*Search for **mycosporine-like amino acids** and fatty acids in Isochrysis galbana and Nannochloropsis gaditana*

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Title: Search for mycosporine-like amino acids and fatty acids in *Isochrysis galbana* and *Nannochloropsis gaditana*

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resumo Neste trabalho duas culturas unialgais, uma de *Isochrysis galbana* (*I. galbana*) e a outra de *Nannochloropsis gaditana* (*N. gaditana*), foram expostas a radiação fotossinteticamente ativa (PAR) suplementadas com radiação ultravioleta (UVR-B) (4h diárias) com um fotoperíodo 16h: 8h, ciclo de luz: escuro. O conteúdo em micosporinas tipo aminoácidos (MAAs) foi determinado através de corrida isocrática em cromatografia líquida de alta precisão (HPLC), com uma coluna C_{18} de fase reversa, em extratos metanólicos e metanólico-aquosos. Não foi possível identificar-se nenhum MAA, com os cinco padrões disponíveis, mas um pico (*composto desconhecido*) com um máximo de absorção (λ_{max}) nos 338 nm foi detectado.

O conteúdo em ácidos gordos (somente para *N. gaditana*) foi determinada por cromatografia gasosa (CG), foram identificados oito ácidos gordos (em dez presentes em *N. gaditana*) e o ácido gordo mais abundante foi o ácido palmítico com 26,86% do total de ácidos gordos.

A fim de identificar novas fontes de antioxidantes seguras e baratas, a atividade antioxidante de diferentes frações de *N. gaditana*, foi avaliada pela capacidade de redução do radical α, α-difenil-β-picrilhidrazil (DPPH). *N. gaditana* foi extraída com *n*-hexano, acetato de etilo e água, através de um processo de extração com três etapas sucessivas. Os resultados variaram entre 0,23±0,46% e 1,23±0,60%, para as três frações, quando comparados com o padrão comercial butil-hidroxitolueno (BHT) que obteve uma capacidade de redução do DPPH de 95,0±2,68%.

Com este trabalho concluiu-se que os procedimentos de extração utilizados não foram adequados na extração deste tipo de compostos, não só em relação às micosporinas tipo aminoácidos e outros compostos de absorção UV, mas também para compostos com propriedades antioxidantes. Na extração de ácidos gordos os resultados obtidos foram bons.

keywords Mycosporine-like amino acids, Fatty acids, High-performance liquid chromatography, Gas chromatography, Ultraviolet radiation, Marine microalgae

abstract In this work two unialgal cultures, one of *Isochrysis galbana* (*I. galbana*) and another of *Nannochloropsis gaditana* (*N. gaditana*), were exposed to photosynthetic active radiation (PAR) plus ultraviolet radiation (UVR-B) (supplemented with 4h daily) in a photoperiod 16h: 8h, light: dark cycle. The content on mycosporine-like amino acids (MAAs) was determined by reverse phase isocratic high performance liquid chromatography (HPLC) employing C_{18} phase, in methanolic and aqueous methanolic extracts. No MAAs were identified, with the five *standards* available, but a peak (*unknown compound*) with absorption maximum (λ_{max}) at 338 nm was detected.

The content on fatty acids (only for *N. gaditana*) was determined by gas chromatography (GC), eight fatty acids were identified (in ten presented in *N. gaditana*) and the most abundant fatty acid was the palmitic acid with 26,86% of the total fatty acids.

In order to identify new sources of safe and inexpensive antioxidants, the antioxidant activity of different fractions of *N. gaditana* was evaluated, by the α,α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging assay. The *N. gaditana* was extracted using *n*-hexane, ethyl acetate and water by a three-step sequential extraction procedure. The results varied between 0,23±0,46% and 1,23±0,60%, for the three fractions, when compared with the commercial standard butylated hydroxytoluene (BHT) that obtained a 95,0±2,68% of capacity for reduction of DPPH.

With this work was concluded that the extracting procedures used were not adequate for the extraction of this kind of compounds, not only for the finding of MAAs or another UV-absorbing compounds, but also for other compounds with antioxidant properties. The results obtained for the extraction procedure for fatty acids showed good results.

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1 Introduction

1.1 Marine environment

The marine environment, with its enormous wealth of biological and chemical diversity, represents a treasure trove of useful materials awaiting discovery. It covers more than 70% of the Earth's surface and contains more than 300 000 described species of plants and animals. Natural products from marine organisms have attracted increasing research interest in recent years (Targett 2002). Since 1975, three major areas of research in aquatic natural products have emerged: toxins, bio products and chemical ecology. Until now, over 15 000 novel compounds have been chemically identified and characterized (Cardozo *et al*. 2007). The number of new marine compounds increased up to almost 4 000 new compounds described between 2001 and 2005 (Klisch and Häder 2008).

A wide variety of products from marine algae present a high actual or potential economic impact (Klisch and Häder 2008).

1.2 Marine microalgae

Microalgae occupy the bottom of the food chain in aquatic ecosystems - they possess the intrinsic ability to take up H_2O and CO_2 - which, with the aid of sunlight, are converted to complex organic compounds that are subsequently kept inside or released from the cell. Microalgae have a worldwide distribution, and are well adapted to survive under a large spectrum of environmental stresses, including heat, cold, salinity, photo-oxidation, osmotic pressure and ultraviolet radiation (UVR) exposure. Microalgae combine, in a balanced way, a few properties typical of higher plants (efficient oxygenic photosynthesis and simple nutritional requirements) with biotechnological attributes proper of microorganisms (such as fast growth rates and the ability to accumulate or secrete primary and secondary metabolites) (Guedes *et al*. 2011). This rather useful combination has led to selection of such microorganisms for applied processes, and represents the basic rationale for the usefulness of microalgal biotechnology.

Yamaguchi (1997) stated that microalgae play an important role in food, food/feed additives and feed supplements for humans and animals. The beneficial value of microalgae could be transferred to humans and animals throughout the food chain. Various microalgal species have become a preferred natural feed for many aquaculture organisms, including oysters, mussels, brine shrimp and fish larvae. Natrah and co-workers (2007) showed that a diet of dried microalgal biomass or extracts could improve the quality of cultured fish, particularly in terms of enhancing resistance to disease and

improvement of flesh quality. Microalgae have high protein content, and for this reason, have been suggested as a possible replacement to animal protein in human consumption, due to the protein shortage in the world (Natrah *et al*. 2007).

1.3 Stratospheric ozone

Greenhouse gases are accumulating dramatically in Earth's atmosphere as a result of human activities and industrialization. The increasing concentration of these gases causes serious global warming increasing the temperatures of the surface air and subsurface ocean. CO_2 is the main greenhouse gas. Many attempts including physical and chemical treatments have been used to recover CO_2 from atmosphere. In biological approach, microalgae appear more photosynthetically efficient than terrestrial plants and are the candidates as efficient CO_2 fixers (Chiu *et al*. 2009).

A marked decrease in the level of the stratospheric ozone, during the past two decades, with the consequent increase in the UVR flow on the terrestrial surface, has been observed throughout the last years (Callone *et al*. 2006; Rastogi *et al*. 2010). This has been attributed to the dispersal of chemicals (primarily chlorofluorocarbons (CFCs) and nitrogen dioxide) from today's society (Wängberg *et al*. 1997). UVR (280-400 nm) has caused a broad spectrum of genetic and cytotoxic effects (lethal DNA and RNA damage, enzyme inhibition, lipid peroxidation, photo-inhibition of photosynthesis, and delay in mitosis are all well described events occurring after exposure to UVR (Dunlap and Yamamoto 1995; Karsten *et al*. 1998)) in aquatic organisms (intertidal epipelagic, planktonic and benthic to depths > 20 m) (Hermando *et al*. 2002; Shick and Dunlap 2002; Callone *et al*. 2006).

The increase of UVR as a consequence of ozone depletion could have a great impact on the photosynthetic carbon fixation by plants and consequently, on the global climate change. Thus, at the present time there is great interest in determining the effects of increased UVR on primary productivity in both terrestrial and aquatic ecosystems (Figueroa *et al*. 2003). As a consequence, the synthesis and accumulation of UV-absorbing compounds in living organisms have been object of intensive research, especially the mycosporine-like amino acids (MAAs) (Callone *et al*. 2006; Klisch and Häder 2008; Rastogi *et al*. 2010). Identification of UV-absorbing compounds in aquatic ecosystems is difficult since there are no textbook guidelines for extraction and analysis (Gröniger *et al*. 2000).

1.4 Mycosporine-like amino acids

The first description and baptizing of mycosporines refers to sporulating terrestrial fungi (Karsten *et al*. 1998; Volkmann and Gorbushina 2006). Wittenburg was the first to report a strong UV-absorbing compound (absorption maximum, λ_{max} = 305 nm) in the gas gland of the epipelagic Portuguese man-of-war (*Physalia physalis*), but the isolated substance (λ_{max} corrected to 310 nm) was never fully characterized (Shick and Dunlap 2002).

Mycosporine-like amino acids are a family of UV-absorbing, intracellular and cytoplasmic compounds, colourless, (polar) water soluble, with high molar extinction coefficients (ε) between 28 100-50 000 M^{-1} cm^{-1} and low molecular-weight (generally < 400 Da), and have a chemical structure based on either a cyclohexenone (mycosporines) or cyclohexenimine (MAAs) ring with amino acid substituents (Wängberg *et al*. 1997; Carreto *et al*. 2001; Shick and Dunlap 2002; Sinha *et al*. 2007; Llewellyn and Airs 2010; Rastogi *et al*. 2010).

The primary MAAs (such as mycosporine-glycine (λ_{max} = 310 nm), mycosporine-glycine-valine (λ_{max} = 335 nm), shinorine (λ_{max} = 334 nm), porphyra-334 (λ_{max} = 334 nm), and mycosporine-2-glycine (λ_{max} = 334 nm)) respond first to increased UVR (Laurion and Roy 2009), they are then gradually transformed into secondary MAAs (other MAAs such as palythine (λ_{max} = 320 nm), asterina-330 (λ_{max} = 330 nm) or palythinol (λ_{max} = 332 nm)), assuming that light stress remains the same (Fig. 1.1). This increased concentration of MAAs should reduce UVR penetration in the cell, and absorbed radiation efficiently as heat without producing reactive oxygen species (ROS), photostability and resistance to several abiotic stressors (Laurion and Roy 2009; Rastogi *et al*. 2010).

There is still a great deal of controversy concerning the precise mechanisms of MAAs biosynthesis (Klisch and Häder 2008; Rastogi *et al*. 2010). Details of the biosynthesis of MAAs in marine algae remain to be demonstrated, but their origin via the shikimate pathway has been a persistent assumption (Shick and Dunlap 2002). The synthesis and accumulation of these UV-absorbing compounds has been recognized as being able to minimize structural and physiological damage caused by UVR exposure in tropical and temperate marine organisms (Hermando *et al*. 2002). Many reports support the view that these substances could act as a protective mechanism against UVR (Dunlap *et al*. 1989; Dunlap and Yamamoto 1995; Karsten *et al*. 1999), while others show that MAAs are unable to provide full protection against UVR effects (Garcia-Pichel *et al*. 1993; Lesser *et al*. 1994).

Mycosporine-like amino acids have a maximum of absorption between 310 and 360 nm (UVR-B: 280-315 nm and UVR-A: 315-400 nm), covering the spectral regions of major biological damage (Wängberg *et al*. 1997; Hermando *et al*. 2002; Callone *et al*. 2006; Volkmann and Gorbushina 2006), particularly UVR-B is detrimental for most sun-exposed organisms, including humans (Rastogi *et al*. 2010). MAAs have been hypothesized to act as sunscreens and thus to reduce harmful effects of UVR (Hermando *et al*. 2002), also play a role by acting as antioxidant molecules scavenging toxic oxygen radicals (only reported for mycosporine-glycine (Montero and Lubián 2003)) (Hermando *et al*. 2002; Llewellyn and Airs 2010), and as compatible osmolytes (Klisch and Häder 2008; Laurion and Roy 2009).

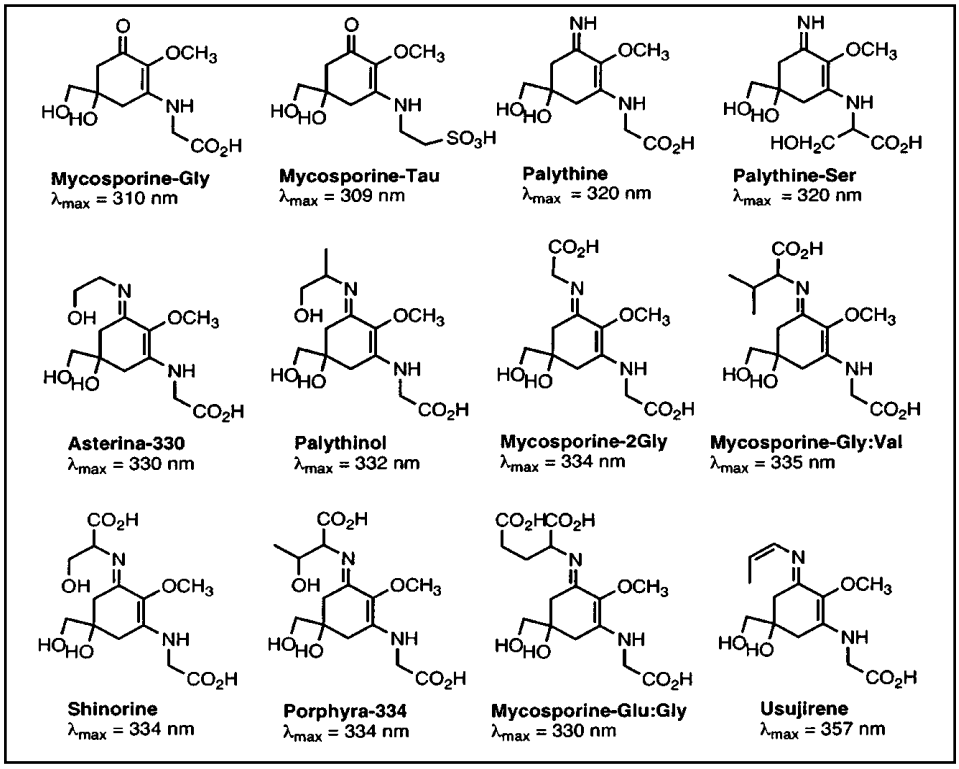

Fig. 1.1 Molecular structures and wavelengths of maximum absorption (λ_{max}) of twelve MAAs in marine organisms (from Shick and Dunlap 2002).

Most marine organisms contain a complex mixture of MAAs where some MAAs increase in concentration during exposure to UVR and the final internal concentration of MAAs, and the particular MAAs composition, reflects the quantity/duration of radiation applied (Hermando *et al*. 2002; Krabs *et al*. 2002). It was proposed that the increase in the concentration of some MAAs was not a direct response to UVR, but represents

accumulation due to seasonal changes, reproductive state or diet (Bandaranayake 1998; Rastogi *et al*. 2010). Some organisms need UVR-A and particularly UVR-B for the synthesis of MAAs, other species like dinoflagellates have high levels of MAAs without being exposed to UVR (Callone *et al*. 2006).

They are found in a wide variety of marine, more than 380 species according to Carreto and co-workers (2001) and Llewellyn and Airs (2010), freshwater and to a smaller degree in terrestrial organisms (Klisch and Häder 2008). The most primitive organisms capable of MAAs biosynthesis are cyanobacteria, MAAs are synthesized in macro- and microalgae, fungi and lichens (Callone *et al*. 2006; Volkmann and Gorbushina 2006). Other marine organisms acquire and metabolize these compounds by trophic transference and by symbiotic or bacterial association (Dunlap and Yamamoto 1995; Callone *et al*. 2006; Cardozo *et al*. 2007).

Many phytoplankton organisms from different regions and taxonomic groups have been found to contain UV-absorbing compounds (Callone *et al*. 2006), MAAs have been reported to occur predominantly in members of the Dinophyceae, Bacillariophyceae and Prymnesiophyceae (Rastogi *et al*. 2010). Geographically, MAAs are distributed ubiquitously, occurring in tropical, temperate and polar aquatic environments (Carreto *et al*. 2001).

About 21 MAAs have been characterized until the present, but this number seems to grow every year as a consequence of the increase in the number of studied organisms and the development of more efficient high-performance liquid chromatography (HPLC) separation techniques (Callone *et al*. 2006; Rastogi *et al*. 2010; Carreto *et al*. 2005), using the distinctive nature of their retention times and absorption spectra. However, certain closely related unknown compounds, have similar absorption maxima and retention times, which still cause difficulties in the identification process (Rastogi *et al*. 2010). Only a few HPLC methods for MAAs separation, in marine organisms, have been reported until now. No method has been able to separate a mixture of over 20 MAAs such as might be found in the most complex samples from marine organisms (Carreto *et al*. 2005).

Commercial applications of these compounds: additives in human skin creams against sun burning, in different non-biological materials as photostabilising agents (in plastics, paint and varnish), as antioxidants (cosmetic and pharmaceutical industries) (Cardozo *et al*. 2007). Indeed, several patents have already been raised on this concern (Montero and Lubián 2003; Rastogi *et al*. 2010), such a product called Helioguard® 365 that contain MAAs from the red alga *Porphyra umbilicalis* (Cardozo *et al*. 2007).

1.5 Fatty acids

Lipids, as one of major fractions of phytoplankton biomass, play an important role in controlling growth and reproduction of many marine animals (Fábregas *et al*. 2004). Microalgal lipids also offer a great promise to generate renewable biofuel, which would meet increasing energy need without hurting natural environments (Lv *et al*. 2010).

Production of lipids in microalgae cells is affected by many environmental conditions such as light and UVR, temperature, salinity, nutrients and CO_2 concentration (Renaud *et al*. 1995; Zhu *et al*. 1997). Moreover, cell growth phase has been found to have a critical effect on cellular lipid yield and composition (Hodgson *et al*. 1991; Lv *et al*. 2010).

Fatty acids have been reported by various researchers to serve important functions in the cosmetics, pharmaceutical, nutraceutical and aquaculture industries. Polyunsaturated fatty acids (PUFAs) have also been shown to play a protective role against free radicals, which are known to have an aging effect on skin (Natrah *et al*. 2007).

Microalgae are a primary source of long-chain polyunsaturated fatty acids (LC-PUFAs), such as docosahexaenoic acid (DHA; 22:6*n*−3) and eicosapentaenoic acid (EPA; 20:5*n*−3), γ-linolenic acid and arachidonic acid which are incorporated in marine animals through the diet and also in humans through consumption of seafood (Mansour *et al*. 2005), are essential for the growth, development, and reduced mortality of marine fish larvae, shrimp and molluscs (Fidalgo *et al*. 1998; Huerlimann *et al*. 2010).

At present, marine fish and their oil are the major commercial sources of PUFAs, but their suitability for human consumption has been questioned from the bio safety perspective (Kumar *et al*. 2010). The current commercial source of EPA is marine fish oil (Fang *et al*. 2004). EPA has been found in a wide variety of marine microalgal classes (Patil *et al*. 2007). EPA is abundant in classes such as diatoms, eustigmatophytes, prymnesiophytes and cryptomonads, but strains rich in DHA are usually limited to the dinoflagellates and some marine heterotrophs, with lower proportions in some prymnesiophytes and cryptomonads (Mansour *et al*. 2005).

Long-chain polyunsaturated fatty acids are essential for normal cell function, and have entered the biomedical and nutraceutical areas as a result of elucidation of their biological role in certain clinical conditions common in Western society such as obesity and cardiovascular diseases. Moreover, LC-PUFAs play key roles in cellular and tissue metabolism, including the regulation of membrane fluidity, electron and oxygen transport, as well as thermal adaptation (Tonon *et al*. 2002).

However, only a few microalgal species have demonstrated industrial production potential, mainly due to the fact that the majority of microalgal cultures present low specific growth rates and low cell densities when grown under conventional photoautotrophic conditions. There is a clear technological need for the development of a safe, sustainable and cheap alternative source of PUFAs for human health and nutrition (Hoshida *et al*. 2005; Kumari *et al*. 2010).

An efficient large-scale cultivation system is needed in order to explore the commercial production of microalgal PUFAs. Despite of photoautotrophic conditions, a number of microalgae are also capable of heterotrophic growth with one or more organic substrates as their sole carbon and energy source. This mode of culture eliminates the requirement for light and, therefore, offers the possibility of greatly increasing cell density and productivity and of being cost effective relative to photoautotrophic growth. Many microalgae have indeed been found to be capable of producing EPA heterotrophically, indicating that heterotrophic microalgal culture may provide an effective and feasible means for the large-scale production of EPA (Cardozo *et al*. 2007).

The accurate quantification of fatty acids in biological samples depends on proper extraction, methylation of fatty acids into fatty acid methyl esters (FAMEs), optimized gas chromatography (GC) run conditions and calculation of their concentration using standards.

1.6 Chromatographic techniques

To speak about HPLC or GC first it is important to define chromatography, and so chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (the stationary phase), while the other (the mobile phase) moves in a definite direction. A mobile phase is described as a fluid, which percolates through the stationary bed in a definite direction. It may be a liquid, a gas or a supercritical fluid, while the stationary phase may be a solid, a gel or a liquid. If a liquid, it may be distributed on a solid, which may or may not contribute to the separation process (Ardrey 2003).

The two components associated with the separation that occurs in a chromatographic system are the mobile and stationary phases. A chromatographic separation occurs if the components of a mixture interact to different extents with the mobile and/or stationary phases and therefore take different times to move from the position of sample introduction to the position at which they are detected (Ardrey 2003).

The identification of analytes is effected by the comparison of the retention characteristic of an unknown with those of reference materials (standards) determined under identical experimental conditions. Often, the retention time is used but, this absolute parameter changes with column length and flow rate and this precludes the use of reference data obtained in other laboratories (Ardrey 2003; Andersen 2005).

1.6.1 High performance liquid chromatography

High performance liquid chromatography is a versatile analytical technology widely used for the analysis of pharmaceuticals, biomolecules, polymers, and many organic and ionic compounds (Dong 2006). The HPLC system consists of solvent pumps, sample injector (manually or an automated injector), analytical column, detector(s) (as diode array detector – DAD) and data recording device, such a computer (Fig. 1.2).

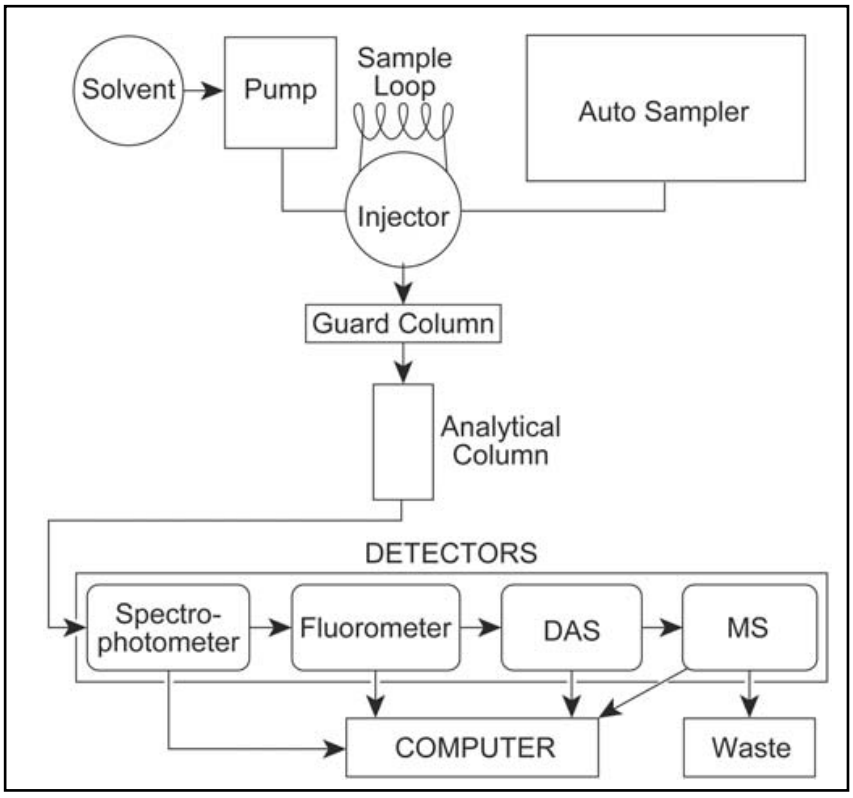

Fig. 1.2 Major components of a HPLC system (DAS - diode array spectrophotometer; MS - mass spectrometer) (from Andersen 2005).

In high performance liquid chromatography, the mobile phase is a liquid delivered under high pressure (up to 400 bar) to ensure a constant flow rate, and thus reproducible

chromatography, while the stationary phase is packed into a column capable of bear the high pressures which are necessary (Ardrey 2003).

Reverse phase isocratic HPLC with UV detection appears to be the most popular method employed in the separation and quantification of MAAs. The separation mechanism in reversed-phase chromatography depends on the hydrophobic binding interaction between the solute molecule in the mobile phase and the immobilised hydrophobic ligand, meaning the stationary phase. The separation is based on analytes partition coefficients between a polar mobile phase and a hydrophobic (non polar) stationary phase (Dong 2006). Water containing approximately 0.1% acetic acid is used as the mobile phase to separate less polar compounds, while mobile phase containing methanol is used for more polar components. In the HPLC analysis, known mycosporines and MAAs have been identified by co-chromatography with authentic standards (Bandaranayake 1998).

The first extraction of a mycosporine was performed using absolute cold ethanol (Dunlap *et al*. 1989). Methanol in concentrations from 20% (Garcia-Pichel *et al*. 1993) up to absolute (Dunlap *et al*. 1989) was performed as well. Reverse phase HPLC employing C_8 phases (Dunlap and Chalker 1986; Whitehead and Vernet 2000) as well as C_{18} phases (Nakamura *et al*. 1982) is the most common method to separate and characterize the MAAs (Chioccara *et al*. 1986). Solvent mixtures have been employed for the isocratic elution of the UV-absorbing compounds in HPLC, but there were also effective separations using gradient acetonitrile elutions (Böhm *et al*. 1995). Most common aqueous methanolic solvents (with the methanol content varying from 10% to 65% have been shown as adequate to elute the UV-absorbing substances from reverse phase columns (Chioccara *et al*. 1986). A solvent mixture of 75: 25 (water: methanol) with 0.1% acetic acid added has been used most commonly to separate isolated MAAs from marine organisms (Karentz *et al*. 1991; Volkmann and Gorbushina 2006).

1.6.2 Gas chromatography

Analysis of the fatty acid content in biological samples has been performed by solvent extraction and GC performs analysis and characterization. A disadvantage to these techniques, in terms of high throughput screening and on-site measurement, is that the steps required to both extract and derivatize the fatty acids for GC analysis are numerous and time consuming. Moreover, adequate amounts of biomass must be cultured for the extraction and derivatization (approximately 10–15 mg wet weight of cells) (Elsey *et al*. 2007). Before the fatty acid components of lipids can be analysed by GC, it is necessary to convert them to low molecular weight non-polar derivatives, such as methyl esters. In

addition, it may be advisable to mask other polar functional groups in a similar manner, or to prepare specific derivatives as an aid to identification. Peak shape and resolution are greatly improved at the same time. It is only possible to identify fatty acids tentatively by GC retention times alone (Christie 1989).

The GC analysis of FAMEs with flame-ionization detector (FID) remains the most frequently used method and the results are often expressed as a relative percentage (%) of total fatty acids. The accurate quantification of fatty acids in biological samples depends on proper extraction, methylation of fatty acids into FAMEs, optimized GC run conditions and calculation of their concentration using external standards (Xu et al. 2010). *Standard* mixtures containing known amounts of methyl esters of saturated, mono and polyunsaturated fatty acids are available commercially from a number of biochemical suppliers. These are invaluable for checking the quantification procedures used, and also for the provisional identification of fatty acids by direct comparison of the retention times of their methyl esters with those of the unknown esters on the same columns under identical conditions (Christie 1989).

1.7 Antioxidant activity

Antioxidant compounds play an important role against various diseases (such chronic inflammation, cancer and cardiovascular disorders) and ageing processes, which explains their considerable commercial potential in medicine, food production and the cosmetic industry (Huerlimann *et al*. 2010; Kumari *et al*. 2010). Antioxidants are effective in protecting the body against damage by ROS, such as peroxide radical (O_2^-) and hydroxyl radical (OH^-). ROS attack biological molecules such as lipids, proteins, DNA and RNA, leading to cell or tissue injury associated with aging, atherosclerosis and carcinogenesis (Duan *et al*. 2006).

Butylated hydroxytoluene (BHT) is one of the most commonly used synthetic food antioxidant, because of its excellent results and low cost (Lee *et al*. 1996). Because it has been shown to cause pathological, enzyme and lipid alterations and have carcinogenic effects, the development of alternative natural antioxidants is of great importance for our health and holds considerable commercial potential (Matsukawa *et al*. 1997). Among natural antioxidants, phenolic antioxidants (e.g. flavonoids) are in the forefront as they are widely distributed in plants (leaves, roots, oilseeds) (Duan *et al*. 2006).

However, natural antioxidants are not limited to terrestrial sources. Some of macroalgae are considered to be a rich source of antioxidants (chlorophylls, carotenoids) (Duan *et al*. 2006). Although macroalgae have received much attention as potential natural

antioxidants, there has been very limited information on antioxidant activity of microalgae (Li *et al*. 2007).

The α,α-diphenyl-β-picrylhydrazyl (DPPH) is a useful reagent for investigating the free radical scavenging activities of compounds. The method is based on the reduction of alcoholic DPPH solution (stable free radical – purple colour, absorption band in ethanol solution centered at 520 nm) in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H (yellow colour) by the reaction (Duan *et al*. 2006). This reaction has been widely used to investigate the ability of extracts and/or pure compounds of those, to act as free radical scavengers or hydrogen donors (Brand-Williams *et al*. 1995; Molyneux 2003; Wang *et al*. 2009).

1.8 Characterization of the species

The biochemical composition of microalgae can be substantially altered in response to environmental factors, including nutrient availability, light, temperature, pH and salinity (Renaud and Parry 1994; Abu-Rezq 1999; Phatarpekar *et al*. 2000). Here two commercial tropical microalgal species were studied:

1. The golden-brown flagellate *Isochrysis galbana*, Parke (1949) (Prymnesiophyceae)(4 μm diameter), has been widely used as a mariculture feed due to its high content of LC-PUFAs (Zhu *et al*. 1997), a source for molluscs larvae, as well as fish and crustaceans in the early stages of growth (Fidalgo *et al*. 1998; Yingying *et al*. 2008), and has been used widely in research into algal physiology, as a model phytoplankter in the development of mathematical models of algal growth (Flynn *et al*. 1994).

2. The green microalgae *Nannochloropsis gaditana*, L.M.Lubián (1982) (Eustigmatophyceae), a unicelular marine eustigmatophyte with polysaccharide walls and contain only one chloroplast (lack of chlorophylls *b* and *c*, a common characteristic of this family, being the chlorophyll *a* the only chlorophyll present) containing a large quantity of EPA as a component of the structure lipids. Is cultivated in many fish hatcheries as the basis of an artificial food chain system (Abu-Rezq 1999; Fang *et al*. 2004; Hoshida *et al*. 2005; Forián *et al*. 2010). *N. gaditana* consists of spherical or slightly ovoid cells of 2 to 4 μm in diameter (Fidalgo *et al*. 1998; Gentile and Blanch 2001).

1.9 Aims of the thesis

The first aim was to screen the presence and identification of MAAs in two microalgal cultures, of *I. galbana* and *N. gaditana* using reverse phase HPLC employing C_{18} phase.

Detection was made by monitoring absorption at 310, 320, 330 and 360 nm. Individual peaks were identified by retention time and by co-chromatography with the five *standards* available. Photosynthetically active radiation (400-700 nm) only (PAR only) and PAR plus UVR-B treatments were tested for their ability to stimulate MAAs production, as a step towards understanding the universality and photoinductibility of this group of UV-absorbing compounds in microalgal cultures.

Also in this study, another aim was to screen in *N. gaditana*, with the PAR plus UVR-B treatment, cultures for fatty acids such as DHA and EPA by GC.

Finally the third aim of the present study was to screen *N. gaditana* for antioxidant activity, by DPPH radical scavenging assay.

2 Material and Methods

2.1 Chemicals used

For the achievement of best results, all labware and plastic material were washed with HCl at 5%, for 15 min and distilled water (dH_2O), for 24 h. All solvents used were HPLC analytical grade: acetonitrile (C_2H_3N) and water (H_2O) from Carlo Erba Reagents (Italy), chloroform ($CHCl_3$) analytical grade stabilized with ethanol; ethyl acetate ($C_4H_6O_2$); *n*-hexane (C_6H_{14}, 96%); *n*-heptane (C_7H_{16}) and acetyl chloride (CH_3COCl) were from Scharlau (Spain), methanol (CH_4O, 99,5%) was from Emplura Merck Chemicals (Germany) and glacial acetic acid ($C_2H_4O_2$) was from Panreac (Spain).

Butylated hydroxytoluene and DPPH were purchased from Sigma-Aldrich (Germany).

2.2 Standards for MAAs

Since standards for MAAs are not commercially available, some of the biological MAAs *standards* (dried powder of crude extracts from different organisms usually replace them) were kindly donated by Prof. Dr. Ulf Karsten, University of Rostock, Germany:

Mastocarpus stellatus, thallus (shinorine),

Porphyra umbilicalis, thallus (porphyra-334),

Lichina pygmaea, thallus (mycosporine-glycine),

Plectropomus leopardus, ocular lenses (asterina-330),

Sprattus sprattus, ocular lenses (palythine).

All of these materials were stored at -20ºC until extraction and analysis.

The extraction procedure was based, with slight modifications, to the procedure of Karsten and co-workers (2005):

Samples of about 6,0 mg dry weight (DW), weighted (Sartorius TE124S, max. 120 g; d=0,1 mg, Germany), were extracted for 2:30 h in screw-capped centrifuge 15 mL tubes (Labbox, Spain) filled with 1 mL 25% aqueous methanol and incubated in a water shaking bath (Julabo SW22, USA) at 45ºC (40 rpm). After centrifugation at 5 000 g for 5 min (Centrifuge 5804 R, Eppendorf, Germany), 800 μL of the supernatant were evaporated to dryness in a rotary evaporator (Rotavac Valve control, Heidolph Instruments, Germany) at 45ºC (60 rpm). Dried extracts were redissolved in 800 μL aqueous solution of 2,5% methanol plus 0,1% glacial acetic acid in water and vortexed for 30 s (30 Hz, Velp Scientifica, Italy). After passing through a 0,2 μm PES syringe filter (Filtres Fioroni, size 25

mm, France) with a syringe without needle 5 mL sterile (Terumo, Belgium), samples were analysed by HPLC, at a final concentration of 50 ppm, as described below (see *2.9 Analysis of MAAs by HPLC*).

2.3 Microalgal species

The two species of marine microalgae used were, *I. galbana* and *N. gaditana*, kindly donated from the collection of Instituto de Investigação das Pescas e do Mar (IPIMAR/CRIPSul), Estação Piloto de Piscicultura, Olhão, Portugal.

2.4 f/2 medium

The microalgae cultures were grown in f/2 medium (Guillard and Ryther 1962) prepared from local natural seawater (adjusted to a salinity of 30 (Ferreira 2009) (seawater refractometer HI96822, HANNA Instruments, Portugal) using dH_2O), from Peniche (39º21'N and 9º24'W, Portugal), filtered through a 50 μm and then through 0,7 μm glass microfibres filters (Filtres Fioroni, size 47 mm, France) under vacuum (Rotavac Valve control, Heidolph Instruments, Germany) and autoclaved at 121ºC for 15 min (Steam Sterilizer Raypa, Spain).

The composition of the f/2 medium used for the cultures was (per liter): 75,0 g $NaNO_3$; 5,0 g NaH_2PO_4 H_2O; 1 mL trace metal solution (3,15 g $FeCl_3$ $6H_2O$, 9,8 g $CuSO_4$ $5H_2O$, 22,0 g $ZnSO_4$ $7H_2O$, 10,0 g $CoCl_2$ $6H_2O$, 180,0 g $MnCl_2$ $4H_2O$, 6,3 g Na_2MoO_4 $2H_2O$, 4,36 g Na_2EDTA $2H_2O$, dH_2O to 1000 mL) (all metals used for the trace metal solution were from Panreac, Spain); and 0,5 mL vitamin solution (all vitamins were from Sigma-Aldrich, Germany) 0,45 μm PES syringe filter (Filtres Fioroni, size 25 mm, France) (1,0 g vitamin B_{12} (cyanocobalamin) dH_2O to 100 mL; 0,1 g vitamin H (biotin) dH_2O to 500 mL, 0,2 g vitamin B_1 (thiamine HCl) dH_2O to 500 mL). Stock solutions and medium were prepared using dH_2O and sterilized by autoclaving at 121 °C for 15 min.

The 500 mL Erlenmeyer flasks, boro glass (Boro 3.0, Normax, Portugal), were filled with 450 mL of local natural seawater plus 1,75 mL of f/2 medium and finally 50 mL of mother culture of each specie was transferred to the new Erlenmeyer flask (which corresponded to 10% of the mother culture).

2.5 Microalgal cultures conditions

Sterile techniques were used for all culture work to minimize bacterial growth.

The procedures were done at Escola Superior de Turismo e Tecnologia do Mar, Instituto Politécnico de Leiria, in Peniche:

1. PAR only treatment: the first procedure was done at 19,5±1ºC, in a plant growth chamber with adjustable light cassettes (KBW 720, Binder, Germany) with four cool daylight tubes (OSRAM 18W/865 - Lumilux, Germany) from above at 15 cm from the center of the 500 mL Erlenmeyer flasks. The microalgal cultures were aerated continuously (Bag tips 1mL, Interscience F-78960, France) by air bubbling (Elite 802, 50 Hz, Hagen, UK). Mixing is the most practical way to dilute radiation evenly to all cells in the culture, improving the light regime (Rocha *et al*. 2003).

2. PAR plus UVR-B treatment (Fig. 2.1): the microalgal cultures were grown in a temperature-controlled room (22±1ºC), under PAR 46,5 μmol photons m^{-2} s^{-1} provided by two 'cool-white' fluorescent tubes at a constant irradiance (CH Lighting F24T5 - Sunlux 10 000 K), 16 h daily (8:00 to 24:00 h), supplemented with 4 h daily UVR-B (12:00 to 16:00 h), in the middle of the light phase, provided by two UV-B tubes (8 W, Viber, France). Experimental irradiance of PAR was measured with a PAR sensor (Apogee Logan, UT), at a single near-center position of the flasks. The radiation measurement was done in air. The PAR tubes were located above the 500 mL Erlenmeyer flasks, 30 cm and the UV-B tubes located in front at 25 cm, positioned horizontally to the cultures flasks. Lamps were preburned for 100 h before beginning the experiments to ensure light emission stability (Laurion and Roy 2009). The microalgal cultures were aerated continuously by air bubbling.

All samplings were done at 16:00 h, after the UVR-B tubes were turned off (Zudaire and Roy 2001). Cell counts were performed daily from 1 mL aliquot of each Erlenmeyer culture (two flasks for each specie), for both procedures. This study was conducted over 1-2 weeks (throughout exponential growth until reached the stationary phase).

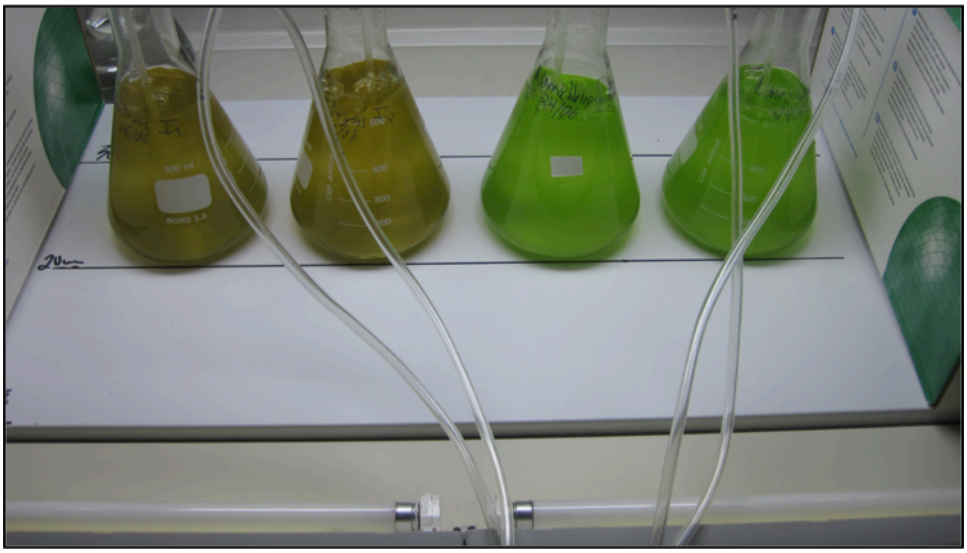

Fig. 2.1 *I. galbana* (left) and *N. gaditana* (right) in the second procedure, PAR plus UVR-B treatment.

2.6 Cell counts

Daily 1 mL subsamples fixed with acid Lugol's iodine solution (10 μL) were taken for determination of cell densities, using a light microscope (Zeiss Axiostar plus, Germany) and a Neubauer haemocytometer (0,0025 mm^2; 0,100 mm depth, Marienfeld, Germany), and the density was determined by averaging three counts (hand tally counter, Japan), until each culture reached the stationary phase. Each sample was diluted which provided a density easy to count.

2.7 Total DW determination

Aliquots of 10 mL (4 x 10 mL for each specie, at the end of exponential growth phase) algal suspension were filtered through preweighed and precombusted (450ºC, 2 h, Nabertherm Controller B170, VWR, USA) 0,7 μm glass microfibres filters. The step of rinsing the edge of the filters was omitted by setting the vacuum before starting the filtration so that liquid could not diffuse to the margin area of the filters. The filters were then dried at 60ºC (Memmert UFB 500, Germany) for 48 h and weighed again (Zhu and Lee 1997).

2.8 Sample extraction for MAAs

At the end of exponential growth phase, cells were harvested to screw-capped centrifuge 15 mL tubes, after gentle mixing, 4 x 10 mL each algal culture of *N. gaditana* and *I. galbana,* by filtration onto 0,7 μm glass microfibres filters at low vacuum pressure and stored at -20ºC until needed (Zudaire and Roy 2001; Tartarotti and Sommaruga 2002), this was done for the five procedures (except for the third).

The five different procedures used to extract MAAs were:

1. This first was with PAR only cultures: samples of *N. gaditana* (day 8) and *I. galbana* (day 5) were extracted in 3 mL absolute methanol at 45ºC (water shaking bath at 99 rpm) for 2:30 h, in the dark. The extracts were centrifuged for 5 min at 15 000 g, then 800 μL of the supernatant was evaporated to dryness using a rotary evaporator (45ºC at 60 rpm). The dry residue was ressuspended in 700 μL in a solution of aqueous 70% methanol plus 0,1% glacial acetic acid (mobile phase). The final solution was filtered with a 0,2 μm PES syringe filter and stored at -20ºC until HPLC analysis (Tartarotti and Sommaruga 2002; Zudaire and Roy 2001).

2. This procedure was with PAR plus UVR-B treatment at (22±1ºC): samples of *N. gaditana* (day 8) and *I. galbana* (day 5) were extracted in 3 mL absolute methanol at 45ºC (water shaking bath at 99 rpm) for 2:30 h, in the dark. The extracts were centrifuged for 5 min at 15 000 g, then 800 μL of the supernatant was evaporated to dryness using a rotary evaporator (45ºC at 60 rpm). The dry residue was ressuspended in 800 μL in a solution of aqueous 2,5% methanol plus 0,1% glacial acetic acid (mobile phase). The final solution was filtered with a 0,2 μm PES syringe filter and also stored at -20ºC until HPLC analysis (Tartarotti and Sommaruga 2002).

3. Samples of *N. gaditana* (day 8) were harvested to screw-capped centrifuge 15 mL tubes, after gentle mixing, and then were centrifuged (x4) for 10 min at 3 000 g and then extracted in 2 mL absolute methanol (sonicated for 1 min at 0ºC at 2 000 rpm (Fisatom 712, 60 Hz, Brazil)) for 2:30 h at 45ºC (water shaking bath at 99 rpm), in the dark. Then the extracts were filtered onto 0,7 μm glass microfibres filters at low vacuum pressure and then was evaporated to dryness using a rotary evaporator (45ºC at 60 rpm) and finally the dry residue was ressuspended in 500 μL H_2O and injected to HPLC (Hermando *et al*. 2002).

4. Samples of *N. gaditana* only (day 8) were extracted in 2 mL absolute methanol for 28 h at -20ºC, in the dark. Then the samples were vortexed for 30 s and the extracts were

clarified by filtration 0,2 μm PES syringe filter, and stored at -20ºC until HPLC analysis (Hannach and Sigleo 1998).

5. Samples of *N. gaditana* only (day 8) were extracted in 2 mL of 20% methanol for 2:30 h at 45ºC, in the dark. Then the samples were centrifuged for 10 min at 15 000 g and the extracts were evaporated to dryness using a rotary evaporator (45ºC at 60 rpm). The dry residue was ressuspended in 100% methanol for 2 min and evaporated to dryness using a rotary evaporator (45ºC at 60 rpm). This last residue was ressuspended in aqueous 0,1% glacial acetic acid then clarified by filtration 0,2 μm PES syringe filter, and stored at -20ºC until HPLC analysis (Gröniger *et al*. 2000).

2.9 Analysis of MAAs by HPLC

2.9.1 Chromatographic apparatus and conditions

Method development for analyses of MAAs was performed using a high-pressure gradient system HPLC ELITE LaChrom (VWR, USA), one pump model L-2130 VWR HITACHI, an auto-sampler injector L-2200 VWR HITACHI and an L-2450 Merck HITACHI diode array detector (DAD) connected via an interface module to a computer running ELITE LaChrom software, used for detection and quantification. A polymer-coated silica reversed phase C_{18} column LiChrospher 100 (5 μm; 4 x 250 mm, Merck), was used for this analysis.

During analysis, column and samples in the auto sampler were kept at 22-26ºC. A volume of 20 μL for each sample was injected into the column. The DAD was continuously scanning from λ = 290 to 400 nm (Karsten *et al*. 2005).

2.9.2 Mobile phase and elution gradient

The mobile phase within the HPLC column consisted of 2,5% aqueous methanol plus 0,1% glacial acetic acid, the flow rate employed was 0,5 mL min^{-1} (Karsten *et al*. 2005), except for the procedure 1.

2.9.3 MAAs identification and quantification

The MAAs were detected at 310, 320, 330 and 360 nm (Karsten *et al*. 2005) and recorded directly on ELITE LaChrom software (Bischof *et al*. 2000; Tartarotti and Sommaruga 2002; Carreto *et al*. 2005). Individual peaks were identified by the absorption spectra, retention time and by co-chromatography with purified *standards* kindly provided by Prof. Dr. Ulf Karsten.

2.10 Extraction of fatty acids

Fatty acids were extracted according to a modified method reported by Takagi and co-workers (2006):

The microalgal cells (about 30 mL) were collected at the end of exponential growth phase (day 8) and centrifuged at 2 500 g for 10 min (HERMLE Labortechnik Universal High Speed Centrifuge Z 323, Germany). The procedure was repeated twice, in screw-capped centrifuge 50 mL tubes (2 mL, Labbox, Spain). Then the pellet was transferred to 2 mL microcentrifuge tubes (Eppendorf, USA) and centrifuged for 10 min at 13 400 g (5ºC), three times (refrigerated microcentrifuge 5415 R, Eppendorf, USA). This transferred procedure from 50 mL to microcentrifuge tubes suffered losses in microalgal biomass. The sample was mixed with methanol/chloroform solution (2: 1) (10 mL: 5 mL) homogenized for 3 min at 2 000 rpm and sonicated for 30 min (Ultrasonic cleaner, VWR, USA). After precipitation of the sample with methanol/chloroform solution, more chloroform and 1% NaCl aqueous solution was added (final ratio of methanol: chloroform: H_2O - 2:2:1 (10 mL: 10 mL: 5 mL). The mixture was centrifuged at 2 500 g for 10 min and the chloroform phase was collected, and this phase was evaporated under vacuum in a rotary evaporator at 40ºC (60 rpm).

Fatty acid methyl esters were prepared according to the procedure of Bandarra and co-workers (2003):

This preparation was carried out using the residue remained for the evaporation, in 5 mL of the acetyl chloride-methanol mixture (1: 19), vortexed for 10 s. The esterification was carried out at 80ºC for 1 h. After cooling (10/15 min), 1 mL of dH_20 and 2 mL of *n*-heptane were added to the mixture, which was vortexed for 1 min. The organic phase was collected and the solvent was removed under a current of nitrogen and the FAMEs were solubilized in 0,1 mL of *n*-heptane.

2.11 Analyses of FAMEs by GC

2.11.1 Chromatographic apparatus and conditions

Fatty acid methyl esters were analysed by GC using a Finnigan Trace Ultra System with an auto-injector AS 3000 (Thermo electron corporation, USA); a capillary column Trace TR-FAME (70% cyanopropyl polysilphenylene-siloxane – highly polar) 0,25 μm film thickness, 30 m x 0,25 mm and fitted with FID at 260ºC.

The oven's programme temperatures started at: 60ºC for 1 min, followed by a continuous increasing of temperature 15ºC min^{-1} for 1 min until 150ºC, then 150-220ºC at 5ºC min^{-1} and held for 10 min before the analysis end (Fábregas *et al*. 2004), the injector was at 250ºC in splitless mode, and 1 μL of sample was used, helium was used as carrier gas running at 1,5 mL min^{-1}. The FID was operating with air at 350 mL min^{-1} and hydrogen at 35 mL min^{-1}. The analytical determinations were carried out in four replicates, and was used a PUFA-3, from menhaden oil, 100 mg, neat; (Supelco, USA), only for qualitative identification only. This menhaden oil was used as a *standard*, and the program for its analysis was the same applied for the sample.

2.12 Antioxidant assay for DPPH radical scavenging activity

The *N. gaditana* cells (day 8) were obtained by centrifugation at 500 g for 10 min, twice. The pellet was weighed (~ 0,05 g) and was extracted with 0,5 mL of *n*-hexane for 30 min at room temperature. The tube was centrifuged at 500 g for 10 min (x2) and the supernatant was recovered. The extraction was repeated and then the two supernatants were combined. The residue was subsequently extracted twice with ethyl acetate (0,5 mL each time) for 30 min at room temperature and the supernatants were combined. Then, the residues were further extracted twice with water (0,5 mL each time) for 30 min at 80ºC, and the supernatants were combined. The *n*-hexane and ethyl acetate fractions were evaporated using a current of nitrogen, and together with the water fraction were stored at -20ºC. The water fraction was directly used in the antioxidant assay, while *n*-hexane and ethyl acetate fractions were diluted in 200 μL ethanol and immediately used in the antioxidant assay (Li *et al*. 2007).

Antioxidant capacity of the three different fractions was evaluated using a visible range scanning spectrophotometer at 517 nm (Duan *et al*. 2006) (B11689 Thermo Spectronic Helios Aquamate, Thermo Scientific, UK).

A DPPH solution was prepared at 0,1 mM with ethanol. Six different concentrations of DPPH were used to calculate the antioxidant capacity of the *N. gaditana*: 30; 10; 3; 1; 0,3 and 0,1 mg mL^{-1} (10 μL of diluted sample were mixed with 990 μL of diluted DPPH) and BHT was used as positive control, then 200 μL of ethanol was added to all samples and after 30 min ate room temperature in the dark, the absorbance was immediately recorded at 517 nm.

The ability to scavenge the DPPH radical was calculated using the follow equation:

Scavenging activity (%)

[*(A*control - *A*sample)/*A*control)] x 100,

*A*control is the absorbance of the control (DPPH ethanolic solution without the sample), and *A*sample is the absorbance of the sample *N. gaditana* with DPPH solution (Wu *et al*. 2005; Duan *et al*. 2006).

2.13 Statistical analysis software

For PAR only and PAR plus UVR-B treatments the results were calculated as mean value ± standard deviation (SD), using GraphPad Prism Software, Inc. for Macintosh.

3 Results and Discussion

3.1 Cell counts

Direct cell counting is a relatively precise method but also time consuming. Additionally, cell density quantification was also achieved by the gravimetric method, DW determination (Rocha et al. 2003). Both methods, cell counting and DW, were suitable for *N. gaditana* and *I. galbana* cell density evaluation.

For this purpose two cultures of *N. gaditana* and two cultures of *I. galbana* cultures, were placed under illumination of 46,5 μmol photons m^{-2} s^{-1} PAR plus UVR-B. All cultures were prepared from a growing *N. gaditana* and *I. galbana* cultures continuously illuminated with 46,5 μmol photons m^{-2} s^{-1} PAR only, for a week, and all of them had the same initial cell density, 10^6 cell mL^{-1}.

Cultures were grown in 500 mL Erlenmeyer flasks 19,5±1ºC (PAR only treatment) and 22±1°C (PAR plus UVR-B treatment) temperatures. Salinity remained at 30 throughout the experiment in both cultures. The variation in cell densities during the experimental period is shown in Fig. 3.1 and 3.2 for PAR only treatment and for PAR plus UVR-B treatment, respectively.

Growth in *I. galbana*: the average initials cell density in the PAR only treatment (*n*=3) was 0,846±0,131 x 10^6 and 1,03±0,114 x 10^6 cells mL^{-1} for PAR plus UVR-B treatment (*n*=6) (Fig. 3.1a and Fig. 3.1b, respectively).

Growth in *N. gaditana*: the average initials cell density was 0,821±0,158 x 10^6 (PAR only treatment, *n*=3) and 2,21±0,160 x 10^6 cells mL^{-1} for PAR plus UVR-B treatment (*n*=6) (Fig. 3.2a and Fig. 3.2b, respectively).

Maximum cell densities were 8,48±2,023 x 10^6 cells mL^{-1} on day 5 (PAR only treatment) and 3,93±1,127 x 10^6 cells mL^{-1} on day 5 (PAR plus UVR-B treatment), for *I. galbana*. The cell density of *N. gaditana* increased to a maximum of 20,92±3,440 x10^6 cells mL^{-1} (PAR only treatment) (day 11) and to 16,91±1,984 x 10^6 cells mL^{-1} (PAR plus UVR-B treatment) (day 8).

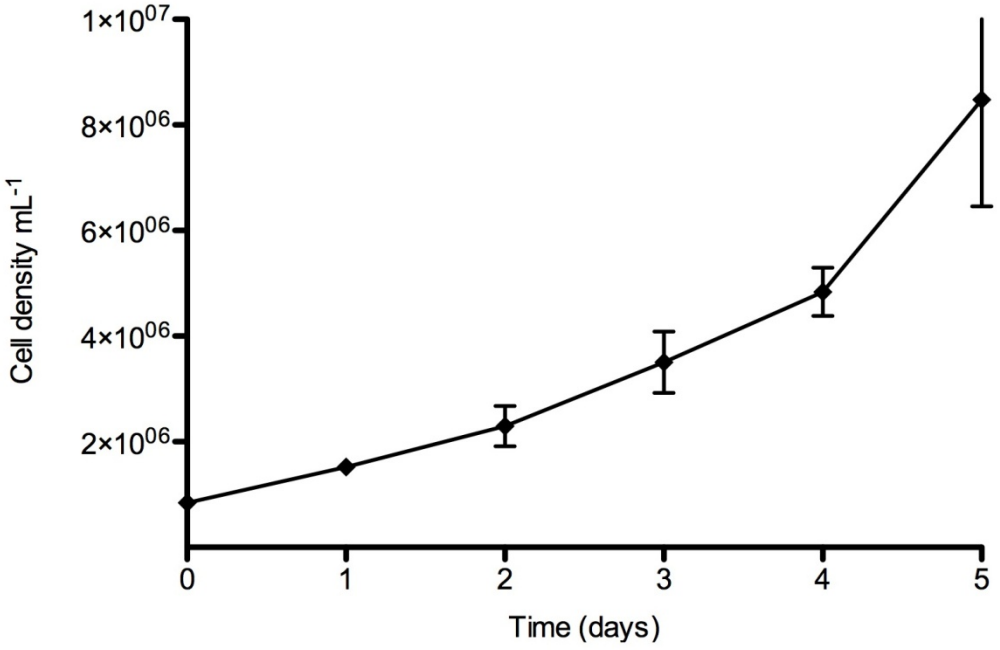

Fig. 3.1a Average growth of *I. galbana* in 500 mL of culture with f/2 medium (PAR only treatment). The vertical bars indicate SD. (*n*=3).

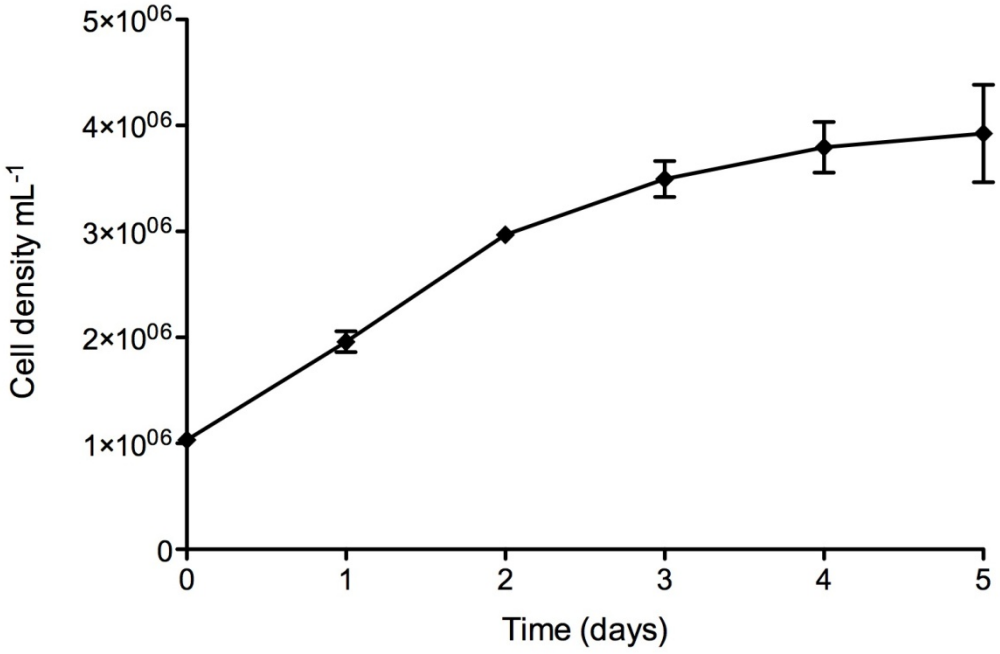

Fig. 3.1b Average growth of *I. galbana* in 500 mL of culture with f/2 medium (PAR plus UVR-B treatment). The vertical bars indicate SD. (*n*=6).

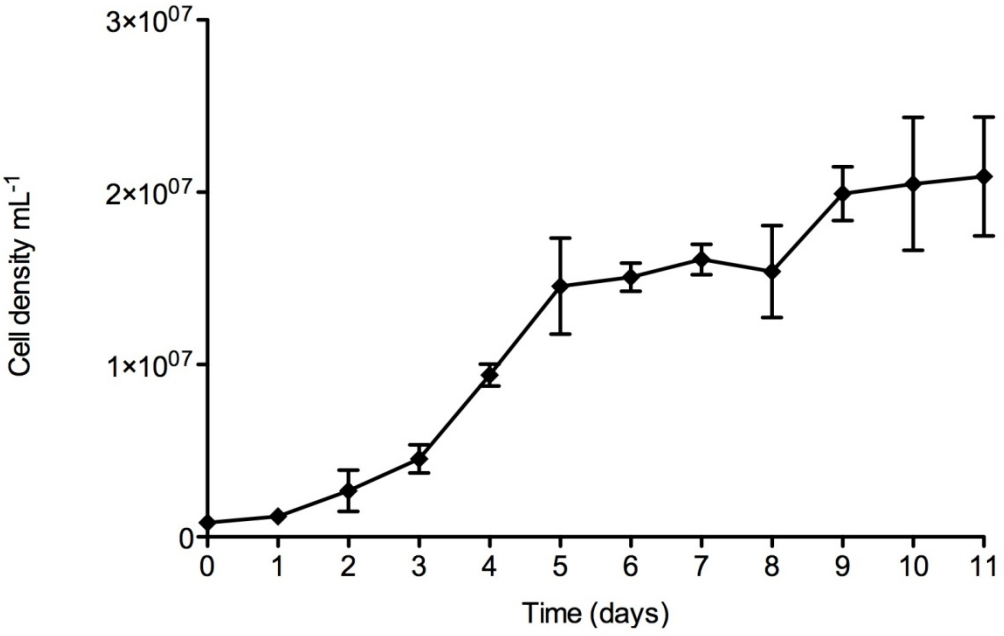

Fig. 3.2a Average growth of *N. gaditana* in 500 mL of culture with f/2 medium (PAR only treatment). The vertical bars indicate SD. (*n*=3).

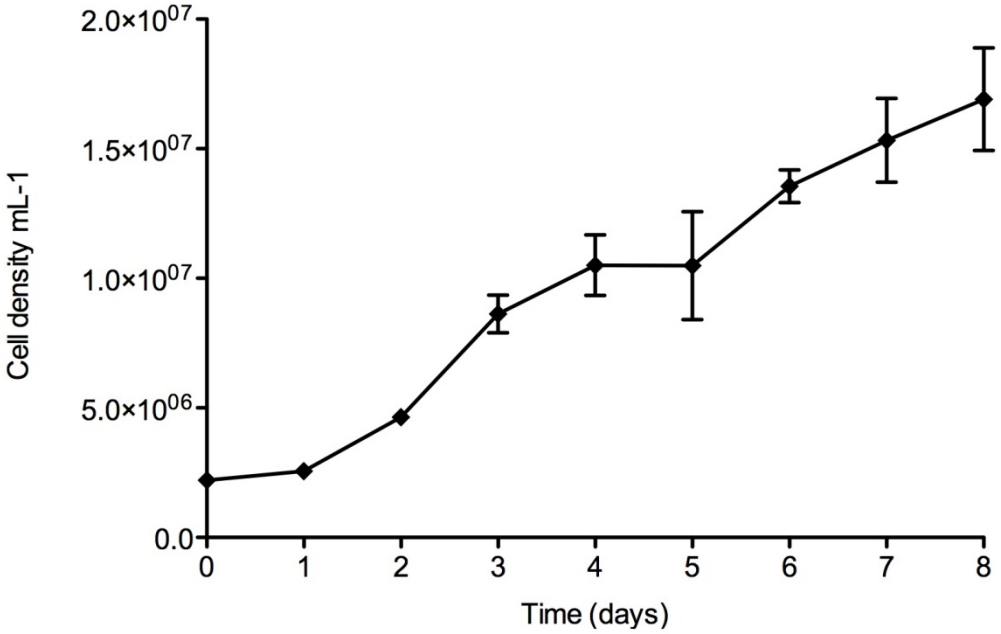

Fig. 3.2b Average growth of *N. gaditana* in 500 mL of culture with f/2 medium (PAR plus UVR-B treatment). The vertical bars indicate SD. (*n*=6).

The growth patterns of the monoculture of the two studied species were different. The monoculture of *I. galbana* grew rapidly until the day 5 in which this specie reached the beginning of the stationary phase. *N. gaditana* had almost a steady growth phase during the exponential phase, during day 5/6 (PAR only treatment) and in PAR plus UVR-B treatment last two days, along day 4/5.

According to the literature, exponential growth generally occurred within the first 8-10 days in *N. gaditana* and in *I. galbana* within the first 4-5 days after inoculation, followed by the onset of stationary phase during the next 1-2 days (Mansour *et al*. 2005). Renaud and co-workers (1994) realized a study with different salinities (range from 10 to 35) in *N. oculata* and *I. galbana*, with a salinity of 30, cells reached the density of 24,9±0,42 x 10^6 cells mL^{-1} and 3,17±0,15 x 10^6 cells mL^{-1}, respectively, in the end of the exponential phase.

According to Phatarpekar and co-workers (2000), that growth in 1L Erlenmeyer flasks (*I. galbana*) under laboratory conditions (20±2ºC, continuous fluorescent illumination and f/2 medium), the end of exponential growth occurred within 6 days. Another study with *I. galbana* (f/2 medium, at 20±1ºC and also a continuous illumination) achieved 5,19 x 10^6 cells mL−1 of cell density in 7 days (Valenzuela-Espinoza *et al*. 2002). Zhu and co-workers (1997) observed for *I. galbana* that a 15ºC the exponential growth phase ends in day 6 and for a temperature of 30ºC this phase ends only in day 12.

For *Nannochloropsis* sp. there is a study from Forján and co-workers, with continuously illumination, PAR plus UVR-A at 25ºC (in f/2 medium), where the cultures achieved the end of the exponential phase in 4 days. Rocha and co-workers (2003) fond results for celll density in *N. gaditana* (f/2 medium and a 12h: 12h light: dark cycle) was 2,0 x 10^7 cells mL^{-1} (day 13).

So as observed in all this different studies, the culturing method, prior culture history and the culture conditions, such as the quality of the light (PAR or UVR-B or UVR-A), the photoperiod, the temperatures applied and the salinities altered the density and duration of the growth curves in both species (Huerlimann *et al.* 2010).

3.3 Analysis of MAAs by HPLC

There is compelling evidence that MAAs shield the organisms from UVR damage (Marchant *et al*. 1991). To verify the contribution of these compounds to the observed increase in UVR-B region in absorption spectra, methanolic (procedures 1, 2, 3, 4) and aqueous methanolic (procedure 5 with 20% methanol) extracts of the cultures were examined with reversed phase HPLC employing C_{18} phase.

Mycosporines-like amino acids have an important UVR photoprotective function in algae because their accumulation is correlated with exposure to UVR in many marine organisms (Dunlap and Chalker 1986; Marchant *et al*. 1991; Llewellyn and Mantoura 1997; Wängberg *et al*. 1997; Hannach and Sigleo 1998; Carreto *et al*. 2005;). There are two important reasons for this: first, the high molar extinction coefficients in the range of environmentally relevant UVR which characterizes MAAs compounds, and second, because they are photo-inducible by high light conditions (Karsten *et al*. 1998; Hermando *et al*. 2002).

The analyses of the methanolic and aqueous methanolic extracts, with these five procedures presented, no presence for MAAs was detected according to the *standards* tested, but in all cases, the same peak was detected (Fig. 3.3 and Fig. 3.4) In both species chromatography of the extracts, yielded one major peak (retention time = 4.17 min), for PAR only and PAR plus UVR-B treatment, with the peak that eluted being always predominant, and its spectrum had a broad absorption band from 290 to 400 nm with a maximum at 338 nm in the HPLC mobile phase (Fig. 3.3 and 3.4, respectively *I. galbana* and *N. oculata*).

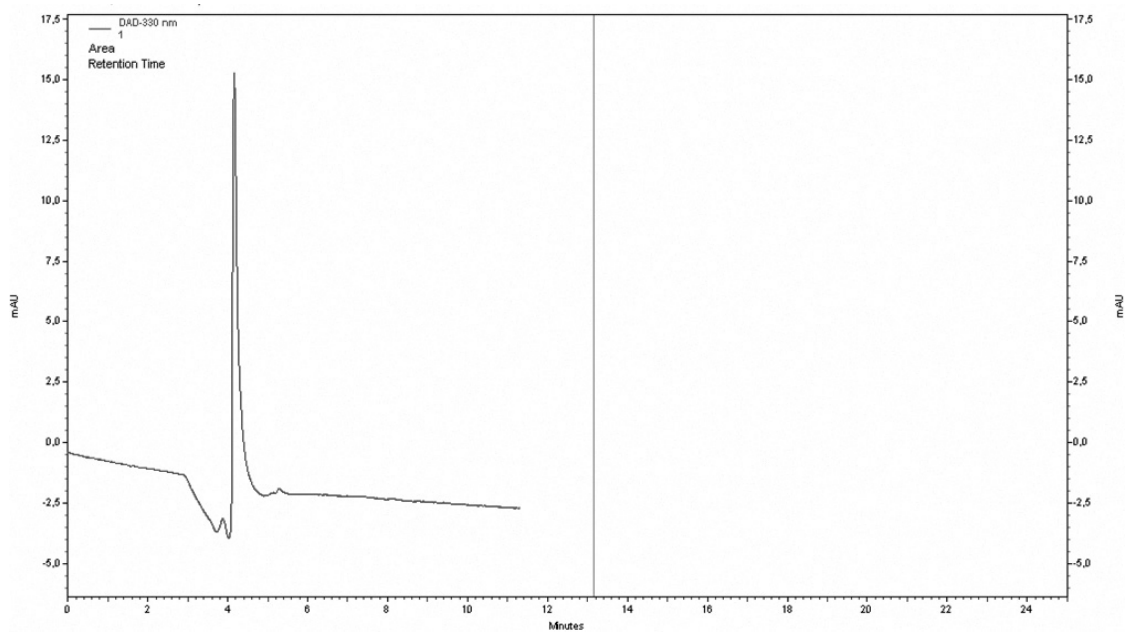

Fig. 3.3 HPLC chromatogram showing the peak and retention time (in min) of the *unknown compound* for *I. galbana* for PAR plus UVR-B treatment, detected at 330 nm.

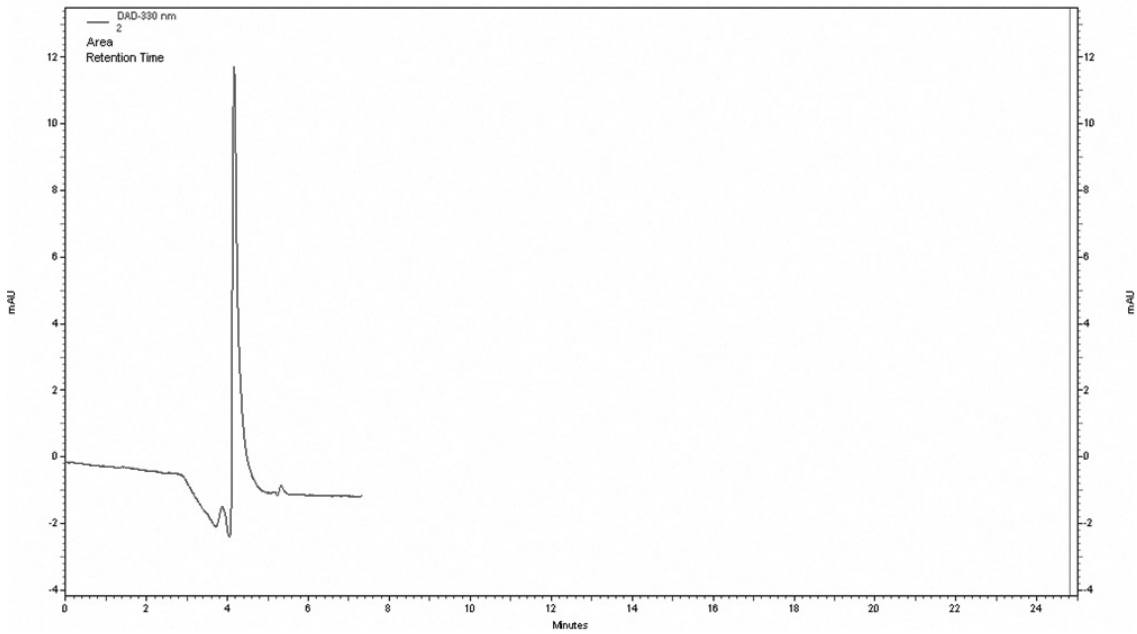

Fig. 3.4 HPLC chromatogram showing the peak and retention time (in min) of the *unknown compound* for *N. gaditana for* PAR plus UVR-B treatment, detected at 330 nm.

On the basis of their retention time and λ_{max}, and comparing with the five *standards* available, the peak detected is *unknown* (Fig. 3.5). As showed in the graphics below, shinorine was detected at 5.79 min (Fig. 3.5a); porphyra-334 was detected at 8.60 min. (Fig. 3.5b); mycosporine-glycine at 8.26 min. (Fig. 3.5c); asterina-330 at 6.13 min. (Fig. 3.5d) and finally palythine was detected at 5.54 min. (Fig. 3.5e). Since the same mobile phase and the conditions of the HPLC run were applied, we can guarantee that the peak observed is not one of the five *standards* used.

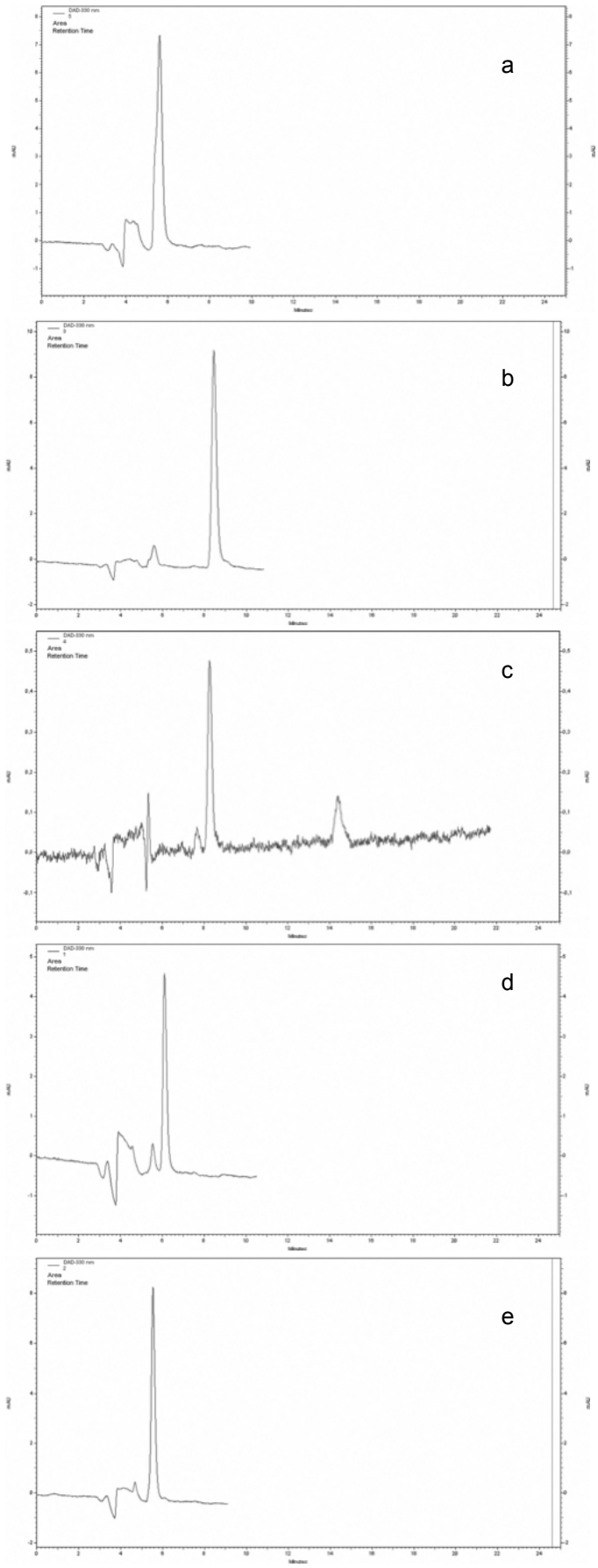

Fig. 3.5 HPLC chromatograms of the five *standards* of MAAs (the biggest peak in each chromatogram), detected at 330 nm (showing the peaks and the retention time): (a) shinorine; (b) porphyra-334; (c) mycosporine-glycine; (d) asterina-330; (e) palythine.

According to Llewellyn and Airs (2010) (in their study was used an anion exchange column with a photodiode array detection (PDA); no supplementary UVR) *I. galbana* contained low concentrations of MAAs (peaks at λ_{max} = 276/278/334 nm) and *N. oculata* also contained low levels of MAAs (three peaks at λ_{max} = 280/294/308 nm). Llewellyn and Airs (2010) found that 12 (of the 33 microalgal cultures studied) did not contain detectable MAAs using HPLC, but UV absorbance when analysed spectrophotometrically, that occurred here in this work at the λ_{max} = 338 nm.

Extraction with methanol or aqueous methanol was used for most MAAs analysis (Nakamura *et al*. 1982; Dunlap and Chalker 1986; Karentz *et al*. 1991; Tartarotti and Sommaruga 2002; Carreto *et al*. 2005). The literature has reported several extraction techniques involving soaking, grinding, or ultrasonic disruption in various solvent combinations, which have had varying degree of success. According to Carreto and co-workers (2005) sonication in 100% methanol appears to be a practical and efficient extraction technique for microalgal cultures. According to Tartarotti and Sommaruga (2002) it is not possible to assure that the most efficient extraction protocol, 25% aqueous methanol at 45ºC, found in their study could be used for all MAAs and biological materials. MAAs such as shinorine and porphyra-334 are chemically stable at 45ºC, but potential modifications of other more labile MAAs cannot be ruled out. In phytoplankton, both temperature and methanol concentration seem to affect the final MAA concentration (Tartarotti and Sommaruga 2002), and this authors also find that in certain organisms extraction of MAAs may be insufficient without serial extractions.

The results obtained with lyophilized organisms, by Carreto and co-workers (2005), showed low extraction efficiency after three serial extractions with 100% methanol. Here the MAAs appears to be sequestered in a bound form not accessible to the methanol. According to Tartarotti and Sommaruga (2002) lyophilized red macroalgae and freshwater phytoplankton assemblages, the mean total concentration of MAAs obtained in 25% aqueous methanol at 45 °C was, respectively, 13 and 3 times higher than in extractions made with 100% methanol at 4 °C, even being for other types of organisms, the procedures done here (procedure 4 and 5) showed the same results as the other three, that MAAs or UV-absorbing compounds were not effectively extracted.

Studies done with Antarctic phytoplankton have shown that diatoms were more resistant to UVR exposure as compared to other phytoplanktonic groups. This resistance to UVR has been hypothesized to be due to the synthesis of UV-absorbing compounds. When cellular MAAs concentrations were low, the inhibition of photosynthesis was significantly greater than when MAA concentrations were high. Although irradiance is the main factor

in the production of MAAs, nutrient availability, salinity and temperature are also known to affect accumulation (Helbling *et al*. 1996).

Another example was in the terrestrial cyanobacterium *Gloeocapsa* sp., exposure to UVR-B in combination with a low-PAR background dosage of 50 μmol m^{-2} s^{-1} resulted in a increase in the intracellular amount of one MAA, which the chemical structure is still unknown (Karsten *et al*. 1998), this could be the case here, but without mass spectrometry and/or nuclear magnetic resonance will not be possible to find if this *unknown compound*, in *I. galbana* and in *N. gaditana*, is really a MAA or an UV-absorbing compound or something else. These findings indicate that phototrophic organisms in low-radiation habitats do not need to synthesize and accumulate MAAs as potential protection against UVR, which could be the case here since low-PAR was used. As a consequence this would save energy, carbon and nitrogen for other physiological purposes (Karsten *et al*. 1998).

The intensity and spectral distribution of radiation has been shown to influence the induction of MAAs (Llewellyn and Airs 2010). Laboratory PAR plus UVR-B with an artificial light source may affect microalgae physiological processes differently from that of solar radiation (Xiong *et al*. 1999). According to Callone and co-workers (2006) and Hannach and Sigleo (1998), some organisms need UVR-A and particularly UVR-B (dinoflagellates such *Alexandrium tamarense*) for the synthesis of MAAs, while other species have high levels of MAAs without been exposed to UVR (such in cyanobacteria that were not stimulated by UVR illumination (Volkmann and Gorbushina 2006), but with the results obtained here for PAR only and PAR plus UVR-B treatment showed no difference on the peak obtained. In opposite and according to the work done by Hannach and Sigleo (1998) UVR-B supplementation approximately doubled the MAAs concentration in *Isochrysis* sp., *Pyramimonas parkeae* and *Amphidinium carterae*, also according to Wängberg and co-workers (1997), UVR-B at ecologically relevant irradiances changes the amount and the pattern of MAA synthesis, both in composition and its dependence on the cell cycle (in methanolic extracts).

Nevertheless, extraction efficiencies of the methods and stability of the more labile MAAs should be determined for the type of organism to be analysed. MAAs are polyfunctional compounds with several ionizable groups (carboxyl, imine, secondary amine), and the retention time and elution sequence of individual MAAs are very sensitive to pH changes (Nakamura *et al*. 1982; Carreto *et al*. 2005), and in this study we did not paid attention to this parameter, so this could be another reason for individual MAAs did not appeared in the chromatograms.

Assuming that the precursor of all MAAs could be mycosporine–glycine, to which an amino acid or amino alcohol is conjugated, each MAA may be synthesized by a separate pathway, and the different pathways may be induced by different wavebands (Krabs *et al*. 2002), this finding could justify the only peak that was observed in the chromatogram. PAR only mainly stimulated the synthesis of porphyra-334, UVR-A and UVR-B predominantly led to the accumulation of shinorine (334 nm) and palythine (320 nm), respectively (Karsten and West 2000), and being the blue light able to induce MAA biosynthesis (Callone *et al*. 2006).

A diode array detector allowed the acquisition of UV absorption spectra, and the spectral characteristics available for MAAs identification were the positions of the λ_{max}. However wavelengths of λ_{max} for some specific MAAs are identical or are only 2 nm apart, which makes it difficult to distinguish these compounds based on absorption spectra only (Carreto *et al*. 2005), so confirmation of peak identity with a high degree of confidence is possible by matching the retention time and UV spectrum with that of authentic standards of MAAs. While this methodology has proven useful, the lack of commercial standards makes identification and quantification of individual MAAs difficult.

With respect to the types of columns used in MAAs extractions, according to Carreto and co-workers (2005), measurements using isocratic elution with acetic acid 0.2% have shown that the retention time of shinorine on CapCell Pak UG was much larger than those obtained on an Alltima column, and that in relation to shinorine, the elution order of palythine and palythine-serine was reversed, so identification here without the other sixteen MAAs *standards* is misaligning because of the type of column used and the lack of more *standards*. According to Volkmann and Gorbushina (2006) and Karsten and co-workers (1998, 1999), their studies with Rhodophyta (red macroalgae), with only one exception in *Devalerea ramentacea*, showed the same chromatogram minus two MAAs as the other species, which they attributed to be column dependent.

However, in natural product chemistry, these properties alone are generally considered insufficient for a secure identification of organic compounds (Carreto *et al*. 2005; Volkmann and Gorbushina 2006). Therefore according to Volkmann and Gorbushina (2006), was suggested the use of precise analytical equipment, like liquid chromatography–mass spectrometry, for accurate identification of MAAs, as well as for their structural characterization and also allows the quantification of individual MAAs without the need for complete resolution.

3.4 Analyses of FAMEs by GC

Dry weight at the end of exponential phase (day 8 and day 5, respectively for *N.gaditana* and *I. galbana*) was 1,165±0,052 g mL^{-1} (*N. gaditana*) and 0,6125±0,241 g mL^{-1} (*I. galbana*). This was calculated only for PAR plus UVR-B treatment.

In order to analyze the fatty acid content of *N. gaditana*, four samples were harvested in the late exponential growth phase (day 8).

Total lipid accumulation in *N. gaditana* (expressed as total FAMEs content) was 9,275±1,91 mg in 30 mL (309,17 mg L^{-1}), which corresponds at a representative value of 0,027% DW for total FAME content. This value was only representative because the 30 mL, collected from the culture, was being lost along the extraction procedure. Ten fatty acids were observed and eight were identified (Fig. 3.6), with the available *standard* of menhaden oil (Fig. 3.7):

The major fatty acids were C16:0, C18:0 (or C18:1 *n*9 or C18:1 *n*7 or C18:2 *n*6), C18:3 *n*3 + *n*4 and the unknown peak (*b*) at 25.14 min., with a minor presence of C12:0, C14:0, C16:2 *n*4, C20:5 *n*3 and C22:5 *n*3 fatty acids, as showed in Table III.I.

The percents of total fatty acids were: saturated fatty acids (SFA) were C12:0 1.18%, C14:0 3.23% and the most abundant C16:0 26.86%. The percents for PUFAs were C16:2 *n*4 7.32%, C18:0 (or C18:1 *n*9 or C18:1 *n*7 or C18:2 *n*6) 30.05%, the identification of this peak was uncertain with the *standard* used, C18:3 *n*3 + *n*4 13.14%, C20:5 *n*3 3.14%, C22:6 *n*3 1.16% and two unknown peaks the first (*a*) with 0.87% at 10.84 min and the second one (*b*) with 12.62% at 25.14 min.

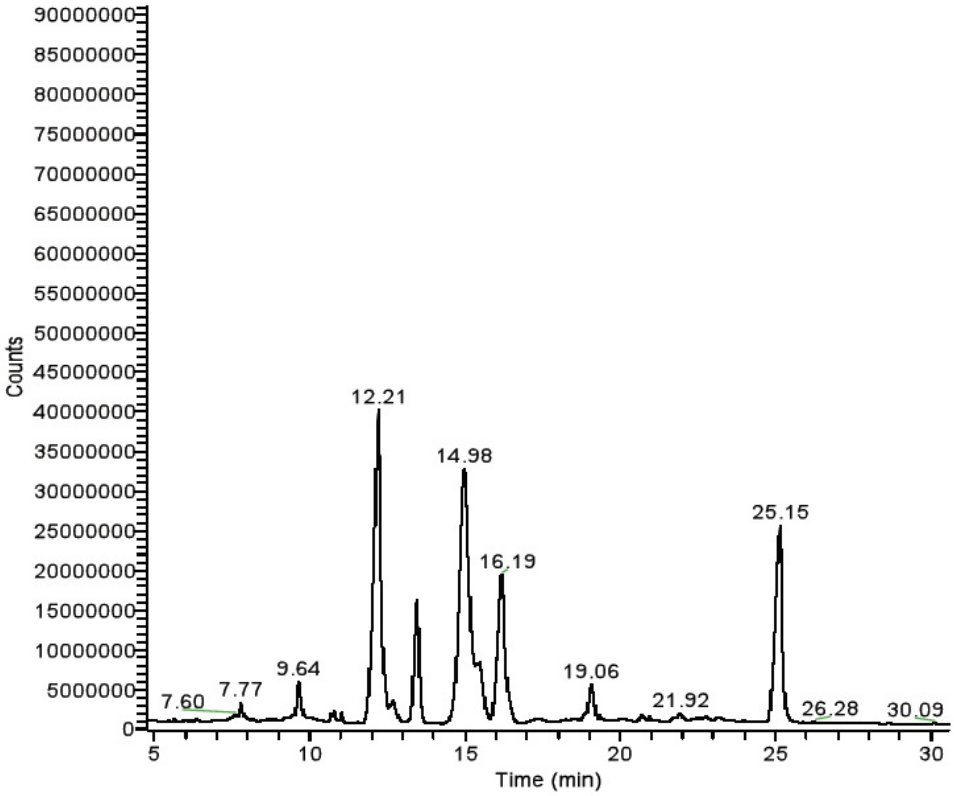

Fig. 3.6 Relative proportions of the major FAME fractions in *N. gaditana* cells culture under PAR plus UVR-B treatment.

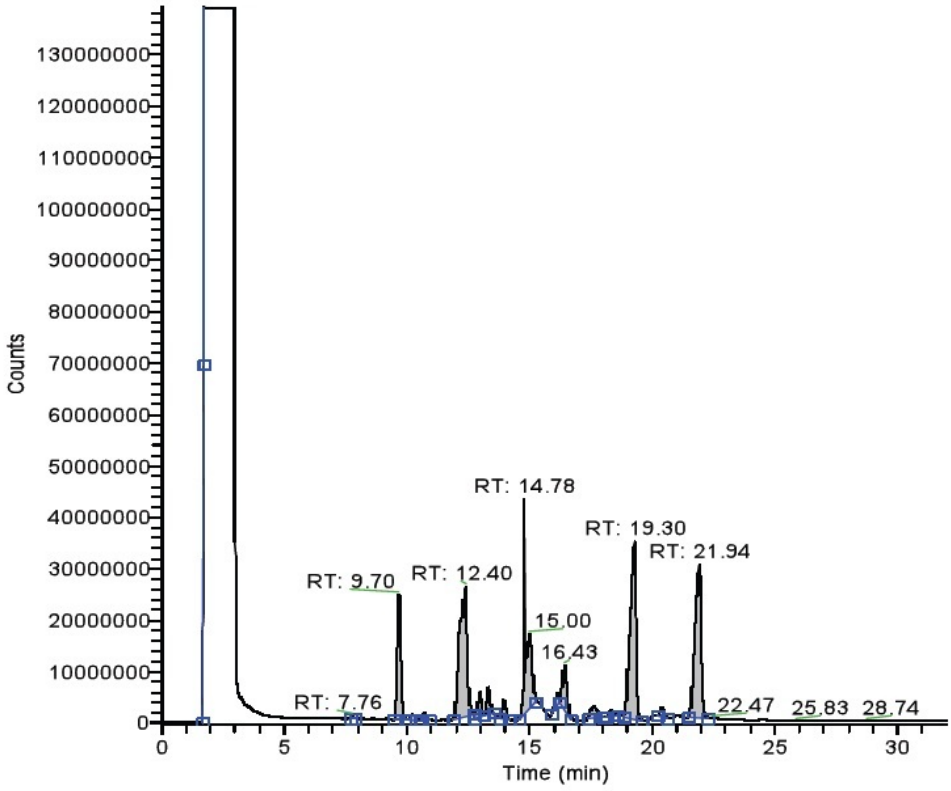

Fig. 3.7 Representative chromatogram with the different peaks and quantities of fatty acids, from menhaden oil (the *standard* used).

Table III.I Fatty acid compositions (as % of total fatty acids) of *N. gaditana* grown in f/2, during late exponential phase of the growth cycle (PAR plus UVR-B treatment).

Fatty acid	Common name	Area %
12:0	Lauric acid	1,18
14:0	Docosapentaenoic acid	3,23
a	-	0,87
16:0	Palmitic acid	26,86
16:1 n7	Palmitoleic acid	-
16:2 n4	Hexadecadienoic acid	7,32
16:3 n4	-	-
18:0 / 18:1 n9 / 18:1 n7 / 18:2 n6	Stearic acid/Oleic acid/Vaccenic acid/Linoleic acid	30,05
18:3 n3 + n4	α-Linolenic acid	13,58
18:4 n3	Stearidonic acid	-
20:1 n9	-	-
20:4 n6 e n3	Arachidonic acid (AA)	-
20:5 n3	Eicosapentaenoic acid (EPA)	3,14
22:5 n3	Docosapentaenoic acid	-
22:6 n3	Docosahexaenoic acid (DHA)	1,16
b	-	12,62
	Total SFA	31,27
	Total PUFA	67,86

Nannochloropsis sp. is a source for PUFAs, mainly EPA (20:5 *n*3), but also myristic and palmitic. They are the three most abundant fatty acids in *Nannochloropsis* sp. (Forján et al. 2011). In this study, linoleic acid is by far the most abundant unsaturated fatty acid in *N. gaditana* accounting for about 14% of the total content of the three most abundant fatty acids in *N. gaditana* (palmitic (27%) and linoleic acid and the *b* peak at 25.14 min, with 13%). The content of fatty acids can be altered by changes in nutritional and environmental parameters, such salt concentration, culture media, CO_2 concentration and

light intensity and many of the changes were species-specific or harvested at different growth stages (Brown *et al*. 1997; Forján *et al*. 2011).

The effect of irradiance on fatty acid composition of *Nannochloropsis* sp. has been described by Fábregas and co-workers (2004), high cellular abundance of EPA was obtained under low light. The results suggest that irradiance is a very useful tool to modify the biochemical composition of *Nannochloropsis* sp., since in our study the EPA was not the most abundant fatty acid, as described in the works of Fábregas and co-workers (2004) and Huerlimann and co-workers (2010). Brown and co-workers (1997) also observed EPA in percentages between 7-34% for Eustigmatophyceae, and in our study only 3% was obtained.

In the study of Huerlimann and co-workers (2010) was obtained for *Nannochloropsis* sp. (f/2 medium, and harvested in late exponential growth phase) 29,2% of total lipid content (% of DW) and at the stationary phase the lipid content was higher (33,2%). The increased accumulation with culture age has been observed in many microalgal taxa, as photosynthetic energy is diverted to lipid production instead of cell division (Huerlimann *et al.* 2010). Low levels of lipid accumulation during the exponential phase can be explained by the strain fulfilling its biotic potential and diverting maximum carbon and energy into cell growth and reproduction (Das *et al*. 2011). On average this class of microalgae contain high proportions of SFA (27%) (Brown *et al*. 1997), namely for palmitic acid with a percentage of 27%, which was also observed in *Nannochloropsis* sp. by Rebolloso-Fuentes and co-workers (2001).

A comparison of biochemical composition of algal cultures from various experiments is therefore difficult due to the differences in culture conditions, analytical methods and the growth phases analysed.

3.5 Antioxidant assay for DPPH radical scavenging activity

In these experiments different extracting solvents were tested in order to evaluate the extracting efficiency of the natural antioxidants from *N. gaditana*. The antioxidants were fractionated to *n*-hexane, ethyl acetate and water, by a three-stage sequential extraction procedure (Li et al. 2007). The antioxidative activity was measured using the DPPH radical scavenging assay according to Duan and co-workers (2006).

The values of the DPPH radical scavenging activities of *N. gaditana*, at the three fractions were presented in Fig. 3.8 These values were compared with those of BHT (10 mg mL^{-1}) 95,0±2,68%.

The capacity for the reduction of DPPH of *n*-hexane fraction was 1,23±0,60%, of ethyl acetate fraction was 0,23±0,46% and finally the water fraction showed a value of 0,60±0,11%. The antioxidant activity of *n*-hexane fraction was higher than that of ethyl acetate and water fraction.

The three different fractions isolated from the crude extract by solvent partition, the *n*-hexane-soluble and ethyl acetate-soluble fractions, as well as the water fraction, revealed no activities in this assay system (Fig. 3.8). None of the fractions were effective at scavenging DPPH radical than the positive control BHT.

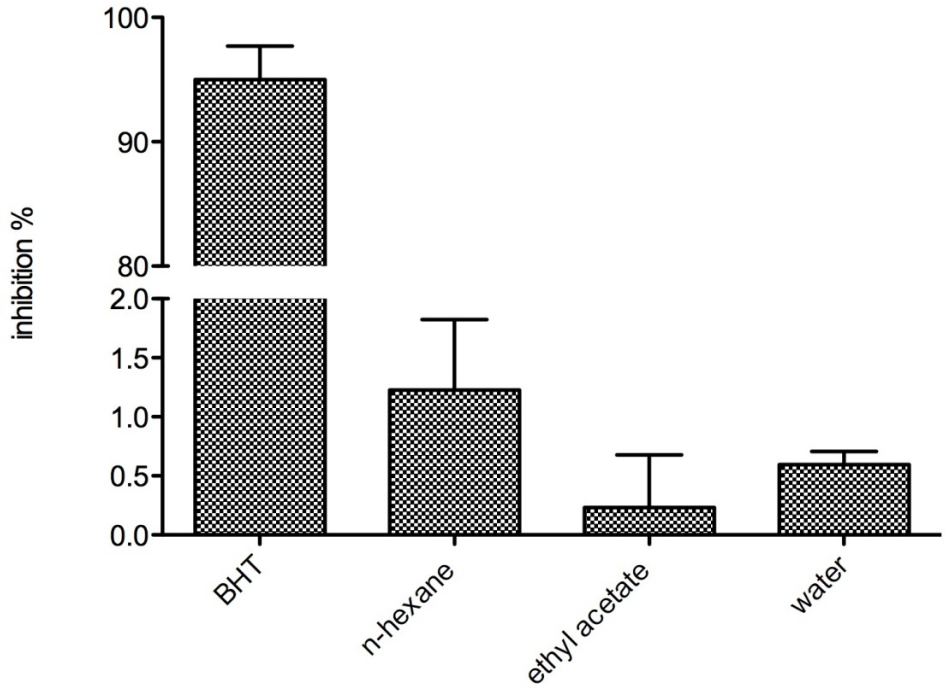

Fig. 3.8 Effects of n-hexane, ethyl acetate and water fractions of N. gaditana on DPPH radical scavenging assay. The results given here are mean values of three replicates and the vertical bars indicate SD.

These results showed that, the process of extraction was not as expected, since according to Li and co-workers (2007) high antioxidant capacities on different fractions for 23 microalgae were observed, using another similar assay for antioxidant activity, the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{+}$) radical decolorisation assay (at 734 nm).

According to Goh and co-workers (2010), and using other assays as well, such as ferric-educing antioxidant power (FRAP) and ferrous-ion chelating (FIC), the results for *Nannochloropsis* sp. with different solvent extracts, showed good antioxidant potential,

namely for dichloromethane fraction that is used to extract carotenoids and tocopherol (the presence of these compounds may contributed to the high scavenging power); for FRAP assay, the dichloromethane fraction attained the most powerful reducing ability; but for the FIC assay was the methanol fraction that had the most chelating ability. Globally, the results showed, that different solvent extracts contained different antioxidant capacities, good antioxidant potential for *Nannochloropsis* sp. also for the DPPH assay (Goh *et al*. 2010).

4 Conclusions/Final remarks

Identification and characterization of different MAAs from various aquatic ecosystems is difficult due to the lack of appropriate information regarding the process of their extraction and analysis. Comparing results from various published studies is made difficult because of the wide range of methodologies used in the separation of MAAs. While a general protocol to extract MAAs from aquatic organisms does not exist, but methanol concentration and temperature is important in estimating the concentration of these substances. A low extraction efficiency of MAAs will change the interpretation of ecological relationships or mask their importance as photoprotective compounds (Tartarotti and Sommaruga 2002). Separation of MAAs in complex samples, such as those found in some marine organisms, requires high resolution, with e.g. HPLC gradient elution methods (Carreto *et al*. 2005) and appropriate standards (many as possible).

For future analysis, it would be useful to do a procedure with a serial of extractions (already explored for Carreto and co-workers (2005)), and also different treatments not only with low-PAR but with high-PAR plus UVR-A (studied for Callone and co-workers) and other irradiance (e.g. stronger, or altering the direction or the distance from the light tubes to the Erlenmeyer flasks) of UVR-B. Also another precise techniques such mass spectrometry and nuclear magnetic resonance is require for the accurate identification and chemical characterisation of the *unknown compound*. Changes in pH were also studied in some groups of research (Nakamura *et al*. 1982; Carreto *et al*. 2005), so in the future this parameter could be taken in to consideration.

The occurrence and physiological importance of MAAs are becoming standard inclusions in studies of the effects of UVR on aquatic organisms. Continuing research on the diversity and abundance of MAAs in necessary to obtain a better understanding of UVR protection in phytoplankton from an ecological perspective and as potential to replace or complement current synthetically derived sunscreen compounds.

For the fatty acid profile, we suggest that samples should be extract in different phases of the growth cycle, not only in the late exponential growth phase but also in the e.g. stationary phase, to see how the content of EPA and other fatty acids will response.

In respect with antioxidant activity, the extraction should be repeated with some aggressive rupture of the cell walls of *N. gaditana*, because no mechanical rupture were applied here, and seems from the results presented that no compounds with antioxidant activity were extracted. Also we proposed the used of other assays (e.g. FRAP, $ABTS^{+}$) and different solvents for the extraction (such methanol and dichloromethane).

The ability to culture microalgae and their great biochemical diversity makes them a valuable potential renewable source of new drugs, growth regulators and other useful chemicals. Although some algal products are already available, continued isolation and screening of microalgae is required as well as studies of algal physiology and biochemistry. Further work in the development of new and better large-scale algal culture systems is required as screening techniques too, for microalgal biotechnology meet the high demands of food, energy and pharmaceutical industries.

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