Università degli Studi di Palermo

AREA QUALITÀ, PROGRAMMAZIONE E SUPPORTO STRATEGICO
SETTORE STRATEGIA PER LA RICERCA
U. O. DOTTORATI

Dottorato in Scienze della Terra e del Mare
Dipartimento di Scienze della Terra e del Mare (DiSTeM)
Settore Scientifico Disciplinare: BIO/06 - Anatomia comparata e citologia

PRODUCTION OF HIGH VALUE-ADDED NUTRACEUTICALS IN A MULTI-TROPHIC AQUACULTURE SYSTEM WITHIN A CLOSED CIRCUIT MARINE HATCHERY (NUTRAQUA)

IL DOTTORE
ELEONORA CURCURACI

IL COORDINATORE
PROF. MARCO MILAZZO

IL TUTOR
DOTT. ANDREA SANTULLI

IL CO TUTOR
PROF.SSA CONCETTA MARIA MESSINA

CICLO XXXIV
ANNO CONSEGUIMENTO TITOLO 2022

Table of contents

Summary

The specific chapters of my dissertation are briefly presented as follows:

Chapter 1

With the aim to optimise the co-cultivation of extractive species in IMTA systems, three microalgae strains, one macroalga and one halophyte have been selected. On the microalgae *Nannochloropsis* sp., *Isochrysis galbana*, *Phaeodactylum tricornutum* have been developed protocols for their cultivation and standardization of yields in lipids, carotenoids and polyphenols, comparing, only for *P. tricornutum*, standard culture condition with nitrogen starvation condition (N-).

The macroalga *Gracilaria gracilis* and the halophyte *Arthrocnemum macrostachyum* have been selected to assess the extractive yields and total polyphenols applying innovative extractive techniques (Supercritical Fluid Extraction), compared to the traditional chemical extractive techniques. The antioxidant capacity of microalgae, *G. gracilis* and *A. macrostachyum* extracts have been assessed through specific assays.

Results showed that *Nannochloropsis* sp., *I. galbana* and *P. tricornutum* lipids and bioactive compounds as carotenoids and polyphenols can be standardized and also modulated modifying culture conditions for their cultivation in IMTA systems. As for microalgae, also *G. gracilis* and *A. macrostachyum*, due to their natural polyphenol production and the antioxidant power of the bioactive compounds extracted with green technologies, can be considered good candidates for cultivation in IMTA systems.

Since the application of bioactive compounds in nutraceutical and cosmeceutical fields requires more analysis, *in vitro* tests of the extracts have been conducted to evaluate their cytotoxicity and their potentiality as a source of antioxidants, as described in Chapter 3.

Chapter 2

Part of my research activity was focused on *P. tricornutum*, a model organism that, in relation to the variation of nutritional loads in water, as in aquaculture, can adapt its metabolism in the direction of fatty acids or antioxidant biosynthesis, as a mechanism of stress adaptation. On this diatom, some protocols for the manipulation of lipid and antioxidant biosynthesis, in relation to nitrogen supply or starvation have been assessed. Furthermore, the effect of nitrogen starvation in *P. tricornutum* was investigated at molecular level by genes expression related to lipid biosynthesis and photosynthesis. As the different culture conditions led to metabolic switches in *P. tricornutum* and affected lipid and antioxidant biosynthesis, these

preliminary results could be used as indicators of *P. tricornutum* nitrogen limitation for environmental or industrial monitoring and, moreover, the cultivation of this strain in different culture medium may have markets in the biotechnology sector for the pilot and industrial scale production of value added ingredients, especially for the food sector (**Published chapter: Curcuraci et al. [1]**).

Chapter 3

In this chapter, the bioactivity of halophyte and marine microalgae bioactive compounds extracted was investigated in human and fish cellular model systems (Hs68 and SAF-1 cell lines) to assess their protective effect against induced oxidative damage. Marine microalgae bioactive compounds have also been tested to prevent the bactericidal growth of *Vibrio harveyi* and *Vibrio anguillarum* in aquaculture facilities.

As halophytes are known for being a rich source of bioactive secondary metabolites with photoprotective activity and nutraceutical applications, *A. macrostachyum* extracts have been tested *in vitro* in Hs68 cell line UV-irradiated to evaluate their protective effect.

Regarding microalgae extracts, the experiment was based on previous studies described by Espinoza Ruiz et al. [2] who assessed the protective effect of synthetic antioxidants (gallic acid and *β*-carotene) against a mixture of the emerging contaminants-induced oxidative stress carbamazepine (CBZ), polybrominated diphenyl ether 47 (BDE-47) and cadmium chloride ($CdCl_2$) at sub-lethal doses on SAF-1 cell line.

For these reasons, the bioactivity of *Nannochloropsis* sp., *I. galbana*, *P. tricornutum* and *P. tricornutum* (N-) extracts was tested in SAF-1 cell line induced to oxidative stress with one selected chemical inducer of known pro-oxidant power (BDE-47).

Furthermore, the bioactivity of the microalgal extracts was tested to assess their bactericidal activity against two fish pathogens relevant in the aquaculture sector: *V. harveyi* and *V. anguillarum*.

In vitro tests results showed a protective effect of *A. macrostachyum* extracts against cellular oxidative damage induced by UV radiation in Hs68 cell line and microalgal extracts showed a protective effect in SAF-1 cells exposed to BDE-47.

Finally, microalgal extracts showed a dose-dependently bactericidal activity against the two fish pathogens of the genus *Vibrio* spp.

Those results confirmed the protective *in vitro* efficacy of the extracts of halophytes and microalgal strains.

Chapter 4

Qualitative and quantitative evaluation of lipids and antioxidants production have been carried out on two microalgal strains, previously collected along the Sicilian coast, then isolated and cultivated at lab-scale: *Chlorella* sp. and *Dunaliella viridis*.

In order to assess and optimise their production performances in terms of lipid, carotenoid and polyphenol yields, the two microalgae strains were subjected to thermic and salt stress.

To verify if these strains may be considered for the Sicilian outdoor aquaculture industry, the strains were grown at extreme temperature (whereas in Sicily temperature exceed 40 °C) and salinity (as high-salinity wastewater may limit contamination in aquaculture facilities).

Each stress condition was investigated on the growth performance, the productivity and the biochemical composition of the two autochthonous microalgae to verify if environmental stresses may trigger the production of high-value biomolecules.

Results suggested that *Chlorella* sp. and *D. viridis* tolerated high temperatures and high salinity but a relevant shift in lipid content, on its composition and in carotenoid's composition was observed.

These preliminary results represent a first characterization of the strains considered for possible biotechnological applications at regional-based algal industries (**Published chapter: Arena et al. [3]**).

Other activities:

On the course of the PhD project, the development of techniques for lipids and antioxidants extraction by chemical solvents and green extractive techniques have also been assessed on two other selected matrices. *Calendula* spp. and *Posidonia oceanica*. On those matrices the bioactive properties of the extracts have been tested *in vitro* at cellular level. In particular, laboratory analyses were carried out and two scientific articles have been published on:

- Extraction of polyphenols from four taxa of the genus *Calendula* collected in Sicily (*C. maritima, C. suffruticosa* subsp. *fulgida*, *C. arvensis*, and the hybrid between the first two ones), using chemical solvents (hexane, ethanol 80%, acetone 70%, and water) and by supercritical fluids extraction (SFE). Evaluation of the antioxidant power of the extracts and evaluation of their bioactive properties *in vitro* on Hs68 cell line exposed to oxidative stress, induced by H_2O_2 [4].
- Phenolic yield assessment of beach cast fresh and dried *Posidonia oceanica* leaves extracted with different solvents (ethanol 70% and methanol 80%) at different drying temperature and grinding conditions. The antioxidant bioactivity of the extracts

obtained by ethanol 70% from green leaves dried at 60 °C and ground four times (Gd-E4) was evaluated *in vitro* on Hs68 cell line, subjected to UV induced oxidative stress [5].

References:

[1] **Curcuraci, E.**; Manuguerra, S.; Messina, C.M.; Arena, R.; Renda, G.; Ioannou, T.; Amato, V.; Hellio, C.; Barba, F.J.; Santulli, A. Culture Conditions Affect Antioxidant Production, Metabolism and Related Biomarkers of the Microalgae Phaeodactylum tricornutum. *Antioxidants* 2022, *11*, 411.

[2] Espinosa Ruiz, C.; Manuguerra, S.; **Curcuraci, E.**; Santulli, A.; Messina, C.M. Carbamazepine, cadmium chloride and polybrominated diphenyl ether-47, synergistically modulate the expression of antioxidants and cell cycle biomarkers, in the marine fish cell line SAF-1. *Mar. Environ. Res.* 2020, *154*.

[3] Arena, R.; Lima, S.; Villanova, V.; Moukri, N.; **Curcuraci, E.**; Messina, C.; Santulli, A.; Scargiali, F. Cultivation and biochemical characterization of isolated Sicilian microalgal species in salt and temperature stress conditions. *Algal Res.* 2021, *59*, 102430.

[4] Messina, C.M.; Troia, A.; Arena, R.; Manuguerra, S.; Ioannou, T.; **Curcuraci, E.**; Renda, G.; Hellio, C.; Santulli, A. Species-specific antioxidant power and bioactive properties of the extracts obtained from wild mediterranean Calendula Spp. (Asteraceae). *Appl. Sci.* 2019, *9*, 4627.

[5] Messina, C.M.; Arena, R.; Manuguerra, S.; Pericot, Y.; **Curcuraci, E.**; Kerninon, F.; Renda, G.; Hellio, C.; Santulli, A. Antioxidant Bioactivity of Extracts from Beach Cast Leaves of Posidonia oceanica (L.) Delile. *Mar. Drugs 2021, Vol. 19, Page 560* 2021, *19*, 560.

Introduction

Over the last decades aquaculture has expanded rapidly ensuring the global supply of protein from fish [1]. In 2018, world capture fisheries reached 96.4 million tonnes, compared to 82.1 million tonnes produced by the aquaculture sector (about 46% of total production), which was estimated to be worth US$250 billion [1]. This data showed that around 50% of fish products, in 2018, were ensured by the global aquaculture industry, compensating for the capture fisheries production deficit [1].

In 2020 FAO confirmed that fisheries catches were declining significantly, leading to an increase in the deficit of up to 50-80 million tonnes by 2030 in meeting the global consumer demand for fish products [1]. Aquaculture is therefore playing an increasingly important role in compensating for the inability of fisheries in guaranteeing the global source of animal protein from fish, maximizing the nutritional benefits of its products while maintaining high levels of environmental sustainability [1] (Fig. 1).

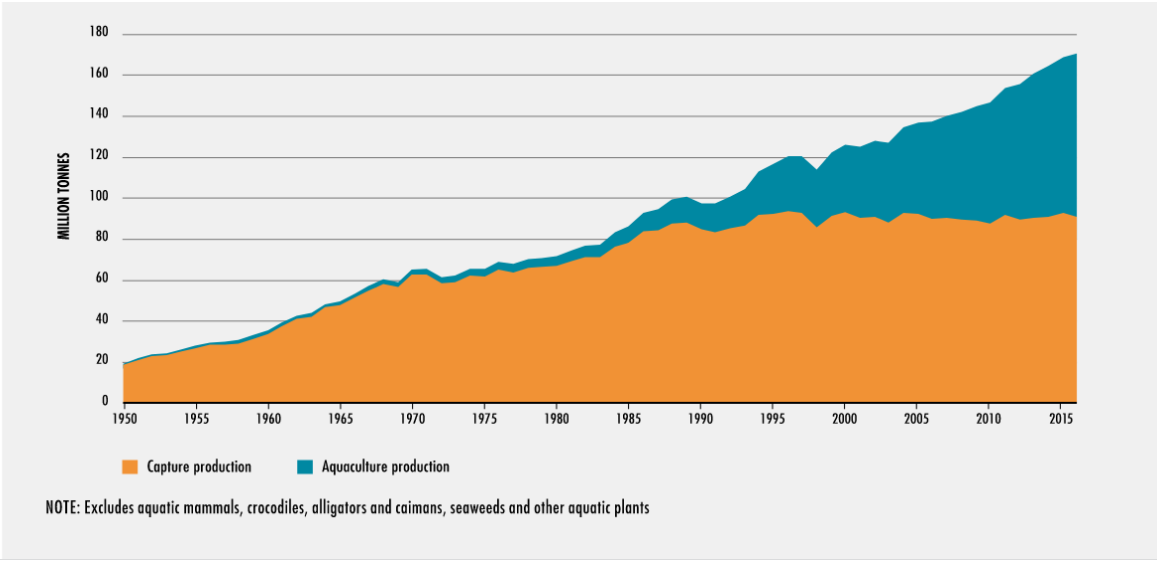


Figure 1. World capture fisheries and aquaculture production. Source FAO 2020 [1]

Aquaculture is mainly carried out in land-based and sea cage farms located along the coast. Over the years there has been strong spatial competition from industries and a consequent deterioration in public perception of this activity [2–4]. To address this competition, FAO has called for the responsible development and management of aquaculture activities in the Mediterranean and the Black Sea to establish offshore aquaculture facilities within Allocated Zones for Aquaculture (AZAs) to reduce water management conflicts and to promote sustainability [2–4].

Depending on farming practices, culture systems can be classified as extensive, semi-intensive and intensive [5]. In extensive systems there is no feeding supplement and low rearing densities are maintained. Due to its low production rates, this practice is slowly disappearing, especially in European countries. Semi-intensive systems are characterized by the addition of supplementary feed and higher densities of farmed species, compared to extensive systems. Whereas, the highest densities and the exclusive use of artificial feeds are practices used in intensive systems [5]. Intensive farming systems ensure high productivity but have a higher impact on the ecosystem. In fact, intensive aquaculture leads to energy loss, with regard to organic matter and in terms of economic costs, proving to be the least sustainable among farming practices [5,6].

In 2018, the European aquaculture was mainly developed in monoculture, dominated by the farming of two marine finfish: seabass (49.2%) and seabream (42.2%) (Figure 2) [7].

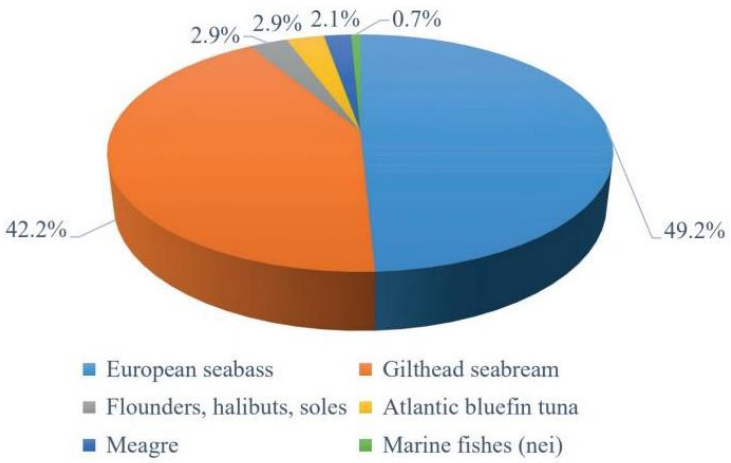


Figure 2. Percentage of European aquaculture production of marine fish species in 2018. Source: Hough 2022 [7]

Nevertheless, recently in the Mediterranean Sea a diversification of farmed species is occurring, with the introduction of new farmed species such as meagre, red porgy (*Pagrus pagrus*) and other seabreams species [7].

Since over the years, a large part of consumers has become very aware and demand increasingly higher guarantees in terms of quality, traceability and production conditions [8], aquaculture development must ensure responsible farming practices that provide food products while maintaining the integrity of aquatic ecosystems and sustainability of the aquaculture industry [1].

To achieve these goals, it is necessary to establish management practices that are efficient, diversified in terms of products and sustainable from both an environmental and a social point of view [6].

Higher levels of sustainability and productivity in the aquaculture sector can be ensured through the development of the Integrated Multi-Trophic Aquaculture (IMTA) (Fig. 3), a polyculture system based on recreating a simplified natural ecosystem that maximises economic and production yields and minimises environmental impacts [9–11].

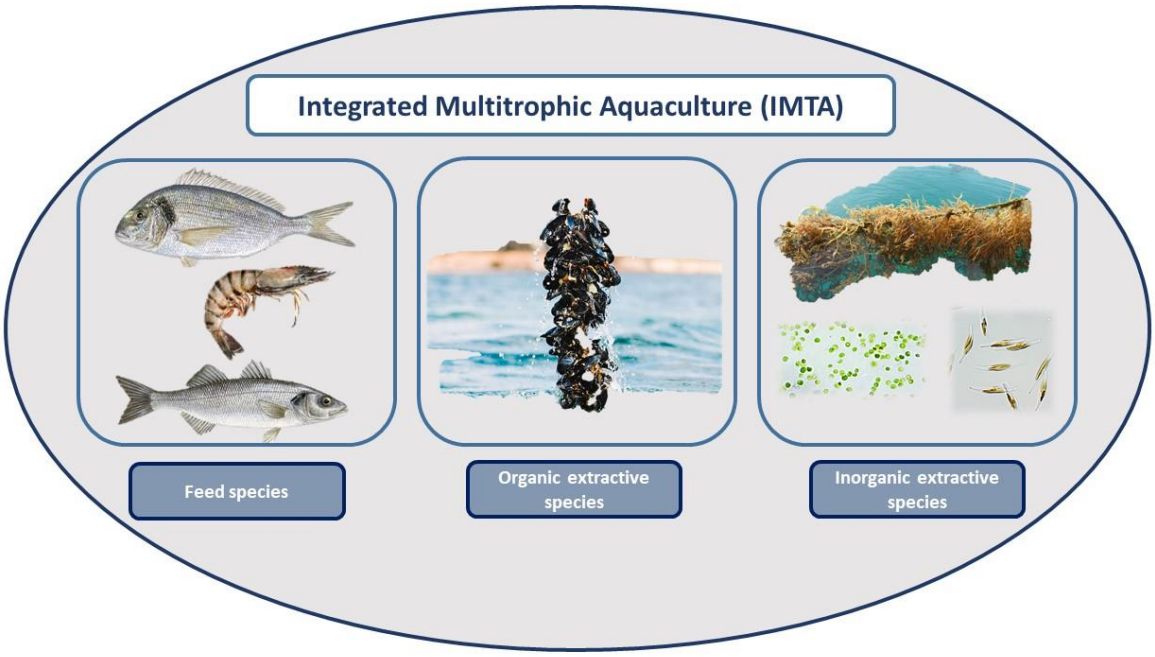


Figure 3. Integrated multitrophic aquaculture (IMTA) scheme

IMTA is an ideal system based on the co-culture of pellet-farmed species with organic (e.g., fish, echinoderms, molluscs) and inorganic (e.g., micro/macro-algae, macrophytes) extractive species [6], recreating the natural food chain through the exploitation of species belonging to different trophic levels and having complementary functions [9–11]. In this way, faces naturally produced by the fish, as well as unconsumed feed (only 25-40% of the nitrogen present in feed is assimilated by the farmed fish species [12]) are reused as nutrients by the co-cultured species in the aquaculture facility [10,11,13]. As in a natural food chain, at every level, in aquatic cultivated organisms there is a loss of energy, uneaten feed, nutrient excretions and faeces. An ecologically engineer balanced IMTA system includes feeding species, inorganic and organic extractive species such as microalgae, macroalgae and plants, deposit-feeders such as polychaetes, sea cucumbers and sea urchins [10,11,14–17] and filter

feeders (mussels) that recover nutrients in the form of particulate organic matter (POM) [17]. IMTA extractive species, acting as living filters, ensures nutrient recycling improving the environmental performance of aquaculture sites [15,18], biomass production as food, feed and as source of high value bioactive molecules [11,15,19]. Indeed, extracted and isolated bioactive compounds and natural pigments of the co-cultivated species, are recognised for their use as functional foods in nutraceuticals and in cosmeceuticals and pharmaceuticals industries, due to their antioxidant and antimicrobial properties which are important for the welfare of farmed species and human health [11,15]. This, as well as having a role in biomitigation of the impact of aquaculture activity, leads to an increase in the commercial role and important economic value of co-cultures, as well as sustainability of the activity, product diversification with a guarantee of higher economic stability and higher consumer acceptability due to the application of sustainable management practices [6,9,20]. Therefore, IMTA increases yields through a more efficient use of resources developing new value chains [15].

In the state of world fisheries and aquaculture by FAO 2020 [1] an increase to 25 million tonnes, in 2018, in the total non-fed aquaculture production has been reported (Fig. 4) of which extractive species production represented the 57.4% of the total global aquaculture production.

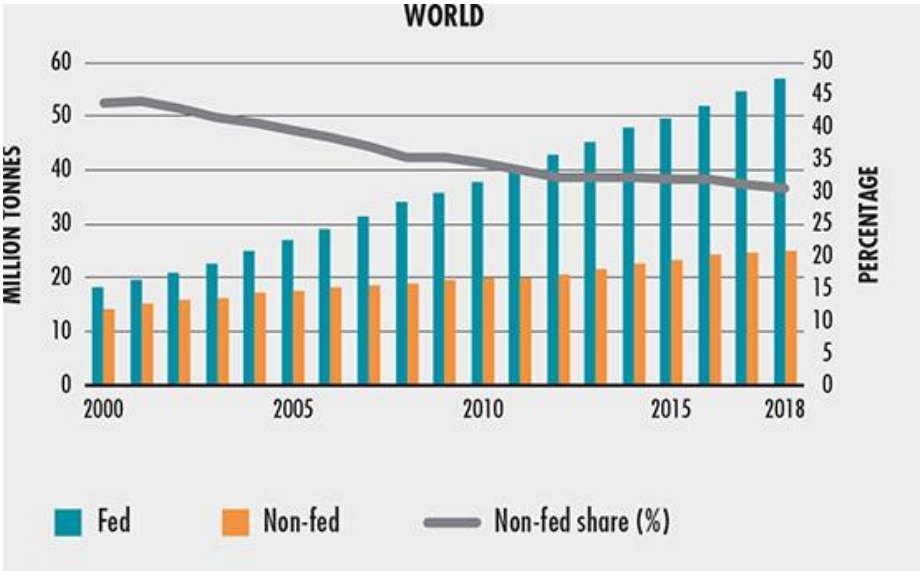


Figure 4. Fed and non-fed aquaculture production, 2000–2018. Source FAO 2020 [1]

IMTA has its origins in Asia where the ratio of fish to extractive aquaculture in 2012 was estimated to be around 1:1 (3.5:1 in Europe) [21,22]. In Asia, extractive organisms such as

molluscs and algae are widely utilized in IMTA's industrial sectors and their high profitability stimulates their production [10,21,22]. Only recently IMTA farming has found a place and attracted interest among producers in Western countries [7], where aquaculture is mainly dominated by monospecific cultivation of fish, which has led to a doubling of the demand for fish oil and fishmeal in the last decade and a low reuse of the waste produced by the aquaculture sector [1,10,23]. This slow adoption of technologies to support IMTA can be attributed to poor legislative, regulatory and social progress and support, consumer's misinformation, economic reasons such as lack of direct financial benefits to the entrepreneur and lack of foresight in responsible management of coastal waters to ensure sustainability and profitability of the adoption of IMTA systems [7,9,10,20].

The legislative framework for IMTA systems is complex and non-harmonised, often focused on monoculture and characterised by low consumer awareness. These conditions could be fixed through closer collaboration between the research sector, through the development of pilot-scale IMTA facilities and subsequent scale-up, through improved regulatory frameworks by legislators and through consumer empowerment for the development of the bioeconomy [3,9,10,15,24].

Over the past 20 years, interest in marine drug discovery (Fig. 5a) and the studies on the bioactivity of metabolites derived from microalgae have increased (Fig. 5b) [25], as well as the interest by industries and consumers in functional foods, defined as "food products fortified with special constituents that possess advantageous physiological effects" [26,27].

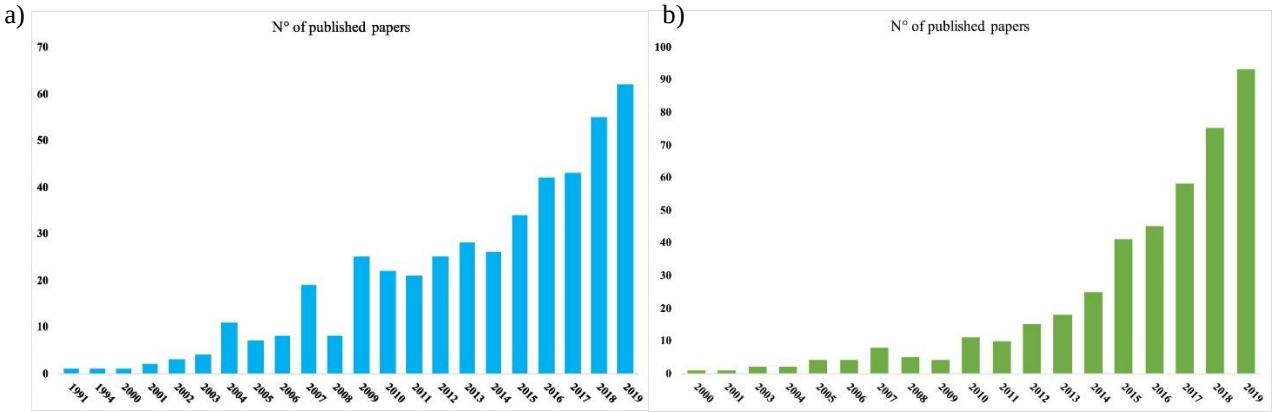


Figure 5. Number of publications per year on the subject of "marine drug discovery", listed in the source index of the Web of Science (Clarivate Analytics) from 1991 to 2019 (a); Number of publications per year on the subject of "microalgae bioactivity" listed in the source index of PubMed.gov database, NCBI, last 20 years (b). Source: Savio et al. 2021 [25]

Functional foods are obtained from traditional foods enriched with nutraceuticals, bioactive compounds extracted from natural plants or animal sources. Since plants, micro and macroalgae produce high value bioactive compounds with proved beneficial effects on human health [27–30], the co-cultivation of extractive species in IMTA systems ensures a higher profitability and productivity.

Aquatic organisms are an important source for the extraction of valuable metabolites, which can be extracted with traditional techniques using chemical solvents or with green innovative extractive methods. Particularly, algae and plants produce biomass rich in value-added products, whit a proved bioactivity [11,31–34]. Valuable metabolites can be divided into primary metabolites and complex secondary metabolites [35]. Among them, carotenoids, antioxidants, fatty acids, enzymes, polymers, peptides, toxins, sterols, and vitamins are compounds suitable for commercialization [36,37].

Primary metabolites are produced by plants and algae converting CO_2 in lipids, carbohydrates and proteins during photosynthesis [36]. Lipids have numerous functions such as energy storage, signalling and structural components of cell membranes. In macroalgae, the lipid content is estimated to be less than 5%, while in microalgae, depending on the strain considered and the culture conditions, it is between 1 and 40% [38]. Microalgae lipids are produced as oil globule starting from the formation of acetyl-CoA by the acetyl-coenzyme A carboxylase (ACCase) gene which catalyzes the *de novo* fatty acid biosynthesis through acetyl CoA carboxylation [35,39] and their production can be enhanced by specific environmental conditions [35,40]. The majority of the lipids obtained from microalgae are long-chain polyunsaturated of the ω-3 series (PUFA), eicosapentaenoic acid (EPA, C 20:5 ω-3) and docosahexaenoic acid (DHA, C 22:6 ω-3) with proved beneficial effect on human health, as well as being essential for farmed species [11,35,40]. Lipid content and fatty acids profile in microalgae are strongly linked to the investigated species and influenced by environmental stressors (such as salinity and temperature) [35,41].

Secondary metabolites are produced from plants and algae for protection and survival [42,43] and are widely recognised to have high antiviral, antibacterial and cytotoxic activity and to act as natural antioxidants [44,45].

- Photosynthetic pigments include carotenoids such as carotenes (e.g. *β*-carotene and lycopene) and xanthophylls (e.g. lutein and zeaxanthin), and chlorophylls (chlorophyll *a* and *b*) are photosynthetic pigments rich in health-beneficial, produced as secondary bioactive metabolites for the adaptation to extreme environmental conditions [43]. These pigments counter the oxidative and photo-

oxidative stress that lead to reactive oxygen species (ROS) generation damaging the photosynthetic apparatus [43,46]. Due to their characteristics, carotenoids and chlorophylls are widely used in the food, nutraceutical and pharmacological industries [47].

- Phenolic compounds which include phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids) and flavonoids, are products of the secondary metabolism of plants and algae (in equal to or less quantities than the minimum observed in terrestrial plants [48]), having a key role as defense against environmental stress [43,49]. Phenolic compounds have one or more aromatic rings bearing hydroxyl substituents [49,50] and are recognized as important healthy natural antioxidant phytochemicals that, inhibiting macromolecular oxidation, have anticancer, anti-microbial, anti-inflammatory, anti-diabetic and antiviral properties [43,48,51].

To maximize the number of microalgae-derived compounds included in clinical trials and the potential of microalgae bioactive compounds, both the physiological and the metabolic status of the microalgal culture have to be considered [25]. The former ensures a better control on biotic and abiotic factors during the cultivation process, the latter detects chemical changes in the composition of the extract, and consequently changes on its bioactivity, by the use of "omics" technologies [25].

The bioavailability and potential beneficial effects of algal metabolites have shown promising results in both *in vivo* and *in vitro* tests [45]. *In vivo* tests include the use of experimental animals. Despite the increase in regulations on the use of chemicals in Europe requiring more testing, the need to reduce *in vivo* tests for ethical, scientific and economic reasons has encouraged to investigate alternative testing methods, replacing vertebrates with invertebrates or with *in vitro* tests [52,53].

In vitro assays are based on the use of cell lines to evaluate the interaction of bioactive molecules with the organism at the cellular level, since the cellular response is the first expression of the effect of the compound, as well as the most sensitive [52,53]. In the last decade, *in vitro* assays with human and fish cell lines have become widely used in assessing the effects induced by natural compound extracts and xenobiotics [25,52,53]. In this view, it is essential, particularly regarding microalgae-derived metabolites, to design bioactivity tests taking into account the selectivity factor of the cell line used to test the compound [25].

Since many technologies have been developed for the extraction of bioactive compounds obtained from different matrices, the growing interest in nutraceutical and cosmeceutical products has directed research towards innovative green extractive methods [54]. Traditional

extractive techniques often require long extraction times, have low yields and are carried out using toxic solvents, whereas SFE is a green separation technology using supercritical fluids as solvent [55]. Since each substance is characterised by a critical temperature (T_c) and pressure (p_c), when temperature and pressure are increased above T_c and p_c simultaneously, the substance is defined as supercritical fluid, a phase with gas and liquid properties with increased extraction efficiency [28,56].

CO_2 used as supercritical fluid, has been widely investigated in SFE technology from the food, pharmaceutical and nutraceutical industries [28,51,55–59]. Due to its T_c (31°C) and p_c (73.8 bar), CO_2 is considered the ideal supercritical fluid for bioactive compounds extraction [28,29,60]. Moreover, CO_2 is non-flammable, non-toxic, non-polluting, is inert and is a low-cost, easily reusable gas, overcoming many of the limitations of traditional chemical solvents extractions [28,29,60].

Among separation techniques, SFE is considered advantageous due to its high selectivity and short extraction times [28,29,56,60] and profitable since the natural bioactive compounds extracted by SFE have found applications in the food, chemical, nutraceutical and pharmaceutical industries [28,29,55,59].

Another important benefit related to the development of IMTA is connected to the capacity of the co-cultivated extractive species to uptake and/or utilize nutrients and feed species by-products, establishing new value chains and more efficient and cost-effective inorganic and organic pollutants removal methods, compared to the conventional treatment techniques [61,62].

In accordance with the principles promoted by the European Commission and by FAO at Rio+20 conference fostering the “Blue Growth” concept, which refers to a green economy and sustainable development of the maritime and coastal sectors [63], it is necessary to implement the concept of reuse of resources by recognising the potentialities of by-products produced within aquaculture facilities [18]. Undigested feed and faeces are some of the main concerns related to intensive aquaculture farming, in fact, particulate and dissolved organic matter exceeding from this activity, may lead to eutrophication condition [64], although the concentrations of dissolved nutrients and solids released in the wastewater depend on the farming practice: cages, ponds, raceways or recirculating aquaculture systems (RAS) [18].

Among farming practices, RAS is an indoor and outdoor system designed to recirculate water between the culture phase and water treatment [18,65,66]. RAS systems consist of a series of units designed for P and N removal (assimilated by cultured extractive organisms), solids removal, and disinfection. Since RAS systems require less water to operate and have a lower

environmental impact, the interest in the use of RAS is steadily increasing, especially in combination with microalgae in aquaculture facilities [18,65,66]. Indeed, cultivation of microalgae in recirculating aquaculture systems not only ensures the removal of nitrogen and phosphorus from wastewater but also produce biomass maximizing the production, proving the increasing profitability of the use of polyculture and multi-trophic systems in the aquaculture sector [18,65,66].

Among dissolved nutrients of aquaculture wastewaters, the most significant are nitrogen (N) and phosphorus (P) [18]. Nitrogen (N) is one of the main causes of eutrophication and represents the 60–90% of dissolved waste produced by aquaculture activities [18,61,66]. Dissolved inorganic nitrogen (DIN) is released as toxic (NH_3) and non-toxic ammonia (NH_4^+). Ammonia can be nitrified to nitrate (NO_3^-) not well tolerated by aquatic species, or reduced to nitrite (NO_2^-), a highly reactive oxidant and toxic, by biological activities [18,66].

Phosphorus (P), that is considered a limiting element in the marine environment, is present in wastewaters as orthophosphate, polyphosphate, pyrophosphate and metaphosphate [67]. It is lost such as feed uneaten and mainly realised as solid with faeces [18].

Nitrogen and phosphorous uptake and recycling from wastewater effluents are at the base of bioremediation, defined as "a sustainable strategy focused on alleviating the negative effects of aquaculture effluents" [68] (Figure 6).

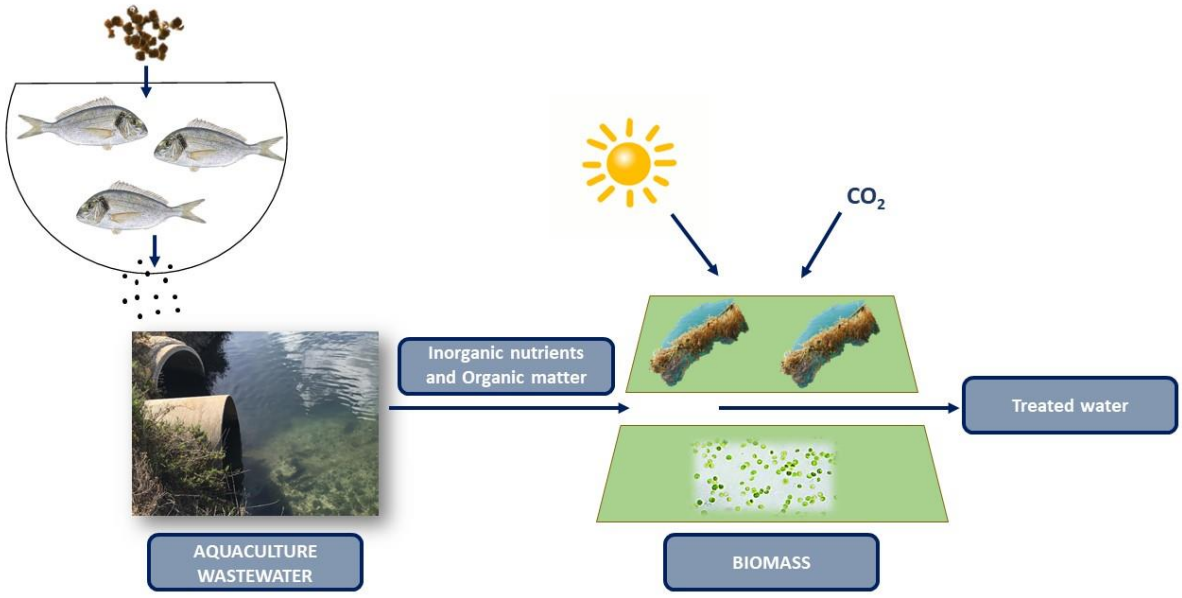


Figure 6. Aquaculture wastewater bioremediation

More specifically, "phytoremediation" refers to the use of plants [61,68] while "phycoremediation" to the use of algae [69] for wastewater remediation, as terrestrial and aquatic plants assimilate nitrogen either as NH_4^+or NO_3^-, especially microalgae show higher photosynthetic efficiency and nitrogen uptake rates [61,64]. Moreover, algae and plants can absorb and store phosphorus, as essential elements for their growth [61].

Bioremediation studies have been conducted by using aquatic plants, halophytes, microalgae and macroalgae [18,64,65,70–74], due to their capacity to reduce chemical and biological oxygen demand, to remove nutrients and heavy metals and to their antibacterial capacity [64]. This make the biofiltering activity of plants and algae on the recycling of nutrients and by-products in aquaculture, a key element in increasing the production of sustainable food products [18].

The state of the art regarding the co-cultivation of extractive species such as microalgae, macroalgae and plants in IMTA systems suggests that the international scientific community and industrial sectors are focusing on promoting IMTA systems since, co-cultivation of extractive and fed species converts by-products of farmed species into resources, mitigating the environmental impact of aquaculture by improving its efficiency and sustainability [8–10,65,72,74,75].

The ability of the microalgae *Nannochloropsis* sp., *Isochrysis galbana*, *Phaeodactylum tricornutum*, *Chlorella* sp. and *Dunaliella viridis*, of the macroalga *Gracilaria gracilis* and of the halophyte *Arthrocnemum macrostachyum* in modulating their lipid and bioactive compounds production in different culture conditions has been largely studied to improve their yield rates and to reduce industrial production costs [36,67,76,77], due to the increasing demand of natural bioactive compounds on the pharmacological, chemical and cosmeceutical industries [67,77] and due to their ability to accumulate exceeding nutrients from wastewaters [78–82]. Microalgae, macroalgae and plants extracts have also demonstrated to have a protective effect, on countering physical and chemical oxidative stresses at cellular level [83–88] and to inhibit the development of pathogenic bacteria in aquaculture facilities [70,89–91].

Although environmental changes are known for their ability to induce a variation in lipid content and antioxidant capacity in microalgae, little is known about molecular mechanisms involved in stress adaptation [39,70]. The use of microalgal local strains for the development of regional-based microalgal industry in Sicily is still uninvestigated [47], as well as the comparison of traditional and green extractive methods of bioactive compounds obtained from the cultivation of *G. gracilis* and *A. macrostachyum* in a seabream and seabass hatchery

pond of a Sicilian aquaculture facility and on the capacity of the extracted bioactive compounds in countering induced oxidative stress and in inhibiting the bactericidal activities .

Objective of the study

The aim of my PhD project entitled "Production of high value-added nutraceuticals in a multi-trophic aquaculture system within a closed circuit marine hatchery (NUTRAQUA)" was: to select, among microalgae, macroalgae and plants, those species considered valuable candidates for the optimization of their cultivation in IMTA systems for the production of high value bioactive molecules and the extraction of these bioactive compounds for their use and industrial scale-up in both cosmeceuticals and nutraceuticals fields.

The project was developed in collaboration with the industrial partner "Aljè Società Agricola S.r.l." interested in the development of multi-trophic aquaculture systems within the "Azienda Ittica San Giorgio Soc. Agr. A R. L.", a land-based Recirculating Aquaculture System (RAS) hatchery of seabream and seabass.

To achieve these goals, different experiments were conducted on three microalgae strains, widely used: *Nannochloropsis* sp., *I. galbana, P. tricornutum* and on two local microalgae strains collected along the Sicilian coast and then isolated: *Chlorella* sp. and *D. viridis.* Specific aims were: a comparative assessment of different culture conditions related to nitrogen deprivation, salinity and temperature variations, through the manipulation of the culture medium on all microalgae strains; the assessment of the total lipid content and fatty acid classes composition; the assessment of the antioxidant activity of the microalgal obtained compounds extracted by ethanol and methanol; to study the combination of biochemical and molecular analyses in *P. tricornutum*. Particularly, the expression of genes involved in lipid metabolism and in photosynthesis was investigated to evaluate how this strain adapts its metabolism in the direction of lipid or antioxidant biosynthesis when cultivated under standard or nitrogen starvation condition, as a mechanism of stress adaptation.

Among seaweeds and plants, *G. gracilis* and *A. macrostachyum* have been selected to assess polyphenols yields quantification, comparing traditional and innovative extractive methods, and to assess the antioxidant activity of the bioactive compounds extracted.

The bioactivity of *Nannochloropsis* sp., *I. galbana, P. tricornutum* extracts was tested *in vitro* utilizing cultivated *Sparus aurata* fibroblast (SAF-1) cell line SAF-1, pretreated with bioactive compounds and exposed to polybrominated diphenyl ether 47 - BDE-47, an environmental contaminant with a high chemical oxidative capacity. Furthermore, the microalgal extracts were used to assess their bactericidal activity against two fish pathogens

of the genera *Vibrio* spp.. Bioactivity of *A. macrostachyum* extracts has been evaluated *in vitro* pretreating human fibroblast (Hs68) cells line, subjected to UV induced oxidative stress.

References

[1] FAO, The State of World Fisheries and Aquaculture 2020. Sustainability in action., Rome, 2020. https://doi.org/10.4060/ca9229en.

[2] P. Sanchez-Jerez, I. Karakassis, F. Massa, D. Fezzardi, J. Aguilar-Manjarrez, D. Soto, R. Chapela, P. Avila, J.C. Macias, P. Tomassetti, G. Marino, J.A. Borg, V. Franičević, G. Yucel-Gier, I.A. Fleming, X. Biao, H. Nhhala, H. Hamza, A. Forcada, T. Dempster, Aquaculture's struggle for space: The need for coastal spatial planning and the potential benefits of Allocated Zones for Aquaculture (AZAs) to avoid conflict and promote sustainability, Aquac. Environ. Interact. 8 (2016) 41–54. https://doi.org/10.3354/aei00161.

[3] B.H. Buck, M.F. Troell, G. Krause, D.L. Angel, B. Grote, T. Chopin, State of the art and challenges for offshore Integrated multi-trophic aquaculture (IMTA), Front. Mar. Sci. 5 (2018) 165. https://doi.org/10.3389/fmars.2018.00165.

[4] FAO, General fisheries commission for the mediterranean commission générale des pêches pour la méditerranée, 2018.

[5] E.A. Baluyut, Aquaculture Methods and Practices : a Selected Review, United Nations Dev. Program. Food Agric. Organ. United Nations. (1989) 1–41.

[6] M. Troell, A. Joyce, T. Chopin, A. Neori, A.H. Buschmann, J.G. Fang, Ecological engineering in aquaculture - Potential for integrated multi-trophic aquaculture (IMTA) in marine offshore systems, Aquaculture. 297 (2009) 1–9. https://doi.org/10.1016/j.aquaculture.2009.09.010.

[7] C. Hough, REGIONAL REVIEW ON STATUS AND TRENDS IN AQUACULTURE DEVELOPMENT IN EUROPE – 2020, FAO Fish. Aquac. Circ. No. 1232/1. (2022).

[8] M.J.M. Bottema, S.R. Bush, P. Oosterveer, Assuring aquaculture sustainability beyond the farm, Mar. Policy. 132 (2021) 104658. https://doi.org/10.1016/j.marpol.2021.104658.

[9] D. Knowler, T. Chopin, R. Martínez-Espiñeira, A. Neori, A. Nobre, A. Noce, G. Reid, The economics of Integrated Multi-Trophic Aquaculture: where are we now and where do we need to go?, Rev. Aquac. 12 (2020) 1579–1594. https://doi.org/10.1111/raq.12399.

[10] M. Thomas, A. Pasquet, J. Aubin, S. Nahon, T. Lecocq, When more is more: taking

advantage of species diversity to move towards sustainable aquaculture, Biol. Rev. 96 (2021) 767–784. https://doi.org/10.1111/brv.12677.

[11] A.T. Mansour, M. Ashour, A.E. Alprol, A.S. Alsaqufi, Aquatic Plants and Aquatic Animals in the Context of Sustainability: Cultivation Techniques, Integration, and Blue Revolution, Sustainability. 14 (2022) 3257. https://doi.org/10.3390/su14063257.

[12] A. Endut, A. Jusoh, N. Ali, Nitrogen budget and effluent nitrogen components in aquaponics recirculation system, Desalin. Water Treat. 52 (2014) 744–752. https://doi.org/10.1080/19443994.2013.826336.

[13] A.G.J. Tacon, Trends in Global Aquaculture and Aquafeed Production: 2000–2017, Rev. Fish. Sci. Aquac. 28 (2020) 43–56. https://doi.org/10.1080/23308249.2019.1649634.

[14] K. Cutajar, L. Falconer, A. Massa-Gallucci, R.E. Cox, L. Schenke, T. Bardócz, A. Sharman, S. Deguara, T.C. Telfer, Culturing the sea cucumber Holothuria poli in open-water integrated multi-trophic aquaculture at a coastal Mediterranean fish farm, Aquaculture. 550 (2022) 1–11. https://doi.org/10.1016/j.aquaculture.2021.737881.

[15] M. Fraga-Corral, P. Ronza, P. Garcia-Oliveira, A.G. Pereira, A.P. Losada, M.A. Prieto, M.I. Quiroga, J. Simal-Gandara, Aquaculture as a circular bio-economy model with Galicia as a study case: How to transform waste into revalorized by-products, Trends Food Sci. Technol. 119 (2022) 23–35. https://doi.org/10.1016/j.tifs.2021.11.026.

[16] S. Albrektsen, R. Kortet, P.V. Skov, E. Ytteborg, S. Gitlesen, D. Kleinegris, L.-T. Mydland, J.Ø. Hansen, E.-J. Lock, T. Mørkøre, P. James, X. Wang, R.D. Whitaker, B. Vang, B. Hatlen, E. Daneshvar, A. Bhatnagar, L.B. Jensen, M. Øverland, Future feed resources in sustainable salmonid production: A review, Rev. Aquac. (2022) 1–23. https://doi.org/10.1111/raq.12673.

[17] M.S. Hargrave, G.M. Nylund, S. Enge, H. Pavia, Co-cultivation with blue mussels increases yield and biomass quality of kelp, Aquaculture. 550 (2022) 737832. https://doi.org/10.1016/j.aquaculture.2021.737832.

[18] C. Campanati, D. Willer, J. Schubert, D.C. Aldridge, Sustainable Intensification of Aquaculture through Nutrient Recycling and Circular Economies: More Fish, Less Waste, Blue Growth, Rev. Fish. Sci. Aquac. 30 (2021) 143–169. https://doi.org/10.1080/23308249.2021.1897520.

[19] T. Chopin, Aquaculture Aquaculture , Integrated Multi-trophic (IMTA) aquaculture integrated multi-trophic (IMTA), in: Sustain. Food Prod., Springer New York, 2013: pp. 184–205. https://doi.org/10.1007/978-1-4614-5797-8_173.

[20] K. Barrington, N. Ridler, T. Chopin, S. Robinson, B. Robinson, Social aspects of the sustainability of integrated multi-trophic aquaculture, Aquac. Int. 18 (2010) 201–211. https://doi.org/10.1007/s10499-008-9236-0.

[21] A.D. Hughes, K.D. Black, Going beyond the search for solutions: Understanding trade-offs in European integrated multi-trophic aquaculture development, Aquac. Environ. Interact. 8 (2016) 191–199. https://doi.org/10.3354/AEI00174.

[22] Fao, The State of world fisheries and aquaculture, 2012, Choice Rev. Online. 50 (2013) 50-5350-50–5350. https://doi.org/10.5860/choice.50-5350.

[23] Fao, The State of World Fisheries and Aquaculture 2018, 2018.

[24] X. Wang, A. Cuthbertson, C. Gualtieri, D. Shao, A review on mariculture effluent: Characterization and management tools, Water (Switzerland). 12 (2020) 1–24. https://doi.org/10.3390/w12112991.

[25] S. Savio, R. Congestri, C. Rodolfo, Are we out of the infancy of microalgae-based drug discovery?, Algal Res. 54 (2021) 102173. https://doi.org/10.1016/j.algal.2020.102173.

[26] I. Siró, E. Kápolna, B. Kápolna, A. Lugasi, Functional food. Product development, marketing and consumer acceptance-A review, Appetite. 51 (2008) 456–467. https://doi.org/10.1016/j.appet.2008.05.060.

[27] A.C. Freitas, D. Rodrigues, T.A.P. Rocha-Santos, A.M.P. Gomes, A.C. Duarte, Marine biotechnology advances towards applications in new functional foods, Biotechnol. Adv. 30 (2012) 1506–1515. https://doi.org/10.1016/j.biotechadv.2012.03.006.

[28] K. Gandhi, S. Arora, A. Kumar, C. Kamal Gandhi, Industrial applications of supercritical fluid extraction: a review, Int. J. Chem. Stud. 5 (2017) 336–340.

[29] R.P.F.F. da Silva, T.A.P. Rocha-Santos, A.C. Duarte, Supercritical fluid extraction of bioactive compounds, TrAC - Trends Anal. Chem. 76 (2016) 40–51. https://doi.org/10.1016/j.trac.2015.11.013.

[30] F. Al Khawli, M. Pateiro, R. Domínguez, J.M. Lorenzo, P. Gullón, K. Kousoulaki, E. Ferrer, H. Berrada, F.J. Barba, Innovative green technologies of intensification for

valorization of seafood and their by-products, Mar. Drugs. 17 (2019) 689. https://doi.org/10.3390/md17120689.

[31] P. Cheng, Y. Li, C. Wang, J. Guo, C. Zhou, R. Zhang, Y. Ma, X. Ma, L. Wang, Y. Cheng, X. Yan, R. Ruan, Integrated marine microalgae biorefineries for improved bioactive compounds: A review, Sci. Total Environ. 817 (2022) 1–11. https://doi.org/10.1016/j.scitotenv.2021.152895.

[32] N.A. ElNaker, A.F. Yousef, L.F. Yousef, A review of Arthrocnemum (Arthrocaulon) macrostachyum chemical content and bioactivity, Phytochem. Rev. 19 (2020) 1427–1448. https://doi.org/10.1007/s11101-020-09686-5.

[33] S. Thanigaivel, N. Chandrasekaran, A. Mukherjee, John Thomas, Seaweeds as an alternative therapeutic source for aquatic disease management, Aquaculture. 464 (2016) 529–536. https://doi.org/10.1016/j.aquaculture.2016.08.001.

[34] S. Bhatia, R. Makkar, T. Behl, A. Sehgal, S. Singh, M. Rachamalla, V. Mani, M.S. Iqbal, S.G. Bungau, Biotechnological Innovations from Ocean : Transpiring Role of Marine Drugs in Management of Chronic Disorders, Mol. Plant. 27 (2022) 1–23.

[35] A.K. Minhas, P. Hodgson, C.J. Barrow, A. Adholeya, A review on the assessment of stress conditions for simultaneous production of microalgal lipids and carotenoids, Front. Microbiol. 7 (2016) 546. https://doi.org/10.3389/fmicb.2016.00546.

[36] Y.H. Park, S. Il Han, B. Oh, H.S. Kim, M.S. Jeon, S. Kim, Y.E. Choi, Microalgal secondary metabolite productions as a component of biorefinery: A review, Bioresour. Technol. 344 (2022) 126206. https://doi.org/10.1016/J.BIORTECH.2021.126206.

[37] R. Perumal Samy, P. Gopalakrishnakone, Therapeutic Potential of Plants as Anti-Microbials for Drug Discovery, Evidence-Based Complement. Altern. Med. 7 (2010) 283–294. https://doi.org/10.1093/ECAM/NEN036.

[38] M. Demarco, J. Oliveira de Moraes, Â.P. Matos, R.B. Derner, F. de Farias Neves, G. Tribuzi, Digestibility, bioaccessibility and bioactivity of compounds from algae, Trends Food Sci. Technol. 121 (2022) 114–128. https://doi.org/10.1016/j.tifs.2022.02.004.

[39] E. Curcuraci, S. Manuguerra, C.M. Messina, R. Arena, G. Renda, T. Ioannou, V. Amato, C. Hellio, F.J. Barba, A. Santulli, Culture Conditions Affect Antioxidant Production, Metabolism and Related Biomarkers of the Microalgae Phaeodactylum

tricornutum, Antioxidants. 11 (2022) 411. https://doi.org/10.3390/antiox11020411.

[40] B. Ramesh Kumar, G. Deviram, T. Mathimani, P.A. Duc, A. Pugazhendhi, Microalgae as rich source of polyunsaturated fatty acids, Biocatal. Agric. Biotechnol. 17 (2019) 583–588. https://doi.org/10.1016/J.BCAB.2019.01.017.

[41] M.R. Gauthier, G.N.A. Senhorinho, J.A. Scott, Microalgae under environmental stress as a source of antioxidants, Algal Res. 52 (2020) 102104. https://doi.org/10.1016/j.algal.2020.102104.

[42] A. Karthikeyan, A. Joseph, B.G. Nair, Promising bioactive compounds from the marine environment and their potential effects on various diseases, J. Genet. Eng. Biotechnol. 20 (2022) 14. https://doi.org/10.1186/s43141-021-00290-4.

[43] L.S.S. Hulkko, T. Chaturvedi, M.H. Thomsen, Extraction and Quantification of Chlorophylls, Carotenoids, Phenolic Compounds, and Vitamins from Halophyte Biomasses, Appl. Sci. 12 (2022) 840. https://doi.org/10.3390/app12020840.

[44] B. Pradhan, P.P. Bhuyan, S. Patra, R. Nayak, P.K. Behera, C. Behera, A.K. Behera, J.-S. Ki, M. Jena, Beneficial effects of seaweeds and seaweed-derived bioactive compounds: Current evidence and future prospective, Biocatal. Agric. Biotechnol. 39 (2022) 102242. https://doi.org/10.1016/J.BCAB.2021.102242.

[45] B. Pradhan, R. Nayak, S. Patra, B.P. Jit, A. Ragusa, M. Jena, Molecules Bioactive Metabolites from Marine Algae as Potent Pharmacophores against Oxidative Stress-Associated Human Diseases: A Comprehensive Review, Molecules. 26 (2020) 1–25. https://doi.org/10.3390/molecules26010037.

[46] D. Nagarajan, S. Varjani, D.J. Lee, J.S. Chang, Sustainable aquaculture and animal feed from microalgae – Nutritive value and techno-functional components, Renew. Sustain. Energy Rev. 150 (2021) 111549. https://doi.org/10.1016/J.RSER.2021.111549.

[47] R. Arena, S. Lima, V. Villanova, N. Moukri, E. Curcuraci, C. Messina, A. Santulli, F. Scargiali, Cultivation and biochemical characterization of isolated Sicilian microalgal species in salt and temperature stress conditions, Algal Res. 59 (2021) 102430. https://doi.org/10.1016/J.ALGAL.2021.102430.

[48] A. Saide, K.A. Martínez, A. Ianora, C. Lauritano, Unlocking the Health Potential of Microalgae as Sustainable Sources of Bioactive Compounds, Int. J. Mol. Sci. 2021,

Vol. 22, Page 4383. 22 (2021) 4383. https://doi.org/10.3390/IJMS22094383.

[49] K.G. Ramawat, J.M. Mérillon, Phenolic compounds: introduction, in: Nat. Prod., 2013: pp. 1544–1580. https://doi.org/10.1007/978-3-642-22144-6.

[50] S. Maqsood, S. Benjakul, A. Abushelaibi, A. Alam, Phenolic Compounds and Plant Phenolic Extracts as Natural Antioxidants in Prevention of Lipid Oxidation in Seafood: A Detailed Review, Compr. Rev. Food Sci. Food Saf. 13 (2014) 1125–1140. https://doi.org/10.1111/1541-4337.12106.

[51] N. Dhouibi, S. Manuguerra, R. Arena, A. Mahdhi, C.M. Messina, A. Santulli, H. Dhaouadi, Screening of antioxidant potentials and bioactive properties of the extracts obtained from two Centaurea L. species (C. kroumirensis coss. and C. sicula L. subsp sicula), Appl. Sci. 10 (2020) 2267. https://doi.org/10.3390/app10072267.

[52] M. Goswami, B.S. Yashwanth, V. Trudeau, W.S. Lakra, Role and relevance of fish cell lines in advanced in vitro research, Mol. Biol. Rep. 49 (2022) 2393–2411. https://doi.org/10.1007/s11033-021-06997-4.

[53] K. Rehberger, C. Kropf, H. Segner, In vitro or not in vitro: a short journey through a long history, Environ. Sci. Eur. 30 (2018) 23. https://doi.org/10.1186/s12302-018-0151-3.

[54] T. Puchkova, S. Khapchaeva, V. Zotov, A. Lukyanov, A. Solovchenko, Microalgae as a source of cosmeceuticals, Mar. Biol. J. 6 (2021) 67–81. https://doi.org/10.1016/B978-0-12-819597-0.00013-1.

[55] F. Sahena, I.S.M. Zaidul, S. Jinap, A.A. Karim, K.A. Abbas, N.A.N. Norulaini, A.K.M. Omar, Application of supercritical CO2 in lipid extraction - A review, J. Food Eng. 95 (2009) 240–253. https://doi.org/10.1016/j.jfoodeng.2009.06.026.

[56] M. Raventós, S. Duarte, R. Alarcón, Application and Possibilities of Supercritical CO2 Extraction in Food Processing Industry: An Overview, Food Sci. Technol. Int. 8 (2002) 269–284. https://doi.org/10.1106/108201302029451.

[57] C.M. Messina, A. Troia, R. Arena, S. Manuguerra, T. Ioannou, E. Curcuraci, G. Renda, C. Hellio, A. Santulli, Species-specific antioxidant power and bioactive properties of the extracts obtained from wild mediterranean Calendula Spp. (Asteraceae), Appl. Sci. 9 (2019) 4627. https://doi.org/10.3390/app9214627.

[58] C.M. Messina, R. Arena, S. Manuguerra, Y. Pericot, E. Curcuraci, F. Kerninon, G.

Renda, C. Hellio, A. Santulli, Antioxidant Bioactivity of Extracts from Beach Cast Leaves of Posidonia oceanica (L.) Delile, Mar. Drugs 2021, Vol. 19, Page 560. 19 (2021) 560. https://doi.org/10.3390/MD19100560.

[59] M. Herrero, A. Cifuentes, E. Ibañez, Sub- and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae - A review, Food Chem. 98 (2006) 136–148. https://doi.org/10.1016/j.foodchem.2005.05.058.

[60] Q. Lang, C.M. Wai, Supercritical fluid extraction in herbal and natural product studies - A practical review, Talanta. 53 (2001) 771–782. https://doi.org/10.1016/S0039-9140(00)00557-9.

[61] H.M. Mustafa, G. Hayder, Recent studies on applications of aquatic weed plants in phytoremediation of wastewater: A review article, Ain Shams Eng. J. 12 (2021) 355–365. https://doi.org/10.1016/J.ASEJ.2020.05.009.

[62] F.R. Pilone, The Waste Management Of Large-Scale Recirculating Aquaculture Systems And Potential Value-Added Products From The Waste Stream, 2021.

[63] A.M. Eikeset, A.B. Mazzarella, B. Davíðsdóttir, D.H. Klinger, S.A. Levin, E. Rovenskaya, N.C. Stenseth, What is blue growth? The semantics of "Sustainable Development" of marine environments, Mar. Policy. 87 (2018) 177–179. https://doi.org/10.1016/j.marpol.2017.10.019.

[64] H. Milhazes-Cunha, A. Otero, Valorisation of aquaculture effluents with microalgae: The Integrated Multi-Trophic Aquaculture concept, Algal Res. 24 (2017) 416–424. https://doi.org/10.1016/J.ALGAL.2016.12.011.

[65] E. Villar-Navarro, C. Garrido-Pérez, J.A. Perales, The potential of different marine microalgae species to recycle nutrients from recirculating aquaculture systems (RAS) fish farms and produce feed additives, Algal Res. 58 (2021) 102389. https://doi.org/10.1016/j.algal.2021.102389.

[66] J. Van Rijn, Waste treatment in recirculating aquaculture systems, Aquac. Eng. 53 (2013) 49–56. https://doi.org/10.1016/j.aquaeng.2012.11.010.

[67] V. Dolganyuk, D. Belova, O. Babich, A. Prosekov, S. Ivanova, D. Katserov, N. Patyukov, S. Sukhikh, Microalgae: A promising source of valuable bioproducts, Biomolecules. 10 (2020) 1–24. https://doi.org/10.3390/biom10081153.

[68] M. Martinez-Porchas, L.R. Martinez-Cordova, J.A. Lopez-Elias, M.A. Porchas-Cornejo, Bioremediation of Aquaculture Effluents, Microb. Biodegrad. Bioremediation. (2014) 542–555. https://doi.org/10.1016/B978-0-12-800021-2.00024-8.

[69] B. Koul, K. Sharma, M.P. Shah, Phycoremediation: A sustainable alternative in wastewater treatment (WWT) regime, Environ. Technol. Innov. 25 (2022) 102040. https://doi.org/10.1016/j.eti.2021.102040.

[70] B. Mishra, A. Tiwari, A.E.D. Mahmoud, Microalgal potential for sustainable aquaculture applications: bioremediation, biocontrol, aquafeed, Clean Technol. Environ. Policy. 1 (2022) 1–13. https://doi.org/10.1007/s10098-021-02254-1.

[71] A. Shahid, S. Malik, H. Zhu, J. Xu, M.Z. Nawaz, S. Nawaz, M. Asraful Alam, M.A. Mehmood, Cultivating microalgae in wastewater for biomass production, pollutant removal, and atmospheric carbon mitigation; a review, Sci. Total Environ. 704 (2020) 135303. https://doi.org/10.1016/j.scitotenv.2019.135303.

[72] L. Mennella, D. Tosco, F. Alberti, L. Cembalo, M. Crescimanno, T. Del Giudice, A. Galati, M. Moglie, A. Scardera, G. Schifani, F. Solfanelli, G. Cicia, Perspectives and challenges of small scale plant microalgae cultivation. Evidences from Southern Italy, Algal Res. 45 (2020) 101693. https://doi.org/10.1016/j.algal.2019.101693.

[73] A. Buhmann, J. Papenbrock, Biofiltering of aquaculture effluents by halophytic plants: Basic principles, current uses and future perspectives, Environ. Exp. Bot. 92 (2013) 122–133. https://doi.org/10.1016/j.envexpbot.2012.07.005.

[74] M. Colette, L. Guentas, P. Gunkel-Grillon, N. Callac, L. Della Patrona, Is halophyte species growing in the vicinity of the shrimp ponds a promising agri-aquaculture system for shrimp ponds remediation in New Caledonia?, Mar. Pollut. Bull. 177 (2022) 113563. https://doi.org/10.1016/j.marpolbul.2022.113563.

[75] G. Biswas, P. Kumar, T.K. Ghoshal, M. Kailasam, D. De, A. Bera, B. Mandal, K. Sukumaran, K.K. Vijayan, Integrated multi-trophic aquaculture (IMTA) outperforms conventional polyculture with respect to environmental remediation, productivity and economic return in brackishwater ponds, Aquaculture. 516 (2020) 734626. https://doi.org/10.1016/J.AQUACULTURE.2019.734626.

[76] S. Hemaiswarya, R. Raja, R.R. Kumar, V. Ganesan, C. Anbazhagan, Microalgae: A

sustainable feed source for aquaculture, World J. Microbiol. Biotechnol. 27 (2011) 1737–1746. https://doi.org/10.1007/s11274-010-0632-z.

[77] W. Levasseur, P. Perré, V. Pozzobon, A review of high value-added molecules production by microalgae in light of the classification, Biotechnol. Adv. 41 (2020) 107545. https://doi.org/10.1016/j.biotechadv.2020.107545.

[78] Y. Zhou, H. Yang, H. Hu, Y. Liu, Y. Mao, H. Zhou, X. Xu, F. Zhang, Bioremediation potential of the macroalga Gracilaria lemaneiformis (Rhodophyta) integrated into fed fish culture in coastal waters of north China, Aquaculture. 252 (2006) 264–276. https://doi.org/10.1016/j.aquaculture.2005.06.046.

[79] Y.F. Yang, X.G. Fei, J.M. Song, H.Y. Hu, G.C. Wang, I.K. Chung, Growth of Gracilaria lemaneiformis under different cultivation conditions and its effects on nutrient removal in Chinese coastal waters, Aquaculture. 254 (2006) 248–255. https://doi.org/10.1016/j.aquaculture.2005.08.029.

[80] N. Spanò, D. Di Paola, M. Albano, A. Manganaro, M. Sanfilippo, C. D'Iglio, G. Capillo, S. Savoca, Growth performance and bioremediation potential of Gracilaria gracilis (Steentoft, L.M. Irvine & Farnham, 1995), Int. J. Environ. Stud. (2021). https://doi.org/10.1080/00207233.2021.1954775.

[81] M. Lopes, A. Sanches-Silva, M. Castilho, C. Cavaleiro, F. Ramos, Halophytes as source of bioactive phenolic compounds and their potential applications, Crit. Rev. Food Sci. Nutr. (2021) 1–24. https://doi.org/10.1080/10408398.2021.1959295.

[82] U. Waller, A.K. Buhmann, A. Ernst, V. Hanke, A. Kulakowski, B. Wecker, J. Orellana, J. Papenbrock, Integrated multi-trophic aquaculture in a zero-exchange recirculation aquaculture system for marine fish and hydroponic halophyte production, Aquac. Int. 23 (2015) 1473–1489. https://doi.org/10.1007/S10499-015-9898-3/TABLES/5.

[83] S. Maqsood, S. Benjakul, A. Abushelaibi, A. Alam, Phenolic Compounds and Plant Phenolic Extracts as Natural Antioxidants in Prevention of Lipid Oxidation in Seafood: A Detailed Review, Compr. Rev. Food Sci. Food Saf. 13 (2014) 1125–1140. https://doi.org/10.1111/1541-4337.12106.

[84] D. Treutter, Significance of flavonoids in plant resistance: a review, Environ. Chem. Lett. 4 (2006) 147–157. https://doi.org/10.1007/s10311-006-0068-8.

[85] G. Surget, V. Stiger-Pouvreau, K. Le Lann, N. Kervarec, C. Couteau, L.J.M. Coiffard,

F. Gaillard, K. Cahier, F. Guérard, N. Poupart, Structural elucidation, in vitro antioxidant and photoprotective capacities of a purified polyphenolic-enriched fraction from a saltmarsh plant, J. Photochem. Photobiol. B Biol. 143 (2015) 52–60. https://doi.org/10.1016/J.JPHOTOBIOL.2014.12.018.

[86] R. Tsao, Z. Deng, Separation procedures for naturally occurring antioxidant phytochemicals, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 812 (2004) 85–99. https://doi.org/10.1016/j.jchromb.2004.09.028.

[87] S.P. Cuellar-Bermudez, I. Aguilar-Hernandez, D.L. Cardenas-Chavez, N. Ornelas-Soto, M.A. Romero-Ogawa, R. Parra-Saldivar, Extraction and purification of high-value metabolites from microalgae: Essential lipids, astaxanthin and phycobiliproteins, Microb. Biotechnol. 8 (2015) 190–209. https://doi.org/10.1111/1751-7915.12167.

[88] Y. Ghasemi, S. Rasoul-Amini, M.H. Morowvat, Algae for the production of pharmaceuticals, in: Bioprocess Sci. Technol., 2011: pp. 163–183.

[89] S. Manuguerra, L. Caccamo, M. Mancuso, R. Arena, A.C. Rappazzo, L. Genovese, A. Santulli, C.M. Messina, G. Maricchiolo, The antioxidant power of horseradish, Armoracia rusticana, underlies antimicrobial and antiradical effects, exerted in vitro, Nat. Prod. Res. 34 (2020) 1567–1570. https://doi.org/10.1080/14786419.2018.1517121.

[90] C.A. Molina-Cárdenas, M. del P. Sánchez-Saavedra, Inhibitory effect of benthic diatom species on three aquaculture pathogenic vibrios, Algal Res. 27 (2017) 131–139. https://doi.org/10.1016/J.ALGAL.2017.09.004.

[91] F. Kokou, P. Makridis, M. Kentouri, P. Divanach, Antibacterial activity in microalgae cultures, Aquac. Res. 43 (2012) 1520–1527. https://doi.org/10.1111/j.1365-2109.2011.02955.x.

Chapter 1 – Production of lipids and antioxidants in three microalgae, in the macroalga *Gracilaria gracilis* and in the halophyte *Arthrocnemum macrostachyum*: preliminary lab experiments

1.1 Abstract

The application of integrated multi-trophic aquaculture systems represents a must nowadays to fulfill the fish products needing in a sustainable way. In this framework, this study was dedicated to the evaluation, at lab scale, of lipid and antioxidant production on two microalgae widely used in the aquaculture sector, *Nannochloropsis* sp. and *Isochrysis galbana*, and on the assessment of lipid and antioxidant production of *Phaeodactylum tricornutum*, by a comparative evaluation of standard culture condition and nitrogen deprivation. Polyphenol yields were compared at lab scale in the macroalga *Gracilaria gracilis* and on the halophyte *Arthrocnemum macrostachyum*, grown at the aquaculture hatchery pond, using different extractive methods as a first step for their cultivation scale-up in IMTA systems.

Regarding microalgae, *Nannochloropsis* sp. showed the highest total lipid content (31.38±0.11%), followed by *I. galbana* (26.31± 0%), *P. tricornutum* cultivated under nitrogen deprivation (N-) (25.31±0.6 %) and *P. tricornutum* cultivated under standard conditions (18.61±0.32 %). In *P. tricornutum* N- a good ratio of omega-3 and omega-6 polyunsaturated fatty acids (PUFA) was maintained (respectively 2.05±0.12 g $100g^{-1}$ and 0.21±0.01 g $100g^{-1}$), compared to *P. tricornutum* cultivated under standard conditions (respectively 2.63±0.19 g $100g^{-1}$ and 0.19 ± 0.01 g $100g^{-1}$). In *P. tricornutum* cultivated under standard conditions showed, in response to a lower lipid content, a higher polyphenol (3.07±0.18 mg GAE g^{-1} DW) and carotenoid content (2.70±0.23 mg g^{-1} DW), resulting in a higher antioxidant activity (DPPH IC50 6.78±1.37 mg DW mL^{-1} and reducing power test EC50 172.71±2.90 mg DW mL^{-1}), compared to other strains considered.

In *G. gracilis* and *A. macrostachyum* extracts the highest antioxidant activity was obtained by green Supercritical Fluid Extraction with co-solvent (SFE+ethanol 96%) (respectively DPPH IC50 10.35±1.05 mg DW mL^{-1} and reducing power test EC50 36.54±5.7 mg DW mL^{-1} in *G. gracilis* and reducing power test EC50 19.95±1.9 mg DW mL^{-1} in *A. macrostachyum*), except for DPPH IC50 value of *A. macrostachyum* which was obtained with solvent N-hexane (DPPH IC50 3.94±1 mg DW mL^{-1}).

Modulation of microalgae culture conditions, as well as evaluation of macroalgae and halophytes lipid and antioxidant yields, are useful preliminary tools for optimising co-cultivation of extractive species in IMTA. Furthermore, as oxidative stress is widely recognised as a potentially harmful condition for all organisms and cellular systems, the extracted bioactive compounds could be then tested in *in vitro* systems for their utilization in nutraceutical and cosmeceutical fields.

KEYWORDS: microalgae, *Gracilaria gracilis, Arthrocnemum macrostachyum*, lipids, bioactive compounds, green extraction, IMTA

1.2 Introduction

The demand for biologically active compounds from the pharmacological, chemical and cosmeceutical industries, from the agricultural and the aquaculture sector, for feed and functional foods production is constantly growing [1,2]. In this context, microalgae are considered a promising resource, especially in the integrated multi-trophic aquaculture (IMTA) sector, thanks to their multiple applications and sustainability [3,4]. In fact, microalgae, that are organisms capable of rapid growth and high biomass productivity [5], are considered the basis of the food chain in the aquaculture sector as, being rich in protein, carbohydrates and lipids [6], they represent an important source of feeding for reared organisms [7]. Furthermore, microalgae play an important role in the control and removal of nutrients [6], in the production of biofuel [5], in preventing pathogens growth and antibiotic overuse [8] and as a source for the production of primary and secondary metabolites [5,9].

In the recent years, the study of microalgal bioactive secondary metabolites has increased exponentially, from the isolation and characterization of bioactive chemicals, to the study of the physiological control mechanisms involved, with a view to improving yields and reducing production costs [10]. The most commonly cultivated and utilized microalgal strains are: *Tetraselmis* sp. as a source of nutraceuticals and for wastewater treatment [11,12], *Chlorella* sp. for its high production of bioactive compounds [11,13], *Isochrysis galbana* for its biochemical profile rich in fatty acids omega-3 and ascorbic acid [11,14], *Nannochloropsis* sp., recognized as a good source of eicosapentaenoic acid (EPA (20:5n3)), is used for feeding rotifers and is a source of several pigments such as chlorophyll a and carotenoids [11,15], *Phaeodactylum tricornutum* for its rapid growth and excellent lipid storage capacity (up to 35%) [11,16–18] and *Dunaliella salina* for its *β*-carotene production ability [19].

Modulation of the microalgae culture medium ensures the production of specific bioactive compounds for nutraceutical, pharmaceutical and cosmeceuticals applications [1,2].

The biochemical composition and physiological state of algal cells can be modified by several environmental factors; as a result, *in vitro* manipulation of the culture medium, can alter the chemical composition and growth characteristics of the microalgae [20]. Microalgae respond to nutrient deprivation stress, in particular with quantitative and qualitative changes in lipid content [21], in fatty acid composition and thus in microalgal growth conditions [16,22,23].

Lipids production and accumulation is a mechanism activated during prolonged environmental nutritional stress [24], thus a modulation of the nutritional deprivation period can lead to a controlled accumulation of lipids [25].

Nutritional stress can induce the generation of free radical species in the cell and changes in antioxidant content, such as the production of reactive oxygen species (ROS), the accumulation of antioxidants and carotenoids [26,27]. An increase in the content of alpha-tocopherol in *Nannochloropsis oculata* [28] and ascorbic acid in *Dunaliella salina* [29] has been observed under nitrogen deprivation stress.

These characteristics make microalgae cultivation a rapidly expanding field in IMTA sector.

Due to the growing interest shown by research in identifying sources of natural compounds with bioactive properties, in order to replace the use of synthetic substances in cosmetics, pharmaceuticals and the agrifood sector [30], interest in macroalgae and halophytes cultivation in IMTA is rapidly increasing [3]. Plant organisms are a very interesting source of bioactive compounds due to their life habits, which have evolved in the production and accumulation of protective substances, known as secondary metabolites, with bioactive properties [31]. These include carotenes and xanthophylls, isoprene compounds with photoprotective and antioxidant functions, such as β-carotene, lycopene and fucoxanthin, polyphenolic compounds such as flavonoids and structural polysaccharides such as agar, alginates and carrageenan [32,33].

The selection of macroalgae to be cultivated is strictly linked to their growth rate, nitrogen availability, on their resistance to epiphytes and pathogens, on their bio-filter capacity and on their bioactive compounds production [34]. Among macroalgae, the red seaweed *Gracilaria* (phylum Rhodophyta) is a genus extensively cultivated, especially in East Asia [35], industrially important for agar extraction [36], for the production of antimicrobial compounds such as acrylic acid and eicosanoids, which are derived from the oxidative metabolism of polyunsaturated fatty acids (PUFA) such as eicosanoic acid and arachidonic acid [36]. Furthermore, due to the remarkable ability to accumulate and utilize dissolved nutrients in

water, *Gracilaria* spp. can be cultivated for bioremediation [37–39]. *Gracilaria* spp., naturally growing in transitional habitats, could be then promoted for its cultivation in IMTA, for both the reduction of organic loads in pond effluents and for the simultaneous production of bioactive compounds.

Halophytes, are salt-tolerant plants that have great potential as their cultivation can take place under drought stress and excess salt conditions and are a good resource for industrial applications [33,40,41]. Their cultivation has been tested in marine recirculating aquaculture systems (RAS), ensuring nutrient recycling and removal [33,42] as well as their ability to produce potent bioactive compounds with antioxidant and antimicrobial potentials [33,41]. In halophytes, phenolic compounds play a crucial role in the defence against ROS and oxidative stress and act as pathogen growth inhibitors [33].

Since halophytes are great sources of functional foods that already have a well-established place in the market [33], these plants can be considered ideal candidates for cultivation in aquaculture systems for the production and extraction of natural bioactive compounds.

In the recent years, the extraction of bioactive compounds by green innovative extractive methods has grown, replacing traditional solvents extraction methods [41,43]. Supercritical fluids extraction (SFE) is a green promising environmental friendly technology that, through the use of CO_2 instead of organic solvent, allows to achieve not toxic high-quality extracts [43,44].

In this chapter a preliminary study was conducted on three microalga strains, one macroalga and one halophyte, selected for their characteristics that make them suitable for co-culture in IMTA for food production, nutrient removal and extraction of bioactive compounds for the nutraceutical and cosmeceutical sectors. Lipid production, total polyphenol content and total carotenoid content under standard condition were evaluated in *Nannochloropsis* sp. (Ochrophyta, Eustigmatophyceae), *I. galbana* (Isochrysidales, Haptophyta) and *P. tricornutum* (Bacillariophyta). In *P. tricornutum* the effects of nitrogen deprivation (N-) on yields, was also evaluated compared to standard conditions.

The antioxidant capacity of the extracts has been assessed through two assay methods: DPPH radical scavenging activity and Reducing power.

On the base of the preliminary studies conducted (Chapter 1), the next step was to evaluate the protective effect of *Nannochloropsis* sp., *I. galbana* and *P. tricornutum* extracts against chemical compounds and their bactericidal activity in *in vitro* experiments with the aim to confirm the ability of these extracts in the nutraceutical and cosmeceutical sectors (Chapter 3). Furthermore, on the macroalga *Gracilaria gracilis* (Stackhouse) Steentoft, L.M. Irvine &

Farnham, 1995 and on the halophyte *Arthrocnemum macrostachyum* (Moric.) C. Koch the global total yields, total polyphenol contents and antioxidant activity were assessed through DPPH radical scavenging activity and Reducing power assay, comparing traditional antioxidants solvent extractions with the green SFE with co-solvent method (SFE+ethanol 96%), for their cultivation scale-up in IMTA systems and to evaluatethe protective effect of *A. macrostachyum* extracts against UV radiation in *in vitro* systems (Chapter 3), for their utilization in the nutraceutical and cosmeceutical industries.

1.3 Materials and Methods

1.3.1 Microalgae

1.3.1.1 Maintenance of microalgal cultures

Monospecific cultures of *Nannochloropsis* sp. (Figure 1A) and *I. galbana* (Figure 1B) have been provided by the PhD industrial partner, "Aljè Società Agricola S.r.l." and cultivated by "Azienda Ittica San Giorgio Soc. Agr. A R. L.", while *P. tricornutum* strain (AC171) was obtained from the Algobank culture collection (France) (Figure 1C). *P. tricornutum* was cultivated under standard condition and under nitrogen deprivation (N-) condition using culture media indicated in Table 1.

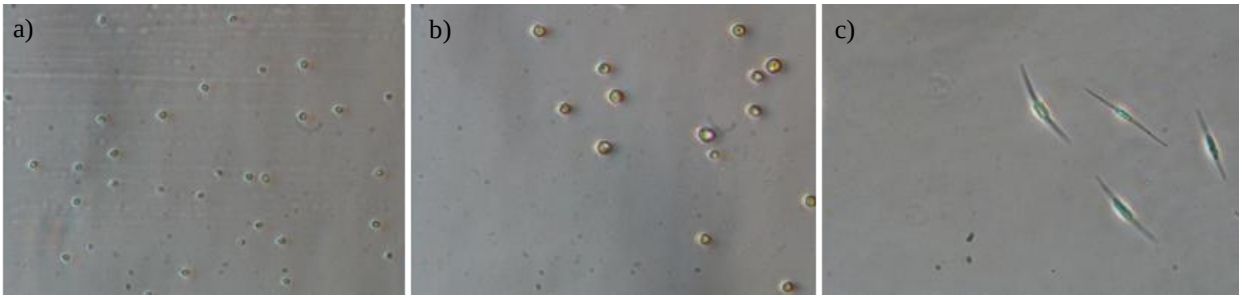


Figure 1. Phase-contrast microscopy images of *Nannochloropsis* sp. (a), *I. galbana* (b) and *P. tricornutum* (c).

Table 1. Microalgal culture media composition

Salts	*Nannochloropsis* sp. and *Isochrysis galbana*	*Phaeodactylum tricornutum*	*Phaeodactylum tricornutum* N-
NaCl	363 mM	363 mM	363 mM
Na_2SO_4	25.0 mM	25.0 mM	25.0 mM
KCl	8.04 mM	8.04 mM	8.04 mM
$NaHCO_3$	2.07 mM	2.07 mM	2.07 mM
KBr	-	725 µM	725 µM
H_3BO_3	-	372 µM	372 µM
NaF	-	65.7 µM	65.7 µM
$MgCl_2$	41.20 mM	41.20 mM	41.20 mM
CaCl2	9.14 mM	9.14 mM	9.14 mM
$SrCl_2$	-	82.0 µM	82.0 µM
$NaNO_3$	-	**549** µM	**10** µM
NaH_2PO_4	-	21.0 µM	21.0 µM
Na_2SiO_3	-	205 µM	205 µM
$CuSO_4$	-	40.0 nM	40.0 nM
Vitamins	50 mM	-	-
TRIS-HCl	-	10.0 mM	10.0 mM

A volume of 150 mL of stock culture was inoculated into 600 mL of each sterile culture medium in duplicate. Microalgae cultures were maintained in aseptic environments at 18-20°C, pH 8 and permanent illumination (LED light).

For nutritional and antioxidant composition analysis, microalgae were recovered by centrifugation for 20 min at 4000 rpm. The recovered pellet was frozen at -80°C and freeze-dried before use.

1.3.1.2 Microalgal total lipids quantification and fatty acids composition

Total lipids were determined according to Folch et al. [45]. The quantification of total lipid content was performed gravimetrically and resuspended in N-hexane. All the samples were analyzed in triplicate (n=3).

Fatty acid methyl esters were determined according to Lepage and Roy [46] and were identified and quantified by gas chromatography. Gas chromatography was performed under the conditions described by Messina et al. [47].

A Perkin Helmer Clarus 580 gas chromatograph equipped with a flame ionisation detector (FID) with a capillary-type column (omegawax 320, Supelco, Bellefonte, PA, USA), 30 m x 0.32 mm x 0.25 *m high-polar phase (polyethylene glycol), was used.

Fatty acids were identified by comparing the chromatogram of the sample with standard fatty acid mixtures (mix PUFA of fish oil, Supelco, Bellefonte, PA, USA). The concentration of the individual acids was calculated from the ratio of the area of the individual peaks to the area of the internal standard (C 23:0 trichosanoic acid) at known concentration.

1.3.1.3 Microalgae antioxidants extraction

Antioxidants were extracted from the microalgal biomass according to Messina et al. [48].
In detail, 5 mL of 80% ethanol was added to 0.5 g of each lyophilised sample.
Samples were homogenised at Ultraturrax (T25 basic, Ika) cold for 5 min and centrifuged at 4000 rpm at 4°C for 10 min.
The supernatant was recovered and stored in the freezer at -20°C until analysis.

1.3.1.5 Total carotenoids content

Determination of carotenoids content was performed spectrophotometrically according to Miazek and Ledakowicz [49] and Maadane et al. [50].
Aliquots of the extracts were prepared at a concentration of 1 mg mL^{-1} in ethanol. The absorbance of the samples was measured at 470, 648 and 664 nm by spectrophotometer (Multiskan, Thermo-Fisher Scientific). Ethanol was used as blank.
Total carotenoids content was calculated using the equations of Lichtenthaler et al. (1) [51]:

$$Chl_a = 13{,}36\ x\ Abs_{664} - 5{,}19\ Abs_{648}$$

$$Chl_b = 27{,}43\ x\ Abs_{648} - 8{,}12\ Abs_{664} \quad (1)$$

$$\textbf{\textit{Total carotenoids}} = \frac{(100\ x\ Abs_{470} - 1{,}63\ x\ Chl_a - 109{,}96\ x\ Chl_b)}{221}$$

1.3.2 Macroalga and halophyte

1.3.2.1 Sample collection and processing

Gracilaria gracilis and *Arthrocnemum macrostachyum* were grown at the aquaculture hatchery pond of the "Azienda Ittica San Giorgio Soc. Agr. A R. L.", located in Petrosino (Trapani, Italy), from the PhD industrial partner "Aljè Società Agricola S.r.l.".

Figure 2. "Azienda Ittica San Giorgio Soc. Agr. A R. L." hatchery pond in Petrosino (Trapani, Italy)

Samples of *G. gracilis* (Fig. 3a) and *A. macrostachyum* (Fig.3b) were collected, in triplicate, between June and July.

a)

b)

Figure 3. Fresh samples of *G. gracilis* (a) and dried sample of *A. macrostachyum* (b)

After collection, samples were cleaned of epiphytes, rinsed with sterile distilled water, dried for 48 h at 40°C, and then homogenized with a blender. Dried powder fraction between 250 and 500 µm of *G. gracilis* and *A. macrostachyum* was extracted by different solvents (Ethanol 96% and Methanol for *G. gracilis*; Acetone 70% and N-hexane for *A. macrostachyum)* and SFE with co-solvent (SFE+ethanol 96%) (ratio 1:10w/v), to assess the yields in total polyphenols.

1.3.2.2 Extraction by solvents

G. gracilis and *A. macrostachyum* phenolic compounds were extracted using solvents with different polarity, such as Ethanol 96%, Methanol, Acetone 70% and N-hexane, according to Messina et al. modified [48]. One gram of dried sample of *G. gracilis* and *A. macrostachyum* was transferred into a flask containing 10 mL of the solvents (Ethanol 96%, Methanol for *G. gracilis* and Acetone 70% and N-hexane for *A. macrostachyum*, respectively). Samples were then homogenized for 5 min at 4 °C with Ultra-Turrax (IKA, Werke Staufen, Germany) at 24.000 rpm.

The extraction yield (w/w) was calculated evaporating on one aliquot of each extract (Ethanol 96%, Methanol, Acetone 70% and N-hexane) in a rotary vacuum evaporator and weighed as described by Messina et al. [48]. The following Equation was used (2):

$$\text{Yield of extract (\%)} = \text{weight of extract/weight of sample} \times 100 \quad (2)$$

1.3.2.3 Supercritical Fluid Extraction

G. gracilis and *A. macrostachyum* phenolic compounds extraction was carried out by SFE technology with co-solvent, according to Messina et al. modified [48].

A supercritical extraction unit (SFE System model HELIX, Applied Separations Allentown, PA, USA), equipped with a CO_2 pump unit and 50 mL steel vessel was used. 1:2 (w/w) of *G. gracilis* and *A. macrostachyum* dried powder (5 g), and hydroscopic dispersing agent (Applied Separations, Allentown, PA, USA) were placed in the extraction vessel sandwiched with defatted glass wool forming a fixed bed in the vessel. The unit was pressurized and the sample was kept in contact with supercritical CO_2 and co-solvent (ethanol 95%), at pre-established conditions of temperature (60 °C) and pressure (150 bar) for 90 min in dynamic mode. Dynamic extraction was carried out with a CO_2 flow of 2 lpm (liter per minute) for *G. gracilis* and 4 lpm for *A. macrostachyum* and a co-solvent flow of 0.25 mL/min for 90 min.

The co-solvent was pumped through the sample using an HPLC pump (Knauer Smartline pump 1050, Berlin, Germany). Extract was obtained in the sample vial collector and stored at -20 °C [48].

1.3.3 Total phenolic content in microalgae, macroalga and halophyte

Polyphenol content of the extracts was determined spectrophotometrically according to Folin and Ciocalteu [52]. The Folin-Ciocalteu reagent develops a colorimetric reaction directly

proportional to the polyphenol content of the extract tested, using gallic acid as a reference standard [53].

The analysis was performed, according to Dhouibi et al. [54], on a 96-well microplate. To 25 µL of extract, 150 µL of H_2O and 12 µL of Folin-Ciolcateu reagent (50%) were added. All samples were analysed in triplicate. The absorbance was measured at 725 nm, using a spectrophotometer (Multiskan, Thermo-Fisher Scientific). The total phenolic contents were expressed as mg of gallic acid mg^{-1} of microalgae weight.

1.3.4 DPPH radical scavenging activity in microalgae, macroalga and halophyte

The determination of the antioxidant activity was evaluated by DPPH radical scavenging activity performed on modified Bernatoniene et al. [55]. The antioxidant compounds reduce picrylhydrazyl (purple radical) to picrylhydrazine (pale yellow hydrazine). The discoloration indicates the free radical scavenging ability of tested extract. This ability was evaluated spectrophotometrically by the determination of the IC50 values (the concentration required to scavenge 50% of DPPH radicals). IC50 value is inversely related to antioxidant capacity of the sample [54].

Forty µL of each extract was loaded into a 96-well microplate at final concentrations of 50, 100, 200, 500, 1000 µg mL^{-1} mixed with 160 µL of a 0.08 mM DPPH solution (Sigma, in 96% aqueous ethanol, VWR Chemicals).

A control was prepared with 40 µL of 80% ethanol and 160 µL of 0.08 mM DPPH solution.

Syntethic antioxidant gallic acid (GAE, Sigma Aldrich) was used as a reference compound and standard curves were prepared for gallic acid at different concentrations (40 µL, at a concentration of 0.05-1 mg mL^{-1}). Each determination was carried out in three replicates. The loaded plates were maintained for 20 min in the dark at room temperature.

The absorbance (Abs) was read at 517 nm after 30 min (Multiskan, Thermo-Fisher Scientific) and the antioxidant activity was calculated from the decrease in absorbance observed following the reduction of the radical; more precisely as the percentage of inhibition of the radical according to the formula (3):

$$\text{Scavenging effect (\%)} = \left[1 - \left(\frac{absorbance\ of\ sample - absorbance\ of\ blank}{absorbance\ of\ control}\right)\right] * 100 \qquad (3)$$

IC50 was calculated through linear regression analysis. Antioxidant activity is expressed as the amount of antioxidant required to achieve 50% inhibition of the radical activity (IC50) [54].

1.3.5 Reducing power test in microalgae, *G. gracilis* and *A. macrostachyum*

Reducing capacity of extracts is reflected by the electron-donation ability, which is considered to be an important strategy to behave as an antioxidant [54]. The reducing power (EC50 value (mg mL^{-1})) was investigated through spectrophotometric detection of Fe^{3+} and Fe^{2+} transformation in the presence of extracts according to Falleh et al. [56]. Samples were incubated at 50°C for 20 min and then centrifuged at 3000 rpm for 10 min. The ferrous ion (Fe^{2+}) can be controlled by measuring the optical density of the formed Perl's Prussian blue at 700 nm. The EC50 value is inversely proportional to reducing power, higher absorbance values indicated a higher reducing power [54].

1.3.6 Statistical analysis

Statistical differences between the groups were assessed by ANOVA analysis, followed by Tukey or Games Howell tests, depending on the homogeneity of the variables. Normality of the variables was confirmed by the Shapiro-Wilk test and homogeneity of variance by Levene's test.

The significance level was 95% in all cases ($P<0.05$). All data were analysed by the computer application SPSS for Windows® (version 20.0, SPSS Inc., Chicago, USA).

1.4 Results and Discussion

1.4.1 Microalgal productive performances

1.4.1.1 Total lipid content and Fatty acid classes composition

In IMTA systems, a proper selection of the microalgal strains to be cultured must take into account the ability of each strain to produce lipids and bioactive molecules that can guarantee improved growth and development of the animals farmed, also ensuring reduced production costs [2,57]. These abilities are closely related to the productive lipid yields and fatty acid profile of each selected strain [58]. Appropriate selection of microalgae strains ensures both their use in aquaculture facilities to enhance animal growth and development and as a source of bioactive compounds, reducing production costs

Nannochloropsis sp., *I. galbana*, *P. tricornutum* cultivated under standard condition and *P. tricornutum* N- showed a variable lipid content (Fig. 4).

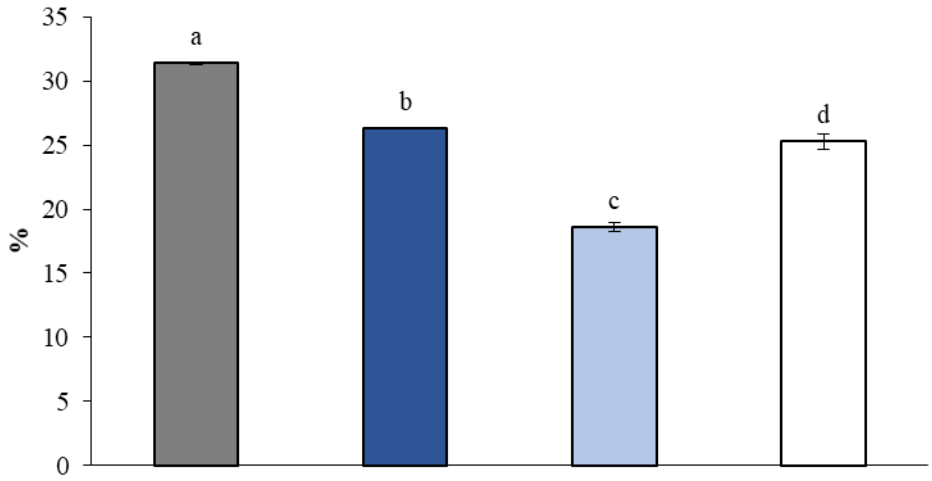


Figure 4. Total lipids (%) content in *Nannochloropsis* sp. (■), *I. galbana* (■), *P. tricornutum* (■) and *P. tricornutum* N- (□). Bars represent the mean ±SEM (n=3) with standard deviations. Treatments that do not share the same letter are significantly different from each other (P<0.05).

In particular, *Nannochloropsis* sp. showed the highest total lipid content (31.38±0.11%), followed by *I. galbana* (26.31± 0%), *P. tricornutum* N- (25.31±0.6 %) and *P. tricornutum* grown under standard conditions (18.61±0.32 %).

A significant enhancement of lipid biosynthesis was obtained in *P. tricornutum* N-, compared to *P. tricornutum* grown under standard conditions (from 18.61±0.32 % to 25.31±0.6 %) (Fig.4).

Fatty acid composition, as expected, showed the highest polyunsaturated fatty acids (PUFAs) of the long chain *n*-3 content (Tot *n*-3) in *I. galbana*, (8.05±0.49 g $100g^{-1}$) followed by *Nannochloropsis* sp. (4.42±0.08 g $100g^{-1}$), *P. tricornutum* grown under standard conditions (2.63±0.19 g $100g^{-1}$) and *P. tricornutum* N- (2.05±0.12 g $100g^{-1}$) (Fig.5).

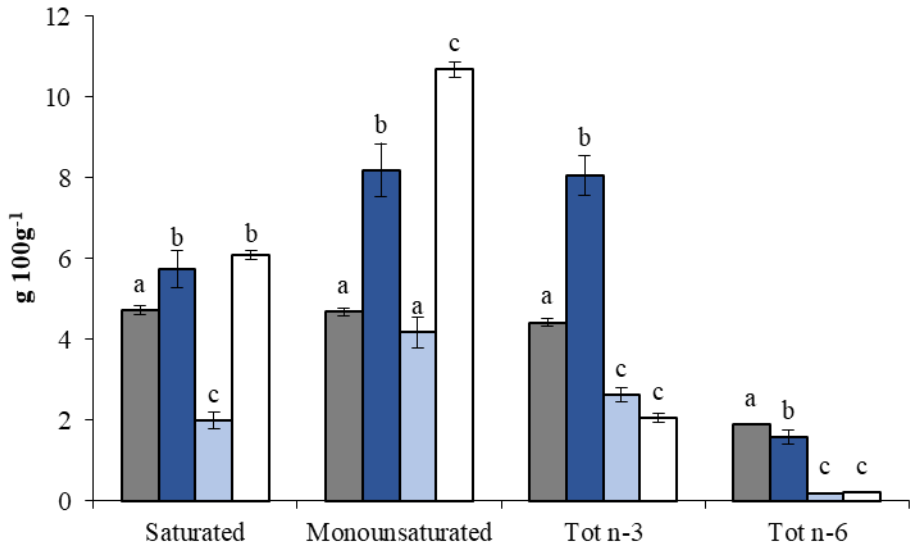


Figure 5. Fatty acid classes composition g fatty acids/100g sample (g $100g^{-1}$) in *Nannochloropsis* sp. (■), *I. galbana* (■), *P. tricornutum* (■) and *P. tricornutum* N- (□). Bars represent the mean ±SEM (n=3) with standard deviations. Treatments that do not share the same letter are significantly different from each other ($P<0.05$).

A comparison between *P. tricornutum* grown under standard condition and *P. tricornutum* N- showed a significant increase in saturated (6.07±0.11 g $100g^{-1}$) and monounsaturated (10.68±0.19 g $100g^{-1}$) fatty acids in N- condition. In contrast, in *P. tricornutum* N- Tot *n*-3 (2.05±0.12 g $100g^{-1}$) and PUFAs of the long chain *n*-6 (Tot *n*-6) (0.21±0.01 g $100g^{-1}$) did not differ significantly from *P. tricornutum* cultivated under standard conditions (respectively 2.63±0.19 g $100g^{-1}$ and 0.19 ± 0.01 g $100g^{-1}$) (Fig.5).

In *P. tricornutum*, despite the starvation condition, a good ratio between total lipids (Fig.4) and Tot *n*-3 (Fig.5) was maintained, as in *P. tricornutum* standard culture.

The cultivation of microalgae to maximize the efficiency for the production of biomass and lipids, needs to take into account *n*-3 fatty acids proportion [59]. In this context, the fatty acid profile has proved to be an important parameter for the selection of cultivable microalgal strains [57,59].

Microalgae accumulate high amounts of lipids and can be an important source of long-chain fatty acids, especially *n*-3 long-chain PUFAs [60,61]. Lipids, in addition to their role as essential nutrients, are known to be good radical scavengers and could be partially responsible for the high antioxidant activity of the considered strains [50].

In this study, *Nannochloropsis* sp. followed by *I. galbana* and *P. tricornutum*, showed the highest lipid content, as already reported in literature [62,63].

Regarding the fatty acid analysis, *I. galbana* showed the highest content for all classes of fatty acids, except Tot *n*-6, followed by *Nannochloropsi*s sp., which maintained a higher Tot *n*-6 content. In the aquaculture sector, *Isochrysi*s sp., is one of the most widely used genera, especially the species *I. galban*a, due to its biochemical profile, rich in PUFAs and ascorbic acid [14], together with the genus *Nannochloropsis*, which is recognised as a good potential source of eicosapentaenoic acid (EPA 20:5 (n3)) and widely used in the aquaculture field for feeding rotifers and in fish hatcheries, also due to its resistance and ease of culture [15].

Microalgae accumulate lipids in the form of triacylglycerols under conditions of environmental stress such as nitrogen limitation [64], indicating that nitrogen starvation results in lipid accumulation directing their metabolism towards the synthesis of reserve compounds, such as lipids and fatty acids, preparing cells at the absence or limitation of nutrients [65,66].

In the present study, significant differences in the lipid profile were observed between *P. tricornutum* grown under standard and nitrogen starvation condition (N-). *P. tricornutum* N- showed a significant increase in total lipids, however, the polyunsaturated fatty acid fraction did not change significantly compared to the standard culture condition. These results suggest that although the metabolism in *P. tricornutum* N- was directed to an increase in the amount of total lipids, the fraction of PUFAs did not decrease significantly [67], thus ensuring an improvement in productive performances maintaining high qualitative fatty acid fractions [68,69].

Nitrogen is a primary constituent of nucleotides, it modulates the synthesis of proteins and it is therefore an essential nutrient for the growth and development of algae [70]. A nitrogen-limited condition therefore leads microalgae to triglycerides accumulation as lipid droplets in the cells [71].

This study confirms that the induction of a stress condition through the modulation of the culture medium, such as the limitation of nitrogen supply, is efficient in improving lipid production, both quantitatively and qualitatively, in *P. tricornutum*.

1.4.1.2 Total polyphenol content

Polyphenols and carotenoids are considered the major contributors to total antioxidant activity in microalgae [72–76], due to their relevant role in switching off reactive oxygen species (ROS) [72,73,77].

The analysed microalgae strains showed significant different total polyphenol content ($P<0.05$) (Fig.6).

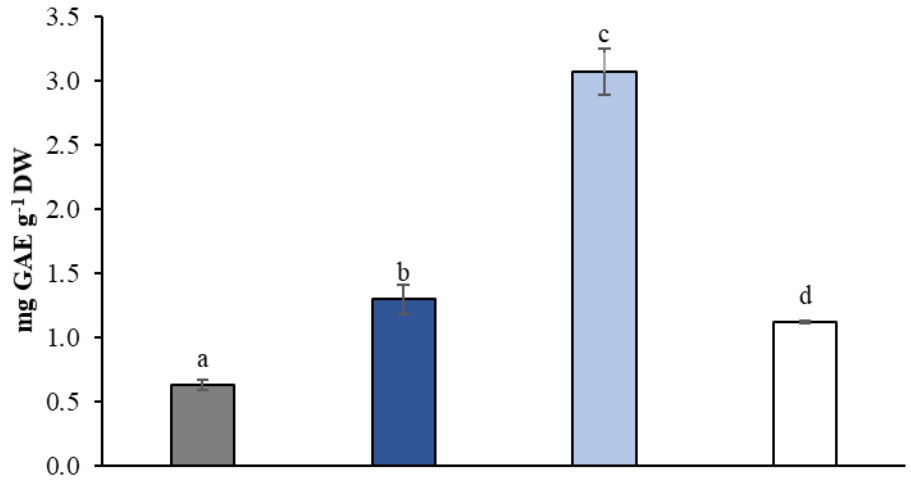


Figure 6. Total polyphenol content (mg GAE g^{-1} DW) in *Nannochloropsis* sp. (■), *I. galbana* (■), *P. tricornutum* (■) and *P. tricornutum* N- (□). Bars represent the mean ±SEM (n=3) with standard deviations. Treatments that do not share the same letter are significantly different from each other ($P<0.05$).

The highest polyphenol content was recorded in *P. tricornutum* (3.07±0.18 mg GAE g^{-1} DW), while the lowest was recorded in *Nannochloropsis* sp. (0.63±0.04 mg GAE g^{-1} DW) (Fig.6).
A significant difference on total polyphenol content was observed between *P. tricornutum* cultivated under standard conditions and *P. tricornutum* N- (1.12±0.01 mg GAE g^{-1} DW) (Fig.6).

1.4.1.3 Total carotenoids

The total carotenoids content (Fig.7) was similar to the total polyphenol content (Fig.6). Significantly higher values were observed in *P. tricornutum* (2.70±0.23 mg g^{-1} DW), followed by *I. galbana* (1.30±0.05 mg g^{-1} DW), *P. tricornutum* N- (0.35±0.01 mg g^{-1} DW) and *Nannochloropsis* sp. (0.27±0.07 mg g^{-1} DW) (Fig.7).

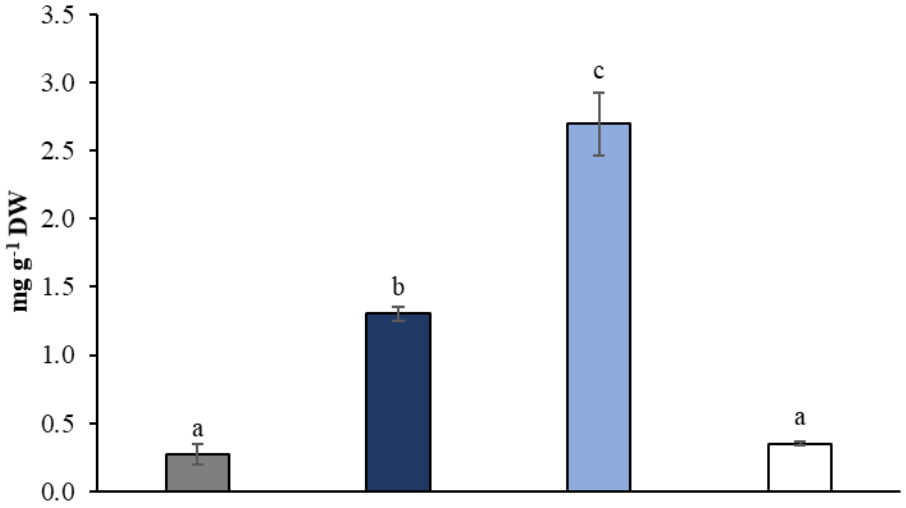


Figure 7. Total carotenoids (mg g^{-1} DW) in *Nannochloropsis* sp. (■), *I. galbana* (■), *P. tricornutum* (■) and *P. tricornutum* N- (□). Bars represent the mean ±SEM (n=3) with standard deviations. Treatments that do not share the same letter are significantly different from each other (P<0.05).

1.4.1.4 Antioxidant activity

The DPPH radical scavenging activity results, expressed as IC50, showed the highest antioxidant activity in *P. tricornutum* (6.78±1.37 mg DW mL^{-1}) (Fig.8a), in accordance with the higher content of polyphenols (Fig.6) and total carotenoids (Fig.7) observed for this strain.

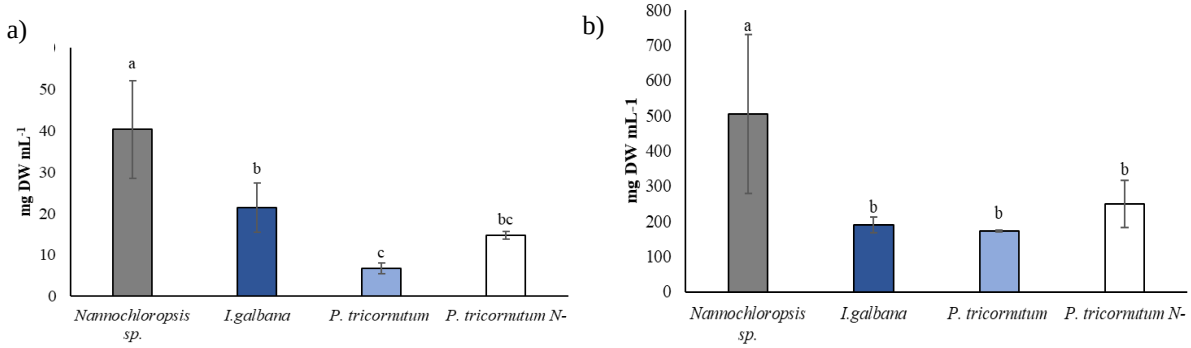


Figure 8. DPPH radical scavenging activity (IC50, mg DW mL^{-1}) and Reducing power (EC50, mg mL^{-1}) in *Nannochloropsis* sp. (■), *I. galbana* (■), *P. tricornutum* (■) and *P. tricornutum* N- (□). Bars represent the mean ±SEM (n=3) with standard deviations. Treatments that do not share the same letter are significantly different from each other (P<0.05).

The lowest antioxidant activity was observed in *Nannochloropsis* sp. (40.34±11.79 mg DW mL^{-1}) (Fig. 8a), concomitant with the lowest content of total polyphenols and carotenoids (Fig.6 and Fig.7).

Regarding the results of reducing power expressed as EC50 (Figure 8b), *P. tricornutum* showed a higher reducing power (172.71±2.90 mg DW mL^{-1}), although no significant differences were recorded compared to *P. tricornutum* N- (249±66.94 mg DW mL^{-1}) and *I. galbana* (189.58±23.25 mg DW mL^{-1}). *Nannochloropsis* sp. showed a significantly lower reducing power (503.89±225.19 mg DW mL^{-1}) (Fig.8b).

Antioxidant content results showed a significantly higher content of polyphenols and total carotenoids in *P. tricornutum* (Fig.6 and Fig.7), with a higher antioxidant activity as evidenced by the DPPH values (Fig.8a) expressed as IC50, and by the reducing power test (Fig.8b) expressed as EC50. Significantly lower values were observed for *Nannochloropsis* sp. for all the studied parameters (Fig.4-8).

Antioxidant content analysis of the three microalgae strains showed that the higher is the lipid content, the lower is the antioxidant content. As confirmed by the DPPH radical scavenging activity test and reducing power test (Fig.8a and b).

In order to assess the antioxidant capacity induced by *Nannochloropsis* sp., *Isochrysis galban*a and *P. tricornutum* extracts, the total carotenoid and polyphenol content was determined in ethanol extracts. In marine algae the production of secondary metabolites is a protective mechanism as a response to stress conditions [5,78,79]. For this reason, the modulation of microalgae growth condition can act as a controlling factor in the production of their secondary metabolites [80].

The obtained results in terms of polyphenol content, carotenoid content and antioxidant activity in *Nannochloropsis* sp., *Isochrysis galban*a and *P. tricornutum* were in agreement with Goiris et al. [81], Maadane et al. [50]. and Safafar et al. [73]. *P. tricornutum* cultivated under standard conditions showed a higher polyphenol and carotenoid content resulting in higher antioxidant activity than the other microalgae strains considered, as evidenced by the IC50 of DPPH (Figure 6) and by the reducing power test (Fig.7) expressed as EC50. Instead, in *P. tricornutum* N- a change in the quantity and quality of microalgal antioxidant production in terms of total polyphenol and carotenoid content occurred.

Nutrient stress and its combination with other stressors can induce lipids accumulation, as reported for the microalgae *Tetraselmis* sp. and *Chlorella vulgaris* [80,82,83] when nutrient stress was combined with salinity stress, with the use of phythormons in *Chlorella sorokiniana* [80,84] and of wastewaters in *C. vulgaris* and *P. tricornutum* [80,83,85].

In agreement with other authors [86–88], *P. tricornutum* N- addressed its metabolism in the accumulation of high-calorie nutrients, such as lipids, to contrast a condition they recognised as energetically disadvantageous.

The used techniques allowed to quantify the lipids and antioxidants production yields of *Nannochloropsis* sp., *Isochrysis galban*a, *P. tricornutum* and *P. tricornutum* cultivated under nitrogen starvation and the transfer of the cultivation techniques on a pilot scale. Furthermore, this study confirmed the ability of *P. tricornutum* to adapt its lipid metabolism according to the nutritional input.

1.4.2 Macroalgal and halophyte global extract yields, antioxidant extraction and antioxidant activity

1.4.2.1 Gracilaria gracilis *extractive methods comparison*

Since seaweeds are receiving significant attention for their potential as natural antioxidants, the total global yield, the total polyphenol contents, the antioxidant activity and the reducing power of the marine red macroalga *Gracilaria gracilis* were determined in this work. Extraction methods using Ethanol 96%, absolute Methanol and SFE (supercritical fluid extraction) were performed and compared.

The results obtained showed a significant effect of the extraction method on the *G. gracilis* global yield. In fact, Methanol extraction was the most efficient (5.35±0.63%), followed by Ethanol 96% (3.81±0.52%) and SFE (0.36±0.05%) (Fig. 9).

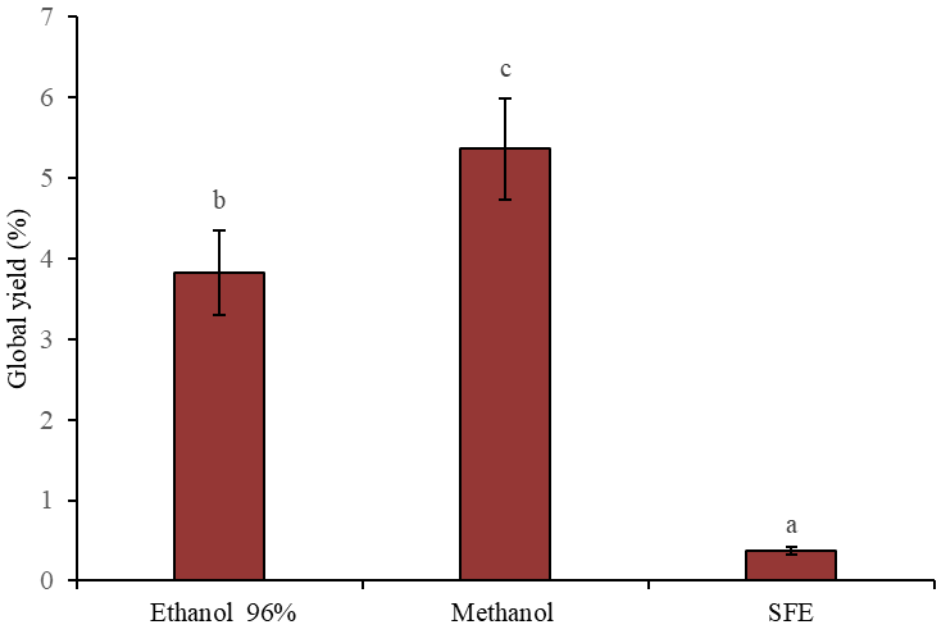


Figure 9. *G. gracilis* global yield (%) (a) extracted by Ethanol 96%, Methanol and SFE. Bars represent the mean ±SEM (n=3) with standard deviations. Treatments that do not share the same letter are significantly different from each other (P<0.05).

The total polyphenol content was significantly higher in the extracts obtained with Ethanol 96% (22.54±1.45 mg GAE g^{-1} DW), followed by Methanol (15.84±1.56 mg GAE g^{-1} DW) and SFE (3.03±0.93 mg GAE g^{-1} DW) (Fig. 10).

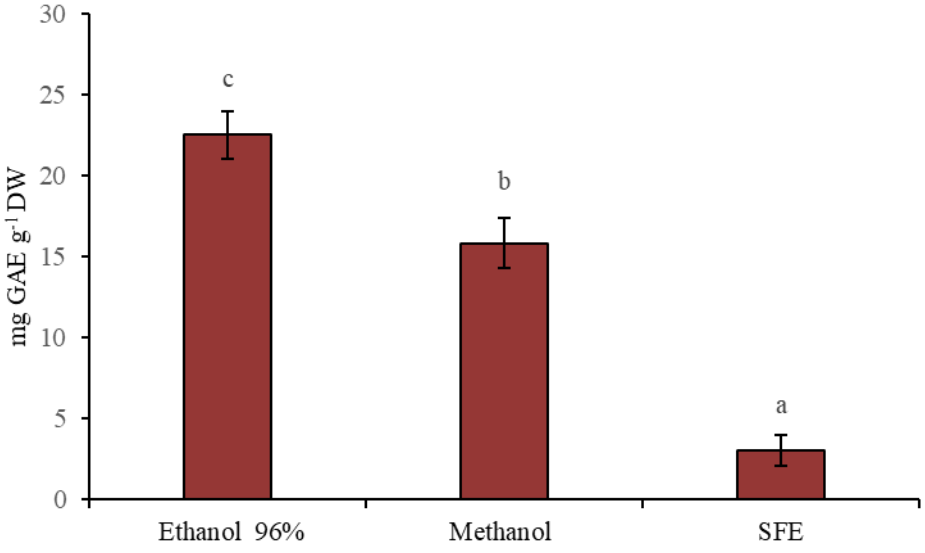


Figure 10. Total polyphenol content (mg GAE g^{-1} DW) in *G. gracilis* extracted by Ethanol 96%, Methanol and SFE. Bars represent the mean ±SEM (n=3) with standard deviations. Treatments that do not share the same letter are significantly different from each other (P<0.05).

However, the antioxidant power of *G. gracilis* extracts obtained by Methanol and SFE with co-solvent (Ethanol 96%) showed higher antioxidant activity (DPPH IC50) (respectively

8.59±1.07 mg DW mL^{-1} and 10.35±1.05 mg DW mL^{-1}) than Ethanol 96% (10.35±1.05 mg DW mL^{-1}) (Fig. 11a); while the highest reducing power value EC50 was obtained by SFE (36.54±5.7) (Fig. 11b).

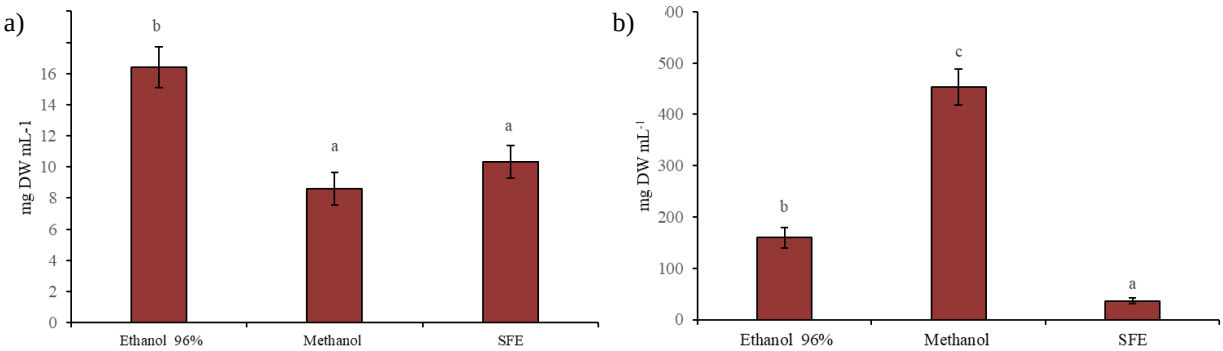


Figure 11. (a) DPPH radical scavenging activity (IC 50, mg DW mL^{-1}) and (b) Reducing power (EC50, mg mL^{-1}) in *G. gracilis* extracted by Ethanol 96%, Methanol and SFE. Bars represent the mean ±SEM (n=3) with standard deviations. Treatments that do not share the same letter are significantly different from each other ($P<0.05$).

The results obtained showed a significant effect of the extraction method on the antioxidant power of *G. gracilis* extracts. Similar results were obtained by Ospina et al. [89] with SFE and co-solvent for *Gracilaria mammillaris* with EtOH 8% (w/w) at 60° C and 100bar pressure, where the extraction yield was 1.40 (%w/w) and the total phenolic content was 2.862 (mg GAE/g).

Chan [90] and Sasadara [91] instead obtained different results, compared to ours, using chemical solvent extractions in *G. changii* and Bulung sangu (*Gracilaria* sp.). In particular, the highest antioxidant activity was obtained with ethyl acetate and Ethanol 100%, suggesting that the species and the antioxidant extraction technique could influence polyphenols antioxidant activity.

The production of polyphenols in *G. gracilis* inhibits ROS-oxidative stress production and have antibacterial properties, as observed by Afonso et al. [92], making those compounds valuable additive for functional fish feed.

Same results were achieved by Passos et al. [93] which, adding *G. gracilis* extracted compounds as diets supplement in *Sparus aurata* feed, improved fish growth, health, and bacterial resistance.

These properties make *Gracilaria* spp. an interesting source for the production of bioactive compounds that have a very wide range of applications: cytotoxic, contraceptive, antimicrobial, antiviral, antifungal, antiparasitic, antihypertensive, antioxidant, anti-inflammatory and analgesic [79] and for bioremediation [39]. These observations highlight

the great potential of the exploitation of these algae with a view to environmental sustainability, sustainable aquaculture development and human health.

1.4.2.2 Arthrocnemum macrostachyum *extractive methods comparison*

Considering the importance of halophyte for their capacity to increase the production of secondary metabolites, such as polyphenolic compounds, under stress conditions [43], *A. macrostachyum* total global yield, total polyphenol contents, antioxidant activity and reducing power were investigated, comparing three extraction methods, using Acetone 70%, N-hexane and SFE (supercritical fluid extraction).

The extraction method had a significant effect on the *A. macrostachyum* global yield, in fact Acetone 70% extraction showed the highest efficiency (21.15±2.3%), followed by N-hexane (15.3±1.3%) and SFE (0.26±0.05%) (Fig. 12).

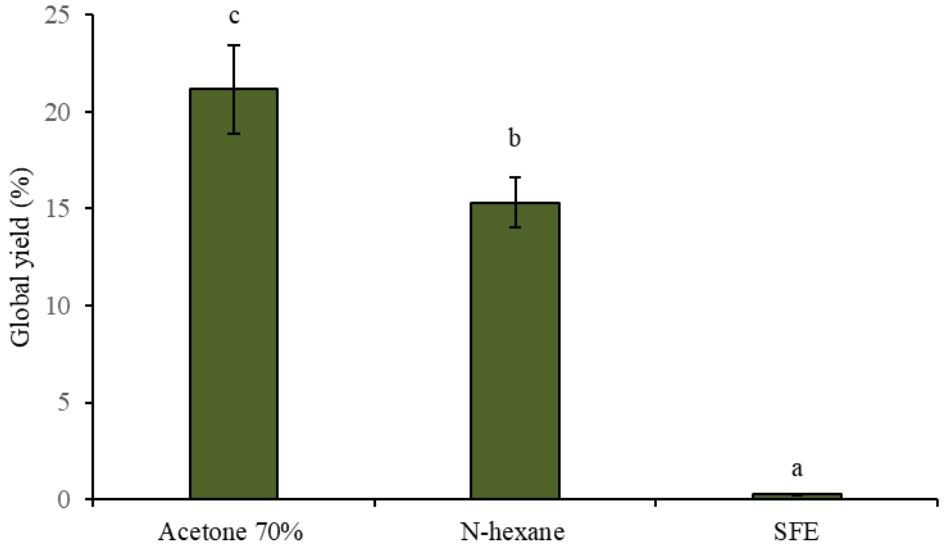


Figure 12. *A. macrostachyum* global yield (%) extracted by Acetone 70%, N-hexane and SFE. Bars represent the mean ±SEM (n=3) with standard deviations. Treatments that do not share the same letter are significantly different from each other ($P<0.05$).

A. macrostachyum total polyphenol content was significantly higher in Acetone 70% extraction (98.53±7.5 mg GAE g^{-1} DW), followed by N-hexane (43.99±3.2 mg GAE g^{-1} DW) and SFE (1.61±0.2 mg GAE g^{-1} DW) (Fig. 13).

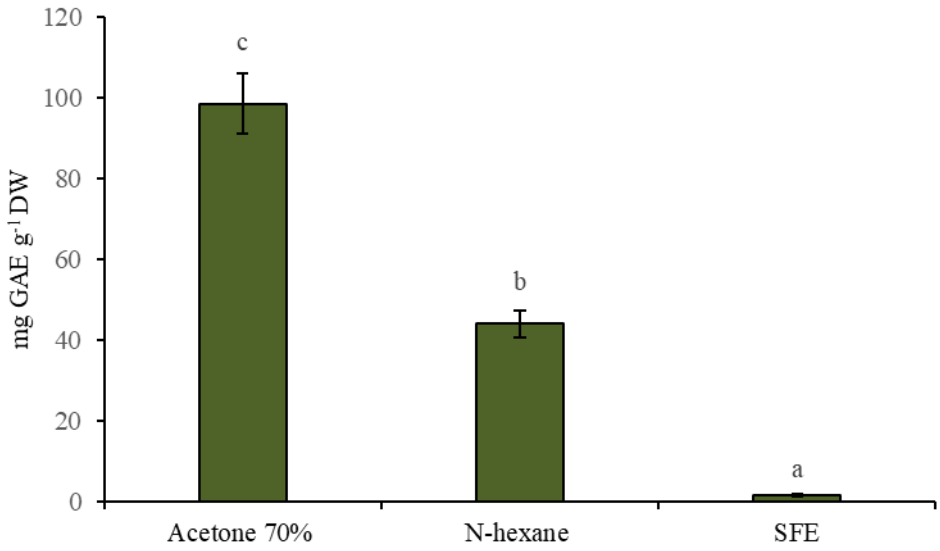


Figure 13. Total polyphenol content (mg GAE g^{-1} DW) in *A. macrostachyum* extracted by Acetone 70%, N-hexane and SFE. Bars represent the mean ±SEM (n=3) with standard deviations. Treatments that do not share the same letter are significantly different from each other (P<0.05).

Despite the extraction technique showed a higher extraction capacity of antioxidants using Acetone 70%, *A. macrostachyum* extracts showed the highest antioxidant activity (DPPH IC50) by N-hexane, followed by SFE (respectively 3.94±1 mg DW mL^{-1} and 27.55±3.4 mg DW mL^{-1}) (Fig. 14a), while the highest reducing power value EC50 was obtained by SFE (19.95±1.9 mg DW mL^{-1}) (Fig. 14b).

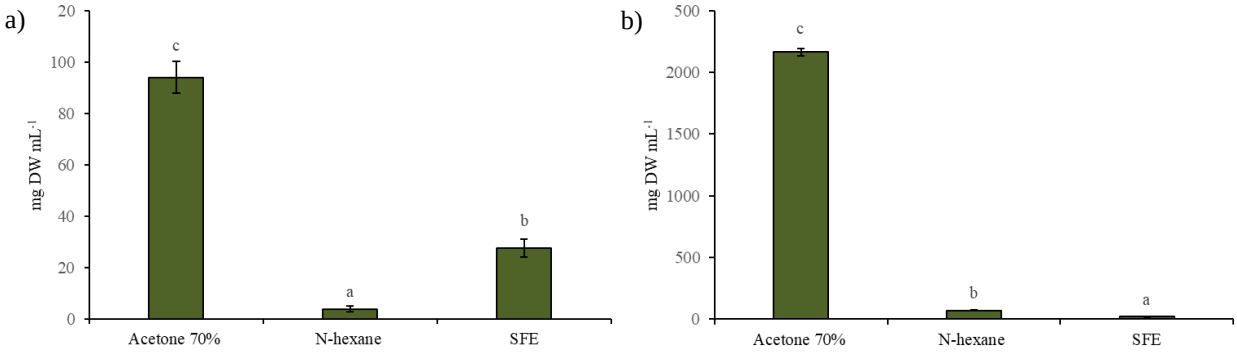


Figure 14. (a) DPPH radical scavenging activity (IC 50, mg DW mL^{-1}) and (b) Reducing power (EC50, mg mL^{-1}) in *A. macrostachyum* extracted by Acetone 70%, N-hexane and SFE. Bars represent the mean ±SEM (n=3) with standard deviations. Treatments that do not share the same letter are significantly different from each other (P<0.05).

The extraction of polyphenols from halophytes is constantly attracting interest from industries due to their high antioxidant and antimicrobial activity, giving these plants increasing popularity for use and application in the agro-food, pharmaceutical and cosmetic industries [33].

It is known that the total phenolic content of *A. macrostachyum*, ranging from 0.09 to 71.6 mg GAE g^{-1} DW, is strictly connected to the extraction method, to the used solvent and to the geographical origin of the plant [94]. With regard to SFE method, there are no data available in the literature for this species. Since both solvents and SFE conditions may vary depending on the species studied, leading to variations in yield and quality of the extracts, as observed by Cristina et al. [95] for the species *Crithmum maritimum* and *Salicornia europaea*, further studies are needed to deepen the knowledge on SFE and co-solvent extractive method for *A. macrostachyum*.

Nevertheless, even if both the extracts of *G. gracilis* (Fig. 9) and *A. macrostachyum* (Fig. 12) obtained with SFE had the lowest yield ($P < 0.05$), compared to extractions with organic solvents, the extracts obtained using SFE eco-friendly technique showed the highest antioxidant activity. SFE could be considered the best extractive technique, among those tested, for the sustainable production of *G. gracilis* and *A. macrostachyum* bioactive compounds.

1.5 Conclusions

The experimental activity described in this chapter was aimed at deepening the basic knowledge on the production performances of two microalgae strains *Nannochloropsis* sp., *Isochrysis galban*a widely used in the aquaculture sector and of the microalga *Phaeodactylum tricornutum* cultivated under nitrogen supply and nitrogen starvation, through a comparative evaluation of lipids and antioxidants of high interest. Moreover, this study was focused on the assessment of polyphenols extraction from the macroalga *Gracilaria gracilis* and the halophyte *Arthrocnemum macrostachyum*, for their use as a source of antioxidants in the nutraceutical and cosmeceutical sectors, comparing traditional extractive methods with green methods.

The obtained results showed that microalgae are a good source of lipids and bioactive compounds as carotenoids and polyphenols and that their production can be modulated through different culture conditions. Regarding *G. gracilis* and *A. macrostachyum*, the obtained results as well as showing a high production of secondary metabolites, the highest antioxidant activity of the extracts was obtained by green Supercritical Fluid Extraction. Therefore, their cultivation in IMTA systems make them good candidates for the extraction of bioactive compounds to be utilized in nutraceutical and cosmeceutical fields.

1.6 References

[1] V. Dolganyuk, D. Belova, O. Babich, A. Prosekov, S. Ivanova, D. Katserov, N. Patyukov, S. Sukhikh, Microalgae: A promising source of valuable bioproducts, Biomolecules. 10 (2020) 1–24. https://doi.org/10.3390/biom10081153.

[2] W. Levasseur, P. Perré, V. Pozzobon, A review of high value-added molecules production by microalgae in light of the classification, Biotechnol. Adv. 41 (2020) 107545. https://doi.org/10.1016/j.biotechadv.2020.107545.

[3] D. Knowler, T. Chopin, R. Martínez-Espiñeira, A. Neori, A. Nobre, A. Noce, G. Reid, The economics of Integrated Multi-Trophic Aquaculture: where are we now and where do we need to go?, Rev. Aquac. 12 (2020) 1579–1594. https://doi.org/10.1111/raq.12399.

[4] M. Thomas, A. Pasquet, J. Aubin, S. Nahon, T. Lecocq, When more is more: taking advantage of species diversity to move towards sustainable aquaculture, Biol. Rev. 96 (2021) 767–784. https://doi.org/10.1111/brv.12677.

[5] Y.H. Park, S. Il Han, B. Oh, H.S. Kim, M.S. Jeon, S. Kim, Y.E. Choi, Microalgal secondary metabolite productions as a component of biorefinery: A review, Bioresour. Technol. 344 (2022) 126206. https://doi.org/10.1016/J.BIORTECH.2021.126206.

[6] B. Mishra, A. Tiwari, A.E.D. Mahmoud, Microalgal potential for sustainable aquaculture applications: bioremediation, biocontrol, aquafeed, Clean Technol. Environ. Policy. 1 (2022) 1–13. https://doi.org/10.1007/s10098-021-02254-1.

[7] D. Nagarajan, S. Varjani, D.J. Lee, J.S. Chang, Sustainable aquaculture and animal feed from microalgae – Nutritive value and techno-functional components, Renew. Sustain. Energy Rev. 150 (2021) 111549. https://doi.org/10.1016/J.RSER.2021.111549.

[8] Q. Lu, P. Han, Y. Xiao, T. Liu, F. Chen, L. Leng, H. Liu, W. Zhou, The novel approach of using microbial system for sustainable development of aquaponics, J. Clean. Prod. 217 (2019) 573–575. https://doi.org/10.1016/j.jclepro.2019.01.252.

[9] B. Ramesh Kumar, G. Deviram, T. Mathimani, P.A. Duc, A. Pugazhendhi, Microalgae as rich source of polyunsaturated fatty acids, Biocatal. Agric. Biotechnol. 17 (2019) 583–588. https://doi.org/10.1016/J.BCAB.2019.01.017.

[10] S. Hemaiswarya, R. Raja, R.R. Kumar, V. Ganesan, C. Anbazhagan, Microalgae: A sustainable feed source for aquaculture, World J. Microbiol. Biotechnol. 27 (2011) 1737–1746. https://doi.org/10.1007/s11274-010-0632-z.

[11] A. Udayan, · Ashutosh, K. Pandey, P. Sharma, N. Sreekumar, · Sunil Kumar, Emerging industrial applications of microalgae: challenges and future perspectives, Syst. Microbiol. Biomanufacturing 2021 14. 1 (2021) 411–431. https://doi.org/10.1007/S43393-021-00038-8.

[12] S. Rakesh, A.M. Alsamman, M.M. Mokhtar, B. Ray, Phycoremediation of aquaculture wastewater and algal lipid extraction for fuel conversion Phycoremediation of aquaculture wastewater and algal lipid extrac-tion for fuel conversion, (2022). https://doi.org/10.36462/H.BioSci.202201.

[13] M.R. Gauthier, G.N.A. Senhorinho, J.A. Scott, Microalgae under environmental stress as a source of antioxidants, Algal Res. 52 (2020) 102104. https://doi.org/10.1016/j.algal.2020.102104.

[14] Y. Sun, H. Wang, G. Guo, Y. Pu, B. Yan, The isolation and antioxidant activity of polysaccharides from the marine microalgae Isochrysis galbana, Carbohydr. Polym. 113 (2014) 22–31. https://doi.org/10.1016/J.CARBPOL.2014.06.058.

[15] J. Camacho-Rodríguez, A.M. González-Céspedes, M.C. Cerón-García, J.M. Fernández-Sevilla, F.G. Acién-Fernández, E. Molina-Grima, A quantitative study of eicosapentaenoic acid (EPA) production by Nannochloropsis gaditana for aquaculture as a function of dilution rate, temperature and average irradiance, Appl. Microbiol. Biotechnol. 98 (2014) 2429–2440. https://doi.org/10.1007/S00253-013-5413-9.

[16] L.T. Guerra, O. Levitan, M.J. Frada, J.S. Sun, P.G. Falkowski, G.C. Dismukes, Regulatory branch points affecting protein and lipid biosynthesis in the diatom Phaeodactylum tricornutum, Biomass and Bioenergy. 59 (2013) 306–315. https://doi.org/10.1016/j.biombioe.2013.10.007.

[17] Z.-K. Yang, Y.-F. Niu, Y.-H. Ma, J. Xue, M.-H. Zhang, W.-D. Yang, J.-S. Liu, S.-H. Lu, Y. Guan, H.-Y. Li, Molecular and cellular mechanisms of neutral lipid accumulation in diatom following nitrogen deprivation, Biotechnol. Biofuels. 6 (2013) 67. https://doi.org/10.1186/1754-6834-6-67.

[18] J. Xue, Y.F. Niu, T. Huang, W.D. Yang, J.S. Liu, H.Y. Li, Genetic improvement of the

microalga Phaeodactylum tricornutum for boosting neutral lipid accumulation, Metab. Eng. 27 (2015) 1–9. https://doi.org/10.1016/j.ymben.2014.10.002.

[19] P. Spolaore, C. Joannis-Cassan, E. Duran, A. Isambert, Commercial applications of microalgae, J. Biosci. Bioeng. 101 (2006) 87–96. https://doi.org/10.1263/jbb.101.87.

[20] A.C. Guedes, F.X. Malcata, Nutritional value and uses of microalgae in aquaculture, in: Aquaculture, 2012: pp. 59–76. https://books.google.it/books?hl=it&lr=&id=wDqZDwAAQBAJ&oi=fnd&pg=PA59&dq=Guedes+AC,+Malcata+FX+(2012b).+Nutritional+Value+and+Uses+of+Microalgae+in+Aquaculture.+In:+Muchlisin+DZ,+editor.+Aquaculture,+59–77.&ots=HLBsgTIvzY&sig=WHAYuLSwFvMxh8AUHhyKaxJQo7s# (accessed February 15, 2022).

[21] L. Rodolfi, G.C. Zittelli, N. Bassi, G. Padovani, N. Biondi, G. Bonini, M.R. Tredici, Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor, Biotechnol. Bioeng. 102 (2009) 100–112. https://doi.org/10.1002/bit.22033.

[22] J.P. Fidalgo, A. Cid, E. Torres, A. Sukenik, C. Herrero, Effects of nitrogen source and growth phase on proximate biochemical composition, lipid classes and fatty acid profile of the marine microalga Isochrysis galbana, Aquaculture. 166 (1998) 105–116. https://doi.org/10.1016/s0044-8486(98)00278-6.

[23] D.L. Alonso, E.H. Belarbi, J.M. Fernández-Sevilla, J. Rodríguez-Ruiz, E.M. Grima, Acyl lipid composition variation related to culture age and nitrogen concentration in continuous culture of the microalga Phaeodactylum tricornutum, Phytochemistry. 54 (2000) 461–471. https://doi.org/10.1016/S0031-9422(00)00084-4.

[24] M. Siaut, S. Cuiné, C. Cagnon, B. Fessler, M. Nguyen, P. Carrier, A. Beyly, F. Beisson, C. Triantaphylidès, Y. Li-Beisson, G. Peltier, Oil accumulation in the model green alga Chlamydomonas reinhardtii: characterization, variability between common laboratory strains and relationship with starch reserves, BMC Biotechnol. 11 (2011) 7.

[25] S.H. Ho, C.Y. Chenb, J.S. Chang, Effect of light intensity and nitrogen starvation on CO2 fixation and lipid/carbohydrate production of an indigenous microalga Scenedesmus obliquus CNW-N, Bioresour. Technol. 113 (2012) 244–252.

[26] S. Boussiba, Carotenogenesis in the green alga Haematococcus pluvialis: Cellular

physiology and stress response, Physiol. Plant. 108 (2000) 111–117. https://doi.org/10.1034/j.1399-3054.2000.108002111.x.

[27] L. Pirastru, M. Darwish, F.L. Chu, F. Perreault, L. Sirois, L. Sleno, R. Popovic, Carotenoid production and change of photosynthetic functions in Scenedesmus sp. exposed to nitrogen limitation and acetate treatment, J. Appl. Phycol. 24 (2012) 117–124. https://doi.org/10.1007/s10811-011-9657-4.

[28] Y. Durmaz, Vitamin E (α-tocopherol) production by the marine microalgae Nannochloropsis oculata (Eustigmatophyceae) in nitrogen limitation, Aquaculture. 272 (2007) 717–722. https://doi.org/10.1016/j.aquaculture.2007.07.213.

[29] H.H. Abd El-Baky, F.K. El Baz, G.S. El-Baroty, Production of Antioxidant by the Green Alga Dunaliella salina, Int. J. Agric. Biol. 6 (2004) 49–57.

[30] T. Vladkova, N. Georgieva, A. Staneva, D. Gospodinova, Recent Progress in Antioxidant Active Substances from Marine Biota, Antioxidants 2022, Vol. 11, Page 439. 11 (2022) 439. https://doi.org/10.3390/ANTIOX11030439.

[31] A. Edreva, V. Velikova, T. Tsonev, S. Dagnon, E. Gesheva, STRESS-PROTECTIVE ROLE OF SECONDARY METABOLITES : DIVERSITY OF FUNCTIONS AND MECHANISMS, Gen. Appl. Plant Physiol. (2008) 67–78.

[32] S. Lomartire, A.M.M. Gonçalves, An Overview of Potential Seaweed-Derived Bioactive Compounds for Pharmaceutical Applications, Mar. Drugs. 20 (2022) 1–32. https://doi.org/10.3390/md20020141.

[33] M. Lopes, A. Sanches-Silva, M. Castilho, C. Cavaleiro, F. Ramos, Halophytes as source of bioactive phenolic compounds and their potential applications, Crit. Rev. Food Sci. Nutr. (2021) 1–24. https://doi.org/10.1080/10408398.2021.1959295.

[34] P. Torres, J.P. Santos, F. Chow, D.Y.A.C. dos Santos, A comprehensive review of traditional uses, bioactivity potential, and chemical diversity of the genus Gracilaria (Gracilariales, Rhodophyta), Algal Res. 37 (2019) 288–306. https://doi.org/10.1016/j.algal.2018.12.009.

[35] FAO, The State of World Fisheries and Aquaculture 2020. Sustainability in action., Rome, 2020. https://doi.org/10.4060/ca9229en.

[36] C.L.F. de Almeida, H. de S. Falcão, G.R. d. M. Lima, C. de A. Montenegro, N.S. Lira, P.F. de Athayde-Filho, L.C. Rodrigues, M.F. V. de Souza, J.M. Barbosa-Filho, L.M.

Batista, Bioactivities from marine algae of the genus Gracilaria, Int. J. Mol. Sci. 12 (2011) 4550–4573. https://doi.org/10.3390/ijms12074550.

[37] Y. Zhou, H. Yang, H. Hu, Y. Liu, Y. Mao, H. Zhou, X. Xu, F. Zhang, Bioremediation potential of the macroalga Gracilaria lemaneiformis (Rhodophyta) integrated into fed fish culture in coastal waters of north China, Aquaculture. 252 (2006) 264–276. https://doi.org/10.1016/j.aquaculture.2005.06.046.

[38] Y.F. Yang, X.G. Fei, J.M. Song, H.Y. Hu, G.C. Wang, I.K. Chung, Growth of Gracilaria lemaneiformis under different cultivation conditions and its effects on nutrient removal in Chinese coastal waters, Aquaculture. 254 (2006) 248–255. https://doi.org/10.1016/j.aquaculture.2005.08.029.

[39] N. Spanò, D. Di Paola, M. Albano, A. Manganaro, M. Sanfilippo, C. D'Iglio, G. Capillo, S. Savoca, Growth performance and bioremediation potential of Gracilaria gracilis (Steentoft, L.M. Irvine & Farnham, 1995), Int. J. Environ. Stud. (2021). https://doi.org/10.1080/00207233.2021.1954775.

[40] Y.A. El-Amier, W. Soufan, K.F. Almutairi, N.S. Zaghloul, A.M. Abd-Elgawad, Proximate composition, bioactive compounds, and antioxidant potential of wild halophytes grown in coastal salt marsh habitats, Molecules. 27 (2022). https://doi.org/10.3390/molecules27010028.

[41] C.M. Messina, G. Renda, V.A. Laudicella, R. Trepos, M. Fauchon, C. Hellio, A. Santulli, From ecology to biotechnology, study of the defense strategies of algae and halophytes (from trapani saltworks, NW sicily) with a focus on antioxidants and antimicrobial properties, Int. J. Mol. Sci. 20 (2019) 881. https://doi.org/10.3390/ijms20040881.

[42] U. Waller, A.K. Buhmann, A. Ernst, V. Hanke, A. Kulakowski, B. Wecker, J. Orellana, J. Papenbrock, Integrated multi-trophic aquaculture in a zero-exchange recirculation aquaculture system for marine fish and hydroponic halophyte production, Aquac. Int. 23 (2015) 1473–1489. https://doi.org/10.1007/S10499-015-9898-3/TABLES/5.

[43] R. Arena, S. Manuguerra, E. Collins, A. Mahdhi, G. Renda, C.M. Messina, A. Santulli, Antioxidant properties of a supercritical fluid extract of the halophyte Mesembryanthemum nodiflorum L. from sicilian coasts: Nutraceutical and cosmeceutical applications, Appl. Sci. 10 (2020). https://doi.org/10.3390/app10072374.

[44] M. Herrero, A. Cifuentes, E. Ibañez, Sub- and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae - A review, Food Chem. 98 (2006) 136–148. https://doi.org/10.1016/j.foodchem.2005.05.058.

[45] J. Folch, M. Lees, G.H.S. Stanley, A simple method for the isolation and purification of total lipids from animal tissues, J. Biol. Chem. 226 (1957) 497–509. https://doi.org/10.1007/s10858-011-9570-9.

[46] G. Lepage, C.C. Roy, Improved recovery of fatty acid through direct transesterification without prior extraction or purification, J. Lipid Res. 25 (1984) 1391–1396.

[47] C.M. Messina, G. Renda, L. La Barbera, A. Santulli, By-products of farmed European sea bass (Dicentrarchus labrax L.) as a potential source of n-3 PUFA, Biol. 68 (2013) 288–293. https://doi.org/10.2478/s11756-013-0148-8.

[48] C.M. Messina, A. Troia, R. Arena, S. Manuguerra, T. Ioannou, E. Curcuraci, G. Renda, C. Hellio, A. Santulli, Species-specific antioxidant power and bioactive properties of the extracts obtained from wild mediterranean Calendula Spp. (Asteraceae), Appl. Sci. 9 (2019) 4627. https://doi.org/10.3390/app9214627.

[49] K. Miazek, S. Ledakowicz, Chlorophyll extraction from leaves, needles and microalgae: A kinetic approach, Int. J. Agric. Biol. Eng. 6 (2013) 107–115. https://doi.org/10.3965/j.ijabe.20130602.0012.

[50] A. Maadane, N. Merghoub, T. Ainane, H. El Arroussi, R. Benhima, S. Amzazi, Y. Bakri, I. Wahby, Antioxidant activity of some Moroccan marine microalgae: Pufa profiles, carotenoids and phenolic content, J. Biotechnol. 215 (2015) 13–19. https://doi.org/10.1016/J.JBIOTEC.2015.06.400.

[51] H.K. Lichtenthaler, C. Buschmann, Chlorophylls and Carotenoids: Measurement and Characterization by UV-VIS Spectroscopy, Curr. Protoc. Food Anal. Chem. 1 (2001) F4.3.1-F4.3.8. https://doi.org/10.1002/0471142913.FAF0403S01.

[52] V. Folin, O., Ciocalteu, Tyrosine and Tryptophane in Proteins, J. Biol. Chem. 73 (1927) 627–650.

[53] V.L. Singleton, J.A. Rossi, Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents, Am. J. Enol. Vitic. 16 (1965) 144–158.

[54] N. Dhouibi, S. Manuguerra, R. Arena, A. Mahdhi, C.M. Messina, A. Santulli, H.

Dhaouadi, Screening of antioxidant potentials and bioactive properties of the extracts obtained from two Centaurea L. species (C. kroumirensis coss. and C. sicula L. subsp sicula), Appl. Sci. 10 (2020) 2267. https://doi.org/10.3390/app10072267.

[55] J. Bernatoniene, R. Masteikova, J. Davalgiene, R. Peciura, R. Gauryliene, R. Bernatoniene, D. Majiene, R. Lazauskas, G. Civinskiene, S. Velziene, J. Muselik, Z. Chalupova, Topical application of calendula officinalis (L.): Formulation and evaluation of hydrophilic cream with antioxidant activity, J. Med. Plants Res. 5 (2011) 868–877.

[56] H. Falleh, R. Ksouri, F. Medini, S. Guyot, C. Abdelly, C. Magné, Antioxidant activity and phenolic composition of the medicinal and edible halophyte Mesembryanthemum edule L., Ind. Crops Prod. 34 (2011) 1066–1071. https://doi.org/10.1016/j.indcrop.2011.03.018.

[57] Fernandes T., Cordeiro N., Microalgae as Sustainable Biofactories to ProduceHigh-Value Lipids: Biodiversity, Exploitation, and Biotechnological Applications, Mar. Drugs. 19 (2021) 1–24.

[58] Yarnold J., Karan H., Oey M., Hankamer B., Microalgal Aquafeeds As Part of a Circular Bioeconomy | Elsevier Enhanced Reader, Trends Plant Sci. 24 (2019) 959–970.

[59] Perdana B.A., Chaidir Z., Kusnanda A.J., Dharma A., Zakaria I.J., Syafrizayanti, Bayu A., Putra M.Y., Omega-3 fatty acids of microalgae as a food supplement: A review of exogenous factors for production enhancement | Elsevier Enhanced Reader, Algal Res. 60 (2021).

[60] D.A. Martins, L. Custódio, L. Barreira, H. Pereira, R. Ben-Hamadou, J. Varela, K.M. Abu-Salah, Alternative Sources of n-3 Long-Chain Polyunsaturated Fatty Acids in Marine Microalgae, Mar. Drugs 2013, Vol. 11, Pages 2259-2281. 11 (2013) 2259–2281. https://doi.org/10.3390/MD11072259.

[61] B. Liu, C. Benning, Lipid metabolism in microalgae distinguishes itself, Curr. Opin. Biotechnol. 24 (2013) 300–309. https://doi.org/10.1016/J.COPBIO.2012.08.008.

[62] E. Ryckebosch, C. Bruneel, R. Termote-Verhalle, K. Muylaert, I. Foubert, Influence of extraction solvent system on extractability of lipid components from different microalgae species, Algal Res. 3 (2014) 36–43.

https://doi.org/10.1016/J.ALGAL.2013.11.001.

[63] L. Li, J. Cui, Q. Liu, Y. Ding, J. Liu, Screening and phylogenetic analysis of lipid-rich microalgae, Algal Res. 11 (2015) 381–386. https://doi.org/10.1016/J.ALGAL.2015.02.028.

[64] Q. Hu, M. Sommerfeld, E. Jarvis, M. Ghirardi, M. Posewitz, M. Seibert, A. Darzins, Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances, Plant J. 54 (2008) 621–639. https://doi.org/10.1111/J.1365-313X.2008.03492.X.

[65] T. Giri, U. Goutam, A. Arya, S. Gautam, Effect of Nutrients on Diatom Growth: A Review, Trends Sci. 19 (2022) 1752. https://doi.org/10.48048/tis.2022.1752.

[66] J. Longworth, D. Wu, M. Huete-Ortega, P.C. Wright, S. Vaidyanathan, Proteome response of Phaeodactylum tricornutum, during lipid accumulation induced by nitrogen depletion, Algal Res. 18 (2016) 213–224. https://doi.org/10.1016/j.algal.2016.06.015.

[67] M.Á. Rincón-Cervera, R. Bravo-Sagua, R.A. Manólio Soares Freitas, S. López-Arana, A.C. de Camargo, Monounsaturated and polyunsaturated fatty acids: structure, food sources, biological functions, and their preventive role against noncommunicable diseases, Bioact. Food Components Act. Mech. Approach. (2022) 185–210. https://doi.org/10.1016/B978-0-12-823569-0.00005-9.

[68] B.R. Kumar, G. Deviram, T. Mathimani, P.A. Duc, Biocatalysis and Agricultural Biotechnology Microalgae as rich source of polyunsaturated fatty acids, Biocatal. Agric. Biotechnol. 17 (2019) 583–588. https://doi.org/10.1016/j.bcab.2019.01.017.

[69] Y. Maltsev, K. Maltseva, Fatty acids of microalgae: diversity and applications, Rev. Environ. Sci. Bio/Technology 2021 202. 20 (2021) 515–547. https://doi.org/10.1007/S11157-021-09571-3.

[70] J. Sharma, S.S. Kumar, N.R. Bishnoi, A. Pugazhendhi, Enhancement of lipid production from algal biomass through various growth parameters, J. Mol. Liq. 269 (2018) 712–720. https://doi.org/10.1016/J.MOLLIQ.2018.08.103.

[71] K. Zienkiewicz, Z. Du, W. Ma, K. Vollheyde, C. Benning, Stress-induced neutral lipid biosynthesis in microalgae — Molecular, cellular and physiological insights, Biochim. Biophys. Acta. 1861 (2016) 1269–1281.

[72] S. Zhang, Y. He, B. Sen, G. Wang, Reactive oxygen species and their applications

toward enhanced lipid accumulation in oleaginous microorganisms, Bioresour. Technol. 307 (2020) 123234. https://doi.org/10.1016/J.BIORTECH.2020.123234.

[73] H. Safafar, J. Van Wagenen, P. Møller, C. Jacobsen, Carotenoids, Phenolic Compounds and Tocopherols Contribute to the Antioxidative Properties of Some Microalgae Species Grown on Industrial Wastewater, Mar. Drugs 2015, Vol. 13, Pages 7339-7356. 13 (2015) 7339–7356. https://doi.org/10.3390/MD13127069.

[74] M. Hajimahmoodi, M.A. Faramarzi, N. Mohammadi, N. Soltani, M.R. Oveisi, N. Nafissi-Varcheh, Evaluation of antioxidant properties and total phenolic contents of some strains of microalgae, J. Appl. Phycol. 22 (2010) 43–50. https://doi.org/10.1007/S10811-009-9424-Y/TABLES/4.

[75] K. Goiris, K. Muylaert, I. Fraeye, I. Foubert, J. De Brabanter, L. De Cooman, Antioxidant potential of microalgae in relation to their phenolic and carotenoid content, J. Appl. Phycol. 24 (2012) 1477–1486. https://doi.org/10.1007/s10811-012-9804-6.

[76] S. Takaichi, Carotenoids in algae: distributions, biosyntheses and functions, Mar. Drugs. 9 (2011) 1101–1118.

[77] S.C. Foo, F.M. Yusoff, M. Ismail, M. Basri, S.K. Yau, N.M.H. Khong, K.W. Chan, M. Ebrahimi, Antioxidant capacities of fucoxanthin-producing algae as influenced by their carotenoid and phenolic contents, J. Biotechnol. 241 (2017) 175–183. https://doi.org/10.1016/J.JBIOTEC.2016.11.026.

[78] I.P.S. Fernando, W.W. Lee, G. Ahn, Marine algal flavonoids and phlorotannins; an intriguing frontier of biofunctional secondary metabolites, Crit. Rev. Biotechnol. 20 (2021) 1–23. https://doi.org/10.1080/07388551.2021.1922351.

[79] B. Pradhan, P.P. Bhuyan, S. Patra, R. Nayak, P.K. Behera, C. Behera, A.K. Behera, J.-S. Ki, M. Jena, Beneficial effects of seaweeds and seaweed-derived bioactive compounds: Current evidence and future prospective, Biocatal. Agric. Biotechnol. 39 (2022) 102242. https://doi.org/10.1016/J.BCAB.2021.102242.

[80] A. Udayan, A.K. Pandey, R. Sirohi, N. Sreekumar, B.-I. Sang, S.J. Sim, S.H. Kim, A. Pandey, Production of microalgae with high lipid content and their potential as sources of nutraceuticals, Phytochem. Rev. (2022) 1–28. https://doi.org/10.1007/s11101-021-09784-y.

[81] K. Goiris, K. Muylaert, I. Fraeye, I. Foubert, J. De Brabanter, L. De Cooman,

Antioxidant potential of microalgae in relation to their phenolic and carotenoid content, J. Appl. Phycol. 24 (2012) 1477–1486. https://doi.org/10.1007/s10811-012-9804-6.

[82] H. Park, D. Jung, J. Lee, P. Kim, Y. Cho, I. Jung, Z.-H. Kim, S.-M. Lim, C.-G. Lee, Improvement of biomass and fatty acid productivity in ocean cultivation of Tetraselmis sp. using hypersaline medium, J. Appl. Phycol. 30 (2018) 2725–2735. https://doi.org/10.1007/s10811-018-1388-3.

[83] S. Mirizadeh, M. Nosrati, & Seyed, A. Shojaosadati, Synergistic Effect of Nutrient and Salt Stress on Lipid Productivity of Chlorella vulgaris Through Two-Stage Cultivation, Bioenergy Res. 13 (2020) 507–517. https://doi.org/10.1007/s12155-019-10077-8.

[84] A. Giridhar Babu, X. Wu, A.N. Kabra, D.P. Kim, Cultivation of an indigenous Chlorella sorokiniana with phytohormones for biomass and lipid production under N-limitation, Algal Res. 23 (2017) 178–185. https://doi.org/10.1016/J.ALGAL.2017.02.004.

[85] X.W. Wang, L. Huang, P.Y. Ji, C.P. Chen, X.S. Li, Y.H. Gao, J.R. Liang, Using a mixture of wastewater and seawater as the growth medium for wastewater treatment and lipid production by the marine diatom Phaeodactylum tricornutum, Bioresour. Technol. 289 (2019) 121681. https://doi.org/10.1016/J.BIORTECH.2019.121681.

[86] Z.K. Yang, Y.F. Niu, Y.H. Ma, J. Xue, M.H. Zhang, W.D. Yang, J.S. Liu, S.H. Lu, Y. Guan, H.Y. Li, Molecular and cellular mechanisms of neutral lipid accumulation in diatom following nitrogen deprivation, Biotechnol. Biofuels. 6 (2013) 1–14. https://doi.org/10.1186/1754-6834-6-67.

[87] N. Yodsuwan, S. Sawayama, S. Sirisansaneeyakul, Effect of nitrogen concentration on growth, lipid production and fatty acid profiles of the marine diatom Phaeodactylum tricornutum, Agric. Nat. Resour. 51 (2017) 190–197. https://doi.org/10.1016/j.anres.2017.02.004.

[88] H. Zhang, W. Yin, D. Ma, X. Liu, K. Xu, J. Liu, Phytohormone supplementation significantly increases fatty acid content of Phaeodactylum tricornutum in two-phase culture, J. Appl. Phycol. 33 (2020) 13–23. https://doi.org/10.1007/s10811-020-02074-8.

[89] M. Ospina, H.I. Castro-Vargas, F. Parada-Alfonso, Antioxidant capacity of Colombian seaweeds: 1. Extracts obtained from Gracilaria mammillaris by means of supercritical

fluid extraction, J. Supercrit. Fluids. 128 (2017) 314–322. https://doi.org/10.1016/j.supflu.2017.02.023.

[90] P.T. Chan, P. Matanjun, S.M. Yasir, T.S. Tan, Antioxidant activities and polyphenolics of various solvent extracts of red seaweed, Gracilaria changii, J. Appl. Phycol. 27 (2015) 2377–2386. https://doi.org/10.1007/s10811-014-0493-1.

[91] M.M.V. Sasadara, I.G.P. Wirawan, Effect of extraction solvent on total phenolic content, total flavonoid content, and antioxidant activity of Bulung Sangu (Gracilaria sp.) Seaweed, in: IOP Conf. Ser. Earth Environ. Sci., IOP Publishing, 2021: p. 012005. https://doi.org/10.1088/1755-1315/712/1/012005.

[92] C. Afonso, A.P. Correia, M. V. Freitas, T. Mouga, T. Baptista, In Vitro Evaluation of the Antibacterial and Antioxidant Activities of Extracts of Gracilaria gracilis with a View into Its Potential Use as an Additive in Fish Feed, Appl. Sci. 2021, Vol. 11, Page 6642. 11 (2021) 6642. https://doi.org/10.3390/APP11146642.

[93] R. Passos, A.P. Correia, I. Ferreira, P. Pires, D. Pires, E. Gomes, B. do Carmo, P. Santos, M. Simões, C. Afonso, T. Baptista, Effect on health status and pathogen resistance of gilthead seabream (Sparus aurata) fed with diets supplemented with Gracilaria gracilis, Aquaculture. 531 (2021) 735888. https://doi.org/10.1016/j.aquaculture.2020.735888.

[94] N.A. ElNaker, A.F. Yousef, L.F. Yousef, A review of Arthrocnemum (Arthrocaulon) macrostachyum chemical content and bioactivity, Phytochem. Rev. 19 (2020) 1427–1448. https://doi.org/10.1007/s11101-020-09686-5.

[95] C. Cristina, P. Lucia, S. Sara, S. Francesco, D. Nobile Matteo Alessandro, C. Amalia, Study of the Efficacy of Two Extraction Techniques from Crithmum maritimum and Salicornia europaea, J. Food Nutr. Res. 6 (2018) 456–463. https://doi.org/10.12691/jfnr-6-7-6.

Chapter 2 - Culture Conditions Affect Antioxidant Production, Metabolism and Related Biomarkers of the Microalgae *Phaeodactylum tricornutum*

2.1 Abstract

Phaeodactylum tricornutum (Bacillariophyta) is a worldwide-distributed diatom with the ability to adapt and survive in different environmental habitats and nutrient-limited conditions. In this research, we investigated the growth performance, the total lipids productivity, the major categories of fatty acids, and the antioxidant content in *P. tricornutum* subjected for 15 days to nitrogen deprivation (N-) compared to standard culture conditions (N+). Furthermore, genes and pathways related to lipid biosynthesis (i.e., glucose-6-phosphate dehydrogenase, acetyl-coenzyme A carboxylase, citrate synthase, and isocitrate dehydrogenase) and photosynthetic activity (i.e., ribulose-1,5-bisphospate carboxylase/oxygenase and fucoxanthin-chlorophyll a/c binding protein B) were investigated through molecular approaches. *P. tricornutum* grown under starvation condition (N-) increased lipids production (42.5 ± 0.19 g/100 g) and decreased secondary metabolites productivity (phenolic content: 3.071 ± 0.17 mg GAE g-1; carotenoids: 0.35 ± 0.01 mg g−1) when compared to standard culture conditions (N+). Moreover, N deprivation led to an increase in the expression of genes involved in fatty acid biosynthesis and a decrease in genes related to photosynthesis. These results could be used as indicators of nitrogen limitation for environmental or industrial monitoring of *P. tricornutum*.

KEYWORDS: *Phaeodactylum tricornutum*; nitrogen stress; gene expression; lipid biosynthesis; photosynthesis; antioxidant activity

2.2 Introduction

From a biotechnological perspective Microalgae are an exceptionally diverse group of photosynthetic marine microorganisms that represent the most numerous forms of life in marine ecosystems. It is known from the literature that the estimated 72 k species of microalgae provide an important contribution to the marine environment and ecosystems [1–4].

These microorganisms can grow in a variety of environments and conditions, from the deepest marine system to the polar, playing an essential role in the evolution of life as the planet's primary producers of oxygen and the most abundant food source for marine animals [2,3,5]. Diatoms are important marine microalgae, interesting not only for their ecological

role but also for their ability to synthesize bioactive compounds useful for both human nutrition and nutraceutical and pharmaceutical applications [4,6–10]. In recent decades, the development of molecular biology and the evolution of "omics" techniques have increased knowledge of marine microorganisms, generating considerable academic and industrial interests [4].

Microalgae are largely used in aquaculture as a food source or as wastewater treatment [5], in biofuel production (limiting nitrogen sources to increase their lipid content) [11], and in the cosmeceutical industry for the production of natural bioactive compounds beneficial to humans health [12]. Cellular biochemical pathways of energy transfer and production of bioactive compounds and metabolic switches in microalgae species are still unexplored [13]. For instance, microalgae are able to alter their metabolism and physiology to survive in unfavorable conditions [11]. Numerous studies have shown that microalgae under stress conditions increase lipid production [14–16] and decrease photosynthetic pigments as reported by Longworth et al. [17] in *Phaeodactylum tricornutum*. Under optimal conditions, microalgae produce antioxidant bioactive compounds (i.e., phenolic compounds and carotenoids) and fatty acids representing a rich source of PUFAs [18].

P. tricornutum exhibits commercial and industrial potentialities, and it is considered a model organism for its adaptable capacity to unfavorable conditions [19] such as nitrogen deprivation. It is widely used for human nutrition thanks to its high-quality protein content and other compounds such as sterols, ω-3 fatty acids useful for human health [20]. Nitrogen is an essential nutrient required by microalgae for their metabolic activities [21]. The reduction of carbon metabolism and the increase in lipid content in microalgae can be associated to a nitrogen deprivation condition [22].

For this reason, the aim of this work was to assess lipid and antioxidant productivity of *P. tricornutum.* The metabolic switches occurring in *P. tricornutum* cultivated under standard and starvation conditions were investigated through molecular markers related to lipid biosynthesis and photosynthesis. The effect of nitrogen stress in *P. tricornutum* was examined at the molecular level by genes related to lipid biosynthesis and photosynthesis: glucose-6-phosphate dehydrogenase (*G6PDH*), acetyl-coenzyme A carboxylase (*ACCase*), citrate synthase (*Cit syn*), isocitrate dehydrogenase (*isocit DH*), ribulose-1,5-bisphospate carboxylase/oxygenase (*RBCL*), and fucoxanthin-chlorophyll a/c binding protein B (*FCP B*) (Figure 1).

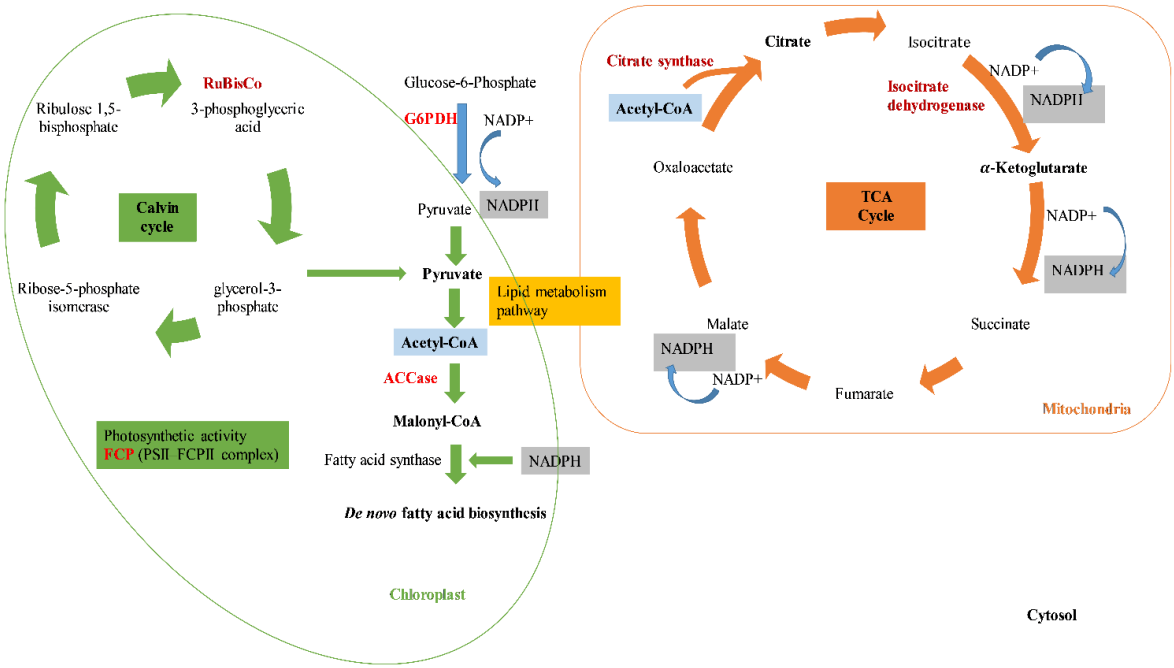


Figure 1. Representative diagram of the enzymes (in red) related to lipid biosynthesis and photosynthesis pathways in *Phaeodactylum tricornutum*. Cytosol: glucose-6-phosphate dehydrogenase (*G6PDH*); chloroplast: acetyl-coenzyme A carboxylase (*ACCase*); citrate synthase (*Cit syn*); isocitrate dehydrogenase (*isocit DH*); mitochondria: ribulose-1,5-bisphospate carboxylase/oxygenase (*RBCL*); fucoxanthin-chlorophyll a/c binding protein B (*FCP B*).

2.3 Materials and Methods

2.3.1 Cultivation Conditions

P. tricornutum strain AC171 was obtained from the Algobank culture collection (France). Growth experiments for each different nutrient conditions, standard (N+) and starvation (N-), were performed in two replicates in 1.5 L flasks, starting from an initial cell density of 1 million cells/mL (T0). The standard culture medium was prepared according to a modified process of Fanesi et al. [23]: 3.3% w/v sea salt containing macro-elements and trace elements, 0.054% v/v $NaNO_3$ (1M), 0.021 % v/v Na_2HPO_4 (0.1M), 0.01% v/v vitamin stock solution (i.e., 297 nM thiamine–HCl, 4.09 nM biotin, and 1.47 nM B_{12}), 0.1% trace metal stock solution I (i.e., 6.56 µM $FeCl_3$ and 6.56 µM Na_2EDTA), and trace metal stock solution II (i.e., 2.42 µM $MnSO_4$, 8.29 µM Na_2EDTA, 254 nM $ZnSO_4$, 5.69 nM $CoSO_4$, 6.10 nM Na_2MoO_4, 1.00 nM Na_2SeO_3, and 6.30 nM $NiCl_2$), 0.2% v/v Na_2SiO_3 (0.105 M), and 1% v/v Tris-HCl (1M) for the maintenance of pH at 8. The starvation culture medium contained the same nutrients, at the same concentrations, as the standard culture, apart from the nitrogen source: $NaNO_3$–, which was only 0.0001% v/v of the total volume. The medium was autoclaved at 120 °C for 20 minutes before inoculation. The aeration of the cultures, which is essential for

the growth and mixing of the cells, was carried out using an aquarium air pump. The airflow was supplied to cultures passing through microfilters (pore size ø = 0.2 µm). Microalgae cultures were kept in aseptic environments to avoid possible contamination at constant temperatures (20 ± 2 °C) and continuous lighting (neon light, 36 W). The cell density was monitored every 3 days using a bright-line hemocytometer (Sigma–Aldrich, Saint Louis, MO, USA) and an optical microscope from LW Scientific, until reaching the plateau phase. Analyses were conducted on the 15th day of culture before reaching the plateau phase.

2.3.2 Biomass Isolation

For the biomass isolation, microalgae cultures were pelleted by centrifugation at 5000 rpm at 4 °C for 20 min (Eppendorf Centrifuge 5430 R). The pellets were recovered, frozen at −80 °C, and lyophilized (Labconco FreeZone25 freeze dryer, equipped with an Edwards vacuum pump oil model R.V 8) for further analysis.

2.3.3 Lipid Content

Total lipids were determined according to Folch's method [24], quantified gravimetrically, and resuspended in n-hexane. All samples were analyzed in triplicate (n = 3). The quantitative determination of fatty acids was conducted from the total lipid content of dried microalgae, according to the method described by Lepage and Roy [25]. Total fatty acids were analyzed as described by Messina et al. [26]. To evaluate the extent of oxidation, the polyene index [27] was determined based on Formula (1):

$$PI = \frac{(EPA + DHA)}{16{:}0} \quad (1)$$

2.3.4 Antioxidant Assays

Extracts from algal biomass were obtained following the protocol previously described by Safafar et al. [28]. Fifty milligrams of freeze-dried biomass, homogenized with 5 mL of 80% ethanol using an Ultraturrax (T25 basic, Ika), were extracted. Then, the extracts were centrifuged at 5000 rpm at 4 °C for 10 minutes, and the supernatant was separated. After, the extracts were evaluated at different concentrations (8–0.25 mg mL^{-1}) for determining antioxidant content. In this study, we performed four assays for the evaluation of the antioxidant activity of microalgae cells.

Polyphenol content was determined spectrophotometrically according to Folin–Ciocalteu [29]. A gallic acid standard solution was prepared in ethanol at concentrations 5–100 mg

mL^{-1}. The analysis was performed on a 96-well plate, and all samples were analyzed in triplicate. The absorbance was measured at 725 nm, using a spectrophotometer (Multiskan-Sky Microplate Reader, Thermo-Scientific™, Waltham, MA, USA). Total phenolics contents are expressed as a mg of gallic acid mg^{-1} of microalgae weight. Total carotenoid content was performed spectrophotometrically according to Maadane et al. [30]. Extracts were prepared at concentration of 1 mg mL^{-1} in ethanol. The samples' absorbances were measured at 470, 648, and 664 nm by spectrophotometer (Multiskan Thermo Fisher Scientific). The total carotenoids content was calculated using Equations (2) of Lichtenthaler et al. [31]:

$$Chl_a = 13{,}36\ x\ Abs_{664} - 5{,}19\ Abs_{648}$$

$$Chl_b = 27{,}43\ x\ Abs_{648} - 8{,}12\ Abs_{664} \quad (2)$$

$$\textbf{Total carotenoids} = \frac{(100\ x\ Abs_{470} - 1{,}63\ x\ Chl_a - 109{,}96\ x\ Chl_b)}{221}$$

For the determination of total antioxidant capacity different assays were carried out. The DPPH radical scavenging activity was performed on modified Bernatoniene et al. [32]. Standard curves were prepared for gallic acid using different concentrations (0.1–0.005 mg mL^{-1}). For this, 40 µL of extracts were mixed with 0.1 mM DPPH radical solution in ethanol (prepared before the analysis) in a 96-well plate. Absorbance was read after 30 min (Multiskan Thermo Fisher Scientific). The percentage of DPPH inhibition was obtained by the following Equation (3):

$$\text{Scavenging effect (\%)} = \left[1 - \left(\frac{absorbance\ of\ sample - absorbance\ of\ blank}{absorbance\ of\ control}\right)\right] * 100 \quad (3)$$

IC 50, which is the concentration of antioxidants reduced at 50%, was calculated through linear regression analysis.

Cellular antioxidant properties by the reducing power assay were determined according to Falleh et al. [33]. After 20 min incubation at 50 °C, the absorbance was read at 700 nm. EC 50 represents the concentration of extracts in which the absorbance is at the half value, and it was used to assess the reducing power of the samples.

2.3.5 Real-Time PCR Analysis

Total RNA was extracted from fresh samples at time zero and after 15 days of monitoring in nitrogen standard and stress cultures. RNA was isolated from samples in PureZOL™ using

the Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad, Hercules, CA, USA) and was measured spectrophotometrically using a Thermo Scientific™ μDrop Plate Spectrophotometer. All samples were analyzed in triplicate. Then, reverse transcription was performed using the 5X iScript Reaction Mix Kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions. The amplification and the relative quantification were performed in triplicate on genes *G6PDH*, *rbcL*, *FCP B*, *ACCase*, *Cit syn*, and *isocit DH* (Table 1). Relative gene expression was evaluated after normalization with the reference genes. Data processing and statistical analysis were performed using CFX Manager Software (Bio-Rad, Hercules, CA, USA). The relative expression of all genes was calculated by the $2^{-\Delta\Delta CT}$ method [34] using *P. tricornutum* RPS (ribosomal protein S1) and TBP (TATA box-binding protein) as the endogenous reference.

Table 1. *Phaeodactylum tricornutum* primer sequences used for real-time PCR.

Gene	Access Number	F/R Primer Sequence (5'–3')	References
G6PDH	MH064127.1	F. GCGAGAAATGGCACAAGG R. GTTCATCGCAGTCGGGAGA	[35]
rbcL		F. CCAAGGTCCTGCTACTGGTG R. TCTCCAACGCATGAAGGGT	
FCP B		F. GCCGATATCCCCAATGGATTT R. CTTGGTCGAAGGAGTCCCATC	[36]
ACCase,		F. GTTGCTTGACGCTGAACTGG R. CCTTCATGCGACCTGTCTTG	[37]
Cit syn		F. TTATGAAGTCATGCCCGACA R. GGTCCCAGTACAGTTGCGAT	[37]
Isocit DH		F. GGGCAGTCATGAAAGACGTT R. ATCCGTCAGCATATCACCGT	[37]
RPS		F. AATTCCTCGAAGTCAACCAGG R. GTGCAAGAGACCGGACATAC	[38]
TBP		F. ATCGATTTGTCAATCCACGAG R. ATACAGATTCTGTGTCCACGG	[38]

2.3.6 Statistical Analysis

Statistical analysis was performed using the computer application SPSS for Windows® (version 20.0, SPSS Inc., Chicago, IL, USA). All the analyses were carried out in triplicate. The results are expressed as the mean ± standard deviation. The homogeneity of variance was confirmed by the Levene test. Data were subjected to one-way analysis of variance (ANOVA), and Student–Newman–Keuls or Games–Howell post hoc tests were performed in order to make multiple comparisons between experimental groups. The significance level was 95% in all cases ($p < 0.05$).

2.4 Results and Discussion

2.4.1 Cell Growth

The obtained results in Figure 2 show a reduction in the growth performance of *Phaeodactylum tricornutum* cultured under starvation (N-) conditions as a common response to nutrient limitations that trigger modifications in primary metabolism [39]. A growth rate exponential phase occurred until day 12 in both treatments (Figure 1) even if, in the first 9 days of culture, a faster growth rate in *P. tricornutum* N+ was observed.

From day 3, a colorimetric change in the cultures was evident. The standard cultures (N+) retained a brown color, while in N-, they turned to yellow due to the degradation of pigments. On day 12, N- cultures reached their maximum growth rate, followed by a decrease from day 13 to 15, while N+ cultures continued their exponential phase until the end of monitoring (day 18) (Figure 2).

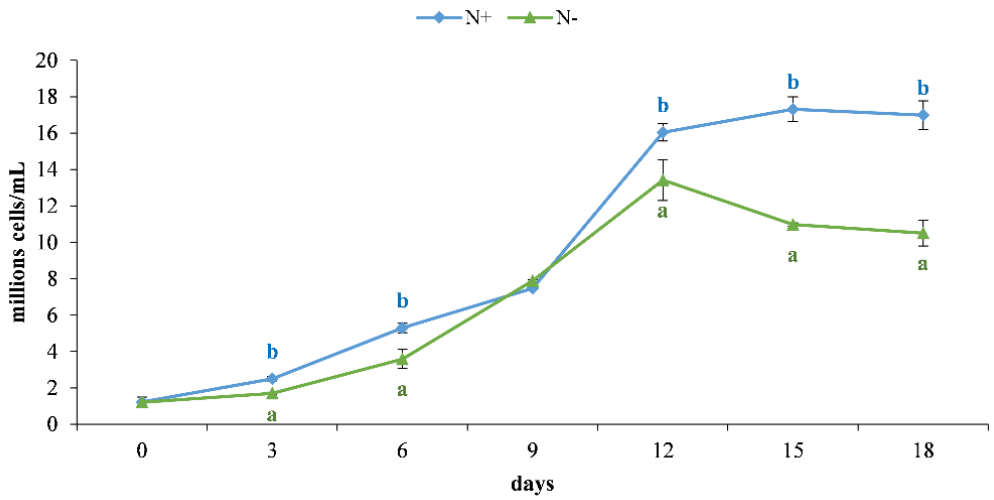


Figure 2. Growth performance of *P. tricornutum* cultivated for 18 days under standard (N+, blue line) and starvation condition (N-, green line). Values are presented as the mean ± SEM (n = 3) with standard deviations. Different letters within the same day indicate significant differences (p < 0.05) within the two treatment conditions (i.e., N+ and N-).

The yields of N– and N+ were, respectively, 0.1574 ± 0.010 g/L and 0.3026 ± 0.0141 g/L. According to Yodsuwan et al. [14], long-term nutrient stress conditions reduced growth performance in *P. tricornutum*. The stress condition resulting from nutrient deficiency led to a decrease in biomass productivity in favor of the production of bioactive microalgal secondary metabolites [40]. No universal or efficient nutrient acquisition strategy of nitrogen limitation exists to date that increases the amount of bioactive secondary metabolites without affecting algal biomass [41,42]. Mixotrophic cultivation of *P. tricornutum* [43] and *Chlorella vulgaris*

(Chlorophyta) [44], in long-term nitrogen-limited cultivations, resulted in improved biomass and biomolecule productivity. Integrative approaches include the synergistic action of different parameters that control both the growth rate and production of bioactive compounds such as light, temperature, and pH [45]. Burch and Franz [46] suggested a combination of nitrogen starvation and low concentration of H_2O_2 as a chemical modulator to stimulate triacylglycerol (TAG) synthesis in the early exponential phase without changing biomass productivity.

2.4.2 Lipid Content

2.4.2.1 Total Lipid Content

Manipulation of microalgae culture medium can induce a variation in lipid concentrations, especially nitrogen limitation conditions lead to an increase in lipid contents [39]. Throughout *P. tricornutum* monitoring for 15 days in N-, the quantity of total lipids increased compared to the initial culture condition (T0) and to the lipid content in N+, as shown in Figure 3, reaching 42.5 ± 0.19 g/100 g of dried algal biomass in N- and 33.35 ± 0.12 g/100 g of dried algal biomass in N+. Our results are in accordance with studies that focused on the cultivation of *P. tricornutum* under nutrient stress conditions [47] on *Nannochloropsis* sp. (Ochrophyta, Eustigmatophyceae) [48], *Conticribra weissflogii (*formerly *Thalassiosira weissflogii*), and *Cyclotella cryptica* (Bacillariophyta) [49] maintained under nitrogen deprivation.

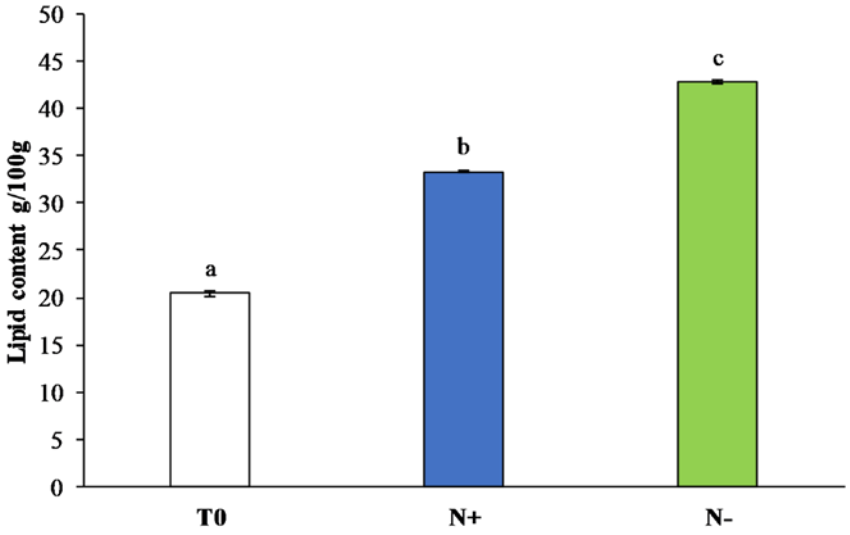


Figure 3. Total lipids (g/100 g) of *P. tricornutum* cultivated for 15 days under initial culture conditions (T0, white bar), under standard conditions (N+, blue bar), and under starvation conditions (N-, green bar). Values are expressed as the mean ± SEM (n = 3) with standard deviations. Different letters indicate statistical differences (p < 0.05) between groups.

2.4.2.2 Fatty Acid Content

The ability of microalgae to survive within a wide range of environmental conditions is largely correlated to the diversity in cellular lipids [50]. Among total lipids, significant differences in fatty acid content were observed between the two different growth conditions (N+ and N-) (Figure 4).

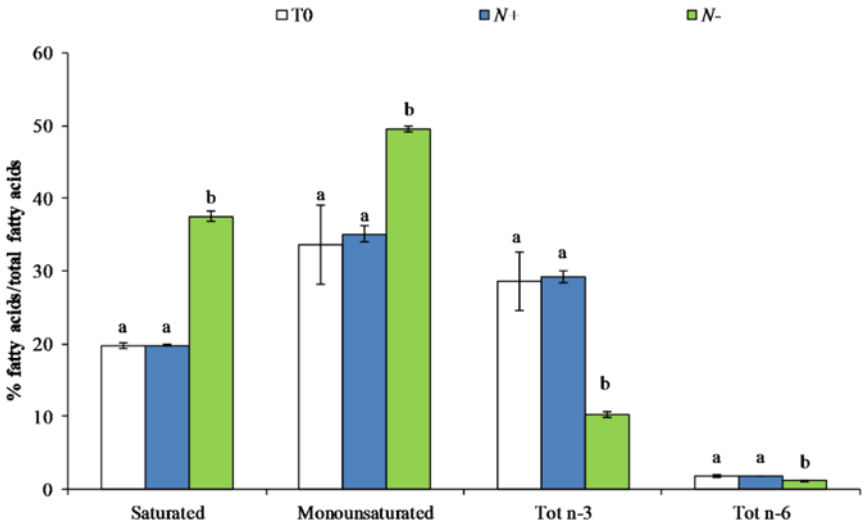


Figure 4. Fatty acids class composition (%, w/w) of *P. tricornutum* cultivated for 15 days under initial culture condition (T0, white bar), under standard conditions (N+, blue bar), and under starvation conditions (N-, green bar). Values are expressed as the mean ± SEM (n = 3) with standard deviations. Different letters indicate statistical differences ($p < 0.05$) between groups.

The maximum percentage of saturated fatty acids, monounsaturated, and polyunsaturated (n-3 and n-6) was observed in N+: 19.79 ± 0.78%, 35.06 ± 4.64%, 29.15 ± 3.12%, and 1.83 ± 0.18%, respectively (Figure 4). In N-, there was a significant increase in the amount of saturated and monounsaturated fatty acids: 37.52 ± 1.92% and 49.56 ± 1.74% (Figure 4). The total percentage of polyunsaturated fatty acids was three-fold reduced in N-. The differences were induced by the extensive increase in palmitic (16:0) and palmitoleic acids (16:1n7) and the decrease in eicosapentaenoic acid (20:5n3–EPA), which was abundant in N+ (Table 2) as reported by similar works [14,51,52].

Table 2. Fatty acids composition (%, w/w) of *P. tricornutum* cultivated for 15 days under standard (N+) and starvation condition (N–).

Fatty Acid	N+	N-
14:0	7.35 ± 0.54 [a]	4.85 ± 0.61 [b]
16:0	11.87 ± 0.72 [a]	31.90 ± 1.57 [b]
16:1n-7	31.12 ± 2.82 [a]	43.35 ± 1.59 [b]
16:2n-4	1.99 ± 0.62 [a]	0.38 ± 0.02 [b]
16:3n-4	12.12 ± 1.51 [a]	1.07 ± 0.10 [b]
18:1n-9	2.63 ± 1.59	4.90 ± 0.34
18:1n-7	1.13 ± 0.21	1.16 ± 0.18
18:2n-6	1.11 ± 0.20 [a]	0.71 ± 0.11 [b]
20:5n-3 EPA	24.43 ± 1.85 [a]	8.30 ± 0.80 [b]
22:5n-3	1.88 ± 0.37 [a]	0.69 ± 0.13 [b]
22:6n-3 DHA	1.62 ± 0.33 [a]	0.71 ± 0.10 [b]

Data are reported as the means (n = 3) with standard deviation. Different letters indicate significant differences between each treatment ($p < 0.05$).

The changes in fatty acids composition caused a significant modification to the polyenoic index (PI) and on the ratio n-3 fatty acids/total fatty acids (n3/tot FA) as shown in Table 3. The polyene index is used to measure the polyunsaturated fatty acids oxidation [53]. EPA and DHA, which represent the main part of polyunsaturated fatty acids (Table 2), were the most susceptible to oxidation. In particular, in N-, EPA and DHA decreased at the increase of 16:0 as a result of the polyene index decreasing significantly. A similar decrease was observed for the total n3/tot FA ratio, caused by a significant decrease in EPA in N-.

Table 3. Unsaturation markers calculated from the fatty acid profile of *P. tricornutum* cultivated for 15 days under standard (N+) and starvation condition (N-).

	N+	N-
Polyene Index	2.21 ± 0.32 [a]	0.28 ± 0.04 [b]
n3/tot FA	0.30 ± 0.04 [a]	0.10 ± 0.01 [b]

Data are reported as the means (n = 3) with standard deviation. Different letters indicate significant differences between each treatment ($p < 0.05$).

Similar results have been reported by Villanova et al. [43] on lipid productivity on the 10th and the 15th days of cultivation of *P. tricornutum* under mixotrophic and phototrophic conditions. *P. tricornutum* is a promising candidate for industrial applications for the production of biofuel and of bioactive molecules, but further studies are essential to identify critical control points and a balance between lipid and biomass productivity of microalgae [54].

2.4.3 Cellular Antioxidant Activity

2.4.3.1 Polyphenol Content

Polyphenols are a very important class of antioxidants that have protective properties associated with cellular defense mechanisms. Although the phenolic content profile varies significantly among species, diatoms adapt differentially to nutritional stress than green algae and plants, reflecting the genetic diversity and complexity of these microorganisms [55]. Significant differences in total polyphenol content between cultivation conditions were observed (Figure 5). Particularly, *P. tricornutum* N+ had a high phenolic compounds content (3.071 ± 0.17 mg GAE/g DW), whereas a lower phenolic content was measured in N- (1.115 ± 0.00 mg GAE/g DW) (Figure 5).

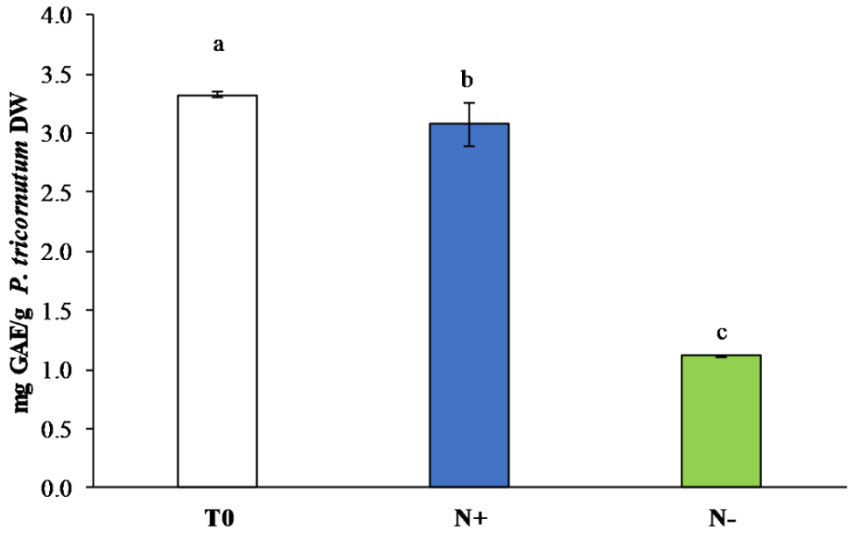


Figure 5. Total polyphenol content (mg GAE/g DW) in *P. tricornutum* extracts cultivated for 15 days under initial culture conditions (T0, white bar), under standard conditions (N+, blue bar), and under starvation conditions (N-, green bar). Values are reported as the means (n = 3), and error bars report the standard deviations. Treatments that do not share the same letter were significantly different from each other ($p < 0.05$).

Under standard conditions, redox reactions of reactive oxygen species (ROS) are produced through photorespiration, and it has been established that oxidative phosphorylation by phenolic compounds do protect cells from damage [55]. ROS are markers of oxidative stress related to lipid accumulation and are frequently observed in microalgae grown under abiotic stresses [56].

In agreement with previous studies [55,56], lower concentrations in total polyphenol content were measured in *P. tricornutum*, *Tetraselmis suecica*, and *Chlorella vulgaris* (Chlorophyta) cultivated under N-limited condition [55]. Similarly, in *Tetradesmus dimorphus* (formerly *Acutodesmus dimorphus*) (Chlorophyta), the total polyphenol content initially increased and

then decreased after 3 days of cultivation under nitrogen deprivation [56]. Knowledge regarding the impact of nutrient stress on the phenolic content of microalgae still remains scarce and species specific [41,55,57].

2.4.3.2 Carotenoids

Our results for *P. tricornutum*, cultivated under standard (N+) and starvation conditions (N-), showed a similar pattern to polyphenols content in the carotenoids content (Figure 6), an important class of bioactive compounds with antioxidant properties.

After 15 days of culture, N–s showed the lowest content of carotenoids (0.35 ± 0.01 mg/g DW) compared to N+ (2.70 ± 0.23 mg/g DW). The lowest content of carotenoids in N- was probably the result of alteration of the photosynthetic system in microalgae grown under conditions of nitrogen deprivation, as a common response of photosynthetic organisms to abiotic stressors [58]. Similar results were observed by Gauthier et al. [59] in *P. tricornutum*, *T. suecica*, and *C. vulgaris* cultures maintained under nitrogen starvation. However, other studies described an increase in carotenoid production in microalgae when cultivated under nutrient starvation [55,60].

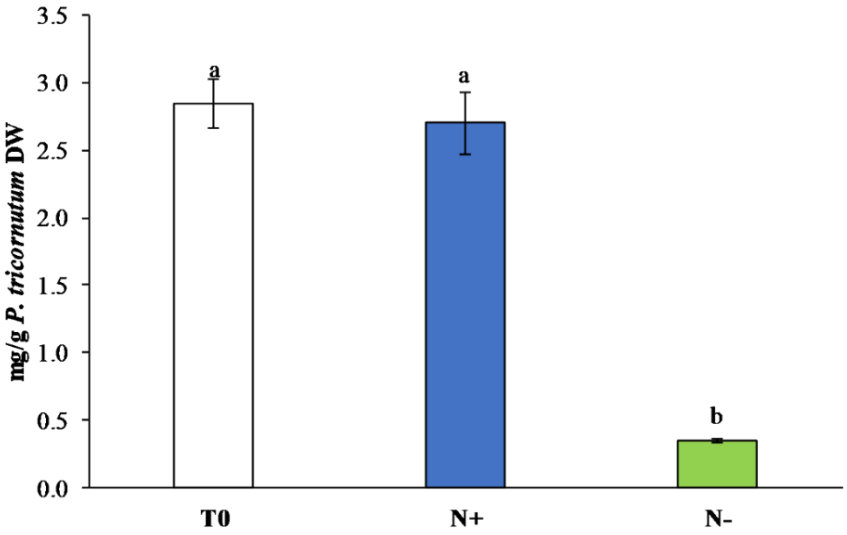


Figure 6. Carotenoid content (mg/g DW) in *P. tricornutum* extracts cultivated for 15 days under initial culture conditions (T0, white bar), under standard conditions (N+, blue bar), and under starvation conditions (N-, green bar). Values are reported as the means (n = 3), and error bars report the standard deviations. Treatments that do not share the same letter were significantly different from each other ($p < 0.05$).

2.4.3.3 Total Antioxidant Assays

The high productivity of *P. tricornutum* antioxidants cultivated under standard condition was confirmed by the results of DPPH assay (Figure 7).

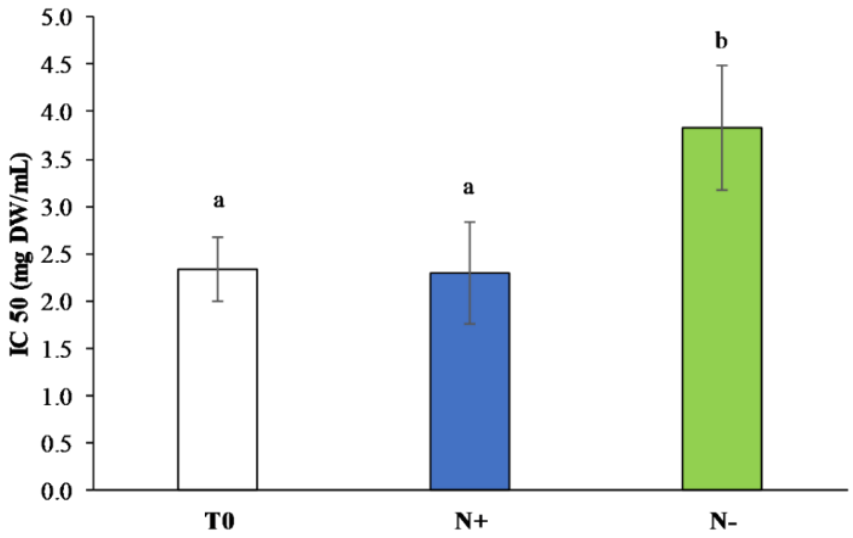


Figure 7. DPPH radical scavenging activity (IC 50, mg DW/mL) in *P. tricornutum* extracts cultivated for 15 days under initial culture conditions (T0, white bar), under standard conditions (N+, blue bar), and under starvation conditions (N-, green bar). Values are reported as the means (n = 3), and error bars report the standard deviations. Treatments that do not share the same letter were significantly different from each other ($p < 0.05$).

In particular, the lowest IC50, 2.29 ± 0.54 mg DW/mL, was recorded in N+. The lowest IC50 corresponded to the highest antioxidant ability. The highest IC50, 3.83 ± 0.66 mg DW/mL, was measured in N-. Considering the pattern observed for polyphenols and carotenoids, these classes of antioxidants are the main contributors to cellular antioxidant activity.

Similar results were obtained by Jeyakumar et al. [61], who observed a higher DPPH scavenging activity in nitrogen-depleted conditions (85%) compared to nitrogen-replete conditions (75%) and control (64%) in *Isochrysis* sp. A study conducted by Singh et al. [62] on *Dunaliella salina* (Chlorophyta) showed similar trends with higher DPPH radical scavenging activity in nitrogen-depleted medium cultures than normal condition. As expected, *P. tricornutum* culture maintained under nitrogen deprivation condition (N-) showed a higher scavenging activity [61].

A further damage on the total antioxidant ability of *P. tricornutum* in N- was demonstrated by a reducing power assay. The results shown in Figure 8 are in line with the DPPH assay results. The highest reducing power was observed in *P. tricornutum* N+ (EC50, 66.54 ± 2.9 mg DW/mL), whereas in N-, the reducing power decreased (EC50, 147.84 ± 9.85 mg DW/mL).

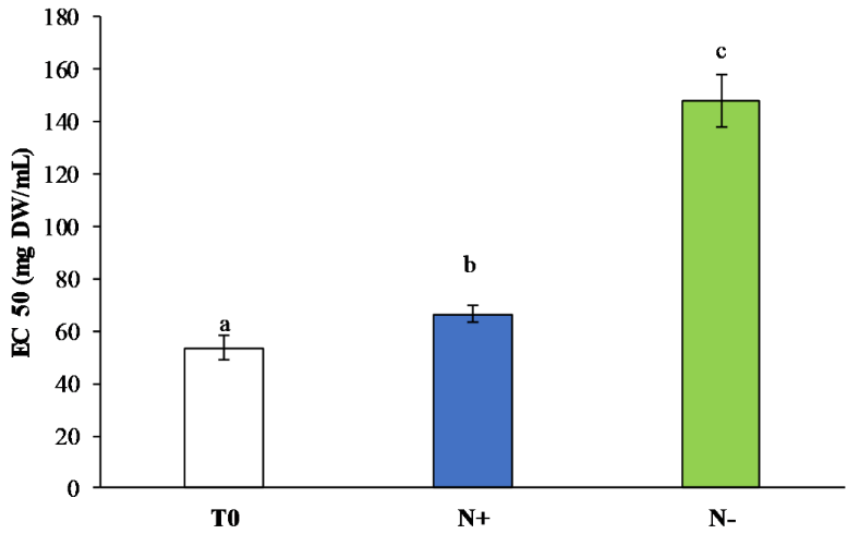


Figure 8. Reducing power (EC50, mg DW/mL) in *P. tricornutum* extracts cultivated for 15 days under initial culture conditions (T0, white bar), under standard conditions (N+, blue bar), and under starvation conditions (N-, green bar). Values are reported as the means (n = 3), and error bars report the standard deviations. Treatments that do not share the same letter were significantly different from each other ($p < 0.05$).

The obtained results are in accordance with Singh et al. [62], who observed a higher reducing power in nitrogen stressed cells of *Dunaliella salina* than those cultivated under normal condition.

2.4.4 Gene Expression

Diatoms show remarkable diversity among species and a great capacity to adapt their metabolism to different nutritional strategies [56,63]. Relevant genes related to lipid biosynthesis (i.e. *G6PDH*, *ACCase*, *Cit syn*, and *isocit DH*) and photosynthetic activity (i.e., *rbcL* and *FCP B*) were analyzed in *P. tricornutum* maintained under two different culture conditions (i.e. N+ and N-) (Figure 9).

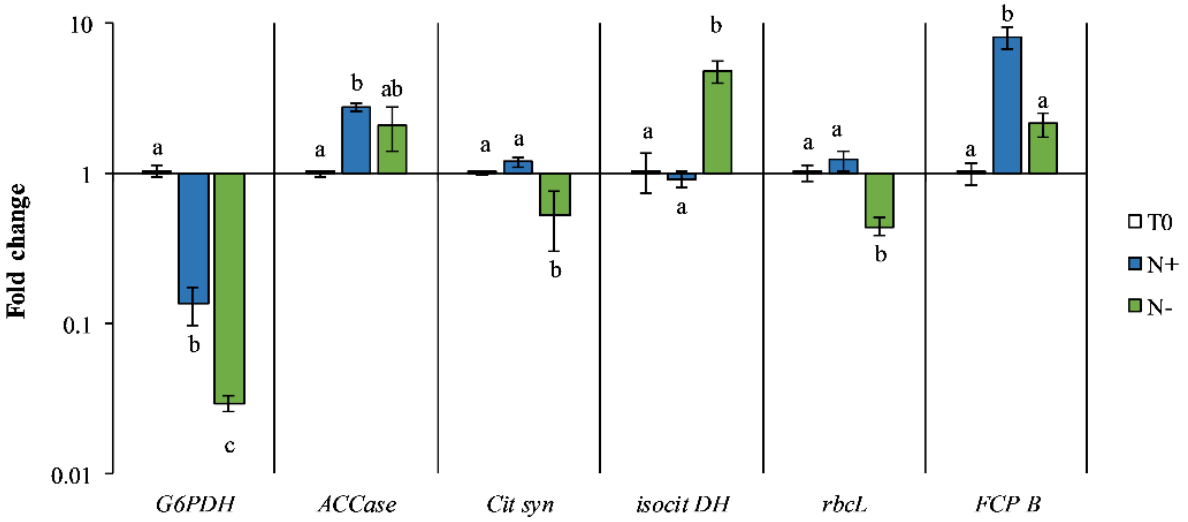


Figure 9. Relative gene expression of genes related to lipid biosynthesis (glucose-6-phosphate dehydrogenase (*G6PDH*), acetyl-coenzyme A carboxylase (*ACCase*), citrate synthase (*Cit syn*), and isocitrate dehydrogenase (*isocit DH*)) and photosynthetic activity (ribulose-1,5-bisphospate carboxylase/oxygenase (large subunit) (*rbcL*) and fucoxanthin-chlorophyll a/c binding protein B (*FCP B*)) in *P. tricornutum* under initial culture conditions (T0, white bars) and after 15 days of cultivation under standard conditions (N+, blue bars) and starvation conditions (N-, green bars). Values are the mean ± SEM (n = 3) with standard deviations. Statistical differences ($p < 0.05$) between groups are indicated by different letters.

G6PDH catalyzes the primary reaction of the pentose phosphate pathway (PPP) and produces a large amount of reducing equivalents in the form of nicotinamide adenine dinucleotide phosphate (NADPH), which are essential for lipid biosynthesis [9]. In our experiment, the G6PDH expression was significantly downregulated in *P. tricornutum* N- compared to N+ ($p < 0.05$) (Figure 9), suggesting that the pathway of PPP was negatively affected by nutrient limitation. These results are in contrast with the literature, where overexpression of G6PDH induced an increase in lipid content in *P. tricornutum*, highlighting its critical role in algal lipid accumulation by enhancing NADPH supply [64]. However, transcription of G6PDH in nitrogen limitation shows significant differences between green microalgae *C. vulgaris* [65], *Chlamydomonas reinhardtii* [66], diatom *Thalassiosira pseudonana* [67], and *Nannochloropsis gaditana* (Eustigmatophyceae) [68]. In fact, modifications at the transcript level of the gene G6PDH suggest that PPP is less active in N– conditions, indicating that NADPH yield could be produced by another metabolic pattern. Further investigations are needed to deeply understand the molecular mechanism of G6PDH under starvation conditions.

De novo fatty acid synthesis is one of the important contributors to the total fatty acid pool in the cell [50]. ACCase catalyzes the first step for the de novo fatty acid biosynthesis, and the

transcriptional pattern of this gene is very crucial for lipid productivity [69,70]. Acetyl-CoA has a significant role in the central metabolic flux, as it is a primary intermediate for biomolecule production and energy metabolism. ACCase catalyzes the conversion of acetyl-CoA to malonyl-CoA, the substrate for the first step of elongation of fatty acids through FAS (fatty acid synthetase). Transcriptomic and proteomic studies suggested that under nitrogen starvation conditions, plastidial acetyl-CoA carboxylase (one of the two isoforms in microalgae) [22,50] is upregulated, and it represents the key enzyme for de novo fatty acid synthesis pathway [50].

In addition, various transcriptomics studies in diatom have revealed the vital role of lipid recycling (as opposed to *de novo* lipid synthesis) in increasing triacylglycerol accumulation during nitrogen deprivation [22]. Our results showed similar responses in both cultivation conditions (Figure 9). We assumed that the lipid content might influence the activity of the enzyme by a retroactive mechanism depending on the amount of lipid in the cell. These results are in agreement with Guerra et al. [37], indicating that in starvation conditions, the recycling of existing fatty acids occurs rather than *de novo* fatty acid biosynthesis.

The analysis of gene expression of two important enzymes participating in the tricarboxylic acid cycle (TCA cycle), *Cit syn* and *isocit DH*, was evaluated. Citrate synthase catalyzes the condensation of Acetyl-CoA and oxaloacetate into citrate [71]. Isocitrate dehydrogenase is involved in a critical irreversible reaction of the TCA cycle associated with carbon and nitrogen metabolism, catalyzing the reaction for the formation of α-ketoglutarate, the precursor for amino acid biosynthesis and NADPH [15,50]. The results showed a downregulation of the *Cit syn* gene in N- compared to N+ (Figure 9), suggesting that carbon was redirected from the TCA cycle to lipid metabolism [72]. Our results are in agreement with studies conducted on the green algae *Chlamydomonas reinhardtii*, where mRNA citrate synthase level under starvation conditions were undetectable, highlighting the key role of this gene in lipid biosynthesis, further confirming the relationship between citrate synthase and lipid accumulation [73]. *Isocit DH* was significantly upregulated in N- ($p < 0.05$) (Figure 9). Our results, in accordance with other studies, indicate the importance of these two regulatory enzymes for metabolic flux checkpoints [50,74,75].

The expression of genes involved in photosynthetic carbon fixation (i.e., *rbcL* and *FCP B*) was investigated. The *rbcL* gene encodes the catalytic large subunit of the enzyme rubisco that catalyzes carbon fixation in the first Calvin cycle's reaction [76]. Carbon dioxide is the exclusive source of carbon in photoautotrophic organisms and is the only pathway for lipid synthesis [76]. *FCP B* is a unique light-harvesting system in diatoms that is scarcely

understood and associated with photosystems II. Fucoxanthin is the most abundant carotenoid in *P. tricornutum*, and it is the most crucial pigment in this pattern [77]. Transcript levels of these vital enzymes showed similar variations, as expected. Both genes were severely downregulated in N- ($p < 0.05$) (Figure 9). The intense downregulation of *FCP B* is in accordance with the decrease in carotenoid content in N- as described in Figure 5. Nitrogen limitation in *P. tricornutum* can lead to high damage of the photosynthetic apparatus and modification of enzymes associated with NADPH, the TCA cycle, and PPP [47,78]. This result indicates that nitrogen starvation decelerates the light-harvesting process in photosynthesis and that the photosynthetical activity inhibits the relevant carbon fixation pathway in favor of lipid biosynthesis [21]. Therefore, alternative metabolic pathways may occur, compensating for carbon assimilation, leading to high lipid accumulation under nitrogen starvation [21].

Even though there have been many attempts dedicated to the quantification of *P. tricornutum*'s lipid content [79], transcriptome/proteome/metabolome analyses [75], and to the optimization of biomass production in nitrogen-limited conditions [14], there is still a considerable amount of ambiguity with regard to the molecular mechanisms and modifications of nutrient limitation in this marine diatom. These genes could be used as indicators for environmental and industrial monitoring of *P. tricornutum*.

2.5 Conclusions

Although lipid productivity is a common strategy for microalgae species to survive under abiotic stress condition, cellular mechanisms involved are not fully understood. Our study focused on the molecular changes between lipid biosynthesis and photosynthesis under nitrogen deprivation conditions, associated with the transcriptional expression pattern of central metabolic regulatory enzymes. We observed substantial differences between lipid content, antioxidant productivity, and gene expression patterns. We reported a reduction in EPA and antioxidant productivity and an increase in TAGs in N- cells. These changes are very important from a biotechnological perspective. The changes in the productivity of important secondary metabolites, such as EPA, antioxidants, and TAGs, make *P. tricornutum* a potential candidate for pilot-scale cultivation in both nutritional strategies. The gene expression modification of the enzymes studied in this report can be used as markers for environmental and industrial monitoring of *P. tricornutum*.

2.6 References

[1] W. Huang, F. Daboussi, Genetic and metabolic engineering in diatoms, Philos. Trans. R. Soc. B Biol. Sci. 372 (2017). https://doi.org/10.1098/rstb.2016.0411.

[2] S. Abu-Ghosh, Z. Dubinsky, V. Verdelho, D. Iluz, Unconventional high-value products from microalgae: A review, Bioresour. Technol. 329 (2021) 124895. https://doi.org/10.1016/j.biortech.2021.124895.

[3] D.S. Andrade, H.F. Amaral, F.Z. Gavilanes, L.R.I. Morioka, J.M. Nassar, J.M. de Melo, H.R. Silva, T.S. Telles, Microalgae: Cultivation, Biotechnological, Environmental, and Agricultural Applications, (2021) 635–701. https://doi.org/10.1007/978-981-15-8999-7_23.

[4] Y. Maltsev, K. Maltseva, Fatty acids of microalgae: diversity and applications, Rev. Environ. Sci. Bio/Technology 2021 202. 20 (2021) 515–547. https://doi.org/10.1007/S11157-021-09571-3.

[5] M. Rizwan, G. Mujtaba, S. Ahmed, K. Lee, N. Rashid, Exploring the potential of microalgae for new biotechnology applications and beyond : A review, Renew. Sustain. Energy Rev. 92 (2018) 394–404. https://doi.org/10.1016/j.rser.2018.04.034.

[6] E. Gallego-Cartagena, M. Castillo-Ramírez, W. Martínez-Burgos, Effect of stressful conditions on the carotenogenic activity of a Colombian strain of Dunaliella salina, Saudi J. Biol. Sci. 26 (2019) 1325–1330. https://doi.org/10.1016/j.sjbs.2019.07.010.

[7] N.T. Eriksen, The technology of microalgal culturing, Biotechnol. Lett. 30 (2008) 1525–1536. https://doi.org/10.1007/s10529-008-9740-3.

[8] M. Vigani, C. Parisi, E. Rodríguez-Cerezo, M.J. Barbosa, L. Sijtsma, M. Ploeg, C. Enzing, Food and feed products from micro-algae: Market opportunities and challenges for the EU, Trends Food Sci. Technol. 42 (2015) 81–92. https://doi.org/10.1016/j.tifs.2014.12.004.

[9] J. Xue, T.T. Chen, J.W. Zheng, S. Balamurugan, Y.H. Liu, W.D. Yang, J.S. Liu, H.Y. Li, Glucose-6-Phosphate Dehydrogenase from the Oleaginous Microalga Nannochloropsis Uncovers Its Potential Role in Promoting Lipogenesis, Biotechnol. J. 15 (2020) 1–9. https://doi.org/10.1002/biot.201900135.

[10] T. Lafarga, Effect of microalgal biomass incorporation into foods: Nutritional and

sensorial attributes of the end products, Algal Res. 41 (2019). https://doi.org/10.1016/j.algal.2019.101566.

[11] B. Ramesh Kumar, G. Deviram, T. Mathimani, P.A. Duc, A. Pugazhendhi, Microalgae as rich source of polyunsaturated fatty acids, Biocatal. Agric. Biotechnol. 17 (2019) 583–588. https://doi.org/10.1016/J.BCAB.2019.01.017.

[12] Ç. Yarkent, C. Gürlek, S.S. Oncel, Potential of microalgal compounds in trending natural cosmetics: A review, Sustain. Chem. Pharm. 17 (2020) 100304. https://doi.org/10.1016/j.scp.2020.100304.

[13] A. Mishra, K. Medhi, P. Malaviya, I.S. Thakur, Omics approaches for microalgal applications: Prospects and challenges, Bioresour. Technol. 291 (2019). https://doi.org/10.1016/j.biortech.2019.121890.

[14] N. Yodsuwan, S. Sawayama, S. Sirisansaneeyakul, Effect of nitrogen concentration on growth, lipid production and fatty acid profiles of the marine diatom Phaeodactylum tricornutum, Agric. Nat. Resour. 51 (2017) 190–197. https://doi.org/10.1016/j.anres.2017.02.004.

[15] Z.K. Yang, Y.F. Niu, Y.H. Ma, J. Xue, M.H. Zhang, W.D. Yang, J.S. Liu, S.H. Lu, Y. Guan, H.Y. Li, Molecular and cellular mechanisms of neutral lipid accumulation in diatom following nitrogen deprivation, Biotechnol. Biofuels. 6 (2013) 1–14. https://doi.org/10.1186/1754-6834-6-67.

[16] H. Zhang, W. Yin, D. Ma, X. Liu, K. Xu, J. Liu, Phytohormone supplementation significantly increases fatty acid content of Phaeodactylum tricornutum in two-phase culture, J. Appl. Phycol. 33 (2020) 13–23. https://doi.org/10.1007/s10811-020-02074-8.

[17] J. Longworth, D. Wu, M. Huete-Ortega, P.C. Wright, S. Vaidyanathan, Proteome response of Phaeodactylum tricornutum, during lipid accumulation induced by nitrogen depletion, Algal Res. 18 (2016) 213–224. https://doi.org/10.1016/j.algal.2016.06.015.

[18] B. Gao, A. Chen, W. Zhang, A. Li, C. Zhang, Co-production of lipids, eicosapentaenoic acid, fucoxanthin, and chrysolaminarin by Phaeodactylum tricornutum cultured in a flat-plate photobioreactor under varying nitrogen conditions, J. Ocean Univ. China. 16 (2017) 916–924. https://doi.org/10.1007/s11802-017-3174-2.

[19] Z. Chen, M.K. Yang, C.Y. Li, Y. Wang, J. Zhang, D.B. Wang, X.E. Zhang, F. Ge,

Phosphoproteomic analysis provides novel insights into stress responses in Phaeodactylum tricornutum, a model diatom, J. Proteome Res. 13 (2014) 2511–2523. https://doi.org/10.1021/pr401290u.

[20] A. Krishna, K. Wayne, K. Rambabu, Y. Tao, D. Chu, P. Show, Food Science and Human Wellness Microalgae : A potential alternative to health supplementation for humans, Food Sci. Hum. Wellness. 8 (2019) 16–24. https://doi.org/10.1016/j.fshw.2019.03.001.

[21] Z.K. Yang, Y.H. Ma, J.W. Zheng, W.D. Yang, J.S. Liu, H.Y. Li, Proteomics to reveal metabolic network shifts towards lipid accumulation following nitrogen deprivation in the diatom Phaeodactylum tricornutum, J. Appl. Phycol. 26 (2014) 73–82. https://doi.org/10.1007/s10811-013-0050-3.

[22] N. Arora, P.T. Pienkos, V. Pruthi, K.M. Poluri, M.T. Guarnieri, Leveraging algal omics to reveal potential targets for augmenting TAG accumulation, Biotechnol. Adv. 36 (2018) 1274–1292. https://doi.org/10.1016/j.biotechadv.2018.04.005.

[23] A. Fanesi, J.A. Raven, M. Giordano, Growth rate affects the responses of the green alga Tetraselmis suecica to external perturbations, Plant, Cell Environ. 37 (2014) 512–519. https://doi.org/10.1111/pce.12176.

[24] J. Folch, M. Lees, G.. Sloane Stanley, A Simple method for the isolation and purification of total lipids from animal tissues, 55 (1956) 999–1033.

[25] G. Lepage, C.C. Roy, Improved recovery of fatty acid through direct transesterification without prior extraction or purification, J. Lipid Res. 25 (1984) 1391–1396.

[26] C.M. Messina, G. Renda, L. La Barbera, A. Santulli, By-products of farmed European sea bass (Dicentrarchus labrax L.) as a potential source of n-3 PUFA, Biol. 68 (2013) 288–293. https://doi.org/10.2478/s11756-013-0148-8.

[27] R. Bustos, L. Romo, K. Yáñez, G. Díaz, C. Romo, Oxidative stability of carotenoid pigments and polyunsaturated fatty acids in microparticulate diets containing krill oil for nutrition of marine fish larvae, in: J. Food Eng., Elsevier, 2003: pp. 289–293. https://doi.org/10.1016/S0260-8774(02)00272-8.

[28] H. Safafar, J. Van Wagenen, P. Møller, C. Jacobsen, Carotenoids, Phenolic Compounds and Tocopherols Contribute to the Antioxidative Properties of Some Microalgae Species Grown on Industrial Wastewater, Mar. Drugs 2015, Vol. 13, Pages

7339-7356. 13 (2015) 7339–7356. https://doi.org/10.3390/MD13127069.

[29] V. Folin, O., Ciocalteu, Tyrosine and Tryptophane in Proteins, J. Biol. Chem. 73 (1927) 627–650.

[30] A. Maadane, N. Merghoub, T. Ainane, H. El Arroussi, R. Benhima, S. Amzazi, Y. Bakri, I. Wahby, Antioxidant activity of some Moroccan marine microalgae: Pufa profiles, carotenoids and phenolic content, J. Biotechnol. 215 (2015) 13–19. https://doi.org/10.1016/J.JBIOTEC.2015.06.400.

[31] H.K. Lichtenthaler, C. Buschmann, Chlorophylls and Carotenoids: Measurement and Characterization by UV-VIS Spectroscopy, Curr. Protoc. Food Anal. Chem. 1 (2001) F4.3.1-F4.3.8. https://doi.org/10.1002/0471142913.FAF0403S01.

[32] J. Bernatoniene, R. Masteikova, J. Davalgiene, R. Peciura, R. Gauryliene, R. Bernatoniene, D. Majiene, R. Lazauskas, G. Civinskiene, S. Velziene, J. Museliк, Z. Chalupova, Topical application of calendula officinalis (L.): Formulation and evaluation of hydrophilic cream with antioxidant activity, J. Med. Plants Res. 5 (2011) 868–877.

[33] H. Falleh, R. Ksouri, F. Medini, S. Guyot, C. Abdelly, C. Magné, Antioxidant activity and phenolic composition of the medicinal and edible halophyte Mesembryanthemum edule L., Ind. Crops Prod. 34 (2011) 1066–1071. https://doi.org/10.1016/j.indcrop.2011.03.018.

[34] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method, Methods. 25 (2001) 402–408. https://doi.org/10.1006/meth.2001.1262.

[35] S. Wu, A. Huang, B. Zhang, L. Huan, P. Zhao, A. Lin, G. Wang, Enzyme activity highlights the importance of the oxidative pentose phosphate pathway in lipid accumulation and growth of Phaeodactylum tricornutum under CO2 concentration, Biotechnol. Biofuels. 8 (2015). https://doi.org/10.1186/s13068-015-0262-7.

[36] M. Siaut, M. Heijde, M. Mangogna, A. Montsant, S. Coesel, A. Allen, A. Manfredonia, A. Falciatore, C. Bowler, Molecular toolbox for studying diatom biology in Phaeodactylum tricornutum, Gene. 406 (2007) 23–35. https://doi.org/10.1016/j.gene.2007.05.022.

[37] L.T. Guerra, O. Levitan, M.J. Frada, J.S. Sun, P.G. Falkowski, G.C. Dismukes,

Regulatory branch points affecting protein and lipid biosynthesis in the diatom Phaeodactylum tricornutum, Biomass and Bioenergy. 59 (2013) 306–315. https://doi.org/10.1016/j.biombioe.2013.10.007.

[38] M. Sachse, S. Sturm, A. Gruber, P. Kroth, Identification and evaluation of endogenous reference genes for steady state transcript quantification by qPCR in the diatom Phaeodactylum tricornutum with constitutive expression independent from time and light, Endocytobiosis Cell Res. J. Int. Soc. Endocytobiol. 24 (2013) 1–7.

[39] B. Chen, C. Wan, M.A. Mehmood, J.S. Chang, F. Bai, X. Zhao, Manipulating environmental stresses and stress tolerance of microalgae for enhanced production of lipids and value-added products–A review, Bioresour. Technol. 244 (2017) 1198–1206. https://doi.org/10.1016/j.biortech.2017.05.170.

[40] B. Sajjadi, W.Y. Chen, A.A.A. Raman, S. Ibrahim, Microalgae lipid and biomass for biofuel production: A comprehensive review on lipid enhancement strategies and their effects on fatty acid composition, Renew. Sustain. Energy Rev. 97 (2018) 200–232. https://doi.org/10.1016/j.rser.2018.07.050.

[41] K. Shi, Z. Gao, T.Q. Shi, P. Song, L.J. Ren, H. Huang, X.J. Ji, Reactive oxygen species-mediated cellular stress response and lipid accumulation in oleaginous microorganisms: The state of the art and future perspectives, Front. Microbiol. 8 (2017) 793. https://doi.org/10.3389/fmicb.2017.00793.

[42] Y. Zhao, H.P. Wang, B. Han, X. Yu, Coupling of abiotic stresses and phytohormones for the production of lipids and high-value by-products by microalgae: A review, Bioresour. Technol. 274 (2019) 549–556. https://doi.org/10.1016/j.biortech.2018.12.030.

[43] V. Villanova, A.E. Fortunato, D. Singh, D.D. Bo, M. Conte, T. Obata, J. Jouhet, A.R. Fernie, E. Marechal, A. Falciatore, J. Pagliardini, A. Le Monnier, M. Poolman, G. Curien, D. Petroutsos, G. Finazzi, Investigating mixotrophic metabolism in the model diatom phaeodactylum tricornutum, Philos. Trans. R. Soc. B Biol. Sci. 372 (2017). https://doi.org/10.1098/rstb.2016.0404.

[44] X.F. Shen, Q.W. Qin, S.K. Yan, J. Le Huang, K. Liu, S.B. Zhou, Biodiesel production from Chlorella vulgaris under nitrogen starvation in autotrophic, heterotrophic, and mixotrophic cultures, J. Appl. Phycol. 31 (2019) 1589–1596. https://doi.org/10.1007/s10811-019-01765-1.

[45] H.S. Kwak, J.Y.H. Kim, H.M. Woo, E.S. Jin, B.K. Min, S.J. Sim, Synergistic effect of multiple stress conditions for improving microalgal lipid production, Algal Res. 19 (2016) 215–224. https://doi.org/10.1016/j.algal.2016.09.003.

[46] A.R. Burch, A.K. Franz, Combined nitrogen limitation and hydrogen peroxide treatment enhances neutral lipid accumulation in the marine diatom Phaeodactylum tricornutum, Bioresour. Technol. 219 (2016) 559–565. https://doi.org/10.1016/j.biortech.2016.08.010.

[47] B. Huang, J. Marchand, V. Blanckaert, E. Lukomska, L. Ulmann, G. Wielgosz-Collin, V. Rabesaotra, B. Moreau, G. Bougaran, V. Mimouni, A. Morant-Manceau, Nitrogen and phosphorus limitations induce carbon partitioning and membrane lipid remodelling in the marine diatom Phaeodactylum tricornutum, Eur. J. Phycol. 54 (2019) 342–358. https://doi.org/10.1080/09670262.2019.1567823.

[48] J. Liang, F. Wen, J. Liu, Transcriptomic and lipidomic analysis of an EPA-containing Nannochloropsis sp. PJ12 in response to nitrogen deprivation, Sci. Rep. 9 (2019) 1–18. https://doi.org/10.1038/s41598-019-41169-2.

[49] G. D'Ippolito, A. Sardo, D. Paris, F.M. Vella, M.G. Adelfi, P. Botte, C. Gallo, A. Fontana, Potential of lipid metabolism in marine diatoms for biofuel production, Biotechnol. Biofuels. 8 (2015) 1–10. https://doi.org/10.1186/s13068-015-0212-4.

[50] S. Nagappan, S. Devendran, P.C. Tsai, H. Jayaraman, V. Alagarsamy, A. Pugazhendhi, V.K. Ponnusamy, Metabolomics integrated with transcriptomics and proteomics: Evaluation of systems reaction to nitrogen deficiency stress in microalgae, Process Biochem. 91 (2020) 1–14. https://doi.org/10.1016/j.procbio.2019.11.027.

[51] J. Valenzuela, R.P. Carlson, R. Gerlach, K. Cooksey, B.M. Peyton, B. Bothner, M.W. Fields, Nutrient resupplementation arrests bio-oil accumulation in Phaeodactylum tricornutum, Appl. Microbiol. Biotechnol. 97 (2013) 7049–7059. https://doi.org/10.1007/s00253-013-5010-y.

[52] T. Řezanka, J. Lukavský, L. Nedbalová, I. Kolouchová, K. Sigler, Effect of starvation on the distribution of positional isomers and enantiomers of triacylglycerol in the diatom Phaeodactylum tricornutum, Phytochemistry. 80 (2012) 17–27. https://doi.org/10.1016/j.phytochem.2012.05.021.

[53] N. Belhaj, E. Arab-Tehrany, M. Linder, Oxidative kinetics of salmon oil in bulk and in

nanoemulsion stabilized by marine lecithin, Process Biochem. 45 (2010) 187–195. https://doi.org/10.1016/j.procbio.2009.09.005.

[54] Y. Cui, S.R. Thomas-Hall, P.M. Schenk, Phaeodactylum tricornutum microalgae as a rich source of omega-3 oil: Progress in lipid induction techniques towards industry adoption, Food Chem. 297 (2019) 124937. https://doi.org/10.1016/j.foodchem.2019.06.004.

[55] K. Goiris, W. Van Colen, I. Wilches, F. León-Tamariz, L. De Cooman, K. Muylaert, Impact of nutrient stress on antioxidant production in three species of microalgae, Algal Res. 7 (2015) 51–57. https://doi.org/10.1016/j.algal.2014.12.002.

[56] K. Chokshi, I. Pancha, A. Ghosh, S. Mishra, Nitrogen starvation-induced cellular crosstalk of ROS-scavenging antioxidants and phytohormone enhanced the biofuel potential of green microalga Acutodesmus dimorphus, Biotechnol Biofuels. 10 (2017) 60. https://doi.org/10.1186/s13068-017-0747-7.

[57] A. Shahid, S. Malik, H. Zhu, J. Xu, M.Z. Nawaz, S. Nawaz, M. Asraful Alam, M.A. Mehmood, Cultivating microalgae in wastewater for biomass production, pollutant removal, and atmospheric carbon mitigation; a review, Sci. Total Environ. 704 (2020) 135303. https://doi.org/10.1016/j.scitotenv.2019.135303.

[58] C. Paliwal, M. Mitra, K. Bhayani, S.V.V. Bharadwaj, T. Ghosh, S. Dubey, S. Mishra, Abiotic stresses as tools for metabolites in microalgae, Bioresour. Technol. 244 (2017) 1216–1226. https://doi.org/10.1016/j.biortech.2017.05.058.

[59] M.R. Gauthier, G.N.A. Senhorinho, J.A. Scott, Microalgae under environmental stress as a source of antioxidants, Algal Res. 52 (2020) 102104. https://doi.org/10.1016/j.algal.2020.102104.

[60] X.M. Sun, L.J. Ren, Q.Y. Zhao, X.J. Ji, H. Huang, Microalgae for the production of lipid and carotenoids: A review with focus on stress regulation and adaptation, Biotechnol. Biofuels. 11 (2018) 272. https://doi.org/10.1186/s13068-018-1275-9.

[61] B. Jeyakumar, D. Asha, P. Varalakshmi, S. Kathiresan, Nitrogen repletion favors cellular metabolism and improves eicosapentaenoic acid production in the marine microalga Isochrysis sp. CASA CC 101, Algal Res. 47 (2020) 101877. https://doi.org/10.1016/j.algal.2020.101877.

[62] P. Singh, M. Baranwal, S.M. Reddy, Antioxidant and cytotoxic activity of carotenes

produced by *Dunaliella salina* under stress, Pharm. Biol. 54 (2016) 2269–2275. https://doi.org/10.3109/13880209.2016.1153660.

[63] D. PATRAS, C. V. MORARU, C. SOCACIU, Bioactive Ingredients from Microalgae: Food and Feed Applications, Bull. Univ. Agric. Sci. Vet. Med. Cluj-Napoca. Food Sci. Technol. 76 (2019) 1. https://doi.org/10.15835/buasvmcn-fst:2018.0018.

[64] Y. Li-Beisson, J.J. Thelen, E. Fedosejevs, J.L. Harwood, The lipid biochemistry of eukaryotic algae, Prog. Lipid Res. 74 (2019) 31–68. https://doi.org/10.1016/j.plipres.2019.01.003.

[65] N. Nordin, N. Yusof, T. Maeda, N.A. Mustapha, M.Z. Mohd Yusoff, R.F. Raja Khairuddin, Mechanism of carbon partitioning towards starch and triacylglycerol in Chlorella vulgaris under nitrogen stress through whole-transcriptome analysis, Biomass and Bioenergy. 138 (2020) 105600. https://doi.org/10.1016/j.biombioe.2020.105600.

[66] J.J. Park, H. Wang, M. Gargouri, R.R. Deshpande, J.N. Skepper, F.O. Holguin, M.T. Juergens, Y. Shachar-Hill, L.M. Hicks, D.R. Gang, The response of Chlamydomonas reinhardtii to nitrogen deprivation: A systems biology analysis, Plant J. 81 (2015) 611–624. https://doi.org/10.1111/tpj.12747.

[67] M.A. Bromke, P. Giavalisco, L. Willmitzer, H. Hesse, Metabolic Analysis of Adaptation to Short-Term Changes in Culture Conditions of the Marine Diatom Thalassiosira pseudonana, PLoS One. 8 (2013) 1–11. https://doi.org/10.1371/journal.pone.0067340.

[68] E. Corteggiani Carpinelli, A. Telatin, N. Vitulo, C. Forcato, M. D'Angelo, R. Schiavon, A. Vezzi, G.M. Giacometti, T. Morosinotto, G. Valle, Chromosome scale genome assembly and transcriptome profiling of nannochloropsis gaditana in nitrogen depletion, Mol. Plant. 7 (2014) 323–335. https://doi.org/10.1093/mp/sst120.

[69] X. Wang, X. Dou, J. Wu, F. Meng, Attenuation pathways of erythromycin and biochemical responses related to algal growth and lipid synthesis in a microalga-effluent system, Environ. Res. 195 (2021) 110873. https://doi.org/10.1016/j.envres.2021.110873.

[70] R. Huerlimann, K. Heimann, Comprehensive guide to acetyl-carboxylases in algae, Crit. Rev. Biotechnol. 33 (2013) 49–65. https://doi.org/10.3109/07388551.2012.668671.

[71] F. Chu, J. Cheng, X. Zhang, Q. Ye, S. Chen, J. Zhou, K. Cen, Transcriptome and key gene expression related to carbon metabolism and fatty acid synthesis of Chlorella vulgaris under a nitrogen starvation and phosphorus repletion regime, J. Appl. Phycol. 31 (2019) 2881–2893. https://doi.org/10.1007/s10811-019-01811-y.

[72] H. Chen, Y. Zheng, J. Zhan, C. He, Q. Wang, Comparative metabolic profiling of the lipid-producing green microalga Chlorella reveals that nitrogen and carbon metabolic pathways contribute to lipid metabolism, Biotechnol. Biofuels. 1 (2017) 1–20. https://doi.org/10.1186/s13068-017-0839-4.

[73] X. Deng, J. Cai, X. Fei, Effect of the expression and knockdown of citrate synthase gene on carbon flux during triacylglycerol biosynthesis by green algae Chlamydomonas reinhardtii, BMC Biochem. 14 (2013) 1–11. https://doi.org/10.1186/1471-2091-14-38.

[74] S. Kaur, J. Hérault, A. Caruso, G. Pencréac'h, M. Come, L. Gauvry, S. Claverol, C. Loiseau, Proteomics and expression studies on lipids and fatty acids metabolic genes in Isochrysis galbana under the combined influence of nitrogen starvation and sodium acetate supplementation, Bioresour. Technol. Reports. 15 (2021) 100714. https://doi.org/10.1016/j.biteb.2021.100714.

[75] I.M. Remmers, S. D'Adamo, D.E. Martens, R.C.H. de Vos, R. Mumm, A.H.P. America, J.H.G. Cordewener, L. V. Bakker, S.A. Peters, R.H. Wijffels, P.P. Lamers, Orchestration of transcriptome, proteome and metabolome in the diatom Phaeodactylum tricornutum during nitrogen limitation, Algal Res. 35 (2018) 33–49. https://doi.org/10.1016/j.algal.2018.08.012.

[76] M. Wan, P. Liu, J. Xia, J.N. Rosenberg, G.A. Oyler, M.J. Betenbaugh, Z. Nie, G. Qiu, The effect of mixotrophy on microalgal growth, lipid content, and expression levels of three pathway genes in Chlorella sorokiniana, Appl. Microbiol. Biotechnol. 91 (2011) 835–844. https://doi.org/10.1007/s00253-011-3399-8.

[77] R. Nagao, M. Yokono, T. Tomo, S. Akimoto, Control mechanism of excitation energy transfer in a complex consisting of photosystem II and fucoxanthin chlorophyll a/c-binding protein, J. Phys. Chem. Lett. 5 (2014) 2983–2987. https://doi.org/10.1021/jz501496p.

[78] L. Alipanah, J. Rohloff, P. Winge, A.M. Bones, T. Brembu, Whole-cell response to nitrogen deprivation in the diatom Phaeodactylum tricornutum, J. Exp. Bot. 66 (2015)

6281–6296. https://doi.org/10.1093/jxb/erv340.

[79] H. Abida, L.J. Dolch, C. Meï, V. Villanova, M. Conte, M.A. Block, G. Finazzi, O. Bastien, L. Tirichine, C. Bowler, F. Rébeillé, D. Petroutsos, J. Jouhet, E. Maréchal, Membrane glycerolipid remodeling triggered by nitrogen and phosphorus starvation in Phaeodactylum tricornutum, Plant Physiol. 167 (2015) 118–136. https://doi.org/10.1104/pp.114.252395.

Chapter 3 – Halophyte and marine microalgae bioactive compounds as a bioavailable source to counter *in vitro* cellular oxidative stress on human (Hs68) and fish (SAF-1) cell lines and to assess *in vitro* bactericidal activity

3.1 Abstract

Green biotechnology development has remarked the importance of the integrated multitrophic aquaculture (IMTA) for the co-cultivation of farmed species and extractive species, such as plants and microalgae, whose extracts have multiple applications, including protecting animal and human health and improving animal welfare.

In this study, bioactivity of halophyte and microalgae extracts have been tested in different cellular model systems. In particular, halophilic plant *Arthrocnemum macrostachyum* extracts were tested to assess their protective effect against UV-induced oxidative damage in human skin fibroblast cell line (Hs68). Regarding microalgae, *Nannochloropsis* sp., *Isochrysis galbana*, *Phaeodactylum tricornutum*, cultivated under standard condition and under nitrogen deprivation (N-), extracts were tested both to assess their protective effect pretreating *Sparus aurata* fibroblast cell line (SAF-1) exposed to increasing concentrations of the flame retardant polybrominated diphenyl ether 47 (BDE-47) and to assess their bactericidal activity against two fish pathogens relevant in the aquaculture sector, *Vibrio harveyi* and *Vibrio anguillarum*.

The results showed that, *A. macrostachyum* extracts had a significant protective effect on Hs68 cell line ($P<0.05$) against UV radiation exposure.

With regard to microalgae, higher concentrations of all tested extracts (0.15-0.30 μg mL^{-1}) had a significant protective effect on SAF-1 cell line ($P<0.05$) against BDE-47 induced toxicity. Furthermore, *P. tricornutum* extracts had the highest significant bactericidal activity ($P<0.05$) at low concentrations (0.05 mg mL^{-1}) against the two pathogens of the genus *Vibrio* spp.

Plants and microalgae extracts represent a bioavailable source of bioactive compounds, efficient on countering cellular oxidative stress and the development of bacterial growth in aquaculture facilities. IMTA systems therefore provide a socio-economic benefit for the development of commercial nutraceutical, cosmeceutical and pharmaceutical products.

KEYWORDS: *Arthrocnemum macrostachyum*, microalgae, bioactive compounds, antioxidant effect, UV, BDE-47, bactericidal activity

3.2 Introduction

The demand for natural products for application in the cosmetics and nutraceutical industries, animal welfare and human health is constantly growing [1,2]. Scientific research has intensively focused on the extraction of bioactive natural compounds from marine organisms [2–5] due to their capacity to prevent harmful oxidative stress conditions caused by exogenous and endogenous factors [4,6]. Oxidative stress induces an overproduction of Reactive Oxygen Species (ROS) [7,8], leading to DNA damage, protein degradation, enzyme inactivation and lipid peroxidation [9–11]. Overproduction of radical species can occur for exposure to UV radiation, causing alterations in genetic material, photoaging and skin cancer [6,8,12]. Antioxidants, such as polyphenols and β-carotene, are able to prevent oxidative stress [13–16].

Among plants, which are known for the production of bioactive compounds showing a significant protective effects against oxidative stress [17,18], halophytes are considered as an important source of these compounds, for their utilization in the nutraceuticals, cosmeceutical and pharmaceutical industries [19]. Halophytes bioactive secondary metabolites, such as flavonoids and phenolic compounds indeed show, for example, a high antioxidant and photoprotective activity against UV radiation [20].

A widely distributed halophyte in European salt marshes is the perennial *Arthrocnemum macrostachyum* (Moric.) K. Koch, known for being a rich source of bioactive compounds with antioxidant properties with proven therapeutic as well as nutraceutical applications [21].

Also chemical contaminants are known to cause oxidative stress, compromising the integrity of all cellular targets [22,23]. Chemical contaminants are constantly found in the marine environment, affecting sediments quality and marine organisms well-being [23–26]. They reach the marine environment mainly from terrestrial sources resulting from agricultural activities, industrial and municipal waste, plastics, pharmaceuticals and their metabolites [24–26]. The emerging contaminant 2,2',4,4'-tetrabromodiphenyl ether (BDE-47), a bioaccumulative flame retardant, highly persistent and carcinogenic, is widely distributed in the marine environment [23,27,28]. In 2019, BDE-47 was included in the list of persistent organic pollutants at the Stockholm Convention [28,29]. Although chemical contaminants are present at low concentrations, from ng/g up to μg/g [23], there is growing concern about the potential effects these compounds may have both on marine organisms and on humans health through the trophic chain [30–32].

Microalgae are known to have a high production rate of several protective chemicals effective against oxidative stresses, such as pigments, polyunsaturated fatty acids, phycosterols,

sulphated polysaccharides and phenolic compounds [33–35]. Microalgae and cianobacteria produce complex polyphenols [36], such as isoflavones, flavanones, flavonols and dihydrocalcones [37], suggesting that unicellular microalgae are a promising alternative source of antioxidants [36,38,39].

It is known that bioactive chemical compounds extracted both from plants [40] and microalgae [41–43], in addition to their significant antioxidant capacity, may have a high capacity for inhibiting aquaculture pathogenic bacteria [40–44].

The rapid expansion of aquaculture has led to the development of intensive culture processes aimed at maximising production yields [45,46]. Indeed, some farming practices, such as a high biomass in the tank, long-term confinement in a limited space and handling, are critical risk factors for the welfare of reared fish, as demonstrated for traditional species, including sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) [45,47]. Such conditions are often associated with epidemics, in which infectious agents, such as bacteria, parasites, viruses and fungi, proliferate more easily. [41–46].

Diseases associated with bacterial infections are the most frequent in fish farming and are a potential cause of significant economic losses [48]. Common pathogenic bacteria include *Vibrio* species, which can infect fish, molluscs and crustaceans [42–44].

Preventive treatment strategies in aquaculture should first aim to increase general welfare status of fish and then to avoid pathogenic infections [46]. Furthermore, considering the potential impact of therapeutic treatments, such as antibiotic treatments, on the environment and human health, the management of fish farm should focus on methods that are environmental friendly, not harmful to human health, and that can have a preventive and long lasting effect on reared fish [44].

Therefore, microalgal cultures will play an important role in the production of additives [49–51] than can be included in the diets of farmed fish exerting an effect in disease prevention alternative to traditional disease treatments, minimizing risks associated with chemical drug utilization [52–55].

Considering halophytes and marine microalgae as potentially cultivable extractive species in integrated multitrophic aquaculture (IMTA) facilities, this study was aimed to examine *in vitro* the protective effect of the extracted bioactive compounds of *A. macrostachyum* tested on UV-irradiated human skin fibroblasts (Hs68) cell line.

Previous results obtained by Espinosa et al. [14] in pretreated *Sparus aurata* fibroblast (SAF-1) cell lines with synthetic antioxidants gallic acid (GAE) and *β*-carotene and then exposed to a mixture of BDE-47, carbamazepine (CBZ) and cadmium chloride ($CdCl_2$), showed the

protective effect of the synthetic antioxidants against the mixture compounds. On these basis, the aim of this study was to assess *in vitro* the antioxidant capacity of microalgal extracts. SAF-1 cells were pretreated with *Nannochloropsis* sp., *I. galbana*, *P. tricornutum* and *P. tricornutum* cultivated under nitrogen starvation (N-) extracts and then induced to oxidative stress using sub-lethal doses of the emerging contaminant BDE-47. In addition, another trial was to assess *in vitro* the bactericidal activity of microalgal extracts against the pathogenic bacteria *Vibrio harveyi* and *Vibrio anguillarum.*

3.3 Materials and Methods

3.3.1 Micro and macroalgae extracts

Microalgae and *A. macrostachyum* extracts were obtained as described in chapter 1 (sections 1.3.1 and 1.3.2).

3.3.2 Human skin fibroblast (Hs68) and *Sparus aurata* fibroblast (SAF-1) cell cultures

Human skin fibroblast (Hs68) cell lines (ECACC n°89051701) (Sigma-Aldrich, St. Louis, MO, USA) were grown as described by Messina et al. [6], as a monolayer in flasks, using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 100 g mL^{-1} penicillin streptomycin, incubated in a humidified atmosphere at 5% CO_2, 95% air and 37°C, under sterile conditions.

Sparus aurata fibroblast (SAF-1) cell lines were cultivated as described by Espinosa Ruiz et al. [14] Cell line SAF-1 (ECACC n°00122301) was seeded in 25 cm^2 plastic tissue culture flasks (Nunc, Germany) cultured in L-15 Leibowitz medium (Sigma, UK), supplemented with 10% fetal bovine serum (FBS, Sigma, UK), 2 mmol L^{-1} L-glutamine (Sigma, UK), 100 i.u. mL^{-1} penicillin (Sigma, UK) and 100 g L^{-1} streptomycin (Sigma, UK).

3.3.3 Evaluation of *A. macrostachyum* extracts protective effect on Hs68 cell line

Hs68 cells were detached when reached 80% of confluence, utilizing a trypsin solution (0.05% of trypsin in PBS, pH 7.2–7.4) and pelleted by centrifugation (1000 rpm, 10 min, 25°C). The cell suspension was dispensed in 96-well plate at a density of 8000 cells/well and incubated for 24 h before the exposure to extracts of *A. macrostachyum* obtained by Supercritical Fluid Extraction with co-solvent (ethanol 96%).

The protective effect of *A. macrostachyum* extracts on Hs68 against oxidative stress induced by UV radiation, was assessed as described in Table 1.

Table1. Experimental scheme of Hs68 cells treatment and UV radiation exposure

Extracts	**Control**	**Control+UV**	**Synthetic antioxidant**	**Synthetic antioxidant +UV**	**Treatment**	**Treatment+UV**
A. macrostachyum	-	-	-	-	+	+
NAC	-	-	+	+	-	-
UV	-	+	-	+	-	+

- Control: Hs68 cells untreated and not stress-induced
- Control+UV: Hs68 cells untreated and stress-induced
- Synthetic antioxidant: Hs68 cells pretreated with the synthetic antioxidant NAC
- Synthetic antioxidant +UV: Hs68 cells pretreated with the synthetic antioxidant NAC and then exposed to UV radiation
- Treatment: Hs68 cells pretreated with *A. macrostachyum* extracts
- Treatment+UV: Hs681 cells pretreated with *A. macrostachyum* extracts and then exposed to UV radiation

Cells were seeded in 96-well plates at a concentration of 7×10^3 cells/well and incubated for 24 h. After 24 h, the cells were treated with the extract dissolved in ethanol at a concentration of 5 μg mL^{-1} in the medium, with a final solvent concentration of 0.1% (*v*/*v*), and left to incubate for 24 h. As already assessed [56] this solvent concentration did not exert any effect on cell viability. After 24 h of incubation, cells were incubated UV-irradiated (Table 1).

To prevent UV light absorption by the cell culture medium, the medium was removed just prior to irradiation and replaced with a thin layer of PBS to cover the cells. All samples were exposed to UV radiation (lamp UV KW 254 nm) at dose rate of 105 erg/mm^2/sec-for 5 min, with a daily increase of 5 min, for a total of 3 days of exposure [58,59]. Cells not treated with *A. macrostachyum* extracts and not UV-irradiated were cultivated as control [6] (Table 1).

N-acetylcysteine (NAC 5 mM), a synthetic antioxidant, that inhibits oxidative stress formation, was used as control [6,56,57] (Table 1).

The viability of cells was measured as described by Messina et al. [6] using the MTT assay according to Mosmann [60].

The optical densities (ODs) at 570 nm with background subtraction at 690 were determined in a microplate reader (Multiscan-Sky Microplate Reader, Thermo-Scientific TM, USA).

The percentage of viability was determined by Formula (1):

$$Viability\ (\%) = \left(\frac{OD\ of\ the\ test\ sample}{OD\ of\ the\ control\ sample}\right) x100 \quad (1)$$

OD measurements were performed in six replicates.

3.3.4 Evaluation of microalgal extracts protective effect on SAF-1 cell line

The protective effect on SAF-1 cells of microalgae extracts against oxidative stress induced by BDE-47 was assessed as described in Table 2.

In particular, untreated cells were cultivated in a specific culture medium used as control (Control, Table 2), while untreated and stress-induced cells (Control+BDE-47, Table 2) were cultivated in a culture medium adding BDE-47 at 10, 50 and 100 μmol L^{-1} concentration. One group of SAF-1 cells was exposed for 24 hours to synthetic antioxidant gallic acid (GAE) and *β*-carotene at the concentrations of 0.07, 0.15 μg mL^{-1} and 0.3 μg mL^{-1} (Synthetic antioxidant, Table 2) [14]. 24 hours after the exposure to synthetic antioxidants, pretreated cells with GAE and *β*-carotene were induced to oxidative stress by 48h exposure to BDE-47 (BDE-47, Table 2) at concentrations of 50 and 100 μmol L^{-1}, according to a standard protocol [7,14,61].

Another group of SAF-1 cells was cultivated adding for 24h increasing concentrations of *Nannochloropsis* sp., *I. galbana*, *P. tricornutum* and *P. tricornutum* N- extracts, at the same concentrations of the synthetic antioxidants (0.07-0.15-0.3 μg mL^{-1}) (Treatment, Table 2). Stock solution of ethanol extracts was diluted to obtain a final solvent concentration, in the culture medium, of less than 0.1% (v/v). Ethanol toxicity, at the concentrations used to dissolve the extract, was previously evaluated on the same cell line. After 24 hours of pretreatment with *Nannochloropsis* sp., *I. galbana*, *P. tricornutum* and *P. tricornutum* N- extracts, SAF-1 cells were induced to oxidative stress by exposure for 48h to BDE-47 (BDE-47, Table 2) at concentrations of 50 and 100 μmol L^{-1}, according to a standard protocol [7,14,61].

Each experiment of viability was carried in six replicates.

Table 2. Experimental scheme of SAF-1 cells treatment and BDE-47 exposure

		Control	Control+ BDE-47	Synthetic antioxidant	Treatment	Treatment+ BDE-47
Microalgal extract	*Nannochloropsis* sp.	-	-	-	+	+
	I. galbana	-	-	-	+	+
	P. tricornutum	-	-	-	+	+
	P. tricornutum N-	-	-	-	+	+
Synthetic antioxidant	*β*-carotene	-	-	+	-	+
	GAE	-	-	+	-	+
	BDE-47	-	+	-	-	+

- Control: SAF-1 cells untreated and not stress-induced
- Control+BDE-47: SAF-1 cells untreated and stress-induced
- Synthetic antioxidant: SAF-1 cells pretreated with GAE and *β*-carotene
- Synthetic antioxidant+ BDE-47: SAF-1 cells pretreated with GAE and *β*-carotene and then exposed to BDE-47
- Treatment: SAF-1 cells pretreated with microalgae extracts
- Treatment+BDE-47: SAF-1 cells first pretreated with synthetic antioxidants or microalgae extracts and then exposed to BDE-47

Cell viability was assessed on SAF-1 by MTT assay, as described by Espinosa Ruiz et al. [14].

3.3.5 Bactericidal activity test

Two fish-pathogenic bacteria, *Vibrio harveyi* and *Vibrio anguillarum* were grown from 1 mL of culture for 24 hours at 25°C in Triptic Soy Broth (TSB) agar (TSA, Difco Laboratories) and supplemented with NaCl to a final concentration of 1.5% (w/v). The bacteria in TSB medium were then cultured at the same temperature with continuous agitation (100 rpm) for 24 hours.

Exponentially growing bacteria were resuspended in sterile PBS and set to 108 c.f.u. mL^{-1} colony forming units. Bactericidal activity was determined following the method of Stevens & Olsen [62] with some modifications. Samples of 20 µL of microalgae extracts (in three replicates) were added to the wells of a 96-well flat-bottomed plate.

PBS solution was added to some wells in place of the extract as positive control.

Aliquots of 20 µL of the previously grown bacteria were added and the plates were incubated for 5 hours at 25°C.

25 µL of MTT (1 mg mL^{-1}) was added to each well and the plates were incubated again for 10 minutes at 25°C to allow the formation of formazan. The plates were centrifuged (2000xg, 10min), and the precipitates, dissolved in 200 µL of dimethylsulfoxide (DMSO), were transferred to a 96-well flat-bottomed plate. Absorbance was measured at 570 nm.

Bactericidal activity was expressed as the percentage of non-viable bacteria cells, calculated as the difference between the absorbance of the surviving bacteria treated with microalgal extracts, compared to the absorbance of the surviving bacteria in the positive control (0% of bactericidal activity).

3.3.6 Statistical Analysis

Statistical differences between groups were assessed by one-way ANOVA analyses, followed by Tukey or Games Howell tests, depending on the homogeneity of the variables. The normality of the variables was confirmed by the Shapiro-Wilk test and the homogeneity of variance by the Levene test. The significance level was 95% in all cases ($P<0.05$). All data were analysed by the computer application SPSS for Windows® (version 20.0, SPSS Inc., Chicago, USA).

3.4 Results and discussions

3.4.1 *A. macrostachyum* extracts protective effect on human skin fibroblast (Hs68) cells UV-irradiated

Hs68 cells were exposed to UV radiation for 5 min, for a total of 3 days, with a daily increase of 5 min. Phase-contrast microscopy indicated that UV radiation led to cellular damage and mortality in untreated Hs68 cells UV-irradiated (Figure 1B), compared to the control (Figure 1A). In contrast, Hs68 cells pretreated with *A. macrostachyum* extracts before UV radiation exposure (Figure 1C) showed no signs of damage after exposure to UV radiation.

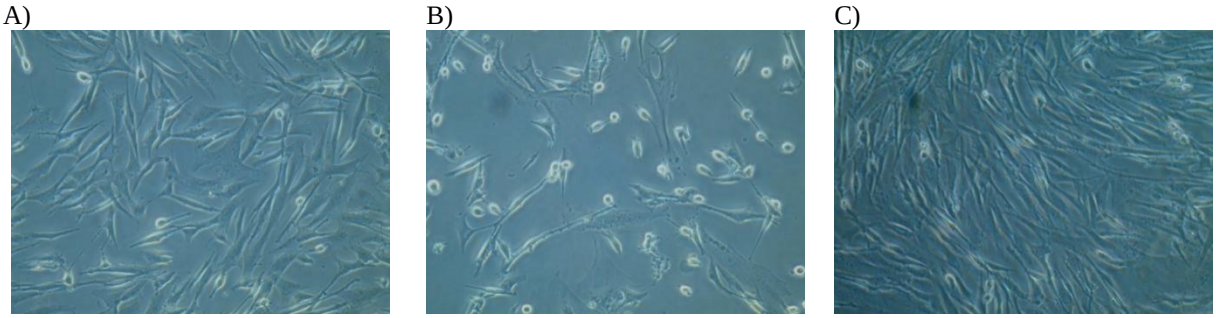


Figure 1. Representative phase-contrast images at 20X magnification. Untreated cells (Control), (A), Hs68 cells UV-irradiated (B) and Hs68 cells pretreated with 5 μg mL^{-1} of *A. macrostachyum* extracts and then exposed to UV radiation (C)

Obtained MTT assay results (Figure 2) confirmed phase-contrast microscopy observations.

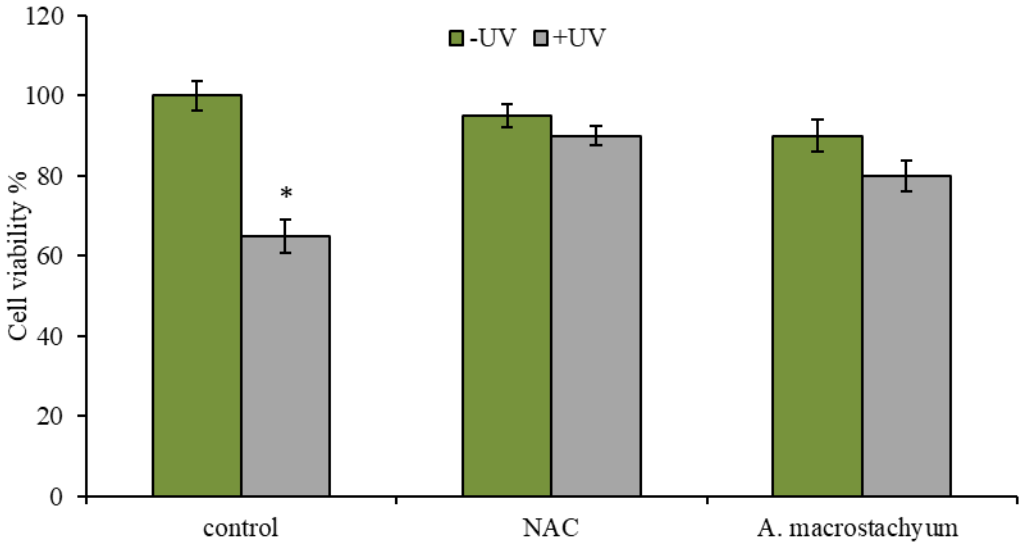


Figure 2. Cell viability test in Hs68 treatment with *A. macrostachyum* extracts (SFE 5 μg mL^{-1}) for 24 h and UV-irradiated. Blue bars: control: fibroblasts maintained in standard culture conditions, NAC: cell pretreated with the synthetic antioxidant N-acetylcysteine (5 mM) and cells pretreated with *A. macrostachyum* SFE extract; red bars Hs68cells exposed to UV radiation. Bars represent the mean ± sd (n = 6). Different superscript letters indicate statistically significant differences (ANOVA; $P < 0.05$) among group.

Results obtained by MTT assay showed that the exposure of not treated Hs68 cells to UV radiation (Control +UV) induced a significant decrease of viability ($P< 0.05$). On the contrary, cells pretreatment with *A. macrostachyum* extracts, before UV radiation exposure (*A. macrostachyum* +UV) led to a photo protective effect, as demonstrated by an increase in cell viability, comparable to cells pretreated with synthetic antioxidant NAC (NAC+UV) (Figure 2). Suggesting that halophytes are an important source of natural compounds with numerous applications, due to their ability to absorb UV radiation and to their antioxidant power [17,20].

Similar results showed that halophyte (*Limonium tetragonum*, *Triglochin maritimum* and *Artemisia scoparia*) red ginseng complex extract inhibited peroxide-induced damage in human keratinocytes (HaCaT) compared to stimulated cells with UV radiation. [63]. Ahn et al. [64] reported that HaCaT treated with different concentrations of libanoridin isolated from the halophyte *Corydalis heterocarpa*, for 24h, after exposure to UVB radiation, inhibited UVB-induced photodamage, in accordance with our results.

Secondary metabolites are naturally produced in response to environment condition variation. Those compounds, such as phenolic acids, non-flavonoids, flavonoids and polyphenols act as UV blockers reducing inflammation and oxidative stress [65]. Phenolic substances are highly

accumulated by saltmarsh halophytes, due to transitional characteristics of their habitat [20,21].

The photoprotective cellular capacity observed in the preliminary *in vitro* tests showed that *A. macrostachyum* might be considered a good candidate for co-cultivation in IMTA systems for the extraction of phytochemicals to be used in nutraceutical and cosmeceutical fields.

3.4.2 Protective effect of microalgal extracts on *S. aurata* fibroblast (SAF-1) cell line exposed to BDE-47

SAF-1 cell line was used as an *in vitro* model to test the protective effect of three microalgae extracts against BDE-47 oxidative stress induction, a highly persistent and easily bioaccumulated carcinogenic flame retardant, considered to be among the most abundant contaminants in the natural environment and for wildlife [28,66,67].

The results of dose-dependent treatment with microalgae extracts on SAF-1 cell line showed that concentrations of 0.07-0.15-0.30 μg mL^{-1} of the microalgae extracts (*Nannochloropsis* sp., *I. galbana*. *P. tricornutum* and *P. tricornutum* N-) and reference synthetic antioxidant (GAE and *β*-carotene) [14] did not exert any significant negative effects on cell viability. For this reason, all three concentrations were chosen for the evaluation of the protective effect at cellular level.

Following a standardised protocol, SAF-1 cells were exposed to BDE-47 (50-100 μmol L^{-1}) for 48 hours. Phase-contrast microscopy obtained images indicated that cells exposed only to the highest BDE-47 concentration (100 μmol L^{-1}) showed cell mortality (Figure 3B), compared to the control (Figure 3A), in contrast to cells pretreated with antioxidants (standard and natural) which showed comparable cellular density and similar characteristics to the control (Figure 3D, F, H and J).

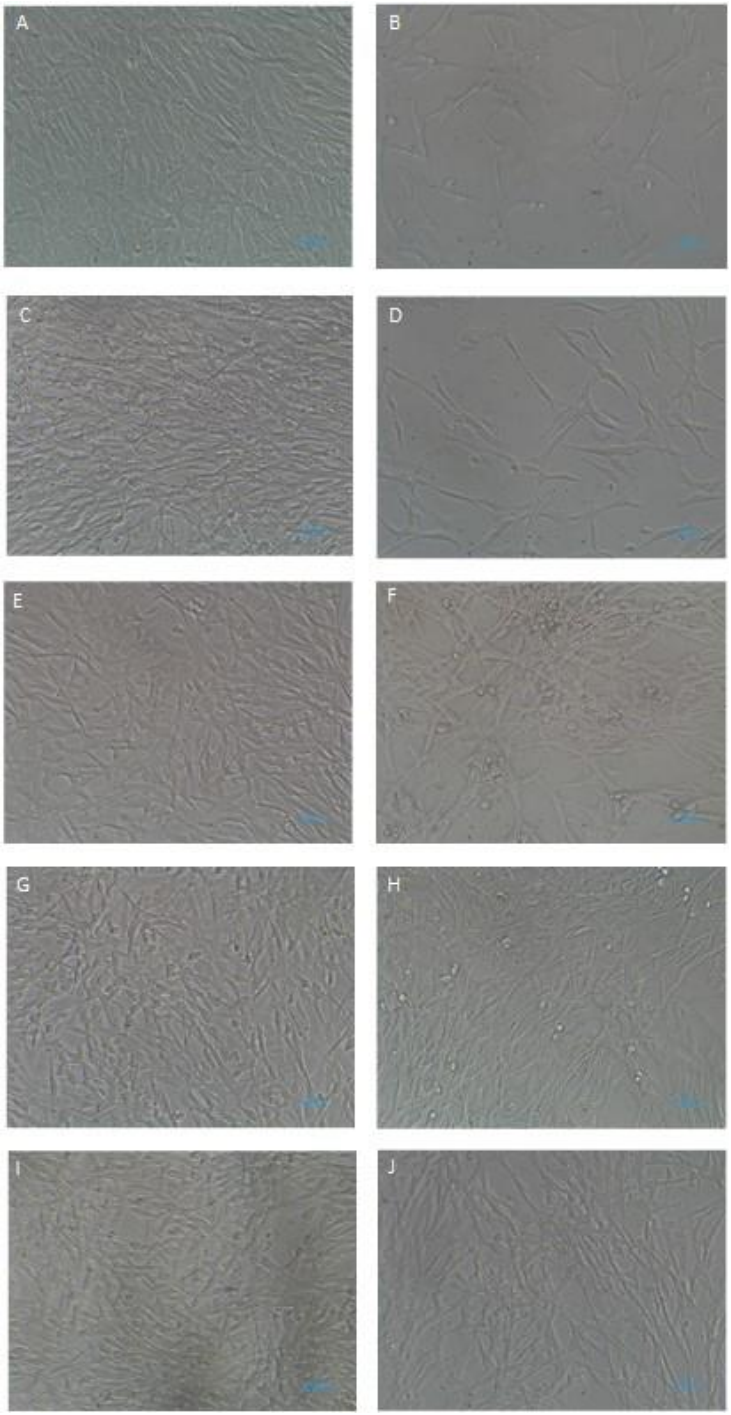
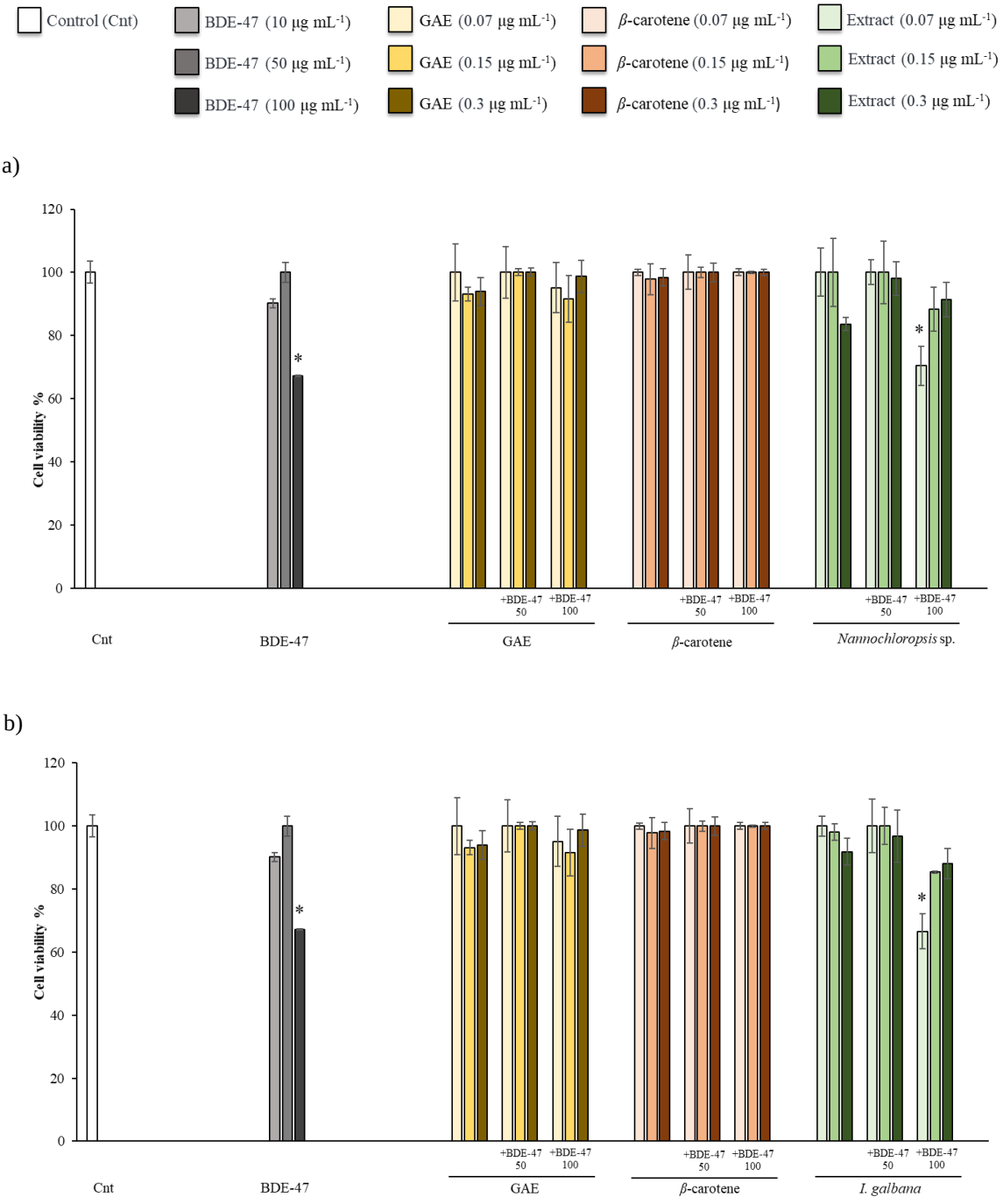


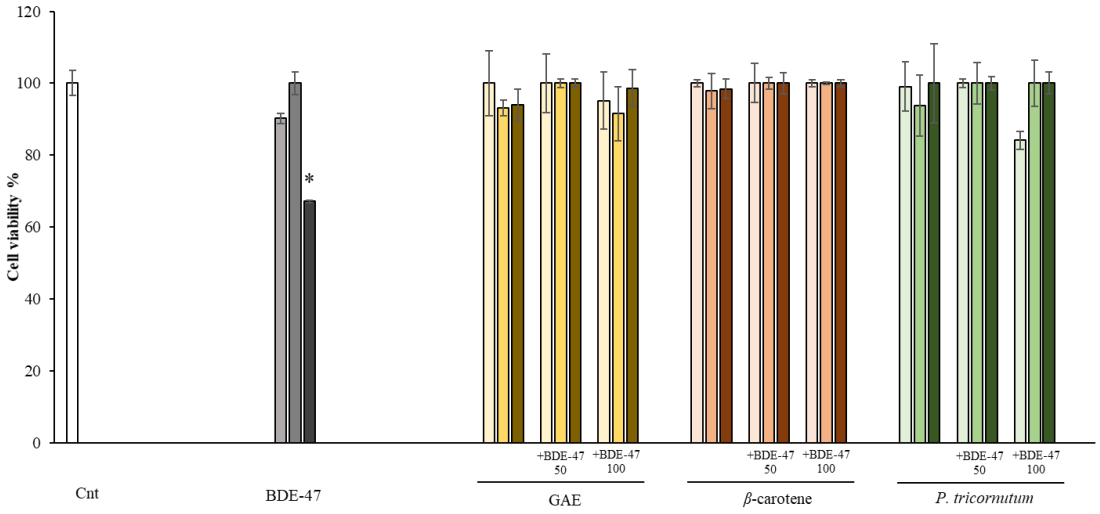
Figure 3. Representative phase-contrast images at 20X magnification. Untreated SAF-1 cells (control) (A), SAF-1 cells exposed to 100 µmol $L^{-1}$of BDE-47 (B) and SAF-1 cells pretreated with 0.15 µg mL^{-1} of microalgal extract and then exposed to BDE-47 100 µmol $L^{-1}$of BDE-47, respectively: *Nannochloropsis* sp. (C), *Nannochloropsis* sp.+BDE-47 (D), *I. galbana* (E), *I. galbana*+BDE-47 (F), *P. tricornutum* (G), *P. tricornutum*+BDE-47 (H), *P. tricornutum* N- (I), *P. tricornutum* N-+BDE-47 (J), 48h after the exposure to the stressor.

Effects on cultured cells showed by phase-contrast microscopy images (Figure 3) were confirmed by MTT test.

Results showed that cell viability of SAF-1 cell line was not affected after 48h incubation with 50 µmol L-1 of BDE-47, whereas exposure to 100 µmol L-1 of BDE-47 resulted in a significant reduction in cell viability (up to 30%) ($P < 0.05$) after 48 hours, compared to the control (Figure 4a-d)



c)



d)

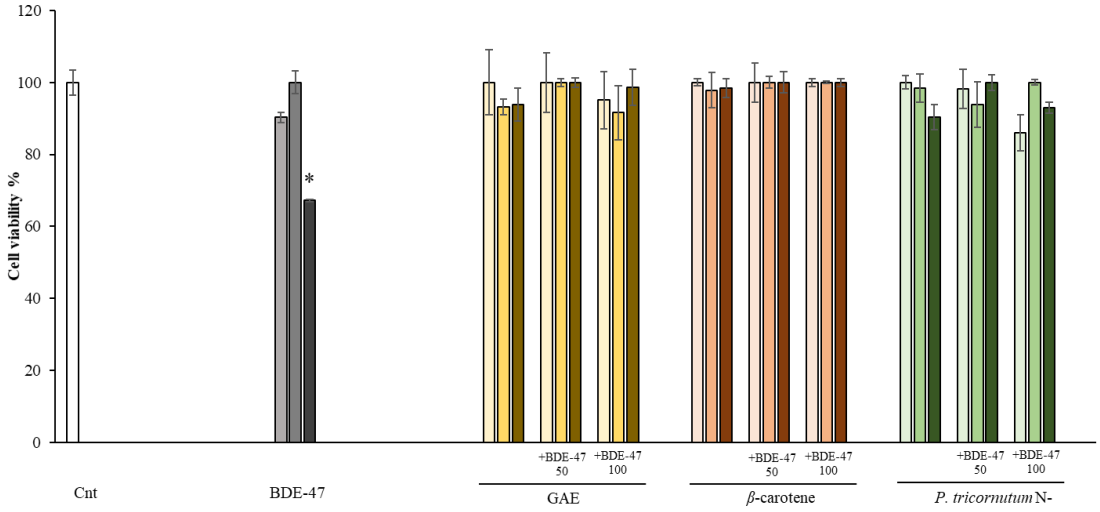


Figure 4. Cell viability (%) of *S. aurata* SAF-1 cells measured comparing control cells (white bars) and cells exposed to different concentrations of BDE-47 (black bars), with cells pretreated for 24h with different concentrations of GAE (yellow bars), *β*-carotene (orange bars), and microalgal extracts (green bars), respectively a) *Nannochloropsis* sp., b) *Isochrysis galbana*, c) *Phaeodactylum tricornutum*, d) *Phaeodactylum tricornutum* N- , and then exposed to different concentrations of BDE-47 (50 µmol L^{-1} and 100 µmol L^{-1}). Bars represent the mean ±SEM (n=6) with standard deviation. Statistical differences (P< 0.05) between groups are indicated by "*".

In SAF-1 cells pretreated with *Nannochloropsis* sp. extracts at 0.07-0.15 and 0.30 µg mL^{-1} the exposure to 50 µmol L^{-1} of BDE-47 did not significantly affect cell viability compared to the control (P<0.05) (Figure 4a). In cells exposed to 100 µmol L^{-1} BDE-47, the lower extract concentration (0.07 µg mL^{-1}) did not protect cells from oxidative stress (mortality reached

30%). In contrast, higher concentrations of *Nannochloropsis* sp. extracts (0.15-0.30 μg mL^{-1}) had a significant protective effect against toxicity induced by BDE-47 (Figure 4a).

SAF-1 cells pretreated with *I. galbana* extracts (Figure 3b) showed similar results as *Nannochloropsis* sp. extracts (Figure 4a). The exposure to 100 μmol L^{-1} of BDE-47 significantly affected cell viability ($P<0.05$) only in those cell lines pretreated with the lowest concentration of microalgal extract (0.07 μg mL^{-1}) (viability rate 66%), compared to the control. In contrast, higher concentrations of *I. galbana* extracts (0.15-0.30 μg mL^{-1}) protected cells from BDE-47-induced damage (Figure 4b).

SAF-1 cells pretreated with extracts obtained from *P. tricornutum* (Figure 4c) and *P. tricornutum* N- (Figure 4d) showed a protective effect in cells exposed to BDE-47 (50-100 μmol L^{-1}), at all tested concentrations (0.07, 0.15 and 0.30 μg mL^{-1}).

The exposure of SAF-1 cells to 100 μmol L^{-1} of BDE-47, pretreated with the highest dose of *P. tricornutum* and *P. tricornutum* N- extracts (0.3 μg mL^{-1}), did not affected cells viability compared to cells exposed to 100 μmol L^{-1} of BDE-47 without any pretreatment (Figure 4c and 4d).

Several studies have shown that the exposure of SAF-1 cells to BDE-47 induces oxidative stress through an overproduction of ROS, which is responsible for cell toxicity and death, changes in biochemical patterns and induction cell transformation [7,14,61,68].

Our results showed that SAF-1 cells pretreatment with *Nannochloropsis* sp., *I. galbana, P. tricornutum* and *P. tricornutum* N- extracts before the exposure to BDE-47 chemical contaminant, were similar to those observed with synthetic antioxidants (GAE and *β*-carotene) used in the trial as a reference (Figure 4).

In fact, the pre-incubation of sea bream cells with microalgal extracts successfully protected cells from BDE-47 oxidative stress-induced, in contrast to not pretreated cells exposed only to the chemical contaminant without pretreatment with synthetic or natural antioxidants. Indeed, BDE-47 at the highest concentration (100 μmol mL^{-1}), by inducing oxidative stress in cells significantly reduced cell viability of not pretreated SAF-1 cells, after 48 hours of exposure [7,14,28,61,66,67].

The protective effect induced at cellular level by the extracts further confirms the antioxidant power of the microalgae used in this experiment, which had already been preliminarily evaluated on a chemical basis (Chapter 1).

The bioactive compounds extracted from *Nannochloropsis* sp., *Isochrysis galbana* and *P. tricornutum* cultivated under standard and starvation condition (N-) showed the same level of protection as synthetic antioxidants (GAE and *β*-carotene) at the same concentrations.

The intrinsic antioxidant power of microalgae extracts therefore is biochemically available at cellular level, enhancing the reducing power of cells and protecting them from the pro-oxidant effect of BDE-47.

The results obtained showed that *P. tricornutum* extracts, followed by *I. galbana* and *P. tricornutum* N- and lastly *Nannochloropsis* sp. extracts, protected SAF-1 cells from BDE-47-induced oxidative stress. The differences observed between the protective effects of the microalgae extracts were probably due to the different antioxidant profile, in agreement with other authors [69–71]. Indeed, the higher levels of carotenoids and polyphenols observed in *P. tricornutum* compared to the other extracts, could explain the obtained results.

Since BDE-47 is responsible for the induction of oxidative stress and production of ROS in different species and environments at sub-lethal doses [7,27,61,72,73], microalgae extracts represent a bioavailable source of antioxidants.

Based on these results, the production of microalgae and the characterisation of their extracts should be improved and standardised.

3.4.3 Evaluation of microalgal extracts bactericidal activity

Vibrionaceae are the main cause of mortality for species farmed in the aquaculture industry [74,75]. Among them, *V. anguillarum* and *V. harveyi* are responsible for infections affecting marine organisms as well as humans [76–78].

For this reason, in this study was investigated the effect of the bioactive compounds extracted from *Nannochloropsis* sp., *I. galbana*, *P. tricornutum* and *P tricornutum* N- against two opportunistic pathogenic bacteria, *V. anguillarum* and *V. harveyi*.

All the microalgal extracts tested showed a dose-dependently bactericidal activity against both bacteria strain utilized.

In the present study only the results obtained using the lowest concentration of extracts are shown.

In particular, *P. tricornutum* extracts had the highest significant bactericidal activity (27.09±9.1%) ($P<0.05$) at low concentrations (0.05 mg mL^{-1}) against *V. anguillarum*, compared to control (Cnt) (Figure 5).

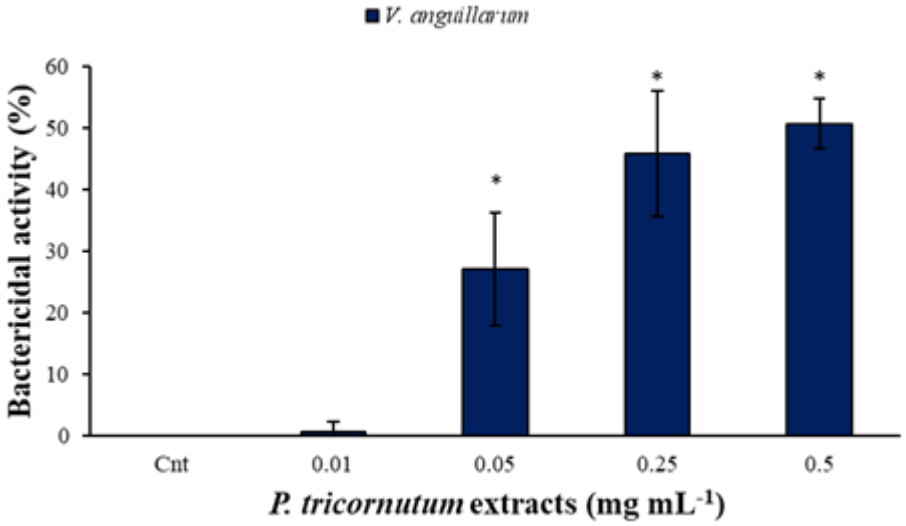


Figure 5. Bactericidal activity (%) against *Vibrio anguillarum* determined using extracts of *P. tricornutum*. Values are the mean ± SEM (n= 3) with standard deviations. Microalgae that show statistical differences (P< 0.05) compared with the control are indicated by "*".

P. tricornutum extracts had also a significant bactericidal activity (10.36±3.69%) (P<0.05) at low concentrations (0.05 mg mL^{-1}) against *V. harveyi*, compared to control (Cnt) (Figure 6).

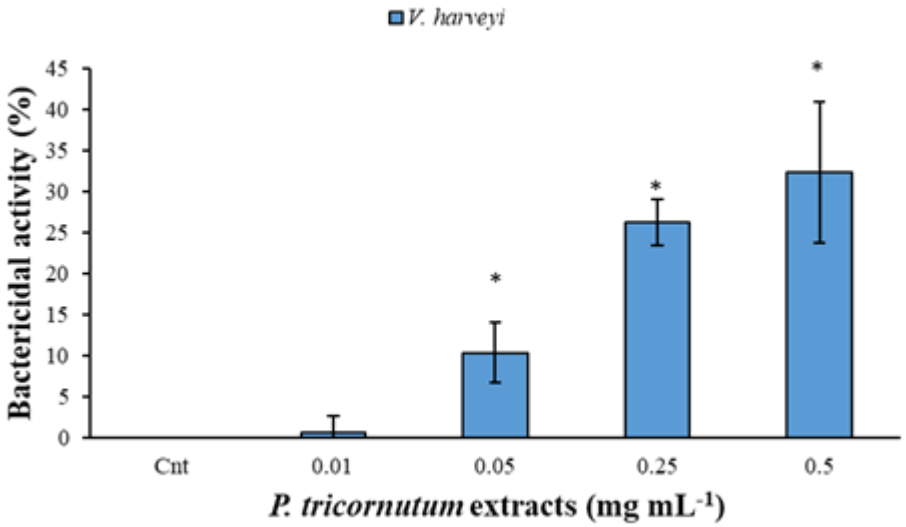


Figure 6. Bactericidal activity (%) against *Vibrio harveyi* determined using extracts of *P. tricornutum*. Values are the mean ± SEM (n= 3) with standard deviations. Microalgae that show statistical differences (P< 0.05) compared with the control are indicated by "*".

In the present study all extracts tested showed dose-dependently bactericidal activity against the two fish pathogens. The different bactericidal activity of the microalgal extracts observed may be due to the composition of each extract.

The ability of bioactive compounds extract from plants, algae and microalgae to inhibit the activity of bacterial pathogens in fish is of great interest as an alternative strategy to the use of drugs, especially in the aquaculture sector [41,79–82]. Algae produce bioactive compounds [83] able to counter environmental stresses [84] with a high biological activity [85,86]. In microalgae the most abundant compounds are flavonoids, in particular phenolic acids and carotenoids, with a high bactericidal activity [44,81,87].

Diatom extracts control the concentration of pathogenic vibrios, as reported by Molina-Càrdenas et al. [43] and Kokou et al. [42], through the production of antibacterial compounds.

Phenolic compounds are essential phytochemicals for the growth and reproduction, produced in defence against pathogens [88]. By binding to bacteria, they form heavy soluble complexes with proteins that inhibit receptor availability on the pathogen's cell surface [88,89].

In this study, *P. tricornutum* extracts showed the highest bactericidal activity, which may be related to the higher polyphenol and *β*-carotene content compared to the other extracts, as described in Chapter 1. *P. tricornutum*, in fact, possess one of the highest antioxidant capacity [90], compared to other cultivated microalgae species [36,70,71,90]. High levels of bioactive compounds in *P. tricornutum* extracts could therefore explain the high level of bactericidal activity against *V. anguillarum* and *V. harveyi*.

3.5 Conclusions

In vitro tests conducted on human skin fibroblast (Hs68) cell line showed a protective effect of *A. macrostachyum* extracts against cellular oxidative damage induced by UV radiation.

I*n vitro* tests on *S. aurata* fibroblast (SAF-1) cell line, pretreated with *Nannochloropsis* sp., *I. galbana*, *P. tricornutum* and *P tricornutum* N- extracts and then exposed to sub-lethal doses of the emerging contaminant BDE-47, showed that all the extracts significantly inhibited oxidative stress induced by the toxic contaminant BDE-47.

Furthermore, all the microalgal extract tested showed a dose-dependently bactericidal activity against two tested fish pathogens of the genus *Vibrio* spp. In particular, *P. tricornutum* extracts showed the highest bactericidal activity, which could be related to the higher content of polyphenols and carotenoids compared to the other extracts.

As oxidative stress is widely recognised as a potentially harmful condition for all organisms and cellular systems, including fish, the possibility of modulating the production of antioxidants in plants and algal strains is a useful research tool to monitor the benefits induced by natural molecules extracted from the co-cultivation of extractive species in IMTA systems.

3.6 References

[1] A. Martins, H. Vieira, H. Gaspar, S. Santos, Marketed Marine Natural Products in the Pharmaceutical and Cosmeceutical Industries: Tips for Success, Mar. Drugs 2014, Vol. 12, Pages 1066-1101. 12 (2014) 1066–1101. https://doi.org/10.3390/MD12021066.

[2] C.A. Espinosa-Leal, S. Garcia-Lara, Current Methods for the Discovery of New Active Ingredients from Natural Products for Cosmeceutical Applications, Planta Med. 85 (2019) 535–551. https://doi.org/10.1055/a-0857-6633.

[3] M.J. Pérez, E. Falqué, H. Domínguez, Antimicrobial action of compounds from marine seaweed, Mar. Drugs. 14 (2016). https://doi.org/10.3390/md14030052.

[4] B. Pradhan, R. Nayak, S. Patra, B.P. Jit, A. Ragusa, M. Jena, Molecules Bioactive Metabolites from Marine Algae as Potent Pharmacophores against Oxidative Stress-Associated Human Diseases: A Comprehensive Review, Molecules. 26 (2020) 1–25. https://doi.org/10.3390/molecules26010037.

[5] W.A.J.P. Wijesinghe, Y.J. Jeon, Biological activities and potential cosmeceutical applications of bioactive components from brown seaweeds: A review, Phytochem. Rev. 10 (2011) 431–443. https://doi.org/10.1007/s11101-011-9214-4.

[6] C.M. Messina, R. Arena, S. Manuguerra, Y. Pericot, E. Curcuraci, F. Kerninon, G. Renda, C. Hellio, A. Santulli, Antioxidant Bioactivity of Extracts from Beach Cast Leaves of Posidonia oceanica (L.) Delile, Mar. Drugs 2021, Vol. 19, Page 560. 19 (2021) 560. https://doi.org/10.3390/MD19100560.

[7] S. Manuguerra, C.E. Ruiz, A. Santulli, C.M. Messina, Sub-lethal doses of polybrominated diphenyl ethers, in vitro, promote oxidative stress and modulate molecular markers related to cell cycle, antioxidant balance and cellular energy management, Int. J. Environ. Res. Public Health. 16 (2019) 588. https://doi.org/10.3390/ijerph16040588.

[8] A.M. Pisoschi, A. Pop, The role of antioxidants in the chemistry of oxidative stress: A review, Eur. J. Med. Chem. 97 (2015) 55–74. https://doi.org/10.1016/J.EJMECH.2015.04.040.

[9] M. Valko, D. Leibfritz, J. Moncol, M.T.D. Cronin, M. Mazur, J. Telser, Free radicals and antioxidants in normal physiological functions and human disease, Int. J. Biochem. Cell Biol. 39 (2007) 44–84. https://doi.org/10.1016/J.BIOCEL.2006.07.001.

[10] B. Halliwell, J.M.C. Gutteridge, Free Radicals in Biology and Medicine, 3rd editio, 1999.

[11] S. Ahsanuddin, M. Lam, E.D. Baron, Skin aging and oxidative stress, AIMS Mol. Sci. 3 (2016) 187–195. https://doi.org/10.3934/molsci.2016.2.187.

[12] J.H. Auh, J. Madhavan, Protective effect of a mixture of marigold and rosemary extracts on UV-induced photoaging in mice, Biomed. Pharmacother. 135 (2021). https://doi.org/10.1016/j.biopha.2020.111178.

[13] C. Espinosa, F. Pérez-Llamas, F.A. Guardiola, M.A. Esteban, M.B. Arnao, S. Zamora, J.A. López-Jiménez, Molecular mechanisms by which white tea prevents oxidative stress, J. Physiol. Biochem. 70 (2014) 891–900. https://doi.org/10.1007/S13105-014-0357-9/FIGURES/3.

[14] C. Espinosa Ruiz, S. Manuguerra, E. Curcuraci, A. Santulli, C.M. Messina, Carbamazepine, cadmium chloride and polybrominated diphenyl ether-47, synergistically modulate the expression of antioxidants and cell cycle biomarkers, in the marine fish cell line SAF-1, Mar. Environ. Res. 154 (2020). https://doi.org/10.1016/j.marenvres.2019.104844.

[15] A. Kornhauser, W.G. Wamer, L.A. Lambert, R.R. Wei, β-Carotene inhibition of chemically induced toxicity in vivo and in vitro, Food Chem. Toxicol. 32 (1994) 149–154. https://doi.org/10.1016/0278-6915(94)90176-7.

[16] M. Tanaka, Y. Kishimoto, M. Sasaki, A. Sato, T. Kamiya, K. Kondo, K. Iida, Terminalia bellirica (Gaertn.) Roxb. Extract and Gallic Acid Attenuate LPS-Induced Inflammation and Oxidative Stress via MAPK/NF- κ B and Akt/AMPK/Nrf2 Pathways, Oxid. Med. Cell. Longev. 2018 (2018). https://doi.org/10.1155/2018/9364364.

[17] S. Maqsood, S. Benjakul, A. Abushelaibi, A. Alam, Phenolic Compounds and Plant Phenolic Extracts as Natural Antioxidants in Prevention of Lipid Oxidation in Seafood: A Detailed Review, Compr. Rev. Food Sci. Food Saf. 13 (2014) 1125–1140. https://doi.org/10.1111/1541-4337.12106.

[18] D. Treutter, Significance of flavonoids in plant resistance: a review, Environ. Chem. Lett. 4 (2006) 147–157. https://doi.org/10.1007/s10311-006-0068-8.

[19] L.S.S. Hulkko, T. Chaturvedi, M.H. Thomsen, Extraction and Quantification of

Chlorophylls, Carotenoids, Phenolic Compounds, and Vitamins from Halophyte Biomasses, Appl. Sci. 12 (2022) 840. https://doi.org/10.3390/app12020840.

[20] G. Surget, V. Stiger-Pouvreau, K. Le Lann, N. Kervarec, C. Couteau, L.J.M. Coiffard, F. Gaillard, K. Cahier, F. Guérard, N. Poupart, Structural elucidation, in vitro antioxidant and photoprotective capacities of a purified polyphenolic-enriched fraction from a saltmarsh plant, J. Photochem. Photobiol. B Biol. 143 (2015) 52–60. https://doi.org/10.1016/J.JPHOTOBIOL.2014.12.018.

[21] L. Barreira, E. Resek, M.J. Rodrigues, M.I. Rocha, H. Pereira, N. Bandarra, M.M. da Silva, J. Varela, L. Custódio, Halophytes: Gourmet food with nutritional health benefits?, J. Food Compos. Anal. 59 (2017) 35–42. https://doi.org/10.1016/j.jfca.2017.02.003.

[22] C.E. Ruiz, S. Manuguerra, A. Cuesta, M.A. Esteban, A. Santulli, C.M. Messina, Sub-lethal doses of polybrominated diphenyl ethers affect some biomarkers involved in energy balance and cell cycle, via oxidative stress in the marine fish cell line SAF-1, Aquat. Toxicol. 210 (2019) 1–10. https://doi.org/10.1016/j.aquatox.2019.02.014.

[23] Y. Jiang, X. Tang, T. Sun, Y. Wang, BDE-47 exposure changed the immune function of haemocytes in Mytilus edulis: An explanation based on ROS-mediated pathway, Aquat. Toxicol. 182 (2017) 58–66. https://doi.org/10.1016/j.aquatox.2016.11.010.

[24] V. Tornero, G. Hanke, Chemical contaminants entering the marine environment from sea-based sources: A review with a focus on European seas, Mar. Pollut. Bull. 112 (2016) 17–38. https://doi.org/10.1016/j.marpolbul.2016.06.091.

[25] D. Álvarez-Muñoz, M. Llorca, J. Blasco, D. Barceló, Contaminants in the Marine Environment, in: Mar. Ecotoxicol., Academic Press, 2016: pp. 1–34. https://doi.org/10.1016/b978-0-12-803371-5.00001-1.

[26] A. Nasri, M. Allouche, A. Hannachi, B. Barhoumi, A. Wahbi, A.H. Harrath, E. Mahmoudi, H. Beyrem, F. Boufahja, Ecotoxicity of polybrominated diphenyl ether (BDE-47) on a meiobenthic community with special emphasis on nematodes: Taxonomic and trophic diversity assessment, Environ. Pollut. 277 (2021) 116727. https://doi.org/10.1016/j.envpol.2021.116727.

[27] Y. Jiang, X. Tang, B. Zhou, T. Sun, H. Chen, X. Zhao, Y. Wang, The ROS-mediated pathway coupled with the MAPK-p38 signalling pathway and antioxidant system plays

roles in the responses of Mytilus edulis haemocytes induced by BDE-47, Aquat. Toxicol. 187 (2017) 55–63. https://doi.org/10.1016/J.AQUATOX.2017.03.011.

[28] Q. Geng, M. Guo, H. Wu, J. Peng, G. Zheng, X. Liu, Y. Zhai, Z. Tan, Effects of single and combined exposure to BDE-47 and PFOA on distribution, bioaccumulation, and toxicity in blue mussel (Mytilus galloprovincialis), Ecotoxicol. Environ. Saf. 228 (2021) 113014. https://doi.org/10.1016/J.ECOENV.2021.113014.

[29] UNEP, Chemicals listed in Annex A. Stockholm Convention for Persistent Organic Pollutants Chatelaine, Switzerland, 2019. http://chm.pops.int/TheConvention/POPsReviewCommittee/RecommendationsofthePOPRC4andPOPRC3/tabid/440/Default.aspx#PentaBDE (accessed February 2, 2022).

[30] A. Herrera, A. Acosta-Dacal, O.P. Luzardo, I. Martínez, J. Rapp, S. Reinold, S. Montesdeoca-Esponda, D. Montero, M. Gómez, Bioaccumulation of additives and chemical contaminants from environmental microplastics in European seabass (Dicentrarchus labrax), Sci. Total Environ. (2022) 153396. https://doi.org/10.1016/J.SCITOTENV.2022.153396.

[31] M. Harmelin-Vivien, X. Bodiguel, S. Charmasson, V. Loizeau, C. Mellon-Duval, J. Tronczyński, D. Cossa, Differential biomagnification of PCB, PBDE, Hg and Radiocesium in the food web of the European hake from the NW Mediterranean, Mar. Pollut. Bull. 64 (2012) 974–983. https://doi.org/10.1016/j.marpolbul.2012.02.014.

[32] X. Bodiguel, J. Tronczynski, V. Loizeau, C. Munschy, N. Guiot, A.M. Le Guellec, N. Olivier, F. Roupsard, C. Mellon, Classical and novel organohalogen compounds (PCBs and PBDEs) in hake (M. merluccius, L.) from the Mediterranean and Atlantic coasts (France), WIT Trans. Ecol. Environ. 110 (2008) 157–166. https://doi.org/10.2495/ETOX080171.

[33] R. Tsao, Z. Deng, Separation procedures for naturally occurring antioxidant phytochemicals, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 812 (2004) 85–99. https://doi.org/10.1016/j.jchromb.2004.09.028.

[34] S.P. Cuellar-Bermudez, I. Aguilar-Hernandez, D.L. Cardenas-Chavez, N. Ornelas-Soto, M.A. Romero-Ogawa, R. Parra-Saldivar, Extraction and purification of high-value metabolites from microalgae: Essential lipids, astaxanthin and phycobiliproteins, Microb. Biotechnol. 8 (2015) 190–209. https://doi.org/10.1111/1751-7915.12167.

[35] Y. Ghasemi, S. Rasoul-Amini, M.H. Morowvat, Algae for the production of pharmaceuticals, in: Bioprocess Sci. Technol., 2011: pp. 163–183.

[36] M. Hajimahmoodi, M.A. Faramarzi, N. Mohammadi, N. Soltani, M.R. Oveisi, N. Nafissi-Varcheh, Evaluation of antioxidant properties and total phenolic contents of some strains of microalgae, J. Appl. Phycol. 22 (2010) 43–50. https://doi.org/10.1007/S10811-009-9424-Y/TABLES/4.

[37] B. Klejdus, L. Lojková, M. Plaza, M. Šnóblová, D. Štěrbová, Hyphenated technique for the extraction and determination of isoflavones in algae: Ultrasound-assisted supercritical fluid extraction followed by fast chromatography with tandem mass spectrometry, J. Chromatogr. A. 1217 (2010) 7956–7965. https://doi.org/10.1016/j.chroma.2010.07.020.

[38] H. Bin Li, K.W. Cheng, C.C. Wong, K.W. Fan, F. Chen, Y. Jiang, Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae, Food Chem. 102 (2007) 771–776. https://doi.org/10.1016/J.FOODCHEM.2006.06.022.

[39] I. Rodriguez-Garcia, J.L. Guil-Guerrero, Evaluation of the antioxidant activity of three microalgal species for use as dietary supplements and in the preservation of foods, Food Chem. 108 (2008) 1023–1026. https://doi.org/10.1016/j.foodchem.2007.11.059.

[40] S. Manuguerra, L. Caccamo, M. Mancuso, R. Arena, A.C. Rappazzo, L. Genovese, A. Santulli, C.M. Messina, G. Maricchiolo, The antioxidant power of horseradish, Armoracia rusticana, underlies antimicrobial and antiradical effects, exerted in vitro, Nat. Prod. Res. 34 (2020) 1567–1570. https://doi.org/10.1080/14786419.2018.1517121.

[41] C. Falaise, C. François, M.A. Travers, B. Morga, J. Haure, R. Tremblay, F. Turcotte, P. Pasetto, R. Gastineau, Y. Hardivillier, V. Leignel, J.L. Mouget, Antimicrobial Compounds from Eukaryotic Microalgae against Human Pathogens and Diseases in Aquaculture, Mar. Drugs 2016, Vol. 14, Page 159. 14 (2016) 159. https://doi.org/10.3390/MD14090159.

[42] F. Kokou, P. Makridis, M. Kentouri, P. Divanach, Antibacterial activity in microalgae cultures, Aquac. Res. 43 (2012) 1520–1527. https://doi.org/10.1111/j.1365-2109.2011.02955.x.

[43] C.A. Molina-Cárdenas, M. del P. Sánchez-Saavedra, Inhibitory effect of benthic diatom species on three aquaculture pathogenic vibrios, Algal Res. 27 (2017) 131–139. https://doi.org/10.1016/J.ALGAL.2017.09.004.

[44] B. Mishra, A. Tiwari, A.E.D. Mahmoud, Microalgal potential for sustainable aquaculture applications: bioremediation, biocontrol, aquafeed, Clean Technol. Environ. Policy. 1 (2022) 1–13. https://doi.org/10.1007/s10098-021-02254-1.

[45] C. Ghittino, M. Latini, F. Agnetti, C. Panzieri, L. Lauro, R. Ciappelloni, G. Petracca, Emerging pathologies in equaculture: Effects on production and food safety, Vet. Res. Commun. 27 (2003) 471–479. https://doi.org/10.1023/B:VERC.0000014204.37722.b6.

[46] P.J. Ashley, Fish welfare: Current issues in aquaculture, Appl. Anim. Behav. Sci. 104 (2007) 199–235. https://doi.org/10.1016/j.applanim.2006.09.001.

[47] J. Mougin, R. Roquigny, C. Flahaut, M. Bonnin-Jusserand, T. Grard, C. Le Bris, Abundance and spatial patterns over time of Vibrionaceae and Vibrio harveyi in water and biofilm from a seabass aquaculture facility, Aquaculture. 542 (2021) 736862. https://doi.org/10.1016/j.aquaculture.2021.736862.

[48] P. Charoonnart, S. Purton, V. Saksmerprome, Applications of microalgal biotechnology for disease control in aquaculture, Biology (Basel). 7 (2018) 24. https://doi.org/10.3390/biology7020024.

[49] Y. Javadzadeh, S. Hamedeyaz, Floating Drug Delivery Systems for Eradication of Helicobacter pylori in Treatment of Peptic Ulcer Disease, in: D.Z. Muchlisin (Ed.), Trends Helicobacter Pylori Infect., InTech, 2014: pp. 59–77. https://doi.org/10.5772/57353.

[50] F.M. Yusoff, S. Banerjee, N. Nagao, Y. Imaizumi, M. Shariff, T. Toda, Use of Microalgae Pigments in Aquaculture, in: Pigment. from Microalgae Handb., Springer, Cham, 2020: pp. 471–513. https://doi.org/10.1007/978-3-030-50971-2_19.

[51] D. Nagarajan, S. Varjani, D.J. Lee, J.S. Chang, Sustainable aquaculture and animal feed from microalgae – Nutritive value and techno-functional components, Renew. Sustain. Energy Rev. 150 (2021) 111549. https://doi.org/10.1016/J.RSER.2021.111549.

[52] E. Ringø, R.E. Olsen, T.Ø. Gifstad, R.A. Dalmo, H. Amlund, G.I. Hemre, A.M. Bakke, Probiotics in aquaculture: a review, Aquac. Nutr. 16 (2010) 117–136.

https://doi.org/10.1111/j.1365-2095.2009.00731.x.

[53] V. Kiron, Fish immune system and its nutritional modulation for preventive health care, Anim. Feed Sci. Technol. 173 (2012) 111–133. https://doi.org/10.1016/j.anifeedsci.2011.12.015.

[54] S. Thanigaivel, N. Chandrasekaran, A. Mukherjee, John Thomas, Seaweeds as an alternative therapeutic source for aquatic disease management, Aquaculture. 464 (2016) 529–536. https://doi.org/10.1016/j.aquaculture.2016.08.001.

[55] M.A.O. Dawood, S. Koshio, M.Á. Esteban, Beneficial roles of feed additives as immunostimulants in aquaculture: a review, Rev. Aquac. 10 (2018) 950–974. https://doi.org/10.1111/raq.12209.

[56] S. Manuguerra, L. Caccamo, M. Mancuso, R. Arena, A.C. Rappazzo, L. Genovese, A. Santulli, C.M. Messina, G. Maricchiolo, The antioxidant power of horseradish, *Armoracia rusticana* , underlies antimicrobial and antiradical effects, exerted *in vitro*, Nat. Prod. Res. 0 (2018) 1–4. https://doi.org/10.1080/14786419.2018.1517121.

[57] M. Abbes, H. Baati, S. Guermazi, C. Messina, A. Santulli, N. Gharsallah, E. Ammar, Biological properties of carotenoids extracted from Halobacterium halobium isolated from a Tunisian solar saltern, BMC Complement. Altern. Med. 13 (2013). https://doi.org/10.1186/1472-6882-13-255.

[58] A. Saija, A. Tomaino, D. Trombetta, M.L. Pellegrino, B. Tita, C. Messina, F.P. Bonina, C. Rocco, G. Nicolosi, F. Castelli, “In vitro” antioxidant and photoprotective properties and interaction with model membranes of three new quercetin esters, Eur. J. Pharm. Biopharm. 56 (2003) 167–174. https://doi.org/10.1016/S0939-6411(03)00101-2.

[59] L. Montenegro, C. Messina, S. Manuguerra, L. Santagati, L. Pasquinucci, R. Turnaturi, C. Parenti, R. Arena, A. Santulli, In Vitro Antioxidant Activity and In Vivo Topical Efficacy of Lipid Nanoparticles Co-Loading Idebenone and Tocopheryl Acetate, Appl. Sci. 9 (2019) 845. https://doi.org/10.3390/app9050845.

[60] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays, J. Immunol. Methods. 65 (1983) 55–63. https://doi.org/10.1016/0022-1759(83)90303-4.

[61] C. Espinosa Ruiz, S. Manuguerra, A. Cuesta, A. Santulli, C. Messina, Oxidative Stress, Induced by Sub-Lethal Doses of BDE 209, Promotes Energy Management and Cell

Cycle Modulation in the Marine Fish Cell Line SAF-1, Int. J. Environ. Res. Public Health. 16 (2019) 474. https://doi.org/10.3390/ijerph16030474.

[62] M.G. Stevens, S.C. Olsen, Comparative analysis of using MTT and XTT in colorimetric assays for quantitating bovine neutrophil bactericidal activity, J. Immunol. Methods. 157 (1993) 225–231. https://doi.org/10.1016/0022-1759(93)90091-K.

[63] J. Shin, J. Park, D. Che, H. Kang, B. Cho, Y. Lim, S. Jang, Protective effects of halophyte complex extract against UVB-induced damage in human keratinocytes and the skin of hairless mice, Exp. Ther. Med. 22 (2021) 1–8. https://doi.org/10.3892/etm.2021.10114.

[64] B.N. Ahn, J.A. Kim, C.S. Kong, Y. Seo, S.K. Kim, Photoprotective effect of libanoridin isolated from Corydalis heterocarpa on UVB stressed human keratinocyte cells, Exp. Dermatol. 22 (2013) 155–157. https://doi.org/10.1111/exd.12085.

[65] N. Saewan, A. Jimtaisong, Natural products as photoprotection, J. Cosmet. Dermatol. 14 (2015) 47–63. https://doi.org/10.1111/jocd.12123.

[66] D. Klinčić, M. Dvoršćak, K. Jagić, G. Mendaš, S. Herceg Romanić, Levels and distribution of polybrominated diphenyl ethers in humans and environmental compartments: a comprehensive review of the last five years of research, Environ. Sci. Pollut. Res. 27 (2020) 5744–5758. https://doi.org/10.1007/S11356-020-07598-7.

[67] P. De Oro-Carretero, J.S. Landaluze, Bioaccumulation And Biotransformation of BDE-47 Using Zebrash Eleutheroembryos (Danio Rerio), (2021). https://doi.org/10.21203/rs.3.rs-760242/v1.

[68] H. Chen, X. Tang, B. Zhou, Z. Zhou, N. Xu, Y. Wang, A ROS-mediated mitochondrial pathway and Nrf2 pathway activation are involved in BDE-47 induced apoptosis in Neuro-2a cells, Chemosphere. 184 (2017) 679–686. https://doi.org/10.1016/J.CHEMOSPHERE.2017.06.006.

[69] A. Maadane, N. Merghoub, T. Ainane, H. El Arroussi, R. Benhima, S. Amzazi, Y. Bakri, I. Wahby, Antioxidant activity of some Moroccan marine microalgae: Pufa profiles, carotenoids and phenolic content, J. Biotechnol. 215 (2015) 13–19. https://doi.org/10.1016/J.JBIOTEC.2015.06.400.

[70] H. Safafar, J. Van Wagenen, P. Møller, C. Jacobsen, Carotenoids, Phenolic Compounds and Tocopherols Contribute to the Antioxidative Properties of Some

Microalgae Species Grown on Industrial Wastewater, Mar. Drugs 2015, Vol. 13, Pages 7339-7356. 13 (2015) 7339–7356. https://doi.org/10.3390/MD13127069.

[71] K. Goiris, K. Muylaert, I. Fraeye, I. Foubert, J. De Brabanter, L. De Cooman, Antioxidant potential of microalgae in relation to their phenolic and carotenoid content, J. Appl. Phycol. 24 (2012) 1477–1486. https://doi.org/10.1007/s10811-012-9804-6.

[72] M.C. Lee, J. Puthumana, S.H. Lee, H.M. Kang, J.C. Park, C.B. Jeong, J. Han, D.S. Hwang, J.S. Seo, H.G. Park, A.S. Om, J.S. Lee, BDE-47 induces oxidative stress, activates MAPK signaling pathway, and elevates de novo lipogenesis in the copepod Paracyclopina nana, Aquat. Toxicol. 181 (2016) 104–112. https://doi.org/10.1016/J.AQUATOX.2016.10.025.

[73] T. Nakari, S. Huhtala, In vivo and in vitro toxicity of decabromodiphenyl ethane, a flame retardant, Environ. Toxicol. 25 (2010) 333–338. https://doi.org/10.1002/TOX.20499.

[74] N. Mohamad, M. Mustafa, M.N.A. Amal, M.Z. Saad, I.S. Md Yasin, N. Al-saari, Environmental Factors Associated with the Presence of Vibrionaceae in Tropical Cage-Cultured Marine Fishes, J. Aquat. Anim. Health. 31 (2019) 154–167. https://doi.org/10.1002/AAH.10062.

[75] A. Nguyen, A. Jacq, Small RNAs in the Vibrionaceae: An ocean still to be explored, Wiley Interdiscip. Rev. RNA. 5 (2014) 381–392. https://doi.org/10.1002/WRNA.1218.

[76] E.C. Amar, J.P.J. Faisan, M.J.S. Aspines-Amar, R.V.J. Pakingking, Temporal changes in innate immunity parameters, epinecidin gene expression, and mortality in orange-spotted grouper, Epinephelus coioides experimentally infected with a fish pathogen, Vibrio harveyi JML1 | Elsevier Enhanced Reader, Fish Shellfish Immunol. 69 (2017) 153–163.

[77] I. Frans, C.W. Michiels, P. Bossier, K.A. Willems, B. Lievens, H. Rediers, Vibrio anguillarum as a fish pathogen: Virulence factors, diagnosis and prevention, J. Fish Dis. 34 (2011) 643–661. https://doi.org/10.1111/J.1365-2761.2011.01279.X.

[78] V. Zammuto, M.G. Rizzo, A. Spanò, G. Genovese, M. Morabito, D. Spagnuolo, F. Capparucci, C. Gervasi, A. Smeriglio, D. Trombetta, S. Guglielmino, M.S. Nicolò, C. Gugliandolo, In vitro evaluation of antibiofilm activity of crude extracts from macroalgae against pathogens relevant in aquaculture, Aquaculture. 549 (2022)

737729. https://doi.org/10.1016/J.AQUACULTURE.2021.737729.

[79] D. Zilberg, A. Tal, N. Froyman, S. Abutbul, N. Dudai, A. Golan-Goldhirsh, Dried leaves of Rosmarinus officinalis as a treatment for streptococcosis in tilapia, J. Fish Dis. 33 (2010) 361–369. https://doi.org/10.1111/J.1365-2761.2009.01129.X.

[80] D. Dubber, T. Harder, Extracts of Ceramium rubrum, Mastocarpus stellatus and Laminaria digitata inhibit growth of marine and fish pathogenic bacteria at ecologically realistic concentrations, Aquaculture. 274 (2008) 196–200. https://doi.org/10.1016/J.AQUACULTURE.2007.11.029.

[81] E. Shannon, N. Abu-Ghannam, Antibacterial Derivatives of Marine Algae: An Overview of Pharmacological Mechanisms and Applications, Mar. Drugs 2016, Vol. 14, Page 81. 14 (2016) 81. https://doi.org/10.3390/MD14040081.

[82] F. Marino, G. Di Caro, C. Gugliandolo, A. Spanò, C. Faggio, G. Genovese, M. Morabito, A. Russo, D. Barreca, F. Fazio, A. Santulli, Preliminary study on the in vitro and in vivo effects of Asparagopsis taxiformis bioactive phycoderivates on teleosts, Front. Physiol. 7 (2016) 459. https://doi.org/10.3389/FPHYS.2016.00459/BIBTEX.

[83] C.C. Hughes, W. Fenical, Antibacterials from the sea, Chem. - A Eur. J. 16 (2010) 12512–12525. https://doi.org/10.1002/CHEM.201001279.

[84] E. Villar-Navarro, C. Garrido-Pérez, J.A. Perales, The potential of different marine microalgae species to recycle nutrients from recirculating aquaculture systems (RAS) fish farms and produce feed additives, Algal Res. 58 (2021) 102389. https://doi.org/10.1016/j.algal.2021.102389.

[85] A.E. AlProl, M.R. Elkatory, The current status of various algal industries, Handb. Algal Biofuels. (2022) 123–147. https://doi.org/10.1016/B978-0-12-823764-9.00010-8.

[86] B. Pradhan, P.P. Bhuyan, S. Patra, R. Nayak, P.K. Behera, C. Behera, A.K. Behera, J.-S. Ki, M. Jena, Beneficial effects of seaweeds and seaweed-derived bioactive compounds: Current evidence and future prospective, Biocatal. Agric. Biotechnol. 39 (2022) 102242. https://doi.org/10.1016/J.BCAB.2021.102242.

[87] L. Picot, T.M. Karpí Nski, M. O˙ Zarowski, R. Alam, M. Łochy´nska, Ł. Łochy´nska, M. Stasiewicz, What Do We Know about Antimicrobial Activity of Astaxanthin and Fucoxanthin?, Mar. Drugs 2022, Vol. 20, Page 36. 20 (2021) 36. https://doi.org/10.3390/MD20010036.

[88] T. Citarasu, Natural antimicrobial compounds for use in aquaculture, Woodhead Publishing Limited, 2012. https://doi.org/10.1016/B978-0-85709-016-4.50016-7.

[89] R. Perumal Samy, P. Gopalakrishnakone, Therapeutic Potential of Plants as Anti-Microbials for Drug Discovery, Evidence-Based Complement. Altern. Med. 7 (2010) 283–294. https://doi.org/10.1093/ECAM/NEN036.

[90] A. Saide, K.A. Martínez, A. Ianora, C. Lauritano, Unlocking the Health Potential of Microalgae as Sustainable Sources of Bioactive Compounds, Int. J. Mol. Sci. 2021, Vol. 22, Page 4383. 22 (2021) 4383. https://doi.org/10.3390/IJMS22094383.

Chapter 4 - Cultivation and biochemical characterization of isolated Sicilian microalgal species in salt and temperature stress conditions

4.1 Abstract

In the last years, the possibility to exploit autochthone microalgae in regional applications has been explored. The regional-based microalgal industry may bring several benefits, as autochthone microalgae are already adapted to the biotic and abiotic stresses of their environment. In this work, this concept was applied to Sicily, in which three microalgal strains were collected from the coastline. Monoalgal strains were then isolated and molecular characterization was performed for the species determination. Three of them, two strains of *Chlorella* and one of *Dunaliella*, were cultivated in lab-scale in four different conditions: Low Temperature-Low Salt (LT-LS), High-Temperature-Low Salt (HT-LS), Low Temperature-High Salt (LT-HS) and High Temperature-High Salt (HT-HS) to investigate the role of each condition on the growth performance, the productivity and the biochemical composition of the microalgal biomass. In particular, lipid, fatty acid composition and antioxidant capacity were assessed. Results indicated that one of the Sicilian strains of *Chlorella* has a better growth performance at a higher temperature while the *Dunaliella* strain is tolerant to high-salt stress. Moreover, the biochemical composition appears to be strongly influenced by temperature and salt stresses: the lipid content decreased in all the strains and a significant shift in fatty acid composition was observed, with an increase in the content of *n*-3 PUFAs in some cases. Results indicated that also the carotenoids content decreased in some of the tested stress conditions. The results obtained in this research represent a first step for developing a regional-based microalgal industry in Sicily by exploiting the natural biodiversity of the Sicilian environment.

KEYWORDS: Microalgal agriculture, biomass, high salinity, heat, PUFAs, carotenoids

4.2 Introduction

Microalgae have nowadays gained considerable attention because of their biotechnological and industrial interest as a result of their wide range of applications such as food, pharmaceuticals, cosmetics, energetics and phycoremediation, among others [1–3]. Albeit their promising application, the microalgal industry is not fully matured as microalgae production is still not economically convenient because of low biomass yields [4]. However, several strategies can be used for the optimization of productivity. With this purpose, some of

the most recent researches rely on the optimization of algal reactor efficiency [5] or the combination of nutrient availability and lightning strategies [6]. Another possible strategy is the "phycoprospecting", *i.e.* the exploitation of selected indigenous microalgae with favourable characteristics for applications in the regional-based production [7]. The cultivation of autochthone microalgae in their environment offers several advantages, being already adapted to both biotic and abiotic stresses [8]. This allows reducing the acclimation process of the strains to a new cultivation condition, leading to economic and time savings. Indeed, native strains are the best candidates for outdoor cultivation [9], which is the cheapest alternative in microalgae industrial production. However, the weakness of microalgae outdoor cultivation is the lower productivity comparing the indoor cultivation where optimal growth condition can be used [10]. For this reason, exploiting the diversity of "Nature's culture collection" [7] and selecting the best microalgae strains for outdoor cultivation can help to pave the way toward industrial algal cultivation. Indeed, several studies focus on the characterisation and isolation of local strains and the investigation of their industrial potential [9,11,12] and some of them have been based in Sicily [13–15]. However, to the authors' knowledge, none of these studies included microalgae biochemical characterization and production rate, combining two stress conditions such as high temperature and salinity. The selection of microalgal strains resistant to both high salinity and temperature is crucial for their industrial cultivation in the outdoor system in Sicily, where the diurnal temperature during summertime may reach 40°C. Moreover, the use of high-salinity wastewater (e.g. deriving from an aquaculture system) could reduce the dependence on freshwater and limit contamination [16].

This work focuses on autochthonous microalgae selection and their characterization for a possible biotechnological application in both aquaculture and food industry. Three microalgal strains, *Chlorella* sp. *Pozzillo*, *Chlorella* sp. *Barcarello* and *Dunaliella viridis*, isolated during the summer from the Sicilian coastline and a salter pond were tested for their tolerance to heat and salt stress by cultivating them for eight days at two different temperatures and salinities. The objective of this work was to verify if these strains can grow in extreme conditions and if salinity and temperature stresses may trigger the production of high-value biomolecules such as lipids, PUFAs and carotenoids.

4.3 Material and Methods

4.3.1 Microalgal strains, isolation, and molecular characterisation

In this study three microalgal strains isolated from different locations on the coastline of Sicily characterised by green or red summer blooms were selected: a) *Dunaliella viridis* (*D. viridis*), previously isolated from Natural Reserve of Saltern ponds, Trapani (TP) [15], b) *Chlorella* sp. *Pozzillo* previously isolated from Pozzillo beach, Palermo (PA) [14], c) *Chlorella* sp. *Barcarello* isolated as *Chlorella* sp. *Pozzillo* [14] from Barcarello beach, Palermo (PA) (GPS coordinates 38°12'28.1"N 13°16'55.6"E). *Chlorella* sp strains were isolated from beaches close to the city with a standard salinity (approx. 21 g L^{-1} NaCl), while *D. viridis* from a salter pond with salinity above the saturation level (>200 g L^{-1} NaCl). *Chlorella* sp. *Barcarello* was isolated by the combination of filtration and dilution methods. The first phase of isolation involved the filtration through wide mesh filters (>200 μm) for removing the impurities from the initial sample (i.e. debris, sand, rock, macroalgae). The filtration was coupled with serial dilutions in 24-well microplates to obtain a single strain per well. Once the individual strain was obtained, the liquid cultures were transferred into a solid medium to obtain individual colonies that were used for molecular characterization by colony PCR. For serial dilutions, liquid and solid f/2 medium with a concentrations of 20 g L^{-1} NaCl was used. The molecular characterization of *Chlorella* sp. *Barcarello* was done as for *Chlorella* sp. *Pozzillo* [14]**.** Molecular characterization was performed by colony polymerase chain reaction (PCR) using Q5 ® high-fidelity DNA Polymerase (NEB), the forward primer A (5′-ACC CTG GTT GAT CCT GCC AG-3′) and primerSSU-inR1 (5′-CAC CAG ACT TGC CCT CCA-3′). For the Colony PCR the following program was used: 95 °C (5 min), 32 cycles of 95 °C (30 s), 55 °C (30 s) and 68 °C (60 s) and a final 7 min extension step of 68 °C. The PCR products were run on 1% of agarose gel (ethidium bromide 0.2 μg/mL) against Jena Bioscience Low Range DNA Ladder, Linear Scale DNA Ladders. The PCR products were purified using the NucleoSpin® Gel and PCR Clean-up and sequenced by BMR service genomics (60 ng of DNA + 0.6 ng of each primer). The sequences obtained were then analysed using online bioinformatics software BLAST for the alignment of sequences and the identification of the belonging species. The sampling site, type of bloom and the reference gene used for the species identification in the selected strains are summarized in Table 1.

Table 1. Sampling site, reference genes for species identification, and type of bloom of *Chlorella* sp. *Pozzillo*, *Chlorella* sp. *Barcarello* and *Dunaliella viridis*.

Microalgal Species	Sampling Site	GPS coordinates	Reference gene	Algal bloom
***Chlorella* sp.**	Pozzillo beach	38°11'01.0"N 13°08'39.0"E	18S rDNA [14]	Green
***Chlorella* sp.**	Barcarello beach	38°12'28.1"N 13°16'55.6"E	18S rDNA	Green
Dunaliella viridis	Saltern pond of Trapani	38°00'51.2"N 12°32'07.4"E	18S rDNA, ITS [15]	Red

4.3.2 Microalgal growth

Microalgal pre-cultures were set up by inoculating 10 mL of a back-up culture in 100 mL of the same liquid medium used for the main experiment. When the cells were in the late exponential phase (around 10 days of cultivation), they were used as inoculum for the main experiment. When the pH was above 8.0, pure CO_2 was supplied until it reached the value of 7.0.

Three microalgal strains, *Chlorella* sp. *Pozzillo*, *Chlorella* sp. *Barcarello* and *D. viridis*, were grown in 1 L Erlenmeyer flasks placed in an oscillating incubator (Corning Lse) under a 127 $\mu E/m^2$ s photon flux in three cultivation conditions: Low Temperature-Low Salt (LT-LS), High-Temperature-Low Salt (HT-LS) and High Temperature-High Salt (HT-HS). Light intensity was measured with a Delta Ohm-HD 9021 quantometer equipped with a Photosynthetic Active Radiation (PAR) probe (Delta Ohm LP 9021 PAR). The medium for the cultivation of *Chlorella* strains in LS conditions is made in artificial seawater (6.3 mM KCl, 2.0 mM $NaHCO_3$, 7.1 mM KBr, 0.36 mM H_3BO_3, 0.024 M Na_2SO_4, 9 mM $CaCl_2$ $2H_2O$, 0.046 M $MgCl_2$ $6H_2O$, 0.35 M NaCl). It is a modified F/2 medium [17] with the following final composition: 3.5 mM $NaNO_3$, 0.036 mM NaH_2PO_4 H_20, 0,12 µM $FeCl_3$ $6H_2O$, 0,12 µM Na_2EDTA, 0.04 µM $CuSO_4$ $5H_2O$, 0.076 µM $ZnSO_4$ $7H_2O$, 0.042 µM $CoCl_2$ $6H_2O$, 0,91 µM $MnCl_2$ $4H_2O$, 0.025 µM Na_2MoO_4 $2H_2O$. The medium for the cultivation of *Dunaliella*, instead, is a modified Ramaraj medium (R.M.) [18] with the following composition: 2.68 mM KCl, 1.5 M NaCl, 0.025 M $NaHCO_3$, 0.15 mM H_3BO_3, 8.07 10^{-4} mM $CaCl_2$ $2H_2O$, 5 M $MgSO_4$ 7 H_2O, 5 mM $NaNO_3$, 0.1 mM NaH_2PO_4, 3.08 10^{-3} mM $FeCl_3$, 8.07 10^{-4} $ZnCl_2$, 3.85 10^{-4} mM $CoCl_2$ 6 H_2O, 10^{-2} $MnCl_2$ 4 H_2O, 3.72 10^{-4} mM $CuSO_4$ 5 H_2O, 2.38 10^{-3} mM Na_2MoO_4 $2H_2O$. The HS conditions consisted of the double concentration of NaCl, resulting in 0.70 M NaCl (about 40 g L^{-1} against 20 g L^{-1} of LS conditions) for *Chlorella* species and 3 M (about 180 g L^{-1} against 90 g L^{-1} of the LS) for *Dunaliella*. All cultures were grown at

24°C in LT conditions and 34°C in HT conditions. In Table 2 there is a clarifying scheme of the employed conditions.

The pH of the F/2 media (both low and high salinity) was around 7, while the one of the R.M. around 9. Electrical conductivity was 45.7 mS/cm in F/2 LS and 73.3 mS/cm in F/2 HS, while it was 113.5 mS/cm in R.M. LS and 192.2 mS/cm in HS condition. Each experiment was carried out for 8 days. The concentration of the microalgal suspension was monitored by reading the absorbance at 750 nm. Then, the values were converted in concentration [g L^{-1}] through calibration curves obtained by filtering a known volume of algal suspension with glass fibre filters (pore size ø = 0.7 µm), washed twice with 10 mL ammonium bicarbonate (0.5 M) and dried at 105°C for 24 h. The dry weight was determined gravimetrically. For each condition, a biological triplicate (n=3) was performed. The value of pH of the cell suspensions was measured at the same moment as optical density by employing a pHmeter (Crison MM 41). Values are reported as mean together with the standard deviation.

Table 2. Scheme of the employed experimental conditions

Condition	*Species*	*T [°C]*	C_{NaCl} *[g* L^{-1}*]*
LS LT	*Chlorella* sp. *Pozzillo*	24	20
LS LT	*Chlorella* sp. *Barcarello*	24	20
LS LT	*Dunaliella viridis*	24	90
HS LT	*Chlorella* sp. *Pozzillo*	24	40
HS LT	*Chlorella* sp. *Barcarello*	24	40
HS LT	*Dunaliella viridis*	24	180
LS HT	*Chlorella* sp. *Pozzillo*	34	20
LS HT	*Chlorella* sp. *Barcarello*	34	20
LS HT	*Dunaliella viridis*	34	90
HS HT	*Chlorella* sp. *Pozzillo*	34	40
HS HT	*Chlorella* sp. *Barcarello*	34	40
HS HT	*Dunaliella viridis*	34	180

4.3.3 Determination of productivity in batch

Batch productivity was calculated by employing the following equation:

$$\mathrm{P} = \frac{X - X_o}{t - t_0} \tag{1}$$

where X_0 is the initial biomass concentration (g L-1) at the initial time t_0 and X the biomass concentration at the final time t. For convenience purposes, a final time of 8 days was adopted for all the cultivation series, which averagely corresponded to the end of the

exponential phase. Data are expressed as an average of three cultivation replicates (n=3) and error bar is reported.

4.3.4 Sample preparation

After 8 days of cultivation, the cell suspension was harvested by centrifugation and the obtained biomass was frozen in liquid nitrogen and freeze-dried for 48 h in a bench lyophilizator (FreeZone 2.5L, LABCONCO, US). The biomass was then stored at -20°C for further analysis.

4.3.5 Total lipids quantification and analysis of total fatty acids

Total lipids were determined gravimetrically according to Folch et al. [19]. Total fatty acid (FA) methyl esters were determined from the total lipid content of dried microalgae, according to the method described by Lepage and Roy [20] and were analysed as described by Messina et al. [21].

4.3.6 Total phenolic content

Folin-Ciocalteu reagent assay [22] was carried out to determine the total phenolic content of samples [23] of microalgae. Gallic acid was used as the standard for calibration (5–500 mg mL^{-1}) and results were expressed as mg of gallic acid equivalents (GAE) per g of microalgae dry weight (DW) [10, 20–22]. The absorbance of each dilution was recorded at 725 nm, using a microplate reader (Multiscan-Sky Microplate Reader, Thermo-Scientific TM, USA). Each sample was analysed in triplicate.

4.3.7 DPPH radical scavenging activity

The free radical scavenging ability of microalgae extracts was determined through the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay as reported previously [13,24,25]. The dried samples were re-suspended in methanol and analysed at various concentrations (from 0.1 to 2 mg mL^{-1}). An aliquot (40 mL) of each extract was mixed with 160 µL of DPPH methanolic solution (0.1 mM). The mixture was vigorously mixed and incubated in darkness for 30 min. Absorbance was recorded at 517 nm. Each sample was measured in triplicate (n=3). The results were reported as IC 50 (i.e., Inhibitory Concentration to cause a 50% decrease of the absorbance). A lower IC 50 value indicates higher antiradical activity.

4.3.8 Beta carotene and chlorophyll HPLC analysis

Beta carotene and chlorophyll were analysed as previously showed [27]. Briefly, ~6 mg of freeze-dried biomass was disrupted together with 50 µL deionized water, 3 mL acetone and 0.7 g glass beads (0.1 mm) by vortex mixing for 2 minutes (Maxi Mixer, Asal). The extraction with acetone was repeated twice. The samples were evaporated and resuspended in methanol before HPLC injection. Carotenoid extracts were analysed with an Infinity 1220 LC (Agilent Technologies, US) equipped with a photodiode array detector and column oven at room temperature. Separation of the compounds was achieved using a Synergi Hydro-RP 18C (5 µm, 250x4 mm, Phenomenex) column with a mobile phase consisting of acetonitrile: water (9:1; v/v) as solvent A and ethyl acetate as solvent B and a constant flow of 1 mL min^{-1}. The gradient program applied was: (i) 0–16 min, 0–60% B; (ii) 16–30 min, 60% B; (iii) 30–32 min 100% B and (iv) 32-35 min 100% A. Method adapted from Couso et al. [28]. The injection volume was 100 µL; chromatograms were recorded at 450 nm. Quantification of chlorophyll *a* and *β*-carotene was performed through calibration curves. Calibration curves were obtained with Chlorophyll *a* (analytical standard, Sigma-Aldrich) in the concentration range between 8 and 500 µg/mL and with β-carotene (synthetic, ≥ 95%, Sigma-Aldrich) in the concentration range between 2 and 500 µg/mL Each sample was analysed in triplicate.

4.3.9 Statistical analysis

All tests were carried out in triplicate and expressed as a mean ± standard deviation (SD). Data were analysed by a one-way variance analysis (ANOVA) using the SPSS 20.0 statistical software program and deviations were considered significant at $P < 0.05$. Different letters were used to indicate significant differences among various treatment in each species at $P<0.05$.

Non-metric multidimensional scaling (nMDS) [29] was used to evaluate patterns in polyphenols, DPPH, lipid content, SFA, MUFA, PUFA, PUFA *n*-3, PUFA *n*-6, *n*-3/*n*-6 chlorophyll *a* and *β*-carotene, as a function of salinity and temperature variables. In the nMDS representation, increasing distances among points in the ordination plot, represent decreasing similarity among the same points. The Euclidean distance on the normalized data was used as a distance measure. Pearson product correlations for the relative proportions of the variables are shown in the vector loading plot. The length and direction of the vectors indicate the strength of the correlation, while the direction and size of each vector indicate the relative abundance of that variable in the samples.

Multivariate data analysis was conducted using the PRIMER 6 v.6.1 software package (PRIMER-E Ltd., Plymouth).

4.4 Results and Discussion

4.4.1 Molecular identification of *Chlorella* sp. *Barcarello*

The Colony PCR using the universal primers for the amplification of 18S gave the single product corresponding to the expected size, as shown in Figure S1 of supplementary material. The obtained sequence was deposited in Genbank database with the name *Chlorella* sp. (Barcarello) and the code MT259187.1. Table 3 shows the percentage of identity of the DNA sequence of 18S rRNA gene from *Chlorella* sp. *Barcarello* with other known *Chlorella* strains.

Table 3. Identity of the DNA sequence of 18S rRNA gene from *Chlorella* sp. *Barcarello* with other known *Chlorella* strains

Isolate species	*Target*	*Identity*	*Accession*
Chlorella sp. Barcarello	*18S* ribosomal RNA gene	100%	MT259187.1
Chlorella sp. Pozzillo	*18S* ribosomal RNA gene	99.6%	MT259188.1
Chlorella vulgaris isolate UMT-M1	*18S* ribosomal RNA gene	99.6%	KJ561358.1
Chlorella kessleri strain SAG 211-11g	*18S* ribosomal RNA gene	98.8%	X56105.1

4.4.2 Microalgal growth and productivity

Chlorella sp. *Pozzillo*, *Chlorella* sp. *Barcarello* and *D. viridis* were grown in four different conditions: LT-LS, LT-HS, HT-LS, HT-HS. Growth curves are reported in Figure 1. At the end of the cultivation, *Chlorella* strains reached a final biomass concentration ranging between 0.3 and 1 g L^{-1}, similar values were observed by other authors using the same genus [30,31]. The final biomass concentration of *D. viridis* was, instead, around 0.3 g L^{-1}, as observed also by other authors [32].

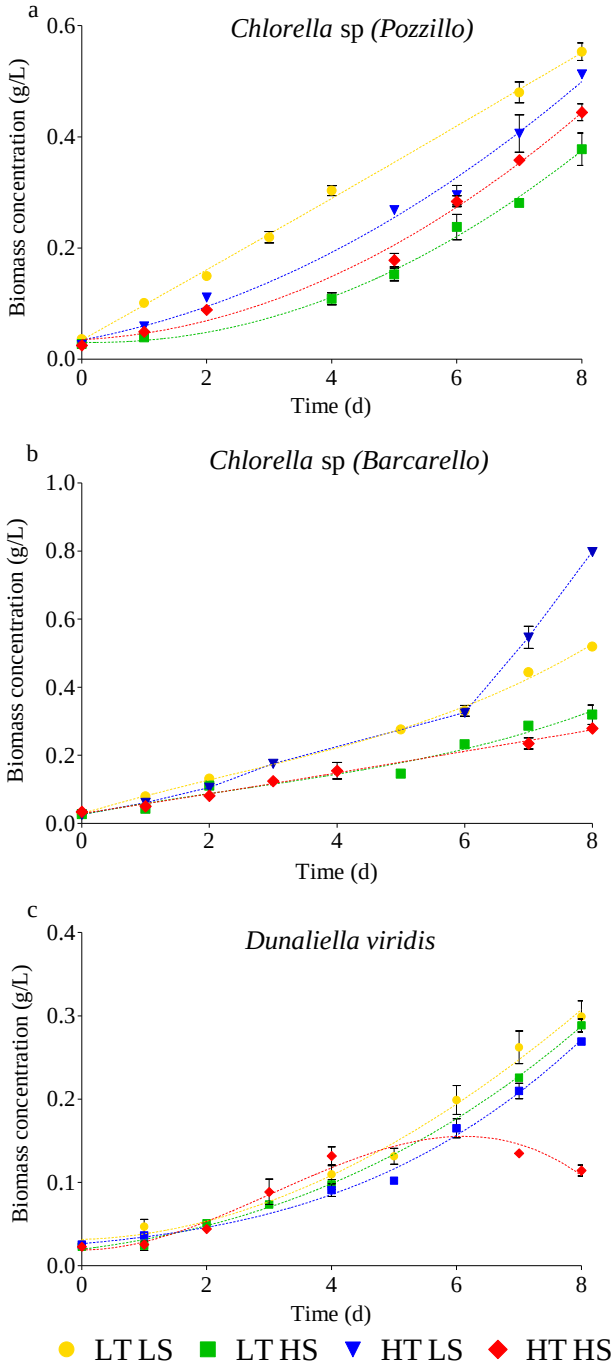


Figure 1. Growth curves of *Chlorella* sp. *Pozzillo* (a), *Chlorella* sp. *Barcarello* (b) and *Dunaliella viridis* (c) at Low Temperature-Low Salt (LT-LS), Low Temperature-High Salt (LT-HS), High-Temperature-Low Salt (HT-LS) and High Temperature-High Salt (HT-HS). Data are reported as means (n=3) with s.d. as error bars.

As shown in Figure 1 a, *Chlorella* sp. *Pozzillo* displayed the best growth performance when cultured in LT-LS condition, while it progressively decreased in HT-LS, in HT-HS and LT-HS. On the other hand, *Chlorella* sp. *Barcarello* (Figure 1b), showed an improved growth compared to the others in the HT-LS condition, while the LT-LS condition was similar to the

other *Chlorella* strain. The same strain showed lower growth performances (and similar to each other) in LT-HS and HT-HS conditions. *D. viridis* (Figure 1c) showed similar initial growths in all the tested conditions, while a decrease of growth was observed in the last days of the HT-HS one. Batch productivities were also calculated from the data for all 8 days, and the results are reported in Figure 2. In *Chlorella* genus the productivity ranged from 0.04 to 0.15 g $L^{-1}d^{-1}$, and the only condition in which there was an evident increase was in *Chlorella* sp. *Barcarello* HT-LS condition, while *D. viridis* showed productivity around 0.03 g L^{-1} in almost all the tested conditions except for HT-HS, case in which the combination of the two stresses made the productivity to decrease. These results are in line with other studies [33,34]. It is worth noting that the reported productivity is calculated on the base of batch data after 8 days of growth (averagely the duration of the exponential phase), while industrial cultivation is usually based on continuous mode. Furthermore, the productivity may significantly vary when scaling up on industrial systems. Even if the data do not yet indicate a commercially viable scenario, they may be compared with literature data obtained in outdoor photobioreactors [35–37] in similar conditions.

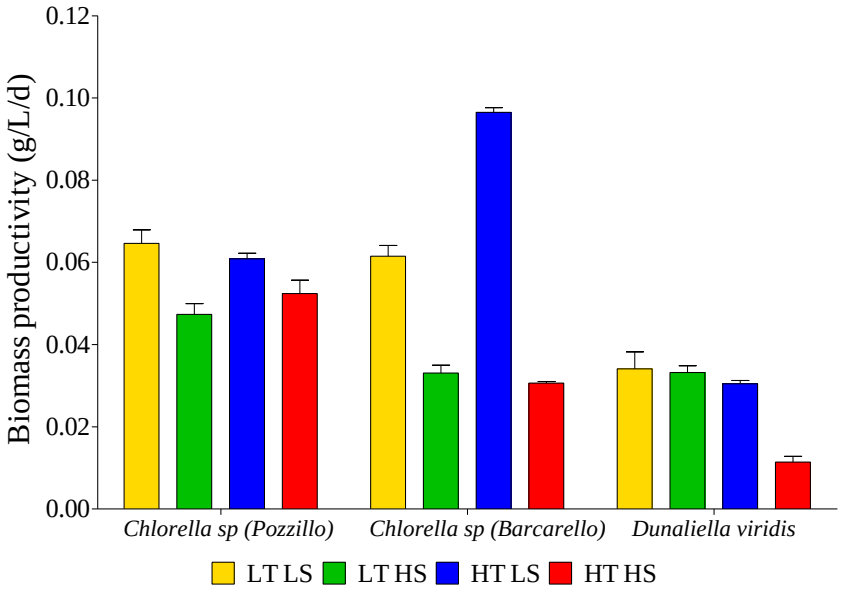


Figure 2. Batch productivities of *Chlorella* sp. *Pozzillo*, *Chlorella* sp. *Barcarello* and *Dunaliella viridis* grown at Low Temperature-Low Salt (LT-LS), Low Temperature- High Salt (LT-HS), High-Temperature-Low Salt (HT-LS) and High Temperature-High Salt (HT-HS). Data are reported as mean (n=3) and error bars report the standard deviations.

Chlorella genus was reported to respond differently when exposed to different temperatures. For example, Lu et al. found that the optimum growth temperature for a strain of *Chlorella* sp.

was 25 °C [38] while for *Chlorella vulgaris* it was found at about 30°C [39]. The variety is highlighted when considering the high range of optimum temperatures: Zhang et al. found the optimum growth for a strain of *Chlorella* sp at 18°C [40] while Turon et al. at 35°C for *Chlorella sorokiniana* [41]. The response of *Chlorella* strains shown in this study at different temperatures is, therefore, well inserted in the context of the abovementioned studies, in both cases of *Chlorella* sp. *Pozzillo* and *Barcarello.* Furthermore, a *Chlorella* strain isolated from the Mediterranean Sea was found to have a better growth response at the highest tested temperature compared to lower temperatures by Pulina et al. [42]. Even if the maximum temperature of the cited study was 17°C, far from the 34°C tested in the present study, we may assume that the trend is similar and that increasing the temperature leads to an advantage in terms of growth performance. This may be related also to the location from which the strains were harvested, in both cases the Mediterranean Sea. *D. viridis*, on the other hand, was reported to have a similar growth performance at 25°C and 35°C also by other authors [43] confirming our findings. The increase of the growth performance of *Chlorella* sp. *Barcarello* with temperature is an advantage if considering the high costs associated with temperature control system in outdoor facilities and that in Sicily external temperature during summer may reach 40°C.

The effect of salinity on *Chlorella* sp. growth was also previously reported by Asem et al. [44], who observed an optimal growth at a salinity of 30 g L^{-1} NaCl. This concentration is comparable with the LS condition of the present study, and the decrease of growth performance of both *Chlorella* strains HS condition found in this study is therefore in line with the abovementioned research. *D. viridis* is known as a halotolerant strain, following the locations where it was selected, a salter pond in Sicily. Byrd et al. reported, for several *Dunaliella* strains, the optimum salinity [45]. The optimum salinity for *D. viridis* is 60 g L^{-1} NaCl, close to the concentration of the LS condition in this study (90 g L^{-1}) (Table 1). We may conclude that this specific strain of *D. viridis*, isolated from a pond where NaCl was above 200 g L^{-1}, has a wide range of salt tolerance and has a similar response at very high salt concentrations. This trait may result in an advantage if considering that it may be cultivated also in brines coming from wastes from industry or natural sources.

4.4.3 Total lipids quantification

After the growth, microalgal biomass was harvested and freeze-dried and the biomass was then employed to perform total lipids quantification. The lipid content of the analysed algal strain is shown in Figure 3.

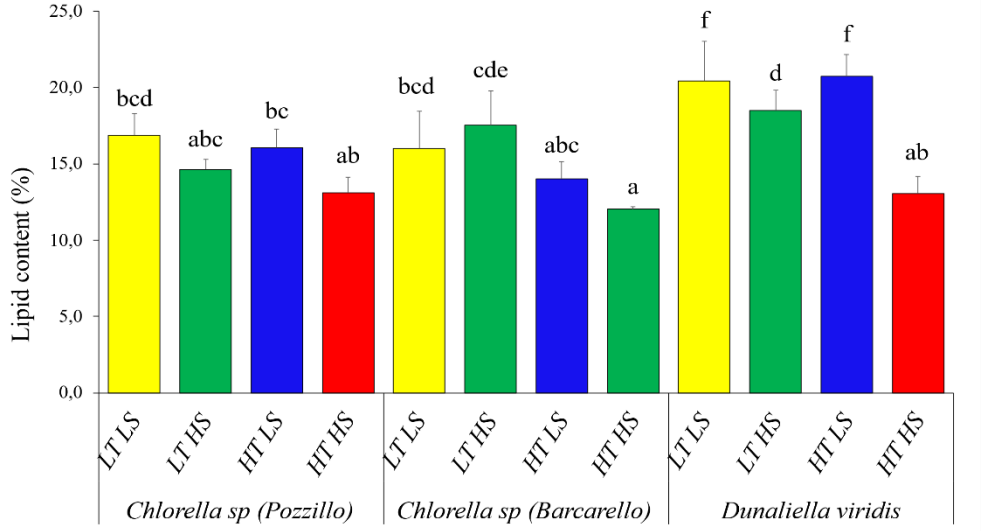


Figure 3. Total lipid content on dry weight (DW) in the strains *Chlorella* sp. *Pozzillo* in *Chlorella* sp. *Barcarello* and *Dunaliella viridis*. Values are reported as means (n=3) and error bars report the standard deviations. Different letters indicate significant differences among various treatment in each species at P<0.05

Chlorella strains showed a lipid content between 12.03±0.15% (*Chlorella* sp. *Pozzillo* HT-HS) and 17.53±2.24% (*Chlorella* sp. *Barcarello* LT-HS), while *D. viridis*, lipid content ranged between 13.51±1.14% (HT-HS) and 20.74±1.42% (HT-LS) accordingly to the literature [46,47].

D. viridis grown in low salinity conditions, both at low and high temperature, showed the highest lipid content (20.41±2.63% and 20.74±1.42%, respectively), while the lowest lipid content was observed when both high temperature and high salinity (HT-HS) are present.

In *Chlorella* strains, at low salinity conditions (LS, 0.35 M) (Table 1), the lipid content was 16.86±1.42% (LT-LS) and 16.06±1.24% (HT-LS) in *Chlorella* sp. *Pozzillo*, and 15.98±2.48% (LT-LS) and 14.00±1.14% (HT-LS) in *Chlorella* sp. *Barcarello*. The values observed are comparable to the values obtained by Rismani and Shariati [48] in *Chlorella vulgaris* at the salinity of 0.2 M. At high salinity conditions (HS, 0.7 M) *Chlorella* sp. *Pozzillo* always showed a decrease in lipid content compared to the relevant low salinity condition. Similar results were obtained by Campennì et al. [49], who found that the lipid content in *Chlorella protothecoides* tends to decrease when increasing the salinity above 0.35 M. A different result

was found in *Chlorella* sp. *Barcarello,* for which, at LT-HS conditions, a slight increase in lipid content was observed. Similar results were obtained by other authors [50,51] who observed an increase in lipid content in *Chlorella vulgaris* and *Chlorella sorokiniana* respectively. Finally, at HT-HS conditions, *Chlorella* sp. *Barcarello* showed a decrease in the lipid content as observed in all the other species.

As regards temperature, in the present work the stress caused by the high temperature tended to decrease the lipid content in all the *Chlorella* strains; this result is confirmed by several studies showing that the cultivation of *Chlorella vulgaris* at a temperature of 25° C led to a lipid content larger than those cultivated at 30° C. [46,52,53]. A slightly different behaviour was observed in *D. viridis*, where, at LS conditions, similar lipid contents were obtained at low and high temperature. Finally, similarly to *Chlorella* strains, when the two stressing conditions are simultaneously present (HT-HS), the lowest value in lipid content is observed. This result is in line with the ones detected by Rismani and Shariati [48] on the same species in high salinity conditions (NaCl 3 M).

In this work, all species showed a tendency to reduce lipid content as stress increased and salinity seemed to have the most discriminating effect.

As for industrial applications, both growth performances and lipid production need to be considered. If we look at the obtained results, the best parameters were observed in the strains *Chlorella* sp Barcarello HT-LS (18.47±0.23 mg L^{-1} d^{-1}) and in *Chlorella* sp Pozzillo LT-LS (11.97±0.09 mg L^{-1} d^{-1}) (Table S1 Supplementary Material). Also in this case, the lipid productivity may be compared with the one obtained from outdoor photobioreactors [35–37].

4.4.4 Analysis of fatty acids

Fatty acids were analysed starting from total lipid extract. Results are shown in Table S2 of Supplementary Material and Figure 4. In *Chlorella* strains, saturated fatty acids (SFAs) ranged from 23.57±1.73% total fatty acids (TFAs) (*Chlorella* sp. *Barcarello* LT-LS) to 29.98±1.31% TFAs (*Chlorella* sp. Barcarello LT-LS) while in *D. viridis* they ranged from 17.07±1.42% TFAs (LT-LS) to 41.56±0.16% TFAs (HT-HS) (Table S2 of Supplementary Material and Figure 4). Monounsaturated fatty acids (MUFAs) in *Chlorella* strains varied from 2.57±1.14% TFAs (*Chlorella* sp. Barcarello HT LS) to 5.97±0.12% TFAs (*Chlorella* sp. *Barcarello* HT-HS) and in *D. viridis* from 5.85±0.05% TFAs (LT-LS) to 9.13±0.07% TFAs (LT HS) (Table S2 of Supplementary Material and Figure 4). Lastly, polyunsaturated fatty acids (PUFAs) in *Chlorella* strains ranged from 65.78±0.57% TFAs (*Chlorella* sp. Pozzillo HT-HS) to 73.12±1.92% TFAs (*Chlorella* sp. Barcarello LT-LS), while in *D. viridis* from

46.77±0.14% TFAs (HT-HS) to 64.91±0.22% TFAs (HT-LS) (Table S2 of Supplementary Material and Figure 4). These results are in line with other works in literature [48,54,55].

Microalgae are a source of *n*-3 fatty acids. In recent years, many studies have been undertaken to evaluate the effect of various treatments on the quantity and quality of lipids obtained from microalgae, in particular, the omega-3 fatty acids including alpha-linolenic acid (ALA) (C18:3 *n*-3), eicosapentaenoic acid (EPA) (C20:5 *n*-3), docosahexaenoic acid (DHA) (C22:6 *n*-3) [56]. This interest is justified by their industrial importance caused by the significant health benefits they provide [57] and consequently their high market value [58].

In general, it can be observed that *Chlorella* strains (both Pozzillo and Barcarello) showed a higher PUFA *n*-6 content and the predominant fatty acid is linoleic acid, 18:2 *n*-6; while in *D. viridis* the PUFA *n*-3 were the most representative PUFA class, with the highest concentration of 18:3*n*-3 (Table S2 of Supplementary Material).

As demonstrated by Pandit et al., the fatty acid profile of *C. vulgaris* varies with saline stress [59]. Our results confirmed their findings: in particular, we observed that the combination of HS and HT factors caused a significant decrease of the PUFA class in all the analysed strains: from 72.75±0.28 to 65.78±0.58 *in Chlorella* sp. *Pozzillo*, from 73.12±1.92 to 68.94±0.23 in *Chlorella* sp. *Barcarello* and from 63.08±3.50 to 46.77±0.14 in *D. viridis*. In particular, in *D. viridis* the decrease of PUFA is linked to an increase of SFA, which reached 41.54±0.71% (HT-HS) with an increase of 16:0 (36.51±0.21). Also, a reduction in the ratio *n*-3/*n*-6 is observed for PUFA, as it significantly 18:3n3 decreases (9.14±0.24) and 18:2*n*-6 increases (25.74±0.28). This condition demonstrated how the synergistic effect of the two factors (HT-HS) resulted in a significant shift in fatty acid composition.

It is important to underline that the content of *n*-3 PUFA shows the same trend of the total lipids in *D. viridis* (Figure 4). On the other hand, the strains of *Chlorella* showed in both the analysed strains a different behaviour as *n*-3 PUFA increased when the cells were cultured in high salt condition (LT-HS) (Figure 4 and Table S2 of Supplementary Material). Furthermore, the production of some classes of significant fatty acids (EPA and DHA) were triggered in temperature stress conditions in *Chlorella* sp. *Pozzillo* and *D. viridis* (Table S2 of Supplementary Material), although in both *Chlorella* strains total *n*-3 PUFA class decreased in HT conditions.

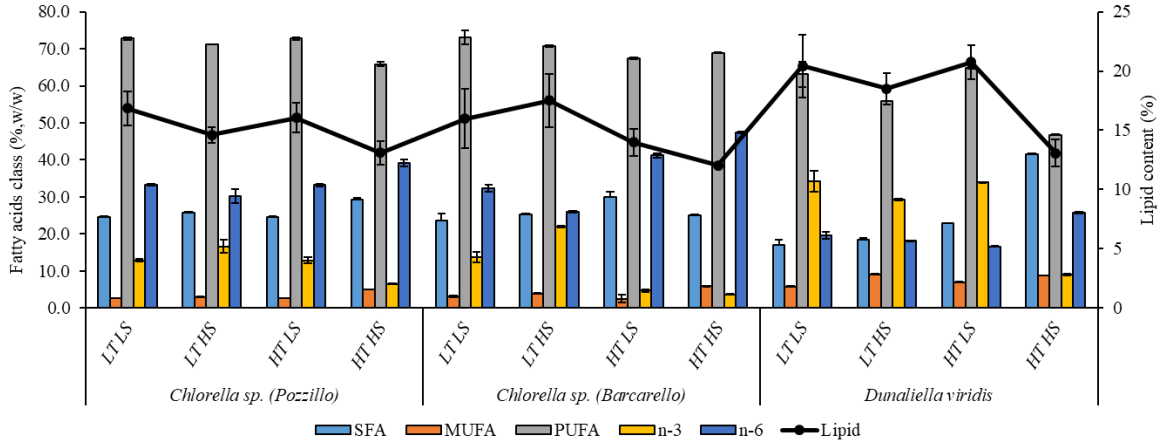


Figure 4. Fatty acids class composition (%, w/w) and total lipids *of Chlorella* sp. *Pozzillo*, *Chlorella* sp. *Barcarello* and *Dunaliella viridis* grown at Low Temperature-Low Salt (LT-LS), Low-Temperature-High Salt (LT-HS), High-Temperature-Low Salt (HT-LS) and High Temperature-High Salt (LT-HS). Data are reported as means (n=3) with standard deviation.

4.4.5 Total phenolic content and DPPH radical scavenging activity

The total phenolic content and anti-oxidative activity of microalgae, the latter evaluated through the DPPH assay, are shown in Figure 5. The total phenolic content (Figure 5a) varied significantly amongst the microalgae species examined ($P<0.05$), with *D. viridis* HT LS accumulating the highest concentration (6.32±0.04 mg GAE g^{-1} DW) and *Chlorella* sp. *Pozzillo* LT HS and *D. viridis* HT HS the lowest (2.96±0.05 and 1.62±0.04 mg GAE g^{-1} DW, respectively).

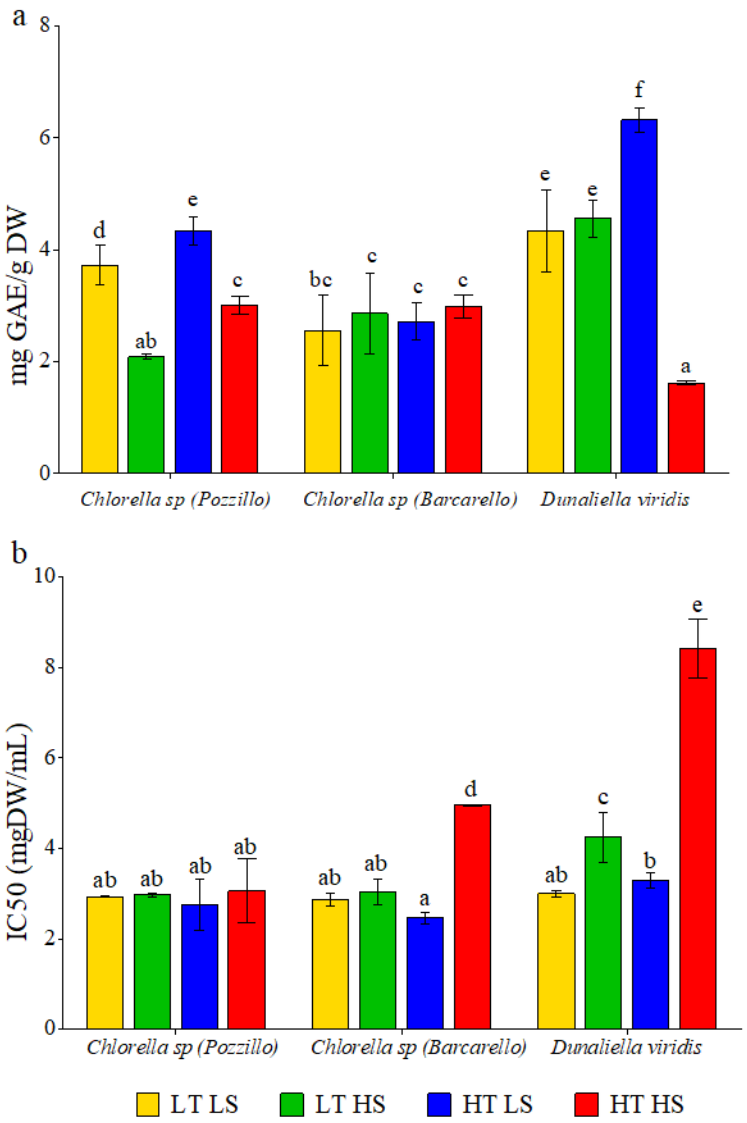


Figure 5. Total polyphenol content (mg GAE g^{-1} DW) (a) and DPPH radical scavenging activity (IC 50, mg DW mL^{-1}) (b) in the strains *Chlorella* sp. *Pozzillo* in *Chlorella* sp. *Barcarello* and in *Dunaliella viridis*. Values are reported as means (n=3) and error bars report the standard deviations. Different letters indicate significant differences among various treatment in each species at $P<0.05$

As regards the anti-oxidative activity of microalgae, it is expressed through the IC_{50} parameter and results are shown in Figure 5b. The anti-oxidative activity is expressed as the quantity of antioxidant (biomass) needed to have the inhibition of 50% of radical DPPH activity, consequently, low values of IC_{50} correspond to a higher antioxidative activity of microalgal biomass. In general, in the strains of *Chlorella* values of IC_{50} ranged between 2.46±0.01 and 4.96±0.01 mg DW mL^{-1} (*Chlorella* sp. *Barcarello* HT-LS and HT-HS, respectively), while in *D. viridis* between 3.00±0.07 and 8.41±0.65 mg DW mL^{-1}.

The different concentration of polyphenols is an intrinsic peculiarity of each species, in fact Maadane et al. [60] showed that different species of microalgae have different polyphenolic

content and that the genus *Dunaliella* presents a higher polyphenolic content than *Chlorella* sp. [60], confirming the result of this work.

Studies on the simultaneous effect of temperature and salinity have barely been performed [61] and the results on the polyphenol content further highlight the combined effect of the two factors salinity and temperature (HT-HS). This condition caused in *D. viridis* a significant decrease in polyphenols and also the lowest antioxidant activity (Figure 5b), with the values of IC 50 at 8.41±0.65 mg DW mL^{-1}.

Studies conducted by Singh et al. [62] on the congeneric *Dunaliella salina*, have shown an increase in antioxidant activity under different stress conditions (increased temperature or salinity) [62]. Our data showed that the increase in temperature under conditions of low salinity maintains a high antioxidant activity (IC 50 3.29±0.16 mg DW mL^{-1}) and that the combination of the two factors is the trigger that significantly reduces antioxidant activity.

This synergistic effect is also observed in *Chlorella* sp. *Barcarello* where the combination of HT and HS led to a lower antioxidant activity (IC 50 4.96±0.01 mg DW mL^{-1}), while HT LS showed the best antioxidant activity (IC 50 2.46±0.13 mg DW mL^{-1}).

4.4.6 Beta carotene and chlorophyll analysis

Figure 6 shows the total content of chlorophyll *a* and *β*-carotene.

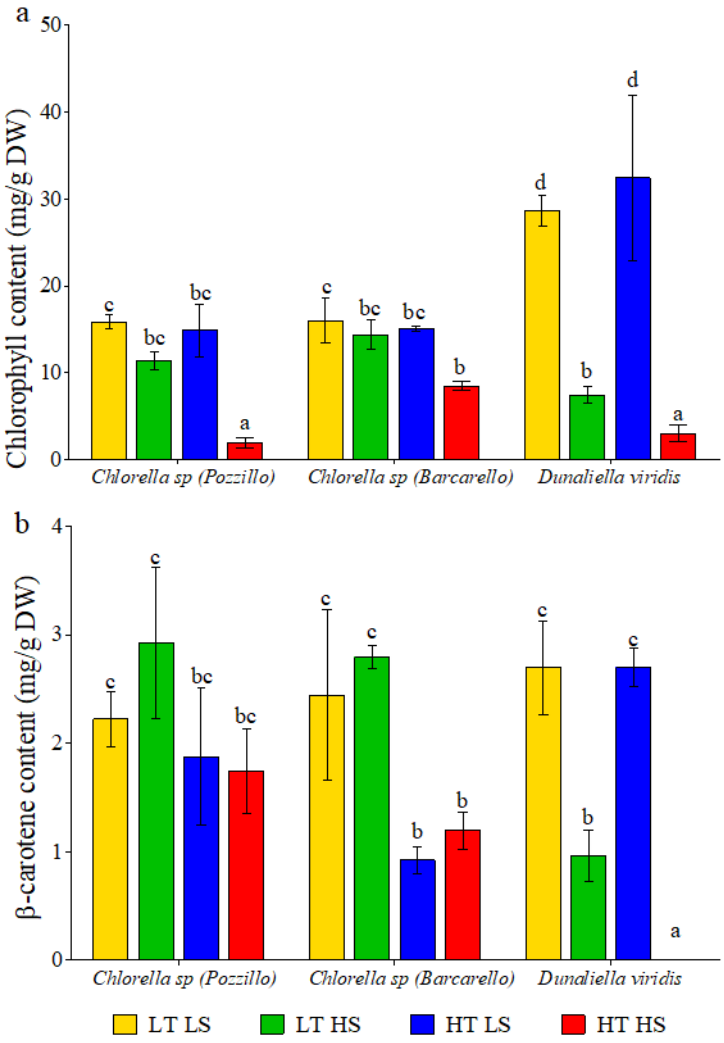


Figure 6. Chlorophyll *a* (mg g^{-1} DW) (a) and β-carotene (mg g^{-1} DW) (b) in the strains *Chlorella* sp. *Pozzillo* in *Chlorella* sp. *Barcarello* and in *Dunaliella viridis*. Values are reported as means (n=3) and error bars report the standard deviations. Different letters indicate significant differences among various treatment in each species at P<0.05

In general, chlorophyll *a* in *Chlorella* strains ranged between 1.88±0.63 and 16.01±2.55 mg g^{-1} DW respectively in *Chlorella* sp. *Pozzillo* HT-HS and *Chlorella* sp. *Barcarello* LT-LS, while in *D. viridis* from 3.00± 0.94 and 32.45± 9.54 mg g^{-1} DW in HT-HS and HT-LS conditions (Figure 6a). These values are in line with other works in literature regardless of temperature variations; for example, Ani Azaman et al. [63] have obtained values of 17.93 (mg g^{-1} DW) and 15.69 (mg g^{-1} DW) of chlorophyll *a* in *C. sorokiniana* and *C. zofingiensis* respectively [63]. A significant decrease is observed in *Chlorella* sp. *Pozzillo* HT-HS and in *Chlorella* sp. *Barcarello* HT-HS (Figure 6a).

In *D. viridis* a significant increase in chlorophyll *a* content was observed with temperature stress (Figure 6a), as also observed by Srirangan et al. [43] which observed that in *D. viridis*

the chlorophyll content was positively influenced by the increase in temperature from 25 to 35°C [43].

As demonstrated for both lipids (Figure 3) and antioxidant activity (Figure 5), the increase of salinity in *D. viridis* induced a significant decrease in chlorophyll *a* production; a similar result was observed by Jiménez and Niell [64] who detected that the content of chlorophyll *a* and *b* significantly decreased with increasing salinity [64].

As reported in Figure 6b, the β-carotene content in *Chlorella* strains varied between 0.93±0.12 and 2.79±0.11 mg g^{-1} DW in *Chlorella* sp. *Barcarello* HT-LS and LT-HS, respectively, while in *D. viridis* it ranged between 0.00, 2.70±0.43 and 2.70±0.17 mg g^{-1} DW in HT-HS, LT-LS and HT-LS respectively. The values of *β*-carotene determined are in line with values obtained by other authors [44].

As shown in Figure 6 b, *β*-carotene content in *Chlorella* strains was negatively affected by the temperature increase. The *β*-carotene content increased from 2.22±0.26 (*Chlorella* sp. *Pozzillo* LT-LS) to 1.88±0.63 mg g^{-1} DW (*Chlorella* sp. *Pozzillo* HT-LS) and from 2.44±0.79 (*Chlorella* sp. *Barcarello* LT-LS) to 0.93±0.12 mg g^{-1} DW (*Chlorella* sp. *Barcarello* HT-LS). The production of β-carotene seems not to have been influenced by the salinity factor for both *Chlorella* strains, as also observed by Ko et al. [61].

On the contrary, in *D. viridis* salinity caused a significant reduction in β-carotene content. While there are no significant differences when temperature varied, high salinity led to a drastic decrease in *β*-carotene content, which combined with high temperatures, leads to an inability of the microalgae to produce *β*-carotene (Figure 6b).

We can further state that the combination of temperature and salinity stresses (HT-HS condition) resulted in a drastic reduction of chlorophyll *a* and *β*-carotene content in all the microalgal strains, as showed also by Ko et al. [61]. In all the stress conditions a correlation between antioxidant activity and *β*-carotene production was observed as demonstrated by Singh et al. [62] in *D. salina*; these results suggest that under stress conditions the antioxidant effect may be due to an increase in *β*-carotene production [62]. There is no correlation between the antioxidant capacity and phenolic content, indicating that these compounds may not contribute significantly to the antioxidant activity of these microalgae [60].

Considering their properties, carotenoids have various industrial applications in food, nutraceutical and pharmacological industries [65]. Therefore, possible use of these microalgal strains in the industrial sector should consider that the highest productivity of chlorophyll *a* was obtained in *Chlorella* sp. *Barcarello* HT-LS (Table S1 supplementary materials). As

regards *β*-carotene the optimum productivity was obtained in *Chlorella* sp. *Pozzillo* LT-LS and LT-HS (0.16±0.03 and 0.47± 0.03 mg L^{-1} d^{-1}) (Table S1 supplementary materials).

4.4.7 nMDS and Pearson's correlation

The nMDS and Pearson's correlation studies (Figure 7) revealed that the assembling of the three microalgae strains is affected both by every single abiotic variable (salinity and temperature) and by the combination of the two abiotic variables. When *D. viridis* is subjected to high temperatures (HT) and high salinity (HS), it shows a different behaviour compared to the same species not subjected to the synergistic effect of the two stresses (LT-LS, LT-HS and HT-LS).

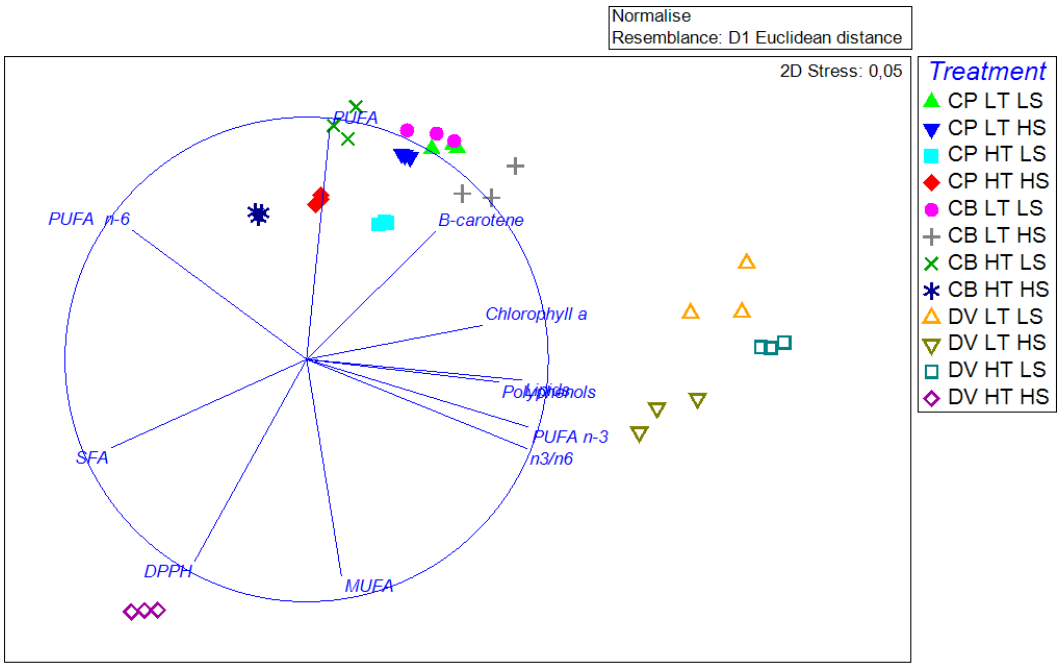


Figure 7. nMDS and Pearson's correlation used to evaluate patterns in polyphenols, DPPH, lipid content, SFA, MUFA, PUFA, PUFA *n*-3, PUFA *n*-6, *n*-3/*n*-6 chlorophyll *a* and *β*-carotene, in relation to salinity and temperature variables in the strains *Chlorella* sp. *Pozzillo* (CP) in *Chlorella* sp. *Barcarello* (CB) and in *Dunaliella viridis* (DV). LT: low temperature, LS: low salinity, HT: high temperature, HS: high salinity.

D. viridis and both *Chlorella* sp. strains are clearly separated from each other while *Chlorella* sp. *Pozzillo* and *Barcarello* show a similar trend between themselves. This proves that the three strains are not only influenced by the intrinsic characteristics of the species but even by the abiotic stressors.

4.5 Conclusions

In this work, two strains of *Chlorella* and one of *Dunaliella* were cultivated on a lab scale in four different conditions: Low Temperature-Low Salt (LT-LS), High-Temperature-Low Salt (HT-LS), Low Temperature-High Salt (LT-HS) and High Temperature-High Salt (HT-HS). Kinetic parameters were estimated together with biochemical parameters.

Results indicated that high salinity decreased the productivity of both *Chlorella* strains and *D. viridis*. The increase of temperature enhanced the productivity in *Chlorella* sp. *Barcarello*, while the combination of HT and HS worsened the growth performance in all strains.

The increase of temperature and salinity decreased the lipid content in all the strains and caused a significant shift in fatty acid composition, leading, in *Chlorella* strains cultivated in LT-HS condition, to an interesting increase in the production of *n*-3 PUFAs. Results indicated that also the carotenoids composition is influenced by the stress environmental conditions in which microalgae are cultivated, with a decrease in chlorophyll *a* and *β*-carotene in many of the stressed conditions.

Although microalgae resulted able to tolerate high temperatures and high salinity, microalgal growth was significantly affected by those environmental stressors. For this reason, the kind of application is a major factor to consider when choosing the growth parameter from an industrial perspective. For example, in Sicily, the diurnal temperature during summer may reach 40°C and in an outdoor facility, one of the main costs is the cooling system, not necessary when employing heat-tolerant strains with the appropriate application. To this respect, the microalgae *Chlorella* sp. *Barcarello* resulted in markedly suitable for being cultivated at high temperature. It is worth noting the relevant shift in lipid content and its composition, together with the changes in carotenoid's composition, that may be interpreted as an adaption to the stress condition in which the cells grew.

The data obtained from this preliminary research lay the foundation for the development of a regional-based algal industry that exploits a local resource, the microalgal strains, to produce biomass and other high-value compounds.

4.6 References

[1] N.F.P. Silva, A.L. Gonçalves, F.C. Moreira, T.F.C.V. Silva, F.G. Martins, M.C.M. Alvim-Ferraz, R.A.R. Boaventura, V.J.P. Vilar, J.C.M. Pires, Towards sustainable microalgal biomass production by phycoremediation of a synthetic wastewater: A kinetic study, Algal Res. 11 (2015) 350–358. https://doi.org/10.1016/j.algal.2015.07.014.

[2] M.B. Ariede, T.M. Candido, A.L.M. Jacome, M.V.R. Velasco, J.C.M. de Carvalho, A.R. Baby, Cosmetic attributes of algae - A review, Algal Res. 25 (2017) 483–487. https://doi.org/10.1016/j.algal.2017.05.019.

[3] T. Lafarga, Effect of microalgal biomass incorporation into foods: Nutritional and sensorial attributes of the end products, Algal Res. 41 (2019). https://doi.org/10.1016/j.algal.2019.101566.

[4] M. Musa, G.A. Ayoko, A. Ward, C. Rösch, R.J. Brown, T.J. Rainey, Factors Affecting Microalgae Production for Biofuels and the Potentials of Chemometric Methods in Assessing and Optimizing Productivity, Cells. 8 (2019) 851. https://doi.org/10.3390/cells8080851.

[5] E.G. Nwoba, D.A. Parlevliet, D.W. Laird, K. Alameh, N.R. Moheimani, Light management technologies for increasing algal photobioreactor efficiency, Algal Res. 39 (2019) 101433. https://doi.org/10.1016/j.algal.2019.101433.

[6] S. Lima, V. Villanova, F. Grisafi, A. Brucato, F. Scargiali, Combined effect of nutrient and flashing light frequency for a biochemical composition shift in Nannochloropsis gaditana grown in a quasi-isoactinic reactor, Can. J. Chem. Eng. 98 (2020) 1944–1954. https://doi.org/10.1002/cjce.23776.

[7] A.C. Wilkie, S.J. Edmundson, J.G. Duncan, Indigenous algae for local bioresource production: Phycoprospecting, Energy Sustain. Dev. 15 (2011) 365–371. https://doi.org/10.1016/j.esd.2011.07.010.

[8] Indrayani, Haslianti, Asriyana, Isolation and screening of marine microalgae from kendari waters, southeast sulawesi, Indonesia suitable for outdoor mass cultivation in hypersaline media, AACL Bioflux. 11 (2018) 1445–1455.

[9] J. Umamaheswari, S. Shanthakumar, Phycoremediation of paddy-soaked wastewater

by indigenous microalgae in open and closed culture system, J. Environ. Manage. 243 (2019) 435–443. https://doi.org/10.1016/j.jenvman.2019.05.023.

[10] S. Van Den Hende, V. Beelen, G. Bore, N. Boon, H. Vervaeren, Up-scaling aquaculture wastewater treatment by microalgal bacterial flocs: From lab reactors to an outdoor raceway pond, Bioresour. Technol. 159 (2014) 342–354. https://doi.org/10.1016/j.biortech.2014.02.113.

[11] O. Komolafe, S.B. Velasquez Orta, I. Monje-Ramirez, I.Y. Noguez, A.P. Harvey, M.T. Orta Ledesma, Biodiesel production from indigenous microalgae grown in wastewater, Bioresour. Technol. 154 (2014) 297–304. https://doi.org/10.1016/j.biortech.2013.12.048.

[12] H. Pereira, L. Custódio, M. Rodrigues, C. de Sousa, M. Oliveira, L. Barreira, N. Neng, J. Nogueira, S. Alrokayan, F. Mouffouk, K. Abu-Salah, R. Ben-Hamadou, J. Varela, Biological activities and chemical composition of methanolic extracts of selected autochthonous microalgae strains from the Red Sea, Mar. Drugs. 13 (2015) 3531–3549. https://doi.org/10.3390/md13063531.

[13] C.M. Messina, G. Renda, V.A. Laudicella, R. Trepos, M. Fauchon, C. Hellio, A. Santulli, From ecology to biotechnology, study of the defense strategies of algae and halophytes (from trapani saltworks, NW sicily) with a focus on antioxidants and antimicrobial properties, Int. J. Mol. Sci. 20 (2019) 881. https://doi.org/10.3390/ijms20040881.

[14] S. Lima, V. Villanova, F. Grisafi, G. Caputo, A. Brucato, F. Scargiali, Autochthonous microalgae grown in municipal wastewaters as a tool for effectively removing nitrogen and phosphorous, J. Water Process Eng. 38 (2020) 101647. https://doi.org/10.1016/j.jwpe.2020.101647.

[15] V. Villanova, C. Galasso, F. Fiorini, S. Lima, M. Brönstrup, C. Sansone, C. Brunet, A. Brucato, F. Scargiali, Biological and chemical characterization of new isolated halophilic microorganisms from saltern ponds of Trapani, Sicily, Algal Res. 54 (2021) 102192. https://doi.org/10.1016/j.algal.2021.102192.

[16] S.J. Takouridis, D.E. Tribe, S.L. Gras, G.J.O. Martin, The selective breeding of the freshwater microalga Chlamydomonas reinhardtii for growth in salinity, Bioresour. Technol. 184 (2015) 18–22. https://doi.org/10.1016/j.biortech.2014.10.120.

[17] R.R.L. Guillard, Culture of phytoplankton for feeding marine invertebrates, in: W.L. Smith, M.H. Chanley (Eds.), Cult. Mar. Invertebr. Anim., Springer US, Boston, MA, 1975: pp. 29–60. https://doi.org/10.1007/978-1-4615-8714-9_3.

[18] R. Sathasivam, N. Juntawong, Modified medium for enhanced growth of Dunaliella strains, Int J Curr Sci. 5 (2013) 67–73. https://doi.org/10.13140/2.1.1362.4326.

[19] J. Folch, M. Lees, G.H.S. Stanley, A simple method for the isolation and purification of total lipids from animal tissues, J. Biol. Chem. 226 (1957) 497–509. https://doi.org/10.1007/s10858-011-9570-9.

[20] G. Lepage, C.C. Roy, Improved recovery of fatty acid through direct transesterification without prior extraction or purification, J. Lipid Res. 25 (1984) 1391–1396.

[21] C.M. Messina, G. Renda, L. La Barbera, A. Santulli, By-products of farmed European sea bass (Dicentrarchus labrax L.) as a potential source of n-3 PUFA, Biol. 68 (2013) 288–293. https://doi.org/10.2478/s11756-013-0148-8.

[22] V.L. Singleton, J.A. Rossi, Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents, Am. J. Enol. Vitic. 16 (1965) 144–158.

[23] D. Krishnaiah, R. Sarbatly, R. Nithyanandam, A review of the antioxidant potential of medicinal plant species, Food Bioprod. Process. 89 (2011) 217–233. https://doi.org/10.1016/j.fbp.2010.04.008.

[24] C.M. Messina, A. Troia, R. Arena, S. Manuguerra, T. Ioannou, E. Curcuraci, G. Renda, C. Hellio, A. Santulli, Species-specific antioxidant power and bioactive properties of the extracts obtained from wild mediterranean Calendula Spp. (Asteraceae), Appl. Sci. 9 (2019) 4627. https://doi.org/10.3390/app9214627.

[25] S. Manuguerra, L. Caccamo, M. Mancuso, R. Arena, A.C. Rappazzo, L. Genovese, A. Santulli, C.M. Messina, G. Maricchiolo, The antioxidant power of horseradish, *Armoracia rusticana* , underlies antimicrobial and antiradical effects, exerted *in vitro*, Nat. Prod. Res. 0 (2018) 1–4. https://doi.org/10.1080/14786419.2018.1517121.

[26] T. Oki, M. Masuda, s. Furuta, y. Nishiba, N. Terahara, I. Suda, Involvement of Anthocyanins and other Phenolic Compounds in Radical-Scavenging Activity of Purple-Fleshed Sweet Potato Cultivars, J. Food Sci. 67 (2002) 1752–1756. https://doi.org/10.1111/j.1365-2621.2002.tb08718.x.

[27] L.M. Schüler, T. Santos, H. Pereira, P. Duarte, N.G. Katkam, C. Florindo, P.S.C.

Schulze, L. Barreira, J.C.S. Varela, Improved production of lutein and β-carotene by thermal and light intensity upshifts in the marine microalga Tetraselmis sp. CTP4, Algal Res. 45 (2020) 101732. https://doi.org/10.1016/j.algal.2019.101732.

[28] I. Couso, M. Vila, J. Vigara, B.F. Cordero, M.Á. Vargas, H. Rodríguez, R. León, Synthesis of carotenoids and regulation of the carotenoid biosynthesis pathway in response to high light stress in the unicellular microalga Chlamydomonas reinhardtii, Eur. J. Phycol. 47 (2012) 223–232. https://doi.org/10.1080/09670262.2012.692816.

[29] K.R. Clarke, R.M. Warwick, Change in Marine Communities: An Approach to Statistical Analysis and Interpretation. 2nd Edition, Plymouth, 2001. doi: 1.

[30] M. Kula, M. Rys, K. Możdżeń, A. Skoczowski, Metabolic activity, the chemical composition of biomass and photosynthetic activity of *Chlorella vulgaris* under different light spectra in photobioreactors, Eng. Life Sci. 14 (2014) 57–67. https://doi.org/10.1002/elsc.201200184.

[31] D. Singh, M. Puri, S. Wilkens, A.S. Mathur, D.K. Tuli, C.J. Barrow, Characterization of a new zeaxanthin producing strain of Chlorella saccharophila isolated from New Zealand marine waters, Bioresour. Technol. 143 (2013) 308–314. https://doi.org/10.1016/j.biortech.2013.06.006.

[32] Y.-C. Lai, A.L. Karam, H.W. Sederoff, J.J. Ducoste, F.L. de los Reyes, Relating nitrogen concentration and light intensity to the growth and lipid accumulation of Dunaliella viridis in a photobioreactor, J. Appl. Phycol. 31 (2019) 3397–3409. https://doi.org/10.1007/s10811-019-01897-4.

[33] C.J. Hulatt, D.N. Thomas, Productivity, carbon dioxide uptake and net energy return of microalgal bubble column photobioreactors, Bioresour. Technol. 102 (2011) 5775–5787. https://doi.org/10.1016/j.biortech.2011.02.025.

[34] J.P. Díaz, C. Inostroza, F.G. Acién, Scale-up of a Fibonacci-Type Photobioreactor for the Production of Dunaliella salina, Appl. Biochem. Biotechnol. 193 (2021) 188–204. https://doi.org/10.1007/s12010-020-03410-x.

[35] I.A. Nascimento, S.S.I. Marques, I.T.D. Cabanelas, S.A. Pereira, J.I. Druzian, C.O. de Souza, D.V. Vich, G.C. de Carvalho, M.A. Nascimento, Screening Microalgae Strains for Biodiesel Production: Lipid Productivity and Estimation of Fuel Quality Based on Fatty Acids Profiles as Selective Criteria, Bioenergy Res. 6 (2013) 1–13.

https://doi.org/10.1007/s12155-012-9222-2.

[36] R.R. Dias, P. Lasta, R.G. Vendruscolo, R. Wagner, L.Q. Zepka, E. Jacob-Lopes, Mapping the performance of photobioreactors for microalgae cultivation. Part II: equatorial and tropical climate zone, J. Chem. Technol. Biotechnol. 96 (2021) 613–621. https://doi.org/10.1002/jctb.6574.

[37] S.F. Siqueira, M.M. Maroneze, R.R. Dias, R.G. Vendruscolo, R. Wagner, C.R. de Menezes, L.Q. Zepka, E. Jacob-Lopes, Mapping the performance of photobioreactors for microalgae cultivation: geographic position and local climate, J. Chem. Technol. Biotechnol. 95 (2020) 2411–2420. https://doi.org/10.1002/jctb.6423.

[38] B.L. Lu, L. Qi, M.X. Li, Effects of Temperature on the Growth and Product Accumulation of *Chlorella sp.*, Adv. Mater. Res. 712–715 (2013) 428–432. https://doi.org/10.4028/www.scientific.net/AMR.712-715.428.

[39] A.W. Mayo, Effects of temperature and pH on the kinetic growth of unialga Chlorella vulgaris cultures containing bacteria, Water Environ. Res. 69 (2013) 64–72.

[40] Q. Zhang, J. Zhan, Y. Hong, The effects of temperature on the growth, lipid accumulation and nutrient removal characteristics of Chlorella sp. HQ, Desalin. Water Treat. 57 (2016) 10403–10408. https://doi.org/10.1080/19443994.2015.1038112.

[41] V. Turon, E. Trably, E. Fouilland, J.-P. Steyer, Growth of Chlorella sorokiniana on a mixture of volatile fatty acids: The effects of light and temperature, Bioresour. Technol. 198 (2015) 852–860. https://doi.org/10.1016/j.biortech.2015.10.001.

[42] S. Pulina, A. Brutemark, S. Suikkanen, B.M. Padedda, L.M. Grubisic, C.T. Satta, T. Caddeo, P. Farina, N. Sechi, A. Lugliè, Effects of warming on a Mediterranean phytoplankton community, Web Ecol. 16 (2016) 89–92. https://doi.org/10.5194/we-16-89-2016.

[43] S. Srirangan, M.-L. Sauer, B. Howard, M. Dvora, J. Dums, P. Backman, H. Sederoff, Interaction of Temperature and Photoperiod Increases Growth and Oil Content in the Marine Microalgae Dunaliella viridis, PLoS One. 10 (2015) e0127562. https://doi.org/10.1371/journal.pone.0127562.

[44] A. Asem, M. Fathi, Investigating the impact of NaCl salinity on growth, β-carotene, and chlorophyll a in the content life of halophytes of algae Chlorella sp AACL BIOFLUX Investigating the impact of NaCl salinity on growth, β-carotene, and

chlorophyll a in the content life , 2013.

[45] S. Mixson Byrd, J.M. Burkholder, P. V. Zimba, Environmental stressors and lipid production by Dunaliella spp. I. Salinity, J. Exp. Mar. Bio. Ecol. 487 (2017) 18–32. https://doi.org/10.1016/j.jembe.2016.11.004.

[46] A. Converti, A.A. Casazza, E.Y. Ortiz, P. Perego, M. Del Borghi, Effect of temperature and nitrogen concentration on the growth and lipid content of Nannochloropsis oculata and Chlorella vulgaris for biodiesel production, Chem. Eng. Process. Process Intensif. 48 (2009) 1146–1151. https://doi.org/10.1016/j.cep.2009.03.006.

[47] F.J.L. Gordillo, M. Goutx, F.L. Figueroa, F.X. Niell, Effects of light intensity, CO2 and nitrogen supply on lipid class composition of Dunaliella viridis, J. Appl. Phycol. 10 (1998) 135–144. https://doi.org/10.1023/A:1008067022973.

[48] S. Rismani, M. Shariati, Changes of the total lipid and Omega-3 fatty acid contents in two microalgae Dunaliella Salina and Chlorella vulgaris under salt stress, Brazilian Arch. Biol. Technol. 60 (2017). https://doi.org/10.190/1678-4324-2017160555.

[49] L. Campenni', B.P. Nobre, C.A. Santos, A.C. Oliveira, M.R. Aires-Barros, A.M.F. Palavra, L. Gouveia, Carotenoid and lipid production by the autotrophic microalga Chlorella protothecoides under nutritional, salinity, and luminosity stress conditions, Appl. Microbiol. Biotechnol. 97 (2013) 1383–1393. https://doi.org/10.1007/s00253-012-4570-6.

[50] R. Kakarla, J.-W. Choi, J.-H. Yun, B.-H. Kim, J. Heo, S. Lee, D.-H. Cho, R. Ramanan, H.-S. Kim, Application of high-salinity stress for enhancing the lipid productivity of Chlorella sorokiniana HS1 in a two-phase process, J. Microbiol. 56 (2018) 56–64. https://doi.org/10.1007/s12275-018-7488-6.

[51] C.-J. Yun, K.-O. Hwang, S.-S. Han, H.-G. Ri, The effect of salinity stress on the biofuel production potential of freshwater microalgae Chlorella vulgaris YH703, Biomass and Bioenergy. 127 (2019) 105277. https://doi.org/10.1016/j.biombioe.2019.105277.

[52] S. Hiremath, P. Mathad, Impact of Salinity on the Physiological and Biochemical Traits of Chlorella vulgaris Beijerinck, 2010.

[53] A. Juneja, R.M. Ceballos, G.S. Murthy, Effects of environmental factors and nutrient availability on the biochemical composition of algae for biofuels production: A review,

Energies. 6 (2013) 4607–4638. https://doi.org/10.3390/en6094607.

[54] D.G. Kim, S.B. Hur, Growth and fatty acid composition of three heterotrophic Chlorella species, Algae. 28 (2013) 101–109. https://doi.org/10.4490/algae.2013.28.1.101.

[55] R.W. Evans, M. Kates, Lipid composition of halophilic species of Dunaliella from the dead sea, Arch. Microbiol. 140 (1984) 50–56. https://doi.org/10.1007/BF00409771.

[56] R. Vazhappilly, F. Chen, Eicosapentaenoic acid and docosahexaenoic acid production potential of microalgae and their heterotrophic growth, J. Am. Oil Chem. Soc. 75 (1998) 393–397.

[57] J.L. Breslow, n−3 Fatty acids and cardiovascular disease, Am. J. Clin. Nutr. 83 (2006) 1477S-1482S. https://doi.org/10.1093/ajcn/83.6.1477S.

[58] A. Ismail, Marine lipids overview: Markets, regulation, and the value chain, OCL - Ol. Corps Gras Lipides. 17 (2010) 205–208. https://doi.org/10.1684/ocl.2010.0321.

[59] P.R. Pandit, M.H. Fulekar, M.S.L. Karuna, Effect of salinity stress on growth, lipid productivity, fatty acid composition, and biodiesel properties in Acutodesmus obliquus and Chlorella vulgaris, Environ. Sci. Pollut. Res. 24 (2017) 13437–13451. https://doi.org/10.1007/s11356-017-8875-y.

[60] A. Maadane, N. Merghoub, T. Ainane, H. El Arroussi, R. Benhima, S. Amzazi, Y. Bakri, I. Wahby, Antioxidant activity of some Moroccan marine microalgae: Pufa profiles, carotenoids and phenolic content, J. Biotechnol. 215 (2015) 13–19. https://doi.org/10.1016/j.jbiotec.2015.06.400.

[61] K. Ko, C.-H. Lee, H.-N. Moon, Y.-J. Lee, J. Yang, K. Cho, D. Kim, I.-K. Yeo, Simultaneous effect of salinity and temperature on the neutral lipid and starch accumulation by oceanic microalgae Nannochloropsis granulata and Chlorella vulgaris, J. Korean Soc. Mar. Environ. Energy. 19 (2016) 236–245. https://doi.org/10.7846/JKOSMEE.2016.19.3.236.

[62] P. Singh, M. Baranwal, S.M. Reddy, Antioxidant and cytotoxic activity of carotenes produced by *Dunaliella salina* under stress, Pharm. Biol. 54 (2016) 2269–2275. https://doi.org/10.3109/13880209.2016.1153660.

[63] S.N.A. Azaman, N. Nagao, F.M. Yusoff, S.W. Tan, S.K. Yeap, A comparison of the morphological and biochemical characteristics of Chlorella sorokiniana and Chlorella

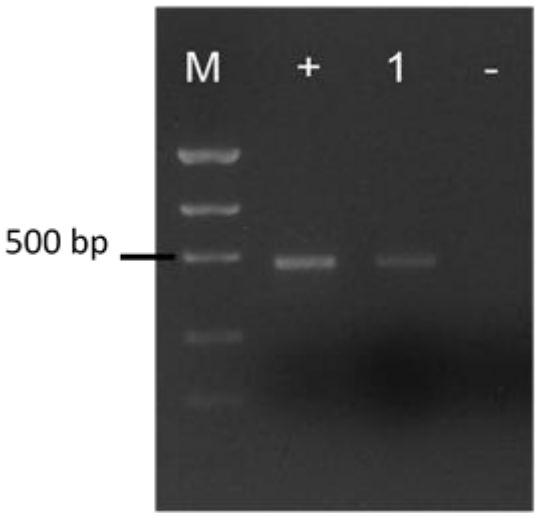
zofingiensis cultured under photoautotrophic and mixotrophic conditions, PeerJ. 5 (2017) e3473. https://doi.org/10.7717/peerj.3473.

[64] C. Jiménez, F.X. Niell, Influence of high salinity and nitrogen limitation on package effect and C/N ratio in Dunaliella viridis, Hydrobiologia. 492 (2003) 201–206. https://doi.org/10.1023/A:1024859819172.

[65] V. Henríquez, C. Escobar, J. Galarza, J. Gimpel, Carotenoids in Microalgae, in: Subcell. Biochem., Springer New York, 2016: pp. 219–237. https://doi.org/10.1007/978-3-319-39126-7_8.

4.7 Supplementary materials

Supplemental figure S1. Gel electrophoresis of PCR product on 1% agarose gel; M: molecular marker, +: positive control, 1: 18S rDNA product of *Chlorella* sp. *Barcarello*, - : negative control.



Supplemental table S1. Batch productivities of chlorophyll, beta-carotene and lipids in *Chlorella* sp. *Pozzillo*, *Chlorella* sp. *Barcarello* and *Dunaliella viridis* grown at Low Temperature-Low Salt (LT-LS), Low Temperature- High Salt (LT-HS), High-Temperature-Low Salt (HT-LS) and High Temperature-High Salt (HT-HS). Data are reported as as means (n=3) with s.d.

		Productivity (mg/L/d)		
Microalgae	Condition	Chlorophyll	Beta-carotene	Lipids
Chlorella sp *(Pozzillo)*	LT LS	1.13 ± 0.14	0.16 ± 0.03	11.97 ± 0.09
Chlorella sp *(Pozzillo)*	LT HS	0.54 ± 0.17	0.14 ± 0.03	6.88 ± 0.07
Chlorella sp *(Pozzillo)*	HT LS	0.92 ± 0.20	0.12 ± 0.04	9.93 ± 0.02
Chlorella sp *(Pozzillo)*	HT HS	0.10 ± 0.03	0.09 ± 0.02	6.97 ± 0.03
Chlorella sp *(Barcarello)*	LT LS	1.05 ± 0.17	0.16 ± 0.05	10.48 ± 0.01
Chlorella sp *(Barcarello)*	LT HS	0.55 ± 0.04	0.11 ± 0.01	6.76 ± 0.11
Chlorella sp *(Barcarello)*	HT LS	2.04 ± 0.34	0.12 ± 0.02	18.97 ± 0.23
Chlorella sp *(Barcarello)*	HT HS	0.32 ± 0.05	0.05 ± 0.01	4.58 ± 0.01
Dunaliella viridis	LT LS	1.01 ± 0.18	0.09 ± 0.01	7.19 ± 0.12
Dunaliella viridis	LT HS	0.25 ± 0.01	0.03 ± 0.01	6.37 ± 0.04
Dunaliella viridis	HT LS	0.99 ± 0.29	0.08 ± 0.01	6.35 ± 0.01
Dunaliella viridis	HT HS	0.03 ± 0.01	0.00 ± 0.00	1.45 ± 0.02

Supplemental table S2. Fatty acids Composition (%, w/w) of *Chlorella* sp (*Pozzillo*) CP, *Chlorella* sp (*Barcarello*) (CB) and *Dunaliella viridis* (DV) grown at Low Temperature-Low Salt (LT-LS), Low-Temperature-High Salt (LT-HS), High-Temperature-Low Salt (HT-LS) and High Temperature-High Salt (LT-HS). Data are reported as means (n=3) with standard deviation. Different letters indicate significant differences among various treatment in each species at $p<0.05$.

	Chlorella sp *Pozzillo*				*Chlorella* sp *Barcarello*				*Dunaliella viridis*			
	LT LS	LT HS	HT LS	HT HS	LT LS	LT HS	HT LS	HT HS	LT LS	LT HS	HT LS	HT HS
14:00	1.30±0.10^{de}	1.23±0.00^{cd}	1.91±0.02^{g}	1.23±0.13^{cd}	1.20±0.12^{cd}	1.07±0.01^{b}	1.36±0.02^{ef}	0.86±0.05^{a}	1.30±0.03^{de}	1.15±0.09^{bc}	1.17±0.05^{bcd}	1.44±0.01^{f}
15:00	0.40±0.00^{a}	0.30±0.10	0.33±0.00^{a}	0.26±0.02^{a}	0.31±0.19^{a}	0.32±0.02^{a}	0.31±0.12^{a}	0.28±0.05^{a}	0.41±0.05^{a}	0.40±0.00^{a}	0.43±0.06^{a}	0.56±0.02^{b}
16:00	18.89±0.56^{cd}	19.81±0.01^{d}	21.60±0.22^{e}	22.23±0.30^{e}	18.57±1.92^{cd}	19.17±0.21^{d}	24.15±1.86^{f}	17.31±0.06^{bc}	14.04±1.66^{a}	16.12±0.21^{b}	19.85±0.15^{d}	36.51±0.21^{g}
16:1*n*-7	1.11±0.04^{bc}	1.00±0.01^{bc}	1.94±0.02^{d}	2.33±0.12^{d}	1.30±0.05^{bc}	1.21±0.01^{c}	0.74±0.78^{b}	2.75±0.04^{e}	0.41±0.04^{a}	0.78±0.01^{b}	1.10±0.06^{bc}	2.63±0.01^{e}
16:2*n*-4	19.97±0.42^{g}	16.15±0.58^{d}	18.40±0.19^{f}	18.66±0.26^{f}	19.83±0.76^{g}	12.61±0.50^{c}	21.38±0.15^{h}	17.63±0.06^{e}	2.36±0.01^{a}	2.22±0.01^{a}	1.92±0.05^{a}	8.60±0.50^{b}
16:3*n*-4	6.65±0.24^{e}	8.15±0.62^{g}	0.17±0.00^{a}	1.42±0.03^{b}	7.20±0.23^{f}	10.26±0.11^{h}	0.18±0.09^{a}	0.19±0.05^{a}	6.99±0.28^{f}	6.20±0.29^{d}	12.44±0.07^{i}	3.29±0.11^{c}
18:00	0.95±0.08^{cd}	1.09±0.02^{e}	1.69±0.02^{h}	1.66±0.00^{h}	0.91±0.02^{c}	1.20±0.01^{f}	1.01±0.15^{d}	1.91±0.04^{i}	0.52±0.04^{a}	0.51±0.00^{a}	0.79±0.06^{b}	1.36±0.01^{g}
18:1*n*-9	0.77±0.03^{a}	1.25±0.08^{b}	3.65±0.04^{g}	2.25±0.07^{d}	0.75±0.19^{a}	1.47±0.02^{c}	1.47±0.35^{c}	2.46±0.04^{e}	3.73±0.23^{g}	6.31±0.05^{i}	3.48±0.03^{f}	4.29±0.02^{h}
18:1*n*-7	0.86±0.04^{c}	0.79±0.02^{c}	0.50±0.01^{b}	0.45±0.02^{b}	1.16±0.04^{d}	1.28±0.01^{e}	0.35±0.02^{a}	0.75±0.05^{c}	1.72±0.22^{f}	2.05±0.02^{h}	2.44±0.04^{i}	1.91±0.01^{g}
18:2n-6	33.20±0.21^{f}	30.29±1.83^{e}	41.10±0.42^{h}	39.19±1.00^{g}	32.31±0.99^{f}	25.92±0.28^{d}	41.19±0.59^{h}	47.40±0.25^{i}	19.58±0.93^{c}	18.19±0.14^{b}	16.68±0.11^{a}	25.74±0.28^{d}
18:3*n*-3	12.92±0.25^{c}	16.65±1.74^{d}	3.22±0.03^{a}	3.73±0.06^{a}	13.78±1.46^{c}	21.90±0.24^{e}	4.70±0.35^{a}	3.73±0.03^{a}	34.15±2.85^{g}	29.25±0.23^{f}	29.41±0.25^{f}	9.14±0.24^{b}
20:00	2.96±0.05^{e}	3.32±0.01^{f}	0.26±0.00^{a}	3.82±0.07^{h}	2.68±0.52^{d}	3.58±0.04^{g}	3.15±0.30^{ef}	4.74±0.02^{i}	0.81±0.21^{b}	0.37±0.00^{a}	0.66±0.06^{b}	1.70±0.04^{c}
20:5*n*-3			1.04±0.01^{b}								0.74±0.06^{a}	
22:5*n*-6			0.13±0.00^{a}	0.64±0.02^{b}							1.38±0.05^{c}	
22:6*n*-3			4.07±0.98^{b}	2.15±0.04^{a}							2.35±0.04^{a}	

Concluding remarks

One of the main goal of the European Blue Growth strategy, to whom this industrial PhD project is related, is the contribution to the development of the aquaculture sector also by marine biotechnology approach, to increase the economic and environmental sustainability of this sector and the consumer acceptability [1].

In particular, based on the proved environmental and economic benefit of the development of integrated multi-trophic aquaculture (IMTA) [2], the NUTRAQUA project was addressed to develop this sector at regional level through: the identification of co-cultivable extractive species within a sea bass and sea bream hatchery recirculating aquaculture system (RAS), the optimization of the productive performance of the co-cultivated species, the extraction of high commercial value nutraceuticals, and the development of monitoring systems.

The study involved five microalgal strains, *Nannochloropsis* sp., *Isochrysis galbana* and *Phaeodactylum tricornutum*, *Chlorella* sp. e *Dunaliella viridis*, cultured under different media conditions to induce nutritional, thermal, and salt stress; one macroalga species, *Gracilaria gracilis* and one halophyte plant, *Arthrocnemum macrostachyum,* cultivated in the hatchery pond. On these cultivated species lipid, phenolic compounds and carotenoids content were assessed by chemical analyses; the bioavailability of the extracted compounds at cellular level were assessed *in vitro* by testing the antioxidant power of the compounds against induced oxidative stress and the its bactericidal activity.

The assessment of lipid yields, total polyphenols and total carotenoids and the analysis of the antioxidant potential in microalgae confirmed that the production of lipids is species-specific, as well as related to the culture conditions to which the microalgae are subjected.

The study also focused on the molecular changes occurring on microalgae as a response to nutrient imbalances, common in aquaculture facilities, revealing that different culture conditions result in different modulations of target genes. Therefore, this study highlighted the validity of the molecular approach in monitor the metabolic status of microalgal cultures.

The bioactivity of the antioxidants obtained from microalgae, cultivated in IMTA, was tested on *Sparus aurata* fibroblast cell line (SAF-1) induced to oxidative stress with the chemical inducer BDE-47; the obtained results showed that the pre-treatment of cells with the antioxidants exerted a protective capacity at cellular level preventing toxicity, attesting the potential of application of the obtained bioactive compounds system, in both nutraceutical, cosmeceutical, and pharmaceutical fields.

In addition, the obtained microalgal extracts tested against fish pathogens showed a dose-dependently bactericidal activity and thus its potential as natural substitutes to antibiotics in the aquaculture sector.

Another experiment was conducted on the isolation and identification of local Sicilian microalgal strains for the development of the Sicilian outdoor aquaculture, taking into account the high temperatures reached in outdoor tanks during the summer season in Sicily. Through this study, ideal conditions for optimizing the production yields in lipids and antioxidants in native microalgal strains were investigated for their use in Sicilian regional outdoor aquaculture.

Finally, another study conducted on the polyphenol yields of the macroalga *G. gracilis* and on the halophyte *A. macrostachyum* grown in the aquaculture pond, revealed that these species, cultivated under the hatchery pond growing conditions, represent an important source of natural antioxidants. Furthermore, *in vitro* studies on human skin fibroblast cell line (Hs68) exposed to oxidative stress by UV irradiation, showed that the halophyte bioactive compounds, extracted by Supercritical Fluid Extraction (SFE), protect against oxidative stress, suggesting the utilization of these compounds in the nutraceutical, pharmaceutical and cosmeceutical sectors.

In conclusion, through this multidisciplinary study that included the use of different culture techniques for microalgae, one macroalgae and one halophyte, biochemical analysis, innovative extraction methods, molecular biology analysis and *in vitro* tests, it has been highlighted how the selection of co-cultivable species for the development on a regional scale of the IMTA industrial sector ensures high levels of productivity as the co-cultivated extractive species produce biomass rich in bioactive compounds that have antioxidant and antimicrobial properties that can find applications in the nutraceutical, cosmeceutical and pharmaceutical industries. Furthermore, the projects demonstrated to have meet the Blue Growth strategy goal as, thanks to the obtained results and their transfer to the enterprise, it could contribute to increase its economic and environmental sustainability and the consumer acceptability on aquaculture sector.

References

[1] Eikeset, A.M.; Mazzarella, A.B.; Davíðsdóttir, B.; Klinger, D.H.; Levin, S.A.; Rovenskaya, E.; Stenseth, N.C. What is blue growth? The semantics of “Sustainable Development” of marine environments. *Mar. Policy* 2018, *87*, 177–179.

[2] Knowler, D.; Chopin, T.; Martínez-Espiñeira, R.; Neori, A.; Nobre, A.; Noce, A.; Reid, G. The economics of Integrated Multi-Trophic Aquaculture: where are we now and where do we need to go? *Rev. Aquac.* 2020, *12*, 1579–1594.

Acknowledgments

Once I read that “a good mentor is a tutor for a project, while a great mentor is a guide for life”.
For always inviting me *to look out the window*, I would like to express my gratitude to my great mentor and guide for life, Dr Andrea Santulli.

Special thanks go to Professor Concetta Maria Messina for strongly believing in my potential and abilities and for pushing me to pursue new and formative experiences.

To Professor Claire Hellio, for her assistance, help and support at every stage of my PhD research project, I would like to extend my sincere thanks.

To the entire staff of the Laboratory of Marine Biochemistry and Ecotoxicology of Trapani I want to say thank you. Particularly to Laura La Barbera for leading my steps in the lab from the very beginning, to Rosaria Arena, Simona Manuguerra and Giuseppe Renda for teaching me, for encouraging me and, above all, for laughing with me on each day of this journey.

To Emanuela Voutsinas, passionate biologist and marine taxonomist for being, above all, a dear friend.

To my mom, my dad and my sister, for always showing me the way when I was getting lost.

To my nephew Samuele and my niece Clara, for inspiring me to ensure a more sustainable use of resources.

To the *living Soil*, for blending into the *Sea.*

Thanks to all of you!

La borsa di dottorato è stata cofinanziata con risorse del
Programma Operativo Nazionale Ricerca e Innovazione 2014-2020 (CCI 2014IT16M2OP005),
Fondo Sociale Europeo, Azione I.1 "Dottorati Innovativi con caratterizzazione Industriale"

UNIONE EUROPEA
Fondo Sociale Europeo

Ministero dell'Istruzione, dell'Università e della Ricerca

PON
RICERCA E INNOVAZIONE
2014 - 2020