Enrichment of Bioactive Compounds in Microalgae for Aquaculture

MASTER DISSERTATION

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MASTER IN APPLIED BIOCHEMISTRY

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Tese apresentada à Universidade da Madeira com vista à

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Abstract

Microalgae are promising microorganisms for the production of food and fine chemicals. Several species of microalgae are used in aquaculture with the purpose of transfer bioactive compounds up to the aquatic food chain. The main objective of this project was to develop a stress–inducement strategy in order to enhance the biochemical productivity of *Nannochloropsis gaditana*, *Rhodomonas marina* and *Isochrysis* sp. for aquaculture purposes having in account their growth and organizational differences. In this regard, two experiments were design: the first one consisted on the alteration of overall nutrient availabilities in growth medium; and the second one comprised changes in nitrogen and sulfur concentrations maintaining the concentrations of the other nutrients present in a commercial growth medium (Nutribloom plus), which is frequently used in aquaculture. Microalgae dried biomass was characterized biochemically and elemental analysis was also performed for all samples. In first experimental design: linear trends between nutrient availability in growth media and microalgae protein content were obtained; optimum productivities of eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) were attained for both *R. marina* and *N. gaditana* in growth media enriched with 1000 μL L^{-1} of nutrient solution whereas for *Isochrysis* sp. the double of Nutribloom plus was needed; the decrease of glucans and total monosaccharides with nutrient availability for *R. marina* and *Isochrysis* sp. showed the occurrence of a possible depletion of carbohydrates towards lipids and proteins biosynthesis. Second experimental desing: *N. gaditana* exhibited the highest variation in their biochemical composition against the applied perturbation; variations observed for microalgae in their biochemical composition were reflected in their elemental stoichiometry; in *N. gaditana* the highest nitrogen concentrations lead to overall maximum productivities of the biochemical parameters. The results of the present work show two stress-inducement strategies for microalgae that may constitute a base for further investigations on their biochemical enhancement.

Keywords: Microalgae, bioactive compounds, glucans, EPA, DHA, PUFA

Resumo

As microalgas são microrganismos promissores para a produção de alimentos e química fina. Diversas espécies de microalgas são utilizadas na aquacultura com o objectivo de transferir compostos bioactivos até ao topo da cadeia trófica aquática. O principal objectivo deste projecto consistiu no desenvolvimento de uma estratégia indução–stress de forma a aumentar a produtividade bioquímica da *Nannochloropsis gaditana*, *Rhodomonas marina* e *Isochrysis* sp. para aquacultura, tendo em consideração o crescimento e diferenças organizacionais. Neste contexto, duas experiências foram desenvolvidas: a primeira consistiu na alteração do teor total de nutrientes disponíveis no meio de crescimento; e a segunda compreendeu alterações nas concentrações de azoto e enxofre mantendo as concentrações dos restantes nutrientes presentes no meio de cultivo comercial (Nutribloom plus), frequentemente utilizado em aquacultura. A biomassa das microalgas foi caracterizada bioquimicamente e a análise elementar foi também efectuada para todas as amostras. Na primeira experiência: foram obtidas relações lineares entre a disponibilidade de nutrientes no meio de cultivo e o total de proteínas das microalgas; as produtividades máximas dos ácidos eicosapentaenoico (EPA) e docosahexaenoico (DHA) foram atingidas, para a *R. marina* e *N. gaditana*, nos meios de cultivo enriquecidos com 1000 $\mu L\ L^{-1}$ de solução nutritiva, enquanto que para a *Isochrysis* sp. foi necessário o dobro para tal; o decréscimo dos glucanos e do total de monosacáridos com a disponibilidade de nutrientes demonstrou a ocurrência de um possível consumo dos carbohidratos para a biosíntese de lípidos e proteínas. Na segunda experiência: *N. gaditana* demonstrou ter uma maior variação na sua composição bioquímica face ao stress aplicado; as variações observadas relativamente à composição bioquímica das microalgas reflectiram-se na sua estequiometria elementar; na *N. gaditana* concentrações superiores de azoto tiveram como consequência o aumento das produtividades para os parâmetros bioquímicos em geral. Por fim, os resultados do presente trabalho demonstram duas estratégias indução–stress para as microalgas que podem servir de base para investigações futuras visando o melhoramento da sua composição bioquímica.

Palavras–chave: Microalgas, compostos bioactivos, glucanos, EPA, DHA, PUFA

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List of Acronyms

a – Constant

AA – Arachidonic Acid

ALA – Alpha linolenic acid

ATP – Adenosine triphosphate

Chl – Chlorophyll

DHA – Docosahexaenoic acid

DW – Dry biomass weight

EPA – Eicosapentaenoic Acid

FA – Fatty acid

FAME – Fatty acid methyl esther

GLA – Gamma linolenic acid

HUFA – Highly unsaturated fatty acid

K – Carrying capacity

LA – Linolenic acid

LT – Leukotriene

MUFA – Monounsaturated fatty acid

N – Cell concentration

$NADPH_2$ – Nicotinamide–adenine dinucleotide phosphate

PG – Prostaglandin

P_x – Productivity being x the biochemical parameter

PUFA – Polyunsaturated fatty acid

PSI – Photosystem I

PSII – Photosystem II

r – Specific growth rate

SDA – Stearidonic acid

SFA – Saturated fatty acid

TL – Total lipid

TM – Total monosaccharide

TP – Total protein

TX – Tromboxanes

ω3 – Omega 3

ω6 – Omega 6

CHAPTER I. General introduction

1. Microalgae

1.1. Microalgae biodiversity and taxonomy

Algae are a highly diversified group of primarily oxygen–releasing photosynthetic organisms (1). In divergence to plants these organisms have a simple body plan in which roots, stems or leaves are absent (1, 2). Algae can be divided into two main categories: macroalgae (which have complex multicellular structures) and microalgae (that encompass unicellular or simple multicellular structures) (1, 3). The latest comprises prokaryotic (e.g. Cyanobacteria) and eukaryotic microorganisms which are present not only in aquatic environments but also in terrestrial environments (3). Microalgae represent a big variety of species across the planet and it's estimated to comprise more than 50,000 species despite of only 30,000 have been studied and analyzed (3).

Microalgae play a very important role in aquaculture as primary producers of bioactive compounds that can enhance fish and fish oils quality through up the food chain; since fish diet composition has a marked influence on their growth, development of the key organs, and on their tissues chemistry (4, 5). Thus several microalgae genus are used to fulfill fish dietary requirements, being *Nannochloropsis*, *Isochrysis*, *Scenedesmus*, *Dunaliella*, *Spirulina*, *Phaeodactylum*, *Pavlova*, *Tetraselmis*, *Skeletonema*, *Chlorella* and *Thalassiora*, the most commonly used (6).

Three microalgae species, *Nannochloropsis gaditana*, *Rhodomonas marina* and *Isochrysis* sp., often used in aquaculture will be subject of study in this work. The taxonomic description are shown below alongside with their morphological appearance (Fig. 1 a–c).

Nannochloropsis gaditana (7)	*Rhodomonas marina* (7)	*Isochrysis* sp.(7)
Empire: Eukaryota	**Empire:** Eukaryota	**Empire:** Eukaryota
Kingdom: Chromista	**Kingdom:** Chromista	**Kingdom:** Chromista
Division: Heterokontophyta (Ochrophyta)	**Division:** Cryptophyta	**Division:** Haptophyta
Class: Chrysophyceae	**Class:** Chryptophyceae	**Class:** Coccolithophyceae
	Order: Pyrenomonadales	**Order:** Isochrysidales

Order: Eustigmatales
Family: Monodopsidaceae
Genus: *Nannochloropsis*
Species: *Nannochloropsis gaditana*

Family: Pyrenomonadaceae
Genus: *Rhodomonas*
Species: *Rhodomonas marina*

Family: Isochrysidaceae
Genus: *Isochrysis*

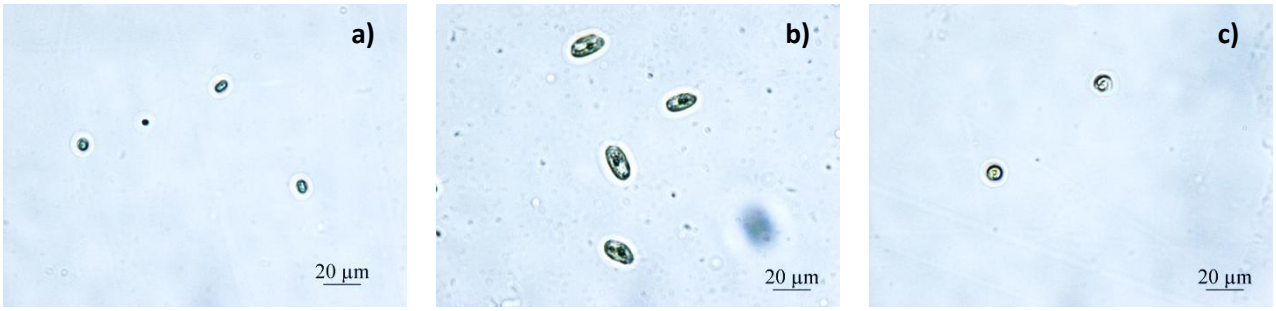

Figure 1. Photomicrograph of a) *N. gaditana*; b) *R. marina*; c) *Isochrysis* sp. at a total magnification of 600×.

1.2. Microalgae trophy – Photosynthetic processes

Microalgae are adapted to scavenge their environments for resources, assuming many types of trophy which can be summarized into two main classes: autotrophy (where microalgae utilize CO_2 as the carbon source) and heterotrophy (in which organic compounds, produced from other biomass sources, are used instead of CO_2) (3, 8, 9). Autotrophs include chemoautotrophs (source of energy obtained from inorganic compounds) and photoautotrophs (source of energy obtained from light) (9). Like plants, algae convert light into chemical energy through photosynthesis reactions (3). Some microalgae (e.g. *Ochromonas*) can assume both phototrophic and heterotrophic growth utilizing both photosynthesis and organic compounds from the environment to fulfill their nutritional needs (mixotrophic growth), namely in low light environments (1, 2).

The photosynthetic processes (Fig. 2) which convert the solar energy into biomass comprise several steps: *i)* Capture of light energy by a light harvesting antennae; *ii)* Conversion of solar energy into chemical energy (ATP and $NADPH_2$): Transfer of electrons removed from water molecules (oxidized at photosystem II – PSII) to a terminal electron acceptor $NADP^+$ - which is then reduced at photosystem I (PSI) to $NADPH_2$; Development of a pH gradient, from proton translocation, which drives the ATP synthesis catalyzed by the protein complex ATP synthase; *iii)* ATP and $NADPH_2$

produced feed the light–independent reactions of photosynthesis (Calvin cycle) that are responsible for CO_2 fixation into carbohydrates, catalyzed by ribulose-1,5-bisphosphate carboxylase oxygenase (RUBISCO) (10-12). The fixed carbon may be stored into reserve molecules which can be broken down in order to provide the energy (ATP) or carbon skeletons needed for cell reactions (12).

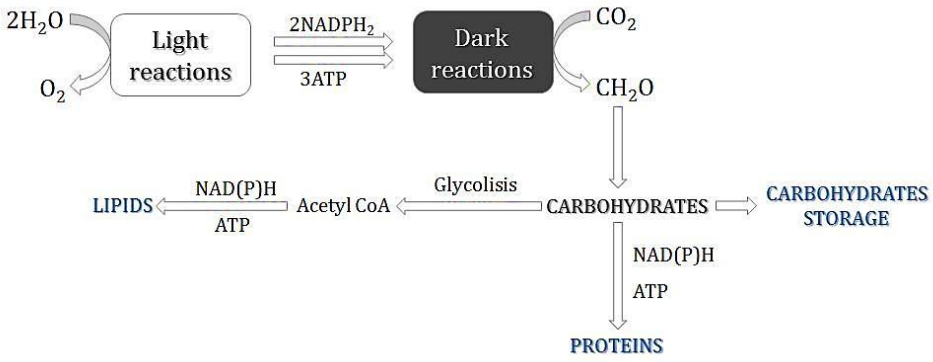

Figure 2. Schematic representation of photosynthetic processes that leads to carbon fixation and metabolites formation. Based on references (11, 13).

The configuration of core metabolic networks is highly varied across distinct algae classes (14). This diversity, alongside with organizational differences in the photosynthetic apparatus scheme might affect key processes such as photosynthesis, carbon allocation and accumulation of high value metabolites by microalgae (14). Although all photosynthetic microorganisms have organic pigments for light harvesting, the quantity of these molecules as well as the presence of accessory pigments in the pigment – protein antennae systems can vary with their phylogeny (2, 14).

The representation of the molecular structures of some pigments forming the light harvesting antennae complexes are displayed in Figure 3. Pigments can be divided into three major classes: Chlorophylls (Chl) which include Chl a, b, c and d; Carotenoids that comprise carotenes (hydrocarbons) and xantophylls (oxygenated hydrocarbons); and Phycobilins (11).

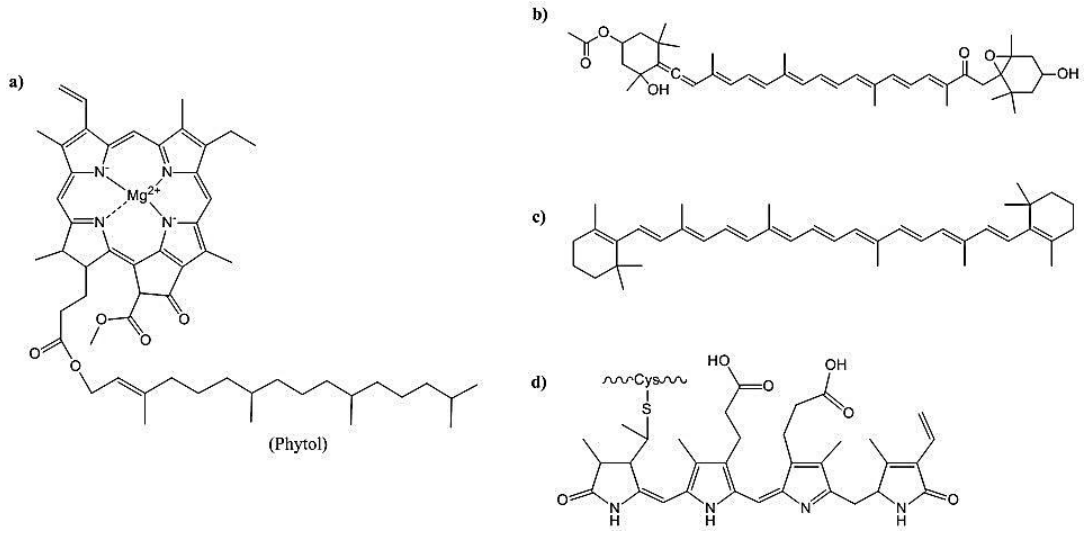

Figure 3. Photosynthetic pigments of light harvesting antennae complexes a) Chlorophyll a; b) Fucoxanthin; c) *β* – Carotene and d) Phycoerythrobilin.

Chlorophylls are molecules characterized for having a tetrapyrrole ring with a central magnesium ion and a linear terpene alcohol (except for Chl c); whereas phycobilins comprise only an open tetrapyrrole in their molecular structure, Fig. 3a and Fig. 3c respectively (11). It should be noted, that carotenoids (Fig. 3 b–c), phycobilins and Chl b, c and d function as accessory light-harvesting pigments, once they offset the range of light absorption not covered by Chl a present in all oxygenic photoautothrophs (11).

Organizational differences in microalgae had been shaped by endosymbiotic acquisitions and evolutionary selection (14). The main groups of algae, classified as divisions, are stablished having in account their characteristics, such as: microalgae ultrastructure, pigmentation, photosynthetic membranes organization and storage products (2). Table 1, shows the main pigments of the algal divisions here studied, where it's possible to visualize that only the Cryptophyta division has phycobilins and alloxanthin. Other differences in xantophylls and carotenes nature are present in Table1.

Table 1. Main pigments of Cryptophyta, Heterokontophyta and Haptophyta, adapted from reference (2).

			Carotenoids	
Division	**Chlorophylls**	**Phycobilins**	**Carotenes**	**Xanthophylls**
Cryptophyta	Chl a, Chl c	Phycoerythrin-545 *r*-Phycocyanin	*α*-, *β*-, *ε*-Carotene	Alloxanthin
Heterokontophyta	Chl a, Chl c	Absent	*α*-, *β*-, *ε*-Carotene	Fucoxanthin, Violaxanthin
Haptophyta	Chl a, Chl c	Absent	*α*-, *β*-Carotene	Fucoxanthin

The limitation of inorganic nutrients in algae growth media affect photosynthetic energy conversion once their presence is crucial for photosystems photochemistry (e.g. Mg^{2+} in chlorophylls) and fundamental enzymatic reactions that influence the downstream metabolic reactions (15).

1.2.1. Nutrient requirements – Medium enrichment

Algae biomass is built up by six major elements: carbon (C), oxygen (O), hydrogen (H), nitrogen (N), sulfur (S) and phosphorus (P) (16). These elements alongside with others crucial for cells viability and physiology are present in algae cells in a species specific structural ratio (Redfield ratio) which determines species nutrient requirements (16-18). The extended Redfield formula obtained by Ho (17) is presented in equation (1); this average stoichiometry may vary in response to changes on the microalgae growth environment (17). Moreover, this formula has been used as a starting point to quantify possible nutrient limitations in microalgae and to determine optimum nutrient ratios – in which the concentration of one nutrient is such that it won't affect the supply of another (19).

$$(C_{124}N_{16}P_1S_{1.3}K_{1.7}Mg_{0.56}Ca_{0.5})_{1000}Sr_5Fe_{7.5}Zn_{0.8}Cu_{0.38}Co_{0.19}Cd_{0.21}Mo_{0.03} \qquad \text{Eq. (1)}$$

Furthermore, the inorganic media components can be divided, according to the relative amounts required by cells, into two main classes: macronutrients (those that must be supplied in large amounts g L^{-1}) and micronutrients (those that must be supplied in small quantities mg L^{-1}) (19-21). Macronutrients comprise the elements previously referred as the building blocks elements of algae biomass plus calcium (Ca), potassium (K), sodium (Na), magnesium (Mg) and iron (Fe) (16). Whereas micronutrients comprise the trace metals, essential as catalytically active cofactors in enzymes, protein stabilizers and for their enzyme-activation functions (16, 21).

1.2.1.1. Nutrient uptake and assimilation

Nutrients are uptaken by microalgae through the plasma membrane which ensures the entry of essential nutrients and metabolites into the alga cell by transporting them through diffusion or by a variety of transmembrane transporters (e.g. channel proteins) (22, 23). As represented in Figure 4 nutrients can be acquired by microalgae in

several forms, being some more energy dispending than others (24). For instance, nitrogen can be acquired as nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+) and urea; albeit NH_4^+ assimilation has less energy costs for algae cells (22, 24). Moreover from the inorganic forms of nitrogen, NO_3^- is the most thermodynamically stable form in the aquatic environments liable to oxidation (25).

Fig. 4 highlights nitrogen and sulfur uptake, reduction and assimilation alongside with their key role in metabolic processes as essential components of proteins. It should be noted that nitrogen and sulfur can only be assimilated in the most reduced forms, NH_4^+ and sulfide (S^{2-}), respectively (22).

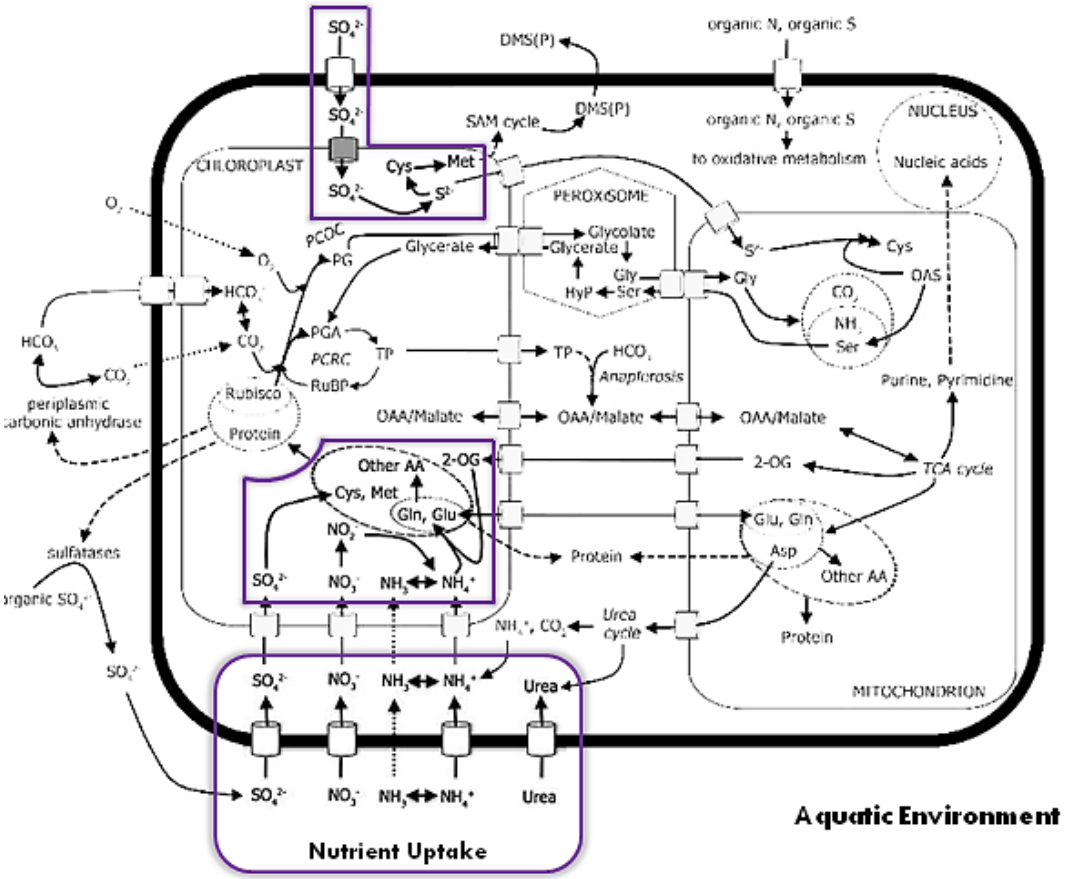

Figure 4. Nitrogen and sulfur uptake, reduction and assimilation parts that are highlighted. In the background are the metabolic pathways in which these elements and carbon are involved. Adapted from reference (22).

The uptake of the essential nutrients from the aquatic environment depends on several parameters such as: the bioavailable nutrient concentration (previously referred), and light regime and concentrations of other nutrients (17). Cells stoichiometry has a tendency to homeostasis across environments and taxa (Eq. 1). However, changes in the uptake rates of the chemical elements may lead to direct repercussions in cell stoichiometry and consequently, in the organic composition of cells, as well as in the

size of metabolic pools (26). Through the background of Fig. 4 is possible to visualize the interconnections and some of the metabolic pathways in which nitrogen, sulfur and carbon are involved.

1.3. Stress responses

The survival of microalgae in variable environments is dependent on their ability to readily adapt to and tolerate stress (27). Understanding the response mechanisms that led to a successful adaptation is important in order to explain not only the patterns of microalgae diversity, but also their flexibility under different environments (27).

Figure 5 shows a hypothetical performance curve for a biological system exposed to an increasing perturbation. Stress is a physiological response derived from an input that led to a deviation from normal operating conditions producing a positive (higher productivity and growth – subsidy effects) or negative output (28, 29).

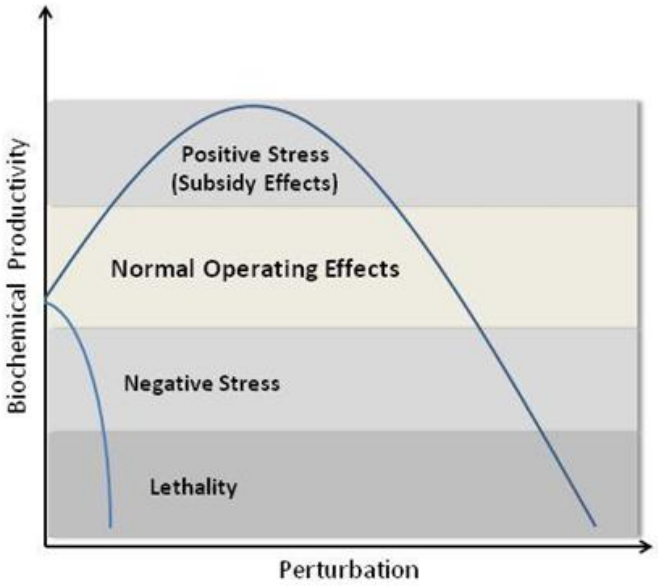

Figure 5. Hypothetical performance curve for biochemical productivity of a microalgae system upon an increasing perturbation. Adapted from references (28, 29).

Biological systems have a set of physiological processes that maintain organism *status quo* upon a perturbation, counteracting any tendency to imbalance (Homeostasis) (26). However, when this primarily cellular feature doesn't work several responses can be taken up by organisms in order to minimize deviations from normality (26). These responses can be categorized into three sets: *i)* regulation – involves changes in the functioning of pre–existing catalysts and as such is dependent on the pre–existing pool

of these molecules, occurs in seconds to minutes; *ii)* acclimation – involves quantitative and/or qualitative alterations of the expressed proteome, occurs in minutes to hours; *iii)* adaptation – responses mediated by change in genome occurs after several generations (26).

Figure 6 is a schematic representation of homeostasis and acclimation response that have been triggering much attention as a strategy to enhance microalgae composition for industrial and commercial purposes. Through this figure is possible to see that the perturbation in homeostasis didn't influence the metabolic pools maintaining their amounts and proportions in cells despite of an alteration in nutrient uptake rates had occur, moreover C/N stoichiometry remains constant (26). In acclimation a diversion from homeostasis occurs, in order to minimize the impact of environmental perturbation. In this case is possible to note alterations in proteins, carbohydrates and lipids pools along with C/N stoichiometry as perturbation starts (26). However, when perturbation ends cells metabolic pools and cell stoichiometry go back to their composition prior to perturbation occur (26).

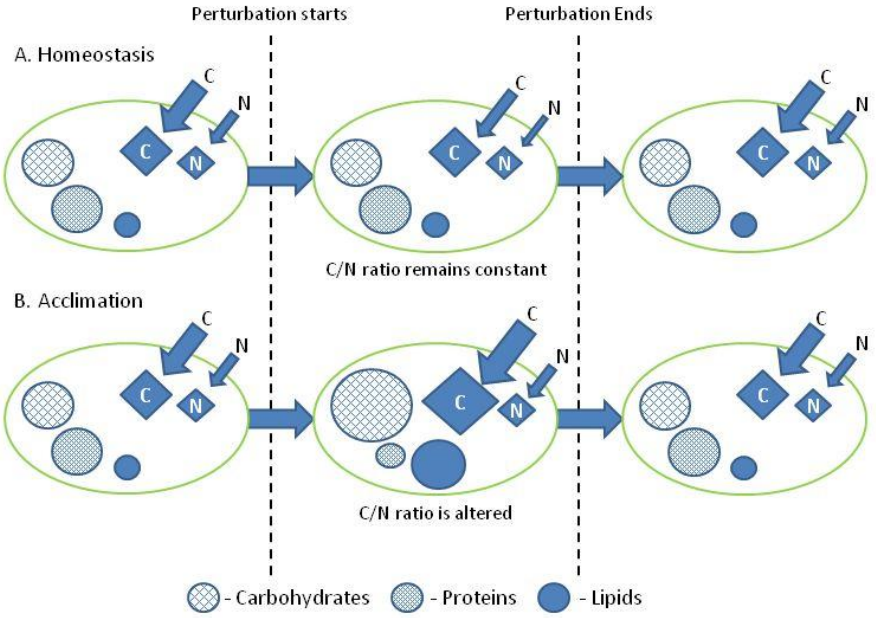

Figure 6. Schematic representation of homeostatic and acclimation responses to an environmental perturbation. Adapted from reference (26).

1.4. Microalgae applications

Microalgae have been attracting much attention from the industry and scientific communities for being promising biomachineries for the production of fine chemicals

(30). Thus, the use of microalgae as "green energy carriers" is of increasing interest since comprises a solution for a sustainable production of renewable energy (3, 30). The advantages of these microorganisms are due to their fast growth, efficient carbon dioxide fixation, not competing for arable lands and potable water and metabolic flexibility (easy acclimation and accumulation of high amounts of special metabolites) (31). Furthermore, the great potential of marine microalgae for several areas of application is related with their biomass and/or biochemical composition. Table 2 shows some products that can be synthesized by microalgae species and their applications.

Table 2. Applications and biological activity of compounds synthesized by microalgae, based on references (30-32).

Compound	Biological activity	Application
β-Carotene	Food additive; pro-vitamin A; antioxidant	Health food supplement; pharmaceuticals
Phycoerythrin		Immunofluorescence techniques
Proteins		Health food supplement
Vitamins	Antioxidant; blood cell formation; blood clotting mechanism	Immune system
Saturated and Monounsaturated fatty acids; Hydrocarbons		Biofuel production
Eicosapentaenoic acid (EPA)	Nutraceutical; antimicrobial; anti-inflammatory	Health food supplement; Therapeutics; Immune system
Arachidonic acid (AA)	Aggregative and vasoconstrictive of platelets	
Docosahexaenoic acid (DHA)	Nutraceutical; brain development	
β-1,3-glucan	Immune-stimulator; antioxidant; reducer of blood cholesterol	Pharmaceuticals; Food technology
Carbohydrates		Bioethanol production; Food technology

1.4.1. Polyunsaturated fatty acids (PUFAs)

PUFAs, namely the subset HUFA (highy unsaturated fatty acids), are important in regulating membranes function and have been recognized essential for processes such as brain and eye development at fetus (30, 33). Moreover, some of these fatty acids are crucial in determining several physiological and pathological processes since they are precursors of pro-inflamatory (prostaglandins – PGs, tromboxanes – TXs; leukotrienes – LTs) and/or anti-inflamatory molecules (lipoxins, resolvins) (33). The following figure (Fig. 7) displays the metabolic pathway of polyunsaturated fatty acids (PUFAs) in human and their relation with pathological processes.

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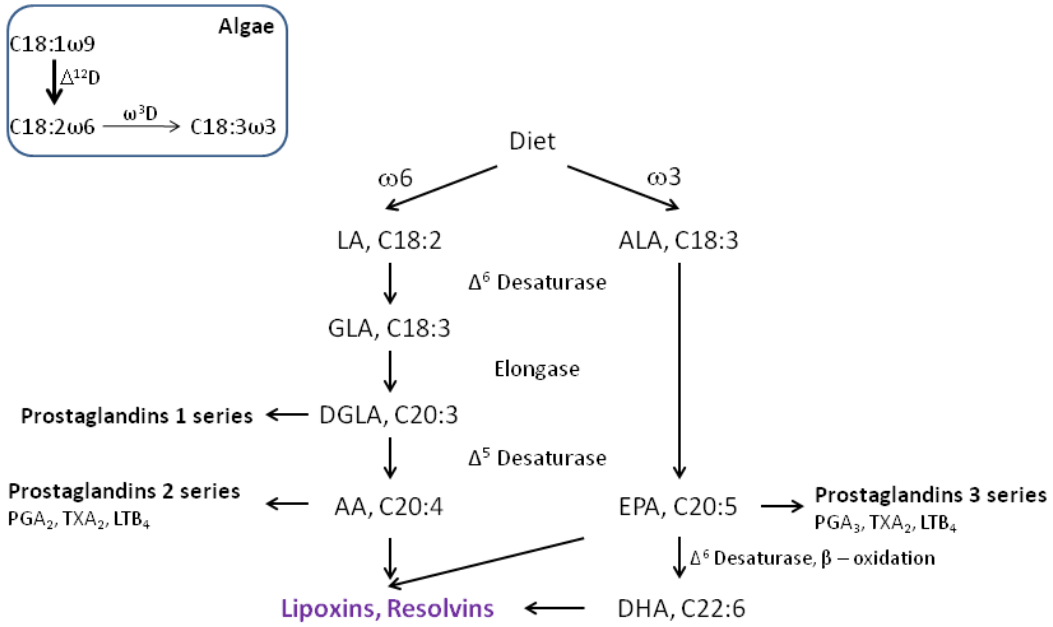

Figure 7. Polyunsaturated fatty acids (PUFAs) synthesis in animals and their relation with the precursors of inflammatory responses, the key process of desaturation of oleic acid (C18:1ω9) into the PUFA precursors, made in algae, is highlighted. Based on references (33, 34).

The nutritional requirements of animals in terms of PUFAs is due to their inability to synthesize the essential fatty acids: linoleic (LA, C18:2ω6) and alpha linolenic (ALA, C18:3ω3) acids, precursors of long chain fatty acids of ω3 and ω6 series respectively, through the monounsaturated fatty acids desaturation (33). Although the human metabolism can proceed to the elongation and desaturation of essential fatty acids into their long chain metabolites this process is not very efficient decreasing even more with age (33, 35). On the other hand microalgae like plants can convert oleic fatty

acid into the precursors of ω3 and ω6 series which is catalyzed by delta 12 desaturase and are known for being rich sources of LC–PUFA (34).

1.5. Objectives of the work

The main goal of this work was exploit organizational differences between *N. gaditana*, *R. marina* and *Isochrysis* sp. to develop a stress–inducement strategy in order to enhance their biochemical productivity for aquaculture purposes. In more detail:

i) The first objectives (Chapter II) were to evaluate the impact of nutrient availability on the biochemical composition and growth of microalgae, as well as the determination of the optimal ranges of nutrient availability for these species. In this chapter, microalgae were exposed to five different nutrient availabilities in growth media, in a batch cultivation system, and biochemical analysis was performed;

ii) The second goals (Chapter III) were to assess the effect of growth media on the qualitative and quantitative fatty acid content as well as the enhancement of EPA and DHA contents in microalgae. Samples had the same experimental design of chapter II, however, biochemical assessment encompassed fatty acid analysis alongside with their productivities;

iii) The third objectives (Chapter IV) were to evaluate the impact of nutrient availability on the carbohydrate profile of microalgae and link the monosaccharides composition with the carbohydrate of origin based on phylogenetic data. Samples had the same experimental design of chapter II and monosaccharides were analysed as alditol acetate;

iv) The fourth objectives (Chapter V) were to investigate the effect of nitrogen and sulfur availabilities in growth media on biochemical composition, growth and elemental stoichiometry of microalgae and to determine the optimal growth conditions that lead to the maximum EPA and DHA productivities. In this chapter, microalgae were exposed to different nitrogen and sulfur concentrations in a batch cultivation system. Biochemical analysis

was performed comprising proteins, lipids, fatty acid content and productivities analysis.

CHAPTER II. Influence of growth medium nutrient availability on the biochemical composition of *Nannochloropsis gaditana*, *Rhodomonas marina* and *Isochrysis* sp. – three marine microalgae

1. Abstract

This study aimed to enhance the gross biochemistry of microalgae in protein and lipids for aquaculture purposes and study their behaviour upon different nutrient conditions. Experiments were conducted with three marine microalgae, *N. gaditana*, *R. marina* and *Isochrysis* sp., commonly used in aquaculture that were subjected to a range of nutrient availabilities in a batch cultivation system. Overall, maximum cell densities were achieved at highest growth media nutrient availabilities and were obtained linear trends between protein content and nutrient availability. In general, highest lipid content were observed at high growth media nutrient availability (*N. gaditana*: T5 – 5 % DW; *Isochrysis* sp.: T5 – 26 % DW) and lipid fractions revealed high percentages of glycolipids in all microalgae (*N. gaditana*: 49 – 57 % TL; *R. marina*: 62 – 73 % TL; *Isochrysis* sp.: 56 – 61 % TL). Towards higher concentrations *Isochrysis* sp., *R. marina* and *N. gaditana* channelled their metabolic fluxes for protein accumulation. The behaviour of lipid content according to nutrient availability was different for *R. marina* supporting the belive/hypothesys that the response of biological systems at environmental stresses is species-specific.

Keywords: Nutrient availability, Biochemical composition, *N. gaditana*, *R. marina*, *Isochrysis* sp.

2. Introduction

In aquaculture several species of microalgae have been used with the purpose of zooplankton enrichment in bioactive compounds (like proteins and neutral lipids) for feeding fish (5). The selection of microalgae strains for aquaculture is based on parameters such as size, digestibility (e.g. cell wall), non-toxicity and nutritional value (5, 6). Meeting these criteria *Nannochloropsis* sp., *Rhodomonas* sp. and *Isochrysis* sp. are cultured as feed for farmed organisms such as bivalves, crab larvae and zooplankton that is then fed for crustacean and fish larvae (5, 6, 36).

The microalgae nutritional value is related with their gross biochemical composition (lipid, protein and carbohydrate) (37, 38). In order to modulate this parameter several strategies have been taken for enhancing the quality of these primary producers as food for aquatic organisms (39, 40). The manipulation of biotic and abiotic factors related with algae culture conditions, for example temperature, nutrient availability and salinity, can induce changes in growth and biochemical composition of microalgae (37, 41). It is noteworthy that the response to changes at different environmental conditions are species-specific (38).

Through photosynthesis microalgae can convert atmospheric CO_2 along with water and light, into organic matter being carbohydrates the major products (31). Carbohydrates and lipids are energy-rich molecules that in stressful conditions are often used as an alternative energy source for the production of raw materials required by cell (31, 42). In addition, proteins can function as nitrogen storage in some organisms and changes in this biochemical parameter can reflect the metabolic rate of actively growing/dividing cells (1, 43). The biochemical assessment can provide some insights about the structural modifications or molecular mechanisms that can lead to a successful adaptation and an accumulation of certain bioactive compounds that can be transferred up to the food chain (42, 43).

In biological systems nutrient availability has a large impact on the intracellular metabolite cycling influencing the biomass chemical composition (29). As with lipid synthesis, lipid component are known to be influenced by numerous factors (e.g. nitrogen starvation, high salinity), especially neutral lipids (44). This way the present study aims to evaluate the impact of nutrient availability on the biochemical

composition and growth of three marine microalgae (*N. gaditana*, *R. marina* and *Isochrysis* sp.), with special emphasis on their lipid classes, as well as the determination of the optimal ranges of nutrient availability for these species commonly used in aquaculture.

3. Materials and Methods

3.1. Chemicals

Methanol, acetone, sodium sulfate and Silica gel were all acquired from Sigma-Aldrich (St. Louis, MO, USA). Chloroform, potassium chloride and dichloromethane were acquired from VWR (Carnaxide, Portugal). Lugols solution was purchased from bioMérieux (Linda-a-Velha, Portugal). All the reagents used for analytical procedures had analytical grade.

3.2. Microorganisms and culture conditions

The *Isochrysis* sp., *R. marina* and *N. gaditana* were supplied by Mariculture Center of Calheta (Madeira, Portugal). The cultivation of each microalgae species was performed by inoculating 75 mL of starter cultures into 425 mL of enriched seawater with commercial culture medium Nutribloom plus (Necton, Portugal). The natural seawater used, for media preparation, was previously adjusted to a salinity of 25 g L^{-1} and sterilized in an autoclave (Uniclav 88) at 121 ºC for 15 min. Five different volumes of nutrient solution, 250 (T1), 500 (T2), 1000 (T3), 2000 (T4) and 4000 (T5) µL L^{-1}, were used for the preparation of growth medium. The nutrient concentrations in the final growth medium are presented in Table 3.

Table 3. Components of the growth medium culture and respective concentrations in final growth medium (mg L^{-1}) for the experiment carried out.

Component	Concentration in final growth medium (mg L^{-1})				
	T1	T2	T3	T4	T5
$NaNO_3$	43	85	170	340	680
KH_2PO_4	3	7	14	27	54
EDTA	2	4	8	15	31
$FeCl_3.6H_2O$	1	3	5	11	22
$ZnCl_2$	0.03	0.07	0.14	0.27	0.54
$ZnSO_4$	0.07	0.14	0.29	0.57	1.15
$MnCl_2.2H_2O$	0.04	0.08	0.16	0.32	0.65
$Na_2MoO_4.2H_2O$	0.01	0.01	0.02	0.05	0.10
$CoCl_2.6H2O$	0.01	0.01	0.02	0.05	0.10
$CuSO_4.5H_2O$	0.01	0.01	0.03	0.05	0.10
$MgSO_4.7H_2O$	0.12	0.25	0.49	0.98	1.97
Vitamins					
Tiamine	0.01	0.02	0.04	0.07	0.14
Biotin	0.001	0.003	0.005	0.010	0.020
B_{12}	0.001	0.002	0.003	0.006	0.012

Experiments were conducted at a temperature of 23 ± 2 ºC, with a photoperiod of 18:6 h light/dark cycles, pH ranging 7 – 9, at a light intensity of 3841 ± 560 lux and compressed air was used for aeration of cell cultures. The microalgae were harvested at stationary phase by centrifugation (centrifuge Labofuge 200 - Heraeus) for 5 min. at 4500rpm and washed with distilled water. Duplicates were conducted for all the experiments.

3.3.Cell concentration and specific growth rate determination

Microalgae growth was monitored daily by counting cells with a 0.1-mm-deep improved Neubauer haemocytometer (Marienfield–Superior) and a light microscope (Olympus BX41) using a 40x magnification; for cell counting, cells were fixed with lugol. A logistic model was used to describe algal growth, as previously presented by Xin (41), Eq. (2):

$$N = \frac{K}{1+e^{a-rt}} \qquad \text{Eq.(2)}$$

Where K (cells mL^{-1}) is the carrying capacity, N (cells mL^{-1}) is the cell concentration in time t (days), a is a constant that refers to the position of the origin and

r (d^{-1}) is the specific growth rate. The specific growth rate was calculated by the linearization of the logistic model.

3.4.Analytical Procedures

For the determination of CHNS content in all experiments an elemental analyser Truspec 630-200-200 was used. Total protein was assessed by multiplying the nitrogen content for 6.25 as described by Kim (39).

Extraction of total lipids was performed according to modified Bligh & Dyer (45). Briefly, to dried algal biomass were added 3mL of a methanol: chloroform mixture (2:1 v/v) followed by 400µL of a saturated solution of KCl and 2mL of cloroform. After homogenization, 2 mL of distilled water were added and the mixture was left stirring for 15 min. Then the sample was let to set and the organic phase was removed and dried in Na_2SO_4 filters. At the end, solvent was evaporated in a Büchi rotavapor R-200, in order to proceed to lipids quantification. Lipid content was quantified gravimetrically. Lipids and protein contents are presented relatively to dry biomass weight (DW) as average of at least two replicates.

For lipid class determination the total lipids were solubilized in dichloromethane and fractionated in activated silica (100 ºC) chromatography column. The separation of lipid classes was made according to Guckert (46) and Smith (47) procedures being the elution sequence as follows: 5 mL of dichloromethane, 5 mL of acetone and finally 10 mL of methanol. These elution's allows the separation of the different lipid fractions: neutral lipids, phospholipids and polar lipids (glycolipids).

3.5.Statistical analysis

Values are represented as means ± standard deviations. In algal growth curves non-linear regression trend lines where fit to data with Solver. Statistical analysis of the data was carried out using the software IBM SPSS Statistics 23. Differences between growth medium nutrient availability treatments were assessed by one-way analysis of variance (ANOVA) followed by a Bonferroni's Post Hoc analysis; p–values < 0.05 were considered to be statistically significant.

4. Results and discussion

4.1.Algal Growth

Growth was monitored daily with the aim to evaluate the impact of the experiment conducted in microalgae growth dynamic. Thus, in Figure 8 the growth curves according to growth medium nutrient availabilities applied, for the microalgae studied, are displayed. Through this figure is possible to visualize that maximal cell densities were achieved in T4 growth media. The highest cell density reached by *N. gaditana* was approximately 62.9 × 10^6 cells mL^{-1} [Fig. 8 (a)] which is closer to the values found by Rocha (48). In the treatments where nutrient availability was lower (T1 – T3) maximum specific growth rates (0.90 – 0.81 d^{-1}) were obtained for *N. gaditana* as well as for *R. marina* (1.22 – 1.56 d^{-1}) (Table 3). *R. marina* maximum cell density was approximately 34.9 × 10^5 cells mL^{-1} [Fig. 8b] which is two times higher than the value reported by Lafarga-De la Cruz (49) with modified f/2 medium. *Isochrysis* sp. revealed a different behaviour regarding to the specific growth rate, where the maximum values achieved 1.16 and 1.05 d^{-1} appear to have no direct relation with nutrient availability. For this microalga, treatments that induced long term growth (T3 – T5) reached higher cell densities. The maximum cell density value attained was approximately 32.1 × 10^6 cells mL^{-1} (Fig. 8 c) which is closer to that previously obtained by Fidalgo (40).

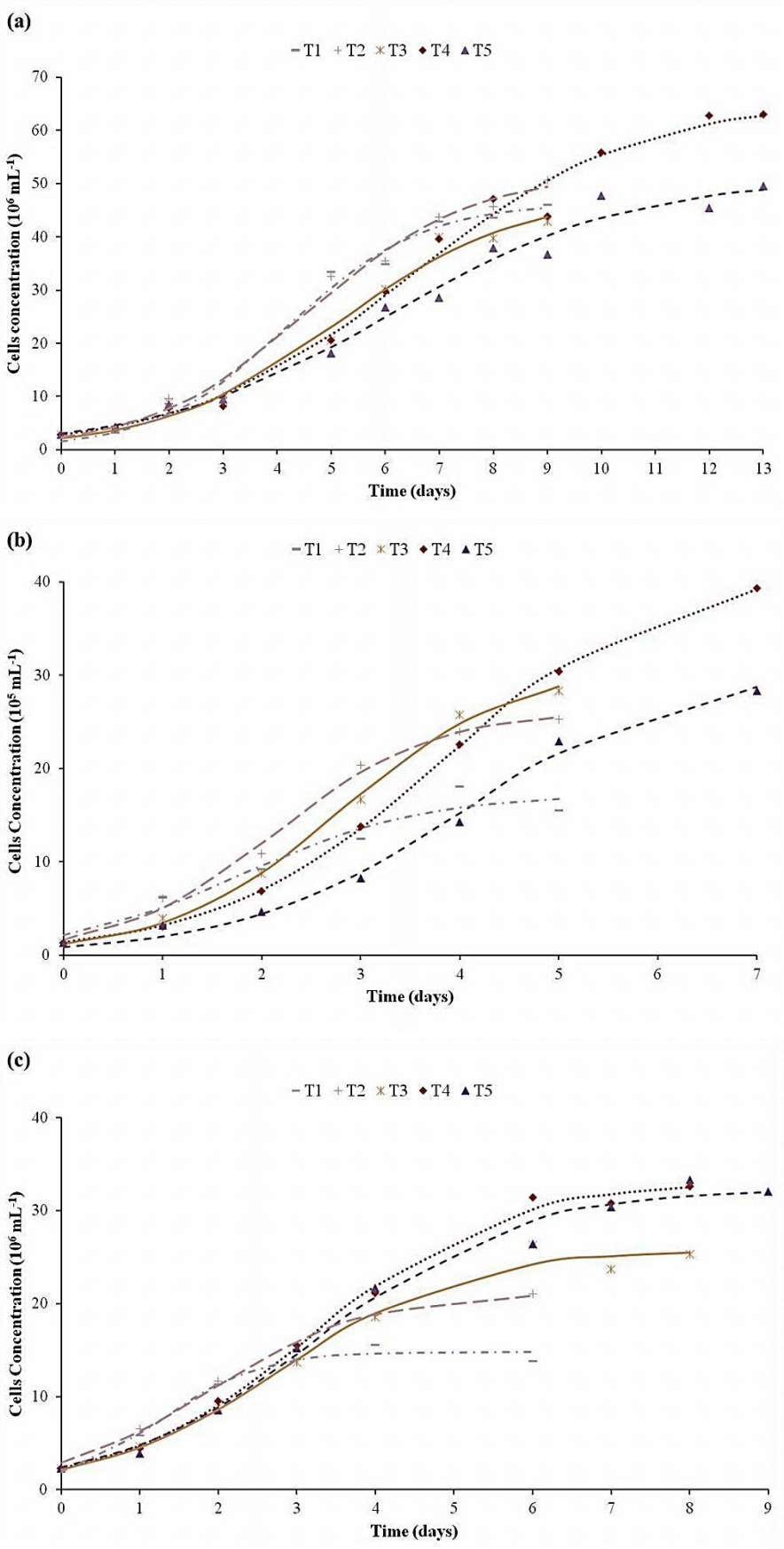

Figure 8. Growth curves for *N. gaditana* (a), *R. marina* (b) and *Isochrysis* sp. (c) upon different nutrient availabilities in growth media.

4.2.Biochemical Composition

Algal culture medium comprises inorganic salts, macro and micronutrients, which some are rate limiting for algal growth and others crucial for the enzymatic reactions that are responsible for the biosynthesis of many metabolites (18). Thus it is important to assess the impact of nutrient availability over the nutrient uptake and the enhancement of the nutritional quality of these primary producers.

C/N ratios presented in Table 4 indicates that the biochemical profile was affected by the nutrient availability. In the three marine microalgae studied the C/N ratio was higher in treatments with the lower nutrient concentrations (T1 and T2). This behaviour was previously observed for many microalgae strains which in nutrient deficient conditions could transform, within the cell, nitrogenous compounds (proteins or peptides) into storage compounds (lipids or carbohydrates) (31, 40). On higher nutrient concentrations C/N ratio presented the lowest values indicating an increase in protein synthesis supported by total protein content presented in Table 4. In nutrient saturation conditions (T4 – T5), the C/N ratios obtained for *R. marina* and *Isochrysis* sp. were closer to those referred in Seixas (50) for another strain of *Rhodomonas* (4.4) and for *Isochrysis galbana* (8.8). Regarding to *N. gaditana*, the C/N ratios (6.68 and 6.85) achieved in nutrient saturation conditions were closer to the Redfield ratio (6.6) (50).

Table 4. Effect of nutrient availability in the biochemical composition of *N. gaditana*, *R. marina* and *Isochrysis* sp.

Parameter	Microalgae	Volume of Nutrient Solution (mL)				
		T1	T2	T3	T4	T5
r (d^{-1})	*N. gaditana*	0.90	0.81	0.81	0.52	0.47
	R. marina	1.22	1.56	1.41	1.01	0.93
	Isochrysis sp.	0.74	1.16	0.74	1.05	0.76
Protein (%DW)	*N. gaditana*	2.82 ± 0.35^{a}	3.17 ± 0.18^{a}	8.58 ± 0.10^{b}	18.81 ± 0.05^{c}	19.70 ± 0.03^{d}
	R. marina	18.69 ± 0.27^{a}	26.68 ± 0.72^{b}	44.48 ± 0.31^{c}	58.85 ± 0.78^{d}	46.34 ± 0.38^{c}
	Isochrysis sp.	12.12 ± 0.46^{a}	13.85 ± 1.13^{a}	25.09 ± 0.52^{b}	36.89 ± 1.51^{c}	36.35 ± 0.38^{c}
Lipid (%DW)	*N. gaditana*	1.88 ± 0.01^{a}	1.54 ± 0.02^{a}	3.18 ± 0.16^{b}	4.94 ± 0.22^{c}	5.15 ± 0.04^{c}
	R. marina	19.04 ± 0.33^{a}	20.54 ± 0.26^{a}	18.92 ± 0.10^{a}	20.24 ± 0.56^{a}	15.75 ± 1.48^{b}
	Isochrysis sp.	16.80 ± 1.31^{a}	14.72 ± 1.29^{a}	23.01 ± 1.58^{b}	24.83 ± 2.05^{b}	25.52 ± 0.32^{b}
C/N (mol/mol)	*N. gaditana*	13.65 ± 0.22^{a}	10.85 ± 0.14^{b}	8.60 ± 0.27^{c}	6.68 ± 0.02^{d}	6.85 ± 0.08^{d}
	R. marina	11.70 ± 0.22^{a}	9.73 ± 0.02^{b}	5.69 ± 0.04^{c}	5.34 ± 0.04^{cd}	5.18 ± 0.01^{d}
	Isochrysis sp.	14.63 ± 0.10^{a}	12.15 ± 0.38^{b}	10.68 ± 0.08^{c}	8.78 ± 0.36^{d}	8.79 ± 0.07^{d}

Values (means ± standard deviations) in the same row, not sharing a common superscript are significantly different ($p < 0.05$). r – specific growth rate (d^{-1}); DW – Dry biomass weight.

Nitrogen is an essential component for microalgae growth and is associated with protein biosynthesis (31). In nutrient-rich conditions some organisms can assimilate the excess nitrogen in culture medium and store it into protein (1). In this regard, linear trends between the total protein content in dry biomass and the nutrient availability were follow for the first four concentrations assessed (T1 – T4), presenting determination coefficients (R^2) between 0.95 – 0.99. To nourish bivalve larvae, the microalgae species must contain at least 12% of protein in their composition despite of the optimum range being 30 – 60 % of protein in dry biomass (51). Thus, for the three microalgae assessed, only *Isochrysis* sp. and *R. marina* were within the optimum range being the highest values obtained of 36.89 – 36.35 % and 44.48 – 58.85 % of dry weight, respectively (Table 4). However the maximum protein obtained for *N. gaditana* at T4 and T5, 18.81–19.70 % of dry biomass, also makes it suitable for bivalve diet.

The main strategies often applied for channel metabolic fluxes towards lipid accumulation are mainly nitrogen and phosphorus starvation (52). The response of *N. gaditana* and *Isochrysis* sp. at low nutrient concentrations (T1 and T2) revealed that lipid content in terms of dry biomass was lower, rising 69 and 107 % and 37 and 56 %, respectively, with nutrient availability, values were stipulated in regard to T3. Furthermore lipid accumulation reached a saturation point at the highest concentration applied. In *R. marina* lipid content remained constant reaching a turning point at the maximum nutrient concentration applied (T5) where after that the lipid content decreased 16.75% of dry biomass when compared to T3. According to Courchesne (52), microalgae under nutrient limitation accumulate lipids when cellular mechanisms for photosynthesis are active and the energy source along with carbon source are abundantly available. In this study, not only the concentration of nitrogen and phosphorus were changed in culture medium, but also the availability of other nutrients which have a key role in photosynthesis reactions, explaining the results observed for *N. gaditana* and *Isochrysis* sp.(18).

4.3. Lipid Class Content

Algal lipids can be divided into two major classes: neutral/nonpolar lipids – that are known as the metabolic energy reserves of cell, and polar lipids – that play a structural role as components of biomembranes (53, 54). In algae, environmental stresses can induce fluctuations in the fluidity of cell membranes (55). Although these

fluctuations are not fully understood, it is often admitted that they are needed to alter physiological properties of biomembranes in order to maintain normal cell processes such as ion permeability and photosynthesis (55).

Through the experiment, a higher content of polar lipid (sum of glycolipids and phospholipids) was observed over the neutral/non polar lipids, being glycolipids the main fraction in all experiments performed for the three microalgae. Glycolipids percentages of total lipid were 49 – 57 % for *Isochrysis* sp., 62 – 73 % for *R. marina* and 56 – 61 % for *N. gaditana* . It should be noted that algae grown under normal conditions often possess large amounts of polar lipids and that glycolipids, predominantly located in photosynthetic membranes, maintain the stability of photosynthetic apparatus that is crucial for microalgae metabolic activities (55, 56).

Like total lipid, lipid class content of dry biomass varied in function of nutrient availability [Fig. 9]. Changes in the lipid fractions proportions evidenced in *N. gaditana* [Fig. 9a] a mobilization of phospholipid to neutral lipids with the increase of nutrient concentrations. Moreover, the highest phospholipid content (29 and 36 % of total lipid) was obtained at the lowest growth medium nutrient availabilities (T1 and T2) by this microalga. Phospholipid content in *R. marina* [Fig. 9b] and *Isochrysis* sp. [Fig. 9c] were within the values previously reported by Kumari (55) as common for algae, approximately 10 – 20 % of total lipid.

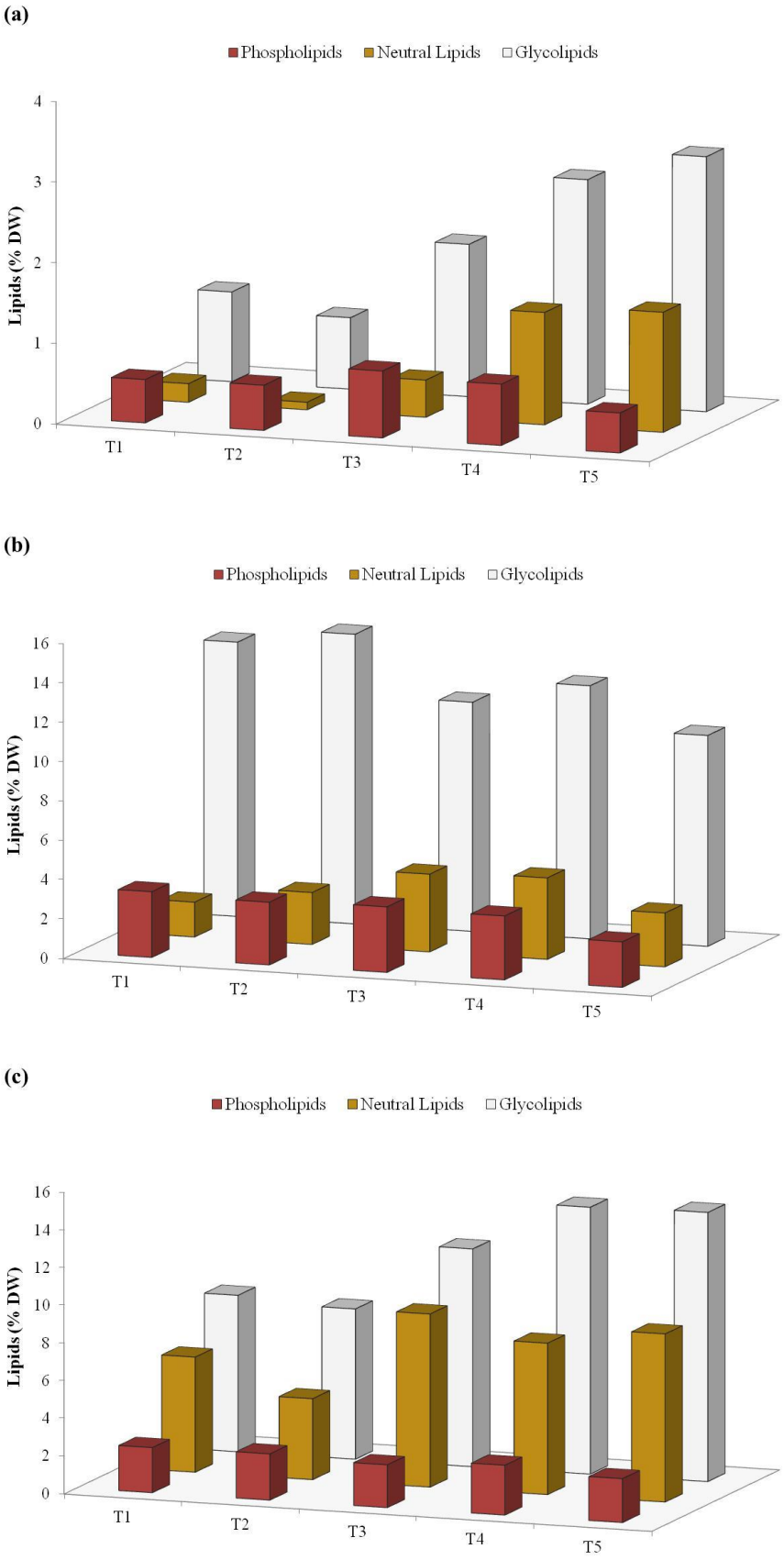

Figure 9. Lipid class content of (a) *N. gaditana*, (b) *R. marina* and (c) *Isochrysis* sp. against growth medium. DW – Dry biomass weight.

5. Conclusion

Nutrient availability had an impact in algal growth dynamics for the three microalgae studied. Towards high nutrient concentrations, C/N ratios indicate that all microalgae channelled their metabolic fluxes to protein accumulation. In the overall, highest protein and lipid content were reached at bigger growth media nutrient availabilities, suggesting that the biochemistry of microalgae is suitable for aquaculture. Different patterns were observed for the three microalgae, namely, the lipid content evolution in regard to the nutrient concentrations applied in *R. marina* and the mobilization of lipid fractions in *N. gaditana* in regard to nutrient availability. These observations support the assumption that the response to environmental stresses is species – specific. Lipid class content was rich in glycolipids which is the main constituent of photosynthetic membranes.

CHAPTER III. Effect of growth medium nutrient availability on the fatty acid profile of *Nannochloropsis gaditana*, *Rhodomonas marina* and *Isochrysis* sp.

1. Abstract

Several species of microalgae are used in aquaculture with the purpose of transfer bioactive compounds, such as eicosapentaenoic (EPA) and docosahexaenoic acids (DHA), up to the aquatic food chain. In order to maximize and enhance the nutritional value of *N. gaditana*, *R. marina* and *Isochrysis* sp. a range of nutrient concentrations were applied to the growth medium. In general microalgae protein, EPA and DHA contents had a similar pattern response to the nutrients input. A mobilization of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) towards polyunsaturated fatty acids (PUFA) synthesis, for *N. gaditana* and *R. marina*, was depicted by a decrease in their ratios with growth medium nutrient availabilities. Optimum EPA and DHA productivities were reached by *R. marina* (EPA: 15.11 mg g^{-1} DW d^{-1}; DHA: 11.55 mg g^{-1} DW d^{-1}) and *N. gaditana* (EPA: 0.28 mg g^{-1} DW d^{-1}) at growth medium with 1000 μL L^{-1} of commercial nutrient solution (Nutribloom plus) whereas for *Isochrysis* sp. were attained at growth medium with 2000 μL L^{-1} of Nutribloom plus (EPA: 0.52 mg g^{-1} DW d^{-1}; DHA: 17.04 mg g^{-1} DW d^{-1}). The observations made for *N. gaditana* indicate that the commercial medium used for the current experiment wasn't suitable for this microalga. The results obtained pointed that the strategy here applied constitutes an advantage for modulating the biochemical composition of microalgae.

Keywords: Nutrient availability, EPA, DHA, *N. gaditana*, *R. marina*, *Isochrysis* sp.

2. Introduction

Microalgae have the ability to grow very fast and produce a variety of compounds that can be used for several areas of application (30, 37). For instance, synthesis of bioactive compounds like polyunsaturated fatty acids (PUFAs), sterols, pigments and vitamins, makes them suitable to use in fish farming and human health (32). While saturated and monounsaturated fatty acids along with hydrocarbons are products synthetized by microalgae strains suitable for biodiesel production (30, 57).

Long chain PUFAs play a key role as membrane constituents of organisms and as precursors of a variety of signalling molecules (e.g. leukotrienes and eicosanoids), which comprises multiple physiological and pathological responses (33, 35). Hence, the intake of PUFAs by humans, namely omega3 (ω3), can prevent and improve cardiovascular disease, hypertension and arthritis (32). Fish and their oils are known as dietary sources of ω3 PUFAs, in particular eicosapentaenoic (EPA – 20:5ω3) and docosahexaenoic (DHA – 22:6ω3) acids (58-60). Nevertheless, as with humans, fish lack the ability of synthesize efficiently ω3 or ω6 (omega6) LC-PUFAs which are required for their normal growth and development, and therefore they acquire them through their diet (30, 35, 61).

Microalgae are the primary sources of PUFAs in the marine food chain being used for aquaculture with the purpose of supplementing them to zooplankton and other aquatic organisms (5, 30). Consequently, the fatty acid composition is an essential parameter in selecting microalgae strains for aquaculture (40). Some of the most commonly used strains are *Nannochloropsis* sp. (EPA – producer) and *Isochrysis* sp. [*Isochyrsis aff. galbana*: DHA – producer; *Isochrysis galbana* (Parke): rich in both EPA and DHA] (35, 40, 62).

Although these photosynthetic organisms naturally produce high amounts of PUFAs, their fatty acid content and composition can be enhanced by exposing them to changes in their growth conditions (35, 62). In this regard, the manipulation of environmental parameters such as composition of the growth medium or the presence of critical media components have influence on the nature, amount and the composition of the desired products (18). The aim of this study was to assess the effect of growth media on the qualitative and quantitative fatty acid content, as well as the enhancement of EPA

and DHA contents in three marine microalgae – *N. gaditana*, *R. marina* and *Isochrysis* sp. (used in aquaculture feeding) by applying a range of different nutrient concentrations.

3. Materials and methods

3.1. Chemicals

The chemicals used had analytical grade. Methanol and heptane were acquired from Sigma-Aldrich (St. Louis, MO, USA). Ethyl acetate was from Merck (Darmstadt, Germany).

3.2. Algal growth and experiment design

The experimental design, cell densities and specific growth rate (r, $days^{-1}$) determinations are described in sections 3.2. and 3.3. of chapter II.

3.3. Determination of specific growth rate and fatty acid productivities

The fatty acid productivities were estimated, according to Hoffmann (57), after harvest, in stationary phase, by the following equation:

$$P_x = r \times c_x \qquad \text{Eq. (3)}$$

Where P_x represents the productivity (mg g^{-1} day^{-1}) of a specific fatty acid (x), r is the growth rate ($days^{-1}$) and c_x is the concentration of the fatty acid.

3.4. Determination of fatty acids

The fatty acid composition of algal dried biomass was determined as fatty acid methyl esters (FAMEs) as previously described by Lepage & Roy (63), modified by Cohen (64). Briefly, fatty acids were converted to FAMEs by adding a mixture of ethyl acetate-methanol (1:19 v/v) to dry biomass that was then left at 80ºC for 1h and further extracted in the heptane fraction. FAMEs were analysed by gas chromatography (Agilent HP 6890) equipped with a mass selective detector (Agilent 5973) and a

capillary column DB-225 J&W (30 m × 0.25 mm inner diameter, 0.15 µm film thickness) from Agilent. The chromatographic conditions were as follows: oven initial temperature was 35 ºC for 0.5 min; increasing 25 ºC min^{-1} to 195 ºC, 3 ºC min^{-1} to 205 ºC and 8 ºC min^{-1} until reach the final temperature of 230 ºC for 3 min.; injector temperature 250 ºC; transfer line temperature 280 ºC; split ratio, 1:100. Helium was used as the carrier gas with a flow rate of 2.6 mL min^{-1}. FAMEs identification was made by comparing the retention times and mass spectra fragmentation to those of known standards (bacterial acid methyl esters CP mix and supelco 37 component FAME mix from supelco). Four replicates were performed for each GC analysis being the results presented as the mean value ± standard deviation (SD) of FAME expressed in mg g^{-1} of dry biomass weight (DW). The internal standard used was the heneicosanoic acid (C21:0).

3.5.Statistical Analysis

Statistical analysis of the data was carried out using the software IBM SPSS Statistics 23. Differences between growth medium nutrient availability treatments were assessed by one-way analysis of variance (ANOVA) followed by a Scheffe's Post Hoc analysis; p–values < 0.05 were considered to be statistically significant.

4. Results and Discussion

4.1.Protein and highly unsaturated fatty acids synthesis (HUFAs)

Microalgae are natural food resources of protein and HUFAs, namely eicosapentaenoic (EPA, C20:5ω3) and docosahexaenoic acids (DHA, C22:6ω3), for higher levels of marine food web (65). EPA and DHA are crucial for maintaining the biomembranes and cellular functions (e.g. cell signalling) (66, 67). Proteins are involved in the metabolic mechanisms as biological catalysts of crucial reactions for cell growth (68). Since sets of proteins like desaturases and elongases, which rely on protein expression, are responsible for keep fatty acid unsaturation reactions, an examination of protein against EPA and DHA behaviour is important (35). Figure 10 shows the effect of the nutrient availability on protein and fatty acids contents.

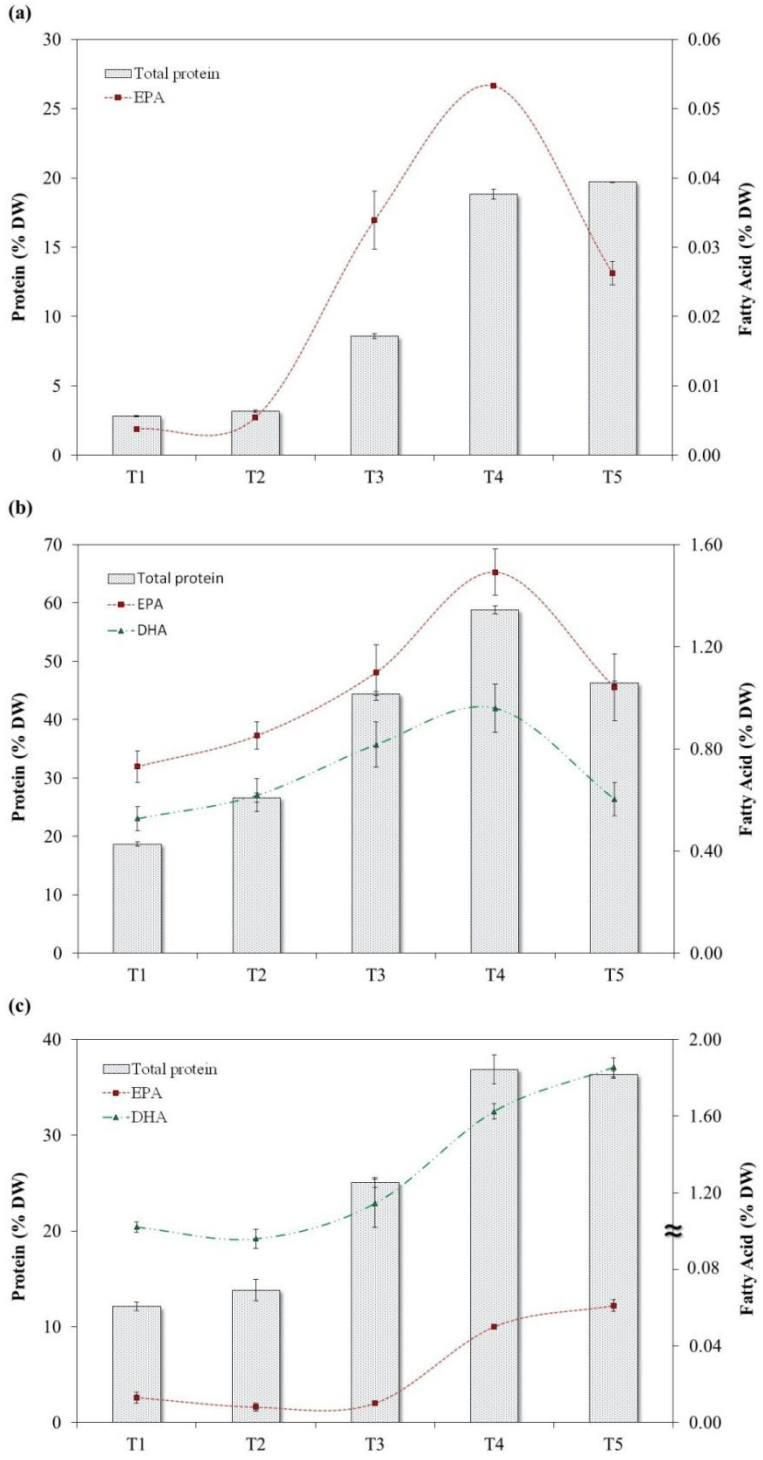

Figure 10. Effect of growth media nutrient availability on protein, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) contents in dry biomass weight (DW) of (a) *N. gaditana*, (b) *R. marina* and (c) *Isochrysis* sp.

Fig. 10a shows that in *N. gaditana* EPA and protein contents progressed in the same way with regard to nutrient availability until T4. In T5 EPA content decreased (51 % DW) in contrast to protein that remained constant in respect to T4. This might suggest that when the growth medium nutrients were too high *N. gaditana* responded by

accumulating nitrogen-related metabolites (proteins) and possibly reducing their membrane permeability associated with DHA and EPA contents (69, 70).

Fig. 10b shows that *R. marina* presented an enhancement of EPA, DHA and protein content with the increase of nutrient availability from T1 – T4, reaching a saturation point in the latter. Further nutrient input (T5) seemed to induce a toxic effect, as showed from a decrement in the maximum cell density achieved and biochemical parameters studied. In *Isochrysis* sp., these biomolecules presented the same trend in regard to nutrient concentrations as displayed in Fig. 10c. Several factors are known to influence the activities of fatty acid elongases and desaturases, namely the availability of vitamins and inorganic cofactors which can enhance or inhibit the activity of these enzymes (33). Thus, the previous observations suggest that higher growth medium nutrient availabilities comprising high levels of cofactors enhanced the activity of desaturases and elongases as well as their expression.

4.2.Fatty acid profile

According to Renaud (71) HUFAs are associated with high growth rates of aquaculture organisms. Knowing that the fatty acid composition of microalgae isn't constant and varies with environmental factors, such as nutrient availability; we aimed to improve microalgae fatty acid content for aquaculture by inducing changes in their growth environment and by exploiting their physiological potential as high productivity strains (35, 65).

Through the analysis of Table 5 is possible to note that fatty acid (FA) composition of *N. gaditana* varied between growth medium nutrient availabilities. The four major fatty acids in *N. gaditana* were linoleic acid (LA, C18:2ω6), alpha linolenic acid (ALA, C18:3ω3), palmitic acid (C16:0) and monounsaturated stearic acid (C18:1), the latter substituted by hexadecatrienoic acid (C16:3ω3) in T3 and by monounsaturated palmitic acid (C16:1) in T4 and T5. In *N. gaditana* the major FA accounted for 75 – 89 % of total FA.

Table 5. Fatty acid profile of *N. gaditana* grown under different growth medium nutrient availabilities.

Fatty Acids (mg g^{-1} DW)	Treatment				
	T1	T2	T3	T4	T5
C14:0	0.07 ± 0.00^{ab}	0.06 ± 0.00^{a}	0.11 ± 0.02^{b}	0.19 ± 0.02^{c}	0.22 ± 0.02^{c}
C16:0	1.84 ± 0.02^{a}	1.25 ± 0.03^{b}	1.81 ± 0.02^{a}	2.79 ± 0.11^{c}	3.20 ± 0.11^{d}
C18:0	0.05 ± 0.01^{a}	0.03 ± 0.00^{a}	0.02 ± 0.00^{a}	0.03 ± 0.00^{a}	0.31 ± 0.04^{b}
Total – SFA[2]	1.98 ± 0.03^{a}	1.36 ± 0.04^{b}	2.00 ± 0.02^{a}	3.12 ± 0.13^{c}	3.84 ± 0.19d
C16:1[1]	0.18 ± 0.01^{a}	0.20 ± 0.03^{a}	0.76 ± 0.04^{b}	2.10 ± 0.12^{c}	1.94 ± 0.27^{c}
C18:1[1]	2.73 ± 0.04^{a}	1.01 ± 0.02^{b}	0.42 ± 0.03^{c}	0.50 ± 0.05^{c}	0.73 ± 0.12^{d}
Total – MUFA[2]	2.91 ± 0.04^{a}	1.21 ± 0.06^{b}	1.17 ± 0.05^{b}	2.60 ± 0.17^{a}	2.67 ± 0.39^{a}
C16:3ω3	0.26 ± 0.01^{a}	0.35 ± 0.01^{a}	1.11 ± 0.08^{b}	0.97 ± 0.18^{bc}	0.80 ± 0.02^{c}
C18:2ω6	0.66 ± 0.03^{a}	0.58 ± 0.03^{a}	1.63 ± 0.04^{b}	2.27 ± 0.18^{c}	2.66 ± 0.01^{d}
C18:3ω3	0.70 ± 0.02^{a}	0.89 ± 0.02^{a}	2.82 ± 0.17^{b}	3.00 ± 0.54^{b}	2.45 ± 0.18^{b}
C18:4ω3	n.d.	n.d.	0.04 ± 0.00^{a}	0.01 ± 0.00^{b}	0.01 ± 0.00^{b}
C20:5ω3 - EPA	0.04 ± 0.00^{a}	0.05 ± 0.00^{a}	0.34 ± 0.04^{b}	0.53 ± 0.00^{c}	0.26 ± 0.02^{d}
Total – PUFA[2]	1.74 ± 0.06^{a}	1.98 ± 0.07^{a}	6.46 ± 0.34^{b}	7.54 ± 1.17^{b}	7.19 ± 0.14^{b}
∑ω3	1.00 ± 0.03^{a}	1.29 ± 0.03^{a}	4.31 ± 0.30^{b}	4.39 ± 0.93^{b}	3.52 ± 0.22^{b}
∑ω6	0.66 ± 0.03^{a}	0.58 ± 0.03^{a}	1.63 ± 0.04^{b}	2.27 ± 0.18^{c}	2.66 ± 0.01^{d}
∑ω3/∑ω6	1.51 ± 0.04^{a}	2.22 ± 0.09^{b}	2.64 ± 0.12^{c}	1.92 ± 0.27^{b}	1.33 ± 0.08^{a}
ω3 HUFA	0.04 ± 0.00^{a}	0.05 ± 0.00^{a}	0.34 ± 0.04^{b}	0.53 ± 0.00^{c}	0.26 ± 0.02^{d}
(SFA+MUFA)/ PUFA	2.81 ± 0.09^{a}	1.30 ± 0.02^{b}	0.49 ± 0.03^{c}	0.77 ± 0.15^{d}	0.91 ± 0.10^{d}
Total[2]	6.62 ± 0.08^{a}	4.54 ± 0.15^{b}	9.63 ± 0.32^{c}	13.26 ± 1.13^{d}	13.70 ± 0.44^{d}

Values (means ± SD of four replications) in the same row, not sharing a common superscript are significantly different ($p < 0.05$). [1]Contains ω9 and ω7 isomers; [2]Contains some minor components not shown; n.d. – non detected; SFA – Saturated fatty acid; MUFA – Monounsaturated fatty acid; PUFA – Polyunsaturated fatty acid; HUFA – Highly unsaturated fatty acid.

Table 6 shows the fatty acid composition of *R. marina*. In *R. marina* the nutrient availability in the growth medium induced a shift on the major fatty acids detected. Therefore in T1 and T2, *R. marina* major fatty acids were C16:0, LA, ALA and stearidonic acid (SDA, C18:4ω3) representing 61 – 64 % of total fatty acids. While in T3 – T5 the C16:0 and LA fatty acids were replaced by DHA and EPA as predominant fatty acids, that together with ALA and SDA accounted for 73 – 79 % of total FA.

Table 6. Fatty acid composition of *R. marina* according to growth medium nutrient availabilities.

Fatty Acids (mg g^{-1} DW)	Treatment				
	T1	T2	T3	T4	T5
C14:0	9.43 ± 0.73^{a}	7.36 ± 1.08^{b}	5.32 ± 0.42^{c}	4.13 ± 0.45^{cd}	3.02 ± 0.27^{d}
C16:0	22.00 ± 1.26^{a}	13.00 ± 1.45^{b}	3.70 ± 0.21^{c}	3.15 ± 0.28^{c}	2.45 ± 0.16^{c}
C18:0	4.04 ± 0.26^{a}	1.86 ± 0.14^{b}	0.32 ± 0.02^{c}	0.34 ± 0.03^{c}	0.25 ± 0.06^{c}
Total – SFA[2]	42.40 ± 2.72^{a}	30.62 ± 4.01^{b}	15.44 ± 0.99^{c}	8.65 ± 0.79^{d}	6.26 ± 0.36^{d}
C16:1[1]	2.28 ± 0.20^{a}	1.52 ± 0.13^{bc}	1.28 ± 0.09^{b}	2.22 ± 0.26^{a}	1.87 ± 0.11^{ac}
C18:1[1]	17.66 ± 1.24^{a}	11.87 ± 1.05^{b}	5.66 ± 0.17^{c}	5.57 ± 0.25^{c}	4.48 ± 0.16^{c}
Total – MUFA[2]	19.98 ± 1.40^{a}	13.41 ± 1.17^{b}	6.94 ± 0.19^{c}	7.79 ± 0.51^{c}	6.36 ± 0.27^{c}
C18:2ω6	19.30 ± 1.33^{a}	12.21 ± 0.86^{b}	4.02 ± 0.09^{c}	4.83 ± 0.20^{c}	1.98 ± 0.22^{d}
C18:3ω3	32.75 ± 1.98^{a}	28.44 ± 1.98^{b}	26.88 ± 0.39^{bc}	23.82 ± 1.12^{c}	16.52 ± 0.15^{d}
C18:4ω3	22.08 ± 1.54^{a}	18.70 ± 1.12^{b}	26.86 ± 0.72^{c}	32.53 ± 0.66^{d}	23.50 ± 0.98^{a}
C20:4ω6 - AA	0.07 ± 0.02^{ab}	0.09 ± 0.02^{a}	0.01 ± 0.00^{c}	0.05 ± 0.02^{bc}	n.d.
C20:5ω3 - EPA	7.31 ± 0.62^{a}	8.52 ± 0.54^{ab}	10.99 ± 1.09^{c}	14.92 ± 0.90^{d}	10.41 ± 1.31^{bc}
C22:6ω3 - DHA	5.27 ± 0.47^{a}	6.18 ± 0.64^{a}	8.17 ± 0.88^{b}	9.59 ± 0.95^{b}	6.03 ± 0.65^{a}
Total – PUFA[2]	87.63 ± 5.69^{a}	74.61 ± 4.01^{b}	77.03 ± 2.55^{bc}	85.98 ± 1.82^{ac}	58.46 ± 3.28^{d}
∑ω3	67.41 ± 4.52^{ab}	61.84 ± 3.23^{ac}	72.89 ± 2.54^{bd}	80.87 ± 1.70^{d}	56.46 ± 3.06^{c}
∑ω6	20.22 ± 1.40^{a}	12.77 ± 0.87^{b}	4.13 ± 0.09^{c}	5.12 ± 0.17^{c}	2.00 ± 0.22^{d}
∑ω3/∑ω6	3.34 ± 0.15^{a}	4.85 ± 0.17^{a}	17.63 ± 0.69^{b}	15.81 ± 0.40^{b}	28.31 ± 1.54^{c}
ω3 HUFA	12.58 ± 1.07^{a}	14.71 ± 1.18^{a}	19.16 ± 1.97^{b}	24.52 ± 1.82^{c}	16.44 ± 1.96^{ab}
DHA/EPA	0.72 ± 0.02^{a}	0.72 ± 0.03^{a}	0.74 ± 0.01^{a}	0.64 ± 0.03^{b}	0.58 ± 0.01^{c}
(SFA+MUFA)/ PUFA	0.71 ± 0.03^{a}	0.59 ± 0.05^{b}	0.29 ± 0.02^{c}	0.19 ± 0.01^{d}	0.22 ± 0.01^{d}
Total[2]	150.01 ± 9.24^{a}	118.64 ± 8.72^{b}	99.40 ± 2.04^{c}	102.43 ± 2.34^{c}	71.08 ± 3.19^{d}

Values (means ± SD of four replications) in the same row, not sharing a common superscript are significantly different ($p < 0.05$). [1]Contains ω9 and ω7 isomers; [2]Contains some minor components not shown; n.d. – non detected; SFA – Saturated fatty acid; MUFA – Monounsaturated fatty acid; PUFA – Polyunsaturated fatty acid; HUFA – Highly unsaturated fatty acid.

Table 7 shows that the fatty acid profile of *Isochrysis* sp. varied across treatments applied. *Isochrysis* sp. major fatty acids, were the myristic acid (C14:0), monounsaturated stearic acid (C18:1), SDA and DHA, which together accounted over 70 % of the total fatty acids in all treatments. It should be noted that the microalgae *Isochrysis* sp. and *R. marina*, presenting the highest contents of HUFAs, have SDA as major FA. Between species, is possible to note qualitative and quantitative differences in their FA profiles.

Table 7. Fatty acid profile of *Isochrysis* sp. grown under different growth medium nutrient availabilities.

Fatty Acids (mg g^{-1} DW)	Treatment				
	T1	T2	T3	T4	T5
C14:0	5.97 ± 0.18^{a}	4.64 ± 0.09^{b}	9.48 ± 0.69^{c}	13.97 ± 0.51^{d}	14.48 ± 0.43^{d}
C16:0	3.91 ± 0.12^{a}	2.98 ± 0.10^{b}	5.34 ± 0.40^{c}	6.77 ± 0.21^{d}	6.78 ± 0.16^{d}
C18:0	0.01 ± 0.00^{a}	n.d.	n.d.	0.03 ± 0.00^{b}	0.02 ± 0.01^{b}
Total – SFA[2]	10.14 ± 0.29^{a}	7.71 ± 0.14^{b}	15.06 ± 1.10^{c}	21.26 ± 0.65^{d}	21.89 ± 0.61^{d}
C16:1[1]	3.44 ± 0.16^{a}	2.36 ± 0.20^{b}	4.63 ± 0.51^{c}	6.49 ± 0.16^{d}	7.27 ± 0.43^{d}
C18:1[1]	7.42 ± 0.27^{a}	4.64 ± 0.32^{b}	8.02 ± 0.84^{a}	9.30 ± 0.32^{c}	10.10 ± 0.23^{c}
Total – MUFA[2]	10.89 ± 0.43^{a}	7.03 ± 0.51^{b}	12.68 ± 1.34^{a}	16.12 ± 0.50^{c}	17.72 ± 0.62^{c}
C16:3ω3	0.07 ± 0.02^{ab}	0.08 ± 0.02^{ab}	0.01 ± 0.00^{a}	0.15 ± 0.04^{bc}	0.17 ± 0.05^{c}
C18:2ω6	2.24 ± 0.15^{ab}	1.81 ± 0.20^{a}	2.91 ± 0.52^{b}	6.30 ± 0.22^{c}	7.33 ± 0.23^{d}
C18:3ω3	3.10 ± 0.10^{a}	2.88 ± 0.23^{a}	5.47 ± 0.47^{b}	7.64 ± 0.15^{c}	8.49 ± 0.25^{d}
C18:4ω3	14.11 ± 0.41^{a}	14.27 ± 0.62^{a}	25.24 ± 1.33^{b}	35.43 ± 0.62^{c}	38.90 ± 0.73^{d}
C20:5ω3 - EPA	0.13 ± 0.03^{a}	0.08 ± 0.02^{a}	0.10 ± 0.01^{a}	0.50 ± 0.01^{b}	0.61 ± 0.03^{c}
C22:6ω3 - DHA	10.20 ± 0.27^{ab}	9.59 ± 0.49^{a}	11.44 ± 1.26^{b}	16.25 ± 0.39^{c}	18.54 ± 0.51^{d}
Total – PUFA[2]	30.10 ± 0.99^{a}	28.86 ± 1.59^{a}	45.44 ± 3.28^{b}	67.80 ± 1.44^{c}	76.09 ± 1.92^{d}
∑ω3	27.61 ± 0.78^{a}	26.90 ± 1.34^{a}	42.25 ± 2.69^{b}	59.91 ± 1.08^{c}	66.66 ± 1.42^{d}
∑ω6	2.31 ± 0.17^{ab}	1.87 ± 0.23^{a}	2.92 ± 0.52^{b}	7.20 ± 0.30^{c}	8.57 ± 0.38^{d}
∑ω3/∑ω6	11.99 ± 0.56^{a}	14.52 ± 1.10^{b}	14.68 ± 1.91^{b}	8.33 ± 0.21^{c}	7.79 ± 0.19^{c}
ω3 HUFA	10.33 ± 0.30^{ab}	9.67 ± 0.50^{a}	11.53 ± 1.28^{b}	16.69 ± 0.50^{c}	19.10 ± 0.60^{d}
DHA/EPA	83.85 ± 16.71^{a}	119.35 ± 19.11^{ab}	134.44 ± 30.34^{b}	33.14 ± 0.59^{c}	30.66 ± 1.32^{c}
(SFA+MUFA)/ PUFA	0.70 ± 0.01^{a}	0.51 ± 0.01^{b}	0.61 ± 0.02^{c}	0.55 ± 0.01^{d}	0.52 ± 0.01^{bd}
Total (mg/g)[2]	51.13 ± 1.49^{a}	43.60 ± 2.14^{b}	73.18 ± 5.14^{c}	105.18 ± 1.81^{d}	115.70 ± 2.98^{e}

Values (means ± SD of four replications) in the same row, not sharing a common superscript are significantly different ($p < 0.05$).
[1]Contains ω9 and ω7 isomers; [2]Contains some minor components not shown; n.d. – non detected; SFA – Saturated fatty acid; MUFA – Monounsaturated fatty acid; PUFA – Polyunsaturated fatty acid; HUFA – Highly unsaturated fatty acid.

In microalgae two pathways can lead to HUFA formation being LA the common precursor which is posteriorly converted to ALA or to gamma linolenic acid (GLA, C18:3ω6) (35). In *Isochrysis* sp. both LA and ALA are presented in concentrations below 10 mg g^{-1}, of dry biomass in contrast with the final highly unsaturated product DHA. These results are within those previously obtained by Huerlimann (35) for *Isochrysis aff. Galbana* (T. ISO). Moreover, the highest content of LA observed in T1 and T2 for *R. marina* can be explained by the downregulation of the desaturases and elongases, induced by the low nutrient availability. This is confirmed by a reduction in ω3 HUFA content and the increase of intermediate FA concentration. The experiment results suggest that *N. gaditana* media wasn't the most appropriate for HUFA accumulation since the highest ω3 and ω6 FA obtained were LA and ALA previously

referred as substrates for desaturation and elongation pathway that lead to HUFA synthesis (35).

The three marine microalgae presented ∑ω3/∑ω6 ratios greater than 1 independently of growth medium nutrient availability treatments applied. This suggests that ω3 pathway is more active than the ω6. For T5 *R. marina* achieved the highest ratio comprising 28 times more ω3 FA than ω6 FA. According to Huerlimann (35) this can be explained by the substrate specificity of the enzymes set (namely desaturases) that link ω3 and ω6 pathways which can be more active and preferentially diverted towards ω3 FA synthesis. Furthermore, *R. marina* fatty acid profile exhibited an increase of ∑ω3/∑ω6 ratio along with the nutrient input whereas *Isochrysis* sp. revealed an opposite trend. The previous observation support the assumption that the responses of species to equal inputs are species-specific and suggest that the nutrient availability affected the biosynthesis of polyunsaturated fatty acids possibly by turning the enzymes specificity towards ω6 FA synthesis, in *Isochrysis* sp., or ω3 FA synthesis, in *R. marina*.

Saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) are produced by *de novo* FA synthesis in chloroplast which posteriorly provides the substrates needed for PUFA biosynthesis in the endoplasmic reticulum, Figure 11 (35, 69). Through the experiment conducted with *N. gaditana* and *R. marina* the (SFA+MUFA)/PUFA ratio showed a mobilization of SFA and MUFA production towards PUFA synthesis with the increase of nutrient availability. In, overall the values observed for (SFA+MUFA)/PUFA ratio in the marine microalgae studied were below 1 with the exception of *N. gaditana* in T1 and T2 with 2.81 and 1.30, respectively.

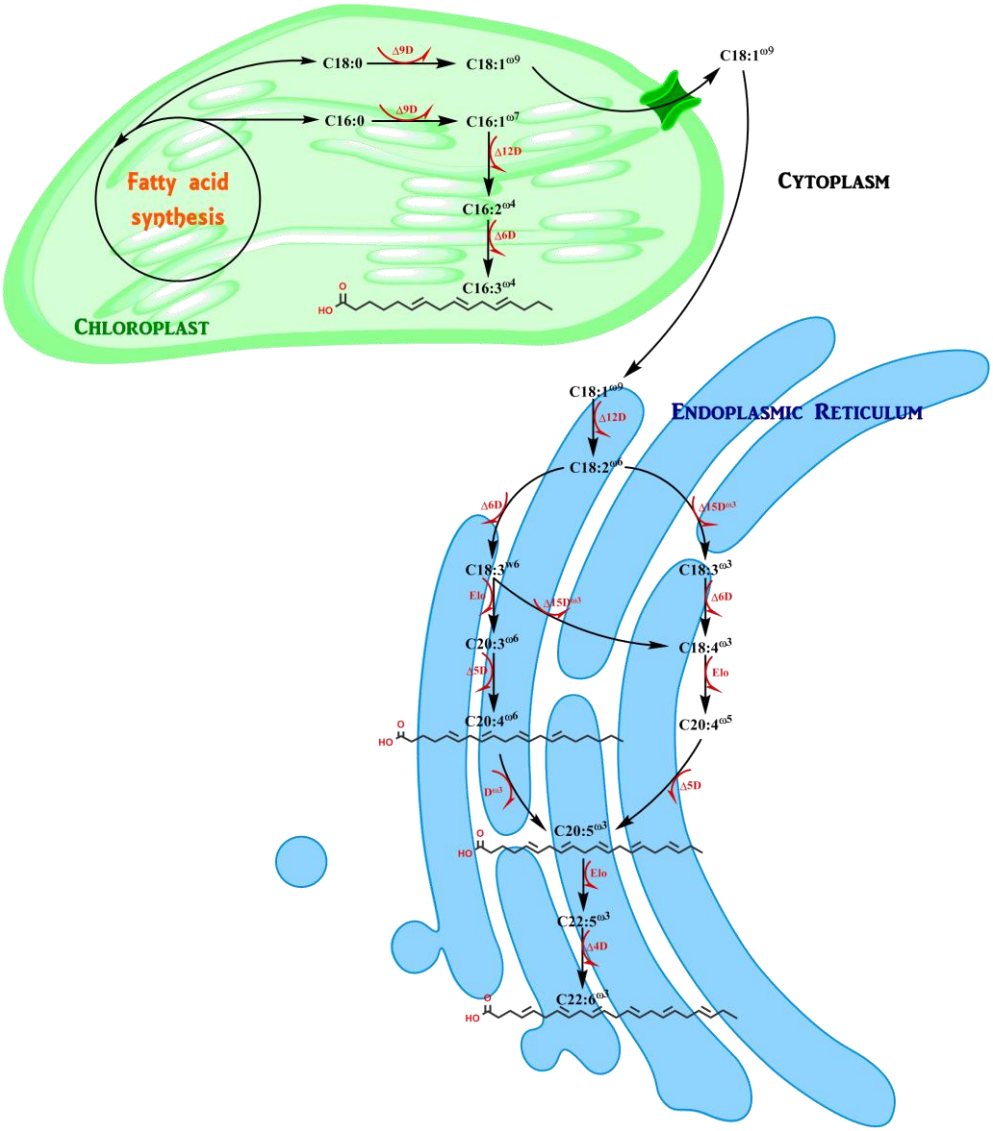

Figure 11. Schematic representation of fatty acids biosynthesis in chloroplast (SFA and MUFA) and endoplasmic reticulum (PUFA), based on Muhlroth (62).

Algae diets with low (SFA+MUFA)/PUFA ratio and ω3/ω6 ratio higher than 2 (∑ω3/∑ω6 > 2) are optimal for feeding larvae and juvenile oysters (72). In this order, the FA profile presented in Tables 5 – 7, shows that both *R. marina* and *Isochrysis* sp., in all treatments, exceeded the earlier recommended minimal along with (SFA+MUFA)/PUFA ratios inferior to 1. In opposition, ∑ω3/∑ω6 ratio in *N. gaditana* were lower than 2 with the exception of those subjected to treatments T2 and T3, making these treatments suitable for the production of this microalga for aquaculture.

4.3.EPA and DHA productivities

The implementation of strategies that induce the accumulation of specific compounds with high value may differentially influence microalgae growth (68). In order to improve microalgae production for commercial applications the productivity of the cultivation systems must be taken into account (65).

According to the productivities presented in Table 6 the growth mediums with the highest EPA and DHA contents along with the highest specific growth rates are T3 for *R. marina* and *N. gaditana*, and T4 for *Isochrysis* sp. Since *N. gaditana* demonstrated similar productivities in T3 and T4 the selection of the best productivity has to be made having in account the costs, essential in terms of commercial applications. DHA productivity isn't applied to *N. gaditana* microalgae once they don't present this specific fatty acid in their fatty acid profile.

Table 8. EPA and DHA **productivities** for *N. gaditana*, *R. marina* and *Isochrysis* sp. according to growth medium nutrient availability.

Experiment	Microalgae	Productivities (mg g^{-1} DW d^{-1})	
		P_{EPA}	P_{DHA}
T1	*N. gaditana*	0.04 ± 0.00	-
	R. marina	9.65 ± 0.75	6.43 ± 0.57
	Isochrysis sp.	0.09 ± 0.02	7.52 ± 0.20
T2	*N. gaditana*	0.04 ± 0.00	-
	R. marina	13.27 ± 0.84	9.63 ± 1.00
	Isochrysis sp.	0.09 ± 0.02	11.10 ± 0.56
T3	*N. gaditana*	0.28 ± 0.03	-
	R. marina	15.54 ± 1.54	11.55 ± 1.25
	Isochrysis sp.	0.08 ± 0.01	8.46 ± 0.93
T4	*N. gaditana*	0.28 ± 0.00	-
	R. marina	15.11 ± 0.92	9.71 ± 0.96
	Isochrysis sp.	0.52 ± 0.01	17.04 ± 0.41
T5	*N. gaditana*	0.12 ± 0.01	-
	R. marina	9.65 ± 1.21	5.58 ± 0.60
	Isochrysis sp.	0.47 ± 0.02	14.16 ± 0.39

P_{EPA} – eicosapentaenoic acid productivity; P_{DHA} – docosahexaenoic acid productivity; DW – Dry biomass weight.

The productivities of *N. gaditana* were low compared to that obtained for the other microalgae mainly due to their low fatty acid content observed in all the experiments conducted.

5. Conclusion

The manipulation of growth medium influenced fatty acid pattern and protein enabling the improvement of biochemical composition of the three microalgae species for aquaculture purposes. The displayed mobilization of fatty acid groups with nutrients input constitutes an advantage for modelling the biochemical composition of microalgae focusing on the area of application. Lowest nutrient concentrations (T1 and T2) induced the interruption of synthetic pathways (elongation and desaturation) resulting in the decrease of nutritional quality of microalgae as food resource for aquaculture. In the current experiment the best growth mediums for the accumulation of the desired products were T3 for both *R. marina* and *N. gaditana* and T4 for *Isochrysis* sp. Concluding, *Isochrysis* sp. is the microalgae species with the highest requirement of nutrient concentration to promote FA accumulation and finally, the commercial medium used in this experiment wasn't suitable for the accumulation of EPA in *N. gaditana*.

CHAPTER IV. Assessment of growth medium nutrient availability impact on marine microalgae carbohydrate

1. Abstract

The metabolic flexibility of microalgae allow the enhancement of their composition to suit their use or application. Therefore, it's essential to understand the downstream cellular processes which are activated under stressful conditions and lead to the desired carbon allocation. Since organizational differences within microalgae influence the carbon allocation, this study aimed to evaluate the impact of nutrient availability on the carbohydrate profile of three marine microalgae (*N. gaditana*, *R. marina* and *Isochrysis* sp.) and link the monosaccharides composition with the carbohydrate of origin based on phylogenetic data. The microalgae species were grown in a batch culture system upon different growth medium nutrient availabilities. Monosaccharides were analysed as alditol acetates. In *N. gaditana* glucose was the major monosaccharide (63 – 75 % of total monosaccharides) for all the study growth media. For both *R. marina* and *Isochrysis* sp. glucose was the principal sugar only in cultures with lowest growth medium nutrient availabilities (76 – 98 % and 61 – 79 %, respectively). The highest contents of glucans in *N. gaditana* and *Isochrysis* sp. indicated the presence of *β*-D-glucans (Chrysolaminarin) and cellulose whereas in *R. marina* might be explained by starch production. *N. gaditana* presented the highest diversity of monosaccharides detected. The decrease of glucans with nutrient availability observed for *R. marina* and *Isochrysis* sp. indicates the occurrence of a possible degradation of carbohydrates towards lipids and proteins biosynthesis. This study demonstrated an effective strategy to enhance biochemical composition for the marine microalgae studied. *Isochrysis* sp. and *R. marina* were the microalgae with the highest potential in regard to carbohydrates applications.

Keywords: Microalgae, Monosaccharide composition, Nutrient availability, *N. gaditana*, *R. marina*, *Isochrysis* sp.

2. Introduction

Microalgae are photosynthetic organisms known for being potential sources of natural compounds which can be applied in therapeutics and biotechnological applications (73). The ability of these microorganisms to readily adapt to growth fluctuating conditions, attributed to their metabolic flexibility, constitutes an advantage in modulating their biomass composition for commercial purposes (11, 30). In this regard, strategies such as the manipulation of parameters like media nutrient concentrations can be taken in order to modify the nature, amount and composition of the products synthesized (18).

Carbohydrates are the main products of photosynthetically-fixed carbon which can be stored intracellularly in multiple forms, such as starch, or deposited into structural polysaccharides (31, 74, 75). Monosaccharides are the building-blocks of the latest molecules and their composition can give an insight of polysaccharide predominance in microalgae (76-78). This is a crucial factor when selecting species for aquaculture feeding and for biotechnological applications, since polysaccharides composition determines the microalgae digestibility and their breakage, thus conditioning the extraction of cell wall coated valued products (77, 79).

The polysaccharides synthesized by microalgae species can be applied in several areas (30). For instance, starch and cellulose can be anaerobically converted to bioethanol, being the former more easily hydrolysable, whereas β-D-glucans (e.g. laminarans) have been attracting increasing attention due to their potential therapeutic applications (30, 31). Therefore β-D-glucans are known for enhancing the host immune system by binding to β-glucan receptors of cells involved in immune responses, such as macrophages and neutrophils, being their biological activity dependent on the ratio of $1\rightarrow3$ and $1\rightarrow6$ linkages and chain length (73, 80). Furthermore, sulphated polysaccharides have multiple biological activities, namely antiviral, antioxidant and anti-inflammatory (81). In addition to these applications polysaccharides can be used in food technology as emulsifiers and also as stabilizers in various food products (30).

To enhance microalgae compounds productivity it's essential to understand the downstream cellular processes that leads to the partitioning of carbon precursors into the multiple forms of carbon storage (14, 31, 82). Nevertheless, organizational

differences within microalgae species might affect processes such as photosynthesis and carbon flux through metabolic networks (14). Therefore, the present study aimed to evaluate the impact of nutrient availability on the carbohydrate profile of *Nannochloropsis gaditana*, *Rhodomonas marina* and *Isochrysis* sp. microalgae and link the monosaccharides composition with the carbohydrate of origin based on phylogenetic data.

3. Materials and Methods

3.1.Chemicals

Ammoniac, sodium borohydride and 1-methylimidazole were acquired from Merck (Darmstadt, Germany). Glacial acetic acid and acetic anhydride were purchased at Riedel-de Haën (St. Louis, MO, USA). Sulfuric acid was acquired from sigma-Aldrich (St. Louis, MO, USA) and dichloromethane from VWR (Carnaxide, Portugal). All the reagents used had analytical grade.

3.2.Algal Growth

The experimental design, cell densities and specific growth rate determinations are described in sections 3.2. and 3.3. of chapter II.

3.3.Neutral Carbohydrate Composition

3.3.1. Acid hydrolysis and alditol acetate derivatization

Monosaccharides were analysed as alditol acetate according to modified Blakeney (83). Briefly, 10 mg of dried algal biomass were exposed to a two stage sulphuric acid hydrolysis (3 h at 20°C in 72% sulfuric acid, followed by 2.5 h at 100°C, after water addition, in an oil bath). After cooling to room temperature, 200 µL of internal standard (2–deoxyglucose – 20 mg/mL) was added to the hydrolysate. To 1 mL aliquot of hydrolysate mixture, 200 µL of 25% ammoniac were added and the reduction of monosaccharides to alditol was performed. The reduction procedure involved the addition of 100 µL of 3M ammoniac solution containing 150 mg/mL of sodium borohydride and the incubation at 30°C for 1 h in a water bath. Then two additions of 50 µL of glacial acetic acid, followed by homogenization, were carried out. Alditol

acetylation was performed by the addition of 0.45 mL of 1-methylimidazole and 3 mL of acetic anhydride to 0.3 mL of the previous mixture. Next, the solution was incubated at 30ºC for 30 min in a water bath. The derivatized monosaccharides (alditol acetate) were extracted with dichloromethane being posteriorly washed several times with water. The solvent was evaporated under a nitrogen atmosphere. The standard solutions were also derivatized prior to GC–MS analysis. At least two replicates were made.

3.3.2. GC–MS Analysis

Alditol acetates were analysed by gas chromatography (Agilent HP 6890) equipped with a mass selective detector (Agilent 5973) and a capillary column DB-225 J&W (30 m x 0.25 mm inner diameter, 0.15 μm film thickness) from Agilent. The inlet temperature was 220°C and the column temperature was held at 220°C for 5min, ramped at 10°/min. to 230°C and kept in this temperature for 6min. The transfer line temperature was 280°C, the split ratio was 1:30 and Helium was used as the carrier gas with a flow rate of 1.2 mL/min. The derivatized monosaccharides were identified by comparing the retention times and mass spectra fragmentation with that obtained through injection of the standards. The quantification of neutral monosaccharides was made through the calculated response factor of each standard towards the internal standard. The standards used were 2–deoxyglucose, L(+)arabinose, D(+)xylose, D(+)galactose, D(+)glucose, D(+)mannose, D(+)rhamnose, D(+)fucose purchased at Sigma-Aldrich (St. Louis, MO, USA). Four replicates were performed for each GC–MS analysis being the results presented as the mean value ± standard deviation (SD) of alditol acetate expressed in mg g^{-1} of dry biomass weight (DW).

3.4.Statistical Analysis

Statistical analysis of the data was carried out using the software IBM SPSS Statistics 23. Differences between growth medium nutrient availability treatments were assessed by one-way analysis of variance (ANOVA) followed by a Tukey's Post Hoc analysis; p–values < 0.05 were considered to be statistically significant.

4. Results and Discussion

Carbohydrates are the major products of photosynthesis and play a structural role as main components of cell walls and organelles membranes (e.g. glycolipids), and as storage materials providing the energy necessary for the metabolic processes in organisms (31, 79, 84). In photosynthetic systems, nutrient availability is crucial for the viability, physiology and metabolic processes of organisms (18). In this regard, the assessment of the nutrient availability effect on microalgae is essential to give an insight of the structural and functional adaptations that lead to a successful adaptation.

Monosaccharide compositional analysis can give an overview about the original polysaccharide structure, cell wall composition and storage products based on phylogeny information from the literature (31, 85). The data in Table 9 show some variation in the neutral monosaccharides detected among the three marine microalgae studied. Results show significant differences ($p < 0.05$) in the amount and composition of monosaccharides in the three microalgae grown under different growth medium nutrient availabilities. The predominant sugar in *N. gaditana* was glucose accounting 63 – 75 % of total monosaccharides (TM), regardless the treatments applied. This observation is according the previous results obtained by Brown (77) for another strain of *Nannochloropsis* (*N. oculata*) cultured in f/2 medium, where glucose accounted for 68% of total monosaccharides. The large amount of this monosaccharide is likely to be derived from chrysolaminarin ((1→3)-*β*-glucan) or cellulose ((1→4)-*β*-glucan) which encompasses the major glucans in microalgae belonging to Heterokontophyta division (86).

Table 9. Monosaccharide profile of *N. gaditana*, *R. marina* and *Isochrysis* sp. according to growth medium nutrient availability.

	Monosaccharide (mg g^{-1} DW)	Treatment				
		T1	T2	T3	T4	T5
N. gaditana	Fucose	0.58 ± 0.05^{a}	0.23 ± 0.03^{b}	0.61 ± 0.04^{a}	0.71 ± 0.06^{a}	1.31 ± 0.15^{c}
	Xylose	1.20 ± 0.09^{a}	0.76 ± 0.02^{a}	2.37 ± 0.34^{b}	5.43 ± 0.25^{c}	6.05 ± 0.31^{d}
	Rhamnose	0.55 ± 0.06^{a}	0.46 ± 0.03^{a}	3.42 ± 0.30^{b}	7.77 ± 0.40^{c}	8.62 ± 0.36^{d}
	Mannose	0.26 ± 0.03^{a}	0.55 ± 0.04^{b}	0.30 ± 0.01^{a}	1.76 ± 0.16^{c}	2.64 ± 0.09^{d}
	Galactose	2.25 ± 0.07^{a}	2.09 ± 0.12^{a}	4.33 ± 0.39^{b}	10.15 ± 0.50^{c}	11.02 ± 0.62^{c}
	Glucose	13.52 ± 0.43^{a}	12.37 ± 1.04^{a}	24.01 ± 0.66^{b}	46.81 ± 0.67^{c}	50.15 ± 1.18^{d}
	Total	18.57 ± 0.63^{a}	16.46 ± 1.18^{a}	35.04 ± 1.36^{b}	72.64 ± 1.41^{c}	79.79 ± 1.61^{d}
R. marina	Fucose	n.d.	n.d.	n.d.	0.06 ± 0.00	n.d.
	Rhamnose	0.64 ± 0.05^{a}	n.d.	n.d.	n.d.	2.20 ± 0.09^{b}
	Mannose	n.d.	n.d.	n.d.	n.d.	0.91 ± 0.05
	Galactose	2.23 ± 0.28^{a}	12.21 ± 0.99^{b}	8.93 ± 1.17^{c}	1.74 ± 0.10^{a}	n.d.
	Glucose	142.66 ± 2.04^{a}	195.29 ± 3.61^{b}	27.78 ± 1.08^{c}	0.25 ± 0.02^{d}	n.d.
	Total	145.53 ± 2.33^{a}	207.50 ± 4.59^{b}	36.71 ± 2.11^{c}	2.06 ± 0.10^{d}	3.11 ± 0.12^{d}
Isochrysis sp.	Arabinose	3.62 ± 0.17^{a}	3.80 ± 0.60^{a}	4.59 ± 0.28^{b}	4.31 ± 0.15^{ab}	4.59 ± 0.15^{b}
	Xylose	0.29 ± 0.03^{a}	0.25 ± 0.02^{a}	0.57 ± 0.06^{b}	0.51 ± 0.04^{bc}	0.44 ± 0.06^{c}
	Mannose	8.97 ± 0.91^{a}	8.99 ± 0.95^{a}	11.96 ± 0.92^{b}	17.32 ± 0.87^{c}	17.94 ± 1.03^{c}
	Galactose	23.48 ± 0.96^{a}	23.79 ± 1.97^{a}	31.44 ± 1.10^{b}	30.74 ± 1.12^{b}	33.46 ± 2.49^{b}
	Glucose	136.77 ± 2.80^{a}	107.78 ± 3.54^{b}	76.91 ± 1.87^{c}	35.37 ± 1.78^{d}	35.41 ± 1.43^{d}
	Total	173.13 ± 3.81^{a}	144.61 ± 5.64^{b}	125.48 ± 4.03^{c}	88.24 ± 1.54^{d}	91.84 ± 4.82^{d}

Values (means ± standard deviations of four replications) in the same row, not sharing a common superscript are significantly different ($p < 0.05$). n.d. – non detected

In *R. marina* a variation in the neutral monosaccharides composition was observed with the nutrient availability, comprising an alteration of the predominant sugars. For T1 – T3 the predominant sugar was glucose (76 – 98 % of TM) changing for galactose at T4 (85 % of TM) and Rhamnose at T5 (71 % of TM). The predominance of glucose in T1 – T3 suggests that these treatments induced an increase in the storage of polysaccharides namely starch ((1→4)-*α*-glucan) (86). Although changes in the monosaccharide pattern indicates that the increase of dissolved minerals in growth medium probably induced an osmotic stress. Once, some microalgae respond to salt stresses by breaking down carbohydrates and accumulating molecules deriving from these, such as glycerol (84).

For *Isochrysis* sp. both galactose and glucose were the predominant sugars representing 75 – 93 % of TM. However their proportions changed with nutrient availability. This might be due to the polysaccharide scales constituted of cellulose-like polymers coating cells and chrysolaminarin storage explaining the predominance of glucans, whereas galactose predominance can be derived from the glycolipid content,

presented previously in section 4.3. of chapter II (86-88). In Brown (77) glucose was the main monosaccharide for two strains of *Isochrysis* genus in f/2 medium accounting 70 and 77% of TM.

Results from Table 9 show that *N. gaditana* total monosaccharides content increased in response to nutrient input. Having in account that the increase in monosaccharide content wasn't made at the expense of lipids and proteins (Table 4 chapter II), the pattern observed might be explained by the accumulation of low molecular weight carbohydrates which may act as osmoregulators or as energy reserves (84).

The matrix and fibrillar fractions of cell wall possess other polysaccharides than glucans, which interconnect cellulose micro fibrils (89). Figure 12 displays the variation of monosaccharide proportions in *N. gaditana*. Here the decrease of glucose proportion in contrast to rhamnose was observed. This might suggest that *N. gaditana* under high growth medium nutrient availabilities displace preference towards rhamnans production for structural purposes and/or for suppress the effects of salt stress. Hereof, the presence of fucose indicates a possible occurrence of sulphated fucans which were evidenced in Heterokonts cell wall (90).

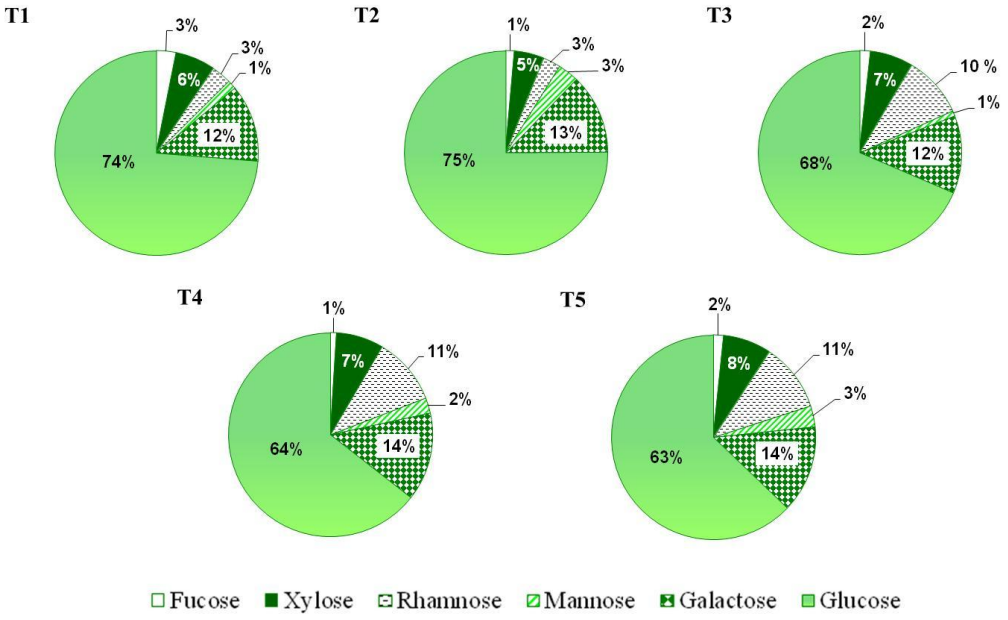

Figure 12. *N. gaditana* monosaccharide proportions regarding growth medium nutrient availability.

For *R. marina* the lowest growth medium nutrient availabilities presented highest contents of monosaccharides 21 – 15 % of dry biomass. According to Silva (91)

a low rate of photosynthetic activity induced an increase in the carbohydrate content for *Rhodomonas* sp. Base on that, the previous observation support the assumption that under stressful conditions affecting microalgae photosynthesis efficiency, the metabolic processes are channelled up towards carbohydrate accumulation in order to fulfil cells requirements. Since treatments with low nutrient concentrations, encompass low concentration of cofactors needed for the maintenance of photosynthetic reactions (18). On the other hand, the highest nutrient availability (T5) seemed to act as a toxic input for *R. marina*, once glucose was absent in monosaccharide composition and a decrease in other parameters such as lipids, proteins and cells concentration were observed in section 4.2. of chapter II.

Isochrysis sp. total monosaccharides composition at low nutrient availabilities displayed an analogous trend to *R. marina* presenting the highest contents (17 – 14 % of DW) at low growth medium nutrient availabilities. However, this microalga appears to be more resistant to the stress input, used in this experiment, because a decrease in the other biochemical parameters wasn't observed (Table 4 chapter II). Furthermore, the decrease of total monosaccharides content with the increase of nutrient availability can be attributed to the use of carbohydrates as substrates for lipid and/or protein synthesis. Once nitrogen assimilation might trigger the addition of nitrogen to carbon skeletons provided by carbohydrates, which are the carbon sink of organisms (31, 91).

Figure 13 exhibits the variation of the monosaccharide composition pattern of *I. galbana* along with nutrient availability in the growth medium. Attending to monosaccharides, glucose was inversely related with the nutrient input (R^2 = 0.94) whereas galactose presented a positive linear relationship with this parameter (R^2 = 0.95). The highest contents of glucose at low growth medium nutrient availabilities might be explained by the accumulation of soluble linked ((1→3)-*β*-glucan (chrysolaminarin) as storage compounds in cells (86).

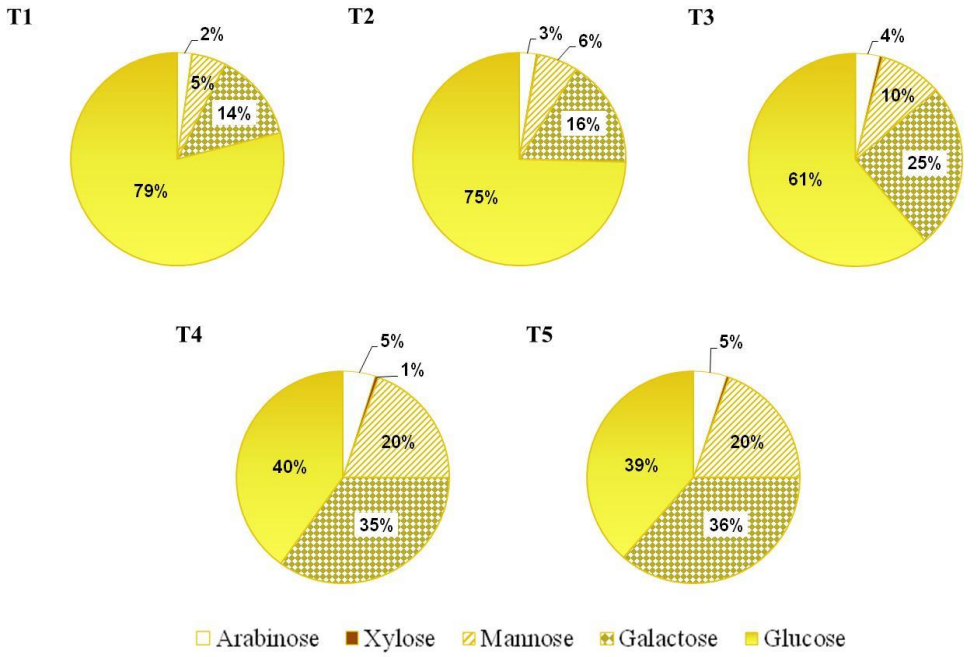

Figure 13. *Isochrysis* sp. monosaccharide proportions regarding growth medium nutrient availabilities.

Moreover the increase of mannose and galactose proportions with growth medium nutrient availability, Fig. 13, can be explained by the analogous behaviour of these monosaccharides and glycolipid content in regard to nutrient availability which suggest that glycolipids comprise large concentrations of galactolipids (e.g. monogalactosyl-diacylglycerol, digalactosyl-diacylglycerol) and mannose glycoconjugates (85). Likewise, the presence of arabinose could be derived from arabinogalactans proteins which make up the cell wall (85).

In general, *Isochrysis* sp. and *R. marina* cultured at the lowest growth medium nutrient availabilities (T1 and T2) contained more glucose-based carbohydrates making these cultures the most suitable for bioethanol production (31). Furthermore, in a previous study, a highly branched (1→3, 1→6)-*β*-D-glucan extracted from *Isochrysis galbana* was demonstrated to inhibit the proliferation of tumor cells (73). Thus the highest content of glucans previously mentioned for *Isochrysis* sp. might comprise highest content of this bioactive compound. Nevertheless, according to phylogenetic information *N. gaditana* also store carbon in polysaccharides of this class of compounds (*β*-D-glucan).

5. Conclusion

Nutrient availability influenced neutral monosaccharide pattern and highlighted the taxonomic differences between species. Under low growth medium nutrient availabilities carbon was stored mainly as carbohydrates for both *R. marina* and *Isochrysis* sp., in contrast to *N. gaditana*. Regarding to carbon partitioning, changes in the nutrient concentrations of growth medium influenced on the lipids/carbohydrates/proteins accumulation. This study demonstrated an effective strategy to enhance biochemical composition for the three marine microalgae. The monosaccharides behaviour with regard to nutrients input constitutes an advantage in modelling the monosaccharide content of microalgae concerning the biotechnological application. In this context *Isochrysis sp.* and *R. marina* were the microalgae with highest potential in regard to carbohydrates applications.

CHAPTER V. The influence of nitrogen and sulfur availabilities on the growth, biochemical and fatty acid composition of *Nannochloropsis gaditana*, *Rhodomonas marina* and *Isochrysis* sp. microalgae

1. Abstract

Fluctuations of nutrient supply is a stress-inducement strategy often applied for optimising microalgae composition for industrial purposes. However, few information is found about nitrogen and sulfur acquisition and metabolisms interactions in microalgae. The aims of the present study were to investigate the effect of nitrogen and sulfur growth medium availabilities on biochemical composition, growth and elemental stoichiometry of *N. gaditana*, *R. marina* and *Isochrysis* sp. The optimal growth condition that lead to the maximum eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) productivities in these three microalgae was determined. Experiments were conducted in a batch cultivation system and a range of nitrogen and sulfur availabilities were applied to the growth medium. *N. gaditana* exhibited the highest variation in their biochemical composition. At the highest growth medium nitrogen availabilities, maximum productivities were achieved for overall biochemical parameters (Protein: 10.33 mg g^{-1} DW d^{-1}; Lipids: 4.71 mg g^{-1} DW d^{-1}; EPA: 1.05 mg g^{-1} DW d^{-1}). Slowest growth was observed at highest growth medium sulfur availabilities despite of highest protein and lipid levels were attained in these treatments. In both *Isochrysis* sp. and *R. marina*, maximum EPA and DHA productivities were reached in control (2000 μL L^{-1} of commercial nutrient solution) and threshold sulfur concentrations were achieved in the treatment with the highest sulfur input. The variations observed for microalgae biochemical composition were reflected in their elemental stoichiometry. These results suggest that *R. marina* responded to changes in growth media nutrient supply by homeostatic control of its elemental and biochemical composition and that the stress-inducement strategy applied for rising EPA productivities was effective for *N. gaditana*.

Keywords: Microalgae, EPA, DHA, elemental stoichiometry, nitrogen; sulfur

2. Introduction

Microalgae are known for being primary sources of essential nutrients that display important functions in human metabolism (30). In this regard, polyunsaturated fatty acids (PUFAs) are acknowledged by their structural and physiological roles (e.g. biosynthesis of eicosanoids) (30). Moreover, the fact of being involved on the mitigation of inflammatory processes, cardiovascular health and cancer prevention makes these components suitable for therapeutic and pharmaceutical applications (30). In aquaculture field, microalgae are used by their high amounts of PUFAs in order to incorporate them into fish lipids (30). Therefore their enhancement in polyunsaturated fatty acids (PUFAs), namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), is crucial for the use of microalgae for aquaculture purposes (30, 40).

The statement that microalgae nutritional value can be manipulated through changes in their growth conditions has been the focus of industrial and scientific developments (40). Changes in growth media, namely in nutrient supply comprise a stress-inducement strategy often applied with the aim of optimising microalgae composition for commercial and aquaculture purposes (29, 92, 93). Nitrogen and sulfur are both macronutrients crucial for protein and vitamin biosynthesis and chlorophyll production (N), which are essential to meet growing cells requirements (22, 68, 94). Moreover, their availabilities in growth medium may affect the amount and activity of coenzymes (e.g. biocytin, coenzyme A and thiamine pyrophosphate) since they are essential components of these macromolecules (95). Coenzymes affect the exit point of Calvin cycle which stimulates the synthesis of carbohydrates as the metabolic pathways that are closely linked to these (31, 95). Thus the presence and concentration of these nutrients can influence the growth, biochemical composition and product yield of microalgae (18).

Although, nitrogen and sulfur had been reported has the most abundant elementary components in microalgae little information is taken about the interaction of nitrogen with sulfur metabolisms (22, 96). Nitrogen and sulfur can be obtained as nitrate and sulphate by photosynthetic cells (+VI) being their assimilation tightly regulated and dependent on their own availabilities alongside with their ratios relatively to the other medium components (22, 94, 97). For instance, sulphate assimilation is reported to be induced by its deficiency in the growth medium in contrast to nitrate (22).

When microalgae are exposed to changes in their growth conditions they may respond in two ways: maintain the cell composition and functional activities at the expense of growth (homeostasis) or modulate their metabolic strategies and cell composition (acclimation) (98). For instance, nutrients fluctuations may comprise alterations in their acquisition and metabolism leading to changes in microalgae elemental composition (98). The way, in which microalgae, respond is linked with their phylogeny, gene expression and metabolic regulation (22). Thus, the aims of the present study were to investigate the effect of nitrogen and sulfur growth medium availabilities on biochemical composition, growth and elemental stoichiometry of *Rhodomonas marina*, *Nannochloropsis gaditana* and *Isochrysis* sp. and to determine the optimal growth conditions that lead to maximum EPA and DHA productivities.

3. Materials and Methods

3.1.Chemicals

Methanol, heptane, sodium nitrate and sodium sulfate were acquired from Sigma-Aldrich (St. Louis, MO, USA). Chloroform and potassium chloride were acquired from VWR (Carnaxide, Portugal). Ethyl acetate was from Merck (Darmstadt, Germany). Lugols solution from bioMérieux (Linda-a-Velha, Portugal). All the chemicals used had analytical grade. Lugols solution was aquired from bioMérieux (Linda-a-Velha, Portugal). All the chemicals used for analytical purposes had analytical grade.

3.2.Growth conditions and experimental design

The *Isochrysis* sp., *R. marina* and *N. gaditana* were supplied by the Mariculture Center of Calheta (Madeira, Portugal). The cultivation of each microalga was performed by inoculating starter cultures into 500 mL of enriched seawater. The inoculation cell number was maintained at 2.5×10^6 cells mL^{-1} and 1.4×10^5 cells mL^{-1} for *R. marina*. The natural seawater used, for media preparation, was previously adjusted to a salinity of 25 g L^{-1} and sterilized in an autoclave (Uniclav 88) at 121 ºC for 15 min.

Data of chapter II showed that *N. gaditana* and *R. marina* growth was reduced in treatments with growth medium nutrient availabilities higher than 2 mL L^{-1} of seawater enriched with commercial Nutribloom puls (Necton, Portugal). This suggesting that was the critical concentration where a significant increase in nutrient might lead to reduced growth and overall low productivity. For assess the effect of nitrogen concentration additions of 4.48 and 6.72 mg of $NaNO_3$ L^{-1}, were made to the seawater previously enriched with 2 mL L^{-1} of Nutribloom plus medium. These additions corresponded to N/S molar ratios of 654:1 (N1) and 658:1 (N2).

To study the effect of sulfur concentration on the growth and biochemical composition of microalgae, different cultures were performed at three N/S molar ratios: 645:1 (NS, control), 190:1 (S1), 111:1 (S2), which corresponded to initial sulfur concentrations of 0.40, 1.34, 2.3 mg L^{-1}. In both experiments, the concentrations of the other nutrients were those found in the Nutribloom plus medium; established has the control (NS).

All the experiments were conducted at a temperature of 23 ± 1 ºC, with a photoperiod of 18:6 h light/dark cycles, pH ranging 7 – 9, a light intensity of 3841 ± 560 lux and compressed air was used for aeration of cell cultures. The microalgae were harvested at stationary phase, centrifugated (centrifuge Labofuge 200 – Heraeus) for 5 min. at 4500 rpm and washed with distilled water. Duplicates were conducted for all the experiments.

Microalgae growth was monitored daily with a Neubauer–improved counting chamber (Marienfield–Superior) and a light microscope (Olympus BX41) with a 40x magnification. The specific growth rate was estimated as the slope of the regression line of the logistic model described by Xin (41) and productivity was calculated based on Hoffmann (57). These procedures are briefly described in section 3.2. of chapter II and section 3.3. of chapter III.

3.3.Analytical procedures

Determination of CHNS content was made by an elemental analyser Truspec 630-200-200. Total protein was assessed by multiplying the nitrogen content for 6.25 as described by Kim (39). Extraction of total lipids was performed according to modified Bligh & Dyer (45) (section 3.4. of chapter II).

3.4.Fatty acid determination

The fatty acid composition of algal dried biomass was determined as fatty acid methyl esters (FAMEs) as previously described by Lepage & Roy (63), modified by Cohen (64). FAMEs were analysed by gas chromatography (Agilent HP 6890) equipped with a mass selective detector (Agilent 5973) and a fused silica capillary column DB-5MS (30 m x 0.25 mm inner diameter, 0.25 μm film thickness) from J&W scientific. The chromatographic conditions were as follows: oven initial temperature was 150 ºC for 2 min; increasing 3 ºC min^{-1} to 205 ºC and kept for 2 min., 3 ºC min^{-1} to 230 ºC and 30 ºC min^{-1} until reach the final temperature of 300 ºC for 5 min; transfer line temperature 260 ºC; detector temperature, 270 ºC; split ratio, 40:1. Helium was used as the carrier gas with a flow rate of 1mL min^{-1}. The fatty acid methyl esters identification was accomplished by comparing the retention times and mass spectra fragmentation to those of known standards (bacterial acid methyl esters CP mix and supelco 37 component FAME mix from supelco). Four replicates were performed for each GC

analysis being the results presented as the mean value ± standard deviation (SD) of FAME expressed in mg g^{-1} of dry biomass weight (DW). The internal standard used was the heneicosanoic acid (C21:0).

3.5. Statistical analysis

Statistical analysis of the data was carried out using the software IBM SPSS Statistics 23. Differences between growth media nutrient availability treatments were assessed by one-way analysis of variance (ANOVA) followed by a Tukey's Post Hoc analysis; p–values < 0.05 were considered to be statistically significant.

4. Results and Discussion

4.1. Microalgae growth and productivities

Algae culture media comprises macronutrients that influence growth and biochemical composition of microalgae (99). Since nitrogen and sulfur are involved as catalysts components in the downstream cellular processes that lead to fatty acid synthesis, the manipulation of their availabilities on growth medium can induce changes on growth rate, protein and lipid levels (62, 99). Thus, it is important to evaluate the impact of N/S ratios over the maximization of EPA and DHA production alongside with the sensitivity of microalgae towards variations in nitrogen content.

The productivity of microalgae valued compounds is dependent on factors, such as: cellular concentrations, carbon fixation efficiency and conversion of photosynthate into the desired products (14, 57). Table 10 displays the microalgae growth rate, total lipids, total proteins, EPA and DHA productivities according to the different growth medium nitrogen and sulfur availabilities.

Table 10. Specific growth rates (r), biochemical composition (TL – total lipids; TP – total proteins), EPA and DHA productivities for cultures of *N. gaditana*, *R. marina* and *Isochrysis* sp.

	Treatment	r (d^{-1})	TL (% DW)	TP (% DW)	P_{EPA} (mg g^{-1} DW d^{-1})	P_{DHA} (mg g^{-1} DW d^{-1})
N. gaditana	S2	0.49	6.79 ± 0.11	14.86 ± 0.35	0.54 ± 0.01	-
	S1	0.53	7.11 ± 0.12	15.56 ± 0.36	0.71 ± 0.01	-
	NS	0.75	4.27 ± 0.38	11.23 ± 0.26	0.82 ± 0.01	-
	N1	0.67	6.33 ± 0.50	12.16 ± 0.28	0.88 ± 0.00	-
	N2	0.78	6.04 ± 0.46	13.24 ± 0.31	1.05 ± 0.04	-
R. marina	S2	0.96	11.79 ± 0.44	28.98 ± 0.68	7.06 ± 0.09	4.96 ± 0.14
	S1	1.01	15.02 ± 0.41	36.61 ± 0.86	10.25 ± 0.05	7.99 ± 0.24
	NS	1.12	12.09 ± 0.31	34.66 ± 0.81	13.57 ± 0.59	9.36 ± 0.61
	N1	1.04	13.14 ± 0.14	36.86 ± 0.86	12.36 ± 0.18	8.87 ± 0.17
	N2	1.06	15.01 ± 0.34	39.16 ± 0.91	11.68 ± 0.87	9.22 ± 0.42
Isochrysis sp.	S2	0.76	10.44 ± 0.37	11.73 ± 0.27	0.18 ± 0.01	3.98 ± 0.13
	S1	0.54	15.52 ± 0.42	18.06 ± 0.42	0.23 ± 0.01	5.19 ± 0.18
	NS	0.81	13.48 ± 0.02	17.04 ± 0.40	0.35 ± 0.01	8.20 ± 0.19
	N1	0.44	20.34 ± 0.13	22.99 ± 0.54	0.25 ± 0.01	5.21 ± 0.11
	N2	0.66	15.28 ± 0.39	21.97 ± 0.51	0.31 ± 0.01	6.87 ± 0.16

Through data analysis, the growth of *N. gaditana* seems to be affected by the different growth media. Microalgae grown in S1 growth media presented the highest maximum cell densities (36.54×10^6 cells mL^{-1}) whereas microalgae grown in N1 and

N2 growth media presented a decrease of 20 % in respect to the cell densities achieved in NS treatment, depicted in [Annex 1a]. Total lipid (TL) content ranged between 4 – 7 % of DW while total protein (TP) accounted 11 – 16 % of DW. These values aren't in accordance with those previously reported by the literature (100) which were three and two times higher regarding maximum TL and TP, respectively, obtained for *Nannochloropsis* sp. grown in f/2 medium. Moreover in the present study, treatments with highest sulfur concentrations presented highest lipid and protein contents in DW along with lower growth rates (0.53 and 0.49 d^{-1}) which in turn lead to lower overall productivity.

In *N. gaditana*, maximum EPA productivity was reached in growth medium that comprised the highest nitrogen input (N2), which means that under nitrogen–replete conditions an increased cell growth was coupled with a higher EPA content. This observation might be due to EPA structural role as a key component of organelles and cell membranes in *Nannochloropsis* (69).

Although *Isochrysis* sp. grown in N1 growth media presented higher lipid and protein contents, maximum EPA and DHA productivities were reached in the NS treatment (control). This is due to the reduced cell growth rate in N1 which consequently lead to overall low productivities. Furthermore, high lipid content is often offset by lower growth rates in microalgae (72).

In *R. marina* both growth and biochemical parameters didn't vary greatly in contrast with *Isochrysis* sp. and *N. gaditana*. However, an accentuated decrease in EPA and DHA productivities as well as a reduction of 21 % in maximum cell density [Annex 1b] and 16 % in protein levels regarding to NS, were observed in cultures with the lowest N/S ratio (S2). This observations can be explained by the highest sulfur input at S2 since as with nutrient limitation, nutrient oversupply can also constitute a stress and sometimes a toxic input in which an increase in nutrient concentrations leads to reduced growth rates and overall lowest productivities (19). Likewise *Isochrysis* sp., maximum EPA and DHA productivities were achieved in control.

4.2. Fatty acid composition

4.2.1. N. gaditana

Table 11 displays the fatty acid profile pattern upon a range of nitrogen and sulfur growth medium availabilities for *N. gaditana*. It's possible to note that the amount of individual fatty acids presented significant differences ($p < 0.05$) regarding to the treatments applied. The majoritarian fatty acids observed for this microalga were palmitic acid (C16:0) and alpha linolenic acid (ALA, C18:3ω3), which together accounted over 40 % of total fatty acids detected.

Table 11. *N. gaditana* fatty acid profile under several nitrogen and sulfur availabilities in growth media.

Fatty Acids (mg g^{-1} DW)	Treatment				
	S2	S1	NS	N1	N2
C14:0	0.28 ± 0.04^{a}	0.41 ± 0.01^{b}	0.29 ± 0.02^{a}	0.38 ± 0.02^{b}	0.40 ± 0.02^{b}
C16:0	5.07 ± 0.27^{a}	6.36 ± 0.13^{b}	4.54 ± 0.21^{c}	6.02 ± 0.07^{b}	6.18 ± 0.26^{b}
C18:0	0.16 ± 0.01^{a}	0.22 ± 0.00^{b}	0.16 ± 0.00^{a}	0.27 ± 0.00^{c}	0.26 ± 0.01^{c}
Total – SFA[2]	6.07 ± 0.31^{a}	7.74 ± 0.15^{b}	5.45 ± 0.24^{c}	7.36 ± 0.10^{b}	7.58 ± 0.31^{b}
C16:1[1]	1.43 ± 0.19^{ab}	1.52 ± 0.05^{a}	1.08 ± 0.04^{c}	1.24 ± 0.04^{cb}	1.23 ± 0.04^{c}
C18:1[1]	1.58 ± 0.15^{a}	2.24 ± 0.14^{b}	1.62 ± 0.09^{a}	2.70 ± 0.13^{c}	2.80 ± 0.08^{c}
Total – MUFA[2]	3.15 ± 0.14^{a}	3.95 ± 0.16^{b}	2.84 ± 0.12^{c}	4.15 ± 0.15^{b}	4.25 ± 0.10^{b}
C16:3ω3	1.40 ± 0.17^{a}	1.48 ± 0.03^{ab}	1.58 ± 0.07^{ab}	1.60 ± 0.01^{ab}	1.65 ± 0.08^{c}
C18:2ω6	3.22 ± 0.25^{e}	4.27 ± 0.07^{b}	2.76 ± 0.11^{a}	3.80 ± 0.03^{c}	3.86 ± 0.19^{c}
C18:3ω3	4.15 ± 0.35^{a}	4.85 ± 0.21^{ab}	4.66 ± 0.23^{ab}	4.88 ± 0.26^{ab}	5.43 ± 0.65^{b}
C18:4ω3	0.31 ± 0.00^{a}	0.25 ± 0.01^{b}	0.28 ± 0.01^{c}	0.23 ± 0.00^{b}	0.24 ± 0.01^{b}
C20:3ω6	0.12 ± 0.02^{a}	0.16 ± 0.00^{b}	0.08 ± 0.00^{c}	0.12 ± 0.00^{a}	0.13 ± 0.01^{a}
C20:4ω6 - AA	0.11 ± 0.02^{a}	0.15 ± 0.00^{b}	0.07 ± 0.00^{c}	0.11 ± 0.00^{a}	0.10 ± 0.00^{a}
C20:5ω3 - EPA	1.11 ± 0.03^{a}	1.35 ± 0.02^{b}	1.09 ± 0.02^{a}	1.31 ± 0.01^{b}	1.35 ± 0.06^{b}
Total – PUFA[2]	11.88 ± 0.37^{a}	14.29 ± 0.26^{b}	11.71 ± 0.41^{a}	13.56 ± 0.30^{b}	14.27 ± 1.00^{b}
ω3	7.18 ± 0.58^{a}	8.28 ± 0.21^{b}	7.90 ± 0.28^{ac}	8.37 ± 0.27^{bc}	9.02 ± 0.77^{bc}
∑ω6	3.51 ± 0.29^{e}	4.70 ± 0.07^{b}	2.97 ± 0.12^{a}	4.11 ± 0.03^{c}	4.18 ± 0.20^{c}
∑ω3/∑ω6	2.06 ± 0.34^{ab}	1.76 ± 0.04^{a}	2.66 ± 0.07^{c}	2.04 ± 0.06^{ab}	2.16 ± 0.10^{b}
ω3 HUFA	1.32 ± 0.07^{a}	1.69 ± 0.02^{b}	1.38 ± 0.02^{a}	1.66 ± 0.01^{b}	1.72 ± 0.06^{b}
EPA/AA	10.45 ± 2.96^{ab}	8.80 ± 0.09^{b}	15.59 ± 0.79^{c}	12.52 ± 0.17^{a}	12.99 ± 0.29^{ac}
Total[2]	21.10 ± 0.72^{a}	25.98 ± 0.47^{b}	20.00 ± 0.74^{a}	25.07 ± 0.42^{b}	26.11 ± 1.30^{b}

Values (means ± SD of four replications) in the same row, not sharing a common superscript are significantly different ($p < 0.05$). [1]Contains ω9 and ω7 isomers; [2]Contains some minor components not shown. SFA – Saturated fatty acid; MUFA – Monounsaturated fatty acid; PUFA – Polyunsaturated fatty acid; HUFA – Highly unsaturated fatty acid.

In order to counterbalance the nitrogen input in treatments N1 and N2, a raise in EPA content (20 % and 24 % DW) was observed. The same pattern was observed in treatment S1 where sulfur was added to growth media instead of nitrogen, having EPA

content increased 24% in DW. Furthermore, growth medium nutrient availability treatments presenting highest EPA contents shared highest C16:0, monounsaturated stearic acid (C18:1) and linoleic acid (LA, C18:2ω6) contents (Table 1). C16:0 and C18:1 are both products of plastidial *de novo* FA synthesis in chloroplast and can be further synthesized into polyunsaturated fatty acids (PUFAs) in the endoplasmic reticulum (35, 69). Thus, their enhancement along with polyunsaturated fatty acids (PUFAs) can be explained by increased plastidial production possibly induced by nitrogen inputs and sulfur growth medium availabilities in treatments (35). In this sense, the increase of EPA contents with chloroplast activity might be due to the physiological role of this PUFA as major component of organelles (e.g. thylakoid) and cell membranes (69).

Nitrogen and sulfur availabilities are known to influence photosynthesis efficiency and hence carbon assimilation and allocation (101). According to Mizuno (102) sulfur decrease leads to an increase in starch and lipid contents along with changes in the fatty acid composition of *Chlorella lobophora* and *Parachlorella kessleri*. However, these changes involve the increase in palmitic and stearic acids coupled with a decrease in PUFAs (102). In contrast to sulfur depletion conditions, which induce a rapid decrease in photosynthesis efficiency, chloroplast transcriptional activity proceeds normally in sulfur repleted media (94). Regarding the nitrogen input, previous studies corroborate the enhancement of EPA production by *Nannochloropsis* sp. under nitrogen–replete environments at saturating levels (68, 103).

4.2.2. R. marina

The fatty acid profile of *R. marina* was similar across treatments presenting some small quantitative differences ($p<0.05$) between media (Table 12). ALA, stearidonic acid (SDA, C18:4ω3), EPA and DHA were the main fatty acids accounting together over 65% of total fatty acids detected. This observation might suggest that the nitrogen and sulfur concentrations present in NS media are within the optimal concentration zone where growth as well as biochemical composition, namely, fatty acid composition, is little affected by the addition of more nutrients (19). The total fatty acids decrease in treatment S2 might indicate that the sulfur concentration applied in growth media exceeded the threshold concentration.

Table 12. *R. marina* fatty acid composition according to growth medium nutrient availability.

Fatty Acids (mg g^{-1} DW)	Treatment				
	S2	S1	NS	N1	N2
C14:0	1.87 ± 0.06^{a}	3.15 ± 0.13^{b}	2.97 ± 0.19^{b}	2.92 ± 0.08^{b}	2.96 ± 0.13^{b}
C16:0	2.49 ± 0.05^{a}	4.22 ± 0.16^{b}	4.03 ± 0.27^{b}	4.07 ± 0.08^{b}	3.88 ± 0.24^{b}
C18:0	0.27 ± 0.02^{a}	0.59 ± 0.01^{b}	0.48 ± 0.04^{c}	0.54 ± 0.01^{cb}	0.53 ± 0.07^{cb}
Total – SFA[2]	4.66 ± 0.11^{a}	8.05 ± 0.30^{b}	7.54 ± 0.49^{b}	7.60 ± 0.16^{b}	7.47 ± 0.44^{b}
C16:1[1]	0.77 ± 0.05^{a}	0.67 ± 0.02^{a}	0.97 ± 0.15^{b}	0.67 ± 0.03^{a}	0.83 ± 0.09^{ab}
C18:1[1]	2.11 ± 0.07^{a}	4.38 ± 0.11^{b}	4.12 ± 0.27^{b}	4.99 ± 0.13^{c}	4.29 ± 0.40^{b}
Total – MUFA[2]	2.87 ± 0.10^{a}	5.05 ± 0.12^{b}	5.09 ± 0.42^{b}	5.66 ± 0.10^{b}	5.12 ± 0.43^{b}
C18:2ω6	3.79 ± 0.05^{a}	9.34 ± 0.22^{b}	7.10 ± 0.46^{c}	8.20 ± 0.23^{d}	8.23 ± 0.55^{d}
C18:3ω3	14.10 ± 0.25^{a}	19.32 ± 0.41^{b}	20.41 ± 1.13^{b}	19.18 ± 0.35^{b}	18.84 ± 1.18^{b}
C18:4ω3	15.49 ± 0.22^{a}	17.12 ± 0.30^{b}	18.46 ± 1.03^{b}	17.66 ± 0.28^{b}	17.71 ± 1.17^{b}
C20:4ω6 - AA	0.03 ± 0.00^{a}	0.25 ± 0.03^{bc}	0.19 ± 0.02^{c}	0.28 ± 0.01^{b}	0.31 ± 0.09^{b}
C20:5ω3 - EPA	7.33 ± 0.09^{a}	10.12 ± 0.05^{b}	12.10 ± 0.53^{c}	11.88 ± 0.17^{cd}	10.98 ± 0.82^{bd}
C22:6ω3 - DHA	5.15 ± 0.15^{a}	7.89 ± 0.24^{b}	8.34 ± 0.54^{bc}	8.53 ± 0.16^{c}	8.67 ± 0.39^{c}
Total – PUFA[2]	46.06 ± 0.51^{a}	64.51 ± 0.73^{b}	66.89 ± 3.62^{b}	66.12 ± 1.14^{b}	65.20 ± 4.22^{b}
∑ω3	42.07 ± 0.47^{a}	54.45 ± 0.58^{b}	59.31 ± 3.13^{c}	57.24 ± 0.91^{bc}	56.22 ± 3.53^{bc}
∑ω6	3.99 ± 0.06^{a}	10.07 ± 0.20^{b}	7.57 ± 0.49^{c}	8.88 ± 0.23^{d}	8.98 ± 0.69^{d}
∑ω3/∑ω6	10.55 ± 0.09^{a}	5.41 ± 0.08^{b}	7.84 ± 0.09^{c}	6.45 ± 0.07^{d}	6.27 ± 0.10^{d}
ω3 HUFA	12.48 ± 0.24^{a}	18.01 ± 0.26^{b}	20.44 ± 1.07^{c}	20.41 ± 0.32^{c}	19.67 ± 1.22^{c}
DHA/EPA	0.70 ± 0.01^{a}	0.78 ± 0.02^{b}	0.69 ± 0.02^{a}	0.72 ± 0.01^{a}	0.79 ± 0.02^{b}
EPA/AA	229.31 ± 30.09^{a}	40.14 ± 4.13^{b}	64.42 ± 6.26^{b}	41.99 ± 2.26^{b}	37.87 ± 9.11^{b}
Total[2]	53.59 ± 0.70^{a}	77.61 ± 1.10^{b}	79.52 ± 4.50^{b}	79.38 ± 1.38^{b}	77.78 ± 5.07^{b}

Values (means ± SD of four replications) in the same row, not sharing a common superscript are significantly different ($p < 0.05$).
[1]Contains ω9 and ω7 isomers; [2]Contains some minor components not shown. SFA – Saturated fatty acid; MUFA – Monounsaturated fatty acid; PUFA – Polyunsaturated fatty acid; HUFA – Highly unsaturated fatty acid.

4.2.3. *Isochrysis* sp.

The fatty acid profile of *Isochrysis* sp. showed significant differences in the relative amounts of fatty acid detected (Table 13). The major fatty acids were myristic acid (C14:0), SDA and DHA which together accounted over 50% of total fatty acid content. Through Table 13 is possible to observe that N1 growth media had lead to an overall fatty acid content increase, 29% in DW. The fatty acid pattern of this microalga was similar when grown in NS and S1 culture medium. Moreover, at S2 *Isochrysis* sp. fatty acid pattern presented an analogous behavior to *R. marina*.

Table 13. *Isochrysis* sp. fatty acid composition according to growth medium nutrient availability.

Fatty Acids (mg g^{-1} DW)	Treatment				
	S2	S1	NS	N1	N2
C14:0	5.29 ± 0.28^{a}	7.56 ± 0.16^{b}	8.31 ± 0.36^{c}	9.68 ± 0.23^{d}	9.11 ± 0.09^{e}
C16:0	2.64 ± 0.13^{a}	4.11 ± 0.09^{b}	4.57 ± 0.15^{c}	5.69 ± 0.11^{d}	4.73 ± 0.05^{c}
C18:0	0.03 ± 0.00^{a}	0.05 ± 0.00^{a}	0.20 ± 0.01^{b}	0.08 ± 0.00^{c}	0.10 ± 0.03^{c}
Total – SFA[2]	8.34 ± 0.43^{a}	12.51 ± 0.27^{b}	13.70 ± 0.51^{c}	16.73 ± 0.36^{d}	14.78 ± 0.14^{e}
C16:1[1]	2.21 ± 0.13^{a}	3.62 ± 0.11^{b}	3.53 ± 0.12^{b}	4.70 ± 0.09^{c}	4.06 ± 0.04^{d}
C18:1[1]	2.68 ± 0.12^{a}	4.33 ± 0.11^{b}	4.74 ± 0.20^{c}	6.41 ± 0.19^{d}	5.00 ± 0.03^{c}
Total – MUFA[2]	5.16 ± 0.26^{a}	8.37 ± 0.24^{b}	8.71 ± 0.33^{b}	11.81 ± 0.27^{c}	9.55 ± 0.06^{d}
C18:2ω6	1.50 ± 0.06^{a}	2.27 ± 0.06^{b}	2.31 ± 0.09^{bc}	3.01 ± 0.05^{d}	2.41 ± 0.04^{c}
C18:3ω3	2.41 ± 0.22^{a}	5.38 ± 0.36^{b}	4.28 ± 0.26^{c}	6.83 ± 0.41^{d}	5.55 ± 0.40^{b}
C18:4ω3	8.23 ± 0.31^{a}	14.52 ± 0.39^{b}	14.23 ± 0.57^{b}	19.20 ± 0.44^{c}	15.86 ± 0.25^{d}
C20:4ω6 - AA	0.05 ± 0.01^{a}	0.08 ± 0.01^{b}	0.08 ± 0.00^{b}	0.11 ± 0.01^{c}	0.08 ± 0.01^{b}
C20:5ω3 - EPA	0.23 ± 0.02^{a}	0.42 ± 0.02^{b}	0.43 ± 0.01^{ab}	0.56 ± 0.02^{c}	0.46 ± 0.02^{a}
C22:5ω6	0.67 ± 0.06^{a}	1.05 ± 0.04^{b}	1.03 ± 0.08^{b}	1.21 ± 0.05^{c}	1.15 ± 0.08^{bc}
C22:6ω3 - DHA	5.24 ± 0.17^{a}	9.58 ± 0.32^{b}	10.07 ± 0.23^{bc}	11.97 ± 0.26^{d}	10.35 ± 0.24^{c}
Total – PUFA[2]	19.49 ± 0.71^{a}	35.03 ± 1.05^{b}	34.63 ± 0.93^{b}	44.99 ± 1.11^{c}	38.01 ± 0.94^{d}
∑ω3	16.11 ± 0.63^{a}	29.90 ± 0.90^{b}	29.01 ± 0.67^{b}	38.57 ± 1.01^{c}	32.22 ± 0.78^{d}
∑ω6	3.27 ± 0.08^{a}	5.00 ± 0.15^{b}	5.45 ± 0.27^{c}	6.24 ± 0.12^{d}	5.61 ± 0.19^{c}
∑ω3/∑ω6	4.93 ± 0.103^{a}	5.99 ± 0.07^{ab}	5.33 ± 0.14^{c}	6.19 ± 0.11^{a}	5.74 ± 0.11^{b}
ω3 HUFA	5.47 ± 0.18^{a}	10.00 ± 0.35^{b}	10.50 ± 0.24^{cb}	12.54 ± 0.28^{d}	10.81 ± 0.25^{c}
DHA/EPA	22.53 ± 1.35^{ac}	22.74 ± 0.47^{ac}	23.18 ± 0.62^{a}	21.24 ± 0.33^{c}	22.26 ± 0.37^{ac}
EPA/AA	5.12 ± 0.25^{a}	5.66 ± 0.35^{a}	5.42 ± 0.18^{a}	5.35 ± 0.21^{a}	5.91 ± 0.28^{a}
Total[2]	32.99 ± 1.29^{a}	55.91 ± 1.48^{b}	57.03 ± 1.71^{b}	73.53 ± 1.69^{c}	62.35 ± 1.10^{d}

Values (means ± SD of four replications) in the same row, not sharing a common superscript are significantly different ($p < 0.05$).
[1]Contains ω9 and ω7 isomers; [2]Contains some minor components not shown. SFA – Saturated fatty acid; MUFA – Monounsaturated fatty acid; PUFA – Polyunsaturated fatty acid; HUFA – Highly unsaturated fatty acid.

The results obtained in N1 cultures might suggest that the nitrogen addition in this treatment promoted the synthesis of chlorophylls which lead to an increase in photosynthetic activity (92). In *Isochrysis zhangjiangensis* cultures exposed to high nitrate concentrations channeled their metabolic fluxes into lipid accumulation at the expense of carbohydrates (92). Moreover, even though N1 had lead to an increase in total fatty acid content, changes in their relative proportions weren't observed. This indicates that growth medium nutrient availabilities applied didn't cause an effect in the fatty acid desaturases regulation, in contrast to that observed to an *Isochrysis* strain by Huerliman (35) in which fatty acid desaturases were all upregulated when nitrogen was abundant.

Environmental conditions may affect differently cellular growth and biochemical composition (68). Thus, the conditions needed to maximize EPA content

can be different from those required for EPA productivity maximization (68). In *Isochrysis* sp., growth medium nutrient availability treatments that lead to higher EPA and DHA productivities weren't the same that presented highest EPA contents. On the other hand, *N. gaditana* maximum EPA content and productivity were achieved in microalgae cultured in N2 growth media.

The presence of EPA, DHA and AA is essential in selecting microalgae for aquaculture purposes (40, 104). However, due to the competitive interactions between them the optimal requirements for each individual fatty acid must be taken into account when feeding live-prey in aquaculture (66). In this regard, all microalgae cultures, with the exception of *N. gaditana* in S1, exceeded the recommended minimal ratios of EPA/AA > 5, for flatfish larvae nutrition, and $\sum\omega3/\sum\omega6 > 2$, for larval and juvenile oysters nutrition (72, 104). Regarding to DHA/EPA ratio only *Isochrysis* sp. was above the recommended value (DHA/EPA > 2) for flatfish larvae nutrition (104).

Figure 15 shows the behavior of major fatty acids sets in regard to control. In this figure is possible to note that *N. gaditana* [Fig. 15a] and *Isochrysis* sp. [Fig. 15b] presented distinct responses towards nitrogen inputs and sulfur growth medium availabilities. In *N. gaditana*, cultures N1 and N2, exposed to highest nitrogen inputs, increased their content in all fatty acid groups being the highest increase achieved by monounsaturated fatty acids (MUFAs) (N1 – 46 %; N2 – 50 %). Even though *N. gaditana* had presented a similar behavior in the prior treatments, *Isochrysis* sp. only presented considerable rises (over 10 %) in treatment N2.

Regarding sulfur growth medium availabilities, cultures with highest sulfur availability (S2) lead to a decrease (39 – 44 %) in all fatty acids sets for *Isochrysis* sp. In *N. gaditana* S1 growth media lead to an increase in all fatty acid sets namely saturated fatty acids (SFAs) and MUFAs, with 39 and 42 % respectively.

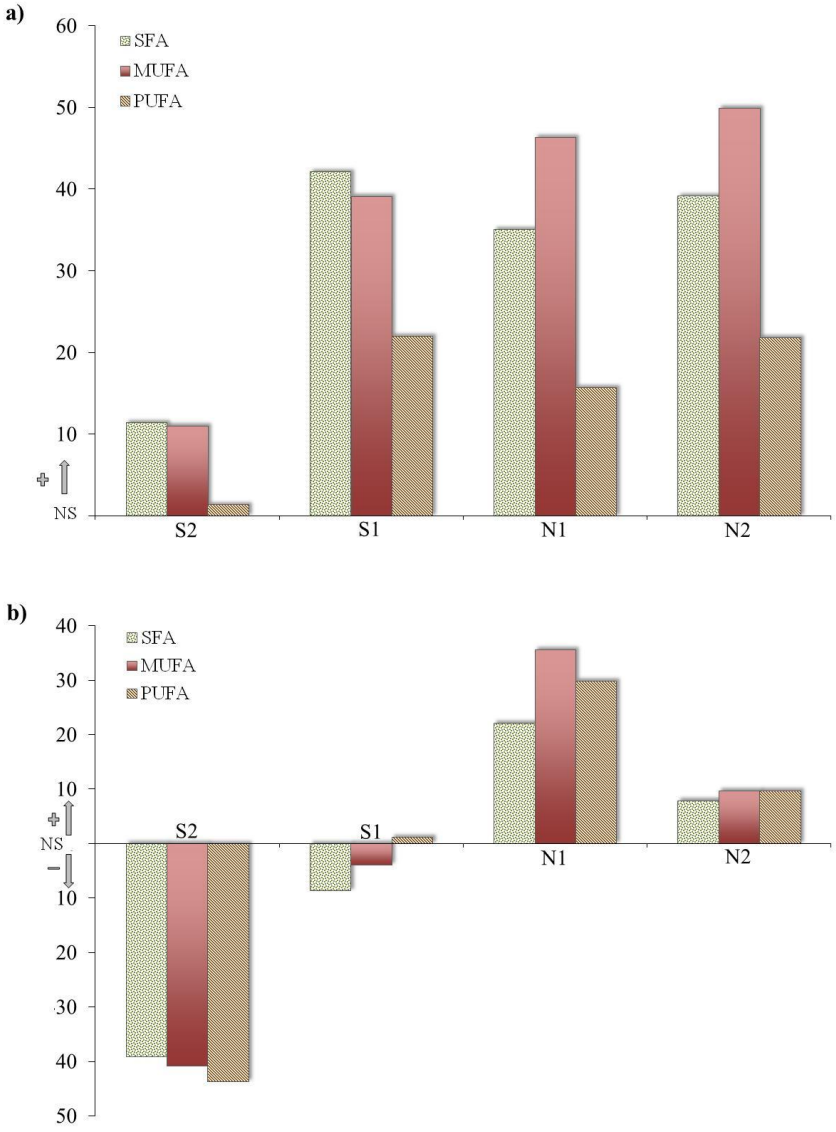

Figure 14. Fatty acid nature variation upon the different growth medium nutrient availability treatments a) *N. gaditana* and b) *Isochrysis* sp.

4.3.Microalgae elemental composition

Microalgae can counteract environment imbalances with acclimation and homeostatic responses (26, 98). The former comprises the modification of the expressed proteome whereas the later maintains a balanced cell composition upon changes in external conditions (26, 98). Moreover, nutrient uptake is mainly influenced by the nutrient amount in its bioavailable form along with other major and trace elements concentrations in growth medium (17).

Figure 16 displays the molar stoichiometry variation of the three microalgae studied within the different growth medium nutrient availabilities applied. Although

only the highest sulfur availability (S2) had induced changes in *R. marina* elemental ratios, in *N. gaditana* and *Isochrysis* sp. changes in both nitrogen and sulfur growth medium availabilities affected stoichiometric relationships [Fig. 16 a–b]. Furthermore, *N. gaditana* in treatments with higher nitrogen availabilities (NS – N2) presented an highest sulfur content, depicted by lower N/S and C/S ratios, in their elementary composition. These observations are in accordance with acclimation responses were cells change or modulate their metabolic strategies and cellular composition with environmental conditions (98).

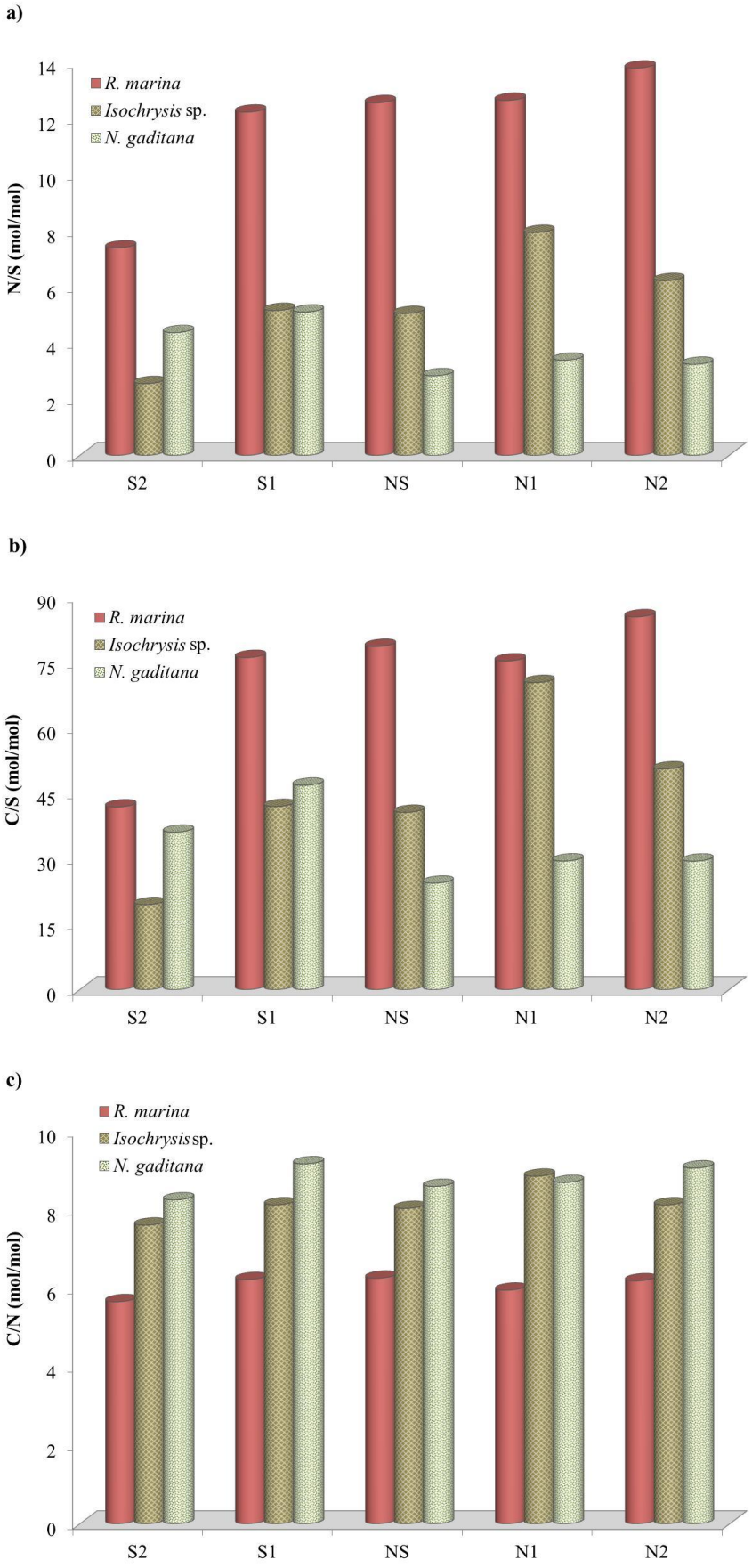

Figure 15. Microalgae molar stoichiometry a) N/S; b) C/S; and C) C/N variation according to growth medium nutrient availabilities applied.

Microalgae elementary composition can vary with changes in growth media composition (105). Nevertheless, in *R. marina* stoichiometric homeostasis was controlled at different nitrogen inputs despite of at highest sulfur growth medium availabilities *R. marina*, like *Isochrysis* sp., had increased their cellular content in sulfur (lowest N/S and C/S ratios). This last observation can be explained by the luxury consumption of the nutrient which may happen when an element is provided in excess by the growth medium (105).

In regard to C/N ratios Fig. 16 c shows that this molar ratio presented an uniform distribution upon the several growth medium nutrient availabilities applied. This indicates that the ratio (carbohydrate and lipid)/protein didn't change regardless the treatment applied.

5. Conclusion

The slight nitrogen inputs in microalgae growth medium significantly influenced the fatty acid composition of *N. gaditana* and *Isochrysis* sp, suggesting that these microalgae, in particular *N. gaditana*, are very sensitive to nitrogen changes. This might constitute an inexpensive stress-inducement strategy that comprises small additions of nitrogen in order to induce the production of a desired output by microalgae. In *N. gaditana*, higher EPA productivities were reached at the highest nitrogen input.

The effect of sulfur on microalgae growth medium significantly influenced the fatty acid composition of microalgae. Threshold sulfur concentrations were achieved at the highest sulfur concentration applied. The nitrogen and sulfur fluctuations didn't influence the elemental stoichiometries of carbon (carbohydrates and lipids) and nitrogen (protein), in contrast, to C/S and N/S molar stoichiometries. In *R. marina* and *Isochrysis* sp. the treatment that comprised higher sulfur acquisitions by microalgae lead to an overall decrease of the biochemical parameters studied. The results show that the *R. marina* responded to changes in growth media nutrient supply by homeostatic control of its elemental and biochemical composition.

Final Conclusions

With the aim to enhance the biochemical composition of *Nannochloropsis gaditana*, *Rhodomonas marina* and *Isochrysis* sp. for aquaculture purposes, the analysis of microalgae biochemical composition along with their growth dynamic was performed. An overview of the conclusions drawn along the experiment sets are highlighted in the following paragraphs.

In Chapter II, it was seen that high nutrient concentrations lead to low C/N ratios showing higher protein accumulation, making the biochemistry of microalgae suitable for aquaculture. For all microalgae studied glycolipids where the main lipid class.

In Chapter III, it was determined the optimum growth mediums for the accumulation of the desired products were those comprising 1000 μL L^{-1} of nutrient solution for both *R. marina* and *N. gaditana* and 2000 μL L^{-1} of nutrient solution for *Isochrysis* sp. Globally it was possible to note that *Isochrysis* sp. had the highest nutrient demand and that the commercial medium used in this experiment wasn't suitable for the accumulation of FA, namely EPA in *N. gaditana*.

In chapter IV, it is demonstrated that neutral monosaccharide pattern highlight microalgae taxonomic differences. Changes in the nutrient concentrations of the growth medium influenced the lipids/carbohydrates/proteins accumulation, demonstrating that the strategy applied was an effective strategy to enhance biochemical composition for the marine microalgae here studied, with both *Isochrysis* sp. and *R. marina* the microalgae with highest potential regarding carbohydrates application.

In chapter V, *N. gaditana* exhibited the highest variation in their biochemical composition reaching higher EPA productivities at the highest nitrogen input. In overall treatments that led to higher sulfur acquisitions by microalgae led to an overall decrease of the biochemical parameters studied. Threshold sulfur concentrations for all microalgae were achieved at the highest sulfur concentration applied. *R. marina* responded to changes in growth media nutrient supply by homeostatic control of its elemental and biochemical composition. Moreover, the stress-inducement strategy here applied for raising EPA productivities was effective for *N. gaditana*.

In summary, microalgae are an excellent chemical platform for several areas of application. Their composition and growth can be modulated in order to achieve the maximum biochemical productivities. The results of the present work show two stress-inducement strategies (alteration of overall nutrient availabilities in growth medium and changes in both nitrogen and sulfur concentrations) that may constitute a base for further investigations.

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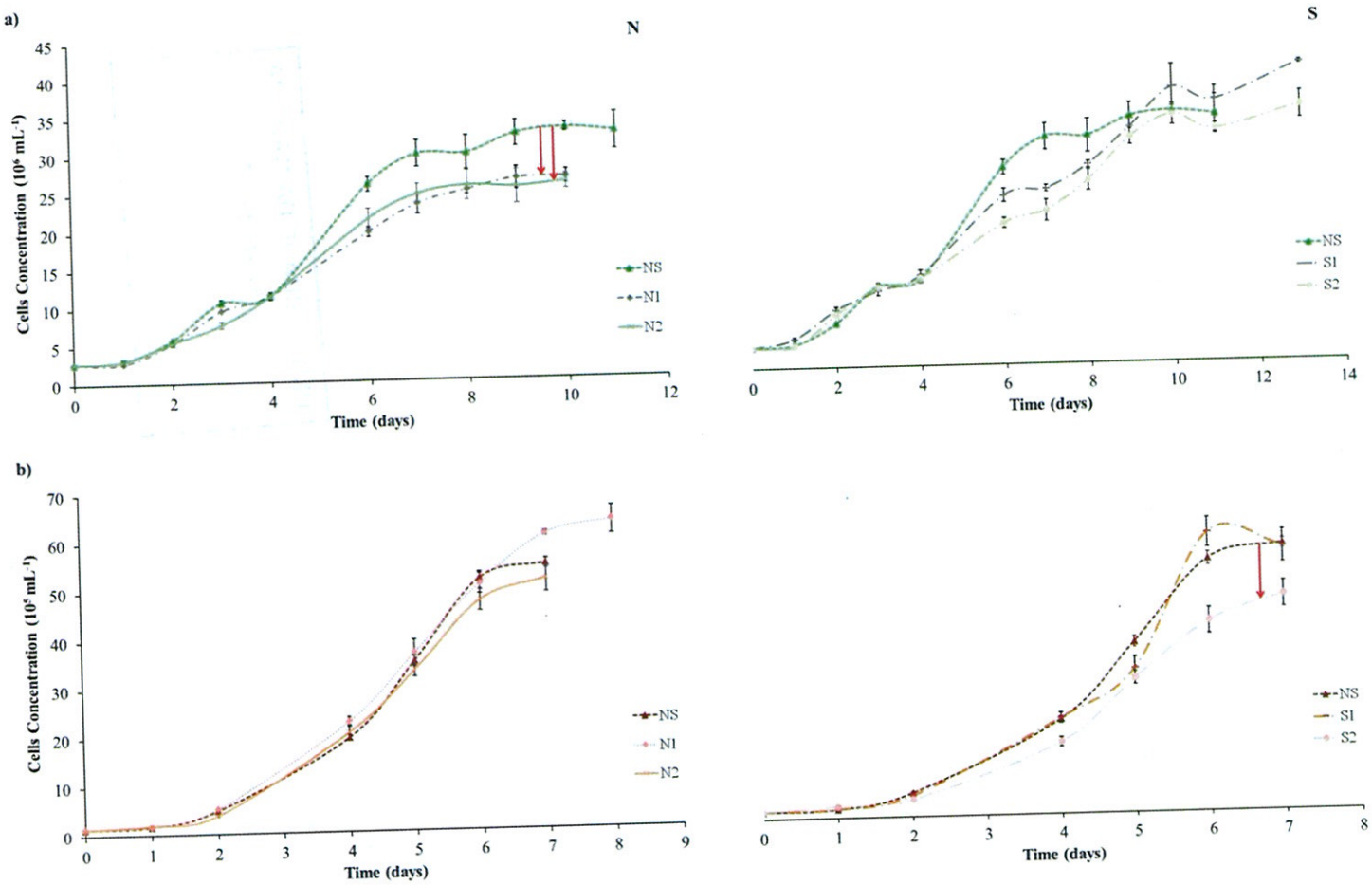
Annex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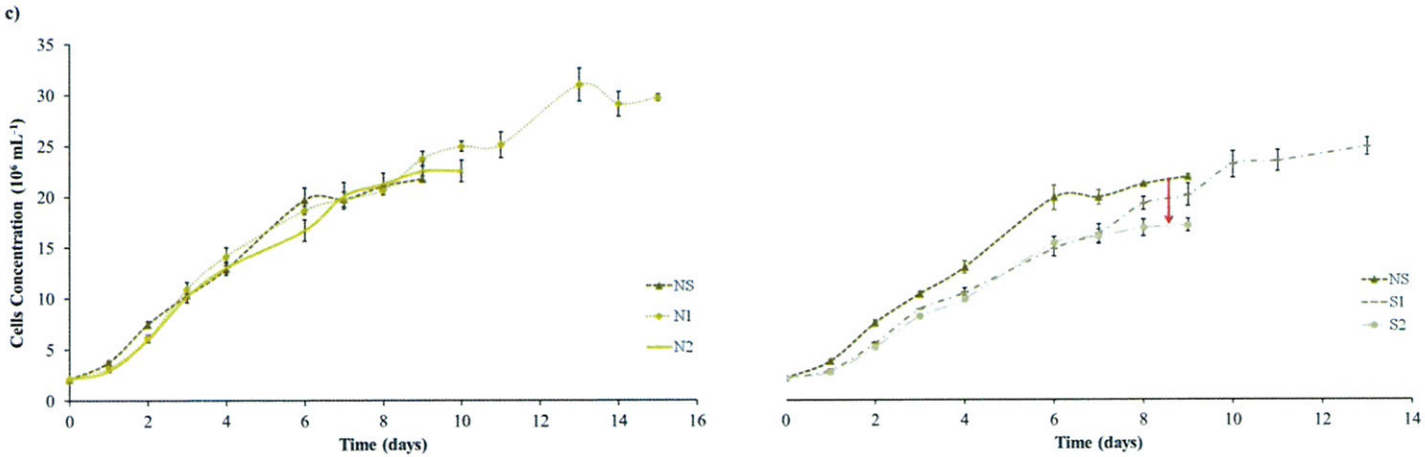

Annex 1. Growth curves for a) *N. gaditana*, b) *R. marina* and c) *Isochrysis* sp. in the nitrogen and sulfur growth medium availabilities experiment (Chapter V). In the figure are highlighted some of the differences observed, the left side corresponds to the nitrogen treatments whereas the right side corresponds to the sulfur treatments.