friendly practices to avoid habitat destruction and pollution (Ahmed and Thompson, 2019).

The use of vegetable raw materials is one of the most used alternatives to FO in aquafeeds formulation. A plethora of studies has been carried out in the last decades on the substitution of marine ingredients with vegetable oils (VO), demonstrating that farmed fish are able to correctly develop with high levels of replacement; up to 60% in some carnivorous species (Abbasi et al., 2020; Álvarez et al., 2020; Izquierdo et al., 2003; Morais et al., 2015; Pérez et al., 2014) or even 100% in Atlantic salmon (Bell et al., 2005). VO are rich in C18 polyunsaturated FA (PUFA), such as linoleic acid (18:2n-6; LA) and α-linolenic acid (18:3n-3; ALA), but poor in the long chain PUFA (LC-PUFA) eicosapentaenoic acid (20:5n-3; EPA), docosahexaenoic acid (22:6n-3; DHA) and arachidonic acid (20:4n-6; ARA) (Sargent et al., 2003) which are considered highly beneficial for health and are produced by marine algae and therefore, abundant in marine origin products (Pérez et al., 2014; Tocher, 2015). It is well known that dietary FA profile affects the FA composition of fish organs and tissues and, consequently, feeding with VO reduces essential LC-PUFA contribution to fish, which can compromise their beneficial effects for consumers (Bell & Waagbø, 2008; Izquierdo et al., 2003; Torstensen et al., 2004). Furthermore, most VO have an elevated unhealthy n-6/n-3 ratio, which increases accordingly in fish flesh (Naylor et al., 2000). In addition, the use of terrestrial raw materials is not the most sustainable alternative when deforestation and energy consumption are taken into account (Tacon et al., 2022).

The search for new alternative ingredients for aquafeeds formulation is therefore essential for the aquaculture sector. Within this context, oils from transgenic plants have been recently tested, although its acceptance is closely linked to ethical and regulatory factors (Ruyter et al., 2019). Farming of fish species with high capacity to biosynthesize LC-PUFA from their C18 precursors abundant in VO (Galindo et al., 2021; Garrido et al., 2019; Marrero et al., 2021, 2022), or the inclusion of micro and macroalgae-origin products rich in n-3 LC-PUFA as possible novel alternatives to marine sources (Byreddy et al., 2019; Dineshbabu et al., 2019; Sprague et al., 2016; Tocher et al., 2019) might be also considered as valuable sustainable strategies for the aquaculture industry and deserve further exploration.

1.3. Functions of lipids and fatty acids

Fats are essential components for living organisms. Lipids play a major role as sources of metabolic energy for fish growth, reproduction, movement and migration (Tocher, 2010).

Its importance is such that lipids, and their main components FA, are along with proteins, the mayor organic constituents of fish (Tocher, 2015). FA molecules present a variable length carbon chain with a methyl terminus and a carboxylic acid head group. FA with two or more double bonds are called PUFA of which those with 20 or more carbon atoms are called LC-PUFA. PUFA can be classified into n-3 or n-6 series, if the first double bond is located in the third or sixth carbon atom from the terminal methyl group, respectively. C18 PUFA such as LA and ALA, are considered essential nutrients for vertebrates because they cannot be synthesized *de novo* (Galindo et al., 2021). Additionally, they are metabolic precursors of the physiologically important LC-PUFA, ARA, EPA, and DHA (Tocher, 2015). PUFA are essential components in diets of all vertebrates, including fish. Furthermore, marine and freshwater ecosystems are usually rich in n-3 LC-PUFA, compared to terrestrial ecosystems, being fish the main source of these physiologically essential FA for humans (Bell & Tocher, 2009; Sargent & Tacon, 1999; Tocher, 2003).

An important function of lipids is the β-oxidation of FA to provide ATP, the main energetic resource for growth, reproduction and swimming in fish (Tocher, 2003). They are also structural compounds of biological membranes, playing a key role to maintain cell integrity. In fish, EPA and DHA are important components of phospholipids, and they are usually more abundant than ARA (Sargent et al., 1999), although ARA is specifically present in phosphatidylinositol (Reis et al., 2020) and particularly abundant in reproductive tissues (Rodríguez et al., 2012). LC-PUFA also play an important role in temperature changes, because its presence, together with the ability to modify their chain length and number of double bonds may change the fluidity and phisiological properties of cell membranes. This is essential for adapting to environmental salinity, temperature and pressure variations (Rodríguez et al., 2012).

C20-22 PUFA also act as precursors of eicosanoids and docosanoids such as prostaglandins, tromboxans, leucotriens, resolvins, protectins, and maresins, through the action of ciclooxigenase and lipooxigenase enzymes (Riediger et al., 2009). ARA derived compounds are more biologically active, being mainly pro-inflammatory, while EPA derived eicosanoids are generally anti-inflammatory (Lee et al., 2016; Sargent et al., 1999). As both compounds compite for the same enzymes in eicosanoids synthesis (Riediger et al., 2009), eicosanoids action is determined by the EPA/ARA ratio, and conditioned by the ingestion of n-3 and n-6 FA (Tocher et al., 2003). Thus, a balanced

intake of FA is crucial, with some diseases being directly related to a higher intake of LA with respect to ALA. Furthermore, a high intake of n-3 FA and lower n-6/n-3 ratios have been related with a lower incidence of some types of cancer (Riediger et al., 2009; Rodríguez et al., 2012; Simopoulos, 2016; Zárate et al., 2017). In fact, due to an increase in cereal, VO, terrestrial herbivorous meat and trans n-6 FA consumption (Simopoulos, 2016), this ratio has increased from 1-2:1 in Paleolitic diets, to 15-20:1 in the tipical Western diet, which is in severe contrast with the recommendations of most National Health Agencies (Ander et al., 2003; Zárate et al., 2017). Finally, PUFA may also affect gene regulation through several mechanisms, including changes in cell membrane composition, eicosanoids production or oxidative stress processes (Tocher, 2015).

DHA plays an essential function in retine photoreceptors, brain and heart tissues in all vertebrates. Its intake has been related to a correct development of brain and vision in infants, and to a good cardiovascular health. ARA is also an important FA in the brain, and together with DHA is needed for its proper development (Ward and Singh, 2005). In fish, n-3 and n-6 FA have the same fuctions as described above. Thus, its unbalance and deficiency have been related to brain and eye disability in larvae, pigment disorders and dysregulated development, reproduction and health condition of farmed fish (Rodríguez et al., 2012; Villalta et al., 2008).

1.4. LC-PUFA biosynthesis

Two types of enzymes mediate the LC-PUFA biosynthesis in vertebrates, including fish. On the one hand, the elongases of very long-chain fatty acids (Elovl) catalyze the condensation reaction of the FA elongation pathway resulting in the extension of the fatty acyl chain in two carbons (Monroig et al., 2018). On the other hand, fatty acyl desaturases (Fads) enzymes introduce a double bond to PUFA substrates to further unsaturate the hydrocarbon chain. There are two types of desaturases to produce PUFA: front-end desaturases and methyl-end desaturases. Front-end desaturases such as Δ4, Δ5, Δ6 or Δ8 introduce double bonds between the carboxylic end of a molecule and a pre-existing double bond. On the contrary, methyl-end desaturases, such as Δ12 and Δ15 (n-3 desaturases) assist in adding a double bond between a pre-existing double bond and the FA methyl end, converting n-6 FA to n-3 FA (Lee et al., 2016; Shi et al., 2021).

All organisms are able to synthesize stearic acid (18:0), from which they can form oleic acid (18:1n-9, OA) by a Δ9 desaturation (Tocher, 2015). However, vertebrates cannot

form LA (18:2n-6) or ALA (18:3n-3) from OA, due to a lack of desaturases Δ12 and Δ15, respectively (Bell & Tocher, 2009; Tocher, 2015). Thus, these two PUFA are considered essential for vertebrates, including fish, and they must be ingested through the diet. ARA and EPA are biosynthesized from 18:2n-6 and 18:3n-3, respectively, by a Δ6 desaturation, an elongation step, and a Δ5 desaturation (Sprecher, 2000). However, DHA synthesis via the "Sprecher pathway" requires two more elongations from EPA to form 24:5n-3, one Δ6 desaturation and a final chain shortening by peroxisomal β-oxidation (Sprecher, 2000). Additionally, DHA synthesis by a Δ4 desaturation over docosapentaenoic acid (DPA, 22:5n-3) has been also reported (Lee et al., 2016). An alternative pathway to produce 20:4n-3 and 20:3n-6 from 18:2n-6 and 18:3n-3, through an elongation and a subsequent Δ8 desaturation, has also been described for some species (Park et al., 2009).

However, because algae possess n-3 desaturases, they do not show the strict separation of n-6 and n-3 pathways typical of mammals and fish. Thus, the presence of a Δ12 desaturase allows LA biosynthesis from OA, and to form ALA after the Δ15 (n-3 desaturase) activity. Finally, ARA, EPA and DHA are biosynthesized following the same pathways as explained for fish, or via n-3 desaturases, linking the n-6 and n-3 pathways, with those routes varying within algae (Meyer et al., 2004; Shi et al., 2021) (Figure 1.3).

The primary producers of PUFAs are photosynthetic organisms, chiefly microalgae, the main source of EPA and DHA (Harwood, 2019). One of the great advantages of algae culture is that their FA content and composition can be enhanced by changes in their growing conditions (Dineshbabu et al., 2019; Fernandes et al., 2016; Lopes et al., 2020). The availability of nutrients such as vitamins and inorganic co-factors can enhance or inhibit Fads and Elovl enzymes. Increased salinity in the external environment has been related to reduced LC-PUFA in some microalgae species. This has been explained as a reduction in membrane fluidity and permeability due to a stress response to increased intracellular osmotic pressure, thereby preventing the diffusion of potentially harmful ions into the cell. CO_2 level is also known to affect FA desaturation, with lower CO_2 availability increasing PUFA content and higher CO_2 increasing saturated FA (SFA) proportions (Fernandes et al., 2016). Microalgal-based heterotrophic production systems can exhibit n-3 FA productivities 2-3 orders of magnitude greater than those of autotrophic production (Oliver et al., 2020). Finally, an increase in environmental temperature decreases algal contents of PUFA, also influencing lipid class composition

(Harwood, 2019), and, in contrast, lower temperatures lead to higher DHA (Oliver et al., 2020).

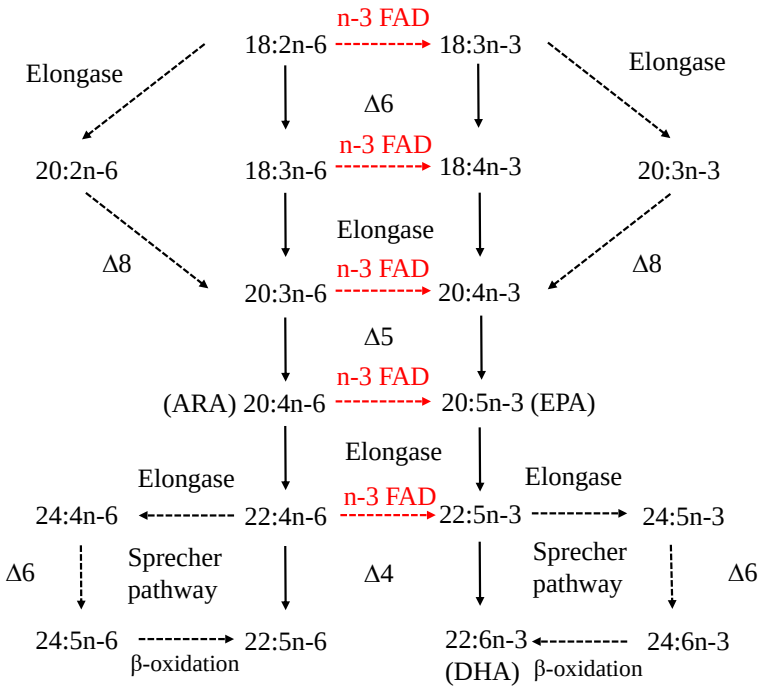


Figure 1.3. PUFA synthesis pathway from C18 precursors to C22 compounds. Modified from Galindo et al. (2021).
In red, n-3 desaturases described for algae and some invertebrates (Monroig and Kabeya, 2018).

1.5. Physiological parameters to assess the status of cultured organisms

Species have different biological and environmental requirements, and consequently, variable responses to rearing conditions. A wide range of physiological, biochemical and behavioral parameters are used to assess welfare and performance, although none of these are considered reliable in isolation, and multiple measures need to be simultaneously taken (Ashley, 2007).

Survival and growth indexes are usually considered primary indicators to evaluate the culture viability of any species. In particular, several factors related to digestion may potentially limit fish growth, including the efficiency of food absorption and conversion,

which depend on meal size and food quality, the availability of digestive enzymes, and the transport capacity in the digestive tract (Lemieux et al., 1999). Thus, digestive enzymes are usually measured to assess the effectiveness of nutritional studies (Gisbert and Mozanzadeh, 2019; Pelusio et al., 2022; Sotoudeh and Mardani, 2018; Vizcaíno et al., 2016). On the other hand, body indexes such as the hepatosomatic (HSI) and viscerosomatic (VSI) indexes are also good indicators of fish health and dietary nutrient utilization (Alam et al., 2020). Finally, as diets have been shown to affect the proximate composition of fish, it is also commonly evaluated changes in protein, lipid, ash and moisture muscle contents, or even fatty acids (Ljubojević et al., 2015) and amino acids profiles (Tu et al., 2020).

Measurement of physiological stress responses is also a valid indicator of animal welfare and health. Stress response is an adaptive function, having an essential role in a short-term perspective. When organisms are not able to cope with chronic stress they may suffer several adverse effects such as reduction in growth, diminished reproductive function, or weakened immune system (Ashley, 2007). It is well known that the primary stress response in fish involves the release of cortisol. Moreover, secondary and tertiary responses cause energy source mobilisation, depletion of glycogen stores, and increase of plasma glucose, together with high muscle activity, anaerobic glycolysis and a rise in plasma levels of lactate. In fact, plasma levels of both glucose and lactate are often used alongside cortisol to assess stress condition (Ashley, 2007; Fazio, 2019).

As oxidative stress and lipid peroxidation analysis are relevant and common procedures in all experiments performed in the present study, a stronger effort has been allocated in this section to explain some aspects regarding oxidative stress and lipid peroxidation status.

Free radicals are molecules with unpair electrons that are unestable and highly reactive towards other molecules. They derive from three elements: oxygen (reactive oxygen species; ROS), nitrogen (reactive nitrogen species) and sulphur (reactive sulphur species). ROS include species such as superoxide radical ($O_2^{\cdot -}$) and hydroxyl radical ($OH^{\cdot}$), nitric oxide (NO), and other species such as hydrogen peroxide (H_2O_2) or singlet oxygen ($^1O_2$), among others (Carocho and Ferreira, 2013; Mesa-Herrera et al., 2019). While reactive nitrogen species derive from the reaction of NO with $O_2^{\cdot -}$, reactive sulphur species are formed by the reaction of ROS with thiols (Carocho and Ferreira, 2013). $O_2^{\cdot -}$ is typically

the first ROS formed, eventually leading to the formation of H_2O_2 and finally to $OH^{\cdot}$ which is, chemically, the most damaging of the ROS (Birnie-Gauvin et al., 2017). While H_2O_2 is not a radical species itself, it has longer life than other oxygen-derived radical species, and it can pass easily through membranes. Furthermore, it can be converted to the highly reactive $OH^{\cdot}$, capable of extracting electrons and protons from macromolecules and thereby, generating more unpaired electrons and radical species (Birnie-Gauvin et al., 2017). In addition, ROS can cause oxidative damage to proteins, lipids, DNA/RNA or sugars. In particular, the presence of double bonds in the LC-PUFA molecule, makes them readily oxidizable, being susceptible to suffering ROS-induced lipid peroxidation (Carocho and Ferreira, 2013). Free radicals are normally produced as part of several phases of aerobic metabolism within the mitochondria, by lipoxygenases and cyclooxygenases during the metabolism of ARA, inflammation processes or physical exercise, among others (Ayala et al., 2014; Carocho and Ferreira, 2013; Pizzino et al., 2017). However, external factors such as increments of temperature, salinity or pollutants may accelerate and promote free radical formation (Birnie-Gauvin et al., 2017; Carocho and Ferreira, 2013). When free radicals are maintained at low or moderate concentrations by the organisms, they play several beneficial roles, having an important function as regulatory mediators in signaling processes (Dröge, 2002). In fact, they are needed to fight pathogens by the host defence system, where phagocytes synthesize and store free radicals, in order to be able to release them and destroy pathogenic microbes (Pizzino et al., 2017).

Homeostasis is controlled by a delicate balance between production and neutralization of ROS by antioxidants (Carocho and Ferreira, 2013). An increased production of ROS or a reduction in antioxidant levels, may cause a disruption in this balance, causing oxidative stress (Ayala et al., 2014; Birnie-Gauvin et al., 2017; Dröge, 2002; Mesa-Herrera et al., 2019). There are several evidences showing that oxidative stress can be responsible, with different degrees of importance, in the origin and/or progression of various human diseases, including cancer, diabetes, metabolic disorders, atherosclerosis, chronic inflammation, and cardiovascular diseases (Dröge, 2002; Pizzino et al., 2017).

To protect themselves against the potential damage caused by ROS, organisms, and particularly their cells, contain various defence systems, including preventing mechanisims formed by antioxidants; either enzymatic or non-enzymatic (Birnie-Gauvin et al., 2017; Mesa-Herrera et al., 2019). Ascorbic acid (vitamin C), glutathione, α-

tocopherol (vitamin E) or carotenoids are primary non-enzymatic antioxidants (Birnie-Gauvin et al., 2017; Carocho and Ferreira, 2013) whereas the main enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPx) or glutathione reductase (GR) (Barata et al., 2005b; Birnie-Gauvin et al., 2017; Carlberg and Mannervik, 1985; Carocho and Ferreira, 2013; Strange et al., 2001) (Figure 1.4). Each enzyme fights against ROS either directly or indirectly. On the one hand, SOD converts O_2^- in H_2O_2; CAT converts H_2O_2 in H_2O; GST acts in the detoxification of endogenous compounds including ROS, or exogenous compounds by glutathione conjugation, and GPx converts H_2O_2 in H_2O, being dependent on glutathione. On the other hand, GR reduces oxidized glutathione (GSSG) to regenerate glutathione (GSH) (Barata et al., 2005b; Birnie-Gauvin et al., 2017; Carlberg and Mannervik, 1985; Carocho and Ferreira, 2013; Strange et al., 2001).

The balance between the production of ROS and antioxidant defences is particularly important in aquatic environments, due to the presence of multitude of stressors such as temperature fluctuations, osmotic stress (anadromous species), changes in oxygen availability, pollution, and other anthropogenic impacts, which can directly affect free radical production (Birnie-Gauvin et al., 2017).

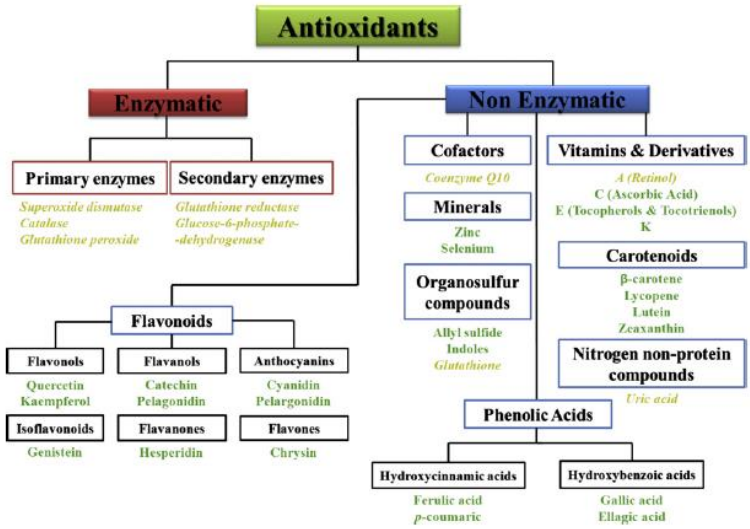


Figure 1.4. Enzymatic and non-enzymatic antioxidants (Carocho and Ferreira, 2013).

A disbalance between ROS and antioxidant defences can cause lipid peroxidation. Lipid peroxidation is a process in which oxidants, including free radicals, attack lipids, especially PUFA, in order to abstract an hydrogen atom from a methylene carbon (Carocho and Ferreira, 2013). Less energy is required for the removal of hydrogen from a carbon double bond than from a methyl carbon, especially when the carbon is between two double-bonds. The hydrogen bonded to this carbon is easily removed and thus lipid peroxidation occurs. The formation of lipid peroxides is not generally affected by the length of the fatty acid chain, being the number of bis-allylic positions the factor that exponentially increases lipid peroxidation (Amaral et al., 2018). Thus, the more double bounds present in the FA the easier it is to remove hydrogen atoms and consequently form a radical; making PUFA (including LC-PUFA) more succestible to radicals than monounsaturated FA (MUFA) and SFA (Carocho and Ferreira, 2013). As a consequence, several compounds such as lipid peroxyl radicals and hydroperoxides are formed (Ayala et al., 2014). Thus, the lipid peroxidation process involves three steps: initiation, propagation and termination (Figure 1.5). In the initiation step, prooxidants, such as free radicals, remove one hydrogen atom from the FA chain, generating a lipid radical. In the second step (propagation), the lipid radical can undergo molecular rearrangement and react with oxygen to form a lipid peroxyl radical that may also abstract hydrogen from other FA, forming new lipid radicals that continues the chain reaction, and lipid hydroperoxides. Termination step occurs when antioxidants donate an hydrogen atom to the peroxyl radical, resulting in the formation of nonradical products (Ayala et al., 2014; Carocho and Ferreira, 2013).

Cells, depending on repair capabilities and metabolic processes, may promote cell survival or induce cell death because of membrane lipid peroxidation. Thus, under subtoxic conditions (low lipid peroxidation) cells maintain their survival, promoting different antioxidant defences and resulting in a stress response. However, under toxic conditions (medium or high lipid peroxidation) and incapability to fight it, cells induce apoptosis or necrosis programmed cell death. Both processes may lead to molecular cell damage that stimulates the development of accelerated aging and pathological states (Ayala et al., 2014).

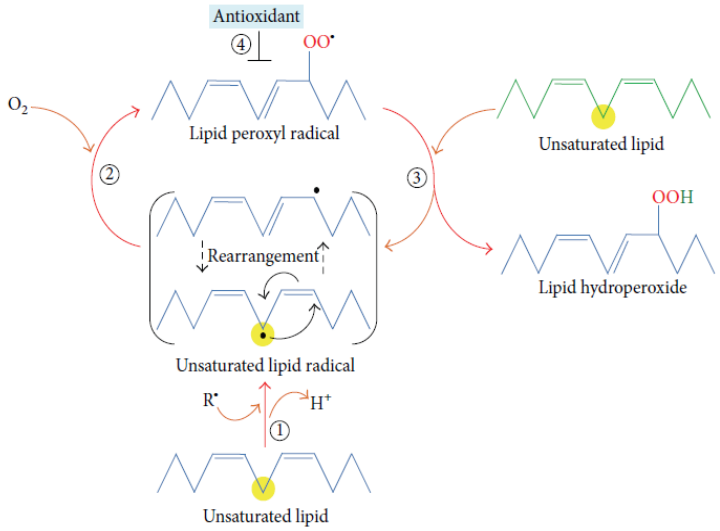


Figure 1.5. Lipid peroxidation process. 1, initiation; 2, propagation; 3, termination; and 4, antioxidant activity (Ayala et al., 2014).

Several oxidation products are produced because of lipid peroxidation, where lipid hydroperoxides are the main and primary products, while aldehydes, including malondialdehyde (MDA) are secondary products formed in the reactions (Ayala et al., 2014; Barata et al., 2005b). Peroxide index (PxI) is a common parameter used to asses primary oxidation products (Yesiltas et al., 2019), while MDA has been traditionally used as a biomarker for lipid peroxidation of FA, because of its facility to react with thiobarbituric acid (TBA). Among other substrates, proteins and DNA are especially vulnerable to changes caused by these aldehydes (Ayala et al., 2014).

1.6. Culture and uses of algae

Algae play an importante role as components of the marine environment, providing many essential ecosystem services. They are key in the uptake of dissolved nutrients and carbon sequestration, also contributing to the global primary production and coastal defence from waves. Furthermore, microalgae are the basis of the marine food-chain being the prime source of n-3 LC-PUFA, while seaweeds are critical habitat-structuring species in coastal ecosystems (Araújo et al., 2021).

Algae are a source of valuable compounds such as polysaccharides, vitamins, minerals, proteins, peptides and amino acids, pigments (chlorophylls, carotenoids, xanthophylls and phycobiliproteins), phenolic compounds, sterols and other lipids like n-3 LC-PUFA (Gómez-Zorita et al., 2020a, 2020b; Lähteenmäki-Uutela et al., 2021; Lopes et al., 2020; Nunes et al., 2020). Several bioactive properties have been attributed to algae or its components. For example, polysaccharides, peptides, carotenoids, and FA have antiaging and antioxidant activities, and mycosporine-like amino acids, present in red algae, have antiphotoaging activity (Lähteenmäki-Uutela et al., 2021). The marine carotenoid fucoxanthin (mainly present in brown algae and diatoms) has been also shown to affect lipid metabolism (Airanthi et al., 2011). Antioxidant, anti-inflammatory, anticancer, antimicrobial and antiobesity properties have been attributed to a wide range of algae (Gómez-Zorita et al., 2020a, 2020b; Lähteenmäki-Uutela et al., 2021).

Macroalgae

In 2018, farmed seaweeds represented 97.1% of total 32.4 million tonnes of wild-collected and cultivated aquatic algae altogether (FAO, 2020). Although 221 species of seaweed are of commercial value, only ten species are intensively cultivated in the world (FAO, 2018). The most cultivated species worldwide are *S. japonica* and *Undaria pinnatifida* (brown algae); *Eucheuma* sp., *Gracilaria* spp., *Kappaphycus* spp., *Porphyra* spp. (red algae), and *Monostroma* spp. and *Enteromorpha* spp. (green algae). Among them, *S. japonica* was the most cultivated seaweed species (33% of total production), followed by *Euchema* spp. (17%) (FAO, 2018; Lähteenmäki-Uutela et al., 2021).

Seaweed farming is practised in a relatively small number of countries, dominated by East and Southeast Asia. However, it is gaining increasing attention in occidental society to develop climate and environmentally friendly bioeconomy. Thus, the world production of seaweeds has more than tripled in the last 20 years, raising up from 10.6 million tonnes in 2000 to 32.4 million tonnes in 2018 (FAO, 2020; Lopes et al., 2020). The rapid growth in the farming of tropical seaweed species in Indonesia as raw material for carrageenan extraction has been the major driver in the rise of farmed seaweed production in the past decade. Despite of this, in the most recent years, global production of farmed algae, dominated by seaweeds, experienced a relative deceleration, mainly due to a slowdown in the output of tropical species and a reduced production in Southeast Asia, while seaweed farming production of temperate and coldwater species was still on the rise (FAO, 2020).

The global seaweed market is lead by Asia (mostly China, Japan and Indonesia), followed by Europe, Latin America and North America. In Asia, cultivation of seaweeds has been practised for decades (Campbell et al., 2019), with China as the main world producer in terms of both value and volume (FAO, 2018) whereas in Europe it has only recently been a commercial activity (Campbell et al., 2019). Information of macroalgal production in Europe is not always reliable. Due to the small volume of seaweed production compared to other biomass sources, usually there are no national legal obligations to report detailed data of production, so available information is frequently fragmented, incomplete, and generally of low quality (Araújo et al., 2021). Including both harvesting from wild stocks and aquaculture, Spain, France and Ireland are the main producers in the European Union by numer of production companies. However, the largest seaweed biomass volumes are produced in Norway, France and Ireland. In Europe, harvesting from wild stocks is the major production method for macroalgae, mainly hand harvesting rather than mechanical. On the contrary, aquaculture production is still at an early stage of development, representing less than 1% of the total seaweed biomass production. The most produced species by aquaculture in Europe is *Saccharina latissima* (considering both production volume and number of companies), while the most collected seaweed by volume is *Laminaria* spp. (Araújo et al., 2021).

An important advantage of macroalgae culture is that their production does not need land, fertilization, or freshwater (Lähteenmäki-Uutela et al., 2021). Furthermore, its production is varied and versatile, and can be used in different formats (fresh, dried, powder, flakes, salted, canned, liquid extracts or as prepared foods) for multiple applications (Buschmann et al., 2017). Hence, farmed seaweeds can be used for direct human consumption, although low-grade products and scraps from processing factories are used for other purposes, including ingredients for food production (Buschmann et al., 2017; FAO, 2020). Algae are also potential candidates to be alternative substrates to several natural resources (Campbell et al., 2019). Interest in macroalgae as raw material is actually increasing by the health-promoting potential as food ingredients and nutraceuticals, and the significant development of natural cosmetics, bio-based materials (biopolymers and bioplastics) and biofuels (Araújo et al., 2021; FAO, 2018; Kumari et al., 2013; Lähteenmäki-Uutela et al., 2021; Tabassum et al., 2017; Verma et al., 2017; Zárate et al., 2020). They are also used for the extraction of pharmaceutical, biomedical and biotechnological resources (alginate, agar-agar and carrageenan), and can be also used as

fertlizer and plant biostimulant (Khan et al., 2009). Beach cast algae and wild macroalgae populations have been utilized as animal feed and as a source of potash, iodine, and algal polysaccharides (Lähteenmäki-Uutela et al., 2021). Moreover, adding seaweeds to livestock feed has been proposed as a novel way to reduce methane emissions from cattle (FAO, 2018). New applications such as bioremediation and biomonitoring are also currently being explored (Araújo et al., 2021).

Nowadays, most production companies in Europe direct their seaweed biomass to food, food-related uses (food supplements, nutraceuticals, hydrocolloid production) and feed components. Cosmetics, fertilizers and biostimulants also contribute to the biomass uses, while biofuels, bioremediation, biomaterials or pharmaceuticals have only a small distribution within European countries (Araújo et al., 2021). The consumer view of seaweed as a type of healthy "superfood", and the increasing body of evidence supporting that consumption of algal food/feed products may have health and nutritional benefits, are primarily responsible for seaweed product innovation (Araújo et al., 2021; Buschmann et al., 2017; FAO, 2018; Galindo et al., 2022). However, the impacts of seasonal and geographical variation on the composition and nutritional value of algal biomass is still an issue. Likewise, digestibility and bioavailability of many 'beneficial seaweed compounds' to humans or animals is not yet clear. This lack of information affects exploitation, storage and processing (Buschmann et al., 2017). Thus, it is necessary to develop a large-scale seaweed aquaculture in Europe to supply its increasing demand as well as to promote research to solve those unanswered issues (Campbell et al., 2019).

The use of algae in feed for marine organisms has been extensively studied in the last few years (Abdala-Díaz et al., 2021; Güroy et al., 2013; Sáez et al., 2020; Sotoudeh and Mardani, 2018; Vizcaíno et al., 2019). An adequate percentage of algae inclusion in aquafeeds has been reported to exert several benefits in some species, including greater growth performance and feed utilisation efficiency, improved protein retention, increase in carcass quality, intestinal microbiota or disease resistance, heightened stress response and modulation of the lipid metabolism. However, high algae inclusion in fish feed might have a negative effect on fish growth and feed efficiency, which have been attributed to certain substances with anti-nutritional activity that may be present in algae, like lectins, tannins, phytic acid, and protease and amylase inhibitors (Lähteenmäki-Uutela et al., 2021; Norambuena et al., 2015). Anti-nutritional substances may hamper the digestibility

and bioavailability of nutrients (Vizcaíno et al., 2019). Thus, a decrease in activity of several digestive enzymes such as alkaline proteases, lipase and pepsin with seaweed inclusion has been described (Peixoto et al., 2016a; Sotoudeh and Mardani, 2018; Vizcaíno et al., 2016; Xuan et al., 2013; Zhu et al., 2017, 2016). However, contradictory results have been reported depending on factors such as fish species and age, dietary inclusion level, type of seaweed, duration of feeding, mode of supplementation and even rearing conditions (Sotoudeh and Mardani, 2018).

Microalgae

Although the farming of microalgae fits into the widely accepted definition of aquaculture, its cultivation tends to be tightly regulated and monitored at the national or local level separately from aquaculture. FAO estimated 87,000 tonnes of farmed microalgae from 11 countries in 2018, of which 86,600 tonnes were reported from China (FAO, 2020). Nonetheless, data understate the real scale of world microalgae production as information from important producers such as Australia, Czechia, France, Iceland, India, Israel, Italy, Japan, Malaysia, Myanmar and the United States of America is unavailable (FAO, 2020). Microalgae production in Europe is lead by Germany, France and Spain. Those three countries and Italy have the largest number of the cyanobacteria *Arthrospira* (Spirulina) spp. producers. Official statistics on microalgae production volumes are almost non-existent at the European scale and the data available are limited and fragmented. In fact, the lack of knowledge on the production of microalgae has been recognized as a limiting factor for evaluating the potential of this sector in Europe (Araújo et al., 2021). In spite of this, Araújo et al. (2021) estimated a production of 182 tons of dry weight (DW) of microalgae and 142 tons of *Arthrospira* spp. However, this information must be taken with caution as they are a rough estimate and some countries and companies could not be included in the study.

The most cultivated species in Europe by number of companies are *Chrolella* spp., *Nannochloropsis* spp., *Haematococcus pluvialis* and the cyanobacteria *Arthrospira* spp., with *Chrolella* spp. and *Arthrospira* spp. being also the most cultivated by volume. These four species are also the most exploited species for biotechnological applications worldwide. *Tetraselmis* sp., *Isochrysis galbana*, *Dunaliella salina*, *Phaeodactylum tricornutum*, *Porphyridium* sp., and *Scenedesmus* sp. are the top produced species in Europe (Araújo et al., 2021; FAO, 2020).

Effects of new microalgae and macroalgae products on live prey and fish performance

Microalgae production has demonstrated environmental benefits, although it also has adverse impacts related to the high water and energy demand, management of wastewater, emission control, land use and risk of microbal contamination. Microalgae are widely cultivated by different production methods and systems, with some commercial plants combining photoautotrophic or heterotrophic cultures. Photobioreactor (PBR) is the most common system used for microalgae production, followed by open ponds and fermenters, while *Arthrospira* spp. is mainly produced in open ponds. PBR are used for the production of high-value low-volume products for nutraceuticals, cosmetics, and pharmaceuticals, contrarily to open ponds, which are commonly used in the production of biomass for low-value applications (Araújo et al., 2021).

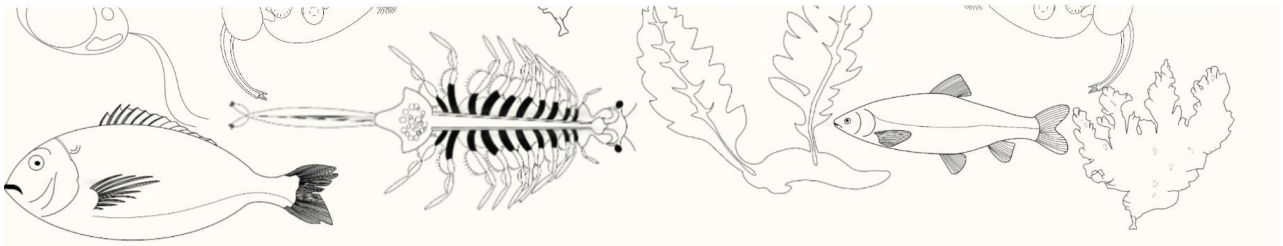
The culture of microalgae is affected by environmental (nutrient availability, temperature, pH, salinity, inorganic carbon, oxygen, light intensity and CO_2), and technical factors (stirring and mixing, width and depth of the bioreactor, harvest frequency or dilution rate) (Khan et al., 2018). Although industrial microalgae production was already established a long time ago, it implies high production costs, and some technological constraints and gaps in the scientific understanding of large scale cultivation, limiting biomass commercialization as high-value products excluding large scale low-cost applications such as fuel (Araújo et al., 2021).

The market value of microalgae is mainly based on their potential as source of high-value bioactive and functional compounds (Araújo et al., 2021; FAO, 2020; Khan et al., 2018). Besides, their biochemical composition can be controlled by environmental conditions. Thus, modifying culture parameters can potentiate microalgae accumulation of proteins, lipids and carbohydrates (Dineshbabu et al., 2019). The lipid content can double or even triple in microalgae exposed to certain nitrogen limitations (Khairy and El-Sayed, 2012). The synthesis of pigments, antioxidants, n-3 LC-PUFA, sterols and vitamins makes microalgae suitable for its use in fish farming, human health, or cosmetic applications, while SFA, MUFA and hydrocarbons are useful for biodiesel production (Dineshbabu et al., 2019; Fernandes et al., 2016). The use of microalgae has been proposed as a source of nutrients and high-value extracts that can be added to food products and feed to increase their nutritional value (Dineshbabu et al., 2019). Several species, including *Chlorella* spp., *Arthrospira* spp., *D. salina* or *H. pluvialis* produce compounds associated with potential health benefits such as antioxidant, antifungal, antiviral, anticarcinogenic, anti-inflammatory, antimicrobial and immune system stimulant. The extracts of some

microalgae species have been used in the cosmetic industry for skin regeneration, moisturizing, anti-aging, and protection from UV radiation. However, further studies are still needed to understand the health-related benefits of these extracts and the bioavailability of its bioactive compounds (Araújo et al., 2021; Khan et al., 2018). Microalgae have been also proposed as a promising feedstock for biofuel production due to their high lipid content. In addition, they do not compete for arable land with food and feed crops, and they can be cultivated in different environments, including sea or fresh water, and even polluted water (Araújo et al., 2021; Chew et al., 2018; Khan et al., 2018). However, further technological developments are imperative to upscale the production volumes and reduce the production costs that can turn biofuel manufacturing from microalgae biomass a reality in the near future (Araújo et al., 2021; Khan et al., 2018).

Chlorella spp., *Nannochloropsis* sp., and *I. galbana* are the most common microalgae species used as feed in aquaculture and animal farming (Araújo et al., 2021; Chew et al., 2018), demonstrating several benefits to bovine, ovine, poultry, and pig industries. In the aquaculture sector, apart from providing valuable nutrients to the aquatic organisms, they also render immunostimulant and disease resistant properties. Their use as fish feed or feed supplement could reduce the burden on small pelagic fish stocks of fishmeal and FO-based aquaculture, and in turn, help reducing the gap between fish production and demand (Dineshbabu et al., 2019). In particular, microalgae are an important source of EPA and DHA, and can be directly incorporated into live preys for larvae and in juvenile feeds. Astaxanthin from *H. pluvialis* is also used as coloring pigment in aquaculture, having a powerful antioxidant effect in marine environments (Araújo et al., 2021). Hence, microalgae have an essential function as feed for zooplankton which serve as live prey for fish, improving their nutritional value (Dineshbabu et al., 2019) and environmental conditions.

Objectives



2. OBJECTIVES

The overall goal of this study was to evaluate different microalgal and macroalgal products for aquaculture live preys production, and as fish feed additives, respectively.

In order to meet this main objective, the following specific objectives were established:

- Test products from *Isochrysis galbana* and *Navicula salinicola* for live preys production, with or without the addition of a lipid emulsion
    - Study the survival of rotifer and *Artemia* fed *I. galbana* and *N. salinicola*.
    - Determine the total antioxidant activity of *I. galbana* and *N. salinicola* and their antioxidant potential on live prey.
    - Analyze the potential of *I. galbana* and *N. salinicola* as enrichment products, through their detailed lipid characterization.
- Characterise the lipid profile of macroalgal species from the Macaronesian region, and assess their potential as food or feed additive.
- Evaluate the use of different inclusion percentages of multispecific and monospecific macroalgal wracks as feed additive for *Ctenopharyngodon idella* and *Sparus aurata* juveniles:
    - Study the effect of macrolagal wracks as feed additives on the performance and biochemical composition of fish.
    - Analyze the antioxidant potential of macroalgal wracks on fish defence system.
    - Investigate the effect of macroalgal wracks on fish digestive capacities.
    - Determine the effect of macroalgal wracks on plasma parameters in *S. aurata*.

Common material and methods

3. COMMON MATERIAL AND METHODS

This section describes the common material and methods performed in biochemical and statistical analysis used along the different chapters. The specific methodology used in each experiment will be detailed in the corresponding section.

Most analysis were carried out at the laboratory of Animal Physiology (Departamento de Biología Animal, Edafología y Geología. Universidad de La Laguna; ULL). The exceptions will be specifically mentioned.

3.1. Lipid composition

3.1.1. Total lipid extraction

The total lipid (TL) of samples was extracted as described by Folch et al. (1957) with small modifications (Christie and Han, 2010). Samples were homogenized in chloroform/methanol (2:1, v/v) using a Virtis rotor homogenizer (Virtishear, Virtis, Gardiner, New York) and a quarter volume of 0.88% (w/v) potassium chloride (KCl) subsequently added to the homogenate. After vigorous shaking, samples were centrifuged under cold conditions at 716 x g for 5 min, the organic solvent collected, filtered through a filter paper (Filter-Lab, Barcelona, Spain), and evaporated under a stream of nitrogen. Lipids were transferred to pre-weighed crystal vials of 1.7 mL capacity, the organic solvent evaporated under a stream of nitrogen and maintained in a desiccator overnight in the dark. The lipid content was determined gravimetrically, resuspended at 10 mg mL^{-1} in chloroform/methanol (2:1, v/v) containing 0.01% (w/v) butylated hydroxytoluene (BHT; Sigma-Aldrich Co., St. Louis, Missouri, USA) as antioxidant, and stored at -20ºC under an inert atmosphere of nitrogen.

3.1.2. Lipid classes

Lipid classes (LC) were analysed by the method of Olsen and Henderson (1989) with minor modifications following Reis et al. (2019). Briefly, a 30 µg aliquot of TL was used to develop a high-performance thin-layer chromatography (HPTLC) in a single-dimensional double-development. Firstly, polar lipids were separated using 1-propanol/chloroform/methyl acetate/methanol/0.25% KCl (5:5:5:2:1.8, v/v), and secondly, a mixture of hexane/diethyl ether/acetic acid (20:5:0.5, v/v) was used for the neutral lipids. The plates were sprayed with 3% (w/v) aqueous cupric acetate containing 8% (v/v) phosphoric acid and burnt at 160ºC. LC were qualitative quantified by calibrated

densitometry using a dual-wavelength flying spot scanner CAMAG TLC Visualizer (Camag, Muttenz, Switzerland), as described by Reis et al. (2019). It was assumed that all LC had the same response factors, using the percentage composition derived directly from the densitometer, as suggested by Olsen and Henderson (1989). LC identification was performed by comparison to external lipid standards (cod roe lipid extract; digalactosyl-diacylglycerol (DGDG), monogalactosyl-diacylglycerol (MGDG), and sulfoquinovosyl-diacylglycerol (SQDG) (Avanti Polar Lipids, Inc., Alabaster, Alabama, USA)) placed on the same HPTLC plate (Figure 3.1).

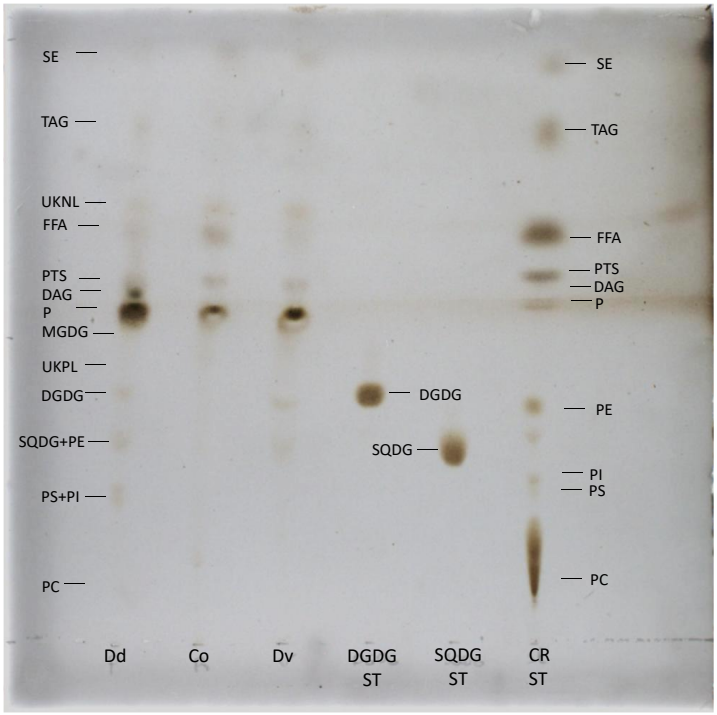


Figure 3.1. Thin layer chromatography of macroalgae lipid classes.
Dd, *Dictyota dichotoma* (Ochrophyta); Co, *Corallina officinalis* (Rhodophyta); Dv, *Dasycladus vermicularis* (Chlorophyta); DGDG ST, digalactosyl-diacylglycerol standard; SQDG ST, sulfoquinovosyl-diacylglycerol standard; CR ST, cod roe standard. SE, sterol esters; TAG, triacylglycerols; UKNL, unknown neutral lipids; FFA, free fatty acids; PTS, phytosterols; DAG, diacylglycerols; P, pigments; MGDG, monogalactosyl-diacylglycerol; UKPL, unknown polar lipids; DGDG, digalactosyldiacylglycerol; PE, phosphatidylethanolamine; SQDG, sulfoquinovosyl-diacylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine.

3.1.3. Fatty acid composition

A 1 mg aliquot of lipid extracts were used to obtain fatty acid methyl esters (FAME) by acid-catalyzed transmethylation using toluene and 1% sulphuric acid in methanol (v/v) for 16 h at 50°C (Christie and Han, 2010). FAME were purified by thinlayer chromatography using 20 x 20 cm plates coated with silicagel (Macherey-Nagel, Duren, Germany) with hexane/diethyl ether/acetic acid (90:10:1, v/v) as solvents (Christie and Han, 2010), and subsequently separated and quantified using a TRACE-GC Ultra gas chromatograph (Thermo Scientific, Milan, Italy) equipped with an on-column injector, a flame ionization detector and a fused silica capillary column Supelcowax® 10 (30 m × 0.32 mm ID, df 0.25 µm) (Supelco Inc., Bellefonte, Pennsylvania, USA). Chromatography conditions were programmed as detailed by Galindo et al. (2022). Helium was used as the carrier gas at 1.5 mL min^{-1} constant flow, and temperature programming was 50 to 150ºC at a rate of 40ºC min^{-1}; from 150ºC to 200ºC at 2ºC min^{-1}; from 200ºC to 214ºC at 1ºC min^{-1} and, finally, up to 230ºC at 40ºC min^{-1}, which was maintained for 3 min. Individual FAME were identified by comparison to a mixture of authentic standards (Mix C4-C24 and PUFA No. 3 from menhaden oil (Supelco Inc.)) and to a well characterized cod roe oil. When necessary, the identity of FAME was confirmed by GC-MS (DSQ II, Thermo Scientific).

Prior to transmethylation, a 5% of nonadecanoic acid (19:0) was added to the lipid fraction as an internal standard. Total FA contents are expressed as mg FA 100 g^{-1} or µg FA mg^{-1} depending on the sample, and as percentage of total FA for individual FA.

3.2. Antioxidant activities and oxidative status

3.2.1. Total antioxidant capacity (TAC)

- Algal biomass extraction and preparation of stock solutions

Samples of macroalgal wracks and different formats of microalgae (fresh, frozen, and spray-dried) were used to obtain extracts for the TAC study. Sequencial extraction with organic solvents was used for seaweed wracks, while microalgae extracts were obtained by a liquid-liquid extraction. All procedures were developed at the “Instituto Universitario de Bio-Orgánica Antonio González” (ULL).

On the one hand, a 25 g sample of each dried seaweed wrack was successively extracted three times each with *n*-hexane, ethyl acetate and ethanol (250 mL x 24 hours) by

maceration at room temperature with continuous stirring, and the filtered solution was concentrated under vacuum to obtain the extracts.

On the other hand, a 25 g of fresh/frozen paste or spray-dried format sample of each microalgae were successively extracted three times with ethanol (250 mL x 24 hours) by maceration at room temperature with continuous stirring, and the filtered solution was concentrated under vacuum to obtain the extract. Subsequently, samples were submitted to a liquid-liquid extraction with *n*-hexane and ethyl acetate (3 times each × 300 mL), and the filtered solutions were also concentrated undern vacuum. Resultant aquaeus extracts were lyophilized and finally weighed.

Dried extracts and a standard solution of Trolox were dissolved in sterile dimethyl sulfoxide (DMSO) using a sonication bath for 3-4 min at a final concentration of 50 mg mL^{-1} (Zárate et al., 2020).

- ABTS radical scavenging assay

Antioxidant activity was assayed by the 2,20-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay (Re et al., 1999; Zárate et al., 2020). Reaction was prepared by mixing 7 mM ABTS (w/v) and 2.4 mM potassium persulfate solution (w/v) for 12-16 h at room temperature in the dark. Resultant solution was then diluted in methanol in order to obtain an absorbance of 0.7 at 734 nm. Stock solutions of samples in DMSO were disolved in methanol at 500 µg mL^{-1}. An initial screening of all samples was developed in 96-well microplates at a final concentration of 250 µg mL^{-1}. A control in which sample was substituted by solvent was also prepared in each plate. ABTS solution was added to the microwells, and after 8 min of incubation, the absorbance was recorded at 750 nm with a BioRad Microplate Reader Model 680 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Percentage of antioxidant activity (% AA) was calculated following the equation (1):

(1) $\% AA = [(Abs_{control} - Abs_{sample})]/(Abs_{control})] \times 100$

where $Abs_{control}$, is the absorbance of ABTS radical + methanol; Abs_{sample}, is the absorbance of ABTS radical + sample/standard.

Samples with more than 50% of AA were selected to test different product concentrations, ranging between 0.244 and 250 µg mL^{-1} in methanol. The same procedure was applied to a standard solution of Trolox (0.098-100 µg mL^{-1}). Concentration yielding 50%

scavenging of ABTS (IC_{50}) of each sample was calculated by interpolation from the % AA *vs*. concentration curve.

- DPPH radical scavenging activity

The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay was conducted following Blois (1958). Samples in DMSO were dissolved in methanol at 500 µg mL^{-1} and an initial screening was carried out testing a final concentration of 250 µg mL^{-1} in methanol. All assays were developed in 96-well microplates, with a control being also added. DPPH dissolved in methanol (45 µg mL^{-1}) was added to the microwells, and the absorbance was measured at 515 nm after 30 min in darkness. % AA was calculated following equation (1), calculating the IC_{50} for samples with % AA higher than 50% (Zárate et al., 2020).

ABTS and DPPH techniques were performed at the Faculty of Sciences and Technology (University of the Azores) under the supervision of Dr. Maria do Carmo Barreto, thanks to a learning stay grant from ULL and CajaSiete.

3.2.2. Lipid peroxides

Peroxide index (PxI) was determined following Shantha and Decker (1994) with small modifications. Briefly, an aliquot of TL (from 1.5 to 4 mg, depending on the sample) was dissolved in 10 mL of chloroform/methanol (7:3, v/v), and 50 µL of ferrous chloride ($FeCl_2$) and hydrochloric acid (HCl) solution, together with 50 µL of ammonium thiocyanate (NH_4SCN) added. After vigorous shaking, and soak for 5 min of reaction, absorbance was measured at 500 nm. The same procedure was applied to a ferric chloride ($FeCl_3$) standard curve in order to quantify PxI concentration. The concentration of lipid peroxides was calculated following equation (2) and expressed as meqO_2 Kg^{-1}.

(2) PxI (meqO_2 Kg^{-1}) = [(Abs- b/a)/(m x Fe atomic weight x 100)]

Where Abs, is the sample absorbance at 500 nm; b, is the intercept; a, is the slope of the pattern line; m, is the lipid mass (g); Fe atomic weight, is 55.845.

3.2.3. TBARS and antioxidant enzymes

Prior to the analysis of thiobarbituric acid reactive substances (TBARS) and antioxidant enzymes, 300 mg of each sample was homogenized using a Virtis rotor homogenizer (Virtishear, Virtis) in an ice-cold 20 mM Tris-Cl (w/v) buffer (pH 7.4) with protease inhibitors (Complete®, Sigma, Madrid, Spain). Muscle, liver or live prey samples were

then centrifuged at 10,000 x g for 5 min at 4ºC, and supernatants aliquoted and stored at -80°C until analysis.

- TBARS assay

TBARS assay was developed in order to determine samples MDA content. A 144 µL sample homogenate was incubated for 60 min at 95ºC in a solution containing 36 µL of 8.1% (w/v) sodium dodecyl sulfate with 0.05% (w/v) BHT, 270 µL of 20% (v/v) acetic acid (pH 3.5) and 270 µL of 0.8% (w/v) thiobarbituric acid (Ohkawa et al., 1979). At the end of this period, samples were cooled in ice, mixed with 180 µL of Milli-Q water together with 900 µL of n-butanol/pryridine (15:1 v/v), and centrifuged at 10,000 x g for 3 min at 4ºC. Finally, 200 µL of supernatant were fluorimetric determined with excitation at 530 nm and emission at 550 nm in a multi-well plate reader (Thermo Scientific Appliskan, Thermo Fisher Scientific, Vantaa, Finland). MDA content was calculated using a standard curve of 1,1,3,3-tetramethoxipropane and expressed as nmol MDA mg protein^{-1}.

- Antioxidant enzyme activities

Superoxide dismutase (SOD, EC 1.15.1.1) catalase (CAT, EC 1.11.1.6), glutathione reductase (GR, EC 1.6.4.2), and glutathione-S-transferase (GST, EC 2.5.1.18) activities were determined. Absorbances were measured in a spectrophotometer (Beckman Coulter DU800 Fullerton, California, USA) and one unit of activity defined as µmol min^{-1} unless otherwise stated.

  - SOD determination

The antioxidant enzyme SOD, represents the first line of defence against ROS (Berwal and Ram, 2018). It catalyzes the conversion of superoxide to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2; equation (3)). Activity was analysed following Mesa-Herrera et al. (2019), which is based on the inhibition of the autooxidation of pyrogallol (1,2,3-trihydroxybenzene) to purpurogallin. Briefly, homogenates were mixed with tris-cacodylic buffer (50 mM Tris-HCl, 50 mM cacodylic acid, 1 mM diethylenetriamine pentaacetic acid, pH 8.2), with 30 mM pyrogallol as substrate. The auto-oxidation of pyrogallol and the inhibition of this reaction were monitored spectrophotometrically at 420 nm every 20 seconds for 10 min at 25ºC. One unit of SOD activity is equivalent to the amount of enzyme that produces a 50% inhibition of the auto-oxidation of pyrogallol.

(3) $O_2^- + O_2^- + 2\ H^+ \longrightarrow H_2O_2 + O_2$

- CAT determination

CAT catalyzes the conversion of H_2O_2 to water and O_2 (equation (4)). This enzyme constitute the second tier of defence against ROS (Tehrani and Moosavi-Movahedi, 2018). Enzymatic activity was analysed following Clairborne (1985), with 485 mM H_2O_2 as substrate. Degradation of H_2O_2 was measured in a 10 mM potassium phosphate buffer (pH 7.0) at 240 nm every 30 seconds and 25ºC during 15 min. CAT activity was calculated using the molar extinction coefficient of H_2O_2 ($\varepsilon = 42.6\ M^{-1}\ cm^{-1}$).

(4) $H_2O_2 + O_2 \longrightarrow H_2O + O_2$

- GR determination

GR enzyme has a key role in regenerating GSSG to GSH via a NADPH-dependent mechanism to maintain the GSH/GSSG balance (equation (5)) (Aydemir et al., 2019). Its activity was measured using a 0.1 M sodium phosphate buffer (pH 7.0), with 1 mM GSSG and 60 µM NADPH as substrates. Oxidation of NADPH was determined at 340 nm, 25ºC during 15 min every 20 seconds. The molar extinction coefficient used was -6.22 $mM^{-1}\ cm^{-1}$ (Chung et al., 1991).

(5) $GSSG + NADPH + H^+ \longrightarrow 2\ GSH + NADP^+$

- GST determination

GST is a key enzyme in several detoxification processes and it is a biomarker of oxidative stress (Biller and Takahashi, 2018; Nadarajapillai et al., 2021). GST was determined as described by Habdous et al. (2002). Briefly, the reaction was developed in a 0.1 M phosphate buffer (pH 6.5) with 5 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as substrates. Activity was determined following the conjugation of GSH with CDNB and the absorbance of the Mesenheimer complex produced measured at 340 nm every 30 seconds for 15 min at 25ºC. GST activity was quantified using the molar extinction coefficient of Mesenheimer complex ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$).

3.3. Digestive enzymes

Panceatic (α-amylase, bile salt-activated lipase (BAL), total alkaline proteases) and gastric (pepsin) enzyme activities were determined in the stomach or gut of fish according to Solovyev et al. (2016). Absorbance was read using a spectrophotometer and specific activity expressed as U mg $protein^{-1}$.

Prior to analysis, stomach and gut samples were homogenized using a Virtis rotor homogenizer (Virtishear, Virtis) in 10 volumes (v/w) of ice-cold Milli-Q water, centrifuged at 3,300 x g for 3 min at 4ºC, and supernatants kept at -80ºC until enzymatic quantification.

- Alpha-amylase (E.C. 3.2.1.1)

Alpha-amylase was assayed using 0.3% soluble starch dissolved in Na_2HPO_4 buffer (pH 7.4) according to Métais and Bieth (1968). The absorbance was measured at 580 nm after stopping the reaction with 1 N HCl and the addition of 2 mL of N/3000 iodine solution (Merck, Darmstadt, Germany). Alpha-amylase activity was defined as the mg of starch hydrolysed at 37ºC per 30 min per mL.

- Bile salt-activated lipase (BAL, E.C. 3.1.1)

BAL activity was analysed by incubation with p-nitrophenyl myristate dissolved in 0.25 mM Tris-HCl, pH 9.0, 0.25 mM 2-methoxyethanol, and 5 mM sodium cholate buffer for 30 min at 30ºC. Acetone/n-heptane (5:2, v/v) was used to stop the reaction. Samples were then centrifuged at 6,000 x g for 2 min at 4°C, and the increase in absorbance of the supernatant determined at 405 nm (Iijima et al., 1998). BAL activity corresponded to the µmol of myristate hydrolysed per min per mL.

- Alkaline proteases

Alkaline proteases activity was determined as described by García-Carreño and Haard (1993). Briefly, samples were incubated at 24ºC for 60 min using azocasein (0.5%) in Tris-HCl 50 nmol L^{-1} (pH 9) as substrate, and the reaction stopped with 20% trichloroacetic acid (TCA). After centrifugation at 10,000 x g for 5 min, the absorbance of the supernatant was read at 366 nm. One unit of activity was defined as 1 µmol of azo dye released per min per mL.

- Pepsin

Pepsin activity was quantified by incubating the extracts with 2% hemoglobin solution in 1 N HCl (pH 2.0) for 60 min at 37°C. The reaction was stopped with 5% TCA, the extract centrifuged at 4,000 x g for 6 min at 4°C, and the absorbance of the supernatant read at 280 nm (Worthington Biochemical Coorporation, 1972). One unit of activity was defined as the µmol of tyrosine released per min per mL.

3.4. Determination of soluble protein

Soluble protein of homogenised samples for analysis of TBARS, antioxidant and digestive enzymes activities was quantified following Bradford (1976), using bovine serum albumin as standard.

3.5. Proximate composition

The moisture content of diets and fish muscle was calculated by drying the samples in an oven at 110ºC until constant weight (AOAC, 2006). Crude protein was determined by conversion of the nitrogen content following Kjeldahl's method. Ash content was estimated by dry-ashing in a furnace at 450ºC for 24 h (AOAC, 2006).

3.6. Plasma parameters

In the experiment with *S. aurata* juveniles, blood was also extracted at the end of the experimental period in order to measure some plasma constituent levels using standard veterinarian clinic assay kits. After blood extraction with a heparinized syringe, the sample was divided into two portions. In the first one, hematocrit was determined by capillary diffusion and centrifugation. The remaining blood was then centrifuged for 20 min at 700 x g and 4°C in a microcentrifuge, and the plasma collected and stored at -80°C until further analysis. Plasma biochemical parameters determined were glucose (BioSystems, Barcelona, Spain) lactate, sodium, chloride (Spinreact, Girona, Spain) and cortisol (Arbor Assays®, Ann Arbor, Michigan, USA).

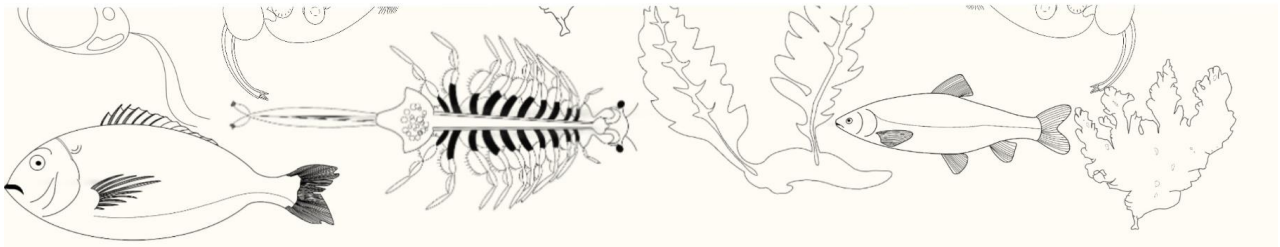
3.7. Statistical analysis

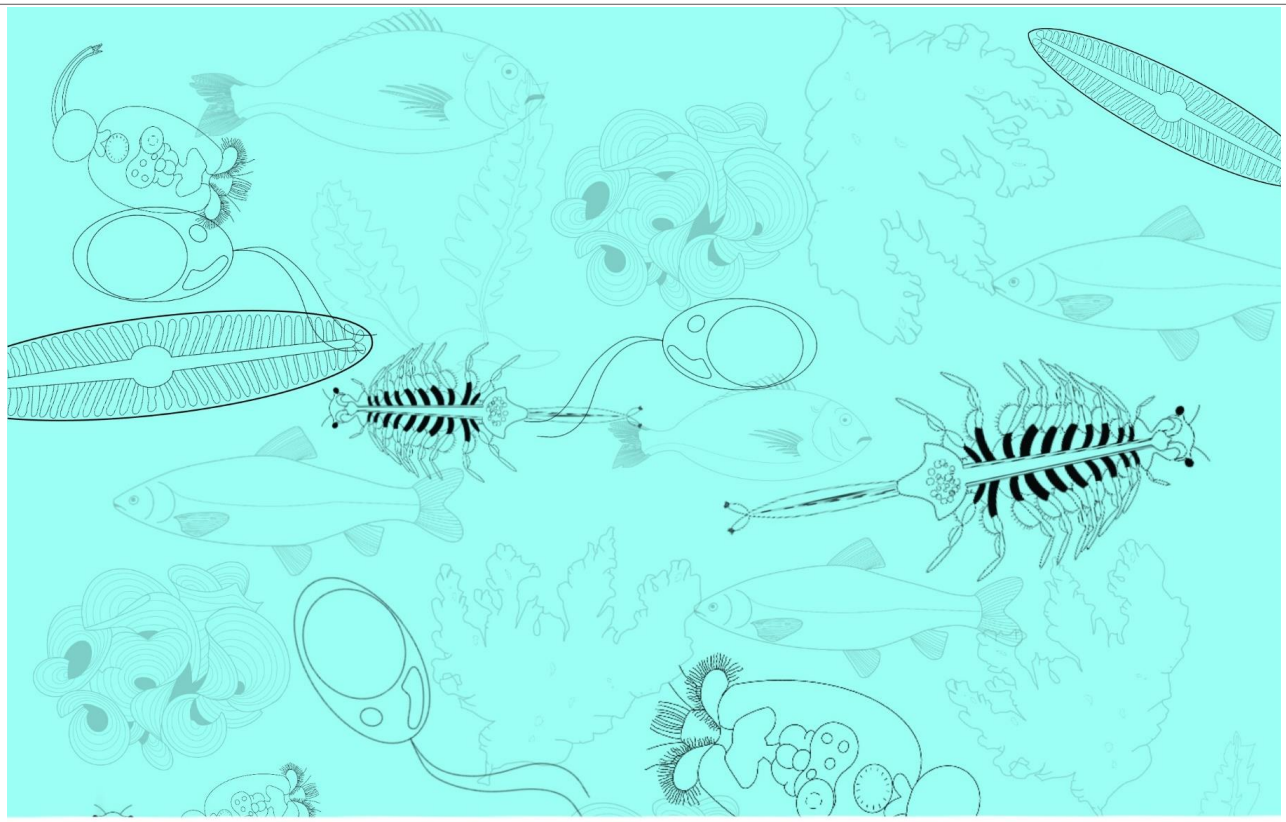
Before analysis, normality and homogeneity of data were confirmed within groups and, where necessary, appropriate variance stabilizing transformations (arcsine and logarithm) were performed. Significant differences in lipid composition (TL, LC and FA composition) as well as peroxide indexes, antioxidant and digestive enzymes activities, plasma parameters, and proximate composition between treatments were tested by one-way ANOVA followed by a Tukey HSD post-hoc test. When transformations did not succeed, Welch test followed by the Dunnett T3 test were performed. Student's t-test or Mann-Whitney tests for normal or non-normal distribution of data, respectively, were also used when necessary.

For the lipid composition of macroalgae from Madeira Archipelago and Gran Canaria Island, two principal component analyses (PCA), one for the LC and the other for the main FA, of all macroalgae were also carried out. Two hierarchical cluster analyses performed with the Ward linkage method and the squared Euclidean distances, subsequently used factor scores to identify macroalgae with similar LC and FA profiles.

Results are presented as means ± standard deviation (SD) and the statistical significance was set at $p<0.05$. All statistical analyses were performed using the IBM® SPSS Statistics 25.0 software package (IBM Corp., New York, USA) for Windows.

Results





Use of microalgae in live prey production

4. RESULTS

4.1. USE OF MICROALGAE IN LIVE PREY PRODUCTION

4.1.1. Specific introduction

After consuming yolk sac, most marine larvae are in an undeveloped state, with a still rudimentary digestive system incapable of processing formulated diets. Live feed is usually required in order to do not compromise larval survival and growth (Bengston, 2003). Live prey have numeorus advantages, including its ability to swim along the water column, contrary to commercial diets, that tend to sink to the bottom, being less accesible than live feed (Samat et al., 2020). Whatsmore, movement of live feed stimulate larval feeding responses in a similar way than in nature. Live prey have been also described to be more palatable and digestible to the larvae compared to formulated diets (Dhont et al., 2013), which additionally deteriorate water quality. However, cultivation and management of live feed is usually costly and unpredictable, being necessary to understand the nutritional requirements of fish larvae in order to facilitate the optimization of diets and feeding protocols (Oliver et al., 2017; Samat et al., 2020).

Zooplankton such as the rotifer *Brachionus plicatilis* and the crustacean *Artemia* are commonly used in aquaculture as live feed for fish and crustaceans larvae (Davis and Hardy, 2022; Eryalçın, 2019; Oliver et al., 2017; Raja et al., 2018; Samat et al., 2020). Their utilization mostly depends on larval mouth size (Eryalçın, 2019). Rotifers are usually used during the earliest stages of larval development due to their small size and slow movements. In the last years, advances in aquaculture hatchery technologies have improved considerably the culture of rotifers (Dhont et al., 2013). On the other hand, *Artemia* nauplii are widely used as live prey in marine fish hatcheries worldwide, due to their appropriate size, availability and digestibility (Viciano et al., 2015). It can be hatched within 24 hours from dormant cysts which can be easily distributed and stored for prolonged periods of time (Dhont et al., 2013).

However, these organisms are not the natural prey of fish larvae, and their nutritional profile might not macth the nutritional requirements of marine larvae (Oliver et al., 2017). Thus, they are deficient in some nutrients including LC-PUFA, compared with their natural preys such as copepods, and consequently, they need to be enriched in order to improve their nutritional value (McEvoy et al., 1995; Viciano et al., 2015). LC-PUFA, especially EPA, DHA and ARA, play significant roles in marine larval development,

being essential for the survival and proper growth of larvae (Salhi et al., 1997; Tocher, 2010). In particular, low levels of 18:2n-6, 18:3n-3, EPA and DHA in the diet of fish larvae promote metabolic disorders, negatively affect their growth and cause skeletal abnormalities (Radhakrishnan et al., 2020). The requirements for these FA depend upon the species, their genetics, and dietary and environmental factors, and must be incorporated through the diet to meet larval needs (Samat et al., 2020).

Nutritional deficiencies in live preys, particularly in LC-PUFA, can be addressed to a certain extent and in a relatively short period of time, by exposing them to a culture medium containing essential nutrients that are passively filtered and incorporated by the zooplankton. These processes are called “bioencapsulation” or “enrichment” (Dhont et al., 2013; Monroig et al., 2003). Thus, improvement of the nutritional value of live feed with essential nutrients to better suit the nutritional needs of the predator has been widely addressed (Cavrois-Rogacki et al., 2020; Dhont et al., 2013; Eryalçın, 2018, 2019; Rodríguez et al., 1997, 1998; Seychelles et al., 2009; Viciano et al., 2015, 2017).

In marine larvae nutrition, the type of lipid in which FA is sterified seems to be particularly relevant (Lund et al., 2018; Reis et al., 2021a). Natural marine phytoplankton and zooplankton is composed mostly of n-3 LC-PUFA-rich phospholipids, while most enrichment methodologies are rich in triacylglycerols (TAG), which usually have lower n-3 LC-PUFA proportions than polar lipids (Morais et al., 2007). Because of this, and in order to supply the high requirements of n-3 LC-PUFA, live prey is frequently enriched with an excessive lipid content, especially its neutral lipid fraction. An excess of neutral lipids have been suggested to negatively influence digestion, absorption and ingestion processes, compromising larval growth (Morais et al., 2007; Reis et al., 2017, 2019). In particular, it has been described that larvae fed TAG-rich diets commonly show an accumulation of lipids vacuoles in the basal zone of enterocytes, which indicates a good digestion and absorption of dietary TAG, but reduced transport capacity (Morais et al., 2007). On the contrary, dietary phospholipids have been related to the lipid transport from enterocytes to tissues. Phospholipids (mainly phosphatidylcholine; PC) are directly involved in lipoprotein production, which are in part responsible for lipid transportation through the circulatory system (Morais et al., 2007).

Feeding with live feed improves growth, survival, disease resistance, and microbial diversity for a variety of aquatic species. However, both reproducibility and predictability

of enrichment processes are crucial in commercial hatcheries. Furthermore, producing enriched live feed with consistent levels of the important nutrients can be complex (Samat et al., 2020). Rotifer are passive filterers, and their nutritional value highly reflects what they consume (Radhakrishnan et al., 2020). Consequently, its biochemical composition can be strongly modified (Ferreira et al., 2009; Pérez et al., 2022). On the contrary, *Artemia* nauplii metabolize phospholipids into TAG (Guinot et al., 2013; Reis et al., 2017, 2019) and retroconvert DHA to EPA (Viciano et al., 2017). Therefore, DHA enrichment of *Artemia* is usually a challenge that has been traditionally assessed through liposomes, microcapsules, marine-oil emulsions, or microalgaes (McEvoy et al., 1995; Reis et al., 2021b; Viciano et al., 2015).

On the other hand, during both early stages of larval development and live prey enrichment protocols, there is a pro-oxidant environment due to the high metabolic activity and the aeration systems, respectively, compromising the appropriate supply of LC-PUFA to fish larvae (Viciano et al., 2017). Although the most common and effective enrichment products for live prey are lipid emulsions (Sorgeloos et al., 2001), they also involve some detrimental effects, such as oxidation of PUFA and bioaccumulation of lipid peroxides which are potentially toxic for fish larvae (Viciano et al., 2015). Oxidation of PUFA during live prey enrichment has been previously studied, even revealing autooxidation in commercial lipid emulsions containing antioxidants (McEvoy et al., 1995; Viciano et al., 2017). Thus, the addition of an external antioxidant to the lipid emulsion has been proposed to prevent PUFA oxidation (Viciano et al., 2017).

Microalgae have been widely used for both the so called green water technique and live prey enrichment. In the green water technique, microalgae contribute to stabilize and improve the water quality and light contrasts, while providing food for the zooplankton. Besides, microalgae can suppose a direct nutrition through larvae active ingestion. It also favours the induction of behavioral processes like initial prey catching, and consequently increases larval prey ingestion. Other positive functions of algae on larval feeding are the increase of survival, regulation of the bacterial population, probiotic effects, immunity stimulation or enhancement of phospholipids. However, these benefits may be species-specific and depend on several factors such as algal species and concentration, or light intensity (Muller-Feuga, 2000; Van der Meeren et al., 2007).

I. galbana is the marine unicelular microalgae mostly used as live feed in aquaculture (El-Tohamy et al., 2021; Sukenik and Wahnon, 1991), together with *Rhodomonas baltica*, *Chlorella* sp., *Nannochloropsis oculata*, *Skeletonema costatum* and *Tetraselmis* sp. (Oliver et al., 2017). Microalgae biomass is a rich source of protein, interesting compounds with antioxidant capacity such as pigments, and n-3 FA, that could be exploited for their nutritional value, nutraceutical and biofuel production (Raja et al., 2018). Furthermore, modification of light intensity or temperature, may modulate the lipid composition of microalgae, being able to increase their LC-PUFA content (Guedes et al., 2010), which is an interesting characteristic for live prey´s enrichment protocols.

In fact, their application in aquaculture is remarkable, where different species are being used for larval nutrition of several groups such as sea cucumbers, molluscs, crustaceans and fish (Eryalçın, 2019; Hemaiswarya et al., 2011; Sibonga et al., 2021). Nevertheless, one of the main problems of microalgae is their high production cost, which can be up to 30-40% of total cost, entailing a serious bottleneck in hatcheries (Eryalçın, 2019; Hemaiswarya et al., 2011). Thus, the high costs associated to their culture, and the difficulty in producing, concentrating and storing live biomass, has encouraged the search for substitutes to live microalgae in recent years (Raja et al., 2018; Samat et al., 2020). Alternatives to live microalgae with better cost-effectiveness include the use of microalgal pastes, dried microalgae, microencapsulates, cryopreserved or flocculated microalgae (Raja et al., 2018).

In addition to changes in the culture facilities, the development of new technologies focused on the post-harvest processing such as spray drying or algal concentration, will facilitate algal biomass distribution to hatcheries, and reduce their related production expenses (Hemaiswarya et al., 2011). Processing microalgae biomass has several advantages such as concentrate bioproducts, obtain high cell densities, ease handling and storage, and efficient downstream processing (Raja et al., 2018). Therefore, microalgae could be readily available in hatcheries in different forms: fresh, paste, frozen, freeze-dried or spray-dried. Commercial algae pastes are cheaper than integrated microalgae cultivation for aquaculture feeds (Raja et al., 2018). While unprocessed pastes should be utilized within a couple of days in hatcheries, freeze-dried formats stand out for their easy utilization, maintainance of original cell shape and texture, and preservation of biochemical properties. Spray-dried microalgae may also be an alternative format, although cells may become smaller and lose quality (Eryalçın, 2019). Nevertheless,

microalgae paste from *N. oculata* and *Chlorella vulgaris* has been recently tested to feed *B. plicatilis* with certain success (Raja et al., 2018; Samat et al., 2020).

In this study, streams of the haptophyte *I. galbana* and the diatom *N. salinicola* present in the Canary Islands waters, were selected to feed rotifer and *Artemia*. *I. galbana* is rich in proteins and lipids (~20% DW of TL), and a highly valuable source of natural bioactive compounds, such as n-3 LC-PUFA (mainly EPA and DHA) and carotenoids (Batista et al., 2013; Bonfanti et al., 2018). It has been extensively used to enrich live preys (Betancor et al., 2017; Chen and Zeng, 2021; Faulk and Holt, 2005) although the effectiveness of a large scale *I. galbana* culture in order to achieve a better lipid production, is not always feasible (Sani et al., 2021).

Diatoms have the capacity to produce biologically active metabolites such as carbohydrates, proteins and lipids (Rachmayanti et al., 2020). For example, some species can accumulate high lipid contents especially under stress conditions (Rachmayanti et al., 2020), while others, greatly accumulate neutral lipids under silicon deficiency (Sukenik and Wahnon, 1991). In particular, *N. salinicola* has been described to have relevant values of EPA, and a desirable combination of relatively large abundance of FA and biomass productivity (Rachmayanti et al., 2020). It tolerates strong salinity variations and high irradiation (Sylvestre et al., 2004), which allows its cultivation in spite of elevated rates of evaporation, and in both sea or brine waters (wastewater), improving water efficiency. *N. salinicola* harvest is easy because of its benthic nature (Rachmayanti et al., 2020), but also because it is a dominant species over other microalgae species, which decreases the probability of cross-contamination with other microalgae cultures (M. Venuleo, Instituto Tecnológico de Canarias (ITC), personal communication). In addition to their capacity to improve water quality in cultivation systems (Khatoon et al., 2007), other application of diatoms is to be used as food for cultured organisms. Particularly, they have been used in the culture of shrimps, sea urchins, and sea cucumbers (Ito and Kitamura, 1997; Xing et al., 2007; Zupo and Messina, 2007). Furthermore, copepods' feeding with diatoms such as *Chaetoceros muelleri* have been previously studied, with this species being one of the most used algal species in commercial aquaculture (El-Tohamy et al., 2021; Puello-Cruz et al., 2009). However, to the best of our knowledge, *N. salinicola* has not been used as feed for live preys to date.

The main objective of this study was to test different post-processing products of these two locally distributed microalgae, *I. galbana* and *N. salinicola*, together or not with lipid emulsions, for rotifer and *Artemia* feeding, and to analyze the zooplankton response to the oxidative stress caused by the enrichment process. Antioxidant potential of the products was also assessed. Furthermore, poblational parameters as well as the lipid composition of rotifers and *Artemia* were determined.

Two set of experiments with rotifers and *Artemia* were developed. In the first experiment, three different formats of *I. galbana* (fresh paste, frozen paste, and spray-dried) were added to rotifers culture media, and a fourth commercial format of *I. galbana* (Phytobloom Prof©) was also used in the *Artemia* assay. In the second experiment, a control group with no microalgae addition was compared to those of live preys receiving fresh, frozen and spray-dried *N. salinicola* formats, and spray-dried *I. galbana*.

4.1.2. Material and methods

4.1.2.1. Experimental conditions

Fresh paste, frozen paste and spray-dried formats of *I. galbana* and *N. salinicola* were produced at ITC, leader of MACBIOBLUE[1] project. Both frozen formats were maintained for approximately 20 days after collection until the beginning of the experiment. Rotifers were obtained at the rearing facilities of Centro Oceanográfico de Canarias from Centro Nacional Instituto Español de Oceanografía (CNIEO-CSIC), while 12-24 hours *Artemia* nauplii were obtained from BF *Artemia* cysts (INVE Aquaculture, Belgium). Both experiments were developed in triplicate at CNIEO-CSIC facilities, under the experimental rearing conditions shown in Figure 4.1.1 and Figure 4.1.2.

4.1.2.2. Experimental design

Experiment 1

The feeding treatments assayed for rotifers were 0.12 g L^{-1} of spray-dried *I. galbana* (ISD) and 1.2 g L^{-1} of either fresh (IFRE) paste or frozen (IFRO) paste of *I. galbana*. A control group fed with baker´s yeast was also developed. However, the lack of microalgae products in this last treatment displayed no coloration in the colorimetric reactions

[1] MACBIOBLUE (MAC/1.1b/086) "Proyecto demostrativo y de transferencia tecnológica para ayudar a las empresas a desarrollar nuevos productos y procesos en el ámbito de la Biotecnología Azul de la Macaronesia" (Interreg MAC 2014-2020 Cooperación Territorial).

performed for oxidative stress meassurements, and results could not be compared with the other treatments (Figure 4.1.1 B).

Similarly, *Artemia* was cultured as described by Reis et al. (2015) and following CNIEO-CSIC protocols. The treatments used for the feeding experiment were 0.1 g L^{-1} of a commercial product based on *I. galbana* (Phytobloom Prof©, Necton, Portugal) as the control group, and the *I. galbana* products at one tenth of the concentration used for rotifers: 0.01 g L^{-1} of ISD, 0.12 g L^{-1} of IFRE and IFRO (Figure 4.1.1 C).

(A)

Experimental conditions		
	Rotifer	***Artemia***
Time	24 h	24 h
Tank volume	5 L	5 L
Temperature	22 ± 0.2ºC	23 ± 0.3ºC
Salinity	28-30 ppt	36 ppt
Density	100 ± 30 individuals mL^{-1}	10 ± 1 individuals mL^{-1}

(B)

(C)

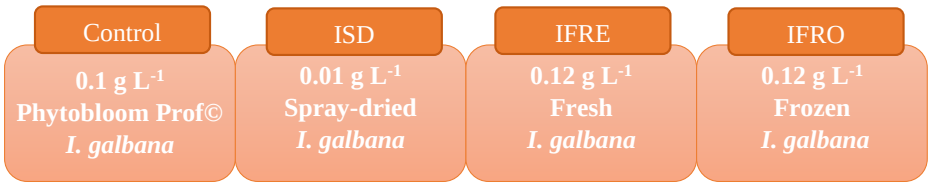


Figure 4.1.1. Experimental conditions (A); experimental dietary **treatments** for rotifer (B) and *Artemia* (C) using different formats of ***Isochrysis galbana***.

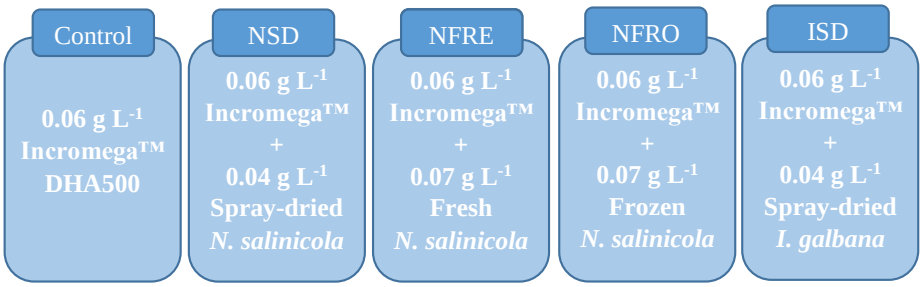
Experiment 2

The experimental rearing conditions of rotifers and *Artemia* nauplii during the experiment are given in Figure 4.1.2 A. The procedure performed in this second experiment mimicked common lipid enrichment protocols for live preys. In this sense, 0.06 g L^{-1} of a commercial lipid based on DHA, Incromega™ DHA500 (Croda, Inc., East Yorkshire, United Kingdom) was used for rotifers enrichment, while 0.6 g L^{-1} of Marine Lecithin LC 60® (PhosphoTech Laboratoires, Saint Herblain, France) as recommended by the manufacturer, was used for *Artemia*. Regardless of the species, the lipid emulsions without microalgae supplementation constituted the control group. In addition, the same lipid emulsion supplemented with one of the three different formats of *N. salinicola* (spray-dried, fresh paste and frozen paste) or spray-dried *I. galbana* were used for the experimental treatments. The significant reduction of rotifers survival fed IFRE in the experiment 1, attributable to an excess of microalgae in the treatment, advised reducing doses of both *I. galbana* and *N. salinicola* formats in this second rotifer experiment. However, the low culture density used for *Artemia*, together with the absence of a negative effect on its survival in experiment 1, allowed the elevation of microalgae concentration to avoid the potential extra oxidative stress caused by the addition of the lipid emulsion. Thus, experimental rotifers were supplemented with either 0.04 g L^{-1} of spray-dried *N. salinicola* (NSD) or *I. galbana* (ISD), and 0.07 g L^{-1} of fresh paste (NFRE) or frozen paste (NFRO) *N. salinicola*, while 0.10 g L^{-1} of NSD and ISD, and 0.20 g L^{-1} of NFRE or NFRO were used for *Artemia* Figure 4.1.2 B and Figure 4.1.2 C.

(A)

Experimental conditions		
	Rotifer	***Artemia***
Time	5 h	5 h
Tank volume	10 L	10 L
Temperature	23 ± 0.0ºC	22 ± 0.2ºC
Salinity	28-30 ppt	36 ppt
Density	180 ± 20 individuals mL^{-1}	10 ± 2 individuals mL^{-1}

(B)



(C)

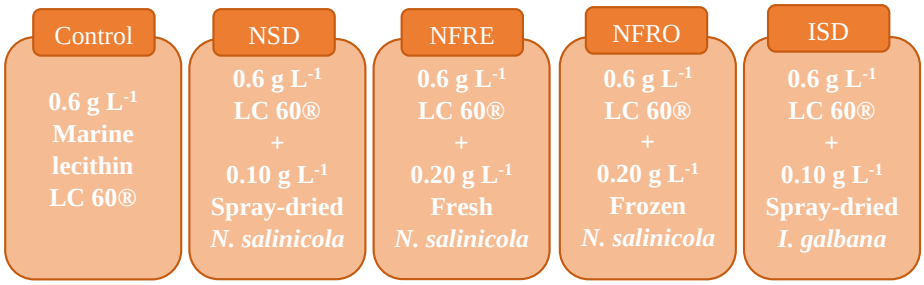


Figure 4.1.2. Experimental conditions (A); experimental dietary **treatments** for rotifer (B) and *Artemia* (C) using different formats of ***Navicula salinicola*** and spray-dried ***Isochrysis galbana*.**

4.1.2.3. Survival and sample preparation

At the beginning and at the end of both experiments, rotifer and *Artemia* nauplii population was volumetrically determined from its culture density (individuals mL^{-1}), and final survival calculated. At the end of the experiments, culture media containing rotifer and *Artemia* was filtered through a 60 or 100 µm mesh, respectively, and washed with clean seawater in order to eliminate remaining microalgae and lipid emulsion. Samples were stored at -80ºC until further analysis.

4.1.2.4. Biochemical analysis

Lipid composition (TL, LC, and FA profile) of the different *I. galbana* and *N. salinicola* formats are displayed in Table 4.1.1 to Table 4.1.4. The FA profile of the lipid emulsions used in the experiment 2 was also assesed (Table 4.1.5). In addition, rotifers and *Artemia* fed with the different microalgae formats and emulsions were also analyzed. In order to determine the potential antioxidant capacity of microalgae formats, TAC of *I. galbana* and *N. salinicola* extracts was evaluated as well as the antioxidant activities (CAT, GST, SOD, GR) and oxidative status (PxI and TBARS) of experimental live prey samples.

Phytoblom Prof© showed the highest TL content of all *I. galbana* formats with a 26% DW (Table 4.1.1). Overall, total neutral lipids (TNL) were the main lipid fraction (61-65%), chiefly formed by free fatty acids (FFA) + phytosterols (PTS) (30-40%) and TAG (12-24%). Pigments were also an important component (19-25%), while MGDG (4-6%) was the main polar lipid.

Table 4.1.1. Total lipid content (% DW) and **lipid class** composition (% of total lipid) of the different formats of the microalgae ***Isochrysis galbana***.

	Phytobloom Prof©	Spray-dried *I. galbana*	Fresh/frozen *I. galbana**
Total lipid	25.60 ± 0.12	16.23 ± 0.18	15.39 ± 0.23
LPC	0.18 ± 0.07	0.31 ± 0.01	0.68 ± 0.08
PC	1.95 ± 0.63	2.44 ± 0.07	2.17 ± 0.30
PS+PI	2.62 ± 0.27	2.25 ± 0.63	1.49 ± 0.28
SQDG	2.27 ± 0.43	2.23 ± 0.93	0.74 ± 0.32
PE	1.54 ± 0.57	0.44 ± 0.12	0.92 ± 0.34
DGDG	2.46 ± 0.20	2.09 ± 0.35	1.31 ± 0.11
MGDG	4.08 ± 0.03	4.74 ± 1.57	6.02 ± 0.82
UKPL	0.73 ± 0.14	1.20 ± 0.31	1.15 ± 0.23
TPL	15.85 ± 0.38	15.70 ± 2.00	14.48 ± 1.25
P	19.43 ± 0.65	20.56 ± 1.06	24.83 ± 1.01
MAG+DAG	3.25 ± 0.38	4.28 ± 0.26	4.26 ± 0.17
FFA+PTS	29.58 ± 0.28	39.55 ± 1.21	34.37 ± 0.46
TAG	24.08 ± 0.62	11.64 ± 1.46	16.33 ± 0.26
SE	6.26 ± 0.49	4.35 ± 0.81	4.37 ± 1.23
UKNL	1.55 ± 0.80	3.92 ± 2.27	1.37 ± 0.48
TNL	64.72 ± 1.03	63.74 ± 3.06	60.69 ± 2.26

Data are presented as means ± SD (n=2). *Fresh and frozen *I. galbana* are presented together as it was not possible to analyse the fresh microalgae. LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; SQDG, sulfoquinovosyl-diacylglycerol; PE, phosphatidylethanolamine; DGDG, digalactosyl-diacylglycerol; MGDG, monogalactosyl-diacylglycerol; UKPL, unknown polar lipids; TPL, total polar lipids; P, pigments; MAG, monoacylglycerols; DAG, diacylglycerols; FFA, free fatty acids; PTS, phytosterols; TAG, triacylglycerols; SE, sterol esters; UKNL, unknown neutral lipids; TNL, total neutral lipids.

TL content and LC profiles of *N. salinicola* formats (spray-dried, fresh, and frozen) and spray-dried *I. galbana* used in experiment 2 are shown in Table 4.1.2. TL of *N. salinicola* formats ranged between 19 and 21% DW, while that of ISD was approximately 15%. TNL was the main fraction in all formats, chiefly formed by FFA+PTS (31-45%), although TAG was especially relevant in NFRO (~30%). Pigments proportion was remarkable high in ISD (~25%).

Table 4.1.2. Total lipid content (% DW) and **lipid class** composition (% of total lipid) of the different formats of the microalgae ***Navicula salinicola*** and of spray-dried ***Isochrysis galbana***.

	Spray-dried *N. salinicola*	Fresh *N. salinicola*	Frozen *N. salinicola*	Spray-dried *I. galbana*
Total lipid	21.07 ± 0.67	20.72 ± 1.30	18.59 ± 1.69	15.39 ± 0.23
LPC	0.55 ± 0.06	nd	0.33 ± 0.07	0.68 ± 0.08
PC	3.72 ± 0.56	1.46 ± 0.71	1.94 ± 0.15	2.17 ± 0.30
PS+PI	1.73 ± 0.36	1.37 ± 0.26	1.01 ± 0.29	1.49 ± 0.28
SQDG	5.24 ± 1.28	1.44 ± 0.06	1.67 ± 0.56	0.74 ± 0.32
PE	4.06 ± 0.42	1.11 ± 0.26	1.67 ± 0.18	0.92 ± 0.34
DGDG	4.26 ± 0.90	3.41 ± 0.16	2.83 ± 0.51	1.31 ± 0.11
MGDG	4.88 ± 1.93	1.86 ± 0.93	1.76 ± 0.31	6.02 ± 0.82
UKPL	3.01 ± 0.99	2.37 ± 0.97	3.15 ± 0.38	1.15 ± 0.23
TPL	27.47 ± 1.38	13.03 ± 2.51	14.35 ± 0.84	14.48 ± 1.25
P	10.51 ± 0.92	13.36 ± 0.43	11.02 ± 1.07	24.83 ± 1.01
MAG+DAG	5.63 ± 1.98	7.72 ± 0.95	7.27 ± 1.86	4.26 ± 0.17
FFA+PTS	44.92 ± 3.55	40.20 ± 2.83	30.59 ± 0.80	34.37 ± 0.46
TAG	5.44 ± 0.14	20.70 ± 0.72	29.73 ± 1.83	16.33 ± 0.26
SE	nd	5.00 ± 0.73	7.04 ± 0.54	4.37 ± 1.23
UKNL	--	--	--	1.37 ± 0.48
TNL	62.02 ± 0.81	73.61 ± 2.66	74.63 ± 0.67	60.69 ± 2.26

Data are presented as means ± SD (n=2). LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; SQDG, sulfoquinovosyl-diacylglycerol; PE, phosphatidylethanolamine; DGDG, digalactosyl-diacylglycerol; MGDG, monogalactosyl-diacylglycerol; UKPL, unknown polar lipids; TPL, total polar lipids; P, pigments; MAG, monoacylglycerols; DAG, diacylglycerols; FFA, free fatty acids; PTS, phytosterols; TAG, triacylglycerols; SE, sterol esters; UKNL, unknown neutral lipids; TNL, total neutral lipids. nd, not detected.

Table 4.1.3 shows the FA profile of *I. galbana* formats (Phytobloom prof©, spray-dried and fresh/frozen). Total PUFA was the most relevant fraction in all formats (37-47%). DHA was especially relevant in ISD (~15%), leading to a relevant total n-3 LC-PUFA proportion of 16% and DHA/EPA ratio of nearly 33.

Table 4.1.3. Total fatty acids (µg mg^{-1} DW) and main **fatty acid** composition (% of total FA) of the different formats of the microalgae ***Isochrysis galbana***.

	Phytobloom Prof©	Spray-dried *I. galbana*	Fresh/Frozen *I. galbana* *
Total FA	99.88 ± 3.80	78.02 ± 0.93	64.22 ± 0.25
Total SFA	31.61 ± 1.54	27.83 ± 1.27	29.35 ± 1.64
14:0	14.82 ± 1.33	15.43 ± 0.66	17.03 ± 1.01
16:0	13.71 ± 0.21	11.60 ± 0.42	11.60 ± 0.41
18:0	1.42 ± 0.29	0.26 ± 0.02	0.18 ± 0.03
Total MUFA	29.95 ± 0.96	23.58 ± 1.33	22.51 ± 3.11
16:1[1]	9.72 ± 0.93	7.15 ± 0.41	7.75 ± 2.88
18:1[1]	18.82 ± 0.25	9.04 ± 0.60	9.50 ± 0.21
20:1[2]	0.82 ± 0.16	7.26 ± 0.14	5.01 ± 0.54
Total n-6 PUFA	11.90 ± 0.80	8.76 ± 0.65	8.95 ± 0.84
18:2	9.74 ± 0.19	3.74 ± 0.03	5.70 ± 0.62
18:3	0.29 ± 0.25	0.59 ± 0.01	0.76 ± 0.03
20:4 (ARA)	0.28 ± 0.09	0.19 ± 0.02	0.16 ± 0.03
22:5	1.23 ± 0.04	3.89 ± 0.45	2.29 ± 0.17
Total n-3 PUFA	24.15 ± 2.08	36.92 ± 1.74	33.42 ± 1.19
18:3	8.98 ± 0.39	6.42 ± 0.01	7.68 ± 0.49
18:4	7.85 ± 0.21	14.70 ± 0.83	13.15 ± 0.49
20:4	0.17 ± 0.01	nd	nd
20:5 (EPA)	0.63 ± 0.06	0.46 ± 0.08	0.47 ± 0.11
22:5	0.38 ± 0.09	0.08 ± 0.10	0.07 ± 0.09
22:6 (DHA)	6.14 ± 1.51	14.99 ± 1.16	11.88 ± 0.31
Total PUFA	36.80 ± 2.88	46.59 ± 2.53	43.39 ± 2.18
DHA/EPA	9.73 ± 1.50	32.56 ± 3.02	26.10 ± 6.65
EPA/ARA	2.35 ± 0.54	2.39 ± 0.11	2.85 ± 0.22
n-3/n-6	2.03 ± 0.04	4.22 ± 0.12	3.74 ± 0.20
Total n-3 LC-PUFA	7.32 ± 1.48	15.80 ± 1.35	12.51 ± 0.25

Data are presented as means ± SD (n=2). *Fresh and frozen *I. galbana* are presented together as it was not possible to analyse the fresh microalgae. FA, fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long chain polyunsaturated fatty acids (≥ C20 and ≥ 2 double bonds). Totals include other minor components not shown. [1] Mainly n-7 isomers, [2] Mainly n-9 isomers. nd, not detected.

The FA profiles of the spray-dried, fresh and frozen *N. salinicola*, and spray-dried *I. galbana* are shown in Table 4.1.4. Total PUFA constituted the main FA family of ISD, whereas it was total MUFA in *N. salinicola* formats. ISD showed high total n-3 PUFA (~37%) and n-3 LC-PUFA (~16%) contents, mainly due to the DHA contribution (~15%). Finally, EPA was remarkable in all *N. salinicola* formats (~10%) together with their EPA/ARA ratios (115-116).

Table 4.1.4. Total fatty acids (µg mg^{-1} DW) and main **fatty acid** composition (% of total FA) of the different formats of the microalgae ***Navicula salinicola*** and of spray-dried ***Isochrysis galbana***.

	Spray-dried *N. salinicola*	Fresh *N. salinicola*	Frozen *N. salinicola*	Spray-dried *I. galbana*
Total FA	142.78 ± 17.82	153.64 ± 17.94	132.65 ± 9.07	78.02 ± 0.93
Total SFA	33.64 ±1.24	34.06 ± 1.07	33.77 ± 1.15	27.83 ± 1.27
14:0	2.70 ± 0.09	2.52 ± 0.08	2.60 ± 0.09	15.43 ± 0.66
16:0	29.21 ± 1.18	29.72 ± 0.94	29.40 ± 0.99	11.60 ± 0.42
18:0	0.44 ± 0.05	0.45 ± 0.02	0.41 ± 0.05	0.26 ± 0.02
Total MUFA	46.43 ± 0.47	45.91 ± 0.18	46.35 ± 0.27	23.58 ± 1.33
16:1[1]	43.83 ± 0.35	43.35 ± 0.22	43.86 ± 0.32	7.15 ± 0.41
18:1[1]	2.08 ± 0.09	2.00 ± 0.05	1.99 ± 0.08	9.04 ± 0.60
20:1[2]	0.12 ± 0.06	0.10 ± 0.09	0.10 ± 0.09	7.26 ± 0.14
Total n-6 PUFA	0.46 ± 0.10	0.49 ± 0.13	0.49 ± 0.12	8.76 ± 0.65
18:2	0.28 ± 0.01	0.25 ± 0.01	0.25 ± 0.01	3.74 ± 0.03
18:3	nd	nd	nd	0.59 ± 0.01
20:4 (ARA)	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.19 ± 0.02
22:5	nd	0.08 ± 0.06	0.06 ± 0.06	3.89 ± 0.45
Total n-3 PUFA	12.95 ± 0.64	12.88 ± 0.54	13.08 ± 0.57	36.92 ± 1.74
18:3	0.55 ± 0.03	0.54 ± 0.02	0.54 ± 0.02	6.42 ± 0.01
18:4	0.87 ± 0.06	0.84 ± 0.10	0.82 ± 0.10	14.70 ± 0.83
20:4	0.22 ± 0.00	0.22 ± 0.01	0.22 ± 0.02	nd
20:5 (EPA)	9.67 ± 0.47	9.66 ± 0.40	9.85 ± 0.40	0.46 ± 0.08
22:5	0.13 ± 0.04	0.15 ± 0.09	0.11 ± 0.02	0.08 ± 0.10
22:6 (DHA)	1.51 ± 0.11	1.48 ± 0.07	1.53 ± 0.07	14.99 ± 1.16
Total PUFA	18.09 ± 0.80	17.86 ± 0.52	17.96 ± 0.64	46.59 ± 2.53
DHA/EPA	0.16 ± 0.01	0.15 ± 0.00	0.16 ± 0.00	32.56 ± 3.02
EPA/ARA	115.47 ± 18.24	114.61 ± 19.16	115.84 ± 9.76	2.39 ± 0.11
n-3/n-6	29.41 ± 6.96	27.45 ± 7.96	28.11 ± 7.53	4.22 ± 0.12
Total n-3 LC-PUFA	11.54 ± 0.57	11.50 ± 0.46	11.71 ± 0.48	15.80 ± 1.35

Data are presented as means ± SD (n=3). FA, fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long chain polyunsaturated fatty acids (≥ C20 and ≥ 2 double bonds). Totals include other minor components not shown. [1] Mainly n-7 isomers, [2] Mainly n-7 isomers for *N. salinicola*, and n-9 for *I. galbana.* nd, not detected.

Finally, LC 60® and Incromega™ oil emulsions were mainly formed by total PUFA (~53 and ~77%, respectively) as a result of high EPA (~13 and ~9%) and DHA (~36 and ~35%) contents (Table 4.1.5).

Table 4.1.5. Main **fatty acid** composition (% of total FA) of the **commercial lipids** LC 60® and Incromega™ oil.

	LC 60®	Incromega™
Total SFA	30.88 ± 0.03	6.16 ± 0.22
14:0	1.25 ± 0.04	0.14 ± 0.01
16:0	25.22 ± 0.01	1.40 ± 0.03
18:0	3.40 ± 0.05	3.33 ± 0.06
Total MUFA	9.92 ± 0.17	14.31 ± 0.25
16:1[1]	1.32 ± 0.04	0.49 ± 0.02
18:1[2]	4.94 ± 0.35	7.82 ± 0.15
20:1[2]	3.44 ± 0.11	2.35 ± 0.04
Total n-6 PUFA	2.84 ± 0.00	7.87 ± 0.24
18:2	0.40 ± 0.01	0.67 ± 0.00
20:2	0.17 ± 0.01	0.39 ± 0.01
20:3	nd	0.18 ± 0.01
20:4 (ARA)	1.89 ± 0.00	2.35 ± 0.02
22:5	0.38 ± 0.00	3.76 ± 0.04
Total n-3 PUFA	49.58 ± 0.07	69.22 ± 0.29
18:3	0.14 ± 0.02	0.34 ± 0.00
18:4	0.19 ± 0.00	0.50 ± 0.00
20:3	0.17 ± 0.01	0.26 ± 0.00
20:4	0.11 ± 0.01	0.70 ± 0.01
20:5 (EPA)	12.99 ± 0.07	9.09 ± 0.02
22:5	0.32 ± 0.01	2.67 ± 0.01
22:6 (DHA)	35.68 ± 0.15	55.17 ± 0.26
Total PUFA	52.51 ± 0.06	76.97 ± 0.15
DHA/EPA	2.75 ± 0.03	6.07 ± 0.02
EPA/ARA	6.87 ± 0.04	3.87 ± 0.04
n-3/n-6	17.46 ± 0.02	8.80 ± 0.32
Total n-3 LC-PUFA	49.26 ± 0.05	68.38 ± 0.29

Data are presented as means ± SD (n=2). FA, fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long chain polyunsaturated fatty acids (≥ C20 and ≥ 2 double bonds). Totals include other minor components not shown. [1] Mainly n-7 isomers; [2] Mainly n-9 isomers. nd, not detected.

4.1.3. Results

Experiment 1

- Survival

Survival of **rotifers** was negatively affected by the fresh *I. galbana* format (39.60 ± 4.41%), while it was higher than 85% (100.00 ± 0.00% and 86.82 ± 16.37%) in rotifers fed the ISD and IFRO *I. galbana*, respectively. On the contrary, ***Artemia*** nauplii did not show differences in survival between treatments, being over 95% in all cases (~96-99%; Figure 4.1.3).

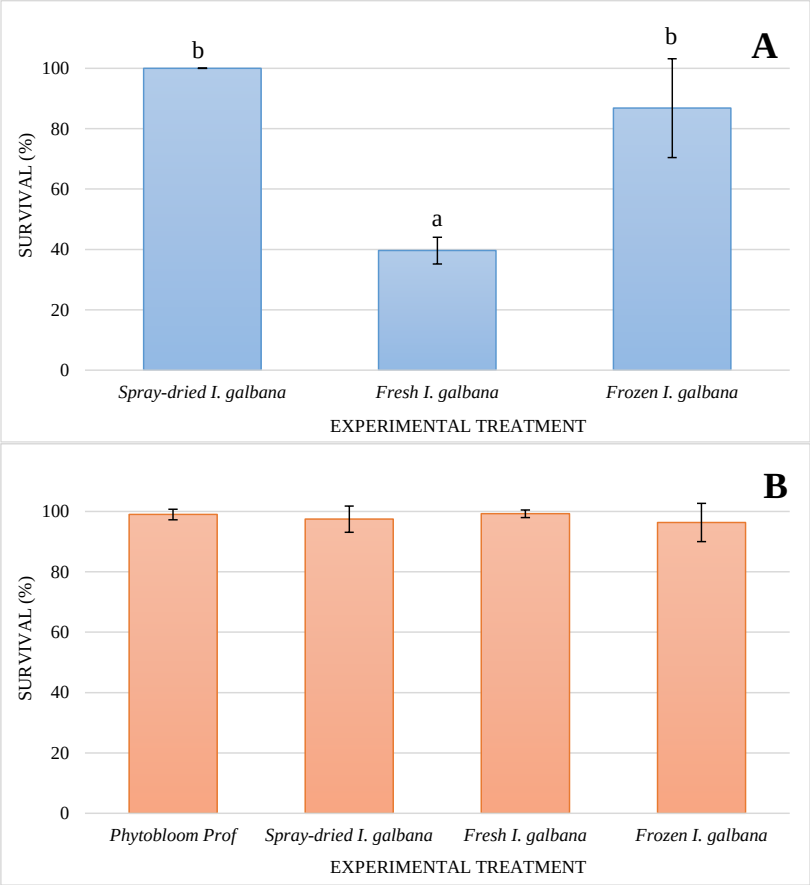


Figure 4.1.3. Survival (%) of **rotifers** (A) and ***Artemia*** (B) fed the different ***Isochrysis galbana*** formats.
Results are presented as means ± SD (n=3). Different letters represent significant differences between dietary treatments ($p<0.05$).

- TL content and LC profile

TL content of **rotifer** remained stable between dietary groups (18-21% DW), while LC greatly varied between treatments (Table 4.1.6). TNL represented 81-83% of TL in rotifers fed spray-dried (ISD) and frozen *I. galbana* (IFRO), and ~87% when receiving fresh *I. galbana* (IFRE). The main components of the neutral lipid fraction were monoacylglycerols + diacylglycerols (MAG+DAG) in ISD and IFRE formats (25-27%); and TAG in IFRO (~32%). FFA were also relevant, being higher in ISD (24.24 ± 2.43%) than in IFRO-rotifers (17.69 ± 2.72%). Both cholesterol (CHO) and sterol esters (SE) were highest in IFRE-rotifers (12.79 ± 0.84% and 11.62 ± 1.76%, respectively).

On the other hand, total polar lipids (TPL) was the lowest in IFRE (~13%). PC was the main LC in the three experimental treatments, with the greatest value present in ISD-rotifers (6.05 ± 0.56%). Phosphatidylethanolamine (PE; 4-5%) and phosphatidylglycerol (PG; 1-2%) were also relevant in all cases, being similar between treatments. Sphingomyelin (SM) and phosphatidylinositol (PI) were more abundant in ISD (0.81 ± 0.12% and 2.82 ± 0.56%, respectively), while IFRO showed the highest value of lysophosphatidylcholine (LPC) (Table 4.1.6).

Artemia TL content did not differ between treatments (14-18% DW; Table 4.1.7), contrarily to the LC profile. TNL, encompassed between 67.54 ± 1.64% in IFRE and 61.27 ± 2.49% in the control Phytobloom Prof-*Artemia*. CHO and TAG were the main neutral lipid fractions (20-23% and 18-25%, respectively), the latter presenting higher proportions in Phytobloom Prof© than in ISD. It is remarkable the high content of MAG+DAG (18.00 ± 3.97%) in the IFRE format, compared to the other treatments (4-9%). ISD-*Artemia* stands out by its content of FFA (10.55 ± 2.14% *vs*. 3-7% in the other formats), while SE was highest in IFRO-*Artemia* (~12%).

The TPL fraction of Phytobloom Prof©-fed *Artemia* was higher (38.73 ± 2.49% of TL), than that of IFRE (32.46 ± 1.64%). PC and PE were the main polar lipids in all treatments, being PC higher in ISD and IFRO (12-13%) than in Phytobloom Prof© and IFRE (9-10%). Nonetheless, PE was higher in Phytobloom Prof© and ISD-*Artemia* (11-12%) than in IFRE and IFRO ones (~9%). Both PI and LPC content were higher in Phytobloom Prof© (~5% and ~9%, respectively), compared to the other treatments (3-4% and 0.2-0.7%, respectively) (Table 4.1.7).

Table 4.1.6. Total lipid content (% DW) and **lipid class** composition (% of total lipid) of **rotifers** fed the different ***Isochrysis galbana*** formats.

	Spray-dried *I. galbana*	Fresh *I. galbana*	Frozen *I. galbana*
Total lipid	20.06 ± 1.56	18.45 ± 0.74	20.76 ± 0.14
LPC	0.03 ± 0.02 ^{a}	0.27 ± 0.10 ^{b}	0.93 ± 0.11 ^{c}
SM	0.81 ± 0.12 ^{b}	0.39 ± 0.06 ^{a}	0.29 ± 0.13 ^{a}
PC	6.05 ± 0.56 ^{b}	3.04 ± 0.45 ^{a}	4.28 ± 0.83 ^{a}
PS	0.16 ± 0.09 ^{a}	0.46 ± 0.08 ^{a}	1.24 ± 0.35 ^{b}
PI	2.82 ± 0.56 ^{b}	1.79 ± 0.24 ^{a}	2.43 ± 0.22 ^{ab}
PG	1.52 ± 0.28	1.24 ± 0.39	2.02 ± 0.25
PE	5.45 ± 0.57	4.13 ± 0.56	5.49 ± 0.73
UKPL	0.46 ± 0.25 ^{a}	1.30 ± 0.50 ^{ab}	2.00 ± 0.61 ^{b}
TPL	17.32 ± 1.46 ^{b}	12.61 ± 1.63 ^{a}	18.68 ± 1.59 ^{b}
MAG+DAG	26.97 ± 2.42 ^{b}	25.28 ± 5.15 ^{b}	13.49 ± 1.27 ^{a}
CHO	9.49 ± 1.58 ^{a}	12.79 ± 0.84 ^{b}	10.87 ± 0.81 ^{ab}
FFA	24.24 ± 2.43 ^{b}	20.45 ± 1.21 ^{ab}	17.69 ± 2.72 ^{a}
TAG	17.23 ± 2.47 ^{a}	17.25 ± 2.00 ^{a}	32.25 ± 1.02 ^{b}
SE	4.74 ± 0.66 ^{a}	11.62 ± 1.76 ^{b}	5.83 ± 3.57 ^{ab}
UKNL	--	--	1.19 ± 0.15
TNL	82.68 ± 1.46 ^{a}	87.39 ± 1.63 ^{b}	81.32 ± 1.59 ^{a}

Results are presented as means ± SD (n=3). Different letters in superscript within the same row represents significant differences between dietary treatments ($p<0.05$). LPC, lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; UKPL, unknown polar lipids; TPL, total polar lipids; MAG, monoacylglycerols; DAG, diacylglycerols; CHO, cholesterol; FFA, free fatty acids; TAG, triacylglycerols; SE, sterol esters; UKNL, uknown neutral lipids; TNL, total neutral lipids.

Table 4.1.7. Total lipid content (% DW) and **lipid class** composition (% of total lipid) of ***Artemia*** fed the different ***Isochrysis galbana*** formats.

	Phytobloom Prof©	Spray-dried *I. galbana*	Fresh *I. galbana*	Frozen *I. galbana*
Total lipid	15.90 ± 2.38	17.63 ± 1.60	16.02 ± 2.18	14.44 ± 3.11
LPC	1.12 ± 0.26 ^{b}	0.22 ± 0.17 ^{a}	0.66 ± 0.12 ^{ab}	0.27 ± 0.10 ^{a}
SM	0.83 ± 0.39	0.58 ± 0.28	0.82 ± 0.21	0.82 ± 0.38
PC	9.42 ± 0.56 ^{a}	11.81 ± 0.61 ^{b}	10.10 ± 0.71 ^{a}	13.20 ± 0.28 ^{b}
PS	2.48 ± 0.11	2.11 ± 0.35	1.58 ± 0.35	2.22 ± 0.03
PI	4.94 ± 0.45 ^{b}	4.39 ± 0.39 ^{ab}	3.52 ± 0.48 ^{a}	3.47 ± 0.03 ^{a}
PG	3.95 ± 0.48	2.83 ± 0.26	2.77 ± 0.76	2.74 ± 0.02
PE	12.48 ± 0.66 ^{b}	11.12 ± 0.92 ^{b}	8.68 ± 0.87 ^{a}	9.24 ± 0.21 ^{a}
UKPL	2.39 ± 0.79 ^{b}	1.16 ± 0.20 ^{a}	4.33 ± 0.64 ^{c}	2.47 ± 0.16 ^{b}
TPL	38.73 ± 2.49 ^{b}	34.23 ± 2.21 ^{ab}	32.46 ± 1.64 ^{a}	34.43 ± 1.21 ^{ab}
MAG+DAG	7.70 ± 1.48 ^{a}	8.77 ± 1.71 ^{a}	18.00 ± 3.97 ^{b}	4.42 ± 0.69 ^{a}
CHO	19.95 ± 2.18	23.12 ± 4.04	19.88 ± 0.40	20.05 ± 1.82
FFA	2.69 ± 0.50 ^{a}	10.55 ± 2.14 ^{c}	3.99 ± 0.90 ^{a}	6.99 ± 0.41 ^{b}
TAG	24.97 ± 1.42 ^{b}	17.86 ± 0.49 ^{a}	22.25 ± 1.88 ^{ab}	21.17 ± 3.08 ^{ab}
SE	4.26 ± 1.25 ^{a}	5.46 ± 0.99 ^{a}	3.42 ± 0.36 ^{a}	11.97 ± 1.15 ^{b}
UKNL	1.71 ± 0.23 ^{b}	--	--	0.96 ± 0.11 ^{a}
TNL	61.27 ± 2.49 ^{a}	65.77 ± 2.21 ^{ab}	67.54 ± 1.64 ^{b}	65.57 ± 1.21 ^{ab}

Results are presented as means ± SD (n=3). Different letters in superscript within the same row represents significant differences between dietary treatments ($p<0.05$). LPC, lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; UKPL, unknown polar lipids; TPL, total polar lipids; MAG, monoacylglycerols; DAG, diacylglycerols; CHO, cholesterol; FFA, free fatty acids; TAG, triacylglycerols; SE, sterol esters; UKNL, unknown neutral lipids; TNL, total neutral lipids.

- FA profile

Total FA content (µg mg^{-1} DW) of **rotifers** remained unchanged regardless of their feeding format. Total MUFA was the main group of FA in all treatments (37-44%), followed by total PUFA (24-28%) and total SFA (23-27%). Oleic acid (18:1n-9) was the main MUFA, being higher in IFRO and IFRE (~23%) than in ISD (~18%) (Table 4.1.8).

PUFA proportions greatly varied between dietary groups. Thus, total n-6 PUFA was highest in ISD-rotifers (8.09 ± 0.65%) and lowest in IFRO-rotifers (5.17 ± 0.41%), mainly due to the 18:2n-6 contribution, which was more abundant in ISD and IFRE (~3%) than in IFRO (~2%). ARA was a minor PUFA in all treatments (~1%). On the other hand, total n-3 levels were also remarkable in ISD-rotifers (~16%), where 18:3n-3 and DHA contents (2.66 ± 0.32% and 6.62 ± 0.87%, respectively) were higher. By contrast, EPA did not exceed 2.5% in any rotifer group. Total n-3 LC-PUFA content was remarkably high in all rotifers, especially in ISD with 9.10 ± 0.82% of total FA.

The highest DHA/EPA ratio was detected in ISD (2.68 ± 0.14 *vs*. 1.6-1.8), while EPA/ARA ratio remained stable between 2.1-2.3. Finally, n-3/n-6 was higher in IFRO (2.55 ± 0.55) than in ISD and IFRE-rotifers (~1.9) (Table 4.1.8).

Table 4.1.9 displays the FA composition of ***Artemia*** fed the control (Phytobloom Prof©) and the three experimental *I. galbana* diets. Total FA was higher in ISD-*Artemia* (90.28 ± 15.74 µg mg^{-1} DW) than in Phytobloom Prof-*Artemia* (31.87 ± 14.78 µg mg^{-1} DW). Regardless of dietary treatments, total MUFA represented the highest proportion of all FA (49-53%), with 18:1 being higher in IFRO (39.43 ± 0.59%) than in the rest of the treatments (36-37%).

IFRE-*Artemia* presented the highest proportion of total PUFA (27.22 ± 1.41%), mainly due to the n-3 PUFA contribution (16.24 ± 1.65%). Both EPA and DHA were similar in all treatments (9-11% and 0.2-1% of total FA, respectively) whereas 18:3n-3 had its highest value in IFRE (4.01 ± 0.18%). In addition, total n-6 PUFA and ARA were stable regardless of the treatment, and 18:2n-6 was higher in IFRE-*Artemia* (5.72 ± 0.23%) than in the other treatments where it stayed slightly below 5%.

Finally, DHA/EPA (0.02-0.11), EPA/ARA (3.1-3.6) and n-3/n-6 (~1.83) ratios remained unvariable regardless the feeding regime (Table 4.1.9).

Table 4.1.8. Total fatty acids (µg mg^{-1} DW) and main **fatty acid** composition (% of total FA) of **rotifers** fed the different ***Isochrysis galbana*** formats.

	Spray-dried *I. galbana*	Fresh *I. galbana*	Frozen *I. galbana*
Total FA	46.43 ± 11.81	54.69 ± 5.88	67.56 ± 7.06
Total SFA	26.79 ± 3.72	22.91 ± 1.68	23.91 ± 1.96
14:0	4.51 ± 0.63	4.76 ± 0.33	5.06 ± 0.19
16:0	11.88 ± 1.26	9.92 ± 0.59	10.30 ± 0.68
18:0	9.11 ± 1.98	7.05 ± 1.48	7.11 ± 2.39
Total MUFA	36.90 ± 2.45	44.43 ± 5.71	43.88 ± 1.70
16:1[1]	13.71 ± 1.00	15.08 ± 2.60	14.42 ± 0.32
18:1[2]	17.83 ± 1.21 [a]	23.28 ± 2.26 [b]	22.68 ± 0.95 [b]
20:1[2]	3.45 ± 0.47	4.04 ± 0.73	4.50 ± 0.68
Total n-6 PUFA	8.09 ± 0.65 [b]	6.27 ± 0.79 [ab]	5.17 ± 0.41 [a]
18:2	3.08 ± 0.06 [b]	3.50 ± 0.52 [b]	2.17 ± 0.23 [a]
20:2	0.97 ± 0.18 [a]	1.59 ± 0.27 [b]	1.19 ± 0.09 [ab]
20:4 (ARA)	1.19 ± 0.25	1.18 ± 0.07	0.96 ± 0.06
22:5	2.86 ± 0.81 [b]	nd	0.86 ± 0.17 [a]
Total n-3 PUFA	15.82 ± 1.22 [b]	11.95 ± 0.89 [a]	13.16 ± 0.88 [a]
18:3	2.66 ± 0.32 [b]	1.89 ± 0.30 [a]	1.88 ± 0.13 [a]
18:4	4.06 ± 0.59 [a]	3.70 ± 0.15 [a]	5.37 ± 0.38 [b]
20:5 (EPA)	2.47 ± 0.05	2.47 ± 0.52	2.15 ± 0.18
22:6 (DHA)	6.62 ± 0.87 [b]	3.89 ± 0.47 [a]	3.75 ± 0.26 [a]
Total PUFA	28.41 ± 2.02 [b]	23.58 ± 0.16 [a]	23.58 ± 1.39 [a]
DHA/EPA	2.68 ± 0.14 [b]	1.59 ± 0.14 [a]	1.75 ± 0.14 [a]
EPA/ARA	2.15 ± 0.45	2.08 ± 0.31	2.26 ± 0.27
n-3/n-6	1.96 ± 0.21 [a]	1.93 ± 0.29 [a]	2.55 ± 0.05 [b]
Total n-3 LC-PUFA	9.10 ± 0.82 [b]	6.36 ± 0.99 [a]	5.90 ± 0.38 [a]

Results are presented as means ± SD (n=3). Different letters in superscript within the same row represents significant differences between dietary treatments ($p<0.05$). FA, fatty acids SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long chain polyunsaturated fatty acids (≥ C20 and ≥ 2 double bonds). Totals include other minor components not shown. [1] Mainly n-7 isomers, [2] Mainly n-9 isomers. nd, not detected.

Table 4.1.9. Total fatty acids (µg mg^{-1} DW) and main **fatty acid** composition (% of total FA) of ***Artemia*** fed the different ***Isochrysis galbana*** formats.

	Phytobloom Prof©	Spray-dried *I. galbana*	Fresh *I. galbana*	Frozen *I. galbana*
Total FA	31.87 ± 14.78 ^{a}	90.28 ± 15.74 ^{b}	64.65 ± 8.92 ^{ab}	64.57 ± 26.02 ^{ab}
Total SFA	21.71 ± 0.70 ^{ab}	22.84 ± 2.00 ^{b}	19.57 ± 0.66 ^{a}	20.25 ± 0.51 ^{ab}
14:0	1.45 ± 0.08 ^{c}	0.96 ± 0.14 ^{ab}	1.20 ± 0.11 ^{bc}	0.88 ± 0.05 ^{a}
16:0	10.63 ± 0.60	10.64 ± 0.66	9.67 ± 0.33	9.87 ± 0.26
18:0	7.03 ± 0.47 ^{a}	8.99 ± 1.35 ^{b}	6.42 ± 0.28 ^{a}	7.37 ± 0.23 ^{ab}
Total MUFA	51.52 ± 1.60	49.31 ± 3.04	49.35 ± 1.15	53.40 ± 0.96
16:1^{1}	13.20 ± 1.80	10.66 ± 2.17	11.52 ± 1.12	12.40 ± 0.53
18:1^{2}	36.71 ± 0.81 ^{a}	36.80 ± 0.70 ^{a}	36.36 ± 0.87 ^{a}	39.43 ± 0.59 ^{b}
20:1^{2}	0.83 ± 0.05	0.81 ± 0.20	0.75 ± 0.07	0.81 ± 0.20
Total n-6 PUFA	7.35 ± 0.66	8.28 ± 0.24	8.88 ± 0.35	8.03 ± 0.50
18:2	4.66 ± 0.35 ^{a}	4.89 ± 0.09 ^{a}	5.72 ± 0.23 ^{b}	4.94 ± 0.13 ^{a}
18:3	nd	0.34 ± 0.01	0.32 ± 0.04	nd
20:4 (ARA)	2.69 ± 0.30	3.05 ± 0.16	2.84 ± 0.14	3.09 ± 0.39
Total n-3 PUFA	13.11 ± 1.26 ^{a}	14.41 ± 0.50 ^{ab}	16.24 ± 1.65 ^{b}	14.23 ± 0.32 ^{ab}
18:3	2.59 ± 0.16 ^{a}	2.86 ± 0.09 ^{ab}	4.01 ± 0.18 ^{c}	3.22 ± 0.12 ^{b}
18:4	0.98 ± 0.13	0.76 ± 0.15	1.97 ± 0.43	1.17 ± 0.25
20:5 (EPA)	9.06 ± 1.33	10.82 ± 0.32	8.97 ± 0.31	9.39 ± 0.31
22:6 (DHA)	0.48 ± 0.12	0.20 ± 0.10	1.00 ± 0.65	0.45 ± 0.28
Total PUFA	22.21 ± 1.79 ^{a}	25.00 ± 0.38 ^{a}	27.22 ± 1.41 ^{b}	24.37 ± 0.48 ^{ab}
DHA/EPA	0.05 ± 0.02	0.02 ± 0.01	0.11 ± 0.07	0.05 ± 0.03
EPA/ARA	3.36 ± 0.25	3.56 ± 0.26	3.17 ± 0.24	3.07 ± 0.38
n-3/n-6	1.78 ± 0.09	1.77 ± 0.10	1.83 ± 0.23	1.78 ± 0.15
Total n-3 LC-PUFA	9.53 ± 1.28	11.02 ± 0.42	10.26 ± 1.12	9.85 ± 0.06

Results are presented as means ± SD (n=3). Different letters in superscript within the same row represents significant differences between dietary treatments ($p<0.05$). FA, fatty acids SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long chain polyunsaturated fatty acids (≥ C20 and ≥ 2 double bonds). Totals include other minor components not shown. [1] Mainly n-7 isomers, [2] Mainly n-9 isomers. nd, not detected.

- Microalgae antioxidant potential and oxidative status of live prey

TAC of microalgae

Extraction yield and antioxidant activities of spray-dried and fresh/frozen *I. galbana* are given in Table 4.1.10. DPPH scavenging capacity of all extracts varied from 25 to 61%. Ethyl acetate extracts showed the highest values in both microalgae formats (39 and 61%), while ethanol extracts the lowest ones (25 and 39%), and no activity was detected in the water extracts. Fresh/frozen *I. galbana* had higher activites in all extracts than spray-dried *I. galbana*. However, only one extract (fresh/frozen *I. galbana*-ethyl acetate) scavenged the DPPH radical by more than 50% at 250 µg mL^{-1}. Thus, IC_{50} was calculated, being almost 30-fold less active than Trolox (IC_{50} values of 204.07 ± 4.35 µg mL^{-1} *vs*. 7.43 ± 0.74 µg mL^{-1}, respectively).

The antioxidant activity of the ABTS assay varied from 23 to 75% (Table 4.1.10). As described for the DPPH method, the most active extracts were those of ethyl acetate (72-75%), while the lowest ones were water in the spray-dried format, and ethanol and water in fresh/frozen *I. galbana*. Three extracts were capable of inhibiting more than 50% of the radicals at 250 µg mL^{-1} (both ethyl acetate extracts and fresh/frozen microalgae-*n*-hexane). However, the three extracts were between 90 and 110-fold less active than Trolox (81.14 ± 1.85, 96.08 ± 2.88 and 96.35 ± 1.73 µg mL^{-1} *vs*. 0.87 ± 0.18 µg mL^{-1}). Furthermore, IC_{50} of ethyl acetate extract from fresh/frozen *I. galbana* was lower than that of the spray-dried format (Table 4.1.10).

Table 4.1.10. Extraction yield (g 100 g^{-1} dried algae), **antioxidant activity** (%) and **IC_{50}** (µg mL^{-1}) of the different ***Isochrysis galbana*** formats and extraction solvents.

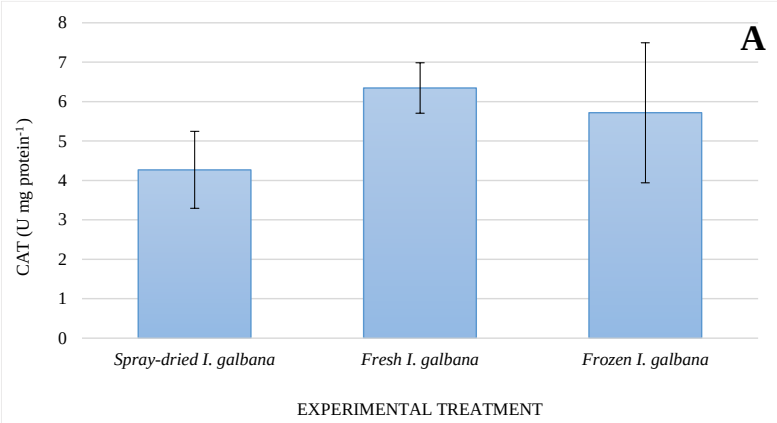
			DPPH		ABTS	
		Extraction yield	Activity	IC_{50}	Activity	IC_{50}
Spray-dried *I. galbana*	Ethanol	0.85	25.31 ± 0.62^{a}	>250	36.22 ± 1.94^{b}	>250
	n-hexane	12.01	36.76 ± 2.46^{ab}	>250	37.11 ± 2.11^{b}	>250
	Ethyl acetate	4.01	38.99 ± 3.99^{b}	>250	74.51 ± 2.35^{c}	96.08 ± 2.88
	Water	28.74	nd	>250	22.68 ± 4.06^{a}	>250
Fresh/frozen *I. galbana*	Ethanol	0.88	38.55 ± 1.34^{a,*}	>250	39.11 ± 5.11^{a}	>250
	n-hexane	9.74	48.77 ± 3.32^{b,*}	>250	58.81 ± 4.12^{b,*}	96.35 ± 1.73
	Ethyl acetate	2.04	60.98 ± 2.58^{c,*}	204.07 ± 4.35	72.49 ± 3.11^{c}	81.14 ± 1.85*
	Water	20.42	nd	>250	42.48 ± 1.09^{ab,*}	>250
Trolox			92.21 ± 0.32	7.43 ± 0.74	83.25 ± 1.28	0.87 ± 0.18

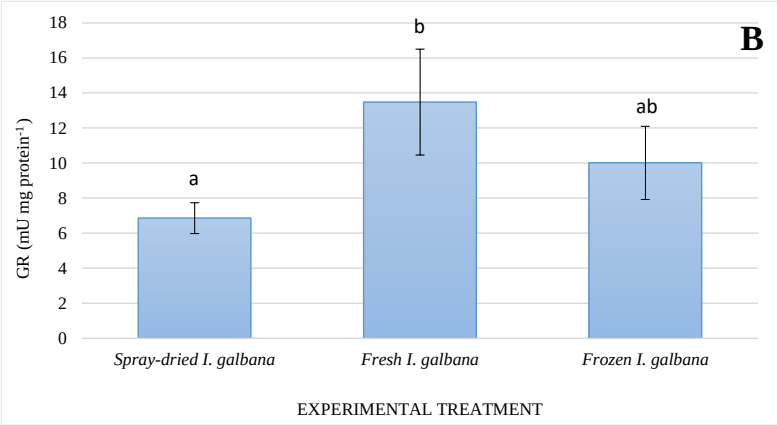
Results are presented as means ± SD. All determinations were carried out in quadruplicate. DPPH, 1,1-diphenyl-2-picryl-hydrazyl; ABTS, 2,20-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); IC_{50}, Concentration yielding 50% scavenging of each radical. ^{a,b,c} Represent significant differences between solvents within the same format of microalgae ($p<0.05$). * Represents significant differences between formats of microalgae for the same solvent ($p<0.05$). nd, not detected. Activity (%) was measured at 250 µg mL^{-1} for microalgae extracts and at 100 µg mL^{-1} for Trolox standard.

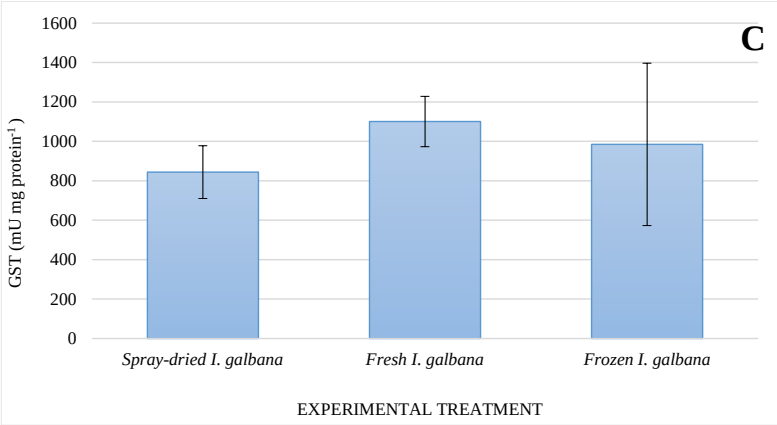
Live prey antioxidant enzymes, PxI and TBARS

Neither antioxidant enzyme activities measured in **rotifers** were affected by the dietary treatment, except GR, which ranged from 6.86 ± 0.88 mU mg protein^{-1} in the ISD-group, to 13.48 ± 3.02 mU mg protein^{-1} in IFRE-rotifers. Thus, CAT, GST and SOD ranged from 4.27 to 6.35 U mg protein^{-1}, 843.76 to 1100.56 mU mg protein^{-1}, and 338.32 to 830.95 U mg protein^{-1}, respectively. Moreover, the format of microalgae given to the rotifers did not influence PxI and TBARS. Thus, PxI ranged between 92.20 and 129.51 meqO_2 Kg^{-1}, and TBARS between 5.27 and 6.00 nmol MDA mg protein^{-1} (Figure 4.1.4).

On the other hand, IFRE-*Artemia* showed the highest values of GR and GST (0.88 ± 0.04 mU mg protein^{-1} and 1736.02 ± 368.19 mU mg protein^{-1}, respectively), while the other experimental treatments ranged from 0.27 to 0.33 mU mg protein^{-1} and 753.26 to 1126.75 mU mg protein^{-1}, respectively (Figure 4.1.5). Nonetheless, CAT and SOD remained unchanged independently of the experimental treatment (3.37-3.95 and 32.14 to 124.59 U mg protein^{-1}, respectively). Regarding lipid oxidation indexes, PxI was the lowest in ISD-*Artemia*, with 15.05 ± 3.85 meqO_2 Kg^{-1}, followed by *Artemia* fed Phytobloom Prof© and IFRO formats (27.93 ± 7.82 and 32.09 ± 14.99 meqO_2 Kg^{-1}, respectively), and that fed IFRE (57.47 ± 16.09 meqO_2 Kg^{-1}). ISD-*Artemia* also had lower TBARS values (1.76 ± 0.16 nmol MDA mg protein^{-1}), than those fed any of the other *I. galbana* formats (3.09-3.38 nmol MDA mg protein^{-1}; Figure 4.1.5).







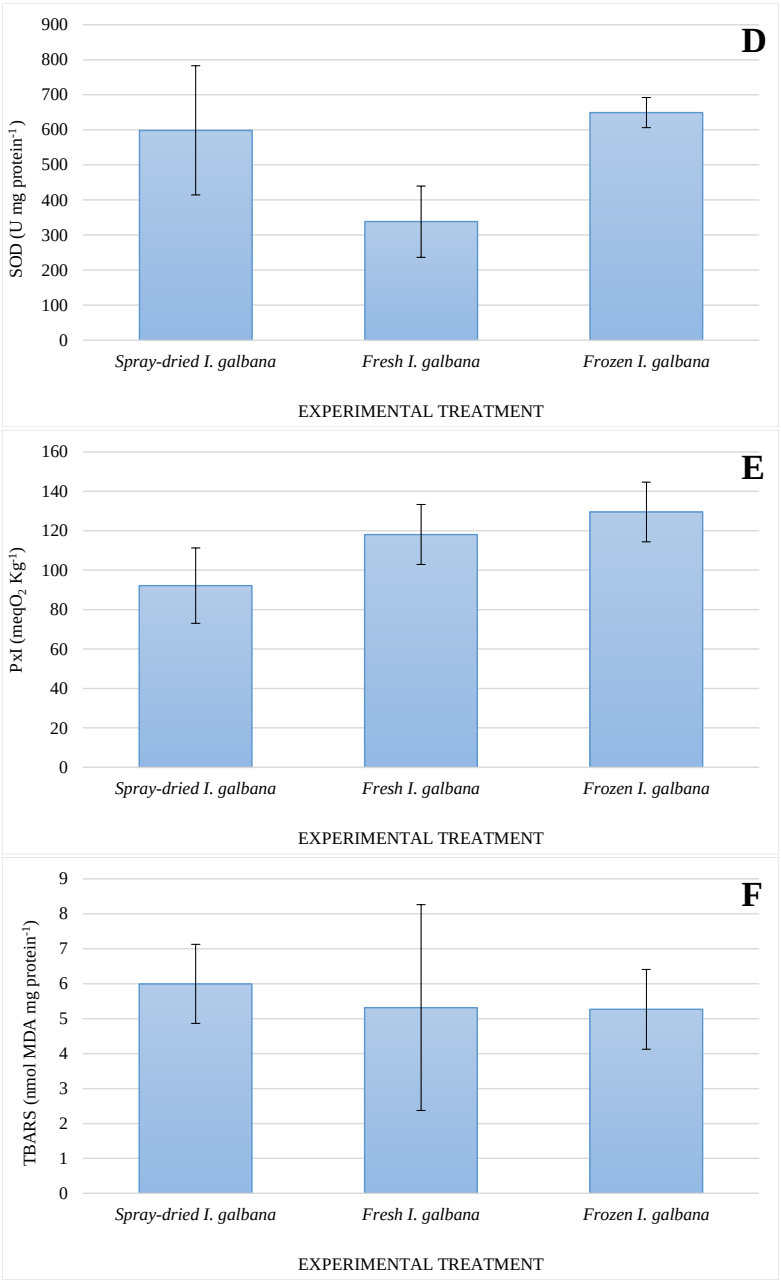
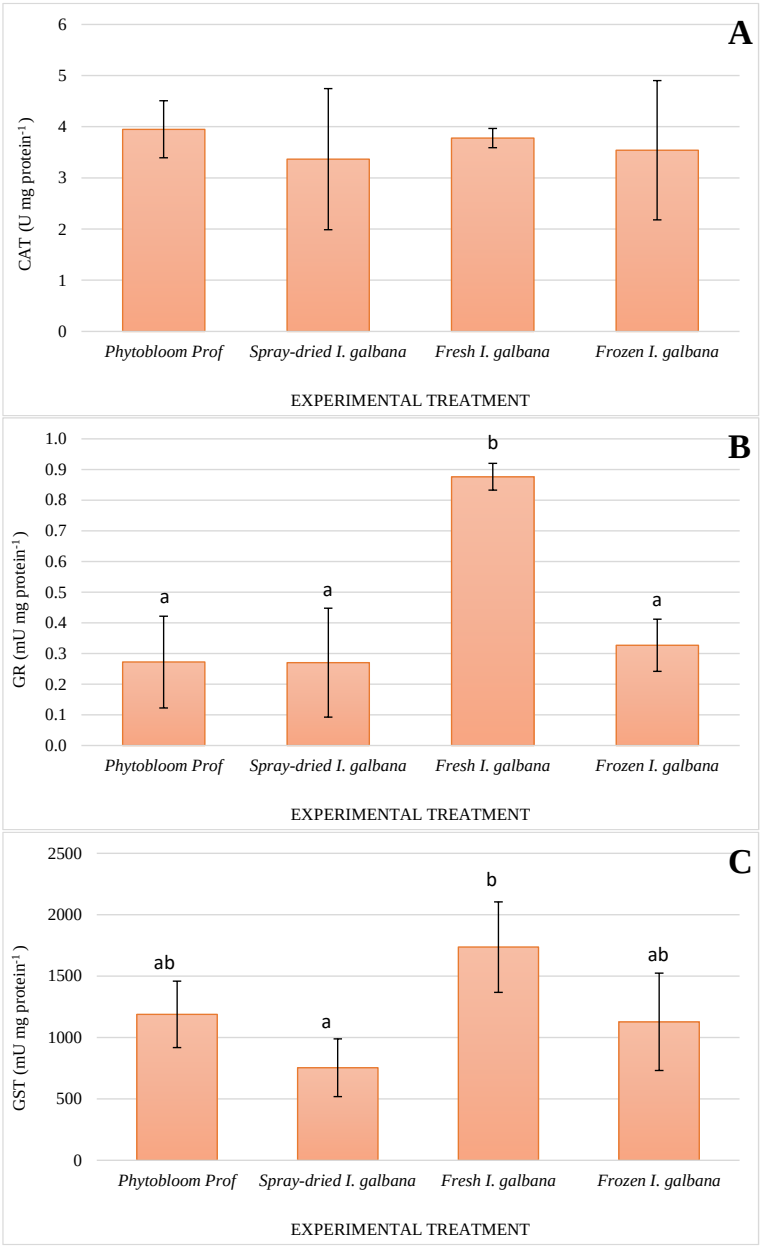


Figure 4.1.4. Antioxidant enzyme activities, peroxide index and **TBARS** of **rotifer** fed the different ***Isochrysis galbana*** formats.
A, catalase (CAT; U mg protein^{-1}); B, glutathione reductase (GR; mU mg protein^{-1}); C, glutathione S-transferase (GST; mU mg protein^{-1}); D, superoxide dismutase (SOD; U mg protein^{-1}); E, peroxide index (PxI; meqO_2 Kg^{-1}) and F, TBARS (nmol MDA mg protein^{-1}). Results are presented as means ± SD (n=3). Different letters represent significant differences between dietary treatments ($p<0.05$).



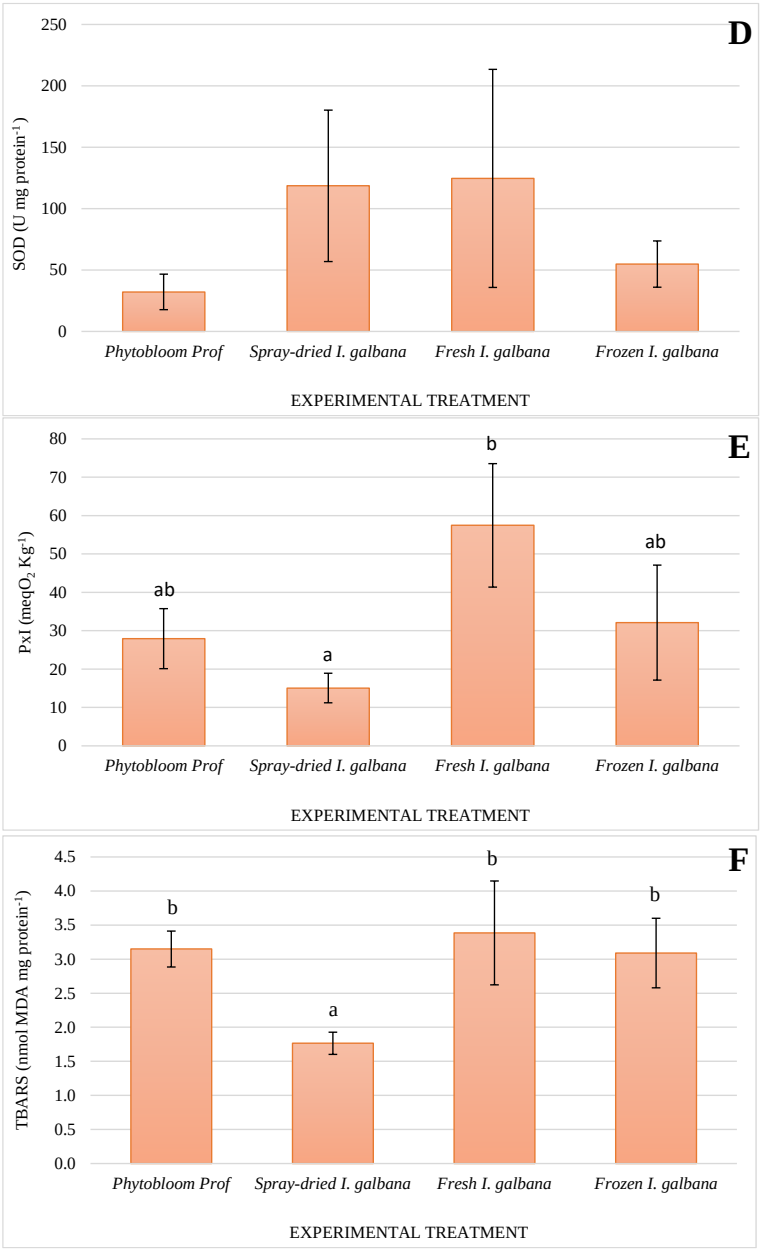


Figure 4.1.5. Antioxidant enzyme activities, peroxide index and TBARS of ***Artemia*** fed the different ***Isochrysis galbana*** formats.
A, catalase (CAT; U mg protein^{-1}); B, glutathione reductase (GR; mU mg protein^{-1}); C, glutathione S-transferase (GST; mU mg protein^{-1}); D, superoxide dismutase (SOD; U mg protein^{-1}); E, peroxide index (PxI; meqO_2 Kg^{-1}) and F, TBARS (nmol MDA mg protein^{-1}). Results are presented as means ± SD (n=3). Different letters represent significant differences between dietary treatments ($p<0.05$).

Experiment 2

- Survival

Neither **rotifer** (69-93%) nor ***Artemia*** (over 92% in all cases) survival was affected by the different formats of *N. salinicola* or spray-dried *I. galbana* (Figure 4.1.6).

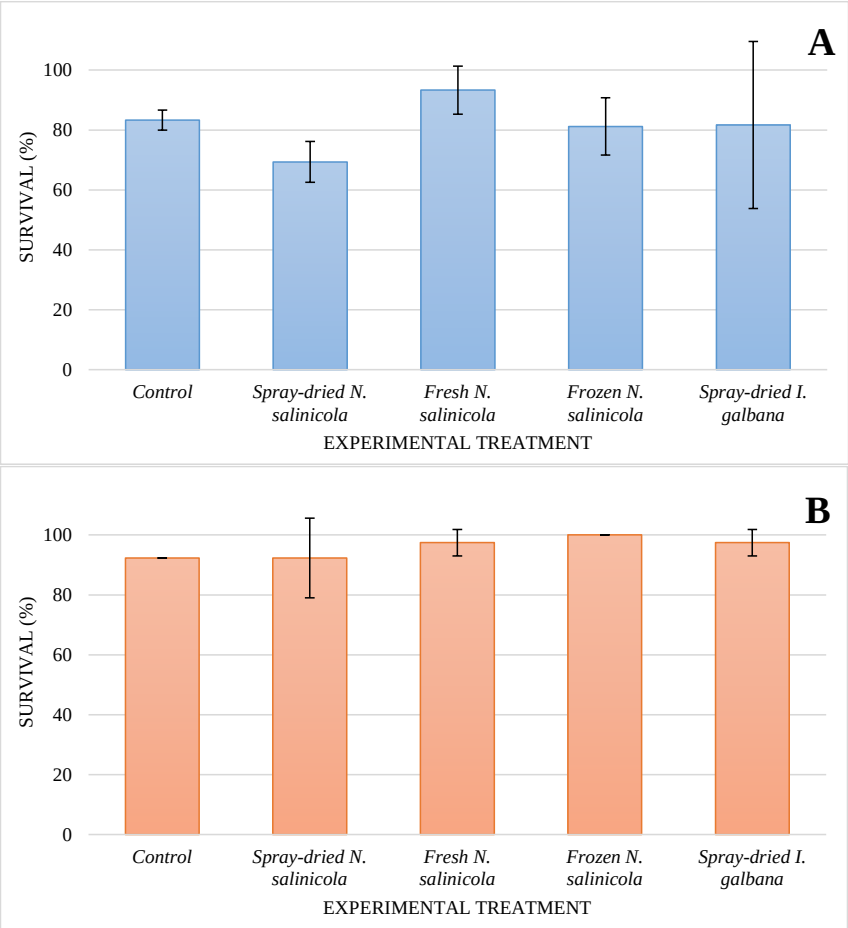


Figure 4.1.6. Survival (%) of **rotifers** (A) and ***Artemia*** (B) fed the different formats of ***Navicula salinicola*** and spray-dried ***Isochrysis galbana*** in combination with a lipid emulsion.
Results are presented as means ± SD (n=3). Different letters represent significant differences between dietary treatments ($p<0.05$).

- TL contents and LC profiles

Table 4.1.11 shows the TL contents and LC profiles of **rotifers** fed with the different dietary treatments. TL content remained unchanged, ranging between 23.01 ± 2.71 and 26.88 ± 6.56% DW in all cases. TNL encompassed between 82-88% of TL, mainly formed by TAG (33-37% of TL) in all cases. The only significant differences between dietary groups were found in CHO and FFA. Thus, CHO was higher in NFRE-rotifers (~15%) than in the control treatment (~12%), while NSD-rotifers showed the highest FFA value (~27%) and NFRO-rotifers the lowest (~18%).

The content of TPL in rotifers represented 12-18%, with both PC and PE (4-5%) being the most dominant polar fractions. However, NFRO-rotifers had the highest values of PG, phosphatidylserine (PS) and LPC+SM (1.93 ± 0.16%, 1.46 ± 0.20% and 1.52 ± 0.19%, respectively), while the lowest values were detected in NSD-rotifers (PG, 1.22 ± 0.47% and LPC+SM, 0.93 ± 0.16%) and NFRE-rotifers (PS, 0.77 ± 0.28% and LPC+SM, 0.88 ± 0.29%; Table 4.1.11).

The feeding treatments did not affect the TL content (18-19% DW) of ***Artemia*** nauplii (Table 4.1.12) that was mainly composed by neutral components in all treatments. The lowest TNL proportion was present in the control treatment (56.84 ± 3.23%), while ISD-*Artemia* showed the highest one with 62.94 ± 2.12% of TL. The main neutral LC were TAG, which was higher in both groups fed the spray-dried microalgae (29-30%) than in the control-*Artemia* (~25%). FFA was highest in NFRE and ISD-groups (~7%), being lowest in NFRO (~4%). SE reached its peak in ISD-*Artemia* (4.06 ± 0.41%), whereas the control group displayed the lowest value (2.65 ± 0.31%). Finally, CHO and MAG+DAG encompassed between 13-16% and 6-7%, respectively.

TPL content represented 43.16 ± 3.23% in the control treatment, followed by the three *N. salinicola* treatments (39-41%) and ISD (37.06 ± 2.12%). PC (10-15%) and PE (10-11%) were the most prominent LC, while NFRO and NSD had the highest PG content (3.56 ± 0.14 and 3.37 ± 0.03%, respectively), and ISD the lowest one (2.89 ± 0.16%)

Table 4.1.11. Total lipid content (% DW) and **lipid class** composition (% of total lipid) of **rotifers** fed the different formats of ***Navicula salinicola*** and spray-dried ***Isochrysis galbana*** in combination with a lipid emulsion.

	Control	Spray-dried *N. salinicola*	Fresh *N. salinicola*	Frozen *N. salinicola*	Spray-dried *I. galbana*
Total lipid	25.67 ± 2.14	25.92 ± 3.37	26.88 ± 6.56	23.01 ± 2.71	23.76 ± 2.50
LPC+SM	1.05 ± 0.30 ^{ab}	0.93 ± 0.16 ^{a}	0.88 ± 0.29 ^{a}	1.52 ± 0.19 ^{b}	0.95 ± 0.05 ^{ab}
PC	4.58 ± 0.24	5.05 ± 0.96	4.52 ± 0.44	4.73 ± 0.92	4.26 ± 0.39
PS	1.30 ± 0.16 ^{ab}	0.89 ± 0.30 ^{ab}	0.77 ± 0.28 ^{a}	1.46 ± 0.20 ^{b}	0.84 ± 0.21 ^{ab}
PI	1.86 ± 0.36	0.96 ± 0.45	0.93 ± 0.43	1.04 ± 0.09	1.07 ± 0.02
PG	1.77 ± 0.19 ^{ab}	1.22 ± 0.47 ^{a}	1.27 ± 0.19 ^{ab}	1.93 ± 0.16 ^{b}	1.28 ± 0.15 ^{ab}
PE	4.81 ± 0.26	4.28 ± 1.21	3.70 ± 0.92	5.05 ± 0.85	4.04 ± 0.22
UKPL	2.24 ± 0.32 ^{b}	--	--	1.15 ± 0.44 ^{a}	0.48 ± 0.15 ^{a}
TPL	17.60 ± 1.34	13.33 ± 3.18	12.08 ± 1.42	16.87 ± 2.61	12.91 ± 0.70
MAG+DAG	5.32 ± 0.06	7.04 ± 0.71	5.44 ± 1.62	7.75 ± 1.91	8.71 ± 1.24
CHO	11.66 ± 1.00 ^{a}	13.80 ± 0.45 ^{ab}	15.29 ± 0.85 ^{b}	13.88 ± 1.38 ^{ab}	14.81 ± 1.93 ^{ab}
FFA	20.57 ± 0.86 ^{ab}	26.60 ± 1.54 ^{b}	24.31 ± 0.89 ^{ab}	18.34 ± 2.96 ^{a}	22.87 ± 4.76 ^{ab}
TAG	37.44 ± 0.77	33.48 ± 3.02	36.12 ± 0.54	35.90 ± 3.54	33.67 ± 1.83
SE	6.03 ± 0.41	5.79 ± 0.69	6.12 ± 0.40	6.89 ± 0.54	6.19 ± 0.91
UKNL	1.40 ± 0.19	--	0.64 ± 0.80	0.37 ± 0.65	0.86 ± 0.38
TNL	82.40 ± 1.34	86.67 ± 3.18	87.92 ± 1.42	83.13 ± 2.61	87.09 ± 0.70

Results are presented as means ± SD (n=3). Different letters in superscript within the same row represents significant differences between dietary treatments ($p<0.05$). LPC, lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; UKPL, unknown polar lipids; TPL, total polar lipids; MAG, monoacylglycerols; DAG, diacylglycerols; CHO, cholesterol; FFA, free fatty acids; TAG, triacylglycerols; SE, sterol esters; UKNL, unknown neutral lipids; TNL, total neutral lipids.

Table 4.1.12. Total lipid content (% DW) and **lipid class** composition (% of total lipid) of ***Artemia*** fed the different formats of ***Navicula salinicola*** and spray-dried ***Isochrysis galbana*** in combination with a lipid emulsion.

	Control	Spray-dried *N. salinicola*	Fresh *N. salinicola*	Frozen *N. salinicola*	Spray-dried *I. galbana*
Total lipid	18.81 ± 1.77	19.49 ± 0.29	19.27 ± 0.70	18.62 ± 1.41	18.33 ± 3.32
LPC+SM	3.03 ± 1.65	2.73 ± 0.06	4.25 ± 0.41	3.43 ± 0.31	2.39 ± 0.51
PC	14.97 ± 3.23	10.98 ± 0.24	10.98 ± 1.24	11.45 ± 1.34	10.08 ± 0.31
PS	3.05 ± 0.20	3.02 ± 0.15	2.81 ± 0.10	3.16 ± 0.39	2.94 ± 0.19
PI	5.02 ± 0.39	4.51 ± 0.38	4.58 ± 0.43	4.82 ± 0.15	4.34 ± 0.40
PG	3.29 ± 0.14 ^{ab}	3.37 ± 0.03 ^{b}	3.15 ± 0.28 ^{ab}	3.56 ± 0.14 ^{b}	2.89 ± 0.16 ^{a}
PE	10.96 ± 0.34	10.81 ± 0.40	10.11 ± 0.18	10.87 ± 0.68	9.83 ± 0.75
UKPL	2.83 ± 0.55	3.77 ± 0.92	3.19 ± 1.17	3.52 ± 0.82	4.60 ± 0.94
TPL	43.16 ± 3.23 ^{b}	39.19 ± 0.93 ^{ab}	39.08 ± 1.01 ^{ab}	40.80 ± 2.22 ^{ab}	37.06 ± 2.12 ^{a}
MAG+DAG	6.35 ± 0.41	5.72 ± 0.39	6.41 ± 0.30	6.37 ± 0.55	6.52 ± 0.89
CHO	16.19 ± 2.14	15.35 ± 0.59	14.84 ± 2.00	15.95 ± 1.04	13.49 ± 1.59
FFA	4.96 ± 0.59 ^{ab}	6.04 ± 0.11 ^{ab}	7.08 ± 1.08 ^{b}	4.06 ± 1.60 ^{a}	7.08 ± 1.41 ^{b}
TAG	25.33 ± 1.39 ^{a}	29.14 ± 0.73 ^{b}	27.42 ± 0.40 ^{ab}	28.05 ± 1.08 ^{ab}	30.06 ± 1.33 ^{b}
SE	2.65 ± 0.31 ^{a}	2.91 ± 0.28 ^{ab}	3.31 ± 0.26 ^{ab}	3.39 ± 0.87 ^{ab}	4.06 ± 0.41 ^{b}
UKNL	1.36 ± 0.06	1.65 ± 0.30	1.86 ± 0.54	1.38 ± 0.88	1.72 ± 0.14
TNL	56.84 ± 3.23 ^{a}	60.81 ± 0.93 ^{ab}	60.92 ± 1.01 ^{ab}	59.20 ± 2.22 ^{ab}	62.94 ± 2.12 ^{b}

Results are presented as means ± SD (n=3). Different letters in superscript within the same row represents significant differences between dietary treatments ($p<0.05$). LPC, lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; UKPL, unknown polar lipids; TPL, total polar lipids; MAG, monoacylglycerols; DAG, diacylglycerols; CHO, cholesterol; FFA, free fatty acids; TAG, triacylglycerols; SE, sterol esters; UKNL, unknown neutral lipids; TNL, total neutral lipids.

- FA profiles

The FA composition of **rotifers** fed the different *N. salinicola* and *I. galbana* formats together with the Incromega™ oil emulsion are displayed in Table 4.1.13. Total FA contents were similar regardless of the dietary treatment (122-140 µg mg^{-1} DW). However, FA profiles highly varied between groups. Total SFA ranged from 9 to 11% of total FA, with NFRE-rotifers having the highest content, while total MUFA represented between 24 and 30% of total FA. The most relevant group was PUFA (56-64% of total FA), being higher in ISD and control-rotifers, followed by NSD, NFRO and lastly, NFRE. A similar pattern can be elucidated in total n-6 (8-9%), total n-3 PUFA (50-58%) and total n-3 LC-PUFA (47-53%).

It is remarkable the great content of 18:2n-6 (3.07 ± 0.05% and 3.04 ± 0.10%), ARA (2.22 ± 0.03% and 2.15 ± 0.03%) and 22:5n-6 (2.91 ± 0.03% and 3.07 ± 0.04%, respectively) in control and ISD-rotifers. Both groups also had the highest values of 22:5n-3 (2.10 ± 0.02% and 2.03 ± 0.04%) and DHA (42.36 ± 0.58% and 42.39 ± 0.69%, respectively) within the n-3 series. EPA, and especially DHA, were present in relevant amounts in all treatments (7-8% and 37-42% of total FA, respectively), with the highest value of EPA found in NSD (8.48 ± 0.04%) (Table 4.1.13).

All rotifers showed high DHA/EPA ratio (5.0-5.6), with control and ISD-rotifers showing the highest values, while EPA/ARA (3.5-4.1) ratio reached its peak in the groups fed NSD and NFRO. Finally, NSD-rotifers showed higher n-3/n-6 ratio (6.58 ± 0.06) with respect to NFRE-rotifers (6.25 ± 0.12) (Table 4.1.13).

FA composition of ***Artemia*** nauplii fed the different *N. salinicola* and *I. galbana* formats in combination with the LC 60® is shown in Table 4.1.14. Total FA contents did not differ regardless of the microalgae format (78-108 µg mg^{-1} DW). *Artemia* total SFA comprised 22 to 29% of total FA, with control treatment having the highest value and ISD and NSD the lowest ones. Palmitic acid (16:0) had remarkable proportions in the control group (18.34 ± 0.40%), and ~14% in NSD, NFRO and ISD-*Artemia*. Total MUFA was higher in NFRO (43.70 ± 1.42%) compared to NSD (41.50 ± 0.67%), control and ISD (38-39%).

PUFA varied between 29.31 ± 0.06% in the control-*Artemia* nauplii and 36.17 ± 0.56% in ISD, of which total n-6 represented being 6-7% and total n-3 a much higher proportion of 22-28%. Total n-6 PUFA, and their main components, 18:2n-6 and ARA were highest

in ISD-*Artemia* (7.11 ± 0.15%, 3.92 ± 0.09% and 2.88 ± 0.06%), respectively. In addition, both experimental groups fed spray-dried formats showed the highest n-3 LC-PUFA (23-24%) and EPA (~13%) contents whereas DHA was more abundant in ISD-*Artemia* (10.80 ± 0.34%) (Table 4.1.14).

DHA/EPA ratio varied from 0.61 ± 0.09 to 0.85 ± 0.02, being higher in ISD-*Artemia* than in control, NFRE and NSD-individuals. On the contrary, EPA/ARA ratio was highest in NSD and NFRO (4.39-4.80), followed by ISD and NFRE (4.21-4.41), and the control group (4.14 ± 0.02). Finally, n-3/n-6 ratio was higher in both groups of *Artemia* nauplii given the spray-dried formats (3.98 ± 0.13 and 4.14 ± 0.08) than in the control, NFRE and NFRO-groups (3.5).

Table 4.1.13. Total fatty acids (µg mg^{-1} DW) and main **fatty acid** composition (% of total FA) of **rotifers** fed the different formats of ***Navicula salinicola*** and spray-dried ***Isochrysis galbana*** in combination with a lipid emulsion.

	Control	Spray-dried *N. salinicola*	Fresh *N. salinicola*	Frozen *N. salinicola*	Spray-dried *I. galbana*
Total FA	140.26 ± 3.35	137.34 ± 12.02	135.82 ± 26.72	122.90 ± 37.49	123.85 ± 12.48
Total SFA	8.67 ± 0.28 ^{a}	9.29 ± 0.04 ^{ab}	11.01 ± 0.86 ^{b}	10.32 ± 0.67 ^{ab}	8.56 ± 1.04 ^{a}
14:0	0.36 ± 0.01 ^{a}	0.50 ± 0.03 ^{ab}	0.60 ± 0.05 ^{b}	0.60 ± 0.03 ^{b}	0.74 ± 0.09 ^{ab}
16:0	3.36 ± 0.11 ^{a}	4.37 ± 0.11 ^{b}	5.90 ± 0.37 ^{c}	5.67 ± 0.42 ^{c}	3.33 ± 0.36 ^{a}
18:0	3.71 ± 0.07 ^{b}	3.26 ± 0.02 ^{a}	3.41 ± 0.21 ^{ab}	3.24 ± 0.14 ^{ab}	3.37 ± 0.39 ^{ab}
Total MUFA	25.50 ± 0.54 ^{a}	28.16 ± 0.92 ^{b}	29.67 ± 0.53 ^{b}	29.80 ± 1.05 ^{b}	23.97 ± 0.43 ^{a}
16:1^{1}	4.50 ± 0.21 ^{a}	9.20 ± 0.63 ^{b}	10.19 ± 0.22 ^{bc}	10.56 ± 0.66 ^{c}	4.39 ± 0.09 ^{a}
18:1^{2}	14.42 ± 0.29 ^{b}	13.13 ± 0.28 ^{a}	13.86 ± 0.33 ^{ab}	13.62 ± 0.39 ^{ab}	13.54 ± 0.40 ^{ab}
20:1^{2}	3.54 ± 0.08 ^{b}	3.16 ± 0.03 ^{a}	3.23 ± 0.13 ^{ab}	3.16 ± 0.07 ^{a}	3.36 ± 0.17 ^{ab}
Total n-6 PUFA	8.57 ± 0.06 ^{b}	7.88 ± 0.08 ^{a}	7.74 ± 0.11 ^{a}	7.77 ± 0.05 ^{a}	8.59 ± 0.12 ^{b}
18:2	3.07 ± 0.05 ^{c}	2.81 ± 0.05 ^{a}	2.88 ± 0.06 ^{ab}	2.96 ± 0.08 ^{abc}	3.04 ± 0.10 ^{bc}
20:2	0.36 ± 0.02 ^{abc}	0.35 ± 0.01 ^{ab}	0.39 ± 0.02 ^{bc}	0.40 ± 0.01 ^{c}	0.33 ± 0.02 ^{a}
20:4 (ARA)	2.22 ± 0.03 ^{c}	2.05 ± 0.03 ^{b}	1.95 ± 0.04 ^{a}	1.96 ± 0.02 ^{a}	2.15 ± 0.03 ^{c}
22:5	2.91 ± 0.03 ^{c}	2.67 ± 0.08 ^{b}	2.52 ± 0.06 ^{ab}	2.45 ± 0.11 ^{a}	3.07 ± 0.04 ^{c}

Table 4.1.13. (Cont)

	Control	Spray-dried *N. salinicola*	Fresh *N. salinicola*	Frozen *N. salinicola*	Spray-dried *I. galbana*
Total n-3 PUFA	56.35 ± 0.70 ^{cd}	53.90 ± 0.89 ^{bc}	50.33 ± 1.18 ^{a}	50.96 ± 1.32 ^{ab}	58.05 ± 1.63 ^{d}
18:3	0.45 ± 0.02 ^{a}	0.50 ± 0.01 ^{a}	0.43 ± 0.06 ^{a}	0.48 ± 0.06 ^{a}	0.85 ± 0.08 ^{b}
18:4	0.44 ± 0.01 ^{a}	0.54 ± 0.01 ^{b}	0.50 ± 0.02 ^{ab}	0.52 ± 0.02 ^{b}	1.89 ± 0.24 ^{c}
20:4	0.68 ± 0.02 ^{ab}	0.66 ± 0.02 ^{ab}	0.62 ± 0.01 ^{a}	0.68 ± 0.05 ^{ab}	0.72 ± 0.06 ^{b}
20:5 (EPA)	7.69 ± 0.13 ^{a}	8.48 ± 0.04 ^{b}	7.41 ± 0.27 ^{a}	7.74 ± 0.24 ^{a}	7.60 ± 0.49 ^{a}
22:5	2.10 ± 0.02 ^{c}	1.97 ± 0.04 ^{ab}	1.87 ± 0.05 ^{a}	1.88 ± 0.07 ^{a}	2.03 ± 0.04 ^{bc}
22:6 (DHA)	42.36 ± 0.58 ^{b}	39.33 ± 0.79 ^{a}	37.19 ± 0.82 ^{a}	37.35 ± 1.09 ^{a}	42.39 ± 0.69 ^{b}
Total PUFA	62.69 ± 0.66 ^{cd}	59.73 ± 0.94 ^{bc}	56.11 ± 1.25 ^{a}	56.77 ± 1.35 ^{ab}	64.48 ± 1.72 ^{d}
DHA/EPA	5.51 ± 0.06 ^{b}	4.64 ± 0.08 ^{a}	5.02 ± 0.10 ^{a}	4.83 ± 0.16 ^{a}	5.59 ± 0.28 ^{b}
EPA/ARA	3.46 ± 0.07 ^{a}	4.13 ± 0.05 ^{b}	3.80 ± 0.19 ^{ab}	3.96 ± 0.09 ^{b}	3.53 ± 0.19 ^{ab}
n-3/n-6	6.32 ± 0.12 ^{ab}	6.58 ± 0.06 ^{b}	6.25 ± 0.12 ^{a}	6.31 ± 0.13 ^{ab}	6.51 ± 0.11 ^{ab}
Total n-3 LC-PUFA	53.24 ± 0.71 ^{c}	50.81 ± 0.87 ^{bc}	47.45 ± 1.12 ^{a}	48.01 ± 1.31 ^{ab}	53.15 ± 1.29 ^{c}

Results are presented as means ± SD (n=3). Different letters in superscript within the same row represents significant differences between dietary treatments ($p<0.05$). FA, fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long chain polyunsaturated fatty acids (≥ C20 and ≥ 2 double bonds). Totals include other minor components not shown. [1] Mainly n-7 isomers, [2] Mainly n-9 isomers.

Table 4.1.14. Total fatty acids (µg mg^{-1} DW) and main **fatty acid** composition (% of total FA) of ***Artemia*** fed the different formats of ***Navicula salinicola*** and spray-dried ***Isochrysis galbana*** in combination with a lipid emulsion.

	Control	Spray-dried *N. salinicola*	Fresh *N. salinicola*	Frozen *N. salinicola*	Spray-dried *I. galbana*
Total FA	94.75 ± 20.78	78.09 ± 12.12	97.70 ± 31.44	85.59 ± 13.16	108.11 ± 24.23
Total SFA	28.61 ± 0.39 ^{d}	21.86 ± 0.58 ^{a}	25.16 ± 0.13 ^{c}	23.14 ± 0.55 ^{b}	23.01 ± 0.58 ^{a}
14:0	1.17 ± 0.03 ^{b}	1.03 ± 0.01 ^{a}	1.10 ± 0.06 ^{ab}	1.07 ± 0.02 ^{ab}	1.37 ± 0.07 ^{c}
16:0	18.34 ± 0.40 ^{c}	13.67 ± 0.48 ^{a}	15.86 ± 0.12 ^{b}	14.49 ± 0.59 ^{a}	13.94 ± 0.58 ^{a}
18:0	6.70 ± 0.03 ^{b}	5.22 ± 0.09 ^{a}	5.99 ± 0.09 ^{b}	5.55 ± 0.03 ^{b}	5.66 ± 0.09 ^{b}
Total MUFA	39.49 ± 0.44 ^{a}	41.50 ± 0.67 ^{ab}	41.91 ± 0.42 ^{bc}	43.70 ± 1.42 ^{c}	37.82 ± 0.31 ^{a}
16:1^{1}	7.46 ± 0.05 ^{ab}	11.40 ± 0.46 ^{c}	10.06 ± 0.29 ^{c}	12.91 ± 1.16 ^{bc}	7.19 ± 0.08 ^{a}
18:1^{2}	29.52 ± 0.48 ^{b}	27.86 ± 0.33 ^{a}	29.54 ± 0.44 ^{b}	28.72 ± 0.50 ^{ab}	28.02 ± 0.42 ^{a}
20:1^{2}	1.46 ± 0.02 ^{b}	1.23 ± 0.07 ^{ab}	1.29 ± 0.03 ^{ab}	1.17 ± 0.09 ^{a}	1.33 ± 0.15 ^{ab}
Total n-6 PUFA	6.15 ± 0.04 ^{a}	6.29 ± 0.06 ^{a}	6.34 ± 0.04 ^{a}	6.28 ± 0.05 ^{a}	7.11 ± 0.15 ^{b}
18:2	3.59 ± 0.06 ^{a}	3.61 ± 0.05 ^{a}	3.63 ± 0.06 ^{a}	3.70 ± 0.09 ^{a}	3.92 ± 0.09 ^{b}
20:4 (ARA)	2.56 ± 0.03 ^{a}	2.68 ± 0.06 ^{a}	2.64 ± 0.03 ^{a}	2.58 ± 0.04 ^{a}	2.88 ± 0.06 ^{b}

Table 4.1.14. (Cont)

	Control	Spray-dried *N. salinicola*	Fresh *N. salinicola*	Frozen *N. salinicola*	Spray-dried *I. galbana*
Total n-3 PUFA	21.80 ± 0.14 [a]	26.05 ± 0.73 [b]	22.22 ± 0.30 [a]	22.27 ± 1.41 [ab]	28.25 ± 0.31 [b]
18:3	2.26 ± 0.02 [a]	2.46 ± 0.01 [b]	2.44 ± 0.03 [b]	2.49 ± 0.05 [b]	2.73 ± 0.07 [c]
18:4	0.99 ± 0.02 [a]	1.08 ± 0.03 [a]	1.08 ± 0.06 [a]	1.13 ± 0.06 [a]	1.58 ± 0.08 [b]
20:4	0.20 ± 0.01 [a]	0.24 ± 0.01 [b]	0.23 ± 0.01 [b]	0.24 ± 0.01 [b]	0.22 ± 0.00 [ab]
20:5 (EPA)	10.61 ± 0.07 [a]	12.86 ± 0.34 [c]	11.11 ± 0.15 [ab]	11.32 ± 0.31 [b]	12.69 ± 0.13 [c]
22:5	0.18 ± 0.02	0.21 ± 0.01	nd	0.17 ± 0.03	0.23 ± 0.01
22:6 (DHA)	7.55 ± 0.12 [a]	9.19 ± 0.43 [a]	7.36 ± 0.09 [a]	6.92 ± 1.18 [ab]	10.80 ± 0.34 [b]
Total PUFA	29.31 ± 0.06 [a]	34.03 ± 0.73 [b]	29.99 ± 0.27 [a]	30.35 ± 1.20 [ab]	36.17 ± 0.56 [c]
n-3/n-6	3.54 ± 0.04 [a]	4.14 ± 0.08 [b]	3.51 ± 0.06 [a]	3.55 ± 0.25 [a]	3.98 ± 0.13 [b]
DHA/EPA	0.71 ± 0.01 [a]	0.71 ± 0.02 [a]	0.66 ± 0.00 [a]	0.61 ± 0.09 [ab]	0.85 ± 0.02 [b]
EPA/ARA	4.14 ± 0.02 [a]	4.80 ± 0.14 [b]	4.21 ± 0.01 [ab]	4.39 ± 0.05 [b]	4.41 ± 0.13 [ab]
Total n-3 LC-PUFA	18.55 ± 0.13 [a]	22.51 ± 0.73 [b]	18.70 ± 0.23 [a]	18.66 ± 1.49 [a]	23.95 ± 0.45 [b]

Results are presented as means ± SD (n=3). Different letters in superscript within the same row represents significant differences between dietary treatments ($p<0.05$). FA, fatty acids; SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long chain polyunsaturated fatty acids (≥ C20 and ≥ 2 double bonds). Totals include other minor components not shown. [1] Mainly n-7 isomers, [2] Mainly n-9 isomers. nd, not detected.

- Microalgae antioxidant potential and oxidative status of live prey

TAC of microalgae

Extraction yield and antioxidant activities of *N. salinicola* and *I. galbana* formats used in the experiment 2 are displayed in Table 4.1.15.

DPPH antioxidant activity assay varied greatly between microalgae formats and solvents. Thus, it was low, ranging between 18.82 ± 8.64% and 43.76 ± 1.98%, with neither of the extracts presenting more than 50% of activity at the maximum concentration tested (250 µg mL^{-1}). In spite of their low activity, the highest values were recorded in spray-dried and fresh/frozen *N. salinicola* ethyl acetate extracts (43.76 ± 1.98% and 42.24 ± 2.91%, respectively) and spray-dried *I. galbana n*-hexane extract (36.76 ± 2.46%).

On the other hand, ABTS scavenging capacity of the different extracts ranged between 22.68 ± 4.06% and 77.31 ± 1.53%. ABTS assay showed that 4 extracts were capable of inhibiting more than 50% of the radicals at 250 µg mL^{-1}. Thus, IC_{50} could be calculated in all ethyl acetate extracts, and in the fresh/frozen *N. salinicola* ethanol extract. The lowest IC_{50} was obtained in fresh/frozen *N. salinicola* ethyl acetate extract, near 100-fold less than that of Trolox (86.68 ± 6.27 µg mL^{-1} *vs*. 0.87 ± 0.18 µg mL^{-1}).

Table 4.1.15. Extraction yield (g 100 g^{-1} dried algae), **antioxidant activity** (%) and **IC_{50}** (µg mL^{-1}) of the different formats of ***Navicula salinicola*** and spray-dried ***Isochrysis galbana,*** and extraction solvents.

			DPPH		ABTS	
		Extraction yield	Activity	IC_{50}	Activity	IC_{50}
Spray-dried *N. salinicola*	Ethanol	0.77	$29.52 \pm 0.61^{a,z}$	>250	$36.53 \pm 0.29^{b,y}$	>250
	n-hexane	24.34	$20.71 \pm 7.02^{ab,yz}$	>250	$26.88 \pm 0.72^{a,x}$	>250
	Ethyl acetate	3.10	43.76 ± 1.98^{b}	>250	77.31 ± 1.53^{c}	89.33 ± 3.62^{yz}
	Water	7.46	18.82 ± 8.64^{a}	>250	$28.31 \pm 1.60^{a,y}$	>250
Fresh/frozen *N. salinicola*	Ethanol	1.79	$28.66 \pm 0.65^{a,z}$	>250	$61.89 \pm 4.93^{b,z}$	171.9 ± 11.1^{b}
	n-hexane	19.97	$27.26 \pm 2.69^{a,y}$	>250	$45.32 \pm 1.05^{a,z}$	>250
	Ethyl acetate	1.29	42.24 ± 2.91^{b}	>250	76.76 ± 0.53^{c}	$86.68 \pm 6.27^{a,y}$
	Water	17.35	22.24 ± 8.71^{ab}	>250	$49.78 \pm 1.00^{b,z}$	>250
Spray-dried *I. galbana*	Ethanol	0.85	$25.31 \pm 0.62^{a,y}$	>250	$36.22 \pm 1.94^{b,y}$	>250
	n-hexane	12.01	$36.76 \pm 2.46^{b,z}$	>250	$37.11 \pm 2.11^{b,y}$	>250
	Ethyl acetate	4.01	38.99 ± 3.99^{ab}	>250	74.51 ± 2.35^{c}	96.08 ± 2.88^{z}
	Water	28.74	nd	>250	$22.68 \pm 4.06^{a,x}$	>250
Trolox			92.21 ± 0.32	7.43 ± 0.74	83.25 ± 1.28	0.87 ± 0.18

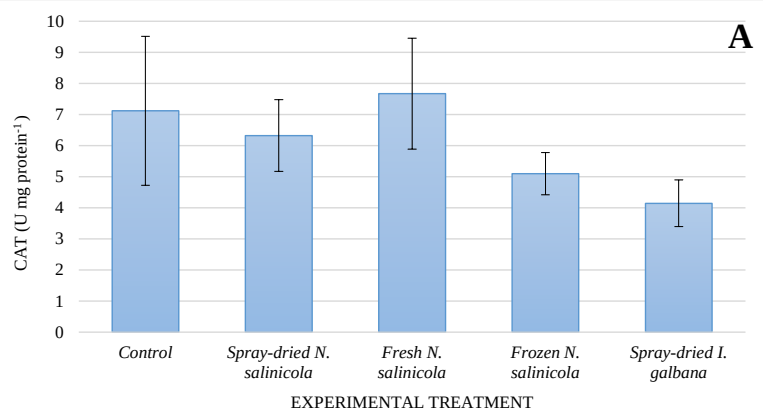
Results are presented as means ± SD. All determinations were carried out in quadruplicate. DPPH, 1,1-diphenyl-2-picryl-hydrazyl; ABTS, 2,20-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); IC_{50}, Concentration yielding 50% scavenging of each radical. ^{a,b,c,d} Represent significant differences between solvents within the same format of microalgae ($p<0.05$). ^{x,y,z} Represent significant differences between formats of microalgae for the same solvent ($p<0.05$). nd, not detected. Activity (%) was measured at 250 µg mL^{-1} for microalgae extracts and at 100 µg mL^{-1} for Trolox standard.

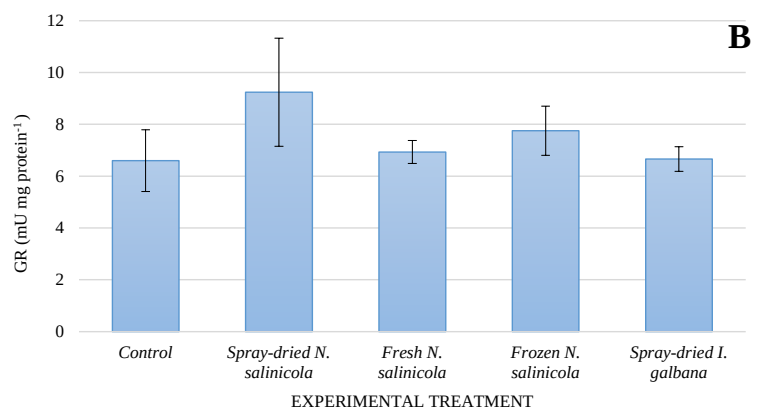
Live prey antioxidant enzymes, PxI and TBARS

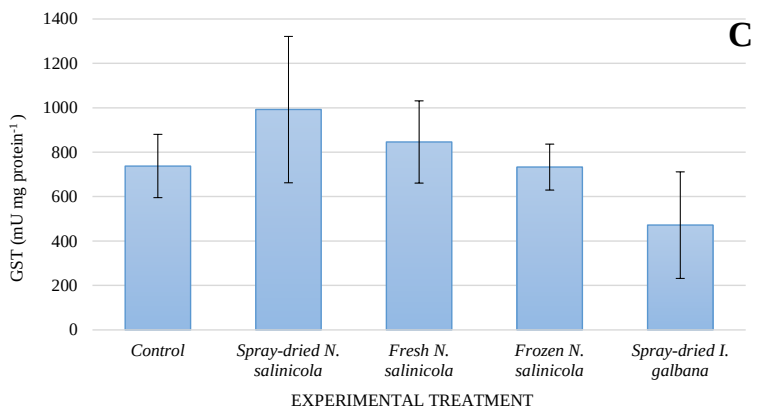
Antioxidant enzyme activities, PxI and TBARS of rotifers and *Artemia* nauplii fed the different formats of *N. salinicola* and ISD are shown in Figure 4.1.7 and Figure 4.1.8.

CAT (4.14-7.67 U mg protein^{-1}), GR (6.60-9.24 mU mg protein^{-1}), GST (736.06-991.95 mU mg protein^{-1}) and SOD (714.15-4135.77 U mg protein^{-1}) in **rotifers** were not affecred by dietary treatments. On the other hand, PxI showed differences in rotifers between the control (lipid emulsion) and the experimental treatments. Thus, PxI was 15.24 ± 2.00 meqO_2 Kg^{-1} in the control treatment, while it ranged between 57.88 and 91.28 meqO_2 Kg^{-1} in *N. salinicola* and ISD. However, TBARS was similar between the control and the ISD treatment (2.02 ± 0.89 and 1.14 ± 0.90 nmol MDA mg protein^{-1}, respectively), but lower than the different formats of *N. salinicola* (4.88-6.05 mg protein^{-1}; Figure 4.1.7).

Regarding ***Artemia*** nauplii (Figure 4.1.8), there were significant differences in the activity of CAT, showing lowest values in both ISD and NFRO (3.22 ± 0.49 and 3.49 ± 0.47 U mg protein^{-1}, respectively), than in the control treatment (6.76 ± 1.82 U mg protein^{-1}). Enzymatic activities of GR (0.40-0.94 mU mg protein^{-1}), GST (1580.11-2237.50 mU mg protein^{-1}), and SOD (48.73-93.11 U mg protein^{-1}) did not vary between dietary treatments. Lipid oxidation indexes PxI and TBARS did neither differ between *Artemia* nauplii (34.66-43.99 meqO_2 Kg^{-1} and 0.84-0.96 nmol MDA mg protein^{-1}, respectively).







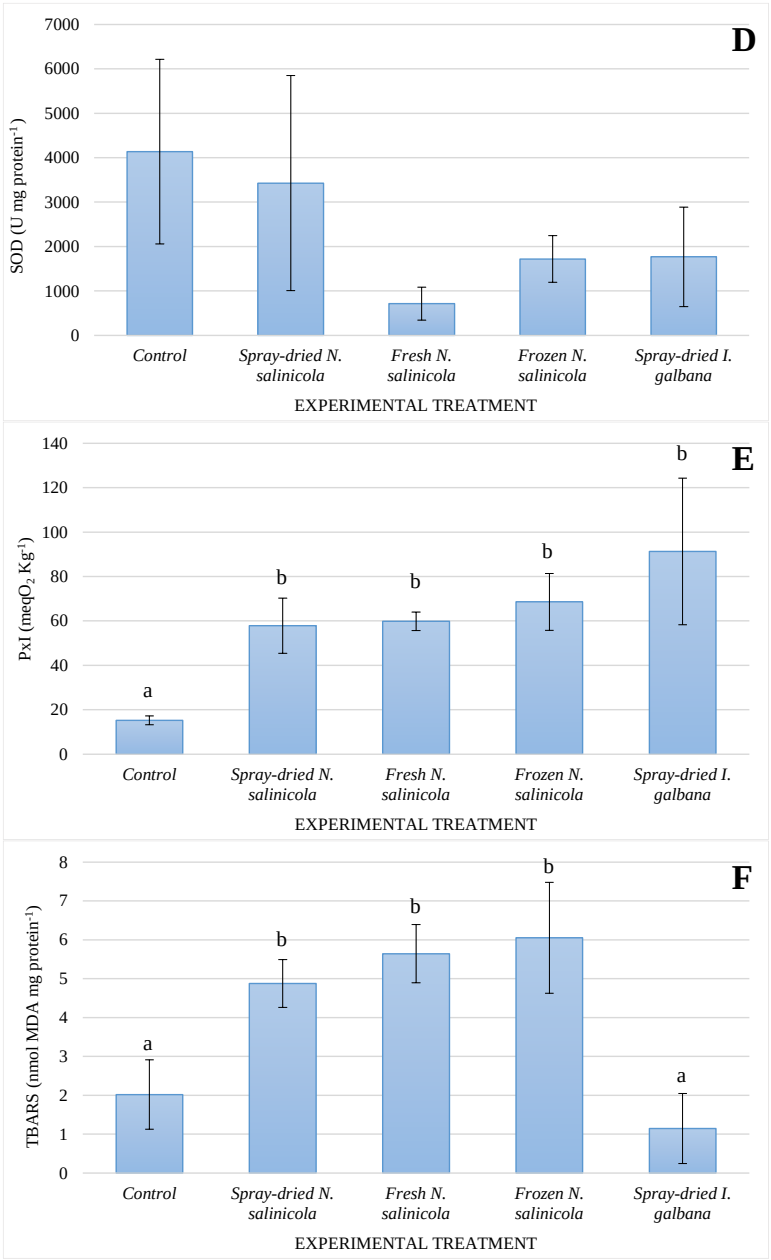
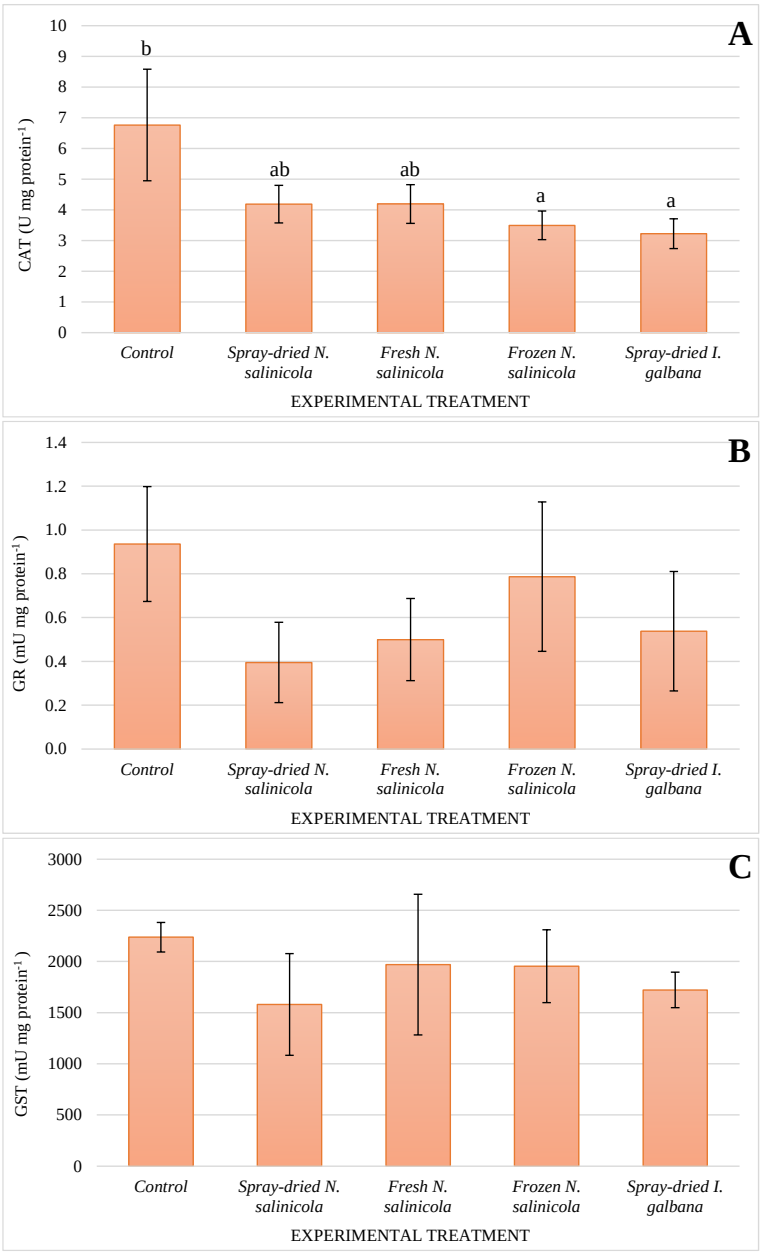


Figure 4.1.7. Antioxidant enzyme activities, peroxide index and TBARS of **rotifer** fed the different formats of ***Navicula salinicola*** and spray-dried ***Isochrysis galbana*** in combination with a lipid emulsion.
A, catalase (CAT; U mg protein^{-1}); B, glutathione reductase (GR; mU mg protein^{-1}); C, glutathione S-transferase (GST; mU mg protein^{-1}); D, superoxide dismutase (SOD; U mg protein^{-1}); E, peroxide index (PxI; meqO_2 Kg^{-1}) and F, TBARS (nmol MDA mg protein^{-1}). Results are presented as means ± SD (n=3). Different letters represent significant differences between dietary treatments ($p<0.05$).



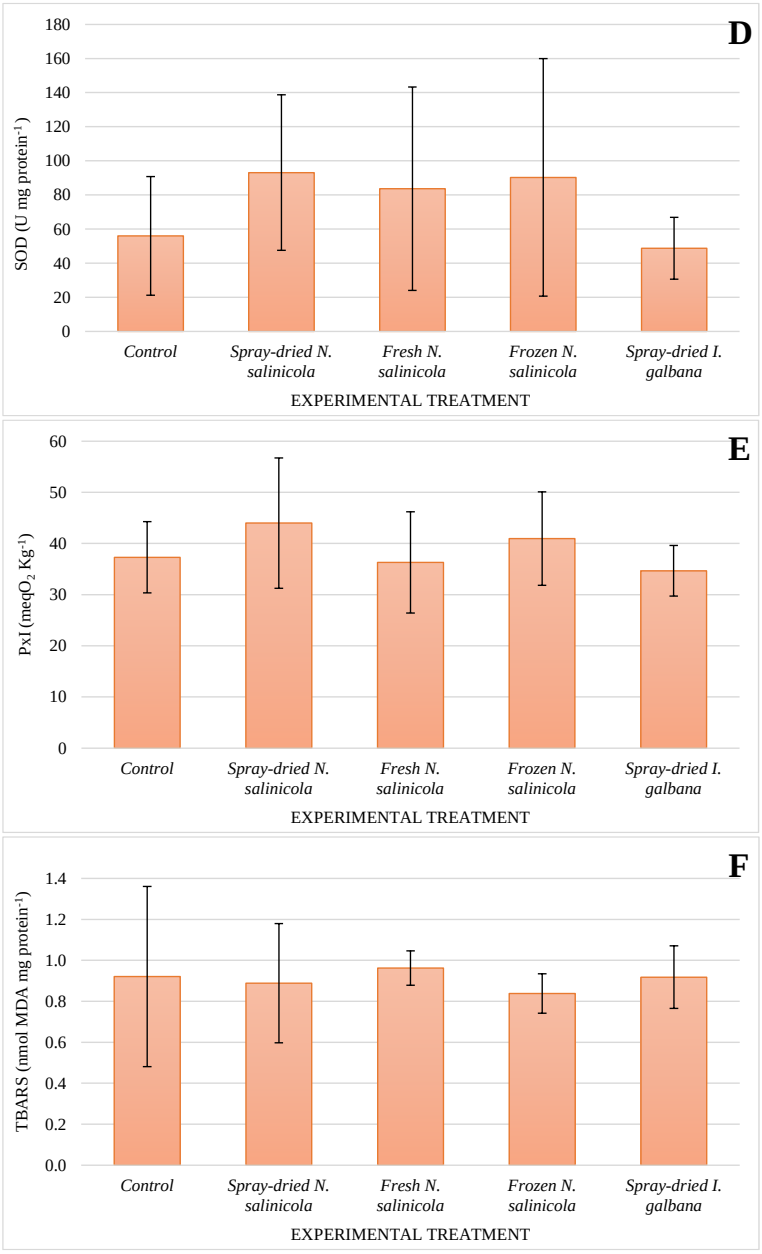


Figure 4.1.8. Antioxidant enzyme activities, peroxide index and TBARS of ***Artemia*** fed the different formats of ***Navicula salinicola*** and spray-dried ***Isochrysis galbana*** in combination with a lipid emulsion.
A, catalase (CAT; U mg protein^{-1}); B, glutathione reductase (GR; mU mg protein^{-1}); C, glutathione S-transferase (GST; mU mg protein^{-1}); D, superoxide dismutase (SOD; U mg protein^{-1}); E, peroxide index (PxI; meqO_2 Kg^{-1}) and F, TBARS (nmol MDA mg protein^{-1}). Results are presented as means ± SD (n=3). Different letters represent significant differences between dietary treatments ($p<0.05$).

4.1.4. Discussion

Rotifers and *Artemia* and rotifers are the most common zooplankton species used as live feed in hatcheries rearing for fish and shellfish larval rearing (Dhont et al., 2013). However, they are deficient in some nutrients such as LC-PUFA, which are essential in the diet of marine organisms, in particular during early life stages, where these compounds are relevant in several tissues such as brain and eye. Therefore, live prey must be enriched with those FA in order to succesfully contribute to larval proper development (Seychelles et al., 2009; Viciano et al., 2015, 2017). The addition of LC-PUFA-rich lipid emulsions, which are highly susceptible to oxidation, must be contrarrested by the inclusion of carotenoids or other antioxidant substances in the commercial products (Viciano et al., 2017). In this sense, a well selected microalgae format can exert several beneficial effects to commercial larval rearing including prey FA enrichment and/or an antioxidant protection (Khairy and El-Sayed, 2012; Turcihan et al., 2021). In this study, different formats of *I. galbana* and *N. salinicola* microalgae alone or together with a LC-PUFA-rich lipid emulsion were tested as enrichment products for both rotifers and *Artemia*.

Survival of *Artemia* was not affected by the format of microalgae used in any of the experiments carried out whereas rotifer's survival was compromised by fresh *I. galbana* consumption in experiment 1. Eryalçın (2019) suggested that fresh *N. oculata* performed worse than spray-dried *N. oculata*, probably related to different physicochemical properties that make the nutrients less available in the former format. Furthermore, algal pastes such as the fresh and frozen formats used in the present work have been described to produce adverse effects over water quality with respect to live microalgae (Dhont et al., 2013). In this experiment, the concentration used for fresh and frozen microalgae were 10 times higher than that of the spray-dried format (1.2 g L^{-1} *vs*. 0.12 g L^{-1}), pointing out to an excess of product added to the water. These doses were adjusted in the second experiment.

TL content in rotifers and *Artemia* was similar to previous studies (Palmtag et al., 2006; Thépot et al., 2016) and did not differ between treatments in either experiment or zooplankton species. However, the FA profiles slightly varied between rotifers fed the different *I. galbana* formats. Thus, total PUFA, including both n-6 and n-3 PUFA were higher in ISD-rotifers, despite the very similar FA profiles of experimental *I. galbana* formats, suggesting that PUFA incorporation into rotifers was enhanced by this dietary

treatment. Thus, ISD format seemed to be the dietary treatment that better enhanced PUFA incorporation into rotifers. Total n-3 LC-PUFA was also higher in this experimental group than in those receiving IFRE or IFRO (Table 4.1.8); mainly due to DHA, with spray-dried *I. galbana* showing the greatest contribution of both total n-3 LC-PUFA and DHA individually (Table 4.1.3). Similarly, rotifers fed both the control and ISD showed the highest total PUFA, n-6 and n-3 PUFA levels in experiment 2 (Table 4.1.13). Furthermore, both NSD and ISD enhanced n-3 LC-PUFA contents in *Artemia*, chiefly because of EPA and DHA percentages, respectively. A higher total n-3 and DHA retention with spray-dried *N. oculata* compared with freshly microalgae formats has also been previously reported in *B. plicatilis* (Eryalçın, 2019).

Although LC-PUFA can be a source of energy in fish, SFA and MUFA are more preferably catabolized (Tocher, 2003). Thus, they are an important source of metabolic energy, and their supply in sufficient amounts is critical, particularly in fast growing larvae with high energetic demands (Morais et al., 2007). Diets containing high levels of SFA and MUFA give a clear advantage to the larvae in terms of energy supply, allowing more valuable essential FA (such as LC-PUFA) to be spared for membrane composition and/or eicosanoid production (Garcia et al., 2008). It has been proposed that rotifers tend to maintain total SFA and total MUFA at constant levels (Rodríguez et al., 1997) in accordance to our first study, where both SFA and MUFA were similar between the *I. galbana* treatments (Table 4.1.8). On the contrary, in the experiment 2, total SFA and total MUFA were higher in the three formats of *N. salinicola* than those present in ISD and control treatments (Table 4.1.13). A higher content of SFA and especially of total MUFA in *N. salinicola* compared to *I. galbana* (Table 4.1.4), seems to favour total SFA and total MUFA retention in rotifers.

Seychelles et al. (2009) analysed the ingestion of fresh and frozen *I. galbana* T-ISO in the FA composition of rotifers. In our study with *I. galbana*, EPA values were lower than those reported by these authors (~2% *vs*. 5-6%), while DHA proportions in ISD were similar (experiment 1). These differences might be explained by the different duration of both experiments, while the enrichment protocol was conducted for 72 h by Seychelles et al. (2009), our study lasted for 24 h. This might also explain that EPA and DHA (in IFRE and IFRO) were incorportared to a lower extent than the reported percentages. Nevertheless, our study demonstrated to be more effective in terms of LC-PUFA incorporation than other microalgae feedings. For example, EPA and DHA were not

detected in rotifers and *Artemia* fed a microalgal mixture of *C. vulgaris* and *Dunaliella salina* for 24 h (Eryalçın, 2018). Moreover, EPA and ARA proportions in *Artemia* nauplii from our both experiments were higher than values reported in *Artemia* fed the commercial Ori-Green™ (algae + lipid emulsion-based product) (Rocha et al., 2017). Differences were probably due to the low n-3 LC-PUFA percentages in *C. vulgaris* and *D. salina* (Guermazi et al., 2010) and Ori-Green™, although the authors did not reported its FA profile. Shorter enrichment periods as those of experiment 2 (5 h), improved DHA retention in *Artemia* fed microalgae + lipid emulsion (Table 4.1.14.) compared to that of Artemia fed *I. galbana* alone (experiment 1; Table 4.1.9) or Ori-Green™ (Rocha et al., 2017). Similar results were obtained in rotifers, with both EPA and DHA being notably higher in our experiment 2 than in rotifers exclusively fed with *I. galbana* (experiment 1) or Ori-Green™ (Rocha et al., 2017). These results indicate that the combination of microalgae + lipid emulsion may be a better enrichment protocol than the commercial Ori-Green™ or *I. galbana* alone. Microalgae, in particular, spray-dried *N. oculata*, which has substantial amounts of EPA (Lubzens et al., 1995), like *N. salinicola* (Table 4.1.4), have been suggested to require a lipid enrichment during cultivation in order to successfully increase n-3 LC-PUFA in *B. plicatilis* (Eryalçın, 2019). According to this, the microalgae *Pavlova* sp. provided in paste format together with the commercial product Algamac 2000® showed an adequate FA profile for Atlantic cod larvae (Garcia et al., 2008). In our study, both rotifers and *Artemia* nauplii improved their n-3 LC-PUFA profile when microalgae were provided together with a lipid emulsion, either LC 60® or Incromega™.

DHA/EPA ratio is an usual indicator to evaluate the nutritional suitability of a diet for marine larvae (Viciano et al., 2017). Regardless of the experiment, both *B. plicatilis* and *Artemia* presented lower DHA/EPA ratios than those present in *I. galbana* (Table 4.1.3) and in the lipid emulsions (Table 4.1.5). The enrichment of *Artemia* with DHA is actually a challenge, due to its capacity to retroconvert DHA to EPA and its apparent inability to retain the C22 n-3 PUFA (Sorgeloos et al., 2001; Viciano et al., 2017). In this sense, a 24 h enrichment period could favour the retroconvertion of DHA to EPA, explaining the high EPA values reported in *Artemia*. Thus, in our experiment, shorter enrichment periods combining a high DHA-microalgae such as *I. galbana* and a lipid emulsion, improves DHA incorporation by *Artemia*. This could be probably due to the higher DHA content in the mixture and/or a decreased DHA retroconversion rate (experiment 2).

On the other hand, rotifers seem to better accumulate EPA than DHA (Rodríguez et al., 1997). Rotifers are freshwater filtering organisms, and freshwater microalgae usually have high amounts of EPA, supporting the preferential incorporation of EPA over DHA. Furthermore, an increased rate of DHA catabolism compared to EPA have been also proposed in rotifers, as it happens in *Artemia* (Rodríguez et al., 1997). In spite of this, and excluding *Artemia* nauplii in experiment 1, dietary ISD better enhanced DHA incorporation into live prey tissues, probably due to its higher content of DHA compared to the other *I. galbana* or *N. salinicola* formats (Tables 4.1.3 and 4.1.4).

Although studies about DHA/EPA ratio requirements in marine finfish suggest that it is species-specific (Park et al., 2006; Rodríguez et al., 1997), the DHA/EPA ratio recommended for marine fish larval proper development is similar or even higher than that present in natural preys such as copepods (~2) (Sargent et al., 1997; Viciano et al., 2017). In the experiment 1, ISD-rotifers had a DHA/EPA ratio of 2.68 (Table 4.1.8), higher than that of rotifers fed fresh or frozen T-ISO (Seychelles et al., 2009), *N. oculata*, or a mixture of *N. oculata* and *C. vulgaris* (Thépot et al., 2016). Furthermore, rotifers fed any *N. salinicola* format and ISD + lipid emulsion had even higher DHA/EPA ratios (4.64-5.69), due to the DHA contribution of the lipid emulsion (Table 4.1.5). However, *Artemia* DHA/EPA ratios were generally low in both experiments, according to other assays with either lipid emulsion or microalgae enrichment (Viciano et al., 2015, 2017). Nevertheless, and although still low, *Artemia* DHA/EPA ratio increased when a DHA-rich lipid emulsion was added together with the microalgae at a short period of enrichment. High DHA/EPA ratios were reported to improve larval growth and pigmentation (Park et al., 2006; Rodríguez et al., 1998; Sorgeloos et al., 2001), while inadequate ratios may alter the EPA and DHA composition of membrane phospholipids, affecting membrane function and larval growth. Furthermore, DHA is especially important in neural tissue phospholipids, playing a critical role in visual and brain processes as fish larvae depend on eyes and brain to identify and efficiently capture live preys. Hence, an appropriate contribution of DHA is of particular relevance at first living stages (Khairy and El-Sayed, 2012; Koven et al., 2018; Rodríguez et al., 1997; Viciano et al., 2017).

In addition to EPA and DHA, ARA is a relevant LC-PUFA in fish nutrition, being important for growth, survival, pigmentation, stress tolerance and egg/larval quality (Bell and Sargent, 2003). Furthermore, ARA, together with EPA, is involved in eicosanoids

production, with both of them compiting for the same enzymes (Sargent et al., 1999). Thus, it has been proposed that both concentration of EPA and ARA, and their ratio are also important in fish nutrition (Bessonart et al., 1999; Sargent et al., 1999). EPA/ARA ratios of 4 have been previously recommended for European seabass (*D. labrax*) and gilthead seabream (*S. aurata*) (Hamre et al., 2013) larvae. In this sense, *Artemia* and rotifers fed *I. galbana*/*N. salinicola* + lipid emulsion had EPA/ARA ratios around 4 (Tables 4.1.13 and 4.1.14). However, live preys fed with *I. galbana* alone gave rise to lower ratios than those recommended by Hamre et al. (2013) (Tables 4.1.8 and 4.1.9).

Several studies have suggested that phospholipids are preferred over TAG as the main lipid source by marine fish larvae (Lund et al., 2021; Park et al., 2006; Reis et al., 2019; Salhi et al., 1999; Turcihan et al., 2021). Diets formulated with TAG caused low growth rates and survival of fish, together with lipid accumulation in the liver and intestine. However, when phospholipids were added to the diet, those effects disappeared. Dietary phospholipids are required for the correct growth and survival of a range of species (Hamre et al., 2013; Lund et al., 2018). The requirement for dietary phospholipids in fish larvae is likely due to a limited ability to biosynthesize them *de novo* for chylomicrons formation and lipid transport out of the enterocytes (Davis and Hardy, 2022). Phospholipids are highly demanded in fast growing stages, as they are structural components of membranes, in addition to being involved in digestion, absorption and transport processes of lipids (Sargent et al., 1999). They also improve stress resistance, skeleton development and flatfish metamorphosis and pigmentation (Hamre et al., 2013). Finally, they are a source of choline and inositol (Sargent et al., 1999).

As PC and PE are the major phospholipids in brain and eggs of most fish species, achieving high values of both phospholipids in live preys is considered essential (McEvoy et al., 1995). In particular, PC improves growth more efficiently than other phospholipids, and enhance feeding activity. PC is also the best phospholipid source to provide LC-PUFA to pikeperch larvae (Lund et al., 2018; Reis et al., 2020). Other structutal component of biomembranes is PI, that presents several functions within the cells, including acting as a precursor of second messengers such as inositol 3 phosphate (IP3), and regulating the entry of calcium ions into the cell from the endoplasmatic reticulum (Hamre et al., 2013). PC and PE were highest in spray-dried *N. salinicola* (Table 4.1.2). but they were differently incorporated by *Artemia* and *B. plicatilis*. In this sense, experiment 1 evidenced that ISD enhanced PC retention in both species, and IFRO only

in *Artemia*, while both ISD and Phytobloom Prof© incremented PE percentages in *Artemia*, and PI in both species (Tables 4.1.6 and 4.1.7). As PC was similar in all *I. galbana* formats used, and spray-dried format contained the lowest PE levels (Table 4.1.1), a higher availability of nutrients in spray-dried formats may be responsible for these results as suggested by Eryalçın (2019).

Despite the above reported benefits regarding phospholipids incorporation in the zooplankton fed ISD, no improvement in the incorporation rates of PC, PE or PI was registered when used along with a lipid emulsion, compared to values obtained with *N. salinicola* + lipid emulsion or with the control treatment (experiment 2). Nevertheless, the effect of the lipid emulsion was noticeable in *Artemia*, which contained widely higher polar lipids when fed with LC 60® (high in phospholipids) than when fed *I. galbana* alone. Recent studies demonstrate that *Artemia* trends to metabolize dietary phospolipids into TAG (Guinot et al., 2013; Reis et al., 2017, 2019), so that our shorter enrichment period of 5 h probably favoured this increment in *Artemia´*s polar lipid contents.

TAG are usually the main neutral lipid, and the most abundant LC in marine organisms (Morais et al., 2007; Sargent et al., 1999). The frozen formats showed the highest TAG and the lowest FFA of all presentations (excluding the commercial Phytobloom Proof®) (Tables 4.1.1 and 4.1.2) pointing out that this post-processing method improves TAG retention by inhibiting lipases action. TAG ranged between 17-32% and 18-25% of TL in rotifers and *Artemia* from experiment 1, respectively. TAG proportions increased in experiment 2 (33-37% in rotifers and 25-30% in *Artemia*), due to the lipid emulsion contribution. This effect was especially high in rotifers, as Incromega™ is based on TAG. On the other hand, CHO has also a key role in modifying the main properties of the lipid bilayer of biological membranes such as fluidity, permeability, and hydrophobicity (Subczynski et al., 2017), and it was also relatively abundant in both species and experiments.

It is essential to provide an adequate balance between energy and essentiality through live prey, which can only really be achieved in practice by a suitable balance of phospholipids and TAG (Sargent et al., 1999). While rotifers and *Artemia* have a natural composition of 30% of polar lipids and 70% of neutral lipids, copepods have a ratio of approximately 50:50 (Hamre et al., 2013). As copepods are considered a valuable food for larvae of marine organisms, enrichment processes should approximate to this ratio. Under our

experimental conditions, *Artemia* showed a polar/neutral lipid ratios of 32-39/68-61 in experiment 1, and 37-43/63-57 in experiment 2, more similar to copepods than that of rotifers (13-19/87-81 in experiment 1, and 12-18/88-82, in experiment 2).

Our results altogether suggest that, as also proposed by others authors, different feed enrichment protocols result in different lipid compositions, and organisms can change their lipid content and profile according to the diet, being this changes species-specific (Rocha et al., 2017).

It must be also considered that encrichment conditions may compromise stability of PUFA during live prey enrichment processes. The vigorous aeration, illumination and temperature in the cultivation tanks promote autoxidation of PUFA and the formation of potentially toxic oxidation products (Viciano et al., 2015, 2017). The concentration of PUFA decreases with longer times of enrichment, especially EPA and DHA, thus reducing enrichment efficiency (McEvoy et al., 1995). Furthermore, as rancid lipids are toxic themselves and affect negatively proteins and vitamins, toxic oxidation products may accumulate and compromise health and survival of larvae. McEvoy et al. (1995) studied enrichment processes with different lipid emulsions during 23 h, and they recommended shorter periods of enrichment in order to reduce the accumulation of potentially toxic compounds and to improve enrichment efficiency, especially in DHA-rich emulsions (Viciano et al., 2015).

Artemia and rotifers naturally posses certain levels of antioxidant enzymes required to metabolize ROS such as CAT, SOD, GST and GR (Viciano et al., 2017; Wang et al., 2015). Antioxidant enzymes prevent, intercept and repair damage caused by free radicals (Viciano et al., 2017), including lipid oxidation. However, these endogenous protective mecanisms may not be enough to prevent oxidative damage, as LC-PUFA are very susceptible to oxidation, especially under enrichment processes (Monroig et al., 2007; Viciano et al., 2017).

SOD is the first defence mechanism in the detoxification process and, along with CAT, is regarded to play an important antioxidant role in aquatic organisms (Barata et al., 2005b). SOD usually increases in early stages of oxidative stress with the increment of ROS. However, if oxidative stress persists, SOD become depleted and unable to fight free radicals (Lukaszewicz-Hussain and Moniuszko-Jakoniuk, 2004; Viciano et al., 2017). In our two experiments and species, SOD was unaffected by the dietary treatment.

The presence of substrates that produce ROS, and thus, the response to oxidative stress, has been related to increased CAT activities. CAT is considered a second line of defence against ROS (Barata et al., 2005a, 2005b). Contrarily to SOD, CAT showed differences in *Artemia* nauplii from experiment 2. CAT activity was higher in *Artemia* fed the control treatment, decreasing in experimental NSD and NFRE groups, and showing its lowest activity in NFRO and ISD-*Artemia* (Figure 4.1.8). As organisms can up-regulate antioxidant defences in response to increasing ROS production (Barata et al., 2005a, 2005b), these results suggest a protective effect of the two last formats compared to the control.

GST are a family of detoxification enzymes that catalize the conjugation of GSH with various electrophilic substances. Thus, it detoxifies endogenous compounds such as peroxidized lipids. GST increases in response to exogenous chemical sources that promote ROS (Barata et al., 2005a, 2005b). In our study, ISD showed the lowest values of GST activity in *Artemia*, followed by IFRO, Phytobloom Prof© and IFRE (experiment 1; Figure 4.1.5), suggesting a better protection effect of ISD, consistently with CAT activity, in experiment 2.

Finally, GR is involved in the GSH-dependent antioxidative system, catalyzing the reduction of GSSG to GSH (Barata et al., 2005b; Lukaszewicz-Hussain and Moniuszko-Jakoniuk, 2004). GSH, is a nonenzymatic antioxidant *per se* and also a substrate for GST enzyme (Wang et al., 2015). Thus, GR is involved in the maintainance of GSH in its reduced form and owing to this, GSH plays its antioxidant functions (Barata et al., 2005b; Lukaszewicz-Hussain and Moniuszko-Jakoniuk, 2004). The increasing production of ROS can deplete the activity of GSH, inactivating GST, and consequently enhancing GR to regenerate GSH and maintan the reduction potential (Wang et al., 2015). The IFRE diet in rotifers from experiment 1 might be producing an excess of ROS, due to the activation of GR, but not of GST (Figure 4.1.4). Nevertheless, in *Artemia*, both GST and GR were enhanced by IFRE treatment (experiment 1), suggesting that GST was probably up-regulated and starting to consume GSH, but without being totally depleted.

It has been described that the efficiency of antioxidant defensive systems to remove ROS and prevent oxidative stress are transient and vary between species, enzymes and stressors, which would explain the disparity of our results between both species and experiments (Barata et al., 2005a, 2005b). The presence of an important antioxidant

carotenoid such as cantaxanthin in *Artemia* (Rønnestad et al., 1998), might be also contributing to the different responses to oxidative stress among the two species. Nevertheless, failure of antioxidant defences to remove ROS, will disrupt the antioxidant/prooxidant balance, leading to oxidative damage (Barata et al., 2005a). ROS have extremely short half-lives, and therefore they are difficult to be directly measured. Oxidative stress can be also assessed through the measurement of several products derived from lipid peroxidation (Viciano et al., 2017). Thus, PxI and TBARS can be used as indicators of oxidative damage to lipids (Shantha and Decker, 1994; Viciano et al., 2017). In the experiment 1, where feeding with *I. galbana* was assayed, IFRE treatment, followed by IFRO and Phytobloom Prof© showed the highest values of PxI and TBARS in *Artemia* (Figure 4.1.5). Relatively high values of both lipid peroxide indexes paralleled with altered patterns of antioxidant enzymes (higher GR in the three treatments and enhanced GST activity in IFRE), which have been previously described by other authors (Barata et al., 2005a). It is important to highlight that ISD palliated oxidative stress even better than the commercial product Phytobloom Prof©. Besides, it has been proposed that, as a consequence of PUFA autoxidation, dissolved oxygen could be reduced in the enrichment media causing mass mortalities of live preys (McEvoy et al., 1995; Viciano et al., 2015). However, and despite the high peroxide indexes, chiefly in IFRE, *Artemia* survival was not significantly decreased under our experimental conditions (Figure 4.1.3), in accordance to the experiment conducted by McEvoy et al. (1995). On the other hand, although lipid peroxidation may affect the enrichment efficiency, particularly that of LC-PUFA, neither ARA, EPA or DHA were affected by the higher PxI or TBARS values in *Artemia*, similarly to data previously reported by Viciano et al. (2017).

In the experiment 2, no antioxidative enzyme activity was enhanced in rotifers. In spite of this, all experimental treatments showed higher PxI and TBARS indexes than the control group, except ISD-TBARS (Figure 4.1.7). TBARS are used to measure MDA content, a final product of lipid peroxidation (Ayala et al., 2014). The fact that ISD showed high values of PxI, but not of TBARS, indicates that (1) it is partially compensating lipid peroxidation, without forming final peroxidation products, or (2) the time of enrichment was enough to generate oxidative stress but not enough to maintain it. Thus, maybe lipid peroxidation would be avoided by reducing the time of enrichment.

Excessive ROS have been described to inactivate SOD and CAT activities in rotifers (Wang et al., 2015). As lipid emulsions tested in experiment 2 promote an extra oxidative

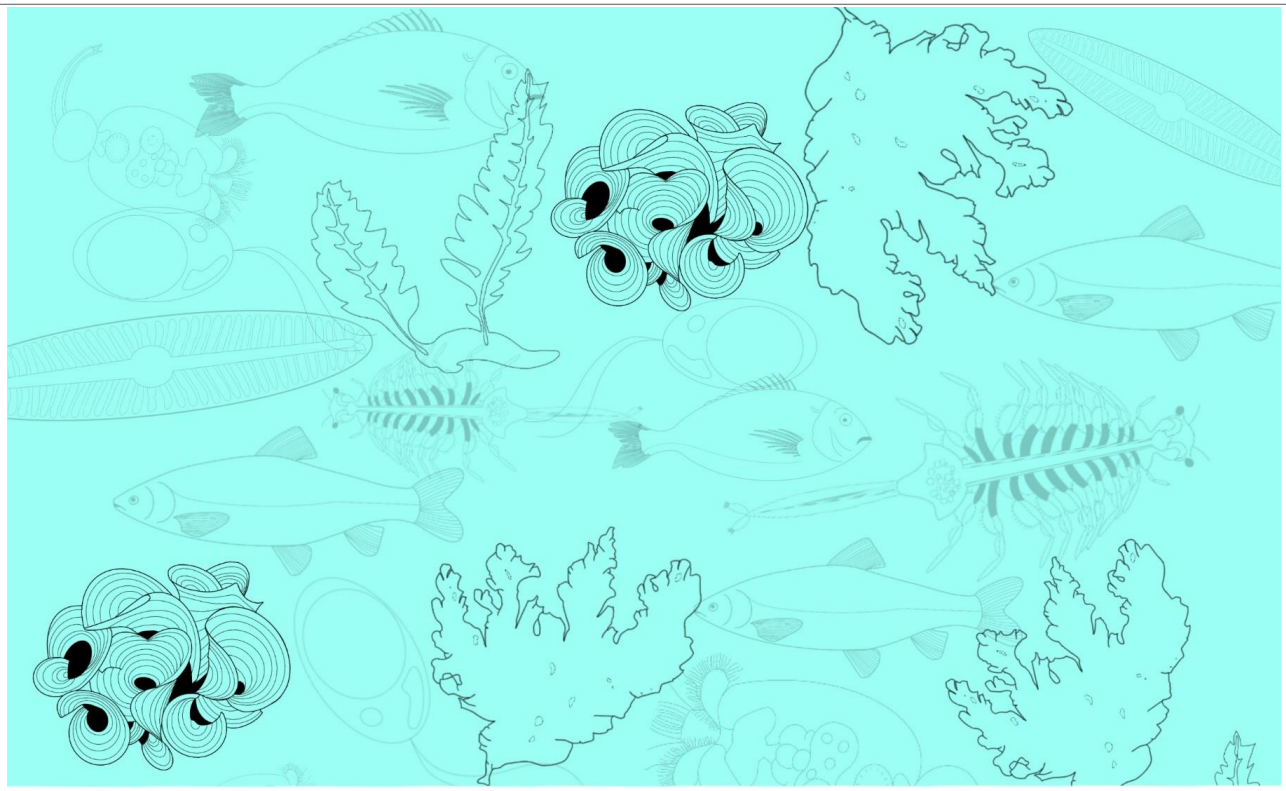
stress (Viciano et al., 2015), higher lipid peroxides values found in rotifers are probably related to a possible depletion of antioxidant enzymes and disability to overcome oxygen-derived free radicals (Lukaszewicz-Hussain and Moniuszko-Jakoniuk, 2004; Viciano et al., 2017). Despite the high peroxide indexes, rotifer survival was not affected by dietary treatments (Figure 4.1.6). However, our results suggest that, as PUFA are especially susceptible to lipid peroxidation, FA composition of rotifers may be altered by the high lipid peroxidation indexes observed in some of the dietary treatments. Thus, high values of TBARS/PxI correlates with lower proportions of DHA (NSD, NFRE and NFRO), EPA and ARA (NFRE and NFRO) in spite of the similar LC-PUFA percentages provided by the different *N. salinicola* formats. High TBARS and low DHA contents in *Artemia* fed a lipid emulsion compared to levels obtained when fed a lipid emulsion + antioxidant have been already stated (Viciano et al., 2017). On the contrary, in spite of the high CAT activity in the control *Artemia*, peroxide indexes did not differ between treatments, indicating that the antioxidant activity was able to efficiently fight against ROS.

Finally, DPPH scavenging capacity in both formats of *I. galbana* (25-61%; Table 4.1.10) was higher than that reported for similar extracts (28-35%; acetone, methanol and hexane) (Saranya et al., 2014). On the contrary, *N. salinicola* extracts showed lower DPPH scavenging activity (22-44%; Table 4.1.5) than that described for *N. salinicola* (~62.8%) (previously known as *Navicula incerta*; Hong et al., 2019) (Affan et al., 2007), although it might also be due to the different solvent used in this study (methanol).

Extracts from fresh/frozen *I. galbana* were usually more active in both DPPH and ABTS assays than the spray-dried extracts. Only ethyl acetate extract from the fresh/frozen format was able to scavenge the DPPH radical by more than 50% at 250 µg mL^{-1}. Nevertheless, the ABTS test is a more sensitive method than the DPPH one, since the neutralization of the ABTS radical can occur by transferring both an electron or a hydrogen atom (Zárate et al., 2020). Thus, the ABTS test yielded more interesting results, with both ethyl acetate and *n*-hexane extracts from fresh/frozen *I. galbana* capable of inhibiting more than 50% of the radicals at 250 µg mL^{-1}. On the other hand, ethyl acetate extracts from both formats and ethanol extract in fresh/frozen *N. salinicola* were also able to neutralize more than 50% of ABTS at 250 µg mL^{-1}. Several metabolites such as FA, carotenoids, glycerophospholipids and sphingolipids have been identified in the ethyl acetate extract from *I. galbana;* with this extract being recommended as the best solvent to obtain most of the valuable FA and carotenoids in this species (Bustamam et al., 2021).

Antioxidant capacity of *I. galbana* has been previously attributed to its carotenoid, polysaccharides and phenolic compounds contents (Saranya et al., 2014; Sun et al., 2014). Interestingly, ISD generally showed better antioxidant results in *Artemia* and rotifers than the other formats of both microalgae, as described above. However, *in vitro* assays can only consider antioxidant activity for their particular reaction system, and their relevance to *in vivo* health protective activities is uncertain. Furthermore, algae products composition is highly complex, and separating each antioxidant compound and studying it individually is costly and inefficient, not withstanding the possible synergistic interactions among the antioxidant compounds (Badarinath et al., 2010).

In conclusion, both *I. galbana* and *N. salinicola* might be considered promising microalgaes to feed *Artemia* and rotifers. Attending to its n-3 LC-PUFA content, DHA/EPA ratio and antioxidant potential, *I. galbana* is more adecuate than *N. salinicola* to feed *Artemia* and rotifers for fish larval rearing. However, in order to successfully enrich live preys, especially *Artemia* in LC-PUFA, a lipid emulsion should be also used in combination to the microalgae. The addition of a lipid emulsion increases the risk of oxidative stress, which is partially compensated by the ISD format, although shorter times of enrichment are recommended in order to avoid DHA retroconversion and lipid peroxidation and consequently, to improve live preys final lipid composition.



Lipid characterization and potential use of macroalgae as food and animal feed additive:

Lipid characterization of macroalgal species from the Macaronesian region

4.2. LIPID CHARACTERIZATION AND POTENTIAL USE OF MACROALGAE AS FOOD AND ANIMAL FEED ADDITIVE

4.2.A. Lipid characterization of macroalgal species from the Macaronesian region

A.1. Specific introduction

Marine macroalgae are fast-growing multicellular, photosynthetic organisms, classified into three major groups based on their pigmentation: green macroalgae (Chlorophyta), red macroalgae (Rhodophyta) and brown macroalgae (Ochrophyta). Seaweeds are traditionally consumed as food in Asia, mainly Japan, China and Korea (Roleda et al., 2018). However, their demand as food has also extended to occidental societies, mainly due to a change in consumer preferences, being increasingly recognized as a type of healthy "superfood" that leads to the production of algal-derived innovative products (FAO, 2018). Marine macroalgae are rich in nutritional and bioactive compounds, including minerals such as iodine, fibre, vitamins, carbohydrates, proteins, lipids, chiefly PUFA, PTS, and phenolic compounds. Seaweeds also possess high contents of pigments that exert antioxidant and anticancer activities (Chandini et al., 2008; Nunes et al., 2020; Stengel et al., 2011; Zárate et al., 2020). In addition, several other compounds of macroalgae, such as carragenans or fucoidans, between others, are described to have potential medical applications, including antitumor, anticoagulant, antiviral, antiprotozoal, antifungal, and antibacterial properties (for details, see Barzkar et al., 2019).

Given the versatility of seaweeds for their application in industries, their global production is expected to increase in the coming decades. Seaweed aquaculture has been practiced for decades in Asian countries (Campbell et al., 2019), especially in China, the main world producer in both value and volume (FAO, 2018). Farmed macroalgae represent 96% of the total global supply (Harwood, 2019), that is almost exclusively used for direct human consumption (FAO, 2018, 2020). In Western countries, seaweeds are mainly exploited for the industrial production of phycocolloids such as alginate, agar-agar and carrageenan (Dellatorre et al., 2020), although its production by aquaculture has been recently established as a commercial activity (Campbell et al., 2019).

Seaweeds are a promising protein source, presenting a higher content of essential amino acids than terrestrial vegetables (Fleurence, 1999). In this regard, it is foreseeable that seaweed consumption increases due to the growing demand for protein sources that can overcome the anticipated challenges of a growing world population and food scarcity, and the demand for alternative proteins in Western countries. In recent years, lipid composition of macroalgae has also raised considerable interest due to their valuable content of n-3 LC-PUFA and of certain type of lipids. In general, marine macroalgae have low lipid levels (<5% DW) (Dellatorre et al., 2020; Schmid et al., 2018), and fluctuating FA profiles, which vary greatly among taxa (Stengel et al., 2011). These variations have been attributed to several factors, including seasonal and geographical changes, environmental parameters, physiological status, and even molecular mechanisms in response to environmental factors (Verma et al., 2017).

Glycolipids and phospholipids are the major LC in algae (Guihéneuf et al., 2015), together with TAG (Harwood, 2019). Glycolipids are mainly located in photosynthetic membranes playing a crucial role in maintaining optimal photosynthetic efficiency (Nakamura and Li-Beisson, 2016), and are predominantly represented by MGDG, DGDG and SQDG. Furthermore, major phospholipids are PC, PE and PG (Guihéneuf et al., 2015), which are mostly localized in non-plastid membranes, except for the latter which is present in the chloroplast envelope (Nakamura and Li-Beisson, 2016). MGDG, DGDG and PG have been described as anti-inflammatory and anti-thrombotic compounds, while PTS are known to lower total and low-density lipoprotein (LDL) cholesterol levels in humans (Ibañez and Cifuentes, 2013). Despite the low lipid level reported in macroalgae (Dellatorre et al., 2020; Schmid et al., 2018), their PUFA content is greater than that of terrestrial plants (Kendel et al., 2015). Within PUFA, the LC-PUFA are physiologically important molecules (Trushenski and Rombenso, 2020) involved in cell membrane structure, transcription, regulation and cellular signalling (Lee et al., 2016; Zárate et al., 2017). Furthermore, a high dietary intake of n-3 LC-PUFA has been shown to prevent some human diseases, including colon and breast cancers, neurodegenerative or inflammatory illnesses, and even to reduce the prevalence of dementia (Harwood, 2019; Lee et al., 2016; Zárate et al., 2017). Particularly, EPA and DHA have been demonstrated to reduce cardiovascular disease and arthritis, and to improve brain function (Harwood, 2019). Consequently, global demand for n-3 FA has significantly increased over the last decades. Fish and other marine products are almost the only natural source of n-3 LC-

PUFA for humans (Zárate et al., 2017). However, the metabolic origin of these FA is generally not fish itself but marine phytoplankton and macroalgae, which form their major dietary source (Colombo et al., 2020). Alternatively, these FA accumulate throughout the marine food chain. Algae possess not just the capacity to synthesize *de novo* ALA and LA, but also LC-PUFA, whose content differs among taxa (Bourgougnon et al., 2011). To date, the feeding of aquatic captive-reared species has relied heavily on fishmeal and FO obtained from wild pelagic fish populations, giving rise to fish stocks currently either fully exploited or overexploited (FAO, 2020). Recently, various plant-based sources have been tested to replace marine ingredients due to their higher availability, sustainability and reduced cost. In fact, diets of most consolidated cultured species include a substancial amount of vegetable meal and VO. Nonetheless, some terrestrial alternatives present low digestibility, contain some antinutritional factors, and are deficient in certain essential amino acids and n-3 LC-PUFA, resulting in a significant reduction of the nutritional quality of the edible product (Welker et al., 2016). By contrast, the inclusion of small amounts of macroalgae in aquafeeds seems to positively affect fish growth performance and feed efficiency due to their high nutritional value and balanced composition (Norambuena et al., 2015).

For all these reasons, a wide variety of seaweeds can be potentially exploited as a main source of n-3 LC-PUFA, not just for direct human consumption but also for animal feed production, offering a continuous and sustainable supply of these essential compounds and contributing to satisfying the world population's needs. **The present study aims** to broadly characterize the lipid and FA profiles of the still understudied, but representative macroalgal species from Madeira Archipelago (14 species), and Gran Canaria Island coasts (12 species). In particular, it is intended to evaluate their potential as sources for both n-3 LC-PUFA and other healthy lipid molecules with marked anti-hypercholesterolemic and anti-hypertriglycerolemic properties for human and animal nutrition.

A.2. Material and methods

A.2.1. Seaweed specimens

Single seaweed samples were collected from representative species of Madeira Archipelago, including two green macroalgal species (Chlorophyta, Ulvophyceae) *Dasycladus vermicularis* (Scopoli) Krasser (Order Dasycladales) and *Ulva* sp. (Order

Ulvales), three species of red macroalgae (Rhodophyta, Florideophyceae) *Corallina officinalis* Linnaeus (Order Corallinales), *Asparagopsis taxiformis* (Delile) Trevisan (Order Bonnemaisoniales) and *Halopithys incurva* (Hudson) Batters (Order Ceramiales), and nine species of brown macroalgae (Ochrophyta, Phaeophyceae) *Cystoseira compressa* (Esper) Gerloff *et* Nizamuddin (Order Fucales), *Cystoseira usneoides* (Linnaeus) M. Roberts (Order Fucales), *Cystoseira humilis* Schousboe ex Kützing (Order Fucales), *Sargassum vulgare* C. Agardh (Order Fucales), *Dictyota dichotoma* (Hudson) J.V. Lamouroux (Order Dictyotales), *Lobophora* J Agardh sp. (Order Dictyotales), *Padina pavonica* (Linnaeus) Thivy (Order Dictyotales), *Halopteris filicina* (Grateloup) Kützing (Order Sphacelariales) and *Halopteris scoparia* (Linnaeus) Sauvageau (Order Sphacelariales). All macroalgae were analysed in triplicate.

The seaweeds were haphazardly harvested, taking the entire algal thallus (between 0.5 and 1 kg) at a maximum depth of 10 m by free diving, from different beaches of Madeira Archipelago including Madeira and Porto Santo Islands (Portugal; Figure 4.2.A.1). The sampling was carried out from March to June 2017, when water temperature ranged from 18.5 to 21ºC. After collection, samples were transported in seawater to the facilities at Madeira University, where they were gently washed with filtered freshwater, frozen at -35ºC and freeze-dried under reduced pressure (4×10^{-4} mbar) with a cooling trap (Scanvac Coolsafe Model 55-4, Labogene, Lynge, Denmark) set at -56ºC for five days. Lyophilized samples were later milled to 200 µm particle size in an electric mill (IKA Werke Model M20, Staufen, Germany), packed with a vacuum sealer (AudionVac Model VMS 153, Derby, UK) and stored at -20ºC until biochemical analysis.

Similarly, 12 macroalgal wracks samples were collected from Las Canteras beach (28°08′24″N 15°26′15″W) in Gran Canaria Island (Spain) in November 2018. The species collected included two green macroalgal species (Chlorophyta, Ulvophyceae) *Cymopolia barbata* (Linnaeus) J.V.Lamouroux (Order Dasycladales) and *Anadyomene stellata* (Wulfen) C.Agardh (Order Cladophorales), six species of red macroalgae (Rhodophyta, Florideophyceae) *Jania rubens* (Linnaeus) J.V.Lamouroux (Order Corallinales), *Jania* sp. (Order Corallinales), *Liagora* sp. (Order Nemaliales), *Asparagopsis* sp. (Order Bonnemaisoniales), *Laurencia* sp. (Order Ceramiales) and *Hypnea spinella* (C.Agardh) Kützing (Order Gigartinales), and four species of brown macroalgae (Ochrophyta, Phaeophyceae) *Stypocaulon* sp. (Order Sphacelariales), *Lobophora* sp. (Order Dictyotales), *Dictyota* sp. (Order Dictyotales) and *Taonia atomaria* (Woodward)

J.Agardh (Order Dictyotales). As explained above, macroalgae were gently washed with filtered freshwater, frozen and freeze-dried in the ITC and stored at -20°C.

Lyophilized samples from both origins were transported to the laboratory of Animal Physiology at the ULL for the biochemical analysis.

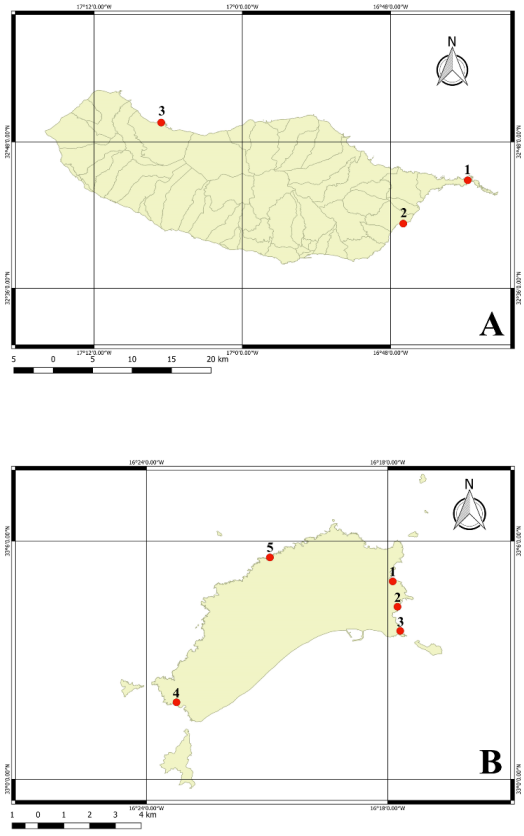


Figure 4.2.A.1. Location of macroalgal **collection** on Madeira Island (A) and Porto Santo Island (B) (Galindo et al., 2022).

Madeira Island (A). Caniçal (1), *Halopteris scoparia*; Santa Cruz (2), *Sargassum vulgare*; Seixal (3), *Padina pavonica* and *Cystoseira humilis*. Porto Santo Island (B). Calhau Serra de Dentro (1), *Dasycladus vermicularis* and *Halopithys incurva*; Abas do Rio (2), *Dictyota dichotoma* and *Halopteris filicina;* Calhau da Baleia (3), *Lobophora* sp.; Praia do Zimbralinho (4), *Corallina officinalis* and *Asparagopsis taxiformis*; Porto das Salemas (5), *Ulva* sp., *Cystoseira compressa* and *Cystoseira usneoides*.

A.2.2. Biochemical analysis

The lipid composition (TL, LC, and FA profile) of the macroalgal species from Madeira Archipelago and Gran Canaria Island was determined as explained in the common material and methods section.

A.2.3. Nutritional indexes

Nutritional quality of macroalgal FA composition was assessed by calculating atherogenicity and thrombogenicity indexes following Cardoso et al. (2017), and the ratio between hypocholesterolemic and hypercholesteloremic FA as described by Santos-Silva et al. (2002):

Atherogenicity index (AI) = [(4 * 14:0) + 16:0 + 18:0]/(∑MUFA + ∑n-6 PUFA + ∑ n-3 PUFA)

Thrombogenicity index (TI) = (14:0 + 16:0 + 18:0)/(0.5 * ∑ MUFA + 0.5 * ∑n-6 PUFA + 3 * ∑n-3 PUFA + n-3/n-6 ratio)

Hypocholesterolemic (h)/hypercholesterolemic (H) ratio (hH) = (18:1n-9 + 18:2n-6 + 20:4n-6 + 18:3n-3 + 20:5n-3 + 22:5n-3 + 22:6n-3)/(14:0 +16:0)

A.3. Results

Seaweed from Madeira Archipelago

- TL content

The lipid content of analysed seaweeds strongly varied among species, from 0.22 ± 0.14% DW (*D. vermicularis*) to 5.23 ± 0.20% DW (*D. dichotoma*) (Table 4.2.A.1).

Table 4.2.A.1. Total lipid content (% DW) of **macroalgae** from **Madeira Archipelago**.

Group/Phylum	Species	TL content
Chlorophyta	*Dasycladus vermicularis*	0.22 ± 0.14 ^{a}
	Ulva sp.	0.88 ± 0.13 ^{ab}
Rhodophyta	*Corallina officinalis*	1.34 ± 0.06 ^{bcd}
	Asparagopsis taxiformis	2.15 ± 0.93 ^{de}
	Halopithys incurva	1.20 ± 0.13 ^{acd}
Ochrophyta	*Cystoseira compressa*	1.79 ± 0.05 ^{bcd}
	Cystoseira usneoides	0.80 ± 0.03 ^{ab}
	Cystoseira humilis	2.93 ± 0.42 ^{e}
	Dictyota dichotoma	5.23 ± 0.20 ^{f}
	Halopteris filicina	0.97 ± 0.18 ^{ac}
	Halopteris scoparia	1.22 ± 0.39 ^{acd}
	Lobophora sp.	1.20 ± 0.08 ^{acd}
	Padina pavonica	0.79 ± 0.02 ^{ab}
	Sargassum vulgare	1.99 ± 0.75 ^{ce}

Results are presented as means ± SD (n=3). Different letters in superscript indicate significant differences among all macroalgal species ($p<0.05$). TL, total lipid.

- LC profiles

All species studied presented higher proportions of TNL (from 31 to 62% of TL) than TPL (from 14 to 37% of TL), except *D. vermicularis* (Chlorophyta) and *P. pavonica* (Ochrophyta) which contained similar levels of both lipid fractions (31-34% of TL). Pigments represented between a fifth and a third of total liposoluble fraction in all seaweeds, except in green macroalgae where it was a 37.08 ± 1.43% and a 15.63 ± 0.78% in *D. vermicularis* and *Ulva* sp., respectively (Table 4.2.A.2 - Table 4.2.A.4).

Ulva sp. contained higher levels of both FFA and TAG (20.23 ± 1.51 and 11.32 ± 0.78%, respectively) than *D. vermicularis* (5.83 ± 1.44 and 4.46 ± 2.03%). Within polar lipids, only MGDG varied significantly among species (Table 4.2.A.2).

Within red macroalgae, TAG was highest in *A. taxiformis* (~30.0%) whereas it was only 4-5% of TL in *C. officinalis* and *H. incurve*. On the other hand, *H. incurva* contained the highest SQDG + PE and DGDG of the three species analysed (Table 4.2.A.3).

PTS was particularly abundant in brown macroalgae (10.89 ± 0.68 - 24.72 ± 3.64% of TL). *C. compressa* had the highest TAG levels (18.86 ± 0.49%), while in the other Ochrophyta analysed, values ranged between 1 and 7% of TL. PS + PI (9.61 ± 1.88%) and SQDG + PE (15.81 ± 3.03%) were remarkably high in *C. usneoides* (Table 4.2.A.4).

The PCA of macroalgal LC showed five components with eigenvalues >1, which accounted for more than 88% of the total variance. Factor loadings and communalities are shown in Supplementary Table 1. According to the dendrogram obtained, the macroalgae were classified into six clusters (Figure 4.2.A.2). Mean factor scores for each cluster of the dendrogram are given in Supplementary Table 2. Thus, Cluster 1 grouped most Ochrophyta species including *C. usneoides*, *H. scoparia*, *Lobophora* sp., *P. pavonica*, *S. vulgare*, and one replicate of *H. ficilina*, all of which were mainly characterized by a high average content of PTS. Two of the three red macroalgae studied, *C. officinalis* and *H. incurva*, formed Cluster 2, with high average proportions of FFA, SE, PC and MGDG. Cluster 3 consisted of the third Rhodophyta species, *A. taxiformis*, and *C. compressa* (Ochrophyta), which contained high average TAG and PS + PI. Cluster 4, which included the rest of the Ochrophyta species, *C. humilis*, *D. dichotoma* and two replicates of *H. ficilina*, showed the highest average percentage of DAG. Finally, the green algae *Ulva* sp. and *D. vermicularis* were the only components of Clusters 5 and 6,

which were characterized by high average SQDG + PE, DGDG and FFA content, and high SQDG + PE, DGDG and pigments, respectively.

Table 4.2.A.2. Lipid class composition of **green macroalgae** (% of total lipid) from **Madeira Archipelago**.

	Dasycladus vermicularis	*Ulva* sp.
PC	2.31 ± 0.55	1.88 ± 0.27
PS+PI	1.12 ± 0.36	1.12 ± 0.19
SQDG+PE	12.77 ± 2.38	15.99 ± 0.80
DGDG	10.52 ± 1.45	9.69 ± 1.38
MGDG	2.31 ± 0.72	1.31 ± 0.45 *
UKPL	2.66 ± 0.70	2.21 ± 0.33
TPL	31.69 ± 4.73	32.19 ± 2.34
P	37.08 ± 1.43	15.63 ± 0.78 *
DAG	6.41 ± 0.76	6.08 ± 0.86
PTS	11.65 ± 3.53	8.96 ± 0.44
FFA	5.83 ± 1.44	20.23 ± 1.51 *
TAG	4.46 ± 2.03	11.32 ± 0.78 *
SE	2.89 ± 1.03	4.95 ± 1.12
UKNL	--	0.64 ± 0.69
TNL	31.23 ± 6.15	52.18 ± 1.56 *

Results are presented as means ± SD (n=3). * Indicates significant differences between species ($p<0.05$). PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; SQDG, sulfoquinovosyl-diacylglycerol; PE, phosphatidylethanolamine; DGDG, digalactosyl-diacylglycerol; MGDG, monogalactosyl-diacylglycerol; UKPL, unknown polar lipids; TPL, total polar lipids; P, pigments; DAG, diacylglycerols; PTS, phytosterols; FFA, free fatty acids; TAG, triacylglycerols; SE, sterol esters; UKNL, unknown neutral lipids; TNL, total neutral lipids.

Table 4.2.A.3. Lipid class composition of **red macroalgae** (% of total lipid) from **Madeira Archipelago**.

	Asparagopsis taxiformis	*Corallina officinalis*	*Halopithys incurva*
PC	3.49 ± 0.08	3.27 ± 0.35	6.17 ± 1.30
PS+PI	1.02 ± 0.61	1.23 ± 0.23	1.29 ± 0.61
SQDG+PE	3.71 ± 1.71 ^{a}	3.34 ± 0.49 ^{a}	7.71 ± 1.80 ^{b}
DGDG	1.79 ± 0.36 ^{b}	1.06 ± 0.11 ^{a}	6.69 ± 0.27 ^{c}
MGDG	1.58 ± 0.82 ^{a}	4.73 ± 0.58 ^{b}	5.60 ± 0.39 ^{b}
UKPL	2.33 ± 0.27	2.60 ± 0.66	1.85 ± 0.59
TPL	13.93 ± 2.49 ^{a}	16.22 ± 1.41 ^{a}	29.32 ± 4.57 ^{b}
P	23.77 ± 3.67	29.16 ± 0.83	29.59 ± 1.09
DAG	5.29 ± 1.33	4.37 ± 1.65	4.58 ± 1.05
PTS	6.17 ± 0.58 ^{a}	14.41 ± 0.41 ^{b}	14.59 ± 1.14 ^{b}
FFA	18.68 ± 6.29 ^{ab}	25.03 ± 2.34 ^{b}	11.13 ± 1.11 ^{a}
TAG	30.02 ± 3.97 ^{b}	4.06 ± 1.79 ^{a}	5.21 ± 2.13 ^{a}
SE	2.15 ± 1.22 ^{a}	6.76 ± 0.77 ^{b}	5.59 ± 2.42 ^{ab}
TNL	62.31 ± 3.94 ^{b}	54.62 ± 1.56 ^{b}	41.09 ± 5.43 ^{a}

Results are presented as means ± SD (n=3). Different letters in superscript within each row indicate significant differences between species ($p<0.05$). PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; SQDG, sulfoquinovosyl-diacylglycerol; PE, phosphatidylethanolamine; DGDG, digalactosyl-diacylglycerol; MGDG, monogalactosyl-diacylglycerol; UKPL, unknown polar lipids; TPL, total polar lipids; P, pigments; DAG, diacylglycerols; PTS, phytosterols; FFA, free fatty acids; TAG, triacylglycerols; SE, sterol esters; TNL, total neutral lipids.

Table 4.2.A.4. Lipid class composition of **brown macroalgae** (% of total lipid) from **Madeira Archipelago**.

	Cystoseira compressa	*Cystoseira usneoides*	*Cystoseira humilis*	*Dictyota dichotoma*	*Halopteris filicina*
PC	1.75 ± 0.37^{abc}	1.26 ± 0.34^{abc}	0.88 ± 0.42^{a}	1.16 ± 0.41^{ab}	2.88 ± 1.10^{bc}
PS+PI	4.51 ± 0.29^{bc}	9.61 ± 1.88^{d}	2.38 ± 0.54^{ab}	4.14 ± 0.49^{bc}	1.06 ± 0.44^{a}
SQDG+PE	8.00 ± 1.41^{abc}	15.81 ± 3.03^{e}	10.63 ± 1.20^{cd}	5.30 ± 0.31^{a}	6.54 ± 1.22^{ab}
DGDG	3.78 ± 0.70	5.85 ± 0.65	5.80 ± 0.57	3.67 ± 0.09	4.34 ± 0.27
MGDG	1.04 ± 0.45^{a}	2.17 ± 0.71^{abc}	1.43 ± 0.56^{ab}	1.41 ± 0.84^{ab}	1.67 ± 0.68^{abc}
UKPL	0.58 ± 0.30	1.94 ± 0.71	2.63 ± 0.37	1.31 ± 0.17	3.52 ± 0.34
TPL	19.66 ± 1.76^{ab}	36.63 ± 6.33^{c}	23.74 ± 2.47^{ab}	16.99 ± 1.37^{a}	20.01 ± 1.79^{ab}
P	23.67 ± 0.75^{ad}	22.71 ± 0.40^{ac}	21.15 ± 0.53^{a}	29.87 ± 0.47^{e}	27.53 ± 3.45^{abcde}
DAG	9.44 ± 0.15^{ce}	2.23 ± 0.61^{a}	12.98 ± 1.24^{de}	14.82 ± 0.93^{e}	8.25 ± 1.65^{abcde}
PTS	10.89 ± 0.68^{a}	24.72 ± 3.64^{d}	16.96 ± 0.59^{bc}	18.90 ± 1.58^{bc}	19.33 ± 1.87^{bc}
FFA	16.42 ± 1.01^{cd}	3.64 ± 1.31^{a}	14.43 ± 1.24^{c}	6.96 ± 0.77^{b}	15.16 ± 1.76^{cd}
TAG	18.86 ± 0.49^{d}	4.65 ± 0.73^{bc}	3.79 ± 0.99^{abcd}	5.17 ± 1.22^{bcd}	6.32 ± 6.10^{abcd}
SE	1.07 ± 0.38^{a}	1.59 ± 0.44^{a}	4.07 ± 1.13^{ab}	1.06 ± 1.11^{ab}	2.47 ± 1.08^{ab}
UKNL	--	3.82 ± 1.16	2.87 ± 1.12	6.23 ± 1.54	0.93 ± 1.14
TNL	56.68 ± 2.01^{d}	40.66 ± 6.40^{abcd}	55.11 ± 2.86^{bd}	53.15 ± 1.31^{cd}	52.46 ± 1.70^{bcd}

Table 4.2.A.4. (Cont)

	Halopteris scoparia	*Lobophora* sp.	*Padina pavonica*	*Sargassum vulgare*
PC	3.01 ± 0.35^{bc}	3.09 ± 0.93^{c}	1.43 ± 1.07^{abc}	2.11 ± 0.36^{abc}
PS+PI	1.19 ± 0.38^{a}	6.04 ± 1.58^{c}	4.80 ± 1.39^{c}	4.98 ± 0.44^{c}
SQDG+PE	8.66 ± 2.09^{abcd}	9.10 ± 1.35^{bcd}	13.14 ± 1.32^{de}	8.68 ± 0.29^{abcd}
DGDG	5.13 ± 1.08	4.58 ± 0.84	6.30 ± 0.55	3.74 ± 0.16
MGDG	2.09 ± 0.81^{abc}	3.06 ± 0.43^{bc}	3.24 ± 0.38^{c}	1.62 ± 0.49^{abc}
UKP	7.63 ± 1.65	3.48 ± 0.41	5.35 ± 1.36	2.59 ± 1.14
TPL	27.71 ± 4.09^{bc}	29.35 ± 3.77^{bc}	34.25 ± 5.15^{c}	23.71 ± 1.91^{ab}
P	25.76 ± 3.07^{abcde}	27.22 ± 0.85^{bde}	31.20 ± 1.80^{cde}	21.22 ± 1.43^{ab}
DAG	4.22 ± 1.49^{abc}	6.73 ± 0.24^{bd}	3.53 ± 0.35^{a}	2.44 ± 0.39^{a}
PTS	15.49 ± 1.29^{b}	21.88 ± 1.93^{cd}	16.28 ± 1.46^{b}	19.63 ± 1.43^{bcd}
FFA	15.54 ± 1.21^{cd}	9.22 ± 0.98^{b}	7.12 ± 1.26^{b}	19.82 ± 1.50^{d}
TAG	6.76 ± 1.04^{c}	1.41 ± 0.33^{a}	2.91 ± 0.20^{ab}	7.40 ± 1.45^{bcd}
SE	4.52 ± 0.59^{b}	2.59 ± 0.01^{ab}	4.71 ± 0.90^{ab}	4.85 ± 1.56^{ab}
UKNL	--	1.59 ± 0.53	--	0.93 ± 0.28
TNL	46.53 ± 1.04^{ab}	43.43 ± 2.98^{abc}	34.55 ± 3.36^{a}	55.07 ± 3.33^{bcd}

Results are presented as means ± SD (n=3). Different letters in superscript within each row indicate significant differences between species ($p<0.05$). PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; SQDG, sulfoquinovosyl-diacylglycerol; PE, phosphatidylethanolamine; DGDG, digalactosyl-diacylglycerol; MGDG, monogalactosyl-diacylglycerol; UKPL, unknown polar lipids; TPL, total polar lipids; P, pigments; DAG, diacylglycerol; PTS, phytosterols; FFA, free fatty acids; TAG, triacylglycerols; SE, sterol esters; UKNL, unknown neutral lipids; TNL, total neutral lipids.

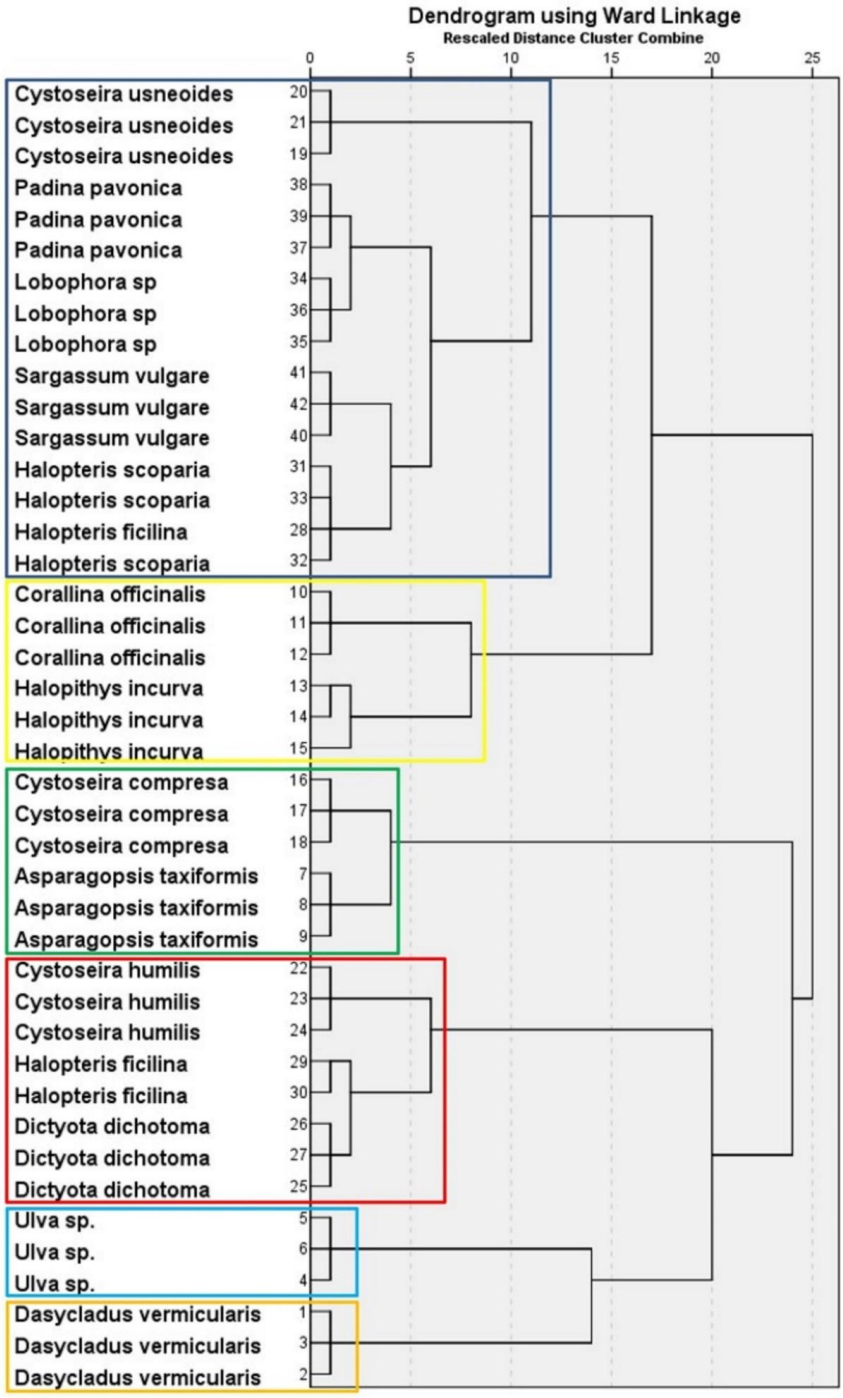


Figure 4.2.A.2. Hierarchical cluster analysis in a dendrogram format showing six clusters of macroalgae from **Madeira Archipelago** according to their **LC composition.**

- FA profile

As expected from their variable lipid contents, total FA of green seaweeds strongly differed among species, with *Ulva* sp. showing the highest content (1.62 ± 0.12 *vs*. 0.17 ± 0.09 µg mg^{-1} DW). The highest proportions of SFA, mainly palmitic acid (16:0), and n-3 PUFA were found in *Ulva* sp., while *D. vermicularis* was relatively richer in MUFA (Table 4.2.A.5). The n-3 LC-PUFA content was low in both species, where DHA represented only 0.48 ± 0.07 and 0.80 ± 0.09% of total FA in *D. vermicularis* and *Ulva* sp., respectively.

Similarly, the total FA contents and FA profiles varied considerably within Rhodophyta species. As expected from its higher lipid content, total FA was highest in *A. taxiformis* (4.94 ± 1.77 µg mg^{-1} DW), and lowest in the coralinacean macroalgae (0.86 ± 0.15 µg mg^{-1} DW). Only total SFA and 16:0 remained unchanged among species, with the latter being the most abundant FA in all of them (Table 4.2.A.6). DHA was highest in *A. taxiformis* (6.64 ± 1.38% of total FA), while *H. incurva* was richer in ARA, ALA and EPA, resulting in higher total n-6 and total n-3 PUFA proportions. The n-6/n-3 ratio was highest in *C. officinalis* (1.62 ± 0.27) and lowest in *A. taxiformis* (0.40 ± 0.03).

Brown macroalgae contained the largest levels of SFA of all species studied (from 34 to 52% of total FA in *D. dichotoma* and *H. scoparia*, respectively), followed by MUFA (26.16 ± 0.19% in *C. usneoides* and 34.24 ± 0.05% in *P. pavonica*) (Table 4.2.A.7). Within Ochrophyta, n-3 PUFA was remarkably high in *D. dichotoma*, which also had the highest TL and total FA contents. The higher values of n-3 PUFA were largely caused by higher EPA (5.01 ± 0.21% of total FA) and stearidonic acid (SDA, 18:4n-3; 8.05 ± 0.21%), while DHA only represented 0.51 ± 0.12% of total FA. Contrarily, *C. compressa* displayed the highest percentage of DHA (3.90 ± 0.38%). Total n-6 PUFA was more abundant in *C. usneoides* and *S. vulgare* due to the high proportions of both LA and ARA.

In order to assess LA, ALA, ARA, EPA and DHA contribution of each seaweed species for their potential application in nutrition, absolute values (mg 100 g^{-1} DW) were calculated from data displayed in Tables 4.2.A.5 to 4.2.A.7, and represented in Figure 4.2.A.3. Thus, within green macroalgae, *Ulva* sp. had the highest LA and ALA contents (11-14 mg 100 g^{-1} DW), while *A. taxiformis* (red algae) contained 31.47 ± 6.45 mg 100 g^{-1} DW of DHA. The brown algae *D. dichotoma* must be highlighted by its ARA and EPA contents (49.44 ± 9.84 and 37.41 ± 9.58 mg 100 g^{-1} DW, respectively), and *C. humilis* by

its amounts of LA, ALA and ARA (47.48 ± 13.35, 37.59 ± 12.42 and 47.11 ± 16.14 mg 100 g^{-1} DW, respectively). *S. vulgare* also showed high contents of LA (48.76 ± 12.39 mg 100 g^{-1} DW).

PCA for seaweed FA profiles revealed that five components had eigenvalues >1 and together accounted for more than 86% of the total variance. Factor loadings and communalities after applying varimax rotation to enhance the interpretability of the results are displayed in Supplementary Table 3. The dendrogram obtained from the hierarchical cluster analysis, which used the factor scores as input variables, revealed that macroalgae could be classified into six clusters (Figure 4.2.A.4). Supplementary Table 4 shows factor scores for each cluster given as means ± SD. Hence, Cluster 1, grouping together *Ulva* sp. (Chlorophyta), and three Ochrophyta species (*C. usneoides*, *C. humilis* and *S. vulgare*), was characterized by the highest average percentage of 16:0 and medium-high average contents of 18:2n-6 and 20:3n-6. Clusters 2-4 comprised only one species each: *H. incurva* (Cluster 2) had the highest average proportion of 18:3n-3, ARA and EPA; *D. dichotoma* (Cluster 3) was characterized by the lowest average percentage of DHA, and high 14:0 and 20:3n-6; *D. vermicularis* (Cluster 4) had the highest average proportions of 15:0, 17:0, 18:0 and 18:2n-6, and low ARA and EPA contents. Cluster 5 contained the red seaweed *A. taxiformis* and the brown *C. compressa*, which had the highest proportions of DHA and low percentages of 18:3n-3, ARA and 18:2n-6. Finally, the red seaweed *C. officinalis*, and the four brown macroalgae (*Lobophora* sp., *H. ficilina*, *H. scoparia* and *P. pavonica*) were grouped in Cluster 6, and were characterized by medium-high average content of all SFA.

Overall, the grouping of macroalgae based on their FA profile did not follow a similar pattern to that described for their LC composition (Figure 4.2.A.2 and Figure 4.2.A.4), except that of pairs *H. scoparia* and *P. pavonica*; *C. usneoides* and *S. vulgare*; and *A. taxiformis* and *C. compressa*. Of all species analysed, *D. vermicularis* (Chlorophyta) had a particular and unique lipid profile.

Table 4.2.A.5. Total fatty acids (μg mg^{-1} DW) and main **fatty acid composition** (% of total FA) of **green macroalgae** from **Madeira Archipelago**.

	Dasycladus vermicularis	*Ulva* sp.
Total FA	0.17 ± 0.09	1.62 ± 0.12 *
Total SFA	28.68 ± 5.02	44.98 ± 0.41 *
14:0	3.57 ± 0.52	1.67 ± 0.08 *
15:0	1.13 ± 0.24	0.74 ± 0.02
16:0	18.69 ± 2.60	38.38 ± 0.14 *
17:0	0.95 ± 0.21	0.49 ± 0.09 *
18:0	4.35 ± 1.60	2.78 ± 0.19
Total MUFA	44.28 ± 1.49	27.23 ± 0.61 *
16:1[1]	15.52 ± 0.77	4.78 ± 0.11 *
18:1[2]	26.75 ± 2.38	21.21 ± 0.70 *
16:2n-4	2.03 ± 0.41	nd
16:3n-4	2.78 ± 0.47	nd
Total n-6 PUFA	14.94 ± 2.25	12.48 ± 0.23
18:2	14.30 ± 2.22	8.53 ± 0.24
20:3	nd	0.70 ± 0.02
20:4 (ARA)	0.64 ± 0.25	1.46 ± 0.05 *
Total n-3 PUFA	3.19 ± 0.66	13.55 ± 0.43 *
16:3	nd	1.70 ± 0.03
18:3	0.71 ± 0.10	6.75 ± 0.09 *
18:4	0.78 ± 0.21	1.87 ± 0.10 *
20:5 (EPA)	1.23 ± 0.30	1.21 ± 0.08
22:6 (DHA)	0.48 ± 0.07	0.80 ± 0.09 *
Total PUFA	22.95 ± 3.89	26.34 ± 0.98 *
DHA/EPA	0.40 ± 0.07	0.66 ± 0.03 *
EPA/ARA	0.82 ± 0.29	0.55 ± 0.06
n-6/n-3	4.71 ± 0.32	0.92 ± 0.02 *
Total n-3 LC-PUFA	1.71 ± 0.37	3.24 ± 0.28 *

Results are presented as means ± SD (n=3). * Indicates significant differences between species ($p<0.05$). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long chain polyunsaturated fatty acids (≥ C20 and ≥ 2 double bonds). Totals include other minor components not shown. [1] Mainly n-7 isomers; [2] Mainly n-9 and n-7 isomers. nd, not detected.

Table 4.2.A.6. Total fatty acids (µg mg^{-1} DW) and main **fatty acid composition** (% of total FA) of **red macroalgae** from **Madeira Archipelago**.

	Asparagopsis taxiformis	*Corallina officinalis*	*Halopithys incurva*
Total FA	4.94 ± 1.77 ^{c}	0.86 ± 0.15 ^{a}	1.89 ± 0.02 ^{b}
Total SFA	46.82 ± 4.31	48.71 ± 1.32	42.32 ± 1.02
14:0	10.07 ± 1.10 ^{b}	4.69 ± 1.59 ^{a}	6.81 ± 0.19 ^{a}
15:0	0.73 ± 0.04 ^{a}	1.62 ± 0.29 ^{b}	0.44 ± 0.10 ^{a}
16:0	32.01 ± 3.24	35.65 ± 1.81	31.99 ± 0.56
17:0	0.62 ± 0.05 ^{a}	1.09 ± 0.19 ^{b}	0.67 ± 0.09 ^{a}
18:0	3.08 ± 0.07 ^{b}	5.02 ± 0.43 ^{c}	2.13 ± 0.30 ^{a}
Total MUFA	32.47 ± 0.87 ^{b}	31.04 ± 1.27 ^{b}	19.98 ± 0.27 ^{a}
16:1^{1}	8.55 ± 0.69 ^{b}	7.88 ± 1.98 ^{ab}	6.11 ± 0.13 ^{a}
18:1^{2}	20.94 ± 1.35 ^{b}	19.78 ± 0.44 ^{b}	12.92 ± 0.30 ^{a}
Total n-6 PUFA	5.46 ± 0.60 ^{a}	9.95 ± 0.90 ^{b}	14.17 ± 0.79 ^{c}
18:2	5.02 ± 0.57 ^{b}	4.86 ± 0.87 ^{b}	2.12 ± 0.10 ^{a}
20:3	nd	nd	0.34 ± 0.02
20:4 (ARA)	0.43 ± 0.04 ^{a}	5.09 ± 0.76 ^{b}	11.71 ± 0.81 ^{c}
Total n-3 PUFA	13.62 ± 2.55 ^{b}	6.22 ± 0.86 ^{a}	21.55 ± 0.71 ^{c}
18:3	1.00 ± 0.10 ^{a}	0.96 ± 0.35 ^{a}	10.90 ± 0.64 ^{b}
18:4	0.82 ± 0.12 ^{b}	0.48 ± 0.00 ^{ab}	0.26 ± 0.10 ^{a}
20:5 (EPA)	2.22 ± 0.39 ^{a}	3.35 ± 0.61 ^{a}	9.31 ± 0.61 ^{b}
22:6 (DHA)	6.64 ± 1.38 ^{c}	1.43 ± 0.24 ^{b}	0.54 ± 0.15 ^{a}
Total PUFA	20.47 ± 3.36 ^{a}	17.68 ± 1.87 ^{a}	35.99 ± 1.18 ^{b}
DHA/EPA	2.98 ± 0.10 ^{c}	0.43 ± 0.03 ^{a}	0.06 ± 0.02 ^{b}
EPA/ARA	15.25 ± 1.79 ^{c}	0.28 ± 0.01 ^{b}	0.05 ± 0.01 ^{a}
n-6/n-3	0.40 ± 0.03 ^{a}	1.62 ± 0.27 ^{c}	0.66 ± 0.02 ^{b}
Total n-3 LC-PUFA	11.80 ± 2.33 ^{b}	4.78 ± 0.84 ^{a}	10.40 ± 0.44 ^{b}

Results are presented as means ± SD (n=3). Different letters in superscript within each row indicate significant differences between species ($p<0.05$). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long chain polyunsaturated fatty acids (≥ C20 and ≥ 2 double bonds). Totals include other minor components not shown. [1] Mainly n-9 and n-7 isomers; [2] Mainly n-9 isomers. nd, not detected.

Table 4.2.A.7. Total fatty acids (μg mg^{-1} DW) and main **fatty acid composition** (% of total FA) of **brown macroalgae** from **Madeira Archipelago**.

	Cystoseira compressa	*Cystoseira usneoides*	*Cystoseira humilis*	*Dictyota dichotoma*	*Halopteris ficilina*
Total FA	4.20 ± 1.65 ^{bd}	1.54 ± 0.31 ^{ab}	6.28 ± 2.07 ^{d}	7.42 ± 1.62 ^{d}	1.56 ± 0.60 ^{ab}
Total SFA	44.71 ± 1.32 ^{bcd}	40.59 ± 0.68 ^{abcd}	44.16 ± 0.75 ^{bc}	34.08 ± 0.93 ^{a}	44.85 ± 0.93 ^{bcd}
14:0	7.81 ± 0.90 ^{bcd}	3.17 ± 1.21 ^{abcd}	6.97 ± 0.23 ^{bc}	10.20 ± 0.36 ^{de}	5.94 ± 0.05 ^{b}
15:0	0.72 ± 0.13 ^{ab}	0.60 ± 0.26 ^{ab}	0.46 ± 0.01 ^{a}	0.59 ± 0.08 ^{ab}	0.71 ± 0.02 ^{b}
16:0	31.47 ± 1.15 ^{bde}	32.89 ± 0.68 ^{bcd}	33.72 ± 0.50 ^{cd}	20.67 ± 0.42 ^{a}	32.77 ± 1.33 ^{cd}
17:0	0.90 ± 0.47 ^{abc}	0.39 ± 0.15 ^{abc}	0.39 ± 0.03 ^{a}	nd	0.92 ± 0.08 ^{b}
18:0	3.12 ± 0.31 ^{b}	1.73 ± 0.24 ^{a}	1.78 ± 0.48 ^{ab}	1.81 ± 0.15 ^{a}	3.86 ± 1.36 ^{abc}
Total MUFA	32.97 ± 0.78 ^{cd}	26.49 ± 0.09 ^{a}	26.62 ± 0.79 ^{ab}	31.43 ± 0.67 ^{bc}	27.78 ± 2.26 ^{abcd}
16:1[1]	8.26 ± 0.19 ^{bcd}	5.70 ± 0.26 ^{a}	5.66 ± 0.04 ^{a}	9.63 ± 0.36 ^{cde}	8.05 ± 0.27 ^{b}
16:1n-5	nd	0.57 ± 0.17 ^{a}	nd	6.11 ± 0.08 ^{e}	0.95 ± 0.05 ^{b}
18:1[2]	21.80 ± 0.72 ^{bcd}	20.63 ± 0.17 ^{b}	20.51 ± 0.76 ^{bcd}	20.98 ± 0.32 ^{b}	18.74 ± 2.05 ^{abcd}
Total n-6 PUFA	8.69 ± 1.42 ^{abc}	19.48 ± 0.44 ^{e}	15.68 ± 0.58 ^{c}	13.22 ± 0.12 ^{b}	13.31 ± 1.23 ^{abcd}
18:2	5.79 ± 0.64 ^{ade}	7.54 ± 0.55 ^{ade}	7.76 ± 0.62 ^{e}	2.69 ± 0.17 ^{a}	6.88 ± 0.06 ^{ce}
20:3	nd	0.99 ± 0.12 ^{b}	0.56 ± 0.03 ^{a}	0.56 ± 0.03 ^{a}	nd
20:4 (ARA)	2.77 ± 0.68 ^{abc}	7.20 ± 0.34 ^{d}	7.46 ± 0.12 ^{d}	6.68 ± 0.13 ^{cd}	3.00 ± 0.34 ^{ab}
Total n-3 PUFA	12.01 ± 1.18 ^{c}	10.91 ± 0.38 ^{bc}	12.12 ± 0.18 ^{c}	18.75 ± 0.41 ^{d}	11.89 ± 0.75 ^{c}
18:3	2.47 ± 0.12 ^{b}	3.42 ± 0.11 ^{d}	5.97 ± 0.03 ^{f}	3.46 ± 0.03 ^{cd}	3.03 ± 0.38 ^{abcde}
18:4	1.70 ± 0.26 ^{ab}	2.94 ± 0.14 ^{d}	3.22 ± 0.14 ^{d}	8.05 ± 0.21 ^{e}	2.22 ± 0.26 ^{bc}
20:5 (EPA)	2.19 ± 0.13 ^{bc}	3.22 ± 0.27 ^{d}	2.16 ± 0.10 ^{bc}	5.01 ± 0.21 ^{e}	3.55 ± 0.24 ^{d}
22:6 (DHA)	3.90 ± 0.38 ^{c}	0.46 ± 0.11 ^{ab}	0.34 ± 0.08 ^{a}	0.51 ± 0.12 ^{ab}	1.08 ± 0.36 ^{b}
Total PUFA	21.51 ± 2.17 ^{b}	30.65 ± 0.57 ^{de}	27.98 ± 0.45 ^{cd}	32.97 ± 0.44 ^{e}	25.41 ± 2.31 ^{c}
DHA/EPA	1.78 ± 0.12 ^{b}	0.14 ± 0.03 ^{a}	0.16 ± 0.04 ^{a}	0.10 ± 0.02 ^{a}	0.31 ± 0.11 ^{a}
EPA/ARA	1.46 ± 0.33 ^{e}	0.06 ± 0.02 ^{a}	0.05 ± 0.01 ^{a}	0.08 ± 0.02 ^{ab}	0.36 ± 0.12 ^{d}
n-6/n-3	0.72 ± 0.08 ^{a}	1.79 ± 0.09 ^{ef}	1.29 ± 0.06 ^{cd}	0.71 ± 0.01 ^{a}	1.12 ± 0.03 ^{bc}
Total n-3 LC-PUFA	7.84 ± 0.93 ^{c}	4.55 ± 0.55 ^{b}	2.93 ± 0.02 ^{a}	7.25 ± 0.25 ^{c}	6.63 ± 0.44 ^{c}

Table 4.2.A.7. (Cont.)

	Halopteris scoparia	*Lobophora* sp.	*Padina pavonica*	*Sargassum vulgare*
Total FA	1.35 ± 0.24 [a]	2.06 ± 0.47 [abc]	1.29 ± 0.24 [a]	4.15 ± 0.98 [cd]
Total SFA	52.32 ± 1.08 [cd]	45.76 ± 0.79 [d]	47.56 ± 1.55 [bcd]	41.81 ± 0.26 [d]
14:0	7.62 ± 0.23 [cd]	11.64 ± 0.48 [e]	8.04 ± 0.22 [d]	3.64 ± 0.05 [a]
15:0	0.78 ± 0.02 [b]	0.83 ± 0.08 [ab]	0.95 ± 0.10 [ab]	0.53 ± 0.04 [ab]
16:0	36.62 ± 1.43 [c]	29.19 ± 0.27 [b]	34.67 ± 0.68 [ce]	30.89 ± 0.40 [bd]
17:0	1.06 ± 0.12 [b]	0.68 ± 0.05 [b]	0.62 ± 0.04 [b]	0.43 ± 0.03 [a]
18:0	4.66 ± 1.06 [abc]	2.24 ± 0.10 [ab]	2.40 ± 0.23 [ab]	5.15 ± 0.28 [c]
Total MUFA	28.93 ± 0.30 [a]	33.30 ± 0.50 [d]	34.24 ± 0.05 [d]	27.48 ± 0.77 [ab]
16:1[1]	9.39 ± 0.18 [de]	10.16 ± 0.51 [ef]	10.23 ± 0.09 [f]	8.17 ± 0.34 [bcd]
16:1n-5	1.33 ± 0.17 [c]	0.64 ± 0.17 [a]	3.41 ± 0.04 [d]	nd
18:1[2]	18.58 ± 0.39 [ac]	22.71 ± 0.27 [d]	24.00 ± 0.09 [d]	16.76 ± 0.38 [a]
Total n-6 PUFA	11.28 ± 1.02 [abc]	10.31 ± 0.53 [ab]	9.23 ± 0.53 [a]	18.79 ± 0.54 [de]
18:2	9.29 ± 1.16 [def]	4.22 ± 0.07 [bd]	3.83 ± 0.51 [abc]	11.70 ± 0.53 [f]
20:3	nd	0.93 ± 0.04 [b]	0.56 ± 0.02 [a]	0.63 ± 0.01 [a]
20:4 (ARA)	1.99 ± 0.23 [a]	4.39 ± 0.24 [b]	2.04 ± 0.19 [a]	6.46 ± 0.02 [cd]
Total n-3 PUFA	5.71 ± 0.65 [a]	9.09 ± 0.50 [b]	7.21 ± 0.58 [a]	10.03 ± 0.46 [bc]
18:3	2.17 ± 0.23 [abcd]	1.52 ± 0.08 [a]	2.41 ± 0.23 [abcd]	4.76 ± 0.16 [e]
18:4	1.18 ± 0.14 [a]	2.62 ± 0.09 [cd]	1.74 ± 0.21 [b]	1.78 ± 0.09 [b]
20:5 (EPA)	1.74 ± 0.42 [b]	3.38 ± 0.14 [d]	1.00 ± 0.09 [a]	2.39 ± 0.16 [c]
22:6 (DHA)	0.63 ± 0.25 [ab]	0.54 ± 0.07 [ab]	0.44 ± 0.23 [ab]	0.49 ± 0.09 [ab]
Total PUFA	17.00 ± 0.79 [a]	19.54 ± 0.77 [ab]	16.44 ± 1.10 [a]	28.82 ± 0.90 [cd]
DHA/EPA	0.37 ± 0.15 [a]	0.16 ± 0.02 [a]	0.44 ± 0.26 [a]	0.21 ± 0.04 [a]
EPA/ARA	0.32 ± 0.12 [cd]	0.12 ± 0.02 [abc]	0.22 ± 0.13 [bcd]	0.08 ± 0.01 [ab]
n-6/n-3	2.00 ± 0.38 [abcdef]	1.13 ± 0.03 [bc]	1.28 ± 0.04 [de]	1.88 ± 0.07 [f]
Total n-3 LC-PUFA	2.37 ± 0.54 [a]	4.95 ± 0.38 [b]	3.06 ± 0.51 [a]	3.48 ± 0.21 [ab]

Results are presented as means ± SD (n=3). Different letters in superscript within each row indicate significant differences between species ($p<0.05$). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long chain polyunsaturated fatty acids (≥ C20 and ≥ 2 double bonds). Totals include other minor components not shown. [1] Mainly n-9 and n-7 isomers; [2] Mainly n-9 isomers. nd, not detected.

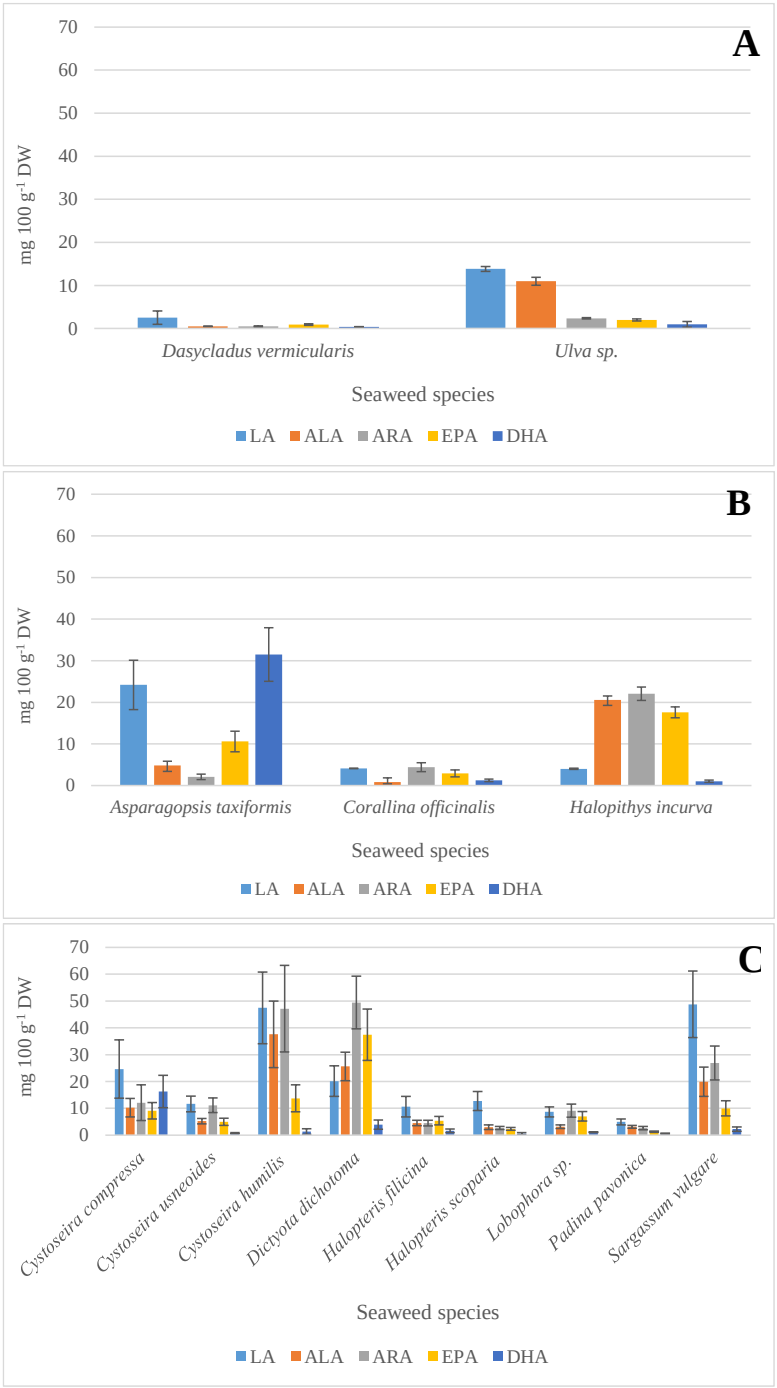


Figure 4.2.A.3. Main **LC-PUFA and their precursors** (mg 100 g^{-1} DW) in green (A), red (B) and brown (C) seaweed species from **Madeira Archipelago**.

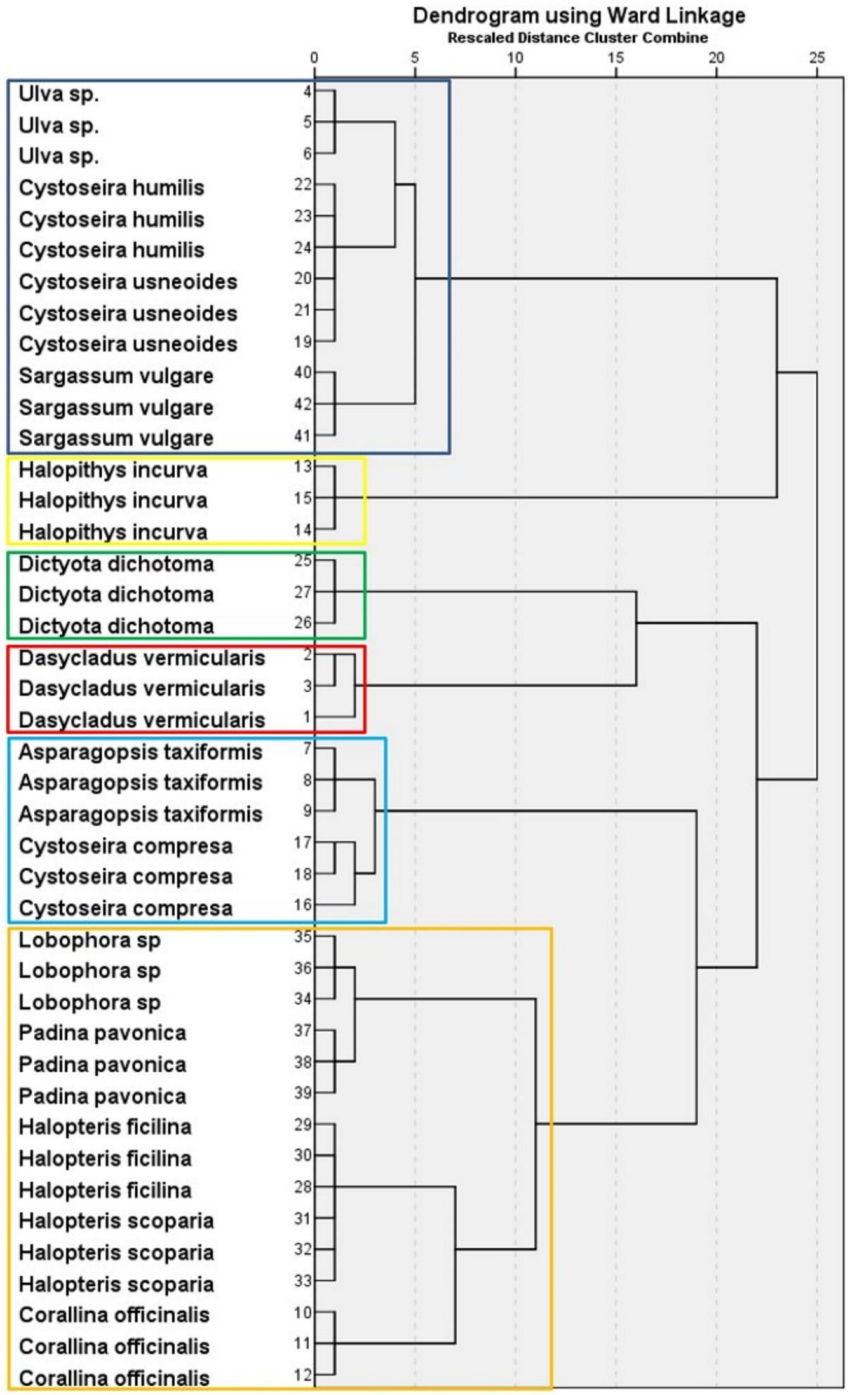


Figure 4.2.A.4. Hierarchical cluster analysis in a dendrogram format showing six clusters of macroalgae from **Madeira Archipelago** according to their **FA composition.**

- Nutritional indexes

D. vermicularis had the lowest AI (0.60 ± 0.14) and *D. dichotoma* the lowest TI (0.41 ± 0.02) whereas both indexes were highest in *H. scoparia* (1.56 ± 0.06 and 1.30 ± 0.06, respectively) (Table 4.2.A.8). hH ratios were lowest in *Ulva* sp., *H. scoparia* and *P. pavonica* (~0.7), but significantly increased in *D. dichotoma*, *S. vulgare* (slightly over 1.2) and *D. vermicularis* (1.71 ± 0.37).

Table 4.2.A.8. Atherogenicity index (AI), **thrombogenicity index** (TI) and **hypocholesterolemic/hypercholesteloremic** fatty acids ratio (hH) of **macroalgae** from **Madeira Archipelago**.

Group/ Phylum	Species	AI	TI	hH
Chlorophyta	*Dasycladus vermicularis*	0.60 ± 0.14 ^{a}	0.69 ± 0.19 ^{bc}	1.71 ± 0.37 ^{e}
	Ulva sp.	0.90 ± 0.03 ^{abc}	0.70 ± 0.02 ^{bc}	0.69 ± 0.01 ^{a}
Rhodophyta	*Asparagopsis taxiformis*	1.48 ± 0.25 ^{fg}	0.74 ± 0.16 ^{bc}	0.84 ± 0.20 ^{ac}
	Corallina officinalis	1.26 ± 0.15 ^{deg}	1.15 ± 0.10 ^{ef}	0.81 ± 0.06 ^{ab}
	Halopithys incurva	1.10 ± 0.05 ^{be}	0.49 ± 0.03 ^{ab}	1.12 ± 0.06 ^{abcd}
Ochrophyta	*Cystoseira compressa*	1.23 ± 0.11 ^{def}	0.73 ± 0.07 ^{bc}	0.94 ± 0.09 ^{ad}
	Cystoseira usneoides	0.84 ± 0.08 ^{ab}	0.67 ± 0.01 ^{bc}	1.18 ± 0.03 ^{cd}
	Cystoseira humilis	1.17 ± 0.03 ^{ce}	0.73 ± 0.02 ^{bc}	1.08 ± 0.03 ^{bcd}
	Dictyota dichotoma	1.00 ± 0.05 ^{bcd}	0.41 ± 0.02 ^{a}	1.27 ± 0.05 ^{d}
	Halopteris filicina	1.14 ± 0.02 ^{be}	0.75 ± 0.03 ^{c}	0.85 ± 0.06 ^{ac}
	Halopteris scoparia	1.56 ± 0.06 ^{g}	1.30 ± 0.06 ^{f}	0.68 ± 0.03 ^{a}
	Lobophora sp.	1.48 ± 0.07 ^{fg}	0.86 ± 0.05 ^{cd}	0.83 ± 0.03 ^{ac}
	Padina pavonica	1.37 ± 0.06 ^{eg}	1.02 ± 0.07 ^{de}	0.71 ± 0.02 ^{a}
	Sargassum vulgare	0.90 ± 0.01 ^{abc}	0.74 ± 0.01 ^{c}	1.22 ± 0.03 ^{d}

Results are presented as means ± SD (n=3). Different letters in superscript in the same column indicate significant differences among all macroalgal species ($p<0.05$).

Seaweed from Gran Canaria Island

- TL content

The lipid content of analysed seaweeds strongly varied among species, from 0.27 ± 0.06% DW (*J. rubens*) to 3.17 ± 0.03% DW (*T. atomaria*) (Table 4.2.A.9).

Table 4.2.A.9. Total lipid content (% DW) of **macroalgae** from **Gran Canaria Island**.

Group/Phylum	Species	TL content
Chlorophyta	*Cymopolia barbata*	2.10 ± 0.16 ^{efg}
	Anadyomene stellata	2.13 ± 0.36 ^{deg}
Rhodophyta	*Jania rubens*	0.27 ± 0.06 ^{ab}
	Jania sp.	0.55 ± 0.08 ^{ab}
	Liagora sp.	0.43 ± 0.00 ^{a}
	Asparagopsis sp.	0.57 ± 0.15 ^{abf}
	Laurencia sp.	0.92 ± 0.01 ^{bd}
	Hypnea spinella	1.58 ± 0.23 ^{defg}
Ochrophyta	*Stypocaulon* sp.	0.62 ± 0.06 ^{abc}
	Lobophora sp.	1.16 ± 0.17 ^{cdef}
	Dictyota sp.	2.02 ± 0.03 ^{ef}
	Taonia atomaria	3.17 ± 0.03 ^{g}

Results are presented as means ± SD (n=3). Different letters in superscript indicate significant differences among all macroalgal species ($p<0.05$). TL, total lipid.

- LC profile

TNL content (39 to 64% of TL) was higher than TPL (13 to 27% of TL) in all species studied.

Among Chlorophyta, *C. barbata* showed greater TPL proportions than *A. stellata*; in particular, its contents of SQDG + PE (11.81 ± 0.80%) and DGDG (8.13 ± 1.05%) were remarkable, and significantly higher. On the contrary, TNL was higher in *A. stellata* due to PTS (19.53 ± 0.55%) and TAG (9.93 ± 0.69%). Pigments were also higher in *A. stellata* (32.38 ± 1.70%) than in *C. barbata* (24.34 ± 2.25%) (Table 4.2.A.10).

Pigments were one the most prominent liposoluble fractions in red macroalgae, comprising from ~20% in *Laurencia* sp. to ~40% in *Liagora* sp. (Table 4.2.A.11). *Laurencia* sp. and *H. spinella* showed the highest FFA contents (31.80 ± 1.24% and 21.26 ± 1.55%, respectively), whereas PTS was remarkable high in *Liagora* sp. (22.97 ± 1.83%), *J. rubens* (21.63 ± 0.59%) and *Asparagopsis* sp. (19.29 ± 2.15%). Within TPL, SQDG + PE was especially prominent in *Asparagopsis* sp. (9.06 ± 2.26%) and *H. spinella* (7.92 ± 0.28%).

Within TNL of brown macroalgae, *T. atomaria* stands out by the greatest proportion of DAG (14.55 ± 1.37%), *Lobophora* sp. by that of PTS (21.84 ± 2.11%), and both *Lobophora* sp. and *Dictyota* sp. by the highest values of SE (2.07 ± 0.40 and 2.16 ± 0.61%, respectively). No relevant variations existed in polar LC between brown macroalgae, with the exception of PC, which was highest in *Stypocaulon* sp. (3.64 ± 1.28%) (Table 4.2.A.12).

PCA for seaweed LC showed five components with eigenvalues >1, which accounted for more than 86% of the total variance. Factor loadings and communalities are shown in Supplementary Table 5. According to the dendrogram obtained, the macroalgae were classified into six clusters (Figure 4.2.A.5). Supplementary Table 6 shows the mean factor scores for each cluster of the dendrogram. Thus, Cluster 1 grouped two of the Rhodophyta species, *J. rubens* and *Liagora* sp., both mainly characterized by a high average content of PTS, and low contents of SQDG+PE, DGDG, PS+PI, DAG and TAG. *A. stellata* (green seaweed), together with most species of brown macroalgae studied (*Dictyota* sp., *Lobophora* sp., *T. atomaria* and one replicate of *Stypocaulon* sp.) comprised Cluster 2, having high percentages of PS+PI and DAG, but low PC and MGDG. Clusters 3-5 comprised only one species each: *Asparagopsis* sp. (Cluster 3) had the highest average

proportions of PC and MGDG, high pigments, and the lowest FFA; *Jania* sp. (Cluster 4) was characterized by the highest average percentage of TAG; and *Laurencia* sp. (Cluster 5) by the highest FFA and SE, and a reduced pigments. Finally, Cluster 6 comprised species from the three groups, with *C. barbata* (green), *H. spinella* (red) and two replicates of *Stypocaulon* sp. containing high proportions of SQDG+PE and DGDG, and low percentages of PTS.

Table 4.2.A.10. Lipid class composition (% of total lipid) of **green macroalgae** from **Gran Canaria Island**.

	Cymopolia barbata	*Anadyomene stellata*
PC	3.36 ± 1.27	1.10 ± 0.31
PS+PI	1.16 ± 0.38	3.41 ± 0.49 *
SQDG+PE	11.81 ± 0.80	3.91 ± 0.45 *
DGDG	8.13 ± 1.05	3.70 ± 0.64 *
MGDG	2.94 ± 1.41	2.17 ± 0.33
TPL	27.40 ± 3.33	14.29 ± 1.52 *
P	24.34 ± 2.25	32.38 ± 1.70 *
DAG	9.07 ± 1.62	8.91 ± 0.35
PTS	12.74 ± 0.51	19.53 ± 0.55 *
FFA	13.75 ± 0.53	14.01 ± 0.39
TAG	6.74 ± 0.07	9.93 ± 0.69 *
SE	3.36 ± 1.06	0.95 ± 0.10 *
UKNL	2.61 ± 0.47	--
TNL	48.26 ± 1.95	53.33 ± 0.79 *

Results are presented as means ± SD (n=3). * Indicates significant difference between species ($p<0.05$). PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; SQDG, sulfoquinovosyl-diacylglycerol; PE, phosphatidylethanolamine; DGDG, digalactosyl-diacylglycerol; MGDG, monogalactosyl-diacylglycerol; TPL, total polar lipids; P, pigments; DAG, diacylglycerols; PTS, phytosterols; FFA, free fatty acids; TAG, triacylglycerols; SE, sterol esters; UKNL, unknown neutral lipids; TNL, total neutral lipids.

Table 4.2.A.11. Lipid class composition (% of total lipid) of **red macroalgae** from **Gran Canaria Island**.

	Jania rubens	*Jania* sp.	*Liagora* sp.	*Asparagopsis* sp.	*Laurencia* sp.	*Hypnea spinella*
PC	2.59 ± 0.31 ^{b}	3.54 ± 0.11 ^{bc}	0.87 ± 0.42 ^{a}	4.30 ± 0.30 ^{c}	3.15 ± 0.88 ^{bc}	3.59 ± 0.83 ^{bc}
PS+PI	1.28 ± 0.15 ^{ab}	1.65 ± 0.35 ^{ab}	0.99 ± 0.35 ^{a}	2.27 ± 0.56 ^{b}	2.15 ± 0.30 ^{ab}	1.98 ± 0.72 ^{ab}
SQDG+PE	3.26 ± 0.16 ^{a}	5.62 ± 0.89 ^{bc}	4.51 ± 0.65 ^{ab}	9.06 ± 2.26 ^{d}	3.16 ± 0.46 ^{a}	7.92 ± 0.28 ^{cd}
DGDG	3.15 ± 0.45 ^{a}	2.84 ± 0.75 ^{a}	4.01 ± 0.39 ^{ab}	3.27 ± 0.64 ^{ab}	3.33 ± 0.64 ^{ab}	4.76 ± 0.33 ^{b}
MGDG	2.62 ± 0.18 ^{a}	3.23 ± 1.16 ^{ab}	2.84 ± 0.72 ^{ab}	5.56 ± 0.13 ^{b}	3.82 ± 0.55 ^{ab}	2.85 ± 0.40 ^{a}
TPL	12.90 ± 0.67 ^{a}	16.88 ± 1.16 ^{ab}	13.21 ± 1.25 ^{a}	24.46 ± 2.95 ^{c}	15.60 ± 1.88 ^{a}	21.10 ± 0.66 ^{bc}
P	31.47 ± 0.59 ^{cd}	29.64 ± 3.32 ^{bc}	40.13 ± 3.48 ^{e}	36.65 ± 1.53 ^{de}	20.26 ± 1.58 ^{a}	24.17 ± 1.78 ^{ab}
DAG	6.58 ± 0.46 ^{abc}	6.28 ± 0.55 ^{ab}	7.92 ± 0.89 ^{bc}	4.20 ± 0.55 ^{a}	5.01 ± 1.23 ^{a}	8.83 ± 1.20 ^{c}
PTS	21.63 ± 0.59 ^{cd}	14.88 ± 2.62 ^{ab}	22.97 ± 1.83 ^{d}	19.29 ± 2.15 ^{bcd}	16.80 ± 1.09 ^{abc}	13.11 ± 1.68 ^{a}
FFA	15.60 ± 1.57 ^{b}	17.74 ± 0.41 ^{b}	9.71 ± 0.93 ^{a}	7.32 ± 1.07 ^{a}	31.80 ± 1.24 ^{d}	21.26 ± 1.55 ^{c}
TAG	6.19 ± 1.15 ^{b}	13.36 ± 2.70 ^{c}	1.35 ± 0.61 ^{a}	5.40 ± 2.36 ^{ab}	3.70 ± 0.29 ^{ab}	6.58 ± 1.77 ^{b}
SE	5.63 ± 0.86 ^{ab}	1.21 ± 0.14 ^{a}	2.47 ± 0.99 ^{ab}	2.67 ± 1.28 ^{ab}	5.79 ± 1.82 ^{b}	4.41 ± 1.88 ^{ab}
UKNL	--	--	2.23 ± 0.69	--	1.04 ± 1.12	0.53 ± 0.92
TNL	55.63 ± 1.87 ^{bc}	53.47 ± 3.77 ^{b}	46.66 ± 4.73 ^{ab}	38.89 ± 4.46 ^{a}	64.14 ± 0.39 ^{c}	54.73 ± 2.41 ^{b}

Results are presented as means ± SD (n=3). Different letters in superscript within each row indicate significant differences between species ($p<0.05$). PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; SQDG, sulfoquinovosyl-diacylglycerol; PE, phosphatidylethanolamine; DGDG, digalactosyl-diacylglycerol; MGDG, monogalactosyl-diacylglycerol; TPL, total polar lipids; P, pigments; DAG, diacylglycerols; PTS, phytosterols; FFA, free fatty acids; TAG, triacylglycerols; SE, sterol esters; UKNL, unknown neutral lipids; TNL, total neutral lipids.

Table 4.2.A.12. Lipid class composition (% of total lipid) of **brown macroalgae** from **Gran Canaria Island**.

	Stypocaulon sp.	*Lobophora* sp.	*Dictyota* sp.	*Taonia atomaria*
PC	3.64 ± 1.28 [b]	1.63 ± 0.35 [ab]	1.25 ± 0.41 [a]	1.30 ± 0.58 [a]
PS+PI	3.00 ± 0.66	3.31 ± 0.89	3.83 ± 0.63	4.09 ± 1.05
SQDG+PE	8.43 ± 2.21	5.66 ± 1.08	4.84 ± 1.01	8.57 ± 3.07
DGDG	5.82 ± 2.04	4.18 ± 0.65	2.66 ± 0.14	3.88 ± 1.86
MGDG	2.83 ± 0.56	2.77 ± 0.92	2.31 ± 0.55	3.32 ± 1.47
TPL	23.72 ± 4.49	17.55 ± 2.74	14.99 ± 2.39	21.15 ± 3.93
P	35.89 ± 3.63	30.59 ± 2.29	33.06 ± 2.90	38.03 ± 2.73
DAG	8.25 ± 0.92 [a]	9.59 ± 0.08 [a]	10.28 ± 0.64 [a]	14.55 ± 1.37 [b]
PTS	16.56 ± 1.75 [a]	21.84 ± 2.11 [b]	18.58 ± 0.78 [ab]	16.16 ± 2.36 [a]
FFA	9.76 ± 1.02	11.45 ± 0.40	14.88 ± 3.36	5.89 ± 1.58
TAG	4.86 ± 0.31	4.49 ± 0.79	6.15 ± 2.10	3.88 ± 0.46
SE	0.97 ± 0.10 [b]	2.07 ± 0.40 [c]	2.16 ± 0.61 [c]	0.33 ± 0.05 [a]
UKNL	--	2.42 ± 1.25	--	--
TNL	40.39 ± 1.66 [b]	51.86 ± 0.69 [a]	52.35 ± 4.43 [a]	40.81 ± 3.97 [b]

Results are presented as means ± SD (n=3). Different letters in superscript within each row indicate significant differences between species ($p<0.05$). PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; SQDG, sulfoquinovosyl-diacylglycerol; PE, phosphatidylethanolamine; DGDG, digalactosyl-diacylglycerol; MGDG, monogalactosyl-diacylglycerol; TPL, total polar lipids; P, pigments; DAG, diacylglycerols; PTS, phytosterols; FFA, free fatty acids; TAG, triacylglycerols; SE, sterol esters; UKNL, unknown neutral lipids; TNL, total neutral lipids.

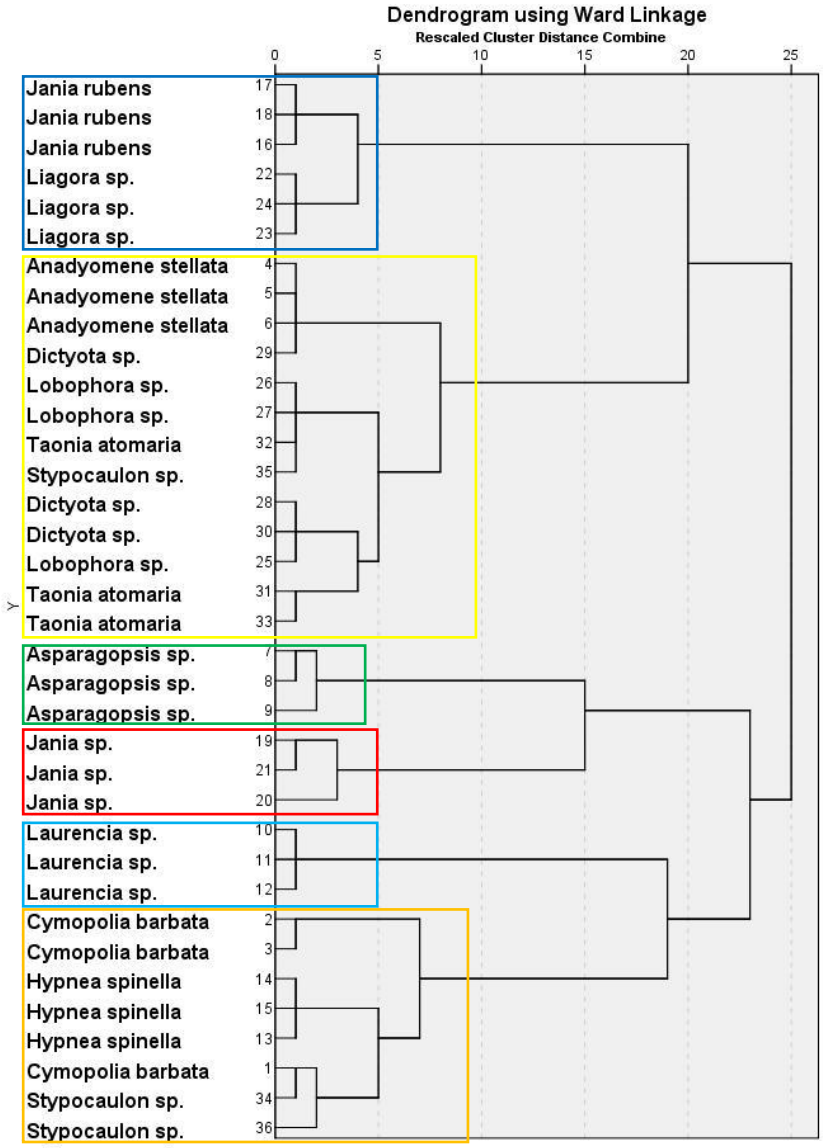


Figure 4.2.A.5. Hierarchical cluster analysis in a dendrogram format showing six clusters of macroalgae from **Gran Canaria Island** according to their **LC composition.**

- FA profile

The FA profiles of green seaweeds greatly varied among species. *C. barbata* stands out by its higher content of total FA (4.79 ± 0.30 *vs.* 2.59 ± 0.70 µg mg^{-1} DW), in spite of their similar lipid contents. *A. stellata* contained higher proportions of SFA, mainly short chain C14 and C16 compounds, and double n-3 PUFA and n-3 LC-PUFA, while *C. barbata* was richer in MUFA. It is remarkable the high amounts of 16:2n-4 and 16:3n-4 detected in *C. barbata* (7.16 ± 0.37 and 1.52 ± 0.09% of total FA, respectively). Finally, *A. stellata* highlighted by its relevant EPA and ARA contents (9.26 ± 0.78% and 6.90 ± 0.81%, respectively), while DHA was higher in *C. barbata* (2.05 ± 0.11% *vs.* 0.97 ± 0.35%) (Table 4.2.A.13).

Similarly, the FA profiles strongly differed among Rhodophyta species (Table 4.2.A.14). C16 SFA was the most prominent FA in all species, ranging from 59.55 ± 1.16% in *Liagora* sp. to 35.68 ± 1.76% in *Jania* sp. By contrast, *Jania* sp. had the highest amount of EPA (6.14 ± 1.19%), DHA (4.28 ± 0.98%), and consequently, of total n-3 LC-PUFA (10.82 ± 2.28%). On the other hand, *H. spinella* was richer in LA (6.61 ± 0.22%) and ARA (5.49 ± 0.13%), leading to the highest total n-6 PUFA contents (12.90 ± 0.04%). This species also showed the highest n-6/n-3 ratio.

Both *Dictyota* sp. and *T. atomaria* had the highest total FA contents (~4 µg mg^{-1} DW) of all brown macroalgae. Besides, SFA varied from 37 to 51% of total FA, with 16:0 representing over 70% of saturates in all cases. Total MUFA was highest in *T. atomaria*, mainly due to its 16:1 isomers, in particular 16:1n-5, with 18.23 ± 0.30% of total FA. EPA content was remarkably high in *Lobophora* sp. (6.12 ± 0.97%), while DHA was not detected in either of the Ochrophyta species analysed. *Lobophora* sp. also had the highest total n-6 PUFA content, mainly composed of 18:2n-6 (8.89 ± 0.21%). Finally, *Dictyota* sp. and *Lobophora* sp. showed high contents of ARA (~6%) (Table 4.2.A.15).

Figure 4.2.A.6 displays LA, ALA, ARA, EPA and DHA contents (mg 100 g^{-1} DW) in all seaweed species from Gran Canaria Island studied. LA was remarkably high in the green macroalgae *C. barbata* (41.61 ± 3.37 mg 100 g^{-1} DW), while *H. spinella* (Rhodophyta) showed relevant amounts of LA and ARA (23.23 ± 2.31 and 19.26 ± 1.51 mg 100 g^{-1} DW, respectively), and *Dictyota* sp. highlighted within brown algae by its ARA content (23.63 ± 3.71 mg 100 g^{-1} DW).

The PCA of macroalgal FA revealed that five components had eigenvalues >1 and together accounted for more than 89% of the total variance. Factor loadings and communalities after applying varimax rotation to enhance the interpretability of the results are displayed in Supplementary Table 7. The dendrogram obtained from the hierarchical cluster analysis, which used the factor scores as input variables, revealed that macroalgae could be classified into six clusters (Figure 4.2.A.7). Factor scores for each cluster is given in Supplementary Table 8. Thus, Cluster 1 grouped together *A. stellata* (Chlorophyta), and two replicates of the Ochrophyta *Lobophora* sp., characterized by the highest contents of 14:0, 18:2n-6, 18:4n-3, ARA and EPA, and the lowest of 16:0, 16:1 and 18:1 isomers. Cluster 2 comprised one single species, the green seaweed *C. barbata*, which had the highest average percentages of 16:1n-7 and 18:1n-7, but low contents of 14:0, 18:0, 18:3n-3, 16:1n-5, 18:1n-5 and 18:1n-9 isomers. *Jania* sp. (Rhodophyta) and one replicate of *Lobophora* sp. formed cluster 3, characterized by reduced proportions of 16:1 isomers. Cluster 4 grouped three red macroalgae (*Asparagopsis* sp., *Laurencia* sp. and *Liagora* sp.) having the highest content of 16:0 and low 18:2n-6, ARA and EPA. Cluster 5 contained the brown seaweeds *Dictyota* sp. and *T. atomaria*, which had the highest proportions of 16:1n-5 and 18:1 isomers. Finally, Cluster 6 comprised two red macroalgal species (*H. spinella* and *J. rubens*) and *Stypocaulon* sp. (brown seaweed), and were characterized by high average contents of 18:0, 18:3n-3 and 16:1 isomers.

FA and LC profiles did not allow to group Gran Canaria Island macroalgae based on their complete lipid profile, as both parameters showed different patterns. Similarities in their LC and FA profiles were only evident in the pairs *A. stellata*-*Lobophora* sp., and *Dictyota* sp.-*T. atomaria*, that were grouped together in both clusters (Figure 4.2.A.5 and Figure 4.2.A.7).

Table 4.2.A.13. Total fatty acids (µg mg^{-1} DW) and main **fatty acid** composition (% of total FA) of **green macroalgae** from **Gran Canaria Island**.

	Cymopolia barbata	*Anadyomene stellata*
Total FA	4.79 ± 0.30	2.59 ± 0.70 *
Total SFA	25.29 ± 1.96	39.47 ± 2.52 *
14:0	1.03 ± 0.11	9.01 ± 0.62 *
15:0	nd	0.70 ± 0.08
16:0	21.55 ± 1.06	27.23 ± 1.96 *
17:0	nd	0.39 ± 0.02
18:0	1.29 ± 0.92	1.40 ± 0.05
Total MUFA	31.84 ± 1.37	20.33 ± 0.25 *
16:1[1]	7.69 ± 0.58	5.35 ± 0.23 *
18:1[2]	23.12 ± 0.87	13.45 ± 0.49 *
16:2n-4	7.16 ± 0.37	nd
16:3n-4	1.52 ± 0.09	0.32 ± 0.20 *
Total n-6 PUFA	19.79 ± 1.12	20.00 ± 1.28
18:2	8.70 ± 0.37	9.78 ± 0.25 *
18:3	9.75 ± 0.69	2.16 ± 0.14 *
20:3	0.35 ± 0.01	0.95 ± 0.10 *
20:4 (ARA)	0.99 ± 0.06	6.90 ± 0.81 *
Total n-3 PUFA	7.93 ± 0.30	15.93 ± 1.35 *
18:3	0.76 ± 0.02	1.02 ± 0.05 *
18:4	2.25 ± 0.06	4.28 ± 0.27 *
20:5 (EPA)	1.70 ± 0.12	9.26 ± 0.78 *
22:6 (DHA)	2.05 ± 0.11	0.97 ± 0.35 *
Total PUFA	37.42 ± 1.56	36.64 ± 2.63
DHA/EPA	1.20 ± 0.03	0.10 ± 0.03 *
EPA/ARA	1.72 ± 0.05	1.35 ± 0.05 *
n-6/n-3	2.50 ± 0.05	1.26 ± 0.04 *
Total n-3 LC-PUFA	4.92 ± 0.27	10.66 ± 1.09 *

Results are presented as means ± SD (n=3). * Indicates significant differences between species ($p<0.05$). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long chain polyunsaturated fatty acids (≥ C20 and ≥ 2 double bonds). Totals include other minor components not shown. [1] Mainly n-11 and n-7 isomers; [2] Mainly n-9 and n-7 isomers. nd, not detected.

Table 4.2.A.14. Total fatty acids (µg mg^{-1} DW) and main **fatty acid** composition (% of total FA) of **red macroalgae** from **Gran Canaria Island**.

	Jania rubens	*Jania* sp.	*Liagora* sp.	*Asparagopsis* sp.	*Laurencia* sp.	*Hypnea spinella*
Total FA	0.60 ± 0.17 ^{a}	1.31 ± 0.08 ^{b}	0.93 ± 0.16 ^{ab}	1.19 ± 0.38 ^{ab}	1.17 ± 0.14 ^{ab}	3.51 ± 0.32 ^{c}
Total SFA	60.33 ± 0.36 ^{bd}	46.59 ± 2.45 ^{a}	71.30 ± 1.13 ^{f}	68.34 ± 2.36 ^{def}	65.48 ± 0.89 ^{ce}	54.43 ± 2.51 ^{abc}
14:0	5.15 ± 0.09 ^{b}	5.57 ± 0.26 ^{bc}	6.18 ± 0.35 ^{c}	12.82 ± 0.64 ^{d}	12.01 ± 0.21 ^{d}	3.66 ± 0.06 ^{a}
15:0	1.55 ± 0.02 ^{c}	1.12 ± 0.07 ^{b}	0.96 ± 0.19 ^{abc}	0.88 ± 0.02 ^{ab}	2.36 ± 0.04 ^{d}	0.79 ± 0.08 ^{a}
16:0	49.05 ± 0.41 ^{c}	35.68 ± 1.76 ^{a}	59.55 ± 1.16 ^{d}	51.05 ± 2.24 ^{bcd}	48.17 ± 0.76 ^{c}	42.35 ± 0.52 ^{ab}
17:0	0.68 ± 0.10 ^{c}	0.57 ± 0.03 ^{bc}	0.43 ± 0.02 ^{a}	0.45 ± 0.01 ^{ab}	0.33 ± 0.01 ^{ab}	0.46 ± 0.02 ^{ac}
18:0	2.80 ± 0.06 ^{bc}	3.03 ± 0.36 ^{c}	2.57 ± 0.33 ^{bc}	2.27 ± 0.07 ^{ab}	1.91 ± 0.20 ^{a}	4.79 ± 0.27 ^{d}
Total MUFA	27.91 ± 0.25 ^{cd}	29.67 ± 1.01 ^{d}	18.88 ± 0.61 ^{a}	22.86 ± 1.46 ^{abc}	21.42 ± 0.23 ^{a}	23.37 ± 0.09 ^{b}
16:1[1]	9.85 ± 0.19 ^{d}	7.56 ± 0.35 ^{b}	8.46 ± 0.15 ^{c}	6.40 ± 0.18 ^{a}	7.00 ± 0.13 ^{b}	5.82 ± 0.19 ^{a}
18:1[2]	15.54 ± 0.45 ^{bc}	17.00 ± 0.52 ^{c}	8.90 ± 0.33 ^{a}	15.48 ± 1.75 ^{abc}	13.36 ± 0.11 ^{b}	17.02 ± 0.30 ^{c}
Total n-6 PUFA	3.87 ± 0.04 ^{ab}	8.92 ± 1.18 ^{cd}	2.35 ± 0.26 ^{a}	2.74 ± 0.27 ^{a}	5.58 ±0.56 ^{bc}	12.90 ± 0.04 ^{d}
18:2	2.58 ± 0.19 ^{b}	3.59 ± 0.28 ^{c}	1.19 ± 0.14 ^{a}	0.98 ± 0.12 ^{a}	2.23 ± 0.35 ^{b}	6.61 ± 0.22 ^{d}
18:3	nd	0.31 ± 0.05	nd	0.21 ± 0.07	nd	0.26 ± 0.01
20:3	nd	0.48 ± 0.08 ^{b}	0.32 ± 0.01 ^{ab}	0.28 ± 0.02 ^{a}	0.35 ± 0.05 ^{ab}	0.43 ± 0.01 ^{b}
20:4 (ARA)	1.04 ± 0.14 ^{a}	3.79 ± 0.70 ^{abc}	0.77 ± 0.03 ^{a}	1.09 ± 0.08 ^{a}	2.70 ± 0.17 ^{b}	5.49 ± 0.13 ^{c}
Total n-3 PUFA	2.38 ± 0.12 ^{b}	12.97 ± 2.54 ^{c}	3.01 ± 0.34 ^{b}	1.60 ± 0.05 ^{a}	3.29 ± 0.30 ^{b}	5.83 ± 0.31 ^{c}
18:3	1.15 ± 0.06 ^{b}	0.99 ± 0.11 ^{b}	0.49 ± 0.04 ^{a}	0.38 ± 0.00 ^{a}	0.44 ± 0.06 ^{a}	2.54 ± 0.06 ^{c}
18:4	0.27 ± 0.03 ^{a}	1.15 ± 0.15 ^{c}	0.47 ± 0.13 ^{ab}	0.39 ± 0.02 ^{ab}	0.47 ± 0.06 ^{b}	1.59 ± 0.11 ^{d}
20:5 (EPA)	0.96 ± 0.06 ^{b}	6.14 ± 1.19 ^{e}	1.53 ± 0.17 ^{c}	0.62 ± 0.07 ^{a}	2.30 ± 0.13 ^{d}	1.25 ± 0.11 ^{bc}
22:6 (DHA)	nd	4.28 ± 0.98 ^{c}	0.52 ± 0.10 ^{b}	0.21 ± 0.09 ^{a}	nd	nd
Total PUFA	6.25 ± 0.09 ^{bc}	21.89 ± 3.73 ^{abcd}	5.56 ± 0.50 ^{ab}	4.76 ± 0.22 ^{a}	9.43 ± 0.24 ^{c}	18.85 ± 0.39 ^{d}
DHA/EPA	---	0.69 ± 0.04 ^{b}	0.34 ± 0.04 ^{a}	0.35 ± 0.19 ^{ab}	---	---
EPA/ARA	0.93 ± 0.07 ^{c}	1.62 ± 0.03 ^{d}	2.00 ± 0.15 ^{e}	0.57 ± 0.03 ^{b}	0.85 ± 0.03 ^{c}	0.23 ± 0.01 ^{a}
n-6/n-3	1.63 ± 0.09 ^{b}	0.69 ± 0.05 ^{a}	0.78 ± 0.07 ^{a}	1.72 ± 0.22 ^{b}	1.70 ± 0.02 ^{b}	2.22 ± 0.12 ^{c}
Total n-3 LC-PUFA	0.96 ± 0.06 ^{a}	10.82 ± 2.28 ^{ab}	2.05 ± 0.27 ^{ab}	0.83 ± 0.03 ^{a}	2.38 ± 0.24 ^{b}	1.71 ± 0.16 ^{b}

Results are presented as means ± SD (n=3). Different letters in superscript within each row indicate significant differences between species ($p<0.05$). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long chain polyunsaturated fatty acids (≥ C20 and ≥ 2 double bonds). Totals include other minor components not shown. [1] Mainly n-11 and n-7 isomers; [2] Mainly n-9 isomers. nd, not detected.

Table 4.2.A.15. Total fatty acids (µg mg^{-1} DW) and main **fatty acid** composition (% of total FA) of **brown macroalgae** from **Gran Canaria Island**.

	Stypocaulon sp.	*Lobophora* sp.	*Dictyota* sp.	*Taonia atomaria*
Total FA	1.10 ± 0.25 ^{a}	1.25 ± 0.27 ^{a}	3.66 ± 0.58 ^{b}	4.31 ± 0.38 ^{b}
Total SFA	50.53 ± 1.68 ^{c}	42.22 ± 0.43 ^{b}	40.34 ± 0.89 ^{b}	36.71 ± 0.89 ^{a}
14:0	5.72 ± 0.26 ^{a}	8.09 ± 1.08 ^{ab}	7.79 ± 0.20 ^{b}	4.95 ± 0.26 ^{a}
15:0	0.96 ± 0.08	0.68 ± 0.15	0.99 ± 0.03	1.05 ± 0.21
16:0	38.31 ± 1.27	29.11 ± 1.07	28.36 ± 0.62	28.35 ± 0.86
17:0	0.47 ± 0.04	0.12 ± 0.20	0.17 ± 0.15	nd
18:0	3.37 ± 0.55 ^{ab}	2.70 ± 1.79 ^{ab}	1.86 ± 0.09 ^{b}	1.18 ± 0.12 ^{a}
Total MUFA	28.60 ± 1.39 ^{a}	25.73 ± 3.81 ^{a}	31.35 ± 0.70 ^{a}	44.77 ± 2.05 ^{b}
16:1	10.58 ± 0.52 ^{b}	7.17 ± 0.21 ^{a}	12.54 ± 0.19 ^{c}	24.80 ± 0.62 ^{d}
16:1n-5	1.42 ± 0.05 ^{b}	0.54 ± 0.01 ^{a}	6.21 ± 0.10 ^{c}	18.23 ± 0.30 ^{d}
18:1[1]	16.52 ± 0.77 ^{a}	17.80 ± 3.39 ^{ab}	18.50 ± 0.31 ^{ab}	19.63 ± 0.88 ^{b}
Total n-6 PUFA	10.24 ± 2.31 ^{b}	17.32 ± 1.10 ^{c}	11.20 ± 0.08 ^{b}	5.80 ± 1.00 ^{a}
18:2	6.47 ± 1.25 ^{c}	8.89 ± 0.21 ^{d}	2.89 ± 0.08 ^{b}	1.03 ± 0.05 ^{a}
18:3	0.29 ± 0.08 ^{a}	1.56 ± 0.09 ^{b}	0.41 ± 0.02 ^{a}	nd
20:3	0.23 ± 0.03 ^{a}	0.89 ± 0.11 ^{b}	0.72 ± 0.03 ^{b}	0.31 ± 0.02 ^{a}
20:4 (ARA)	3.08 ± 0.82 ^{a}	5.99 ± 0.78 ^{b}	6.45 ± 0.03 ^{b}	1.63 ± 0.49 ^{a}
Total n-3 PUFA	5.71 ± 1.39 ^{a}	10.26 ± 1.77 ^{b}	10.61 ± 0.38 ^{b}	5.24 ± 1.32 ^{a}
18:3	2.17 ± 0.45 ^{b}	1.00 ± 0.16 ^{a}	2.62 ± 0.04 ^{b}	0.61 ± 0.09 ^{a}
18:4	1.63 ± 0.41 ^{a}	2.66 ± 0.57 ^{a}	4.28 ± 0.16 ^{b}	2.49 ± 0.59 ^{a}
20:5 (EPA)	1.70 ± 0.36 ^{a}	6.12 ± 0.97 ^{b}	2.66 ± 0.10 ^{a}	1.57 ± 0.43 ^{a}
Total PUFA	15.96 ± 3.70 ^{ab}	28.88 ± 3.00 ^{c}	22.21 ± 0.66 ^{bc}	11.28 ± 2.48 ^{a}
DHA/EPA	---	---	---	---
EPA/ARA	0.56 ± 0.03 ^{b}	1.02 ± 0.03 ^{c}	0.41 ± 0.01 ^{a}	0.97 ± 0.04 ^{c}
n-6/n-3	1.80 ± 0.04 ^{b}	1.71 ± 0.22 ^{ab}	1.06 ± 0.04 ^{a}	1.12 ± 0.12 ^{a}
Total n-3 LC-PUFA	1.91 ± 0.55 ^{a}	6.60 ± 1.05 ^{c}	3.70 ± 0.20 ^{b}	2.15 ± 0.66 ^{ab}

Results are presented as means ± SD (n=3). Different letters in superscript within each row indicate significant differences between species ($p<0.05$). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long chain polyunsaturated fatty acids (≥ C20 and ≥ 2 double bonds). Totals include other minor components not shown. [1] Mainly n-9 isomers. nd, not detected.

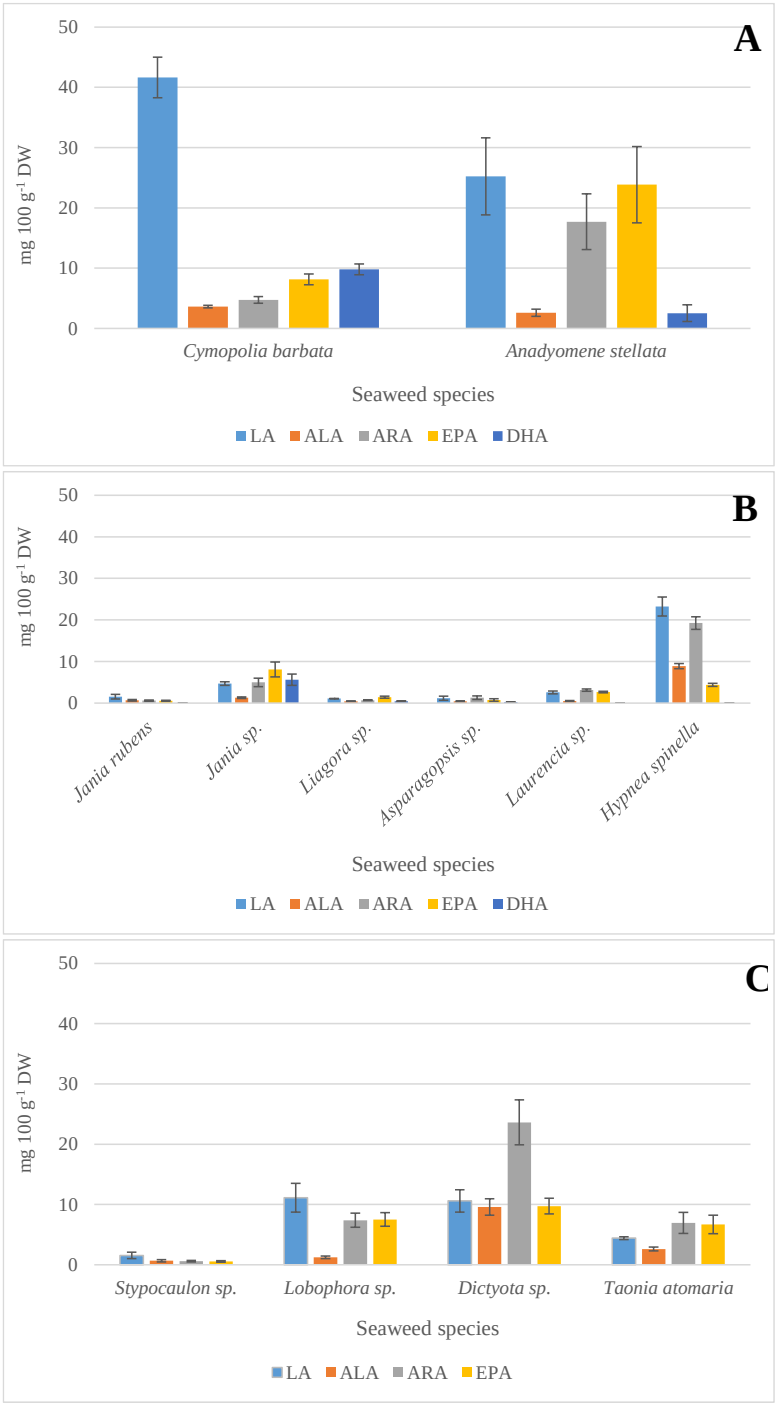


Figure 4.2.A.6. Main **LC-PUFA and their precursors** (mg 100 g^{-1} DW) in green (A), red (B) and brown (C) seaweed species from **Gran Canaria Island**.

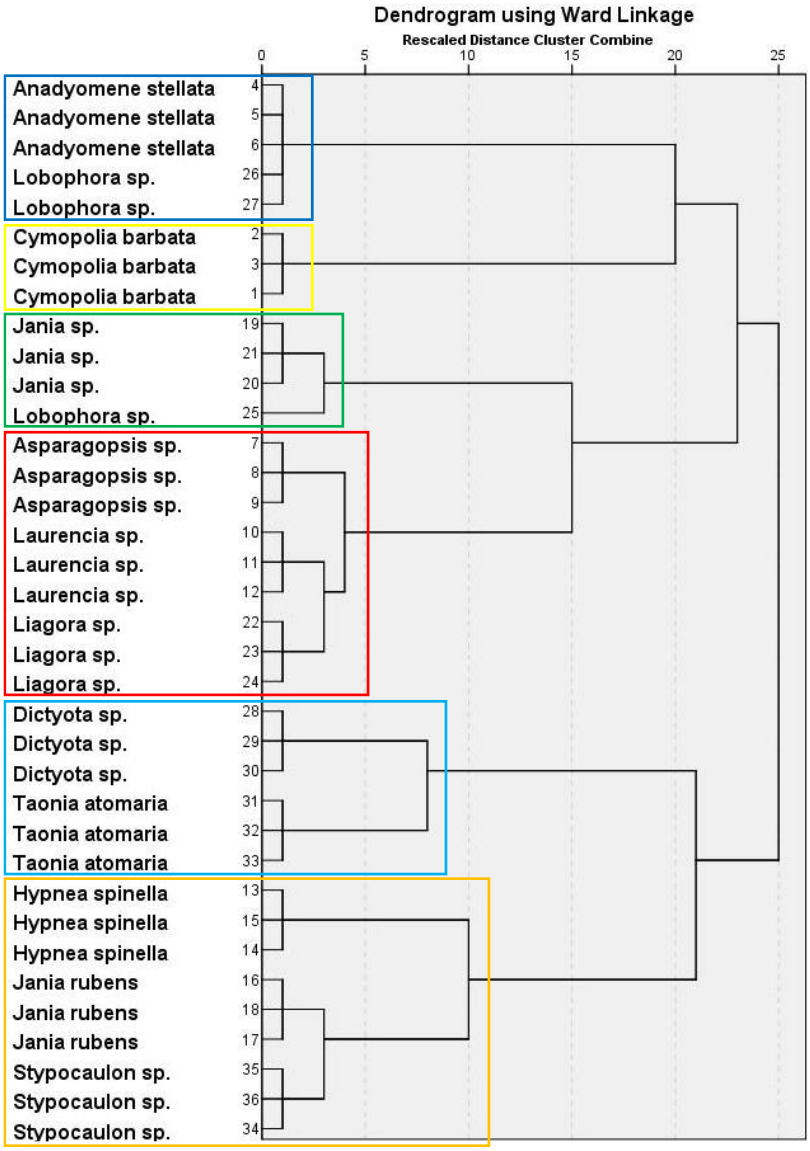


Figure 4.2.A.7. Hierarchical cluster analysis in a dendrogram format showing six clusters of macroalgae from **Gran Canaria Island** according to their **FA composition.**

- Nutritional indexes

C. barbata showed the most favourable AI and TI ratios from a health point of view (0.45 ± 0.06 and 0.48 ± 0.05, respectively). The highest TI were registered in *Liagora* sp. and *Asparagopsis* sp., and these two species, together with *Laurencia* sp. showed the highest AI. Finally, *Liagora* sp. presented the lowest hH ratio (0.16 ± 0.02) (Table 4.2.A.16).

Table 4.2.A.16. Atherogenicity index (AI), **thrombogenicity index** (TI) and **hypocholesterolemic/hypercholesteloremic** fatty acids ratio (hH) of **macroalgae** from **Gran Canaria Island**.

Group/Phylum	Species	AI	TI	hH
Chlorophyta	*Cymopolia barbata*	0.45 ± 0.06 ^{a}	0.48 ± 0.05 ^{a}	0.97 ± 0.07 ^{e}
	Anadyiomene stellata	1.15 ± 0.14 ^{abcd}	0.55 ± 0.08 ^{ab}	1.11 ± 0.12 ^{cef}
Rhodophyta	*Jania rubens*	2.12 ± 0.04 ^{e}	2.41 ± 0.05 ^{e}	0.26 ± 0.01 ^{b}
	Jania sp.	1.19 ± 0.13 ^{bcd}	0.76 ± 0.15 ^{bc}	0.77 ± 0.11 ^{abcdef}
	Liagora sp.	3.59 ± 0.26 ^{ef}	3.28 ± 0.29 ^{f}	0.16 ± 0.02 ^{a}
	Asparagopsis sp.	3.86 ± 0.35 ^{ef}	3.65 ± 0.31 ^{f}	0.20 ± 0.04 ^{ab}
	Laurencia sp.	3.24 ± 0.15 ^{f}	2.60 ± 0.16 ^{e}	0.30 ± 0.02 ^{b}
	Hypnea spinella	1.47 ± 0.03 ^{d}	1.41 ± 0.05 ^{d}	0.69 ± 0.01 ^{ce}
Ochrophyta	*Stypocaulon* sp.	1.45 ± 0.11 ^{bcd}	1.29 ± 0.21 ^{d}	0.53 ± 0.08 ^{abcd}
	Lobophora sp.	1.20 ± 0.09 ^{bcd}	0.76 ± 0.05 ^{bc}	0.97 ± 0.08 ^{ef}
	Dictyota sp.	1.15 ± 0.02 ^{c}	0.70 ± 0.03 ^{abc}	0.86 ± 0.02 ^{df}
	Taonia atomaria	0.88 ± 0.04 ^{b}	0.83 ± 0.10 ^{c}	0.66 ± 0.05 ^{cef}

Results are presented as means ± SD (n=3). Different letters in superscript in the same column indicate significant differences among all macroalgal species ($p<0.05$).

A.4. Discussion

The seaweeds analysed differed greatly in their lipid contents, LC and FA profiles providing evidence of strong interspecific variations. In addition, several others factors have been suggested to affect the biochemical composition of algae, including the part of the macroalgal thallus used for the analysis, seasonal, environmental or even geographical factors, or the impact of species-dependent fatty acid transformation and decomposition (Alsufyani et al., 2014; Pereira et al., 2012). All these factors should be considered in future research to provide a more complete lipid description of the selected macroalgal species.

The TL content of the studied macroalgae were broadly similar to the low levels described in earlier literature (Bourgougnon et al., 2011; Kendel et al., 2015; Mæhre et al., 2014; Nunes et al., 2020; Verma et al., 2017). Nevertheless, some differences detected were probably related to both geographical and seasonal factors. Thus, the two Chlorophyta species from Madeira Archipelago had lower lipid contents than other species of *Ulva*, such as *Ulva lactuca* and *U. reticulata* from India and *U. lactuca* from Hong Kong (Verma et al., 2017; Wong and Cheung, 2000). On the other hand, the lipid level of *Ulva* sp. (Table 4.2.A.1) was slightly higher than that of *U. lactuca* collected in the United Kingdom and *Ulva rigida* from South Africa (Foster and Hodgson, 1998; Marsham et al., 2007). In contrast, *C. barbata* and *A. stellata* from Gran Canaria Island (Table 4.2.A.9) contained higher lipid levels than most *Ulva* species described above, excluding *U. reticulata* from India.

Among Rhodophyta, *A. taxiformis* from Madeira Archipelago and *H. spinella* from Gran Canaria Island stood out from the rest by their higher lipid (~2% of TL in DW; Tables 4.2.A.1 and 4.2.A.9). *C. officinalis* (Madeira) had a higher lipid content than that obtained by Marsham et al. (2007) for the same species. However, *J. rubens* (Gran Canaria Island) showed lower values than those reported for this species in the Egyptian Mediterranean sea (El Maghraby and Fakhry, 2015) but higher than that of the Mediterranean coast of Turkey (Polat and Ozogul, 2009). All these variations evidence the influence of environmental factors in macroalgae lipid composition.

Gosch et al. (2012) described that Ochrophyta, chiefly species from Dictyotales, such as *Dictyota bartayresii*, *D. dichotoma* and *Spatoglossum macrodontum*, had large lipid contents of 10-12%. In accordance, Dictyotales analysed in the present work such as *T.*

atomaria and *Dictyota* sp. from Gran Canaria (Table 4.2.A.9) and *D. dichotoma* from Madeira (Table 4.2.A.1) showed the highest TL values of all brown macroalgae. Furthermore, *D. dichotoma*, highlighted by having the highest lipid content (5.23 ± 0.20%) of all species studied, higher than that cited by Verma et al. (2017), which is probably related to the recognized higher lipid levels of *Dictyota* species in temperate climates (McDermid and Stuercke, 2003).

Despite their overall low lipid levels, the proportions of the physiologically important PUFA in macroalgae analysed are higher than those of most terrestrial plants (Guil et al., 1996; Wielgosz-Collin et al., 2016). C18 PUFA such as LA and ALA are considered essential FA for vertebrates since they cannot be synthesized *de novo*, and therefore, their incorporation through diet becomes necessary. The human capacity to endogenously produce LC-PUFA from their C18 PUFA precursors through successive elongation and desaturation processes was demonstrated to be much lower than presumed (Metherel and Bazinet, 2019). Since nearly 70% of the world's population does not reach the minimum recommended daily intake of n-3 FA, due either to unhealthy nutritional habits or to difficulties accessing them, it is mandatory to include sources of n-3 LC-PUFA in the human diet for general health and wellbeing (D'Angelo et al., 2020; Taha, 2020).

This work demonstrates that the LC and FA composition should not be considered as useful biomarkers for taxonomic studies in seaweeds, due to a large interspecific variability (Figure 4.2.A.2 - Figure 4.2.A.7). However, green macroalgae are often described to be characterized by high amounts of C16 PUFA (including 16:3n-3 and 16:4n-3) and C18 PUFA (LA and ALA, similar to terrestrial plants), with LC-PUFA being usually absent (Kendel et al., 2015; Nakamura and Li-Beisson, 2016; Santos et al., 2019). Likewise, species of green macroalgae analysed here such as *Ulva* sp. (Madeira; Table 4.2.A.5) and *C. barbata* (Gran Canaria; Table 4.2.A.13) contained high proportions of 16:0, OA (18:1n-9), and C18 PUFA, such as LA and ALA. Interestingly, *C. barbata* contained notable proportions of 16:2n-4 and 18:3n-6. Although 16:2n-4 has been previously described to be present in some diatoms (De Carvalho and Caramujo, 2018), its proportion in this species might be suggesting a taxonomic character. On the other hand, 18:3n-6, together with its derived FA, 20:3n-6, have been suggested as important nutraceutical compounds for preventing the development of atherosclerosis (Moss and Ramji, 2016). According to what has been described for green macroalgae, LC-PUFA contents were low, with DHA being found in trace amounts in all species, as previously

reported by McCauley et al. (2016), and was only slightly higher in *C. barbata* (2.05 ± 0.11%). *A. stellata* (Gran Canaria) was also an exception, because of its interesting contents of ARA and EPA (6.90 ± 0.81% and 9.26 ± 0.78%, respectively) for animal and human nutrition.

In contrast, red macroalgae were characterised by high levels of 16:0, OA and ARA, and also constitute an important source of EPA (Sánchez-Machado et al., 2004; Schmid et al., 2018). In the present study, *H. incurva* displayed an unique FA composition within Madeira macroalgae (Figure 4.2.A.4), standing out of the rest for the highest proportions of ARA (~12% of total FA) and EPA (~9% of total FA), while DHA was highest in *A. taxiformis* (7% of total FA) (Table 4.2.A.6). Besides, *H. spinella* collected in Gran Canaria Island (Table 4.2.A.14) showed the highest value of ARA (5.49 ± 0.13%), while both DHA and EPA were also remarkable in *Jania* sp. (4.28 ± 0.98% and 6.14 ± 1.19%, respectively). In spite of the n-3 LC-PUFA values in *Jania* sp., *Jania rubens* showed low amounts of EPA, and DHA was even absent. This result confirms the strong variability in FA composition even between species from the same genus, reinforcing the hypothesis of high inter-specific and intra-generic variations in the FA profiles of macroalgae (Kumari et al., 2013; Lopes et al., 2020).

Therefore, the red macroalgae studied might be considered attractive sources of n-3 LC-PUFA (Sánchez-Machado et al., 2004), potentially promoting animal and human health and wellbeing (Murata and Nakazoe, 2001; Zárate et al., 2017). In particular, *H. incurva* and *A. taxiformis* might be promising candidates to partially substitute marine ingredients in aquafeed formulation, as occurs with other species of red macroalgae (Morais et al., 2020; Younis et al., 2018). However, some safety issues, such as the production of halogenated toxic compounds described in *A. taxiformis* by Machado et al. (2016), must be considered before recommending this seaweed as a food or feed ingredient.

In the present study, the FA profiles of Dictyotales and Fucales were similar to those previously described by Kumari et al. (2010) and Santos et al. (2019) where 14:0, 16:0, OA, and C18-C20 PUFA were reported as the major FA. Among Ochrophyta, *D. dichotoma* (Madeira) presented high n-3 PUFA, especially SDA (18:4n-3) and EPA with 8.05 ± 0.21% and 5.01 ± 0.21% of total FA, respectively. SDA is the first metabolic intermediate in the conversion of ALA into EPA and DHA (Whelan, 2009). The consumption of VO containing high SDA, such as *Echium* oil, was reported to improve

the incorporation of n-3 PUFA, and especially EPA, into human tissues compared with VO containing ALA (Guil-Guerrero, 2007). Nonetheless, the importance of SDA in animal and human health goes beyond its function as a precursor of EPA and DHA, and offers beneficial effects similar to those of EPA (Whelan, 2009). On the other hand, *C. usneoides*, *C. humilis*, *D. dichotoma* and *S. vulgare* from Madeira (Table 4.2.A.7), and *Dictyota* sp. and *Lobophora* sp. from Gran Canaria (Table 4.2.A.15) highlighted by their content in ARA (6-7%). EPA was also relevant in *Lobophora* sp. (Gran Canaria) (~6%). However, DHA was only remarkable (3.90 ± 0.38%) in *C. compressa* from Madeira, while it was not even detected in any of the brown species collected in Gran Canaria Island. Accordingly to our study, brown macroalgae are believed to have relatively high contents of EPA, ARA, LA and SDA (Dellatorre et al., 2020).

Finally, *T. atomaria* from Gran Canaria showed a remarkably high content of 16:1n-5 (18.23 ± 0.30%). This FA was also abundant in both *D. dichotoma* (Madeira) and *Dictyota* sp. (Gran Canaria), with ~6% of total FA. The high content of 16:1n-5 caused that both *Dictyota* sp. and *T. atomaria* from Madeira were grouped together in the same cluster (Figure 4.2.A.7). Although 16:1n-5 is actually considered a quimiotaxonomic FA from genus *Dictyopteris* and *Dictyota* (Khotimchenko, 1995), its high content in *T. atomaria* may be indicating a broader presence in other genus of brown macroalgae.

Despite the interesting FA profile of some seaweed species analysed here, their generally reduced lipid content implies that their supply of certain FA is probably low. However, according to the Regulation (EC) 1924/2006 of the European Parliament, and of the Council of 20 December 2006 on nutrition and health, the nutrition claim “source of n-3 fatty acids” can be made if the product contains at least 0.3 g ALA per 100 g and per 100 kcal, or at least, 40 mg of EPA + DHA per 100 g and per 100 kcal (Lähteenmäki-Uutela et al., 2021). In this sense, *D. dichotoma* and *A. taxiformis* from Madeira, supply 41-42 mg 100 g^{-1} DW of EPA+DHA (Figure 4.2.A.3) and could be considered as sources of n-3 FA.

The n-3 and n-6 PUFA families often show opposite physiological functions, with their relative proportions having implications for animal physiological and pathological mechanisms (Liu et al., 2015; Simopoulos, 2016; Tocher et al., 2019). High n-6/n-3 ratios hamper the biosynthesis of n-3 LC-PUFA (Smink et al., 2012) and impair corresponding eicosanoid and docosanoid derivatives production (Zárate et al., 2017). All the

macroalgae analysed had an n-6/n-3 FA ratio well below 10, as recommended by the World Health Organization (WHO) of the United Nations (Matanjun et al., 2009) for potential human and animal health, and wellbeing applications.

It is widely accepted that a reduction of SFA consumption and an increase in that of PUFA positively impacts human health by decreasing blood cholesterol (Moussa et al., 2014). AI, TI and hH indexes indicate the relationship between pro-thrombogenic (saturated) and anti-thrombogenic (unsaturated) FAs (Özden et al., 2020), and they have been proposed as nutritional fat quality indicators that measure dietary propensity to influence the incidence of coronary heart disease (Moussa et al., 2014; Pérez et al., 2014; Santos-Silva et al., 2002). Therefore, lower dietary AI, TI, and higher hH would prevent the risk of appearance of coronary diseases (Gerasimenko and Logvinov, 2016). Overall, all macroalgal studied showed AI, TI and hH values according to those reported in previous literature (Chen and Liu, 2020). In particular, *D. vermicularis* (green algae), *C. usneoides* and *S. vulgare* (brown algae) from Madeira Archipelago, and *C. barbata* and *A. stellata* (green algae) from Gran Canaria Island displayed the most advantageous values of these cardiovascular health indicators of all species analysed (Tables 4.2.A.8 and 4.2.A.16).

In animal nutrition, the type of lipid in which FA is provided seems to be particularly relevant (Lund et al., 2018; Reis et al., 2021a). Algal lipids can be divided into neutral lipids including TAG as storage compounds, and polar lipids, such as glycolipids and phospholipids, with major structural functions. Betaine lipids, not isolated in our study, are also common lipids in non-plastid membranes of algae, especially in green and brown seaweeds, and are considered as replacement compounds for PC under phosphorus deficiency (Huang et al., 2019). In fact, a reciprocal relationship between certain betaine lipids and PC has been proposed (Künzler and Eichenberger, 1997; Nakamura and Li-Beisson, 2016). Although several studies have reported that Dictyotales, Fucales (Ochrophyta) and Ulvales (Chlorophyta) do not have PC (Wielgosz-Collin et al., 2016), our study supports that of Jones and Harwood (1992), where small amounts of PC were detected in Fucales such as *Fucus vesiculosus* and *Ascophyllum nodosum*. On the other hand, PC is expected in Rhodophyta species, being reported to represent up to 55-75% of total phospholipids (Wielgosz-Collin et al., 2016). PC is an interesting source of LC-PUFA present in rich marine lecithin, which is of increasing interest for cosmetic, food, and pharmaceutical sectors (Alhajj et al., 2020).

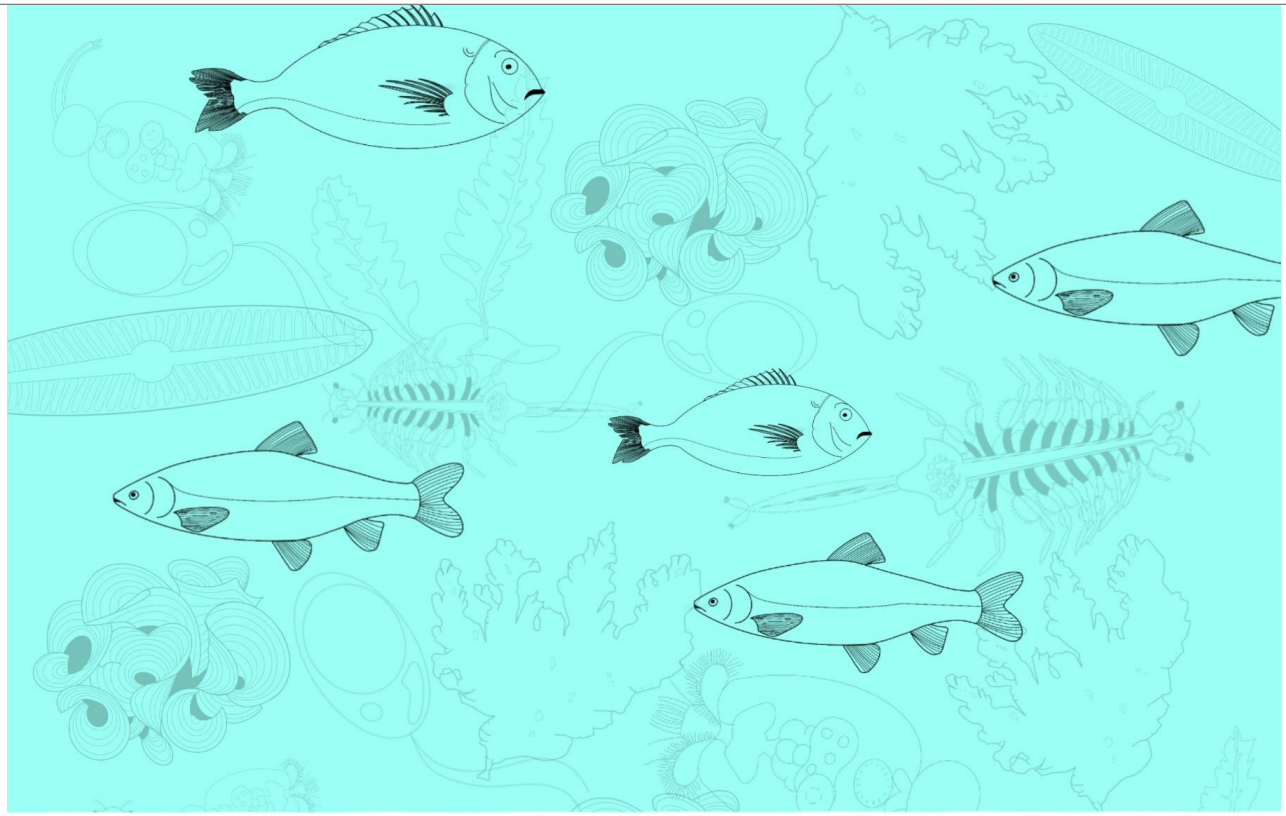
All the macroalgae studied here had a higher content of glycolipids than phospholipids. Thus, Chlorophyta species generally had remarkably high percentages of both SQDG and DGDG, excluding *A. stellata* (Tables 4.2.A.2 and 4.2.A.10), in contrast to other studies where MGDG was higher than SQDG and DGDG in green macroalgae (Khotimchenko, 2002). Furthermore, MGDG and DGDG have been described as the most characteristic glycolipids of Rhodophyta (Wielgosz-Collin et al., 2016), although Khotimchenko (2002) reported a high variability in their content among species. In our study, *H. incurva* had the highest proportions of SQDG, DGDG and MGDG among red seaweeds from Madeira (Table 4.2.A.3), while in Gran Canaria Island, *Asparagopsis* sp. showed the highest content of SQDG and MGDG, and *H. spinella* of DGDG (Table 4.2.A.11). On the other hand, the contents of MGDG in brown macroalgae were low (Tables 4.2.A.4 and 4.2.A.12). On the basis of the above, and in agreement to Wielgosz-Collin et al. (2016), glycolipids do not seem to be a valid taxonomic character since they are present in all brown seaweed species.

The glycolipids MGDG and DGDG from marine organisms have been reported to have antifungal, fibrinolytic and antitumor activities (Gerasimenko and Logvinov, 2016; Kendel et al., 2015; Wielgosz-Collin et al., 2016), which make seaweeds potentially interesting dietary components for human and animal nutrition, in addition to their higher LC-PUFA contents than in terrestrial plants (Sahaka et al., 2020).

C. officinalis, *H. incurva*, *C. usneoides*, *Lobophora* sp., *S. vulgare* (Madeira), and *A. stellata J. rubens*, *Liagora* sp. and *Asparagopsis* sp. (Gran Canaria) presented the highest content of PTS. Macroalgal PTS include several molecules such as fucosterol, stigmasterol, sitosterol and saringosterol, together with variable amounts of cholesterol (Schepers et al., 2020). PTS present benefits for cardiovascular diseases and anti-inflammatory processes (Kendel et al., 2015), and also decrease intestinal cholesterol absorption, reducing LDL cholesterol and therefore, factors of cardiovascular disease risk (Patch et al., 2006). Humans cannot biosynthesize PTS *de novo* (Kendel et al., 2015), again supporting that macroalgae might be a potential source of beneficial compounds for human nutrition.

In conclusion, the present study provides evidence of a high variability in the lipid contents, LC and FA profiles of macroalgae, making a definition of a characteristic pattern within each phylum highly complex. Seasonal, environmental and even

geographical factors affect these lipid profiles and should be considered in future research to ensure biochemical stability or even to identify algal species with a sufficiently high or diverse content of lipid molecules to be of commercial interest. Overall, the species analysed here contained lower lipid levels but higher PUFA proportions than terrestrial plants, and reduced n-6/n-3 FA ratios as recommended by WHO. Some brown and red macroalgae are an attractive source of n-3 LC-PUFA for human consumption and might also be considered as a potential substitute for marine ingredients in aquafeed formulations and production. The high contents of DGDG, MGDG, and PTS, together with the high levels of EPA, and low n-6/n-3 ratios makes *H. incurva* an interesting macroalga from a nutritional point of view. It is also remarkable that *A. taxiformis* (Rhodophyta) contained a high proportion of DHA, *D. dichotoma* (Ochrophyta) an unusually high content of the nutraceutical SDA, and *A. stellata* (Chlorophyta) high EPA proportions. Furthermore, both *A. taxiformis* and *D. dichotoma* showed relevant amounts of EPA+DHA allowing them to be considered as good sources of n-3 LC-PUFA.



Lipid characterization and potential use of macroalgae as food and animal feed additive:

Use of macroalgal wracks as feed for fish species

4.2.B. Use of macroalgal wracks as feed additive for fish species

B.1. Specific introduction

Fish meal and FO have traditionally been used as major ingredients in commercial aquafeeds as the most important sources of protein, amino acids, FA, minerals and energy (Güroy et al., 2007, 2013) for carnivorous species. However, the fluctuating availability of marine ingredients, their sustained price rise and the increment of global aquaculture production have driven the search for more sustainable alternatives (Galindo et al., 2021).

Lipids, mainly formed by FA, are the primary organic components of fish, together with proteins. C18 PUFA such as LA and LNA, are essential nutrients for vertebrates, and metabolic precursors of the physiologically important LC-PUFA including ARA, EPA and DHA (Tocher, 2015). LC-PUFA are involved in key physiological roles including transcription regulation, cellular signalling and cell membrane structure (Tocher, 2015). Although the use of VO from terrestrial plants is one of the most common alternatives to FO in aquafeed formulation, they are deficient in LC-PUFA, reducing the contribution of essential FA to fish flesh (Pérez et al., 2014). Additionally, diverse crop-plant derived protein sources have low digestibility and can only supply scarce amounts of some essential amino acids (Norambuena et al., 2015). Within this context, the increased demand for bioactive products from natural sources has boosted the search of new sustainable dietary ingredients able to preserve or improve the nutritional value of farmed fish for human consumers (Norambuena et al., 2015). In this sense, algae have been proposed as suitable alternative sources for lipids and proteins for farmed fish due to their high nutritional quality and balanced composition, high production rates and potential availability (Güroy et al., 2007; Norambuena et al., 2015).

The inclusion of micro and macroalgae in aquafeeds has been recently studied in both freshwater and marine fish species (Abdala-Díaz et al., 2021; Chen and Zeng, 2021; Ergün et al., 2009; Güroy et al., 2007, 2013; Norambuena et al., 2015; Perez-Velazquez et al., 2018; Sáez et al., 2020). Small dietary amounts of algae (2.5-10%) produced positive effects in fish growth performance, feed efficiency, lipid metabolism, body composition, stress response, liver function, and disease resistance, among others, in so far species studied (Güroy et al., 2007; Norambuena et al., 2015). However, a high algal inclusion (>10%) led to poor growth and reduced feed efficiency in gilthead seabream, *S. aurata* (Wassef et al., 2005) and nile tilapia, *Oreochromis niloticus* (Azaza et al., 2008).

This detriment in growth was attributed to the presence of antinutrients, including saponins, tannins, phytic acid, and protease and amylase inhibitors (Azaza et al., 2008; Vizcaíno et al., 2016), which are present in the vegetative tissues of terrestrial plants, and that have been also suggested in algae (Azaza et al., 2008; Norambuena et al., 2015; Vizcaíno et al., 2016). However, up to 25% inclusion of *U. rigida* in *S. aurata* diet in a short period trial, enhanced the immune system of fish and promoted their protection against the pathogen *Photobacterium damselae* subsp. *piscicida* without growth impairment (Abdala-Díaz et al., 2021). Thus, it is believed that fish response to dietary algal inclusion is species-specific and dependent on seaweed species, dose, and rearing conditions (Guerreiro et al., 2019; Norambuena et al., 2015).

All aerobic organisms, including fish, are susceptible to reactive oxygen species (ROS) attack. Fish tissues contain large amounts of PUFA, which are essential for membrane structure but are highly vulnerable to be oxidized. Consequently, fish must have an effective antioxidant system to prevent PUFA oxidation (Martínez-Álvarez et al., 2005; Rueda-Jasso et al., 2004). To mitigate damage caused by ROS, fish, as other animals, have developed antioxidant defences, including a number of enzymes involved in preserving redox homeostasis such as SOD, CAT, GST, or GR, but also antioxidant molecules like carotenoids, vitamins or peptides (Martínez-Álvarez et al., 2005). Either biotic or abiotic factors may increase or decrease the antioxidative response mechanisms in fish. Thus, phylogeny, age or feeding behaviour, environmental factors such as dissolved oxygen and temperature, parasites, diseases or xenobiotics may influence the risk of oxidative stress (Martínez-Álvarez et al., 2005). Additionally, dietary lipid level, vitamins, minerals and even the type of starch have been also associated with oxidative stress in fish. In this sense, high dietary-PUFA levels have been shown to increase lipid peroxidation in fish tissues. Moreover, a high dietary lipid level enhanced CAT and SOD activities, while diets containing raw carbohydrate increased oxidation rates in senegalese sole, *Solea senegalensis* juveniles (Rueda-Jasso et al., 2004). Antioxidative response of fish may also depend on the dietary supply of antioxidants such as vitamin E, which is the main soluble antioxidant present in animals (Martínez-Álvarez et al., 2005).

In vitro bioactivity testing of extracts from edible marine macroalgae on mammal cell lines have demonstrated their potential benefits as dietary antioxidants (Yuan and Walsh, 2006). In addition, polysaccharides and fucoxanthin (a marine carotenoid found in brown macroalgae and silicified microalgae, i.e. diatoms) have been reported as important

mediators in lipid metabolism and are being increasingly studied in human and animal nutrition (Norambuena et al., 2015). *In vitro* studies have also evidenced antioxidant properties of seaweed, and even algae consumption have been related to an increase in the endogenous antioxidant enzymes SOD and CAT activities in some mammals *in vivo* (Mohamed et al., 2012). Although there is an emerging interest in determining the role of dietary seaweed supplementation on antioxidant and immune responses in fish (Peixoto et al., 2016b), these studies are still scarce (Akbary and Aminikhoei, 2018; Kim et al., 2013; Peixoto et al., 2016b; Valente et al., 2015). Previous investigations suggested that feed supplemented with macroalgae may moderate stress responses and thus improve vitality, illness resistance (Kim et al., 2013; Mohamed et al., 2012), and flesh quality of fish (Valente et al., 2015), important parameters for the aquaculture industry (Peixoto et al., 2016b).

Strandings of macroalgal wracks that regularly detach from offshore seaweed beds and then accumulate on the coasts play a key role in beach ecosystems, preventing coastal erosion and acting as both a source of organic matter and a substrate for several invertebrates (Portillo, 2008). However, this clumping natural biomass is often regarded as an indicator of beach poor quality by bathers, and thought to compromise the aesthetics of the beach, as well as charged to cause unpleasant smell after decomposing. Thus, algae accumulations are usually removed and dumped in local landfills, causing an increased pressure on the handling and management of beach casts (Mossbauer et al., 2012). New uses for this biological biomass are being currently evaluated (Nunes et al., 2020, 2019a; Zárate et al., 2020), trying to reduce the environmental and economic impact linked to the managing of beach casts compared to their simple disposal.

Grass carp (*C. idella*) is an herbivorous, freshwater species, with high growth rate and easy adaption to captivity (Aslam et al., 2021). It was the major fish species produced in the world in the last years (FAO, 2020). On the other hand, gilthead seabream *S. aurata* is a marine carnivorous species with high commercial value and easy adaption to captivity, which is perceived as a quality fish by consumers. Nowadays, it is the most important finfish aquaculture product in the Mediterranean with a total production of 136,000 tons in 2020 (Savoca et al., 2021).

Several studies have been conducted to reduce fish meal in diets for *C. idella* (Aslam et al., 2021; Liu et al., 2019, 2020), including that of Salama et al. (2015) where a 25%

dietary inclusion of the microalgae *Arthrospira* (Spirulina) *platensis* did not significantly affect fish performance and whole body composition. Dietary inclusion of macroalgae has been also studied in *S. aurata* (Abdala-Díaz et al., 2021; Emre et al., 2013; Guerreiro et al., 2019; Martínez-Antequera et al., 2021; Passos et al., 2021; Shpigel et al., 2017; Vizcaíno et al., 2016), where an improvement in growth has been described with a 5-10% dietary inclusion of *Pterocladia capillacea* or *Ulva* sp. (Wassef et al., 2005). However, to the best of our knowledge, the inclusion of macroalgal wracks as dietary supplement has not yet been addressed in *C. idella* or *S. aurata*, selected for the present study due to their importance for the aquaculture sector, their respective herbivorous and carnivorous condition, and their different trophic level.

Thus, the **main objective of the present study** was to evaluate the use of Macaronesian macroalgal wracks as a feasible supplement in aquafeeds for *C. idella* and *S. aurata* juveniles from an eco-physiological and eco-sustainable perspective. Thus, the effect of macroalgal wracks inclusion was evaluated in the survival, growth and fat deposition of fish, proximate and lipid composition of muscle, oxidative status and antioxidant enzyme activities, and digestive capacities. Additionally, antioxidant potential of beach casts was also assessed.

To achieve the proposed objectives, two experiments were developed. In the first experiment, one multispecific macroalgal wrack and one monospecific macroalgal wrack (*Lobophora* sp.) were tested in *C. idella* juveniles. During the second experiment, the use of a multispecific macroalgal wrack and two monospecific macroalgal wracks (*Lobophora* sp. and *Dictyota* sp.) in *S. aurata* juveniles was evaluated.

B.2. Material and methods

All experimental procedures were in accordance with the Spanish Royal Decree 53/2013 of 1st February on the protection of animals for experimentation or other scientific purposes, and were approved by the ULL Ethical Committee CEIBA (Comité de Ética de la Investigación y Bienestar Animal) (CEIBA2015-0165).

B.2.1. Macroalgal wracks collection and pre-treatment

Macroalgal wracks were removed with a bulldozer-like machine from Las Canteras beach (28°08′24″N 15°26′15″W) in Gran Canaria Island (Spain) as part of the ordinary beach management procedure operated by local public administrations. Random samples of this

biomass, with a minimum weight of 20 kg representing at least 1% of the total biomass collected in each cleaning event, were separated from sand, washed with seawater, and dried by the action of continuous natural wind in the shadow. Subsamples of wet biomass were used for taxonomic identification. After 24 h, dried samples were crushed and ground to a fine powder (1 mm) with a rotor beater mill (SR 30; Retsch GmbH, Haan, Germany), and stored at room temperature in the dark (Zárate et al., 2020).

B.2.2. Experimental conditions

Experiment 1

A total of 69 grass carp (*C. idella*) juveniles were obtained from Pisciber Bio Secure Fishes, S.L. (Terrasa, Barcelona, Spain). Before the beginning of the trial, fish, with an initial weight of 33.53 ± 8.02 g, were randomly distributed into three experimental tanks in a rearing system allocated at the facilities of Fundación Neotrópico (Santa Cruz de Tenerife, Tenerife, Spain), and fed with the control diet for 3 weeks to acclimatize to the experimental conditions. The experiment was carried out in 1 m^3 polyethylene tanks under a recirculating aquaculture system (RAS) equipped with an Eheim Biopower 240 (Eheim GmbH & Co. KG, Deizisau, Germany) biofilter, and a recirculation pump Eheim compact+ 3000 (Eheim GmbH & Co. KG), with a water flow rate of 1000 L h^{-1}. In the manufacturing of diets, a basal extruded commercial diet (TI-3 Tilapia, Skretting, Burgos, Spain) was triturated, supplemented or not with the macroalgal wrack powder, and repelletised to avoid texture and palatability differences among diets. Experimental fish were daily fed with a 3-5% of their total biomass, three times a day, with the commercial diet (CD, control group) or with the same diet supplemented with either a 7% of a wind dried powder (1 mm) of multispecific (MU) macroalgal wrack (CD+MU7) or a 7% of *Lobophora* sp. monospecific (MOL) macroalgal wrack (CD+MOL7) (Figure 4.2.B.1). MU and MOL macroalgal wrack composition is shown in Figure 4.2.B.2. The trial was carried out for 100 days under natural photoperiod and ambient daylight of 1500 lux, and the average rearing water conditions in experimental tanks were: temperature (24.7 ± 0.4ºC), dissolved oxygen (6.9 ± 0.1 mg L^{-1}), and pH (7.5 ± 0.1).

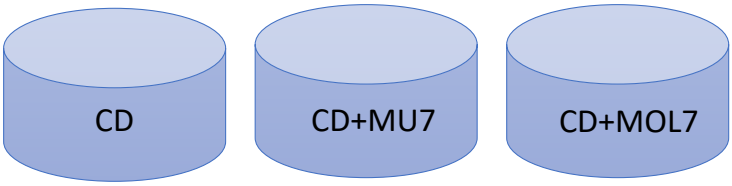


Figure 4.2.B.1. Tank distribution of the dietary treatments for juveniles of ***Ctenopharyngodon idella.***

CD, control diet; CD+MU7, control diet with a 7% multispecific macroalgal wrack inclusion; CD+MU7, control diet with a 7% monospecific *Lobophora* sp. macroalgal wrack inclusion.

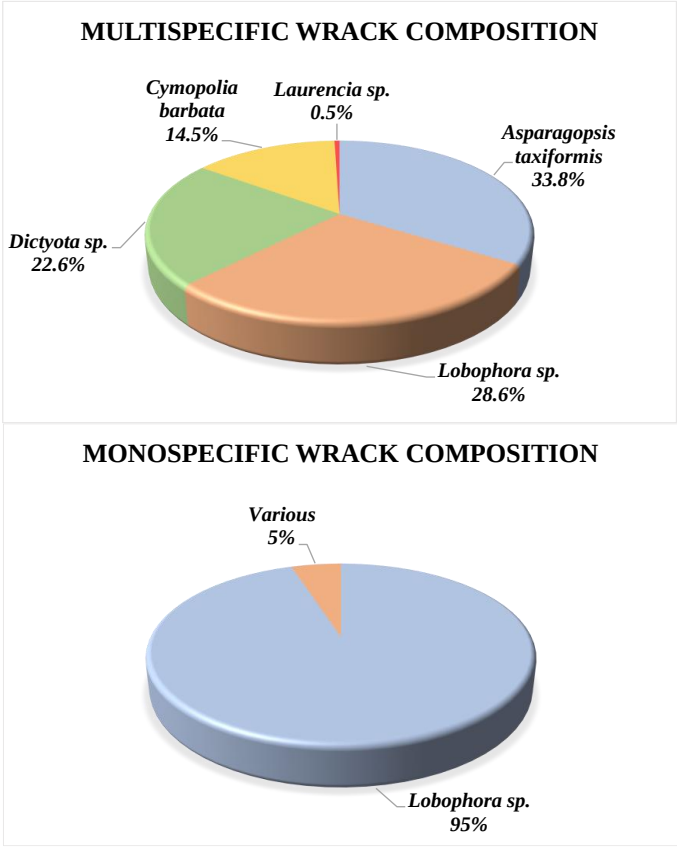


Figure 4.2.B.2. Composition of **multispecific** (MU) and **monospecific** (MOL) macroalgal wracks used as dietary supplements for ***Ctenopharyngodon idella*** juveniles.

Experiment 2

Gilthead seabream, *S. aurata*, juveniles of 18.63 ± 1.00 g initial weight (n=228) were obtained from Geremar (Santa Cruz de Tenerife, Tenerife, Spain). Fish were maintained in the experimental system at the CNIEO-CSIC facilities, and fed with the control diet for 3 weeks to acclimatize to the experimental conditions. In the manufacturing of diets, a basal extruded commercial diet (Inicio Plus 805, Biomar, Palencia, Spain) was triturated, supplemented or not with the macroalgal wrack, and repelletised to avoid texture and palatability differences among diets. Fish were randomly distributed in 12 tanks (by triplicates, 19 individuals in each tank) and fed the commercial diet (CD, control group), the same diet supplemented with either a 7% of a wind dried powder product of MU macroalgal wrack (CD+MU7), a 7% of *Lobophora* sp. MO wrack (CD+MOL7), or a 7% of *Dictyota* sp. MO wrack (CD+MOD7) (Figure 4.2.B.3). The composition of macroalgal wracks is shown in Figure 4.2.B.4. Animals were fed with a 3-5% of their total biomass, three times a day for 93 days. The fish were reared in polyethylene tanks (1 m^3) with a continuous seawater supply of 25 L min^{-1}, under natural photoperiod and ambient daylight of 1600 lux, with the following average rearing water conditions: temperature (20.2 ± 0.4ºC), dissolved oxygen (7.1 ± 0.2 mg L^{-1}), and pH (7.6 ± 0.2).

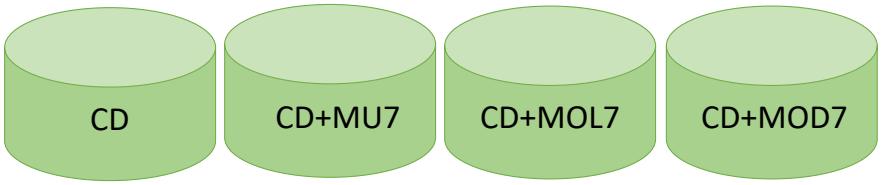


Figure 4.2.B.3. Tank distribution of the dietary treatments for juveniles of ***Sparus aurata.***
CD, control diet; CD+MU7, control diet with a 7% of multispecific macroalgal wrack inclusion; CD+MOL7, control diet with a 7% of monospecific-*Lobophora* sp. macroalgal wrack inclusion; CD+MOD7, control diet with a 7% of monospecific-*Dictyota* sp. macroalgal wrack inclusion.

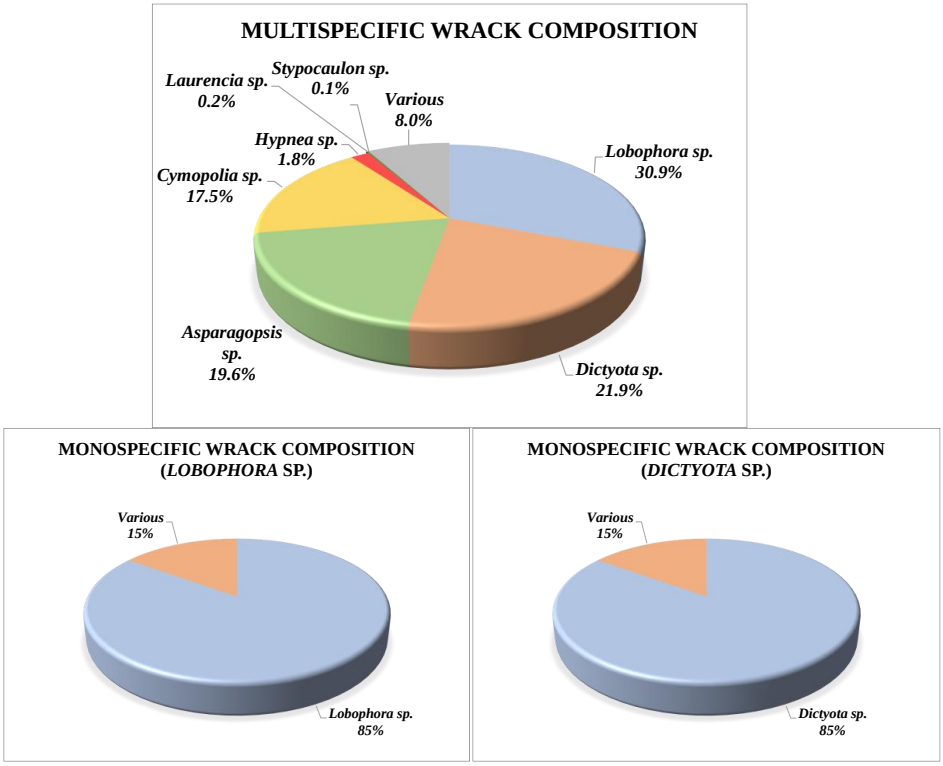


Figure 4.2.B.4. Composition of **multispecific** (MU), **monospecific-*Lobophora* sp.** (MOL) and **monospecific-*Dictyota* sp.** (MOD) macroalgal wracks used as dietary supplements for ***Sparus aurata*** juveniles**.**

B.2.3. Biochemical analysis of macroalgal wracks and diets

The proximate composition (moisture, ash, protein and lipid (TL and FA profiles)) of diets as well as the lipid composition of macroalgal wracks was determined (Table 4.2.B.1 to Table 4.2.B.6).

TL and LC profile of both MU and MOL wracks used for *C. idella* experiment is shown in Table 4.2.B.1. TL content was ~3% DW in both wracks. Neutral fraction (46-58%) was mainly formed by PTS (19-20%), MAG + DAG (12-14%) and FFA (~6% in the MOL wrack and ~14% in the MU one). On the othe hand, MGDG was the main polar lipid in both beach casts, representing ~5% in MU wrack and ~10% in MO wrack. Pigments were also a relevant component with 26-31% of total liposoluble fraction.

Table 4.2.B.1. Total lipid content (% DW) and **lipid class** composition (% of total lipid) of multispecific (MU) and monospecific-*Lobophora* sp. (MOL) **wracks** used as dietary supplement for ***Ctenopharyngodon idella*** juveniles.

	MU wrack	MOL wrack
Total lipid	2.51 ± 0.14	3.49 ± 0.24
PC	0.78 ± 0.13	3.44 ± 1.63
PS+PI	1.48 ± 0.01	4.25 ± 2.00
SQDG+PE	2.15 ± 0.48	4.53 ± 1.38
DGDG	2.02 ± 0.85	4.46 ± 1.02
MGDG	4.56 ± 0.80	9.55 ± 0.30
UKPL	0.63 ± 0.52	1.80 ± 1.32
TPL	11.63 ± 2.73	28.03 ± 7.08
P	30.76 ± 2.54	25.78 ± 0.69
MAG+DAG	13.79 ± 0.85	12.38 ± 1.97
PTS	19.13 ± 1.36	19.82 ± 2.07
FFA	13.56 ± 0.33	6.31 ± 1.60
TAG	8.44 ± 0.42	6.75 ± 3.06
SE	2.70 ± 1.37	0.94 ± 0.44
TNL	57.62 ± 0.33	46.19 ± 6.42

Data are presented as means ± SD (n=3). PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; SQDG, sulfoquinovosyl-diacylglycerol; PE, phosphatidylethanolamine; DGDG, digalactosyl-diacylglycerol; MGDG, monogalactosyl-diacylglycerol; UKPL, unknown polar lipids; TPL, total polar lipids; P, pigments; MAG, monoacylglycerols; DAG, diacylglycerols; PTS, phytosterols; FFA, free fatty acids; TAG, triacylglycerols; SE, sterol esters; TNL, total neutral lipids.

Table 4.2.B.2 displays the FA profile of MU and MOL wracks. Total SFA was the main fraction (39-47%) in both wracks. Total MUFA was a relevant component of MU wrack (~33%), while total PUFA was in the MOL wrack (~35%). EPA reached ~8% of total FA in the MOL wrack and ~2% in the MU one, contrarily to DHA that was a minor component in both beach casts (0.3-0.4%).

Table 4.2.B.2. Main **fatty acid** composition (% of total FA) of multispecific (MU) and monospecific-*Lobophora* sp. (MOL) **wracks** used as dietary supplement for ***Ctenopharyngodon idella*** juveniles.

	MU wrack	MOL wrack
Total SFA	47.18 ± 2.53	39.34 ± 3.10
14:0	6.09 ± 0.28	7.83 ± 0.34
16:0	36.31 ± 2.00	28.17 ± 1.78
18:0	2.00 ± 0.24	1.00 ± 0.23
Total MUFA	32.57 ± 0.88	21.39 ± 0.87
16:1[1]	11.65 ± 1.13	6.56 ± 0.75
18:1[1]	19.88 ± 0.48	13.85 ± 0.35
20:1[1]	0.11 ± 0.20	nd
Total n-6 PUFA	8.30 ± 0.78	19.74 ± 1.63
18:2	2.63 ± 0.44	9.60 ± 0.23
20:4 (ARA)	3.94 ± 0.75	7.08 ± 0.40
Total n-3 PUFA	6.18 ± 0.53	13.50 ± 1.69
18:3	0.97 ± 0.11	1.41 ± 0.19
20:5 (EPA)	2.31 ± 0.42	7.97 ± 1.24
22:5	nd	nd
22:6 (DHA)	0.29 ± 0.09	0.43 ± 0.10
Total PUFA	15.04 ± 1.16	34.53 ± 3.74
DHA/EPA	0.13 ± 0.04	0.06 ± 0.02
EPA/ARA	0.59 ± 0.01	1.12 ± 0.14
n-3/n-6	0.75 ± 0.05	0.68 ± 0.05
Total n-3 LC-PUFA	3.21 ± 0.34	8.64 ± 1.29

Results are presented as means ± SD (n=3). FA, fatty acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids (≥C20 and ≥2 double bonds). Totals include other minor components not shown. [1] Mainly n-9 isomers. nd, not detected.

The proximate composition of the experimental diets used to feed *C. idella* juveniles is given in Table 4.2.B.3. Thus, moisture, protein, ash, and lipid ranged between 10-13%, 34-36%, 7-10% and ~9% DW, respectively. Moreover, a 7% inclusion of macroalgae did not markedly affect dietary FA composition. Thus, total MUFA was the main group of FA (40-41%) in all diets, both total n-6 and total n-3 PUFA were also similar in the three diets (~22% and 10-11%, respectively), while EPA and DHA represented ~3% and ~2% of total FA, respectively.

Table 4.2.B.3. Proximate composition (moisture (%), protein (% DW), ash (% DW) and total lipid content (% DW)), **total fatty acids** (µg mg^{-1} DW) and main **fatty acid** composition (% of total FA) of **diets** for ***Ctenopharyngodon idella*** juveniles.

	CD	CD+MU7	CD+MOL7
Moisture	12.89 ± 0.57	10.22 ± 0.04	12.49 ± 3.02
Protein	35.63 ± 2.29	34.25 ± 1.55	35.20 ± 1.42
Ash	7.30 ± 1.06	10.49 ± 0.04	10.09 ± 0.22
Total lipid	9.19 ± 1.38	8.92 ± 0.15	9.28 ± 0.77
Total FA	70.95 ± 23.27	71.71 ± 18.75	75.16 ± 20.66
Total SFA	24.00 ± 0.53	23.79 ± 0.11	23.76 ± 0.50
14:0	2.23 ± 0.08	2.29 ± 0.02	2.28 ± 0.03
16:0	16.16 ± 0.28	16.03 ± 0.11	15.97 ± 0.35
18:0	4.11 ± 0.06	4.01 ± 0.01	4.03 ± 0.11
Total MUFA	41.01 ± 0.36	40.38 ± 0.31	40.33 ± 0.72
16:1[1]	4.37 ± 0.07	4.49 ± 0.03	4.44 ± 0.06
18:1[2]	34.50 ± 0.27	33.83 ± 0.29	33.94 ± 0.53
20:1[2]	0.95 ± 0.05	0.93 ± 0.03	0.94 ± 0.05
Total n-6 PUFA	22.48 ± 0.58	22.32 ± 0.20	22.31 ± 0.35
18:2	21.38 ± 0.32	21.35 ± 0.08	21.35 ± 0.29
20:4 (ARA)	0.70 ± 0.08	0.74 ± 0.06	0.74 ± 0.03
Total n-3 PUFA	10.41 ± 0.44	11.21 ± 0.24	10.96 ± 0.72
18:3	3.80 ± 0.07	3.88 ± 0.15	4.03 ± 0.30
20:5 (EPA)	3.14 ± 0.17	3.46 ± 0.01	3.30 ± 0.23
22:5	0.53 ± 0.14	0.47 ± 0.01	0.43 ± 0.02
22:6 (DHA)	2.26 ± 0.32	2.47 ± 0.02	2.28 ± 0.16
Total PUFA	34.80 ± 1.00	35.40 ± 0.50	35.06 ± 0.74
DHA/EPA	0.72 ± 0.07	0.71 ± 0.01	0.69 ± 0.01
EPA/ARA	4.42 ± 0.32	4.68 ± 0.37	4.42 ±0.17
n-3/n-6	0.46 ± 0.01	0.50 ± 0.01	0.49 ± 0.03
Total n-3 LC-PUFA	6.11 ± 0.45	6.65 ± 0.06	6.19 ± 0.43

Results are presented as means ± SD (n=3). CD, Control diet; CD+MU7, Control diet supplemented with 7% multispecific macroalgal wrack of *Lobophora* sp.; CD+MOL7, Control diet supplemented with 7% monospecific macroalgal wrack; FA, fatty acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids (≥C20 and ≥2 double bonds). Totals include other minor components not shown. [1] Mainly n-7 isomers; [2] Mainly n-9 isomers.

Table 4.2.B.4 shows the TL and LC profile of MU, MOL and MOD wracks used to supplement *S. aurata* juveniles diet. All wracks had a similar TL content (~3%), of which 24-29% were lipid soluble pigments. TNL ranged between 58 and 60% of TL, with FFA encompassing 25-26%, and TAG and PTS accounting for ~11% and 9-11% of TL, respectively. In the polar fraction (12-18%), SQDG + PE was remarkably high in all wracks (~4%), while DGDG was also important in the MOL wrack (~5%).

Table 4.2.B.4. Total lipid content (% DW) and **lipid class** composition (% of total lipid) of multispecific (MU), monospecific (*Lobophora* sp.; MOL) and monospecific (*Dictyota sp*.; MOD) **wracks** used as dietary supplement for ***Sparus aurata*** juveniles.

	MU wrack	MOL wrack	MOD wrack
Total lipid	2.72 ± 0.10	3.35 ± 0.06	2.86 ± 0.05
PC	1.47 ± 0.23	1.14 ± 0.47	1.37 ± 0.62
PS+PI	2.24 ± 1.00	2.73 ± 0.29	2.18 ± 0.13
SQDG+PE	3.90 ± 1.35	4.07 ± 0.14	3.86 ± 0.13
DGDG	2.47 ± 1.54	4.71 ± 0.43	1.68 ± 0.30
MGDG	2.49 ± 0.76	2.69 ± 0.23	2.03 ± 0.42
UKPL	1.46 ± 1.23	2.63 ± 0.75	1.16 ± 0.84
TPL	14.03 ± 5.64	17.98 ± 1.86	12.29 ± 0.67
P	26.37 ± 0.32	23.77 ± 2.10	29.39 ± 1.01
MAG+DAG	7.64 ± 0.95	6.37 ± 0.14	9.90 ± 0.18
PTS	10.81 ± 0.04	10.03 ± 0.96	9.06 ± 0.28
FFA	25.23 ± 0.74	25.81 ± 1.64	26.13 ± 1.14
TAG	11.32 ± 5.29	11.52 ± 1.99	11.12 ± 1.01
SE	4.59 ± 0.28	4.51 ± 0.99	2.12 ± 0.02
TNL	59.60 ± 5.33	58.25 ± 0.24	58.32 ± 0.34

Data are presented as means ± SD (n=2). PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; SQDG, sulfoquinovosyl-diacylglycerol; PE, phosphatidylethanolamine; DGDG, digalactosyl-diacylglycerol; MGDG, monogalactosyl-diacylglycerol; UKPL, unknown polar lipids; TPL, total polar lipids; P, pigments; MAG, monoacylglycerols; DAG, diacylglycerols; PTS, phytosterols; FFA, free fatty acids; TAG, triacylglycerols; SE, sterol esters; TNL, total neutral lipids.

MU, MOL and MOD wrack FA profiles are given in Table 4.2.B.5. Total SFA varied between 41-44% of total FA. Total MUFA was remarkable in the MU and MOD wrack, with ~31% and ~27%, while total PUFA varied between ~26% in the MOD wrack and ~35% in the MOL one. EPA reached ~8% of total FA in the MOL wrack and ~4% in the MU and MOD wracks. Finally, DHA accounted for ~2% of total FA in the MU wrack, being a minor component in both monospecific wracks (~0.4%).

Table 4.2.B.5. Main **fatty acid** composition (% of total FA) of multispecific (MU), monospecific-*Lobophora* sp. (MOL) and monospecific-*Dictyota sp*. (MOD) **wracks** used as dietary supplement for ***Sparus aurata*** juveniles.

	MU wrack	MOL wrack	MOD wrack
Total FA	4.64 ± 0.27	6.29 ± 2.32	3.79 ± 0.14
Total SFA	41.28 ± 0.79	41.57 ± 3.36	44.30 ± 0.97
14:0	6.28 ± 0.18	8.92 ± 0.17	8.56 ± 0.10
16:0	30.61 ± 0.72	29.73 ± 0.54	31.70 ± 0.13
18:0	2.00 ± 0.16	1.03 ± 0.02	1.52 ± 0.20
Total MUFA	31.33 ± 1.48	20.75 ± 1.31	27.27 ± 0.30
16:1[1]	8.59 ± 1.02	4.79 ± 2.05	6.58 ± 0.53
18:1[1]	21.18 ± 0.10	14.97 ± 0.04	19.18 ± 0.12
20:1	0.62 ± 0.01	0.33 ± 0.46	0.74 ± 0.03
Total n-6 PUFA	14.01 ± 0.57	21.32 ± 1.13	15.69 ± 0.96
18:2	7.11 ± 0.37	9.74 ± 0.25	6.29 ± 0.30
20:4 (ARA)	4.34 ± 0.17	7.33 ± 0.20	6.14 ± 0.47
Total n-3 PUFA	9.85 ± 1.64	12.89 ± 1.39	8.65 ± 0.87
18:3	1.31 ± 0.05	1.14 ± 0.03	1.26 ± 0.08
20:5 (EPA)	4.09 ± 0.41	7.84 ± 0.24	4.41 ± 0.53
22:5	0.26 ± 0.13	0.11 ± 0.01	nd
22:6 (DHA)	1.87 ± 0.77	0.39 ± 0.01	0.39 ± 0.00
Total PUFA	24.89 ± 2.02	35.36 ± 2.39	25.55 ± 2.09
DHA/EPA	0.45 ± 0.14	0.05 ± 0.00	0.09 ± 0.01
EPA/ARA	0.94 ± 0.06	1.07 ± 0.05	1.40 ± 0.06
n-3/n-6	0.70 ± 0.09	0.60 ± 0.03	0.55 ± 0.02
Total n-3 LC-PUFA	6.64 ± 1.51	8.75 ± 1.16	5.32 ± 0.61

Results are presented as means ± SD (n=2). FA, fatty acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids (≥C20 and ≥2 double bonds). Totals include other minor components not shown. [1] Mainly n-9 isomers. nd, not detected.

Table 4.2.B.6 shows the proximate composition and FA profile of experimental diets used for *S. aurata* juveniles. Moisture, protein, ash and lipid proportions varied between 6-7%, 46-49%, 10-12% and 15-18% DW, respectively. Dietary FA composition was similar regardless of the macroalgal wrack inclusion. Thus, total MUFA was the major component (37-38%) in all diets, followed by total PUFA (33-34%). Total n-6 and total n-3 comprised 15 and 17-18% of total FA, respectively, with EPA (4.5-5%) and DHA (8-9%), remaining stable in all experimental diets.

Table 4.2.B.6. Proximate composition (moisture (%), protein (% DW), ash (% DW) and total lipid content (% DW)), **total fatty acids** (µg mg^{-1} DW) and main **fatty acid** composition (% of total FA) of **diets** for ***Sparus aurata*** juveniles.

	CD	CD+MU7	CD+MOL7	CD+MOD7
Moisture	6.15 ± 0.08	6.11 ± 0.06	6.71 ± 0.23	6.63 ± 0.27
Protein	47.95 ± 0.59	48.80 ± 0.40	47.58 ± 0.57	46.20 ± 1.46
Ash	9.66 ± 0.05	12.05 ± 0.11	11.79 ± 0.02	11.64 ± 0.75
Total lipid	17.73 ± 0.84	16.07 ± 0.53	15.06 ± 1.45	16.20 ± 0.90
Total FA	110.44 ± 1.21	107.73 ± 5.46	92.05 ± 12.63	108.17 ± 13.30
Total SFA	27.81 ± 0.17	27.45 ± 0.25	27.73 ± 0.53	27.47 ± 0.97
14:0	3.50 ± 0.03	3.46 ± 0.06	3.50 ± 0.09	3.46 ± 0.16
16:0	18.39 ± 0.15	18.17 ± 0.25	18.27 ± 0.42	18.09 ± 0.67
18:0	4.20 ± 0.02	4.23 ± 0.18	4.25 ± 0.21	4.20 ± 0.17
Total MUFA	37.58 ± 0.27	37.33 ± 0.18	37.26 ± 0.65	37.34 ± 0.67
16:1[1]	5.02 ± 0.08	5.01 ± 0.15	5.02 ± 0.22	4.93 ± 0.13
18:1[2]	27.79 ± 0.24	27.67 ± 0.27	27.55 ± 0.40	27.66 ± 0.57
20:1[2]	2.50 ± 0.01	2.44 ± 0.03	2.46 ± 0.03	2.49 ± 0.09
Total n-6 PUFA	15.35 ± 0.15	15.29 ± 0.18	15.27 ± 0.20	15.25 ± 0.37
18:2	13.80 ± 0.05	13.78 ± 0.19	13.72 ± 0.16	13.72 ± 0.29
20:4 (ARA)	0.81 ± 0.03	0.85 ± 0.01	0.86 ± 0.04	0.84 ± 0.04
Total n-3 PUFA	17.16 ± 0.50	17.68 ± 0.26	17.50 ± 0.78	17.75 ± 1.01
18:3	2.18 ± 0.02	2.20 ± 0.04	2.17 ± 0.07	2.18 ± 0.08
20:5 (EPA)	4.50 ± 0.12	4.63 ± 0.03	4.61 ± 0.18	4.68 ± 0.26
22:5	0.91 ± 0.03	0.94 ± 0.02	0.92 ± 0.03	0.94 ± 0.06
22:6 (DHA)	8.37 ± 0.33	8.68 ± 0.16	8.53 ± 0.45	8.72 ± 0.57
Total PUFA	33.31 ± 0.60	33.81 ± 0.40	33.55 ± 0.99	33.85 ± 1.41
DHA/EPA	1.86 ± 0.02	1.88 ± 0.02	1.85 ± 0.03	1.86 ± 0.03
EPA/ARA	5.53 ± 0.06	5.46 ± 0.02	5.34 ± 0.10	5.54 ± 0.06
n-3/n-6	1.12 ± 0.02	1.16 ± 0.01	1.15 ± 0.04	1.16 ± 0.04
Total n-3 LC-PUFA	14.17 ± 0.49	14.65 ± 0.21	14.46 ± 0.66	14.74 ± 0.90

Results are presented as means ± SD (n=3). CD, Control diet; CD+MU7, Control diet supplemented with 7% multispecific macroalgal wrack; CD+MOL7, Control diet supplemented with 7% monospecific macroalgal wrack of *Lobophora* sp.; CD+MOD7, Control diet supplemented with 7% monospecific macroalgal wrack of *Dictyota* sp.; FA, fatty acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids (≥C20 and ≥2 double bonds). Totals include other minor components not shown. [1] Mainly n-7 isomers; [2] Mainly n-9 isomers.

B.2.4. Fish growth parameters and biochemical determinations

Fish weight was measured at the beginning, during (monthly) and at the end of the experimental period, and final survival, weight increment and specific growth rate (SGR; (ln final weight-ln initial weight)/days × 100) calculated. At the end of the experiment, five specimens were starved for 24 h prior to slaughter, and samples of muscle, liver and digestive tract collected and immediately stored at -80°C until biochemical analysis. Hepatosomatic (HSI; liver weight/body weight × 100), viscerosomatic (VSI; viscera weight/body weight × 100), and visceral-fat indexes (VFI) were also determined. VFI was calculated from visible fat of organs according to the following scale: 1 (low), 2 (medium) or 3 (high).

Fish muscle proximate and lipid composition (TL, LC and FA profile) were assessed. Antioxidant enzymes and TBARS were measured in liver and muscle, while PxI was determined only in muscle due to insufficient sample size. Finally, digestive enzymes were analysed in the stomach and gut of *C. idella* and *S. aurata* juveniles.

In order to determine the potential antioxidant capacity of seaweeds, total antioxidant capacity of MU and MOL macroalgal wracks extracts from both experiments was also analysed.

B.3. Results

Experiment 1

- Survival, growth parameters, body indexes and muscle proximate composition

Survival was greater than 80% in all experimental fish. Preliminary studies performed by our group with *C. idella* juveniles evidenced a detriment in growth with a dietary inclusion of 20% of the same MU tested in the present work (unpublished data). By contrast, a 7% dietary inclusion of either MU or MOL, did not affect fish growth parameters. Additionally, *C. idella* juveniles fed CD+MU7, presented the lowest and healthiest VSI and VFI values (Table 4.2.B.7).

The proximate composition of muscle from *C. idella* juveniles was not affected by dietary composition (Table 4.2.B.7). Thus, moisture (79-80%), protein (77-82% DW), ash (5-6% DW), and TL (6-9% DW) remained unchanged in all fish groups.

Table 4.2.B.7. Growth parameters, **body indexes** and muscle **proximate composition** (moisture, protein, ash and total lipid content) from ***Ctenopharyngodon idella*** juveniles fed the dietary treatments.

	CD	CD+MU7	CD+MOL7
Growth parameters			
Weight increment (g)	15.70	23.20	18.19
SGR (% day^{-1})	0.36	0.68	0.39
Body indexes			
HSI (%)	1.47 ± 0.40	1.30 ± 0.51	1.54 ± 0.62
VSI (%)	7.99 ± 1.25 ^{b}	6.85 ± 0.87 ^{a}	7.74 ± 1.42 ^{ab}
VFI	2.80 ± 0.41 ^{b}	1.93 ± 0.70 ^{a}	2.67 ± 0.49 ^{b}
Proximate composition			
Moisture (%)	79.68 ± 3.38	79.50 ± 1.32	79.00 ± 1.76
Protein (% DW)	81.63 ± 6.31	78.38 ± 7.19	77.26 ± 8.65
Ash (% DW)	5.48 ± 0.71	6.07 ± 1.12	6.05 ± 0.96
Total lipid (% DW)	6.47 ± 0.84	7.31 ± 2.28	8.51 ± 1.89

Results are presented as means ± SD (n=5 for body indexes and proximate composition). Different letters in superscript within each row denote significant differences ($p<0.05$). CD, Control diet; CD+MU7, Control diet supplemented with 7% multispecific macroalgal wrack of *Lobophora* sp.; CD+MOL7, Control diet supplemented with 7% monospecific macroalgal wrack; SGR, specific growth rate; HSI, hepatosomatic index; VSI, viscerosomatic index; VFI, visceral-fat index.

- Muscle LC profile

Regardless of dietary treatment, muscle LC profile was characterized by higher TNL (55-65% of TL) than TPL. TNL were mainly represented by TAG, which accounted for 31-43% of TL, and CHO with 11-14%, whereas PC was the main phospholipid in all fish groups (17-23%), followed by PE (10-11%) and PS + PI (5-6%). Differences between dietary treatments were only significant for SE, which was lower in the CD-fish (3.10 ± 0.71%) than in the experimental fish (4.6-5.5%) (Table 4.2.B.8).

Table 4.2.B.8. Lipid class composition (% of total lipid) of muscle from ***Ctenopharyngodon idella*** juveniles fed the dietary treatments.

	CD	CD+MU7	CD+MOL7
SM	1.74 ± 1.10	2.59 ± 1.19	1.22 ± 0.46
PC	22.65 ± 3.14	19.71 ± 2.93	16.61 ± 4.69
PS+PI	6.49 ± 0.94	6.42 ± 1.30	5.12 ± 1.27
PG	2.30 ± 0.39	2.13 ± 0.63	1.94 ± 0.48
PE	11.42 ± 2.57	11.28 ± 3.12	10.22 ± 3.02
TPL	44.60 ± 3.85	42.14 ± 7.76	35.11 ± 9.13
MAG	2.50 ± 0.42	1.88 ± 0.58	2.59 ± 0.76
DAG	1.47 ± 0.55	1.57 ± 0.27	0.96 ± 0.33
CHO	12.88 ± 1.50	14.45 ± 2.89	11.14 ± 2.40
FFA	2.88 ± 0.85	2.47 ± 0.71	2.47 ± 0.53
TAG	32.58 ± 6.51	31.97 ± 9.93	43.18 ± 11.80
SE	3.10 ± 0.71 [a]	5.52 ± 1.51 [b]	4.55 ± 0.61 [b]
TNL	55.40 ± 3.85	57.86 ± 7.76	64.89 ± 9.13

Data are presented as means ± SD (n=5). Different letters in superscript within each row denote significant differences ($p<0.05$). CD, Control diet; CD+MU7, Control diet supplemented with 7% multispecific macroalgal wrack; CD+MOL7, Control diet supplemented with 7% monospecific macroalgal wrack of *Lobophora* sp.; SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; TPL, total polar lipids; MAG, monoacylglycerols; DAG, diacylglycerols; CHO, cholesterol; FFA, free fatty acids; TAG, triacylglycerols; SE, sterol esters; TNL, total neutral lipids.

- Muscle FA profile

The FA composition of *C. idella* muscle was similar between groups (Table 4.2.B.9). Total MUFA was the most abundant family of FA with 38-43% of total, mainly due to 18:1, which represented approximately an 80% of the group, followed by SFA (25-26% of total FA), with 16:0 as its main component (18-19%). The only difference between dietary groups was the content of total n-6 PUFA, which was higher in CD+MU7-fish (18.13 ± 1.27%) than in CD-fish (15.73 ± 1.15%) (Table 4.2.B.9). Regarding n-3 PUFA (12-14%), DHA was the greatest component, ranging from 6.55 ± 2.88% in CD+MO7-fish to 8.60 ± 3.07% in CD+MU7-fish, while relative amounts of EPA were notably lower (2.31-2.70% of total FA).

Table 4.2.B.9. Total fatty acids (mg FA 100 g of wet weight^{-1}) and main **fatty acid** composition (% of total FA) of muscle from ***Ctenopharyngodon idella*** juveniles fed the dietary treatments.

	CD	CD+MU7	CD+MOL7
Total FA	939.63 ± 156.75	1096.37 ± 387.10	1145.12 ± 312.46
Total SFA	25.88 ± 0.92	25.38 ± 1.34	25.33 ± 0.94
14:0	1.86 ± 0.33	1.47 ± 0.39	1.85 ± 0.34
16:0	18.87 ± 0.61	18.44 ± 0.79	18.70 ± 0.58
18:0	4.75 ± 0.77	4.81 ± 1.02	4.32 ± 0.82
Total MUFA	42.07 ± 5.82	38.27 ± 6.57	42.89 ± 5.99
16:1[1]	8.16 ± 1.43	6.02 ± 1.02	7.82 ± 1.44
18:1[2]	34.17 ± 4.39	31.04 ± 5.36	33.81 ± 4.46
20:1[2]	0.92 ± 0.3	0.97 ± 0.12	1.02 ± 0.11
Total n-6 PUFA	15.73 ± 1.15 [a]	18.13 ± 1.27 [b]	16.12 ± 1.53 [ab]
18:2	9.90 ± 0.79	10.84 ± 1.77	10.58 ± 1.16
20:4	3.67 ± 1.18	4.83 ± 1.53	3.43 ± 1.59
Total n-3 PUFA	12.36 ± 3.56	14.49 ± 3.47	11.92 ± 3.61
18:3	1.83 ± 0.31	1.66 ± 0.43	1.77 ± 0.28
20:5	2.31 ± 0.71	2.70 ± 0.44	2.38 ± 0.76
22:5	0.90 ± 0.20	1.10 ± 0.19	0.89 ± 0.20
22:6	6.84 ± 2.41	8.60 ± 3.07	6.55 ± 2.88
Total PUFA	28.54 ± 4.46	33.06 ± 4.52	28.74 ± 4.88
DHA/EPA	2.92 ± 0.26	3.13 ± 0.74	2.69 ± 0.51
EPA/ARA	0.63 ± 0.05	0.65 ± 0.12	0.76 ± 0.24
n-3/n-6	0.78 ± 0.18	0.79 ± 0.14	0.73 ± 0.15
Total n-3 LC-PUFA	10.52 ± 3.42	12.83 ± 3.63	10.16 ± 3.85

Results are presented as means ± SD (n=5). Different letters in superscript within each row denote significant differences ($p<0.05$). CD, Control diet; CD+MU7, Control diet supplemented with 7% multispecific macroalgal wrack; CD+MOL7, Control diet supplemented with 7% monospecific macroalgal wrack of *Lobophora* sp.; FA, fatty acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids (≥C20 and ≥2 double bonds). Totals include other minor components not shown. [1] Mainly n-7 isomers; [2] Mainly n-9 isomers.

- Macroalgae antioxidant potential and oxidative status of fish

TAC of macroalgal wracks

Total antioxidant capacity of MU and MOL wracks is displayed in Table 4.2.B.10. Every extract from MU wrack showed low activity for DPPH (<40%). Both ethyl acetate and ethanol extracts from MOL wrack scavenged the DPPH radical by more than 50% at 250 µg mL^{-1}, with ethyl acetate surpassing 80%. For these two samples, the IC_{50} values were calculated. The most active extract (ethyl acetate) was near 10-fold less active than Trolox (IC_{50} values of 70.80 µg mL^{-1} *vs.* 7.43 µg mL^{-1}).

All extracts except that of ethanol from MU wrack, exceeded 50% activity after the ABTS assay. Overall, MOL-extracts were more active than MU ones. MOL-ethyl acetate extract was the most active one, with an IC_{50} of 13.17 µg mL^{-1}, over 15-fold higher than that of Trolox (0.87 µg mL^{-1}) (Table 4.2.B.10).

Table 4.2.B.10. Extraction yield (g 100 g^{-1} dried algae), **antioxidant activity** (%) and **IC_{50}** (µg mL^{-1}) of the multispecific and monospecific macroalgal **wracks** used as feed supplement for ***Ctenopharyngodon idella*** juveniles.

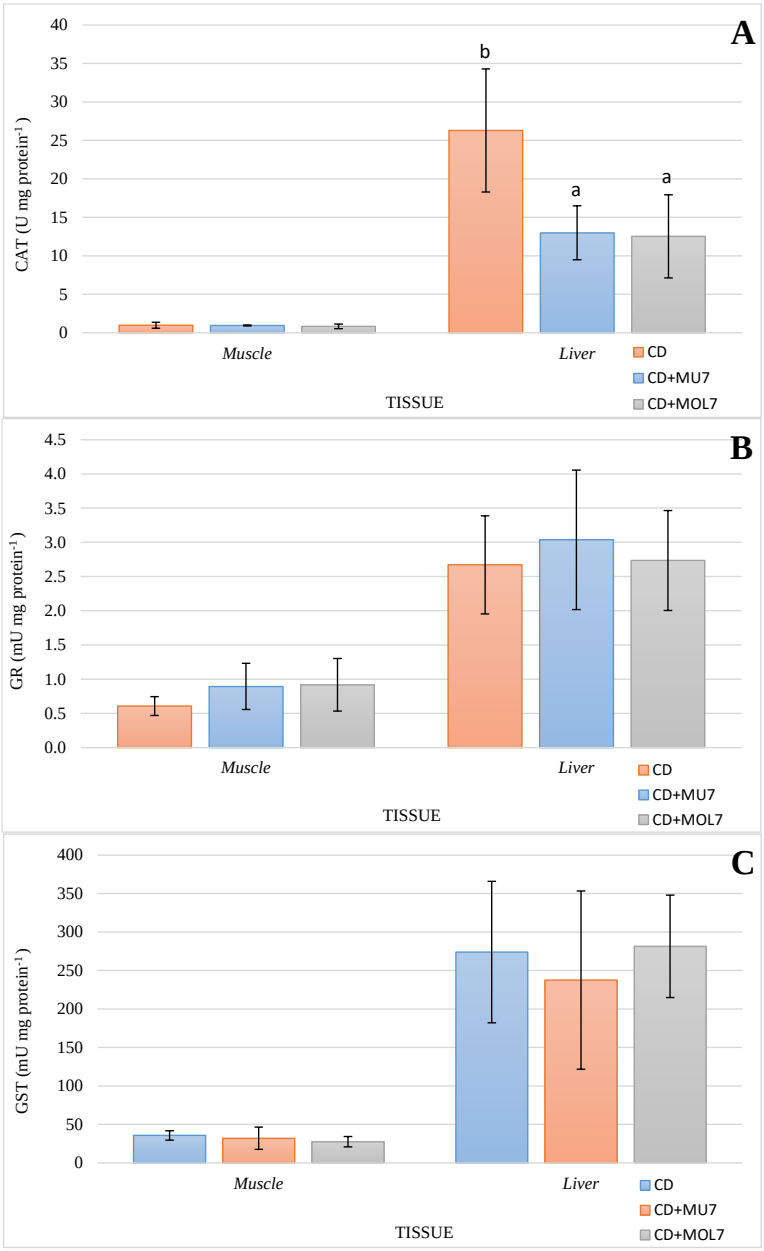
			DPPH		ABTS	
		Extraction yield	Activity	IC_{50}	Activity	IC_{50}
Multispecific macroalgal wrack	*n*-hexane	1.91	12.12 ± 2.47 ^{a,*}	>250	53.24 ± 5.03 ^{b,*}	197.04 ± 11.96 *
	Ethyl acetate	0.87	38.37 ± 1.66 ^{b,*}	>250	79.03 ± 1.57 ^{c}	69.89 ± 1.56 *
	Ethanol	5.58	7.04 ± 0.57 ^{a,*}	>250	27.82 ± 2.14 ^{a,*}	>250
Monospecific macroalgal wrack (*Lobophora* sp.)	*n*-hexane	2.87	24.42 ± 2.15 ^{a}	>250	74.08 ± 0.96 ^{a}	79.83 ± 3.70 ^{c}
	Ethyl acetate	2.56	89.63 ± 2.91 ^{c}	70.80 ± 1.06 ^{a}	78.99 ± 0.95 ^{b}	13.17 ± 0.68 ^{a}
	Ethanol	15.38	62.53 ± 1.58 ^{b}	169.91 ± 4.11 ^{b}	80.20 ± 1.72 ^{b}	43.87 ± 1.36 ^{b}
Trolox			92.21 ± 0.32	7.43 ± 0.74	83.25 ± 1.28	0.87 ± 0.18

Results are presented as means ± SD. All determinations were carried out in quadruplicate. DPPH, 1,1-diphenyl-2-picryl-hydrazyl; ABTS, 2,20-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); IC_{50}, Concentration yielding 50% scavenging of each radical. ^{a,b,c} Represent significant differences between solvents within the same wrack ($p<0.05$). * Represents significant differences between wracks for the same solvent ($p<0.05$). Activity (%) was measured at 250 µg mL^{-1} for macroalgal extracts and at 100 µg mL^{-1} for Trolox standard.

Fish antioxidant enzymes, PxI and TBARS

CAT, GST and GR activities were determined in muscle and liver, while SOD was determined exclusively in the liver of *C. idella* juveniles (Figure 4.2.B.5). All antioxidant activities were higher in the liver than in the muscle. None of the activities differed between treatments, except that of CAT, which was near the half in the liver of both experimental fish groups (12-13 U mg protein^{-1}) than in the control one (~26 U mg protein^{-1}). GST varied between 28 and 36 mU mg protein^{-1}, and 238-281 mU mg protein^{-1} in muscle and liver, respectively. GR ranged between 0.6-0.9 mU mg protein^{-1} in muscle and 2.7-3.0 mU mg protein^{-1} in the liver, while SOD activity was 697-756 U mg protein^{-1} in muscle from *C. idella*.

Finally, PxI and TBARS were determined in order to assess the oxidative status of the fish. PxI remained unchanged in muscle (8-9 meqO_2 Kg lipid^{-1}) whereas TBARS varied between 0.3 and 0.6 nmol MDA mg protein^{-1} in muscle, and 1.0-1.2 nmol MDA mg protein^{-1} in the liver.



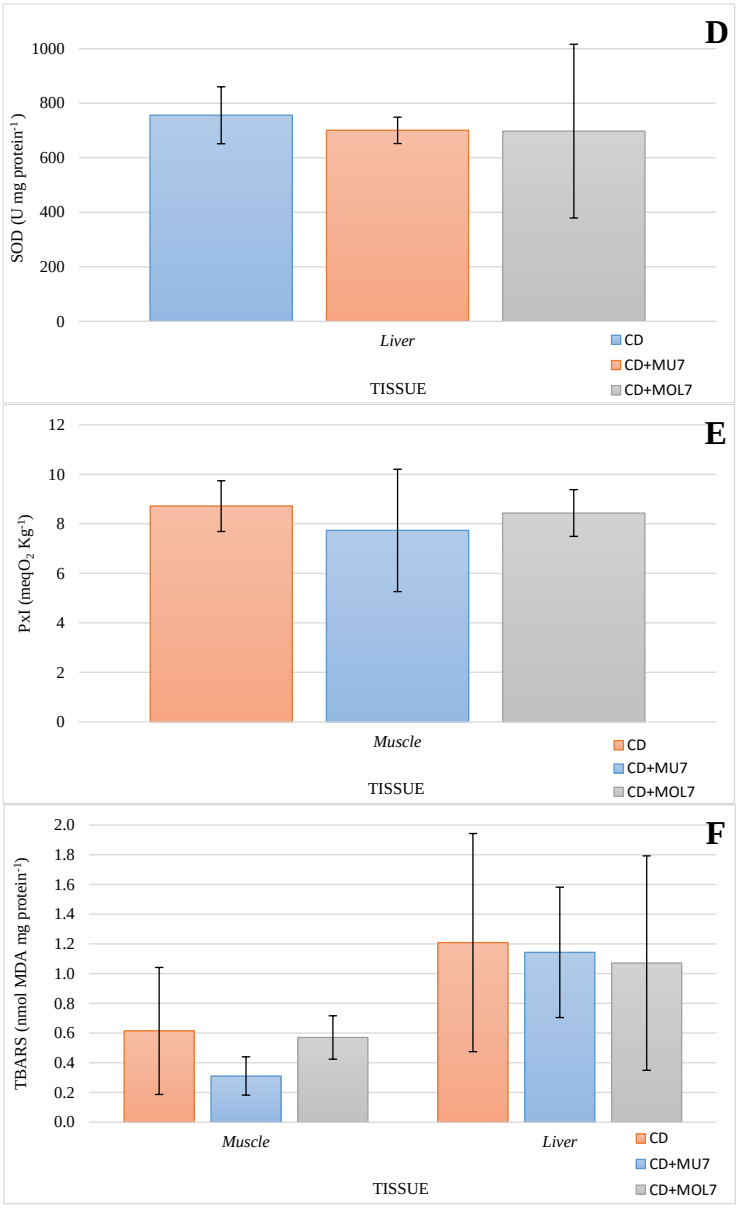


Figure 4.2.B.5. Antioxidant enzyme activities, peroxide index and TBARS of tissues from ***Ctenopharyngodon idella*** juveniles fed the dietary treatments.
A, catalase (CAT; U mg protein^{-1}); B, glutathione reductase (GR; mU mg protein^{-1}); C, glutathione S-transferase (GST; mU mg protein^{-1}); D, superoxide dismutase (SOD; U mg protein^{-1}); E, peroxide index (PxI; meqO_2 Kg^{-1}) and F, TBARS (nmol MDA mg protein^{-1}). All assays were determined in muscle and liver excluding SOD and PxI (muscle). CD, Control diet; CD+MU7, Control diet supplemented with 7% multispecific macroalgal wrack; CD+MOL7, Control diet supplemented with 7% monospecific macroalgal wrack of *Lobophora* sp. Results are presented as means ± SD (n=5). Different letters represent significant differences between dietary treatments ($p<0.05$).

- Digestive enzymes

The activity of the intestinal digestive enzymes (Table 4.2.B.11) did not differ between fish groups. Thus, alkaline protease activity varied between 186 and 256 mU mg protein^{-1}, BAL activity was 13-34 mU mg protein^{-1}, and that of alpha-amylase ranged between 167 and 289 U mg protein^{-1}.

Table 4.2.B.11. Digestive enzymes (alkaline proteases, bile salt-activated lipase and α-amylase) determined in the gastrointestinal tract of ***Ctenopharyngodon idella*** juveniles fed the dietary treatments.

	CD	CD+MU7	CD+MOL7
Alkaline proteases (mU mg protein^{-1})	226.00 ± 94.49	185.94 ± 44.27	255.61 ± 155.16
Bile salt-activated lipase (mU mg protein^{-1})	25.59 ± 14.26	34.22 ± 21.86	13.35 ± 7.92
Alpha-amylase (U mg protein^{-1})	167.21 ± 78.04	289.34 ± 120.90	223.19 ± 46.30

Results are presented as means ± SD (n=5). CD, Control diet; CD+MU7, Control diet supplemented with 7% multispecific macroalgal wrack; CD+MO7, Control diet supplemented with 7% monospecific macroalgal wrack.

Experiment 2

- Survival, growth parameters, body indexes and proximate composition

At the end of the experimental period, survival of *S. aurata* juveniles was 100% in all treatments. In addition, a 7% dietary inclusion of either MU, MOL or MOD, did not affect fish growth parameters with respect to the control. Similarly, there were no significant differences in body indexes between dietary groups (Table 4.2.B.12).

Muscle proximate composition of *S. aurata* juveniles was similar between treatments. Thus, moisture (75-77%), protein (81-83% DW), ash (6-7% DW), and TL (8-9% DW) remained unchanged in all fish groups (Table 4.2.B.12).

Table 4.2.B.12. Growth parameters, body indexes and **proximate composition** (moisture, protein, ash and total lipid content) of muscle from ***Sparus aurata*** juveniles fed the dietary treatments.

	CD	CD+MU7	CD+MOL7	CD+MOD7
Growth parameters				
Weight increment (g)	90.58 ± 1.41	87.07 ± 2.91	83.35 ± 5.98	87.09 ± 8.32
SGR (% day^{-1})	1.43 ± 0.04	1.43 ± 0.02	1.38 ± 0.04	1.45 ± 0.04
Body indexes				
HSI (%)	1.25 ± 0.09	1.46 ± 0.22	1.56 ± 0.15	1.45 ± 0.23
VSI (%)	7.32 ± 0.90	7.69 ± 0.72	8.99 ± 2.55	8.22 ± 0.98
VFI	2.60 ± 0.55	2.40 ± 0.55	2.25 ± 0.96	2.00 ± 0.71
Proximate composition				
Moisture (%)	74.56 ± 1.64	75.70 ± 0.82	77.10 ± 2.51	75.64 ± 0.98
Protein (% DW)	81.21 ± 4.32	81.04 ± 0.93	83.08 ± 4.79	81.53 ± 5.19
Ash (% DW)	6.24 ± 0.80	6.54 ± 0.80	5.86 ± 1.60	6.64 ± 0.81
Total lipid (% DW)	9.25 ± 3.06	9.45 ± 3.48	7.81 ± 2.08	8.21 ± 1.69

Results are presented as means ± SD (n=3 for growth parameters; n=5 for body indexes and proximate composition). CD, Control diet; CD+MU7, Control diet supplemented with 7% multispecific macroalgal wrack; CD+MOL7, Control diet supplemented with 7% monospecific macroalgal wrack of *Lobophora* sp.; CD+MOD7, Control diet supplemented with 7% monospecific macroalgal wrack of *Dictyota* sp.; SGR, specific growth rate; HSI, hepatosomatic index; VSI, viscerosomatic index; VFI, visceral-fat index.

- Mucle LC profile

Regardless of dietary treatment, TNL represented 61-67% of TL in muscle of all seabream juveniles, and were mainly composed by TAG (between 45 and 51% of TL). CHO was also an important neutral lipid fraction with 9-11% of TL, while muscle MAG was higher in CD+MU7 (1.43 ± 0.30% of TL) and CD+MOD7-fish (1.15 ± 0.24%), than in CD+MOL7-fish (0.84 ± 0.34%). With respect to polar lipids, PC (16-20%) and PE (9-11%) highlighted as the major phospholipids (Table 4.2.B.13).

Table 4.2.B.13. Lipid class composition (% of total lipid) of muscle from ***Sparus aurata*** juveniles fed the dietary treatments.

	CD	CD+MU7	CD+MOL7	CD+MOD7
SM	0.83 ± 0.31	0.89 ± 0.43	0.47 ± 0.10	0.48 ± 0.18
PC	17.80 ± 3.50	16.49 ± 4.35	19.89 ± 5.07	16.86 ± 2.54
PS	1.83 ± 0.65	1.72 ± 0.49	2.43 ± 1.62	1.98 ± 0.88
PI	3.75 ± 0.70	3.22 ± 1.14	3.91 ± 1.10	3.89 ± 0.71
PG	1.61 ± 0.47	1.55 ± 0.61	1.68 ± 0.77	1.34 ± 0.27
PE	9.71 ± 2.14	9.19 ± 2.08	10.93 ± 3.11	10.53 ± 2.01
TPL	35.54 ± 6.75	33.07 ± 8.26	39.31 ± 10.40	35.09 ± 5.42
MAG	1.35 ± 0.28 ^{ab}	1.43 ± 0.30 ^{b}	0.84 ± 0.34 ^{a}	1.15 ± 0.24 ^{b}
DAG	0.96 ± 0.24	1.07 ± 0.23	1.19 ± 0.39	1.25 ± 0.16
CHO	9.15 ± 1.38	10.82 ± 1.67	10.65 ± 2.05	10.19 ± 1.50
TAG	49.77 ± 7.20	50.74 ± 9.46	44.67 ± 12.18	48.97 ± 7.18
SE	3.23 ± 0.73	2.87 ± 0.88	3.33 ± 1.30	3.36 ± 0.88
TNL	64.46 ± 6.75	66.93 ± 8.26	60.69 ± 10.40	64.91 ± 5.42

Data are presented as means ± SD (n=5). Different letters in superscript within each row denote significant differences ($p<0.05$). CD, Control diet; CD+MU7, Control diet supplemented with 7% multispecific macroalgal wrack; CD+MOL7, Control diet supplemented with 7% monospecific macroalgal wrack of *Lobophora* sp.; CD+MOD7, Control diet supplemented with 7% monospecific macroalgal wrack of *Dictyota* sp.; SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; TPL, total polar lipids; MAG, monoacylglycerols; DAG, diacylglycerols; CHO, cholesterol; TAG, triacylglycerols; SE, sterol esters; TNL, total neutral lipids.

- Muscle FA profile

FA composition of *S. aurata* muscle is shown in Table 4.2.B.14. Dietary seaweed inclusion did not vary muscle FA profile, excluding total SFA, which was higher in CD+MOL7 and CD+MOD7-fish (~26% of total FA) than in the control group (24.50 ± 0.77%), despite

their main component (16:0) remained unchanged. Total MUFA (36-38%) was the main group of FA, due to 18:1 contribution, followed by total PUFA (35-37%) and finally, total SFA (25-26%). DHA was the main n-3 PUFA (12-13%), more than double of EPA content with ~5%. Finally, ARA was a minor fraction in all treatments (~1% of total FA).

Table 4.2.B.14. Total fatty acids (mg FA 100 g of wet weight^{-1}) and main **fatty acid** composition (% of total FA) of muscle from ***Sparus aurata*** juveniles fed the dietary treatments.

	CD	CD+MU7	CD+MOL7	CD+MOD7
Total FA	1733.3±662.9	1768.0±1004.2	1291.7±386.1	1494.2±269.2
Total SFA	24.50 ± 0.77^{a}	25.50 ± 0.46^{ab}	26.13 ± 0.92^{b}	26.36 ± 0.55^{b}
14:0	2.16 ± 0.19	2.25 ± 0.31	2.09 ± 0.45	2.03 ± 0.19
16:0	17.74 ± 0.49	18.09 ± 0.33	18.50 ± 0.82	18.67 ± 0.43
18:0	4.00 ± 0.47	4.41 ± 0.44	4.73 ± 0.46	4.74 ± 0.33
Total MUFA	36.81 ± 2.62	37.80 ± 3.23	36.34 ± 4.33	37.16 ± 3.37
16:1^{1}	5.11 ± 0.52	5.18 ± 0.54	4.88 ± 0.65	4.97 ± 0.43
18:1^{2}	28.18 ± 1.85	28.90 ± 2.59	27.88 ± 3.17	28.50 ± 2.72
20:1^{2}	1.82 ± 0.20	1.89 ± 0.12	1.79 ± 0.78	1.86 ± 0.16
Total n-6 PUFA	14.19 ± 0.46	13.98 ± 0.59	13.45 ± 0.48	13.25 ± 0.35
18:2	11.02 ± 0.60	11.00 ± 0.31	10.46 ± 0.64	10.40 ± 0.42
20:4	1.18 ± 0.22	1.08 ± 0.18	1.20 ± 0.33	1.16 ± 0.26
Total n-3 PUFA	22.61 ± 2.62	21.07 ± 2.80	22.26 ± 3.97	21.62 ± 3.08
18:3	1.58 ± 0.16	1.58 ± 0.09	1.47 ± 0.17	1.47 ± 0.10
20:5	5.00 ± 0.62	4.57 ± 0.52	4.85 ± 1.05	4.74 ± 0.72
22:5	1.81 ± 0.12	1.70 ± 0.23	1.74 ± 0.12	1.66 ± 0.11
22:6	13.00 ± 2.26	12.05 ± 2.11	13.08 ± 3.10	12.73 ± 2.37
Total PUFA	36.88 ± 2.42	35.14 ± 3.12	35.75 ± 3.72	34.90 ± 3.30
DHA/EPA	2.59 ± 0.18	2.62 ± 0.17	2.69 ± 0.09	2.68 ± 0.13
EPA/ARA	4.27 ± 0.33	4.26 ± 0.28	4.10 ± 0.27	4.13 ± 0.36
n-3/n-6	1.60 ± 0.21	1.50 ± 0.17	1.66 ± 0.33	1.63 ± 0.20
Total n-3 LC-PUFA	20.45 ± 2.84	18.92 ± 2.91	20.27 ± 4.22	19.62 ± 3.17

Results are presented as means ± SD (n=5). Different letters in superscript within each row denote significant differences ($p<0.05$). CD, Control diet; CD+MU7, Control diet supplemented with 7% multispecific macroalgal wrack; CD+MOL7, Control diet supplemented with 7% monospecific macroalgal wrack of *Lobophora* sp.; CD+MOD7, Control diet supplemented with 7% monospecific macroalgal wrack of *Dictyota* sp.; FA, fatty acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain polyunsaturated fatty acids (≥C20 and ≥2 double bonds). Totals include other minor components not shown. [1] Mainly n-7 isomers; [2] Mainly n-9 isomers.

- Macroalgae antioxidant potential and oxidative status of fish

TAC of macroalgal wracks

Total antioxidant capacity of MU, MOD and MOL wracks is displayed in Table 4.2.B.15. Every extract from MU wrack showed low activity for DPPH (<40%). On the contrary, MOL-ethanol and MOD-ethyl acetate extracts scavenged the DPPH radical by more than 50% at 250 µg mL^{-1}. The IC_{50} values were calculated for these two samples. The most active extract (MOD-ethyl acetate) was near 20-fold less active than Trolox (IC_{50} values of 147.54 µg mL^{-1} *vs.* 7.43 µg mL^{-1}).

Excluding MU-ethanol, all extracts exceeded 50% activity after the ABTS assay. Overall, MOL wrack comprised the more active extracts, followed by MOD and MU extracts. Thus, MOL-ethyl acetate was the most active of all extracts, showing an IC_{50} of 17.42 ± 1.13 µg mL^{-1}, 20-fold higher than Trolox (0.87 µg mL^{-1}).

Table 4.2.B.15. Extraction yield (g 100 g^{-1} dried algae), **antioxidant activity** (%) and **IC_{50}** (µg mL^{-1}) of the multispecific and monospecific macroalgal **wracks** used as feed supplement for ***Sparus aurata*** juveniles.

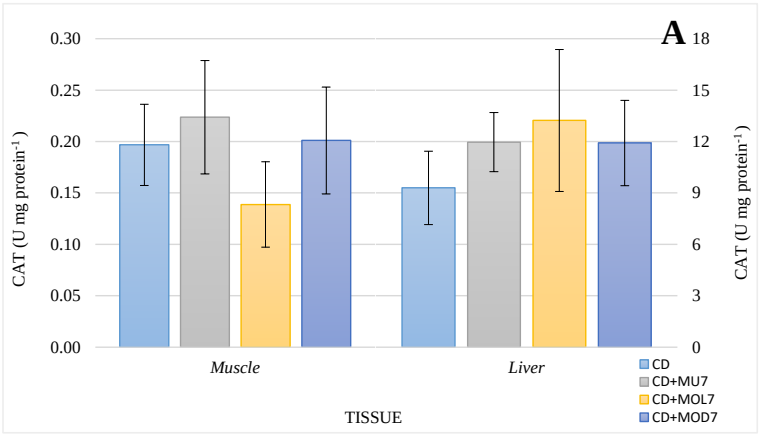
			DPPH		ABTS	
		Extraction yield	Activity	IC_{50}	Activity	IC_{50}
Multispecific macroalgal wrack	*n*-hexane	1.31	19.42 ± 1.27 ^{b,y}	>250	74.80 ± 3.40 ^{b}	77.85 ± 2.63 ^{b,y}
	Ethyl acetate	0.56	42.04 ± 2.26 ^{c,y}	>250	73.47 ± 1.24 ^{b,y}	70.40 ± 2.59 ^{a,z}
	Ethanol	5.50	6.53 ± 4.88 ^{a,x}	>250	31.21 ± 1.80 ^{a,y}	>250
Monospecific macroalgal wrack (*Lobophora* sp.)	*n*-hexane	2.48	15.80 ± 10.56 ^{a,y}	>250	68.48 ± 4.57 ^{a}	132.96 ± 2.85 ^{c,z}
	Ethyl acetate	1.85	38.99 ± 3.99 ^{b,y}	>250	79.97 ± 1.94 ^{b,z}	17.42 ± 1.13 ^{a,x}
	Ethanol	2.01	54.09 ± 0.86 ^{c,z}	207.47 ± 7.31	82.40 ± 1.53 ^{b,z}	40.99 ± 2.56 ^{b,y}
Monospecific macroalgal wrack (*Dictyota* sp.)	*n*-hexane	2.35	30.94 ± 2.62 ^{a,z}	>250	74.11 ± 3.68 ^{a}	74.17 ± 3.20 ^{b,y}
	Ethyl acetate	1.35	69.65 ± 3.70 ^{b,z}	147.54 ± 8.75	74.26 ± 4.13 ^{a,y}	31.67 ± 0.86 ^{a,y}
	Ethanol	6.53	32.06 ± 1.61 ^{a,y}	>250	81.31 ± 1.17 ^{b,z}	80.90 ± 3.71 ^{c,x}
Trolox			92.21 ± 0.32	7.43 ± 0.74	83.25 ± 1.28	0.87 ± 0.18

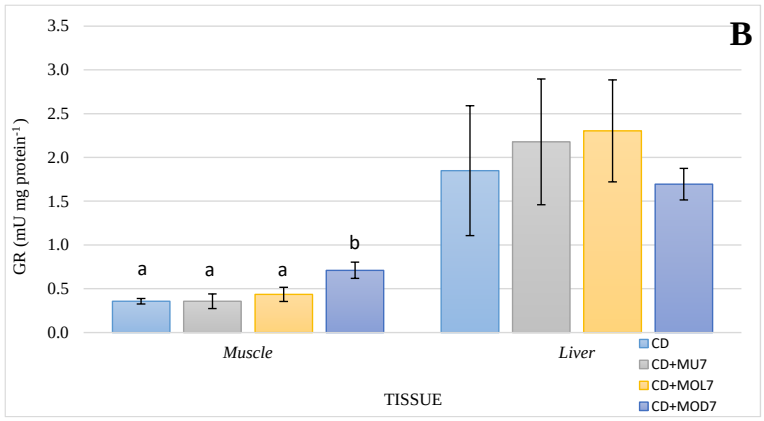
Results are presented as means ± SD. All determinations were carried out in quadruplicate. DPPH, 1,1-diphenyl-2-picryl-hydrazyl; ABTS, 2,20-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); IC_{50}, Concentration yielding 50% scavenging of each radical. ^{a,b,c} Represent significant differences between solvents within the same wrack ($p<0.05$). ^{x,y,z} Represent significant differences between wracks for the same solvent ($p<0.05$). Activity (%) was measured at 250 µg mL^{-1} for macroalgal extracts and at 100 µg mL^{-1} for Trolox standard.

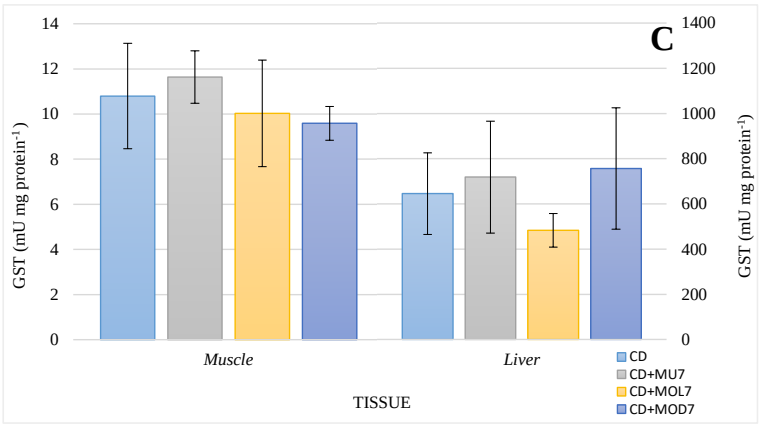
Fish antioxidant enzymes, PxI and TBARS

All antioxidant activities were higher in the liver than in the muscle of gilthead seabream juveniles (Figure 4.2.B.6). Thus, CAT varied from 0.14 to 0.22 U mg protein^{-1} in muscle to 9-13 U mg protein^{-1} in the liver, SOD activity was 75-106 U mg protein^{-1} and 300-400 U mg protein^{-1} in muscle and liver, respectively, and GST ranged between 10-12 mU mg protein^{-1} in muscle and 484-757 mU mg protein^{-1} in the liver. However, there were no significant variations between fish groups for CAT, SOD and GST in any tissue analysed. By contrast, GR activity was highest in muscle of CD+MOD7-fish (0.71 ± 0.09 *vs*. ~0.40 mU mg protein^{-1} in the other experimental groups), while it did not vary in the liver (~2 mU mg protein^{-1}).

The oxidative status of *S. aurata* juveniles was assessed through PxI and TBARS determinations. Both indexes remained unchanged regardless of the diet. PxI comprised 3-4 meqO_2 Kg lipid^{-1} in muscle, and TBARS ~0.2 nmol MDA mg protein^{-1} in muscle, and 0.3-0.4 nmol MDA mg protein^{-1} in the liver (Figure 4.2.B.6).







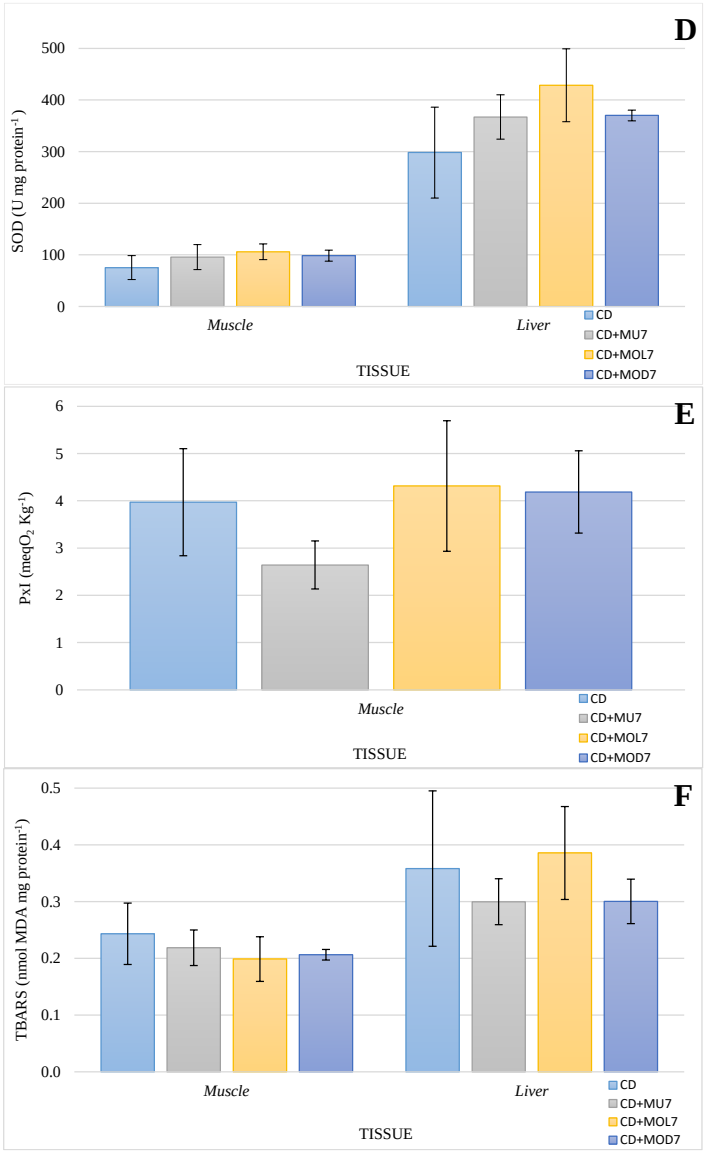


Figure 4.2.B.6. Antioxidant enzyme activities, peroxide index and TBARS of tissues from ***Sparus aurata*** juveniles fed the dietary treatments.
A, catalase (CAT; U mg protein^{-1}); B, glutathione reductase (GR; mU mg protein^{-1}); C, glutathione S-transferase (GST; mU mg protein^{-1}); D, superoxide dismutase (SOD; U mg protein^{-1}); E, peroxide index (PxI; meqO_2 Kg^{-1}) and F, TBARS (nmol MDA mg protein^{-1}). All assays were determined in muscle and liver excluding PxI (muscle). CD, Control diet; CD+MU7, Control diet supplemented with 7% multispecific macroalgal wrack; CD+MOL7, Control diet supplemented with 7% monospecific macroalgal wrack of *Lobophora* sp.; CD+MOD7, Control diet supplemented with 7% monospecific macroalgal wrack of *Dictyota* sp. Results are presented as means ± SD (n=5). Different letters represent significant differences between dietary treatments ($p<0.05$).

- Digestive enzymes

Table 4.2.B.16 displays the activity of digestive enzymes in the gastrointestinal tract of gilthead seabream juveniles. Pepsin activity was highest in CD+MOL7-fish (21.25 ± 11.49 mU mg protein^{-1}), followed by that of CD+MOD7 (15.81 ± 6.12 mU mg protein^{-1}), CD+MU7 (6.19 ± 3.49 mU mg protein^{-1}) and CD-fish (4.14 ± 1.34 mU mg protein^{-1}). Alkaline proteases and BAL also varied between dietary groups. Thus, alkaline proteases showed the lowest activity when fish were fed CD+MU7 and CD+MOL7 diets (12.85 ± 5.81 and 14.07 ± 6.71 mU mg protein^{-1}, respectively), whereas BAL activity was maximal in CD+MU7 and CD+MOD7-fish (7.23 ± 1.34 and 7.11 ± 2.08 mU mg protein^{-1}, respectively). Finally, CD and CD+MU7-fish had the highest alpha-amylase values in the intestine (0.30-0.31 ± 0.10 U mg protein^{-1}, respectively), followed by CD+MOL7 (0.27 ± 0.11 U mg protein^{-1}) and CD+MOD7 (0.13 ± 0.06 U mg protein^{-1}).

Table 4.2.B.16. Digestive enzymes (pepsin, alkaline proteases, bile salt-activated lipase and α-amylase) determined in the gastrointestinal tract of ***Sparus aurata*** juveniles fed the dietary treatments.

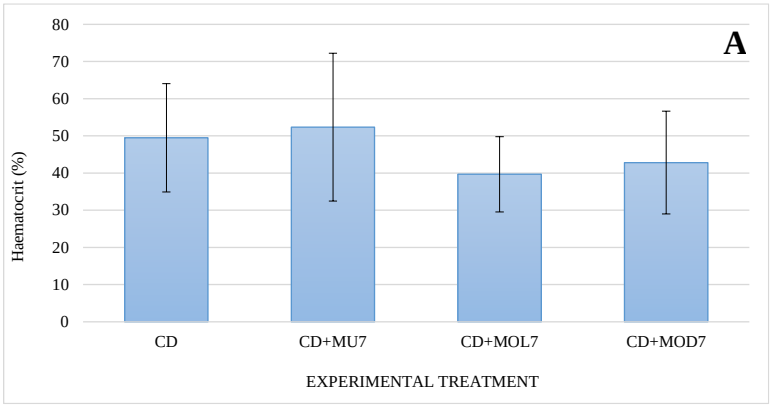
	CD	CD+MU7	CD+MOL7	CD+MOD7
Pepsin (mU mg protein$^{-1)}$	4.14 ± 1.34^{a}	6.19 ± 3.49^{ab}	21.25 ± 11.49^{c}	15.81 ± 6.12^{bc}
Alkaline proteases (mU mg protein$^{-1)}$	47.52 ± 11.83^{b}	12.85 ± 5.81^{a}	14.07 ± 6.71^{a}	68.50 ± 41.35^{ab}
Bile salt-activated lipase (mU mg protein$^{-1)}$	6.33 ± 1.33^{ab}	7.23 ± 1.34^{b}	4.61 ± 0.75^{a}	7.11 ± 2.08^{ab}
Alpha-amylase (U mg protein$^{-1)}$	0.30 ± 0.08^{b}	0.31 ± 0.10^{b}	0.27 ± 0.11^{ab}	0.13 ± 0.06^{a}

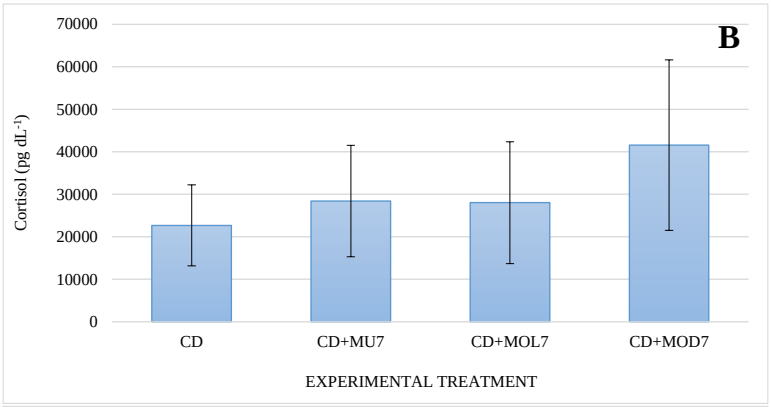
Results are presented as means ± SD (n=5). Different letters in superscript within each row denote significant differences ($p<0.05$). CD, Control diet; CD+MU7, Control diet supplemented with 7% multispecific macroalgal wrack; CD+MOL7, Control diet supplemented with 7% monospecific macroalgal wrack of *Lobophora* sp.; CD+MOD7, Control diet supplemented with 7% monospecific macroalgal wrack of *Dictyota* sp. Pepsin was determined in the stomach while alkaline proteases, bile salt-activated lipase and alpha-amylase were determined in the instestine.

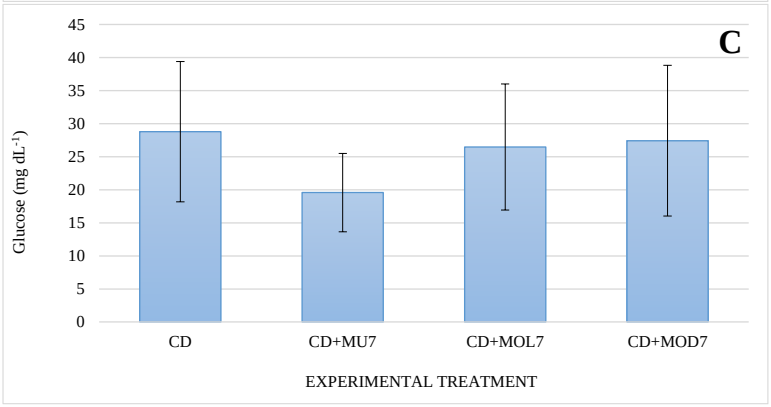
- Plasma parameters

At the end of the experimental period, plasma parameters were determined in blood from *S. aurata* (Figure 4.2.B.7). Any of these parameters significantly varied between dietary treatments. Thus, hematocrit fraction ranged between 39.67 ± 10.12% and 52.33 ± 19.86%, and cortisol between 22,666.97 ± 9,532.45 pg dL^{-1} in CD-fish and 41,577.50 ± 20,073.36 pg dL^{-1} in CD+MOD7-fish.

Similarly, glucose (20-29 mg dL^{-1}), lactate (32-34 mg dL^{-1}), sodium (460-492 mg dL^{-1}), and chlorure (1446-1780 mg dL^{-1}) did not vary between fish groups.







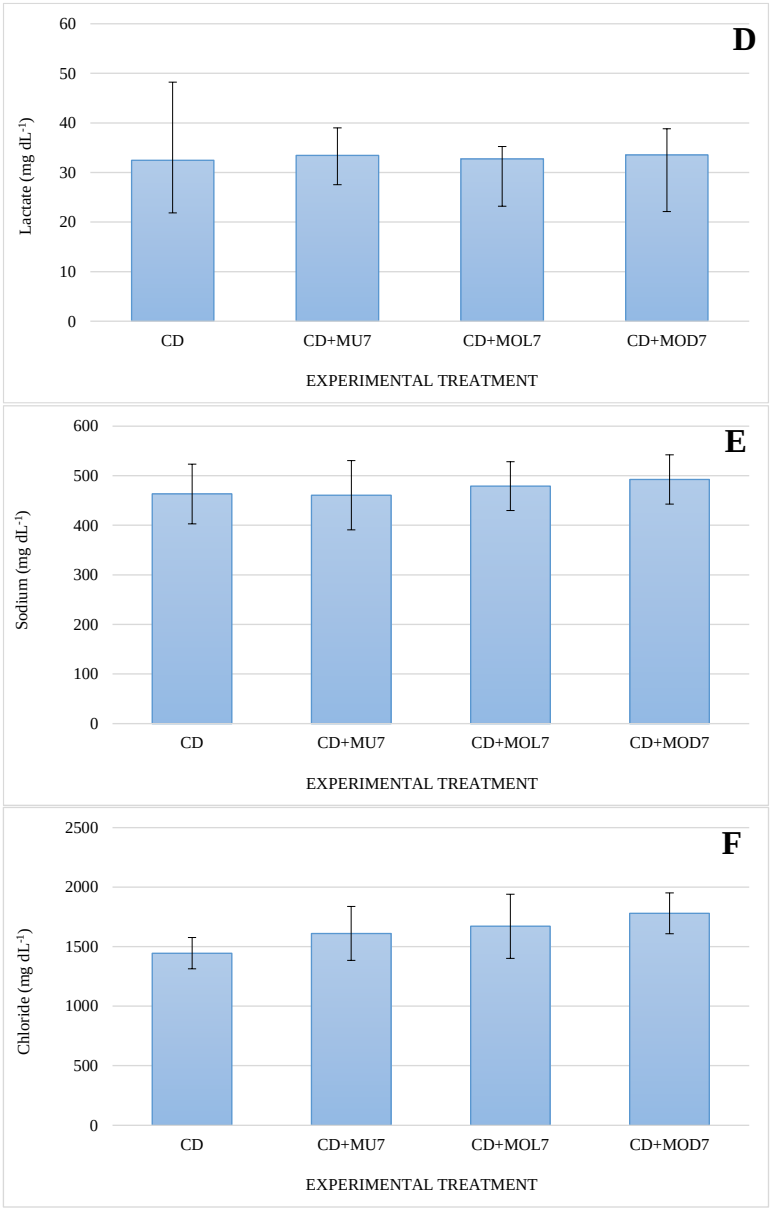


Figure 4.2.B.7. Plasma parameters from ***Sparus aurata*** juveniles fed the dietary treatments.
A, hematocrit (%); B, cortisol (pg dL^{-1}); C, glucose (mg dL^{-1}); D, lactate (mg dL^{-1}); E, sodium (mg dL^{-1}) and F, chloride (mg dL^{-1}). CD, Control diet; CD+MU7, Control diet supplemented with 7% multispecific macroalgal wrack; CD+MOL7, Control diet supplemented with 7% monospecific macroalgal wrack of *Lobophora* sp.; CD+MOD7, Control diet supplemented with 7% monospecific macroalgal wrack of *Dictyota* sp. Results are presented as means ± SD (n=5).

B.4. Discussion

Marine macroalgae have been proposed as a valuable alternatives to terrestrial plants in aquafeed formulation, not only for their potential as protein-nutritive sources, but also because of their content of bioactive-compounds that make them potential candidates to promote fish health and welfare. However, most investigations to date have been focused on a reduced number of carnivorous fish species where *Ulva* (Chlorophyta), *Gracilaria* and *Porphyra* (Rhodophyta) genus are the most studied seaweeds due to their high availability and commercial value. By contrast, dietary inclusion of brown macroalgae has been little studied (Abdala-Díaz et al., 2021; Güroy et al., 2013; Sáez et al., 2020; Sotoudeh and Mardani, 2018; Vizcaíno et al., 2019; Wan et al., 2019).

The inclusion of seaweed and microalgae in fish diets has been shown to improve some growth performance parameters (Wan et al., 2019). Thus, weight increment of *S. aurata* and *D. labrax* was enhanced with a 5 or 10% dietary inclusion of *Ulva* sp. or *P. capillacea* (Wassef et al., 2005, 2013). Moreover, Chen et al. (2021) demonstrated that a low inclusion (5%) of *Chlorella sorokiniana* improved *O. mykiss* growth, while a 10% inclusion did not exert any effect on fish perfromance. Neither *U. lactuca* (2.6% and 7.8%) nor *U. rigida* (4%) caused significant differences in *S. aurata* growth (Emre et al., 2013; Shpigel et al., 2017). By contrast, a 10% inclusion of *U. lactuca* or *Enteromorpha linza* in *O. mykiss* juveniles, and a 5% of *Chondrus crispus*, or 5% of a mix of *U. lactuca* and *C. crispus* resulted in lower growth rates compared to the control diet in *S. aurata* (Guerreiro et al., 2019; Yildirim et al., 2009). Finally, 15% of *U. rigida* and 25% of *Gracilaria cornea* reduced SGR in *S. aurata* (Vizcaíno et al., 2016). These contradictory results demonstrated that dietary seaweed supplementation is dependent on fish, seaweed species and dose (Guerreiro et al., 2019; Norambuena et al., 2015). In fact, the digestibility of macroalgae species included in fish diets may change depending on their level and type of complex polysaccharides that can act as barriers and chelators, hampering their digestion. Nonetheless, herbivorous and omnivorous fish such as carps have been shown to better digest dietary seaweeds (Wan et al., 2019), due to a better capacity to digest cell walls (Tocher et al., 2019). In our study, all macroalgal wracks included at 7% in feed for *C. idella* and *S. aurata* juveniles produced a similar growth performance as the commercial CD (Tables 4.2.B.7 and 4.2.B.12, respectively). The utilization of seaweeds as an ingredient *per se*, at an industrial scale, requires expensive processes that makes it often not economically viable. Thereby, in the last years, the

interest for macroalgae has emphasized on a quality more than on a quantity approach, based on their content in bioactive compounds. Hence, the inclusion of macroalgae in aquafeeds is now more focused on low inclusion levels, than on their use as main ingredients (Sáez et al., 2020).

O. mykiss reduced VSI and HSI indexes with the dietary inclusion of >25% of the microalgae *A. platensis* (Teimouri et al., 2016), while *S. aurata* decreased VSI when fed with a 4% of *U. rigida* (Emre et al., 2013). In our study, the reduction of VSI and VFI with the 7% inclusion of MU in *C. idella* (Table 4.2.B.7) could be attributed to the presence of the carotenoid fucoxanthin in brown algae such as *Lobophora* sp. and *Dictyota* sp., which were part of the MU wrack. Fucoxanthin has been described to present anti-obesity and lipolytic effects (Muradian et al., 2015). The possible synergetic effect between macroalgal species in the MU wrack could be favoring a stronger lipolytic action than *Lobophora* sp. alone (MO wrack). Furthermore, it seems that bioavailability (solubility and adsorption) of fucoxanthin in humans may be affected by the co-presence of certain lipids (Peng et al., 2011). Hence, the better performance of MU over MO may be also related to some favorable combination of lipids in the multispecific wrack. A reduction in VSI may be an economic advantage for fish production as viscera is often discarded, so lower VSI decreases the volume of by-product produced. Additionally, reduced HSI suggests no negative impact of seaweed inclusion on fish health (Teimouri et al., 2016). According to data obtained by Guerreiro et al. (2019), a 5% inclusion of *U. lactuca*, *C. crispus*, or a mix of both, did not vary body indexes in *S. aurata*, in spite of expecting the same lipolytic effect. However, a 10% of *U. ohnoi* (Martínez-Antequera et al., 2021), and 5, 15 and 25% of *G. cornea* and *U. rigida* (Vizcaíno et al., 2016) in feed for *S. aurata* decreased fish HSI. Overall, a more efficient dietary lipid utilization, reducing the accumulation of body lipids has been described in specimens fed on seaweeds (Vizcaíno et al., 2016). Further investigations focused on using higher inclusion percentages of macroalgal wracks in aquafeeds for *C. idella* and *S. aurata* juveniles should be performed in order to establish a better-defined relation between seaweed species, inclusion rates, and reduction of fish fat deposition.

Dietary inclusion of macroalgae has been described to vary proximate composition of fish muscle. Thus, the high protein content of macroalgae meals, has been related to higher muscle protein contents, whereas fish fed diets supplemented with *Ulva* sp. reduced their lipid accumulation in muscle (Sáez et al., 2020). In our study, the absence of differences

between treatments in the proximate composition of both *C. idella* and *S. aurata* muscle could be probably associated with the use of isoproteic and isolipidic diets, and to the low level of macroalgae inclusion. Furthermore, the similar proximate composition, together with the absence of detrimental effects on growth, suggest that the composition of the experimental diets was sufficient to meet the nutritional needs of *C. idella* and *S. aurata* juveniles at the same level as the CD.

Both qualitative and quantitative effects of macroalgae meal on lipid metabolism have been previously reported, including an adverse impact on EPA and DHA levels with seaweed inclusion (Guerreiro et al., 2019). Dietary FA profiles are generally reflected in fish muscle FA composition (Sáez et al., 2020), in accordance to our experiment. Furthermore, due to the low lipid content of the beach cast algae used, and the FA similarities between diets, no significant changes were expected in muscle lipid profile. Nonetheless, MU inclusion seems to slightly enhance a selective retention of n-6 PUFA in muscle from *C. idella* (Table 4.2.B.9), according to the effect described for *N. oculata* in *S. aurata* by Sáez et al. (2022). Similarly, n-3 PUFA was reported to be selectively retained in muscle when *S. senegalensis* were fed diets supplemented with a 5% of *Ulva ohnoi* (Sáez et al., 2020). Here, SFA was increased in muscle from *S. aurata* when fed CD+MOL7 and CD+MOD7 diets (Table 4.2.B.14), as it has been previously described for the same species fed a diet supplemented with 5% of *C. crispus*, or 5% of a mix of *U. lactuca* and *C. crispus* (Guerreiro et al., 2019). SFA also increased in giant grouper when *Schizochytrium limacinum* was included in its diet, due to a higher SFA content in the experimental diets (García-Ortega et al., 2016). However, there were no significant differences in the dietary FA composition of diets in our experiments. Peixoto et al. (2016a) reported that inclusion of *Ulva* sp. reduced lipase activity responsible for TAG hydrolysis in *D. labrax*. As TAG are usually rich in SFA, a reduction in lipase activity induced by a commercial blend of seaweeds have been proposed to reduce SFA is muscle (Bruni et al., 2020). In our study, the higher SFA proportions in muscle from *S. aurata* juveniles could be caused by an increase in the pancreatic lipase activity (not determined in our study), which has TAG as its preferred substrate (Soumanou et al., 2013).

Studies regarding the effect of seaweed on fish LC profile are scarce. Overall, *C. idella* muscle LC profile was not affected by the dietary treatment, except SE, which was more abundant in both groups receiving macroalgae (Table 4.2.B.8). SE are formed from sterol in a normal homeostatic process that can be enhanced after an excess of sterol ingestion.

They are also a storage form of FA (Korber et al., 2017). Marine macroalgae are rich in PTS (Galindo et al., 2022), including several molecules such as fucosterol, stigmasterol, sitosterol and saringosterol, together with variable amounts of CHO (Schepers et al., 2020). The higher contribution of PTS in both diets supplemented with macroalgae, may be enhancing fish SE synthesis/deposition. It is important to stress that PTS are known to lower total and LDL cholesterol levels in humans (Ibañez and Cifuentes, 2013).

Mustafa et al. (1995) described an increase in TAG and phospholipids in the muscle of sea bream fed a 5% of *Ascophyllum nodosum*, *Porphyra yezoensis* and *Ulva pertusa*. However, LC in muscle from *S. aurata* were similar between dietary groups, excluding MAG, which showed the highest values in MU and MOD treatments (Table 4.2.B.13). Unfortunately, we were not able to separate MAG and DAG in the analysis performed over beach cast algae. However, the fraction MAG+DAG was slightly higher in MU and MOD wracks than in the MOL one. Higher contribution of MAG in these two treatments could be leading to higher MAG proportions in *S. aurata* muscle. However, an increase in MAG could be also due to an increase of BAL activity. Both BAL activity and MAG percentages patterns are well correlated, with both of them showing the lowest values in CD+MOL7. Although fish BAL acts towards TAG producing glycerol and FFA as final products, MAG is also produced in the process (Bogevik et al., 2008; Davis and Hardy, 2022). However, in this case, we should also expect an increase in FFA, which was not observed in muscle from *S. aurata*.

Lower HSI in response to *Ulva* inclusion has been related to changes in hepatic lipid metabolism that determined a lower deposit or higher mobilization of certain lipid classes (Martínez-Antequera et al., 2021). Thus, the general absence of significant differences in LC composition of muscle from *C. idella* and *S. aurata* matches with the similar HSI obtained in both species regardless of the treatment. The existence of bioactive compounds in algae capable of influencing lipid metabolism have been described, although the underpinning mechanisms responsible of those effects have not yet been fully elucidated; not even the nature of those substances (Norambuena et al., 2015; Sáez et al., 2022). Hence, the potential effects of macroalgae on fish lipid metabolism deserves future attention (Bruni et al., 2020; Vizcaíno et al., 2016).

The total antioxidant capacity of the beach casts macroalgae used in our work showed promising results. Contrarily to DPPH assays, all extracts, except MU-ethanol in both

experiments, were capable of inhibiting more than 50% of the radicals at 250 µg mL^{-1} in the ABTS assay. The greater sensitivity of the ABTS method compared to that of DPPH would explain these results (Zárate et al., 2020). All extracts from the *Lobophora* sp. wrack (*C. idella* experiment), had the highest antioxidant activities (Table 4.2.B.10). On the other hand, the highest DPPH activities of wracks used in the *S. aurata* experiment were detected in MOL-ethanol and MOD-ethyl acetate, while MOL-ethyl acetate highlighted by its IC_{50}-ABTS values (Table 4.2.B.15). Ito et al. (2018) suggested that the content of polyphenols and tocopherols might be the cause of the higher DPPH activity in *Ochrophyta* species compared to species from other phyla. More specifically, Zárate et al. (2020), described an ethanol extract from a macroalgal wrack formed by 95% *Lobophora* sp. from Gran Canaria Island as a good antioxidant product; although they obtained better results than in our study (IC_{50}-ABTS 6.21 ± 1.03 *vs*. 43.87 ± 1.36 µg mL^{-1}; Table 4.2.B.10). Furthermore, fucoxanthin is a typical pigment from some *Lobophora* species, in particular *Lobophora variegata* (Hegazi, 2002). Several beneficial properties have been attributed to fucoxanthin, including the free radical scavenging and single oxygen species quenching, resulting in antioxidant capacities (Nunes et al., 2019b). Besides fucoxanthin, antioxidant molecules present in brown algae comprise other pigments, phenolic compounds, sulphated polysaccharides and sterols (Zárate et al., 2020). A significant correlation between the phenolic content and the antioxidant activity in seaweed extracts has been proposed (Demirel et al., 2009). Specifically, brown algae contain a special group of phenolics called phlorotannins, which are involved in protection against oxidative damage. Their antioxidant activity is 10 to 100 times more powerful than that of other polyphenols and thus, brown algae posses higher antioxidant activity than green and red algae (Generalić Mekinić et al., 2021). These authors showed a good antioxidant potential of *D. dichotoma*, with the best results obtained in the ethanolic extract (compared to water extract), attributing this activity to flavonoids and tannins. The antioxidant activity of macroalgal wracks is essential since it can potentially contribute to the protection of proteins and lipids, among others, in fish (Zárate et al., 2020). Nonetheless, results from *in vitro* studies assessing macroalgae antioxidant capacity can be hard to extrapolate to complex living organisms (Rosa et al., 2020).

Understanding the effects of seaweed supplementation in fish is particularly important in aquaculture because farming protocols often induce stress conditions that affect the immune system responses, including an increase in ROS production that might

compromise growth performance and animal welfare (Peixoto et al., 2016b). It is well known that seaweed contain bioactive compounds with great antioxidant activity that may mitigate stress responses in fish (Peixoto et al., 2019). The antioxidant capacity of macroalgal wracks studied here, affected antitoxidant activities in fish, in particular, CAT in *C. idella* liver (Figure 4.2.B.5) and GR in *S. aurata* muscle (Figure 4.2.B.6). Thus, CAT activity was decreased in the liver of *C. idella* juveniles fed macroalgae. This depletion has been pointed out as an indicator of a reduced requirement to remove hydrogen peroxide and lipid peroxides from tissues (Sotoudeh and Mardani, 2018). On the other hand, GR catalyzes the reduction of GSSG to GSH, representing the antioxidant restoration potential. Thus, the increment of GR activity in muscle of *S. aurata* receiving MOD supplementation suggests a higher capacity to mold the glutathione metabolism state (Peixoto et al., 2016b). Nevertheless, seaweed supplementation increased lipid peroxidation in the liver of *S. aurata* fed with 5% of *U. lactuca*, 5% of *C. crispus* or a mix of both (2.5% each) (Guerreiro et al., 2019), or up to 5% *Gracilaria gracilis* extract and powder (Passos et al., 2021). Similar results were obtained in *D. labrax* fed 7.5% *Gracilaria* spp. or a mix of *Gracilaria* spp., *Ulva* spp. and *Fucus* spp. (2.5% each) (Peixoto et al., 2016b). This was attributed to an increase in cellular lipid layer degradation that causes a rise in oxidative stress (Guerreiro et al., 2019). In our study, and in spite of the changes in CAT and GR activities, the global oxidative status of muscle or liver did not differ between dietary groups. Although no significantly, TBARS tended to decrease in muscle from CD+7MU-grass carp. Chen et al. (2021) reported that a 10% inclusion of *C. sorokiniana* significantly decreased MDA content in both kidney and liver of *O. mykiss*, while a 5% of inclusion did not have any effect. The absence of significant differences in our study may be related to the herbivorous condition of *C. idella* entailing for a greater tolerance to seaweed inclusion, although the reduced percentage of dietary inclusion cannot be completely ruled out. Furthermore, commercial diets used as base for the experimental diets have antioxidants such as BHT or butylhydroxyanisole (BHA) in their composition. The antioxidant effect of macroalgae inclusion would be probably more evident if commercial diets have not contained those antioxidants.

Feed composition, and, in particular, the dietary inclusion of algae have demonstrated to influence the activity of enzymes involved in digestive and absorptive processes in several fish species (Sáez et al., 2022; Sotoudeh and Mardani, 2018; Vizcaíno et al., 2019). Pepsin activity was only determined in *S. aurata* juveniles as *C. idella* lack

stomach (Gioda et al., 2017). It has been suggested that dietary fibre from seaweed would inhibit pepsin activity, and consequently, also fish growth performance, as in carnivorous fish, the initial stages of digestion occur in stomach (Xuan et al., 2013). However, this effect has been reported with high levels (20%) of *Gracilaria lemaneiformis* inclusion in black sea bream (Xuan et al., 2013), and *Sargassum horneri*, *U. lactuca* and *G. lemaneiformis* in white-spotted snapper (Zhu et al., 2016, 2017), but not with 5, 10 and 15% macroalgae addition. In our study, pepsin activity was not inhibited by 7% of algal inclusion, being even relatively enhanced in CD+MOL7 and CD+MOD7-fish, suggesting some positive effects of these two macroalgal species. On the other hand, the inclusion of *Gracilaria pygmaea* decreased protease activity in *O. mykiss* (Sotoudeh and Mardani, 2018), whereas *G. cornea* and *U. rigida* reduced that of *S. aurata* (Vizcaíno et al., 2016). This same effect was detected in the intestine of *S. aurata* with CD+MU7 and CD+MOL7 dietary treatments, but not for *C. idella* juveniles, suggesting some compensatory mechanism in this species.

Contrary to what was reported by Sotoudeh and Mardani (2018), BAL activity did not increase with macroalgae inclusion in *C. idella* compared to the control. However, slight changes in BAL activity between beach casts were seen in *S. aurata* (Table 4.2.B.16), with MOL wrack showing the lowest activity, and MU wrack the highest one. Although we did not observe significant differences in *S. aurata* fat deposition, the lipolytic activity of the mulstispecific wrack described for the *C. idella* experiment, might be enhancing BAL activity in *S. aurata*. As explained before, BAL is known to hydrolyze TAG, producing MAG in the process (Bogevik et al., 2008; Davis and Hardy, 2022). The higher BAL activity in CD+MU7-juveniles is in accordance to higher values of MAG in this fish group. On the other hand, lower lipase activity between fish fed diets with seaweed supplement has been previously reported in *D. labrax* fed up to 7.5% of *Ulva* spp. supplementation, which was compensated when *Ulva* spp. was included in a mix diet together with *Gracilaria* spp. and *Fucus* spp. (Peixoto et al., 2016a). Variation in the nutritive value of different macroalgae species and their physiological characteristics may cause an increase on non-starch polysaccharides, antinutritional factors and fibre, which Zhu et al. (2016) hypothesized to cause lower lipase activities in fish fed diets with seaweed inclusion. Nonetheless, the lipid source and the level of dietary inclusion have been the most reported modulators of lipase activity (Peixoto et al., 2016a). Thus, differences in lipase activity are believed to be related to a broader preference for PUFA

as substrates (Sotoudeh and Mardani, 2018). In this context, the similar dietary PUFA contribution of our experimental diets might also be responsible for the absence of detectable changes in the lipase enzyme activity of the treatments with algal inclusion compared with CD in both *C. idella* and *S. aurata* juveniles. However, more studies are still needed to better understand the effect of algae inclusion in the lipid metabolism of fish (Vizcaíno et al., 2016).

C. idella is an herbivorous species that have high amylase activities and thus proper digestibility of algal products (Gioda et al., 2017; Norambuena et al., 2015), which could enable higher inclusion levels in feeds (Tocher et al., 2019). This could explain the absence of differences in alpha-amylase activity in this species (Table 4.2.B.11). On the contrary, *S. aurata* showed the lowest alpha-amylase activity with MOD inclusion (Table 4.2.B.16). This species is considered a carnivorous fish, and although it is capable of adapt its diet to other food items such as seaweeds (Guerreiro et al., 2019), carnivorous fish are believed to have a reduced ability to digest starch (Liang et al., 2022). Furthermore, macroalgae have a relatively high content of non-starch polysaccharides such as cellulose, xylans, agar, carrageenan, or alginate, which are antinutritional factors for monogastric animals like fish. This is one reason why >10% seaweed feed inclusion has been related to lower performance of fish. The addition of non-starch polysaccharide-degrading enzymes for seaweed feed supplementation to remove or breakdown complex carbohydrates have been proposed, showing an increase in growth in rabbitfish (Xie et al., 2019). In this sense, our results point out that a 7% of dietary inclusion of CD+MU7 and CD+MOL7 did not negatively affect starch digestibility in *S. aurata*.

In spite of the changes in the digestive capacity of *S. aurata* juveniles outlined above, both *S. aurata* and *C. idella* presented similar growth rates in all experimental groups, in line with other studies suggesting the capacity of both species to adapt to changes in dietary composition (Emre et al., 2013; Shpigel et al., 2017). It is worth mentioning that several studies reporting some beneficial effects of seaweed inclusion (either in growth, fat deposition, oxidative status or digestive capacities), used extruded diets (Chen et al., 2021; Martínez-Antequera et al., 2021; Peixoto et al., 2016a, 2016b) unlike our study. Seaweeds processing and dietary extrusion remove or break some of the complex carbohydrates present in seaweeds allowing fish to access them (Guerreiro et al., 2019). Furthermore, the impact of anti-nutritional factors in digestion and growth permormance has been proposed to be contrarrested by the addition of exogenous enzymes. Besides the

non-starch polysaccharide-degrading enzymes explained above, the most common are phytases, cellullases, hemicellullases, proteases, lipases, amylases and pepsin (Bharathi et al., 2019; Liang et al., 2022). In fact, cellullase enzymatic hydrolysis of *Nannochloropsis gaditana* showed significantly lower TBARS in the liver of *S. aurata* compared to raw microalgae (Sáez et al., 2022).

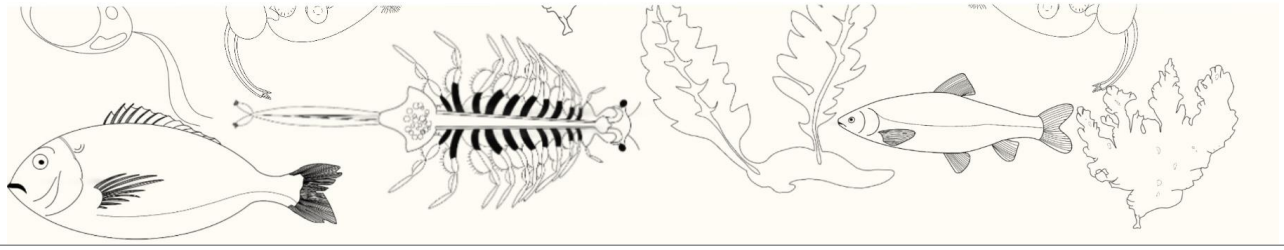
The hematological profile of a cultured fish may be an indicator of its physiological status and health. Plasma parameters, together with other routine diagnostic methods can be used to identify and assess conditions that cause stress and/or diseases that affect production performance (Fazio, 2019). Cortisol and hematocrit values obtained here were in agreement to those previously reported for *S. aurata* (Ashry et al., 2021; Díaz-López et al., 2009). Hematocrit levels are often elevated during stress in order to increase the oxygen supply to the major organs in response to a higher metabolic demand (Cnaani et al., 2004). Furthermore, hematocrit and haemoglobin are often regarded as diagnostic tools to assess the absence of anemic features (Ashry et al., 2021). Although we did not measure haemoglobin content, hematocrit levels were unaffected by seaweed supplementation. On the other hand, cortisol is usually measured as an acute stress indicator in plasma (Sadoul and Geffroy, 2019), while blood glucose and lactate levels are also frequently used as evaluators of stressors in fish. Thus, chronic stress, or repeated acute stress have been proposed to increase lactate and glucose levels (Cnaani et al., 2004; Fazio et al., 2015), as fish faced with repeated stress usually increase energy demand, leading to a higher glucose needs. Anaerobic activity of muscle also increases lactate demand (Fazio et al., 2015). Algae supplementation has been reported to decrease glucose levels in *S. aurata*, being attributed to a lower starch proportion or to a reduction in digestible carbohydrates in the algae (Guerreiro et al., 2019; Vizcaíno et al., 2016). Finally, decrease in the serum levels of potassium, sodium and chloride may indicate an impairment in ionic regulation after stress (McDonald and Milligan, 1997). In this sense, neither cortisol, glucose, lactate, sodium nor chlorure changed in either fish group. Interestingly, the plasma parameters analysed indicate that there is not apparent detriment in physiological status, health or wellbeing in fish fed diets with macroalgal wracks inclusion.

For all above, further studies are needed to test higher dietary inclusion percentages of seaweeds in aquafeeds. The addition of exogenous enzymes together with the macroalgal wracks could also potentially improve their digestibility and bioavailability of beneficial

compounds to fish, allowing to increase seaweed dietary inclusion rates in order to promote the applicability of the results obtained. However, upon being natural resources, macroalgal wracks identity and composition may change depending on seasonality, a limitation that must be taken into account. In our study, different macroalgal wracks were tested in two fish species with different feeding habits, leading to similar results. More studies using beach casts composed by other species would also allow to determine the potential of such ingredients for fish feeding.

In conclusion, the macroalgal wracks from Gran Canaria coasts used in the present work might be considered as a potential feed additive for fish. Thus, a 7% of macroalgae may be included in the diet without detrimental effects on *C. idella* and *S. aurata* survival, growth, proximate composition, FA and LC profile, and antioxidant status. In spite of some minor changes in the digestive capacity of *S. aurata*, it did not cause any apparent detriment in the physiology of the fish. Additionally, the MU wrack enhanced antioxidant activity of CAT in the liver of *C. idella*, leading to a lower and healthier perivisceral fat deposition. A potential capacity to better restore the antioxidant condition of the organism was also detected in muscle from *S. aurata* with MOD dietary supplementation. However, antioxidant activity would depend on the relative abundance of macroalgae species present in the collected wracks, and their conservation status. The utilization of macroalgal biomass originating from beach casts that is usually discharded, might contribute to the sustainable use of ocean resources empowering the blue economy strategy in islands.

Conclusions



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5. CONCLUSIONS

1. Fresh, frozen and spray-dried *I. galbana* and *N. salinicola* are suitable formats to feed *Artemia* without any detrimental effect on its survival rate. By contrast, the fresh format of *I. galbana* decreased rotifer survival, probably due to an excess of product rather than to a negative effect of the format itself.

2. The combination of microalgae and a lipid emulsion for 5 h is a good enrichment protocol for live preys with regard to their n-3 LC-PUFA contents and DHA/EPA ratios.

3. Among all dietary treatments, spray-dried *I. galbana* better enhanced DHA incorporation in both rotifer and *Artemia*, leading to DHA/EPA ratios over 2 in rotifers, highly favourable for marine larval nutrition. Spray-dried *I. galbana* also improved phospholipids retention in both species, suggesting a higher bioavailability of nutrients.

4. The spray-dried format of *I. galbana* partially compensated the increased oxidative stress caused by the addition of a lipid emulsion in the enrichment protocols.

5. Spray-dried *I. galbana* was the best microalgae format used for feeding live preys, due to its lipid profile and antioxidant capacity.

6. Macroalgae are highly variable in their lipid content, LC and FA profiles, making highly complex a definition of a characteristic pattern within each phylum.

7. Fat content of most macroalgae was scarce, with Ochrophyta usually showing the highest values. However, their low n-6/n-3 ratios make most algae analysed attractive from a nutritional point of view, and for the prevention of inflammatory, neurodegenerative and cardiovascular diseases.

8. Overall, *A. taxiformis* and *D. dichotoma* were particularly interesting because of their highest contents of EPA+DHA (41-42 mg per 100 g of macroalgae).

9. Green algae, together with the brown macroalgae *C. usneoides* and *S. vulgare* showed the most favorable values of cardiovascular health promoting nutritional indicators.

10. The high content of DGDG, MGDG and FTS in some of the seaweeds analysed make them a potential beneficial resource for human health due to their antitumor, antifungal and anti-hypercholesterolemic reported properties.

11. A 7% inclusion of multispecific and monospecific macroalgal wracks from Gran Canaria Island coasts could be used in diets for *C. idella* and *S. aurata* juveniles without any detrimental effect on survival, growth, muscle proximate composition, FA and LC profiles, and *S. aurata* plasma parameters.

12. The multispecific wrack caused the lowest fat deposition in *C. idella*, while both multispecific and monospecific beach casts reduced CAT activity in the liver of *C. idella*, indicating some protective effect of macroalgae over the antioxidant capacity of the fish. The monospecific *Dictyota* sp. wrack displayed a remarkably high capacity to mold the glutathione metabolism in *S. aurata* juveniles.

13. Dietary inclusion of macroalgal wracks did affect digestive capacity of *S. aurata* juveniles without impairing fish growth performance or health status, while that of *C. idella* remained unchanged probably because of its herbivorous condition.

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Annex

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7. ANNEX

Supplementary Table 1. Rotated component loadings of the first five principal components for lipid classes of Madeira Archipelago seaweed.

	Rotated component loadings					
	Component					
Lipid class	1	2	3	4	5	Communalities
PC	-0.236	-0.287	0.207	**0.581**	0.459	0.729
PS+PI	0.086	0.512	**-0.733**	0.180	-0.272	0.913
SQDG+PE	**0.903**	0.174	-0.194	0.124	-0.272	0.973
DGDG	**0.962**	-0.058	0.077	-0.050	0.139	0.956
MGDG	-0.080	0.200	0.288	**0.650**	0.542	0.846
P	-0.007	0.113	-0.061	-0.016	**0.911**	0.846
DAG	-0.135	0.004	0.011	**-0.903**	0.152	0.856
PTS	-0.111	**0.893**	-0.303	-0.004	-0.013	0.902
FFA	-0.458	-0.305	**0.623**	0.062	-0.446	0.894
TAG	-0.288	**-0.850**	-0.205	0.023	-0.312	0.944
SE	0.063	0.152	0.**788**	0.463	-0.044	0.865

Major loadings for each lipid class are given in bold.

Supplementary Table 2. Mean factor scores for each cluster of the dendrogram based on lipid classes data of Madeira Archipelago seaweed.

	CLUSTER 1	CLUSTER 2	CLUSTER 3	CLUSTER 4	CLUSTER 5	CLUSTER 6
PC1	0.10 ± 0.62	-0.72 ± 0.89	-0.88 ± 0.48	-0.32 ± 0.54	1.73 ± 0.36	1.76 ± 0.63
PC2	0.63 ± 0.52	0.16 ± 0.50	-1.80 ± 0.76	0.56 ± 0.14	-0.91 ± 0.10	-0.68 ± 0.35
PC3	-0.40 ± 0.96	1.17 ± 0.71	-0.85 ± 0.29	0.13 ± 0.78	1.23 ± 0.22	-0.05 ± 0.25
PC4	0.52 ± 0.41	1.18 ± 0.51	-0.15 ± 0.68	-1.56 ± 0.50	-0.14 ± 0.04	-0.51 ± 0.14
PC5	-0.31 ± 0.74	0.93 ± 0.82	-0.43 ± 0.35	0.20 ± 0.74	-1.61 ± 0.09	1.72 ± 0.30

Results represent mean ± SD. PC1, PC2, PC3, PC4 and PC5 were extracted through principal component analysis of the lipid classes.

Supplementary Table 3. Rotated component loadings of the first five principal components for fatty acids of Madeira Archipelago seaweed.

	Rotated component loadings					
	Component					
Fatty acid	1	2	3	4	5	Communalities
14:0	-0.101	-0.079	**0.817**	-0.233	-0.249	0.801
15:0	**0.743**	-0.432	0.149	0.006	0.311	0.858
16:0	0.064	0.000	0.021	**0.958**	0.004	0.922
17:0	**0.878**	-0.137	-0.034	0.196	-0.103	0.839
18:0	**0.718**	-0.198	-0.405	-0.061	-0.043	0.725
18:2n-6	0.108	-0.313	**-0.894**	-0.188	0.003	0.945
18:3n-3	-0.320	**0.820**	-0.177	0.290	0.062	0.895
18:4n-3	**-0.634**	-0.062	0.298	-0.504	0.273	0.823
20:3n-6	**-0.713**	0.055	0.028	0.155	0.548	0.835
20:4n-6	-0.234	**0.847**	0.114	-0.034	0.307	0.881
20:5n-3	0.012	**0.906**	0.330	-0.148	0.016	0.953
22:6n-3	0.082	-0.230	0.239	0.077	**-0.866**	0.874

Major loadings for each fatty acid are given in bold.

Supplementary Table 4. Mean factor scores for each cluster of the dendrogram based on fatty acids data of Madeira Archipelago seaweed.

	CLUSTER 1	CLUSTER 2	CLUSTER 3	CLUSTER 4	CLUSTER 5	CLUSTER 6
PC1	-0.86 ± 0.35	0.42 ± 0.14	-1.3 ± 0.11	1.11 ± 0.72	0.04 ± 0.52	0.63 ± 0.96
PC2	0.05 ± 0.34	3.23 ± 0.24	0.22 ± 0.01	-0.68 ± 0.11	-0.53 ± 0.30	-0.38 ± 0.43
PC3	-0.91 ± 0.61	0.37 ± 0.08	1.29 ± 0.05	-1.63 ± 0.48	0.55 ± 0.38	0.50 ± 0.70
PC4	0.53 ± 0.68	0.35 ± 0.04	-2.28 ± 0.09	-1.97 ± 0.38	0.13 ± 0.29	0.30 ± 0.45
PC5	0.38 ± 0.35	-0.30 ± 0.13	0.56 ± 0.09	0.09 ± 0.46	-2.03 ± 0.79	0.44 ± 0.63

Results represent mean ± SD. PC1, PC2, PC3, PC4 and PC5 were extracted through principal component analysis of the fatty acids.

Supplementary Table 5. Rotated component loadings of the first five principal components for lipid classes of Gran Canaria Island seaweed.

	Rotated component loadings					
	Component					
Lipid class	1	2	3	4	5	Communalities
PC	-0.191	0.389	**0.677**	-0.300	0.276	0.812
PS+PI	0.157	-0.075	-0.067	**0.919**	-0.013	0.880
SQDG+PE	0.257	**0.881**	0.280	0.131	-0.026	0.938
DGDG	-0.026	**0.850**	-0.195	-0.204	-0.129	0.819
MGDG	0.051	0.017	**0.912**	0.003	-0.160	0.861
P	**0.894**	-0.246	-0.031	0.075	-0.192	0.904
DAG	0.276	0.266	-0.563	**0.597**	-0.132	0.838
PTS	0.355	**-0.709**	-0.174	-0.242	-0.368	0.853
FFA	**-0.936**	-0.150	0.027	-0.102	0.131	0.927
TAG	-0.093	-0.028	-0.035	-0.040	**0.959**	0.932
SE	**-0.710**	-0.139	0.092	-0.434	-0.289	0.803

Major loadings for each lipid class are given in bold.

Supplementary Table 6. Mean factor scores for each cluster of the dendrogram based on lipid classes data of Gran Canaria Island seaweed.

	CLUSTER 1	CLUSTER 2	CLUSTER 3	CLUSTER 4	CLUSTER 5	CLUSTER 6
PC1	-0.40 ± 0.88	0.35 ± 0.44	1.04 ± 0.12	-2.40 ± 0.22	0.38 ± 0.75	0.11 ± 0.09
PC2	1.48 ± 0.88	-0.27 ± 0.59	-0.19 ± 0.57	-0.69 ± 0.26	-0.79 ± 0.22	-0.33 ± 0.29
PC3	-0.40 ± 0.81	-0.57 ± 0.45	2.42 ± 0.71	0.75 ± 0.41	-0.53 ± 0.36	-0.43 ± 0.80
PC4	-0.32 ± 0.55	0.93 ± 0.82	-0.39 ± 0.60	0.17 ± 0.44	-1.30 ± 0.13	0.37 ± 0.42
PC5	0.09 ± 0.16	-0.24 ± 0.75	-0.20 ± 0.45	-0.78 ± 0.24	-0.81 ± 0.65	2.47 ± 0.79

Results represent mean ± SD. PC1, PC2, PC3, PC4 and PC5 were extracted through principal component analysis of the lipid classes.

Supplementary Table 7. Rotated component loadings of the first five principal components for fatty acids of Madeira Archipelago seaweed.

	Rotated component loadings					
	Component					
Fatty acid	1	2	3	4	5	Communalities
14:0	-0.112	-0.100	**0.807**	-0.300	-0.220	0.811
16:0	**-0.841**	-0.331	0.391	0.090	0.003	0.977
16:1n-11	-0.012	0.117	-0.147	0.005	**0.938**	0.914
16:1n-7	-0.412	-0.219	**-0.554**	-0.027	-0.486	0.762
16:1n-5	-0.006	**0.950**	-0.029	-0.151	0.061	0.930
18:0	-0.249	-0.236	-0.028	**0.884**	-0.220	0.949
18:1n-9	0.457	**0.699**	0.190	0.445	-0.191	0.969
18:1n-7	-0.101	-0.327	**-0.826**	-0.329	0.013	0.909
18:1n-5	-0.076	**0.864**	0.141	-0.034	0.303	0.866
18:2n-6	**0.786**	-0.437	-0.279	0.145	0.082	0.916
18:3n-3	0.294	0.110	-0.009	**0.780**	0.423	0.886
18:4n-3	**0.883**	0.281	0.068	-0.016	0.224	0.914
20:4n-6	**0.800**	-0.013	0.421	0.395	0.059	0.976
20:5n-3	**0.787**	-0.219	0.297	-0.156	-0.156	0.804

Major loadings for each fatty acid are given in bold.

Supplementary Table 8. Mean factor scores for each cluster of the dendrogram based on fatty acids data of Madeira Archipelago seaweed.

	CLUSTER 1	CLUSTER 2	CLUSTER 3	CLUSTER 4	CLUSTER 5	CLUSTER 6
PC1	0.60 ± 0.11	1.73 ± 0.39	-1.05 ± 0.29	-0.47 ± 0.61	0.36 ± 0.40	0.30 ± 0.51
PC2	-0.65 ± 0.10	-0.70 ± 0.07	-0.47 ± 0.32	-0.24 ± 0.15	-0.03 ± 0.10	2.00 ± 0.84
PC3	-2.58 ± 0.94	0.88 ± 0.36	-0.75 ± 0.43	-0.30 ± 0.38	-0.39 ± 0.27	0.14 ± 0.48
PC4	-1.05 ± 0.41	-0.66 ± 0.21	-0.60 ± 0.23	1.12 ± 1.06	0.58 ± 0.53	-0.09 ± 0.74
PC5	0.07 ± 0.37	-0.34 ± 0.21	-0.42 ± 0.74	0.80 ± 0.62	-1.90 ± 0.79	0.38 ± 0.62

Results represent mean ± SD. PC1, PC2, PC3, PC4 and PC5 were extracted through principal component analysis of the fatty acids.

ARTICLE

Lipid characterization of 14 macroalgal species from Madeira Archipelago: implications for animal and human nutrition

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Abstract: The lipid and fatty acid profiles of 14 marine macroalgal species from the Madeira Archipelago, including two green (Ulvales and Dasycladales), three red (Corallinales, Bonnemaisoniales, and Ceramiales) and nine brown (Fucales, Dictyotales, and Sphacelariales) species were characterised in order to determine their potential use for animal and human nutrition. The total lipid content of species analysed was generally low, varying from 0.2 to 5.2% of dry weight. All species presented an omega 6/omega 3 (n-6/n-3) ratio lower than 10, as recommended by the World Health Organization for proper human health. Polyunsaturated fatty acids (PUFA), including linoleic acid and alpha-linolenic acid were exceptionally high in the green macroalga *Ulva* sp. Red macroalgae were rich in n-3 long-chain PUFA, particularly *Asparagopsis taxiformis*, which contained 6.6% of docosahexaenoic acid, and *Halopithys incurva* with 9.3% of eicosapentaenoic acid. Within Ochrophyta, *Dictyota dichotoma* is an interesting source of n-3 PUFA due to its high stearidonic acid proportion (8.0%). In addition, *H. incurva* contained a high proportion of both mono- and digalactosyldiacylglycerols. According to their lipid profiles, most macroalgae analysed might be considered of particular interest for their potential exploitation for human nutrition and livestock and aquaculture production.

Keywords: lipid profile; macroalgae; nutrition; polyunsaturated fatty acids.

1 Introduction

Marine macroalgae are fast-growing multicellular, photosynthetic organisms, classified into three major groups based on their pigmentation: green macroalgae (Chlorophyta), red macroalgae (Rhodophyta) and brown macroalgae (Ochrophyta). Seaweeds are traditionally consumed as food in Asia, mainly Japan, China and Korea (Roleda et al. 2018). However, their demand as food has also extended to occidental societies, mainly due to a change in consumer preferences, being increasingly recognized as a type of healthy "superfood" that

leads to the production of algal-derived innovative products (FAO 2018a). Marine macroalgae are rich in nutritional and bioactive compounds, including minerals such as iodine, fibre, vitamins, carbohydrates, proteins, lipids, chiefly polyunsaturated fatty acids (PUFA), phytosterols (PTS), and phenolic compounds. Seaweeds also possess high contents of pigments that exert antioxidant and anticancer activities (Chandini et al. 2008; Nunes et al. 2020; Stengel et al. 2011; Zárate et al. 2020). In addition, several other compounds of macroalgae are described to have potential medical applications, including antitumor, anticoagulant, antiviral, antiprotozoal, antifungal, and antibacterial properties (for details, see Barzkar et al. 2019).

Given the versatility of seaweeds for their application in industries, their global production is expected to increase in the coming decades. Seaweed aquaculture has been practiced for decades in Asian countries (Campbell et al. 2019), especially in China, the main world producer in both value and volume (FAO 2018a). Farmed macroalgae represent 96% of the total global supply (Harwood 2019), and this is almost exclusively used for direct human consumption (FAO 2018a, b). In Western countries, seaweeds are mainly exploited for the industrial production of phycocolloids such as alginate, agar-agar and carrageenan (Dellatorre et al. 2020), although its production by aquaculture has been recently established as a commercial activity (Campbell et al. 2019).

Seaweeds are a promising protein source, presenting a higher content of essential amino acids than vegetables (Fleurence 1999). In this regard, seaweed consumption is expected to increase due to the growing demand for protein sources that can overcome the anticipated challenges of a growing world population and food scarcity, and the demand for alternative proteins in Western countries. In recent years, lipid composition of macroalgae has also raised considerable interest due to their valuable content of omega-3 (n-3) PUFA and of a certain type of lipids. In general, marine macroalgae have low lipid levels (<5% of dry weight, DW) (Dellatorre et al. 2020; Schmid et al. 2018), and fluctuating fatty acid (FA) profiles, which vary greatly among taxa (Stengel et al. 2011). These variations have been attributed to several factors, including seasonal and geographical changes, environmental parameters, physiological status, and even molecular mechanisms in response to environmental factors (Verma et al. 2017).

Glycolipids and phospholipids are the major lipid classes in algae (Guihéneuf et al. 2015), together with triacylglycerols (TAG) (Harwood 2019). Glycolipids are mainly located in photosynthetic membranes playing a crucial role in maintaining optimal photosynthesis efficiency (Nakamura and Li-Beisson 2016), and are predominantly represented by monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG). Furthermore, major phospholipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (Guihéneuf et al. 2015), which are mostly localized in non-plastid membranes, except for the latter which is present in the chloroplast envelope (Nakamura and Li-Beisson 2016). MGDG, DGDG or phosphatidylglycerol have been described as anti-inflammatory and anti-thrombotic compounds, while PTS are known to lower total and low-density lipoprotein cholesterol levels in humans (Ibañez and Cifuentes 2013). Despite the low lipid level reported in macroalgae (Dellatorre et al. 2020; Schmid et al. 2018), their PUFA content is greater than that of terrestrial plants (Kendel et al. 2015). Within PUFA, the long-chain PUFA (LC-PUFA) are physiologically important molecules (Trushenski and Rombenso 2019) involved in cell membrane structure, transcription, regulation and cellular signalling (Lee et al. 2016; Zárate et al. 2017). Furthermore, a high dietary intake of n-3 LC-PUFA has been shown to prevent some human diseases, including colon and breast cancers, neurodegenerative or inflammatory illnesses, and even to reduce the prevalence of dementia (Harwood 2019; Lee et al. 2016; Zárate et al. 2017). Particularly, eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) have been demonstrated to reduce cardiovascular disease and arthritis, and to improve brain function (Harwood 2019). Consequently, global demand for n-3 FA has significantly increased over the last decades. Fish and other marine products are almost the only natural source of n-3 LC-PUFA for humans (Zárate et al. 2017). However, the source of these FA is generally not fish itself but marine phytoplankton and macroalgae, which form their major dietary source (Colombo et al. 2019). Algae possess not just the capacity to synthesize *de novo* alpha-linolenic acid (ALA; 18:3n-3) and linoleic acid (LA; 18:2n-6), but also LC-PUFA, whose content differs among taxa (Bourgougnon et al. 2011). To date, the feeding of aquatic captive-reared species has relied heavily on fishmeal and fish oils obtained from wild pelagic fish populations, whose stocks are currently either fully exploited or overexploited (FAO 2018b). Recently, various plant-based sources have been tested to replace marine ingredients due to their higher availability, sustainability and reduced cost. Nonetheless, terrestrial alternatives present low digestibility, contain some antinutritional factors, and are deficient in certain essential amino acids and n-3 LC-PUFA, resulting in a significant reduction of the nutritional quality of the edible product (Welker et al. 2016). By contrast, the inclusion of small amounts of macroalgae in aquafeeds seems to positively affect fish growth performance and feed efficiency due to their high nutritional value and balanced composition (Norambuena et al. 2015).

For all these reasons, a wide variety of seaweeds can be potentially exploited as a main source of n-3 LC-PUFA, not just for direct human consumption but also for animal feed production, offering a continuous and sustainable supply of these essential compounds and contributing to satisfying the world population's needs. The main objective of the present study was to broadly characterize the lipid and FA profiles of the still understudied, but most representative 14 macroalgal species from the Madeira Archipelago, in order to evaluate their potential as sources for both n-3 LC-PUFA and other healthy lipid molecules with marked anti-hypercholesterolemic and anti-hypertriglycerolemic properties for human and animal nutrition.

2 Materials and methods

2.1 Specimens of seaweeds

Single samples were collected from representative species of the Madeira Archipelago, including two green macroalgal species (Chlorophyta, Ulvophyceae) *Dasycladus vermicularis* (Scopoli) Krasser (Order Dasycladales) and *Ulva* sp. (Order Ulvales), three species of red macroalgae (Rhodophyta, Florideophyceae) *Corallina officinalis* Linnaeus (Order Corallinales), *Asparagopsis taxiformis* (Delile) Trevisan (Order Bonnemaisoniales) and *Halopithys incurva* (Hudson) Batters (Order Ceramiales), and nine species of brown macroalgae (Ochrophyta, Phaeophyceae) *Cystoseira compressa* (Esper) Gerloff *et* Nizamuddin (Order Fucales), *Cystoseira usneoides* (Linnaeus) M. Roberts (Order Fucales), *Cystoseira humilis* Schousboe *ex* Kützing (Order Fucales), *Sargassum vulgare* C. Agardh (Order Fucales), *Dictyota dichotoma* (Hudson) J.V. Lamouroux (Order Dictyotales), *Lobophora* J. Agardh sp. (Order Dictyotales), *Padina pavonica* (Linnaeus) Thivy (Order Dictyotales), *Halopteris filicina* (Grateloup) Kützing (Order Sphacelariales), and *Halopteris scoparia* (Linnaeus) Sauvageau (Order Sphacelariales), and were analysed in triplicate.

The seaweeds were haphazardly harvested, taking the entire algal thallus (between 0.5 and 1 kg) at a maximum depth of 10 m by free diving, from different beaches of the Madeira Archipelago including Madeira and Porto Santo islands (Portugal; Figure 1). The sampling was carried out from March to June 2017, when water temperature ranged from 18.5 to 21 °C. After collection, samples were transported to the laboratory in seawater, where they were gently washed with filtered freshwater, frozen at −35 °C and freeze-dried under reduced pressure (4×10^{-4} mbar) with a cooling trap (Scanvac Coolsafe Model 55-4, Labogene, Lynge, Denmark) set at −56 °C for five days. Lyophilized samples were later milled to 200 µm particle size in an electric mill (IKA Werke Model M20, Staufen, Germany), packed under vacuum with a vacuum sealer (AudionVac Model VMS 153, Derby, UK) and stored at −35 °C until biochemical analysis.

2.2 Lipid extraction

Total lipid (TL) was extracted using 10 ml of chloroform/methanol (2:1, v/v) per 100 mg sample, according to the method described by Folch et al. (1957) with small modifications (Christie and Han 2010). The lipid content was gravimetrically determined after evaporation of the organic solvent under a stream of nitrogen. TL extracts were stored at 10 mg ml^{-1} in chloroform/methanol (2:1, v/v) containing 0.01% (w/v) of butylated hydroxytoluene (Sigma-Aldrich Co., St. Louis, Missouri, USA) as an antioxidant, under an inert atmosphere of nitrogen at −20 °C.

2.3 Lipid classes and fatty acid composition

Lipid classes were separated by one-dimensional double-development high-performance thin-layer chromatography (HPTLC), using 1-propanol/chloroform/methyl acetate/methanol/0.25% potassium chloride (5:5:5:2:1.8, v/v) for polar lipids, and hexane/diethyl ether/acetic acid (20:5:0.5, v/v) for neutral lipids. Lipid classes were then quantified by calibrated densitometry using a dual-wavelength flying spot scanner CAMAG TLC Visualizer (Camag, Muttenz, Switzerland), as described by Reis et al. (2019). Lipid class identification was performed by comparison to external lipid standards (cod roe lipid extract; DGDG and SQDG (Avanti Polar Lipids, Inc., Alabaster, Alabama, USA)) placed on the same HPTLC plate (Supplementary Figure S1).

Fatty acid methyl esters (FAME) were obtained by acid-catalyzed transmethylation of 1 mg of lipid extracts using 1% sulphuric acid in methanol (v/v) for 16 h at 50 °C (Christie and Han 2010). FAME were purified by thin-layer chromatography (TLC) with hexane/diethyl ether/acetic acid (90:10:1, v/v) as solvents (Christie and Han 2010), and subsequently separated and quantified using a TRACE-GC Ultra gas chromatograph (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) equipped with an on-column injector, a flame ionization detector and a fused silica capillary column, Supelcowax TM 10 (30 m × 0.32 mm I.D. × 0.25 µm; Sigma-Aldrich Co., St. Louis, Missouri, USA). Helium was used as carrier gas and temperature programming was 50–150 °C at 40 °C min^{-1} slope, then from 150 to 200 °C at 2 °C min^{-1}, to 214 °C at 1 °C min^{-1} and, finally, up to 230 °C at 40 °C min^{-1}. Individual FAME were identified by reference to a mixture of authentic standards (Mix C4-C24 and PUFA No. Three from menhaden oil (Supelco Inc., Bellefonte, Pennsylvania, USA) and a well characterized cod roe oil (for details, see Supplementary Figure S2), and the identity of FAME confirmed, when necessary, by GC–MS (DSQ II, Thermo Scientific).

2.4 Nutritional indices

Nutritional quality of macroalgal FA composition was assessed by calculating atherogenicity and thrombogenicity indices following Cardoso et al. (2017) and the ratio between hypocholesterolemic and hypercholesteloremic FA as described by Santos-Silva et al. (2002):

$$\text{Atherogenicity index (AI)} = [(4 \times 14{:}\,0) + 16{:}\,0 + 18{:}\,0] / (\sum \text{MUFA} + \sum \text{n} - 6\,\text{PUFA} + \sum \text{n} - 3\,\text{PUFA})$$

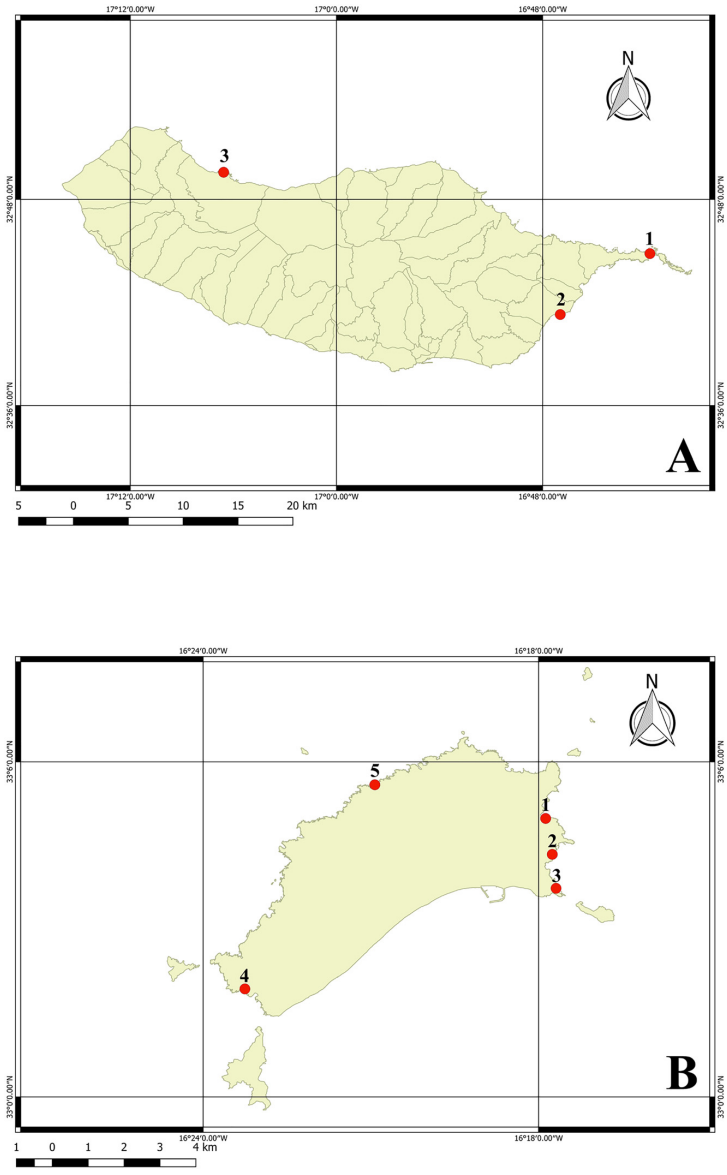

Figure 1: (A) Location of macroalgal sampling collection on Madeira island. Caniçal (1), *Halopteris scoparia*; Santa Cruz (2), *Sargassum vulgare*; Seixal (3), *Padina pavonica* and *Cystoseira humilis*. (B) Location of macroalgal sampling collection on Porto Santo island. Calhau Serra de Dentro (1), *Dasycladus vermicularis* and *Halopithys incurva*; Abas do Rio (2), *Dictyota dichotoma* and *Halopteris filicina;* Calhau da Baleia (3), *Lobophora* sp.; Praia do Zimbralinho (4), *Corallina officinalis* and *Asparagopsis taxiformis*; Porto das Salemas (5), *Ulva* sp., *Cystoseira compressa* and *Cystoseira usneoides*.

$$\text{Thrombogenicity index (TI)} = (14{:}\,0 + 16{:}\,0 + 18{:}\,0)/(0.5 \times \sum\text{MUFA} + 0.5 \times \sum n - 6\,\text{PUFA} + 3 \times \sum n - 3\,\text{PUFA} + n - 3/n - 6\,\text{ratio})$$

$$\text{Hypocholesterolemic (h)/hypercholesterolemic (H) ratio (hH)} = (18{:}\,1n - 9 + 18{:}\,2n - 6 + 20{:}\,4n - 6 + 8{:}\,3n - 3 + 20{:}\,5n - 3 + 22{:}\,5n - 3 + 22{:}\,6n - 3)/(14{:}\,0 + 16{:}\,0)$$

2.5 Statistical analysis

Before analysis, normality and homogeneity of data were confirmed within groups and, where necessary, appropriate variance stabilizing transformations were performed. When transformations did not succeed, Welch test followed by the Dunnett T3 test were used. Significant differences in lipid classes and FA

composition of red and brown macroalgae were assessed by one-way ANOVA followed by the Tukey HSD post-hoc test. Differences between green macroalgae were determined by Student's *t*-test or Mann-Whitney tests for normal or non-normal distribution of data, respectively. In addition, comparisons of TL, AI, TI, and hH indices between all species studied were also determined by one-way ANOVA.

Two principal component analyses (PCA), one for the lipid classes and the other for the main FA, of all macroalgae were carried out. Two hierarchical cluster analyses subsequently used factor scores to identify macroalgae with similar lipid classes and FA profiles. The hierarchical cluster analysis were performed with the Ward linkage method and the squared Euclidean distances.

Results are presented as mean ± standard deviation (SD, $n = 3$) and the statistical significance was set at $p < 0.05$. All statistical analyses were performed using IBM® SPSS Statistics 25.0 software package (IBM Corp., New York, USA) for Windows.

3 Results

3.1 Total lipid content

The lipid content of analysed seaweeds strongly varied among species, from 0.2% DW (*Dasycladus vermicularis*) to 5.2% DW (*Dictyota dichotoma*) ($p < 0.05$; Table 1).

3.2 Lipid class profiles

All species studied presented higher proportions of total neutral lipids (TNL; from 31 to 62% of TL) than of total polar lipids (TPL; from 14 to 37% of TL) (Tables 2–4), except for *Dasycladus vermicularis* (Chlorophyta) and *Padina*

Table 1: Total lipid content (% of dry weight) of macroalgae.

Group/Phylum	Species	TL content
Chlorophyta	*Dasycladus vermicularis*	0.2 ± 0.1^{a}
	Ulva sp.	0.9 ± 0.1^{ab}
Rhodophyta	*Corallina officinalis*	1.3 ± 0.1^{bcd}
	Asparagopsis taxiformis	2.1 ± 0.9^{de}
	Halopithys incurva	1.2 ± 0.1^{acd}
Ochrophyta	*Cystoseira compressa*	1.8 ± 0.0^{bcd}
	C. usneoides	0.8 ± 0.0^{ab}
	C. humilis	2.9 ± 0.4^{e}
	Dictyota dichotoma	5.2 ± 0.2^{f}
	Halopteris filicina	1.0 ± 0.2^{ac}
	H. scoparia	1.2 ± 0.4^{acd}
	Lobophora sp.	1.2 ± 0.1^{acd}
	Padina pavonica	0.8 ± 0.0^{ab}
	Sargassum vulgare	2.0 ± 0.7^{ce}

Results are presented as mean ± SD ($n = 3$). Different superscript letters indicate significant differences among all macroalgal species ($p < 0.05$). TL, total lipid.

Table 2: Main lipid class composition of green macroalgae (% of total lipid).

	Dasycladus vermicularis	*Ulva* sp.
PC	2.3 ± 0.6	1.9 ± 0.3
PS + PI	1.1 ± 0.4	1.1 ± 0.2
SQDG + PE	12.8 ± 2.4	16.0 ± 0.8
DGDG	10.5 ± 1.4	9.7 ± 1.4
MGDG	2.3 ± 0.7	1.3 ± 0.5*
UkPL	2.7 ± 0.7	2.2 ± 0.3
TPL	31.7 ± 4.7	32.2 ± 2.3
P	37.1 ± 1.4	15.6 ± 0.8*
DAG	6.4 ± 0.8	6.1 ± 0.9
PTS	11.7 ± 3.5	9.0 ± 0.4
FFA	5.8 ± 1.4	20.2 ± 1.5*
TAG	4.5 ± 2.0	11.3 ± 0.8*
SE	2.9 ± 1.0	4.9 ± 1.1
UkNL	nd	0.6 ± 0.7
TNL	31.2 ± 6.1	52.2 ± 1.6*

Results are presented as mean ± SD ($n = 3$). *Indicates significant difference between the species ($p < 0.05$). PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; SQDG, sulfoquinovosyldiacylglycerol; PE, phosphatidylethanolamine; DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; UkPL, unknown polar lipids; TPL, total polar lipids;
P, pigments; DAG, diacylglycerols; PTS, phytosterols; FFA, free fatty acids; TAG, triacylglycerols; SE, sterol esters; UkNL, unknown neutral lipids; TNL, total neutral lipids; nd, not detected.

Table 3: Main lipid class composition of red macroalgae (% of total lipid).

	Asparagopsis taxiformis	*Corallina officinalis*	*Halopithys incurva*
PC	3.5 ± 0.1	3.3 ± 0.4	6.2 ± 1.3
PS + PI	1.0 ± 0.6	1.2 ± 0.2	1.3 ± 0.6
SQDG + PE	3.7 ± 1.7^{a}	3.3 ± 0.5^{a}	7.7 ± 1.8^{b}
DGDG	1.8 ± 0.4^{b}	1.1 ± 0.1^{a}	6.7 ± 0.3^{c}
MGDG	1.6 ± 0.8^{a}	4.7 ± 0.6^{b}	5.6 ± 0.4^{b}
UkPL	2.3 ± 0.8	2.6 ± 1.1	1.9 ± 0.6
TPL	13.9 ± 2.5^{a}	16.2 ± 1.4^{a}	29.3 ± 4.6^{b}
P	23.8 ± 3.7	29.2 ± 0.8	29.6 ± 1.1
DAG	5.3 ± 1.3	4.4 ± 1.7	4.6 ± 1.0
PTS	6.2 ± 0.6^{a}	14.4 ± 0.4^{b}	14.6 ± 1.1^{b}
FFA	18.7 ± 6.3^{ab}	25.0 ± 2.3^{b}	11.1 ± 1.1^{a}
TAG	30.0 ± 4.0^{b}	4.1 ± 1.8^{a}	5.2 ± 2.1^{a}
SE	2.1 ± 1.2^{a}	6.8 ± 0.8^{b}	5.6 ± 2.4^{ab}
TNL	62.3 ± 3.9^{b}	54.6 ± 1.6^{b}	41.1 ± 5.4^{a}

Results are presented as mean ± SD ($n = 3$). Different superscript letters within each row indicate significant differences between species ($p < 0.05$). PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; SQDG, sulfoquinovosyldiacylglycerol; PE, phosphatidylethanolamine; DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; UkPL, unknown polar lipids; TPL, total polar lipids; P, pigments; DAG, diacylglycerols; PTS, phytosterols; FFA, free fatty acids; TAG, triacylglycerols; SE, sterol esters; TNL, total neutral lipids.

Table 4: Main lipid class composition of brown macroalgae (% of total lipid).

	Cystoseira compressa	*C. usneoides*	*C. humilis*	*Dictyota dichotoma*	*Halopteris filicina*	*H. scoparia*	*Lobophora sp.*	*Padina pavonica*	*Sargassum vulgare*
PC	1.8 ± 0.4^{abc}	1.3 ± 0.3^{abc}	0.9 ± 0.4^{a}	1.2 ± 0.4^{ab}	2.9 ± 1.1^{bc}	3.0 ± 0.3^{bc}	3.1 ± 0.9^{c}	1.4 ± 1.1^{abc}	2.1 ± 0.4^{abc}
PS + PI	4.5 ± 0.3^{bc}	9.6 ± 1.9^{d}	2.4 ± 0.5^{ab}	4.1 ± 0.5^{bc}	1.1 ± 0.4^{a}	1.2 ± 0.4^{a}	6.0 ± 1.6^{c}	4.8 ± 1.4^{c}	5.0 ± 0.4^{c}
SQDG + PE	8.0 ± 1.4^{abc}	15.8 ± 3.0^{e}	10.6 ± 1.2^{cd}	5.3 ± 0.3^{a}	6.5 ± 1.2^{ab}	8.7 ± 2.1^{abcd}	9.1 ± 1.3^{bcd}	13.1 ± 1.3^{de}	8.7 ± 0.3^{abcd}
DGDG	3.8 ± 0.7	5.8 ± 0.7	5.8 ± 0.6	3.7 ± 0.1	4.3 ± 0.3	5.1 ± 1.1	4.6 ± 0.8	6.3 ± 0.5	3.7 ± 0.2
MGDG	1.0 ± 0.4^{a}	2.2 ± 0.7^{abc}	1.4 ± 0.6^{ab}	1.4 ± 0.8^{ab}	1.7 ± 0.7^{abc}	2.1 ± 0.8^{abc}	3.1 ± 0.4^{bc}	3.2 ± 0.4^{c}	1.6 ± 0.5^{abc}
UkPL	0.6 ± 0.3	1.9 ± 0.7	2.6 ± 0.4	1.3 ± 0.2	3.5 ± 0.3	7.6 ± 2.8	3.5 ± 0.4	5.4 ± 1.4	2.6 ± 1.2
TPL	19.7 ± 1.8^{ab}	36.6 ± 6.3^{c}	23.7 ± 2.5^{ab}	17.0 ± 1.4^{a}	20.0 ± 1.8^{ab}	27.7 ± 4.1^{bc}	29.4 ± 3.8^{bc}	34.3 ± 5.2^{c}	23.7 ± 1.9^{ab}
P	23.7 ± 0.8^{d}	22.7 ± 0.4^{ac}	21.2 ± 0.5^{a}	29.9 ± 0.5^{e}	27.5 ± 3.4^{abcde}	25.8 ± 3.1^{abcde}	27.2 ± 0.9^{bde}	31.2 ± 1.8^{cde}	21.2 ± 1.4^{ab}
DAG	9.4 ± 0.2^{ce}	2.2 ± 0.6^{a}	13.0 ± 1.2^{de}	14.8 ± 0.9^{e}	8.2 ± 1.6^{abcde}	4.2 ± 1.5^{abc}	6.7 ± 0.2^{bd}	3.5 ± 0.3^{a}	2.4 ± 0.4^{a}
PTS	10.9 ± 0.7^{a}	24.7 ± 3.6^{d}	17.0 ± 0.6^{bc}	18.9 ± 1.6^{bc}	19.3 ± 1.9^{bc}	15.5 ± 1.3^{b}	21.9 ± 1.9^{cd}	16.3 ± 1.5^{b}	19.6 ± 1.4^{bcd}
FFA	16.4 ± 1.0^{cd}	3.6 ± 1.3^{a}	14.4 ± 1.2^{c}	7.0 ± 0.8^{b}	15.2 ± 1.8^{cd}	15.5 ± 1.2^{cd}	9.2 ± 1.0^{b}	7.1 ± 1.3^{b}	19.8 ± 1.5^{d}
TAG	18.9 ± 0.5^{d}	4.6 ± 0.7^{bc}	3.8 ± 1.0^{abcd}	5.2 ± 1.2^{bcd}	6.3 ± 6.1^{abcd}	6.8 ± 1.0^{c}	1.4 ± 0.3^{a}	2.9 ± 0.2^{ab}	7.4 ± 1.4^{bcd}
SE	1.1 ± 0.4^{a}	1.6 ± 0.4^{a}	4.1 ± 1.1^{ab}	1.1 ± 1.1^{ab}	2.5 ± 1.1^{ab}	4.5 ± 0.6^{b}	2.6 ± 0.0^{ab}	4.7 ± 0.9^{ab}	4.9 ± 1.6^{ab}
UkNL	nd	3.8 ± 1.2	2.9 ± 1.1	6.2 ± 1.5	0.9 ± 1.1	nd	1.6 ± 0.5	nd	0.9 ± 0.3
TNL	56.7 ± 2.0^{d}	40.7 ± 6.4^{abcd}	55.1 ± 2.9^{bd}	53.1 ± 1.3^{cd}	52.5 ± 1.7^{bcd}	46.5 ± 1.0^{ab}	43.4 ± 3.0^{abc}	34.5 ± 3.4^{a}	55.1 ± 3.3^{bcd}

Results are presented as mean ± SD ($n = 3$). Different superscript letters within each row indicate significant differences between species ($p < 0.05$). PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; SQDG, sulfoquinovosyldiacylglycerol; PE, phosphatidylethanolamine; DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; UkPL, unknown polar lipids; TPL, total polar lipids; P, pigments; DAG, diacylglycerol; PTS, phytosterols; FFA, free fatty acids; TAG, triacylglycerols; SE, sterol esters; UkNL, unknown neutral lipids; TNL, total neutral lipids; nd, not detected.

pavonica (Ochrophyta) which contained similar levels of both lipid fractions (31–34% of TL).

Ulva sp. contained higher levels of free fatty acids (FFA) and TAG (20.2 and 11.3%, respectively) than *Dasycladus vermicularis* (5.8 and 4.5%) ($p < 0.05$). Among polar lipids, only MGDG varied significantly among species (Table 2).

Within red macroalgae, TAG was highest in *Asparagopsis taxiformis* (30.0%) whereas TAG was only 4.1 and 5.2% of TL in *Corallina officinalis* and *Halopithys incurva*, respectively. On the other hand, *H. incurva* ($p < 0.05$) contained the highest SQDG + PE and DGDG (Table 3).

PTS was particularly abundant in brown macroalgae (10.9–24.7% TL; Table 4). *Cystoseira compressa* had the highest TAG levels (18.9%), while in the other Ochrophyta analysed, values ranged between 1.4 and 6.8% of TL. Phosphatidylserine (PS) + phosphatidylinositol (PI) (9.6%), and SQDG + PE (15.8%) were remarkably high in *C. usneoides* ($p < 0.05$; Table 4).

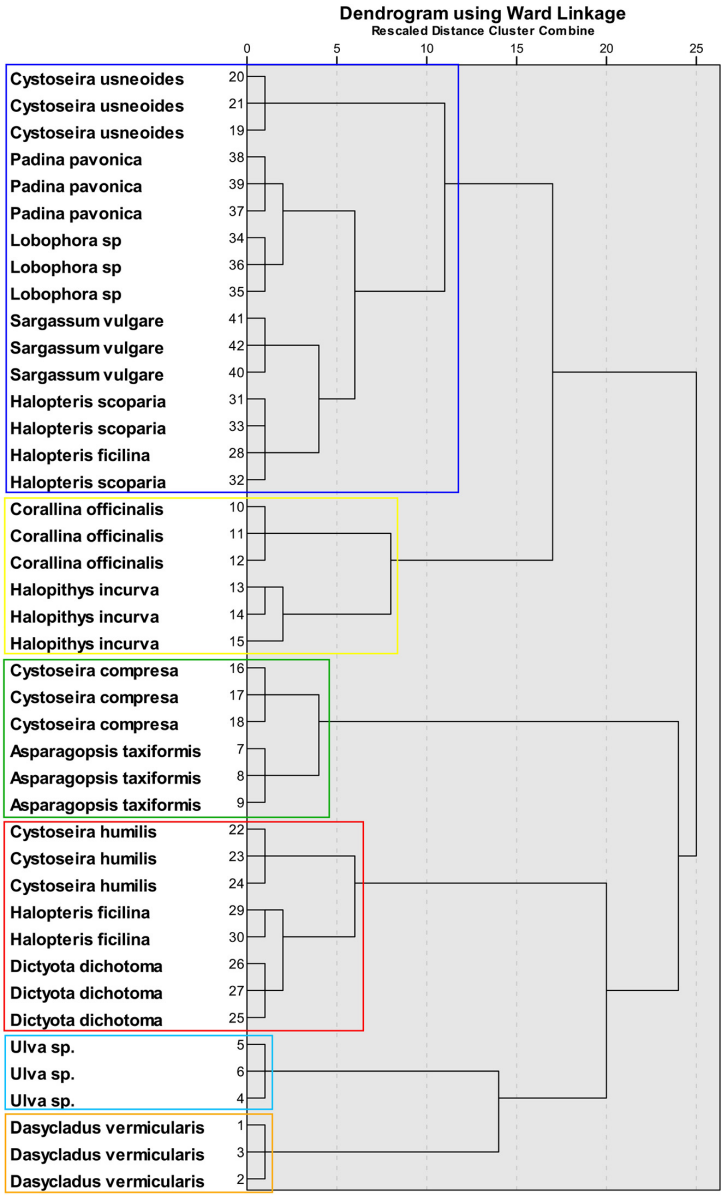

Figure 2: Hierarchical cluster analysis in a dendrogram format showing six clusters of macroalgae according to the lipid class composition.

The PCA of macroalgal lipid classes showed five components with eigenvalues >1, which accounted for more than 88% of the total variance. Factor loadings and communalities are shown in Supplementary Table S1. According to the dendrogram obtained, the macroalgae were classified into six clusters (Figure 2). Mean factor scores for each cluster of the dendrogram are given in Supplementary Table S2. Thus, Cluster one grouped most Ochrophyta species including *Cystoseira usneoides*, *Halopteris scoparia*, *Lobophora* sp., *Padina pavonica*, *Sargassum vulgare*, and one replicate of *H. ficilina*, all of which were mainly characterized by a high average content of PTS. Two of the three red macroalgae studied, *Corallina officinalis* and *Halopithys incurva*, formed Cluster 2, with high average proportions of FFA, sterol esters, PC and MGDG. Cluster three consisted of the third Rhodophyta species, *Asparagopsis taxiformis*, and *C. compressa* (Ochrophyta), which contained high average TAG and PS + PI. Cluster 4, which included the rest of the Ochrophyta species, *C. humilis*, *Dictyota dichotoma* and two replicates of *H. ficilina*, showed the highest average percentage of diacylglycerol. Finally, the green algae *Ulva* sp. and *Dasycladus vermicularis* were the only components of Clusters 5 and 6, which were characterized by high average SQDG + PE, DGDG and FFA content, and high SQDG + PE, DGDG and P, respectively.

3.3 Fatty acid profile

The FA profiles of green seaweeds strongly differed among species. *Ulva* sp. had higher contents of saturated fatty acids (SFA), mainly palmitic acid (16:0), and n-3 PUFA ($p < 0.05$; Table 5), while *Dasycladus vermicularis* was richer in monounsaturated fatty acids (MUFA). The n-3 LC-PUFA content was low in both species, where DHA represented only 0.5 and 0.8% of total FA in *D. vermicularis* and *Ulva* sp., respectively.

Similarly, the FA profiles varied considerably within the Rhodophyta. Only total SFA and 16:0 were not significantly different among the species, with the latter being the most abundant FA in all three species (Table 6). *Asparagopsis taxiformis* had the highest amount of DHA (22:6n-3; 6.6% of total FA), while *Halopithys incurva* was richer in arachidonic acid (ARA, 20:4n-6), ALA and EPA, leading to higher total n-6 and total n-3 PUFA contents. The n-6/n-3 ratio was highest in *Corallina officinalis* (1.6) and lowest in *A. taxiformis* (0.4; Table 6).

Brown macroalgae contained the highest levels of SFA of all species studied (from 34.1 to 52.3% of total FA in *Dictyota dichotoma* and *Halopteris scoparia*, respectively), followed by MUFA (26.2% in *Cystoseira usneoides* and 34.2% in *Padina pavonica*) (Table 7). Within Ochrophyta, n-3 PUFA was remarkably high in *D. dichotoma*, mainly due to the higher levels of EPA (5.0% of total FA) and stearidonic acid (SDA, 18:4n-3; 8.0%), while DHA was only 0.5% of total FA. On the other hand, *C. compressa* showed the highest value of DHA (3.9%). Total n-6 PUFA was more abundant in *C. usneoides* and *Sargassum vulgare* due to the high proportions of both LA and ARA.

PCA for seaweed FA revealed that five components had eigenvalues >1 and together accounted for more than 86% of the total variance. Factor loadings and communalities after applying varimax rotation to enhance the interpretability of the results are displayed in Supplementary Table S3. The dendrogram obtained from the hierarchical cluster analysis, which used the factor scores as input variables, revealed that macroalgae could be classified into six clusters (Figure 3). Supplementary Table S4 shows factor

Table 5: Main fatty acid composition (% of total FA) of green macroalgae.

	Dasycladus vermicularis	*Ulva* sp.
14:0	3.6 ± 0.5	1.7 ± 0.1*
15:0	1.1 ± 0.2	0.7 ± 0.0
16:0	18.7 ± 2.6	38.4 ± 0.1*
17:0	1.0 ± 0.2	0.5 ± 0.1*
18:0	4.3 ± 1.6	2.8 ± 0.2
Σ SFA[1]	28.7 ± 5.0	45.0 ± 0.4*
16:1[#]	15.5 ± 0.8	4.8 ± 0.1*
18:1[##]	26.7 ± 2.4	21.2 ± 0.7*
Σ MUFA[2]	44.3 ± 1.5	27.2 ± 0.6*
18:2n-6	14.3 ± 2.2	8.5 ± 0.2
20:3n-6	nd	0.7 ± 0.0
20:4n-6	0.6 ± 0.3	1.5 ± 0.1*
Σ n-6 PUFA[3]	14.9 ± 2.5	12.5 ± 0.2
16:3n-3	nd	1.7 ± 0.0
18:3n-3	0.7 ± 0.1	6.8 ± 0.1*
18:4n-3	0.8 ± 0.2	1.9 ± 0.1*
20:5n-3	1.2 ± 0.3	1.2 ± 0.1
22:6n-3	0.5 ± 0.1	0.8 ± 0.1*
Σ n-3 PUFA[4]	3.2 ± 0.7	13.5 ± 0.4*
Σ n-3 LC-PUFA[4]	1.7 ± 0.4	3.2 ± 0.3*
Σ PUFA[3,4,5]	22.9 ± 3.9	26.3 ± 1.0*
n-6/n-3	4.7 ± 0.3	0.9 ± 0.0*
DHA/EPA	0.4 ± 0.1	0.7 ± 0.0*
ARA/EPA	0.5 ± 0.1	1.2 ± 0.1*

Results are presented as mean ± SD ($n = 3$). *Indicates significant difference between the species ($p < 0.05$). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long chain polyunsaturated fatty acids (≥C20 and ≥ 2 double bonds); DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ARA, arachidonic acid; nd, not detected. [1]Also includes 22:0; [2]also includes 15:1, 17:1, 20:1n-9 and 22:1; [3]also includes 20:2n-6 and 22:2n-6; [4]also includes 20:4n-3; [5] also includes 16:2n-4 and 16:3n-4. [#]Mainly n-7 isomer; [##]mainly n-9 and n-7 isomers.

Table 6: Main fatty acid composition (% of total FA) of red macroalgae.

	Asparagopsis taxiformis	*Corallina officinalis*	*Halopithys incurva*
14:0	10.1 ± 1.1[b]	4.7 ± 1.6[a]	6.8 ± 0.2[a]
15:0	0.7 ± 0.0[a]	1.6 ± 0.3[b]	0.4 ± 0.1[a]
16:0	32.0 ± 3.2	35.7 ± 1.8	32.0 ± 0.6
17:0	1.6 ± 0.0[a]	1.1 ± 0.2[b]	0.7 ± 0.1[a]
18:0	3.1 ± 0.1[b]	5.0 ± 0.4[c]	2.1 ± 0.3[a]
Σ SFA[1]	46.8 ± 4.3	48.7 ± 1.3	42.3 ± 1.0
16:1[#]	8.5 ± 0.7[b]	7.9 ± 2.0[ab]	6.1 ± 0.1[a]
18:1[##]	20.9 ± 1.3[b]	19.8 ± 0.4[b]	12.9 ± 0.3[a]
Σ MUFA[2]	32.5 ± 0.9[b]	31.0 ± 1.3[b]	20.0 ± 0.3[a]
18:2n-6	5.0 ± 0.6[b]	4.9 ± 0.9[b]	2.1 ± 0.1[a]
20:3n-6	nd	nd	0.3 ± 0.0
20:4n-6	0.4 ± 0.0[a]	5.1 ± 0.8[b]	11.7 ± 0.8[c]
Σ n-6 PUFA	5.5 ± 0.6[a]	9.9 ± 0.9[b]	14.2 ± 0.8[c]
18:3n-3	1.0 ± 0.1[a]	1.0 ± 0.3[a]	10.9 ± 0.6[b]
18:4n-3	0.8 ± 0.1[b]	0.5 ± 0.0[ab]	0.3 ± 0.1[a]
20:5n-3	2.2 ± 0.4[a]	3.4 ± 0.6[a]	9.3 ± 0.6[b]
22:6n-3	6.6 ± 1.4[c]	1.4 ± 0.2[b]	0.5 ± 0.1[a]
Σ n-3 PUFA[3]	13.6 ± 2.6[b]	6.2 ± 0.9[a]	21.6 ± 0.7[c]
Σ n-3 LC-PUFA[3]	11.8 ± 2.3[b]	4.8 ± 0.8[a]	10.4 ± 0.4[b]
Σ PUFA[3,4]	20.5 ± 3.4[a]	17.7 ± 1.8[a]	36.0 ± 1.2[b]
n-6/n-3	0.4 ± 0.0[a]	1.6 ± 0.3[c]	0.7 ± 0.0[b]
DHA/EPA	3.0 ± 0.1[c]	0.4 ± 0.0[a]	0.1 ± 0.0[b]
ARA/EPA	0.2 ± 0.0[a]	1.5 ± 0.1[c]	1.3 ± 0.1[b]

Results are presented as mean ± SD (n = 3). Different superscript letters within each row indicate significant differences between species (p < 0.05). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long chain polyunsaturated fatty acids (≥C20 and ≥ 2 double bonds); DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ARA, arachidonic acid; nd, not detected. [1]Also includes 20:0 and 22:0; [2]also includes 14:1, 15:1, 17:1, 20:1n-9 and 22:1; [3]also includes 20:3n-3, 20:4n-3 and 22:5n-3; [4]also includes 16:2, 16:3n-4, 16:4n-1 and 20:2n-9. [#]Mainly n-7 isomer and n-9 isomers; [##]mainly n-9 isomer.

scores for each cluster given as mean ± SD Hence, Cluster 1, grouping together *Ulva* sp. (Chlorophyta), and three Ochrophyta species (*Cystoseira usneoides*, *C. humilis* and *Sargassum vulgare*), was characterized by the highest average percentage of 16:0 and medium-high average content of LA and 20:3n-6. Clusters 2–4 comprised only one species each: *Halopithys incurva* (Cluster 2) had the highest average proportion of ALA, ARA and EPA; *Dictyota dichotoma* (Cluster 3) was characterized by the lowest average percentage of DHA, and high 14:0 and 20:3n-6; *Dasycladus vermicularis* (Cluster 4) had the highest average proportions of 15:0, 17:0, 18:0 and LA, and low ARA and EPA contents. Cluster five contained the red macroalga *Asparagopsis taxiformis* and the brown *Cystoseira compressa*, which had the highest proportion of DHA and low percentages of ALA, ARA and LA. Finally, the red macroalga *Corallina officinalis*, and the four brown macroalgae (*Lobophora* sp., *Halopteris ficilina*, *H. scoparia* and *Padina pavonica*) were grouped in Cluster 6, and were characterized by medium-high average content of all SFA.

Overall, the grouping of macroalgae based on their FA profile did not follow a similar pattern to that described for their lipid class composition (Figure 2). Only the pairs *Halopteris scoparia* and *Padina pavonica*; *Cystoseira usneoides* and *Sargassum vulgare*; and *Asparagopsis taxiformis* and *C. compressa* were similar in their lipid class and FA profiles. Of all species analysed, *Dasycladus vermicularis* (Chlorophyta) had a particular and unique lipid profile.

3.4 Nutritional indices

Dasycladus vermicularis had the lowest AI (0.6) and *Dictyota dichotoma* the lowest TI (0.4), whereas *Halopteris scoparia* had the highest AI (1.6) and TI (1.3) values (Table 8). *Ulva* sp., *H. scoparia* and *Padina pavonica* showed the lowest hH ratios (0.7), while *D. dichotoma* and *Sargassum vulgare* had the highest values.

4 Discussion

The seaweeds analysed differed greatly in their lipid content, lipid classes and FA profiles providing evidence of strong interspecific variations. Several factors have been suggested to affect the biochemical composition of algae, including the part of the macroalgal thallus used for the analysis (Alsufyani et al. 2014; Pereira et al. 2012), which was strictly controlled in the present study. However, our one-off collection methodology did not allow us to evaluate seasonal, environmental or even geographical factors, or the impact of species-dependent fatty acid transformation and decomposition that should be considered in future research to provide a complete lipid description of the selected macroalgae.

The TL content of the macroalgae studied were broadly similar to the low levels described in earlier literature (Bourgougnon et al. 2011; Kendel et al. 2015; Mæhre et al. 2014; Nunes et al. 2020; Verma et al. 2017). Nevertheless, some differences, probably related to both geographical and seasonal factors, were detected. Thus, the two Chlorophyta species had lower lipid contents than other species of *Ulva*, such as *Ulva lactuca* and *U. reticulata* from India, *U. lactuca* from Hong Kong, and *U. rigida* from South Africa (Foster and Hodgson 1998; Verma et al. 2017; Wong and Cheung 2000). In contrast, the lipid level of *Ulva* sp. was

Table 7: Main fatty acid composition (% of total FA) of brown macroalgae.

	Cystoseira compressa	*C. usneoides*	*C. humilis*	*Dictyota dichotoma*	*Halopteris ficilina*	*H. scoparia*	*Lobophora sp.*	*Padina pavonica*	*Sargassum vulgare*
14:0	7.8 ± 0.9^{bcd}	3.2 ± 1.2^{abcd}	7.0 ± 0.2^{bc}	10.2 ± 0.4^{de}	5.9 ± 0.0^{b}	7.6 ± 0.2^{cd}	11.6 ± 0.5^{e}	8.0 ± 0.2^{d}	3.6 ± 0.1^{a}
15:0	0.7 ± 0.1^{ab}	0.6 ± 0.3^{ab}	0.5 ± 0.0^{a}	0.6 ± 0.1^{ab}	0.7 ± 0.0^{b}	0.8 ± 0.0^{b}	0.8 ± 0.1^{ab}	1.0 ± 0.1^{ab}	0.5 ± 0.0^{ab}
16:0	31.5 ± 1.1^{bde}	32.9 ± 0.7^{bcd}	33.7 ± 0.5^{cd}	20.7 ± 0.4^{a}	32.8 ± 1.3^{cd}	36.6 ± 1.4^{c}	29.2 ± 0.3^{b}	34.7 ± 0.7^{ce}	30.9 ± 0.4^{bd}
17:0	0.9 ± 0.5^{abc}	0.4 ± 0.2^{abc}	0.4 ± 0.0^{a}	nd	0.9 ± 0.1^{b}	1.0 ± 0.1^{b}	0.7 ± 0.1^{b}	0.6 ± 0.0^{b}	0.4 ± 0.0^{a}
18:0	3.1 ± 0.3^{b}	1.7 ± 0.2^{a}	1.8 ± 0.5^{ab}	1.8 ± 0.2^{a}	3.9 ± 1.4^{abc}	4.7 ± 1.1^{abc}	2.2 ± 0.1^{ab}	2.4 ± 0.2^{ab}	5.1 ± 0.3^{c}
Σ SFA[1]	44.7 ± 1.3^{bcd}	40.6 ± 0.7^{abcd}	44.2 ± 0.8^{bc}	34.1 ± 0.9^{a}	44.9 ± 0.9^{bcd}	52.3 ± 1.1^{cd}	45.8 ± 0.8^{d}	47.6 ± 1.5^{bcd}	41.8 ± 0.3^{d}
16:1$^{\#}$	8.3 ± 0.2^{bcd}	5.4 ± 0.6^{a}	5.7 ± 0.0^{a}	9.6 ± 0.4^{cde}	8.0 ± 0.3^{b}	9.4 ± 0.2^{de}	10.2 ± 0.5^{ef}	10.2 ± 0.1^{f}	8.2 ± 0.3^{bcd}
18:1$^{\#\#}$	21.8 ± 0.7^{bcd}	20.6 ± 0.2^{b}	20.5 ± 0.8^{bcd}	21.0 ± 0.3^{b}	18.7 ± 2.0^{abcd}	18.6 ± 0.4^{ac}	22.7 ± 0.3^{d}	24.0 ± 0.1^{d}	16.8 ± 0.4^{a}
Σ MUFA[2]	33.0 ± 0.8^{cd}	26.2 ± 0.3^{a}	26.6 ± 0.8^{ab}	31.4 ± 0.7^{bc}	27.8 ± 2.3^{abcd}	28.9 ± 0.3^{a}	33.3 ± 0.5^{d}	34.2 ± 0.1^{d}	27.5 ± 0.8^{ab}
18:2n-6	5.8 ± 0.6^{ade}	7.5 ± 0.5^{ade}	7.7 ± 0.6^{e}	2.7 ± 0.2^{a}	6.9 ± 0.1^{ce}	9.3 ± 1.2^{def}	4.2 ± 0.1^{bd}	3.8 ± 0.5^{abc}	11.7 ± 0.5^{f}
20:3n-6	nd	1.0 ± 0.1^{b}	0.6 ± 0.0^{a}	0.6 ± 0.0^{a}	nd	nd	0.9 ± 0.0^{b}	0.6 ± 0.0^{a}	0.6 ± 0.0^{a}
20:4n-6	2.8 ± 0.7^{abc}	7.2 ± 0.3^{d}	7.5 ± 0.1^{d}	6.7 ± 0.1^{cd}	3.0 ± 0.3^{ab}	2.0 ± 0.2^{a}	4.4 ± 0.2^{b}	2.0 ± 0.2^{a}	6.5 ± 0.0^{cd}
Σ n-6 PUFA[3]	8.7 ± 1.4^{abc}	19.5 ± 0.4^{e}	15.7 ± 0.6^{c}	13.2 ± 0.1^{b}	13.3 ± 1.2^{abcd}	11.3 ± 1.0^{abc}	10.3 ± 0.5^{ab}	9.2 ± 0.5^{a}	18.8 ± 0.5^{de}
18:3n-3	2.5 ± 0.1^{b}	3.4 ± 0.1^{d}	6.0 ± 0.0^{f}	3.5 ± 0.0^{cd}	3.0 ± 0.4^{abcde}	2.2 ± 0.3^{abcd}	1.5 ± 0.1^{a}	2.4 ± 0.2^{abcd}	4.8 ± 0.2^{e}
18:4n-3	1.7 ± 0.3^{ab}	2.9 ± 0.1^{d}	3.2 ± 0.1^{d}	8.0 ± 0.2^{e}	2.2 ± 0.3^{bc}	1.2 ± 0.1^{a}	2.6 ± 0.1^{cd}	1.7 ± 0.2^{b}	1.8 ± 0.1^{b}
20:5n-3	2.2 ± 0.1^{bc}	3.2 ± 0.3^{d}	2.2 ± 0.1^{bc}	5.0 ± 0.2^{e}	3.6 ± 0.2^{d}	1.7 ± 0.4^{b}	3.4 ± 0.1^{d}	1.0 ± 0.1^{a}	2.4 ± 0.2^{c}
22:6n-3	3.9 ± 0.4^{c}	0.5 ± 0.1^{ab}	0.3 ± 0.1^{a}	0.5 ± 0.1^{ab}	1.1 ± 0.4^{b}	0.6 ± 0.3^{ab}	0.5 ± 0.1^{ab}	0.4 ± 0.2^{ab}	0.5 ± 0.1^{ab}
Σ n-3 PUFA[4]	12.0 ± 1.2^{c}	10.9 ± 0.4^{bc}	12.1 ± 0.2^{c}	18.8 ± 0.4^{d}	11.9 ± 0.8^{c}	5.7 ± 0.6^{a}	9.1 ± 0.5^{b}	6.8 ± 0.5^{a}	10.0 ± 0.5^{bc}
Σ n-3 LC-PUFA[4]	7.8 ± 0.9^{c}	4.6 ± 0.6^{b}	2.9 ± 0.0^{a}	7.2 ± 0.2^{c}	6.6 ± 0.4^{c}	2.4 ± 0.5^{a}	4.9 ± 0.4^{b}	3.1 ± 0.5^{a}	3.5 ± 0.2^{ab}
Σ PUFA[3,4,5]	21.5 ± 2.2^{b}	30.6 ± 0.6^{de}	28.0 ± 0.5^{cd}	33.0 ± 0.4^{e}	25.4 ± 2.3^{c}	17.0 ± 0.8^{a}	19.5 ± 0.8^{ab}	16.4 ± 1.1^{a}	28.8 ± 0.9^{cd}
n-6/n-3	0.7 ± 0.1^{a}	1.8 ± 0.1^{ef}	1.3 ± 0.1^{cd}	0.7 ± 0.0^{a}	1.1 ± 0.0^{bc}	2.0 ± 0.4^{abcdef}	1.1 ± 0.0^{bc}	1.3 ± 0.0^{de}	1.9 ± 0.1^{f}
DHA/EPA	1.8 ± 0.1^{b}	0.1 ± 0.0^{a}	0.2 ± 0.0^{a}	0.1 ± 0.0^{a}	0.3 ± 0.1^{a}	0.4 ± 0.1^{a}	0.2 ± 0.0^{a}	0.4 ± 0.3^{a}	0.2 ± 0.0^{a}
ARA/EPA	1.3 ± 0.3^{abcdef}	2.2 ± 0.2^{de}	3.5 ± 0.1^{f}	1.3 ± 0.1^{b}	0.8 ± 0.1^{a}	1.2 ± 0.2^{abc}	1.3 ± 0.0^{abe}	2.0 ± 0.1^{cd}	2.7 ± 0.2^{df}

Results are presented as mean ± SD (n = 3). Different superscript letters within each row indicate significant differences between species (p < 0.05). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LC-PUFA, long chain polyunsaturated fatty acids (≥C20 and ≥ 2 double bonds); DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ARA, arachidonic acid; nd, not detected. [1]Also includes 20:0, 22:0 and 24:0; [2]also includes 15:1, 20:1n-11, 20:1n-9 and 22:1; [3]also includes 20:2n-6, 22:2n-6 and 22:4n-6; [4]also includes 20:3n-3; 20:4n-3 and 22:5n-3; [5]also includes 16:2, 16:3n-4 and 16:4n-1. [#]Mainly n-7 and n-9 isomers; [##]mainly n-9 isomer.

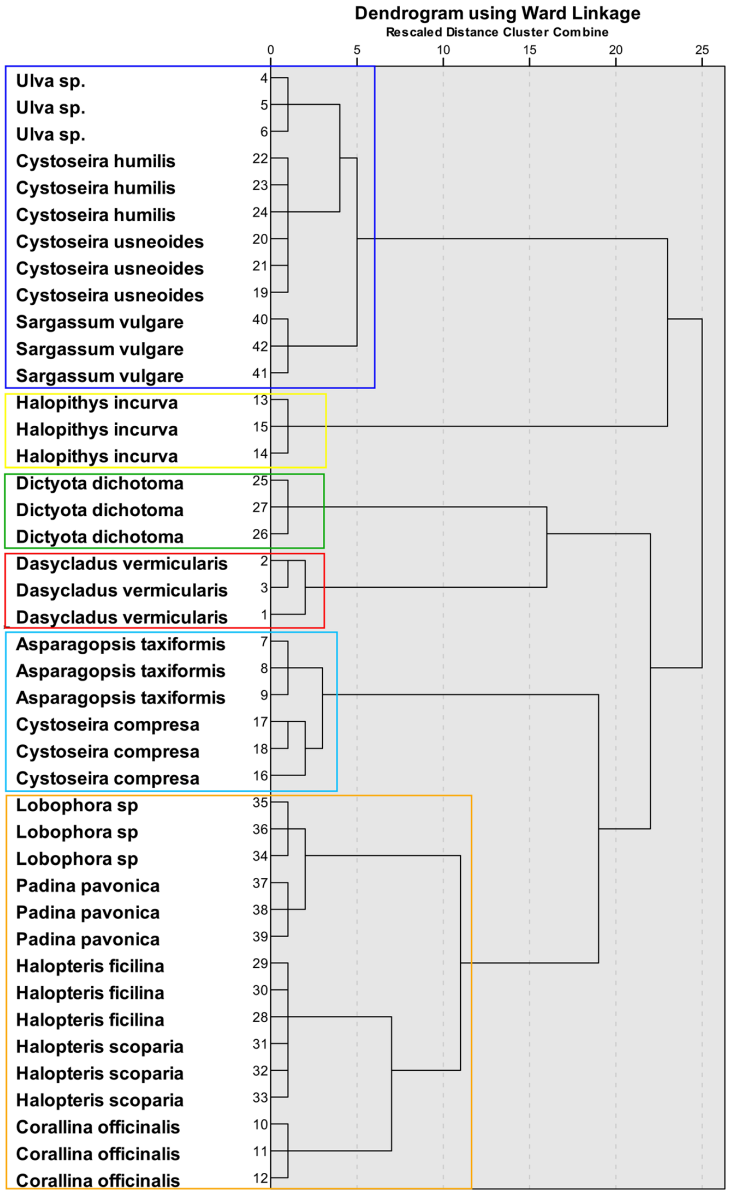

Figure 3: Hierarchical cluster analysis in a dendrogram format showing six clusters of macroalgae according to the fatty acid composition.

slightly higher than that of *U. lactuca* collected in North Yorkshire, UK (Marsham et al. 2007).

Among Rhodophyta, *Asparagopsis taxiformis* stood out from the rest in its high lipid content (~2% of TL in DW) and *Corallina officinalis* had a higher lipid content than that reported by Marsham et al. (2007) for the same species. Gosch et al. (2012) described that Ochrophyta, chiefly species from the Dictyotales, such as *Dictyota bartayresii*, *Dictyota dichotoma* and *Spatoglossum macrodontum*, had large lipid contents of 10–12%. In our present work, *D. dichotoma* had the highest lipid content (5.2%) of all species studied, higher than that cited by Verma et al. (2017), which is probably related to the recognized higher lipid levels of *Dictyota* species in temperate climates (McDermid and Stuercke 2003).

Table 8: Atherogenicity index (AI), thrombogenicity index (TI) and hypocholesterolemic/hypercholesteloremic fatty acids ratio (hH) of macroalgae.

Group/Phylum	Species	AI	TI	hH
Chlorophyta	*Dasycladus vermicularis*	0.6 ± 0.1^{a}	0.7 ± 0.2^{bc}	1.7 ± 0.4^{e}
	Ulva sp.	0.9 ± 0.0^{abc}	0.7 ± 0.0^{bc}	0.7 ± 0.0^{a}
Rhodophyta	*Asparagopsis taxiformis*	1.5 ± 0.3^{fg}	0.7 ± 0.2^{bc}	0.8 ± 0.2^{ac}
	Corallina officinalis	1.3 ± 0.1^{deg}	1.1 ± 0.1^{ef}	0.8 ± 0.1^{ab}
	Halopithys incurva	1.1 ± 0.1^{be}	0.5 ± 0.0^{ab}	1.1 ± 0.1^{abcd}
Ochrophyta	*Cystoseira compressa*	1.2 ± 0.1^{def}	0.7 ± 0.1^{bc}	0.9 ± 0.1^{ad}
	C. usneoides	0.8 ± 0.1^{ab}	0.7 ± 0.0^{bc}	1.2 ± 0.0^{cd}
	C. humilis	1.2 ± 0.0^{ce}	0.7 ± 0.0^{bc}	1.1 ± 0.0^{bcd}
	Dictyota dichotoma	1.0 ± 0.0^{bcd}	0.4 ± 0.0^{a}	1.3 ± 0.1^{d}
	Halopteris filicina	1.1 ± 0.0^{be}	0.7 ± 0.0^{c}	0.8 ± 0.1^{ac}
	H. scoparia	1.6 ± 0.1^{g}	1.3 ± 0.1^{f}	0.7 ± 0.0^{a}
	Lobophora sp.	1.5 ± 0.1^{fg}	0.9 ± 0.0^{cd}	0.8 ± 0.0^{ac}
	Padina pavonica	1.4 ± 0.1^{eg}	1.1 ± 0.1^{de}	0.7 ± 0.0^{a}
	Sargassum vulgare	0.9 ± 0.0^{abc}	0.7 ± 0.0^{c}	1.2 ± 0.0^{d}

Results are presented as mean ± SD ($n = 3$). Different superscript letters in the same column indicate significant differences among all macroalgal species ($p < 0.05$).

Despite their low lipid levels overall, the proportions of physiologically important PUFA in the macroalgae analysed were higher than those of terrestrial plants (Wielgosz-Collin et al. 2016). C18 PUFA such as LA and ALA are considered essential FA for vertebrates since they cannot be synthesized *de novo*, and therefore, their incorporation through diet becomes necessary. The human capacity to endogenously produce LC-PUFA from their C18 PUFA precursors through successive elongation and desaturation processes was reported to be much lower than presumed (Metherel and Bazinet 2019). Since nearly 70% of the world's population does not reach the minimum recommended daily intake of n-3, due either to unhealthy nutritional habits or to difficulties accessing them, it is mandatory to include sources of n-3 LC-PUFA in the human diet for general health and wellbeing (D'Angelo et al. 2020; Taha 2020).

The present work demonstrates that the lipid class and FA composition should not be considered to be useful biomarkers for taxonomic studies in seaweeds, due to the high interspecific variability detected (Figures 2 and 3). However, green macroalgal species are often described as being characterized by high amounts of C16 FA (including 16:3n-3 and 16:4n-3) and C18 PUFA (LA and ALA, similar to terrestrial plants), with LC-PUFA being usually absent (Kendel et al. 2015; Nakamura and Li-Beisson, 2016; Santos et al. 2019). Likewise, the green species analysed here showed high proportions of 16:0, oleic acid (18:1n-9; OA), and C18 PUFA, such as LA in *Dasycladus vermicularis* and ALA in *Ulva* sp., while LC-PUFA contents were low. DHA was found in trace amounts in both species, as previously reported by McCauley et al. (2016). In contrast, red macroalgae were characterised by high levels of 16:0, OA and ARA, which also constitute an important source of EPA (Sánchez-Machado et al. 2004; Schmid et al. 2018). In the present study, *Halopithys incurva* displayed the highest proportions of ARA (~12% of total FA) and EPA (~9% of total FA), while DHA was highest in *Asparagopsis taxiformis* (6.6% of total FA). Therefore, the red macroalgae studied might be considered attractive sources of n-3 LC-PUFA (Sánchez-Machado et al. 2004), potentially promoting animal and human health and wellbeing (Murata and Nakazoe 2001; Zárate et al. 2017). In particular, *H. incurva* and *A. taxiformis* might be promising candidates to partially substitute for marine ingredients in aquafeed formulation, as with other red macroalgal species (Morais et al. 2020; Younis et al. 2018). However, some safety factors, such as the production of the halogenated toxic compounds described in *A. taxiformis* (Machado et al. 2016), must be considered before recommending this seaweed as a food or feed ingredient.

In the present study, the FA profiles of Dictyotales and Fucales were similar to those previously described by Kumari et al. (2010) and Santos et al. (2019) where 14:0, 16:0, OA, and C18-C20 PUFA were reported as the major FA. Among Ochrophyta, *Dictyota dichotoma* presented a high n-3 PUFA, especially SDA (18:4n-3) with 8.0% of total FA. SDA is the first metabolic intermediate in the conversion of ALA into EPA and DHA (Whelan 2009). The consumption of vegetable oils containing high SDA, such as *Echium* oil, was reported to improve the incorporation of n-3 PUFA, and especially EPA, into human tissues compared with vegetable oils containing ALA (Guil-Guerrero 2007).

Nonetheless, the importance of SDA in animal and human health might go beyond its function as a precursor of EPA and DHA, and offer beneficial effects similar to those of EPA (Whelan 2009).

The n-3 and n-6 PUFA families often show opposite physiological functions, with their relative proportions having implications for animal physiological and pathological mechanisms (Liu et al. 2015; Simopoulos 2016; Tocher et al. 2019). High n-6/n-3 ratios hamper the biosynthesis of n-3 LC-PUFA (Smink et al. 2012) and impair eicosanoid and docosanoid production (Zárate et al. 2017). All the macroalgae analysed had an n-6/n-3 FA ratio well below 10, as recommended by WHO (Matanjun et al. 2009) for potential human and animal health and wellbeing applications.

It is widely accepted that a reduction of SFA consumption and an increase in PUFA ingestion impacts human health positively by decreasing blood cholesterol (Moussa et al. 2014). AI, TI and hH indices indicate the relationship between pro-thrombogenic (saturated) and anti-thrombogenic (unsaturated) FAs (Özden et al. 2020), and they have been proposed as both nutritional fat quality indicators and measures of dietary propensity to influence the incidence of coronary heart disease (Moussa et al. 2014; Pérez et al. 2014; Santos-Silva et al. 2002). Therefore, lower dietary AI, TI, and higher hH would prevent the risk of appearance of coronary diseases (Gerasimenko and Logvinov 2016). In this sense, *Dasycladus vermicularis* (green alga), *Cystoseira usneoides* and *Sargassum vulgare* (brown algae), displayed the most favourable values of all species analysed for these cardiovascular health indicators.

In animal nutrition, the type of lipid in which FA is provided seems to be particularly relevant (Lund et al. 2018; Reis et al. 2021). Algal lipids can be divided into neutral lipids as storage compounds and polar lipids, including glycolipids and phospholipids, with major structural functions. Betaine lipids, not isolated in our study, are also common lipids in non-plastid membranes of algae, especially in green and brown seaweeds, and are being considered as replacement compounds for phosphatidylcholine (PC) under phosphorus deficiency (Huang et al. 2019). In fact, a reciprocal relationship between certain betaine lipids and PC has been proposed (Künzler and Eichenberger 1997; Nakamura and Li-Beisson 2016).

Although several studies have shown that Dictyotales, Fucales (Ochrophyta) and Ulvales (Chlorophyta) do not have PC (Wielgosz-Collin et al. 2016), our study supports that of Jones and Harwood (1992), where small amounts of PC were detected in fucoids such as *Fucus vesiculosus* and *Ascophyllum nodosum*. On the other hand, PC is expected in Rhodophyta species, being reported to represent up to 55–75% of total phospholipids (Wielgosz-Collin et al. 2016). PC is an interesting source of LC-PUFA-rich marine lecithin, which is of increasing interest for cosmetic, food, and pharmaceutical sectors (Alhajj et al. 2020).

All the macroalgae studied here had a higher content of glycolipids than phospholipids. Thus, Chlorophyta species had remarkably high percentages of both SQDG and DGDG, in contrast to other studies where MGDG was higher than SQDG and DGDG in green macroalgae (Khotimchenko 2002). Furthermore, MGDG and DGDG have been described as the most characteristic glycolipids in red algae (Wielgosz-Collin et al. 2016), although Khotimchenko (2002) reported high variability in glycolipid content among species. In our study, *Halopithys incurva* had the highest contents of SQDG, DGDG and MGDG among red seaweeds, while brown macroalgae had low contents of MGDG. According to Wielgosz-Collin et al. (2016), glycolipids do not seem to be valuable as a taxonomic character since they are present in all brown species.

The glycolipids MGDG and DGDG from marine organisms have been reported to have antifungal, fibrinolytic and antitumor activities (Gerasimenko and Logvinov 2016; Kendel et al. 2015; Wielgosz-Collin et al. 2016), which make seaweeds potentially interesting dietary components for human and animal nutrition, in addition to their higher LC-PUFA content than in terrestrial plants (Sahaka et al. 2020).

Corallina officinalis, *Halopithys incurva*, *Cystoseira usneoides*, *Lobophora* sp. and *Sargassum vulgare*, had high contents of phytosterols (PTS). Macroalgal PTS include several molecules such as fucosterol, stigmasterol, sitosterol and saringosterol, together with variable amounts of cholesterol (Schepers et al. 2020). PTS present benefits for cardiovascular diseases and anti-inflammatory processes (Kendel et al. 2015), and also decrease intestinal cholesterol absorption, reducing low-density lipoprotein-cholesterol (LDL-C) and therefore, reduce cardiovascular disease risk factors (Patch et al. 2006). Humans cannot biosynthesize PTS *de novo* (Kendel et al. 2015), again suggesting that macroalgae might be a potential source of these beneficial compounds for human nutrition.

5 Conclusions

The present study provided evidence of a high variability in the lipid contents, lipid classes and FA profiles of macroalgae, making a definition of a characteristic pattern within each phylum highly complex. Seasonal, environmental and even geographical factors affect these lipid profiles and should be considered in future research to ensure

biochemical stability or even to identify algal species with a sufficiently high or diverse content of lipid molecules to be of commercial interest.

Overall, the species analysed contained lower lipid levels but higher PUFA proportions than terrestrial plants, and had low n-6/n-3 FA ratios as recommended by WHO. Red macroalgae are an attractive source of n-3 LC-PUFA for human consumption and might also be considered as a potential substitute for marine ingredients in aquafeed formulations and production. The high contents of DGDG, MGDG, and PTS, together with the high levels of EPA, and low n-6/n-3 ratios makes *Halopithys incurva* an interesting macroalga from a nutritional point of view. *Asparagopsis taxiformis* (Rhodophyta) also contained a high proportion of DHA, and *Dictyota dichotoma* (Ochrophyta) an unusually high content of the nutraceutical SDA.

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