UNIVERSIDADE D
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Telma Maria Borrego da Encarnação

MICROALGAE TECNOLOGY

SUSTAINABLE TRANSFORMATION OF EMERGING POLLUTANTS

Tese no âmbito de Doutoramento em Química, ramo de Fotoquímica, orientada pelos Professor Doutor Hugh Douglas Burrows, Professor Doutor Alberto António Caria Canelas Pais e Professora Doutora Maria da Graça Ribeiro Campos e apresentada ao Departamento de Química da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Agosto de 2019

Faculdade de Ciências e Tecnologia
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"Each discovery, each advance, each increase in the sum of human riches, owes its being to the physical and mental travail of the past and the present".

Piotr Kropotkin

A Pedro, Gabriela, Edite e Simone

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List of publications and main outcomes

Published papers and patent that resulted from the research work presented in this Thesis:

Encarnação, T., Pais, A.C., Campos, M.G., Burrows, H.D. Cyanobacteria and microalgae: A renewable source of bioactive compounds and other chemicals; *Science Progress*, (2015) 98, 145-168. DOI: 10.3184/003685015X14298590596266

Arranja, C., Aguiar, A., Encarnação, T., Fonseca, S., Justino, L., Castro, R., Benniston, A., Harriman, A., Burrows, H.D., and Sobral, A.J. Double-tailed long chain BODIPYs-Synthesis, characterization and preliminary studies on their use as lipid fluorescence probes. *Journal of Molecular Structure*, (2017) 1146, 62-69. DOI: 10.1016/j.molstruc.2017.05.119

Encarnação, T., Arranja, C., Cova, T., Pais, A.C., Campos, M.G., Sobral, A.J., and Burrows, H.D. Monitoring oil production for biobased feedstock in microalgae. A novel method combining the BODIPY BD-C12 fluorescent probe and simple image processing. *Journal of Applied Phycology*, (2018) 30, 2273–2285. DOI: 10.1007/s10811-018-1437-y

Encarnação, T. and Burrows, H.D. Provisional Patent nº 110536 PT, Optical or corrective lens.

Encarnação, T., Pais, A.C., Campos, M.G., Burrows, H.D. Endocrine-disrupting chemicals: Impact on human health, wildlife, and the environment; *Science Progress*, (2019) 102, 3-42.

Encarnação, T., Pais, A.C., Campos, M.G., Burrows, H.D. Chapter Microalgal biomass of industrial interest: methods of characterization/ Secondary metabolites analysis " in the book "Biomass, waste and related by-products characterization " Environmental Sciences-Springer.

Papers that resulted from the research work presented in this Thesis and were submitted for publication in 2019:

Palito, C., Encarnação, T., Valente, A., Pais, A.C. Microalgae immobilization and use in bioremediation – a review.

Encarnação, T., Palito, C., Aguiar, A., Pais, A.C., Campos, M.G., Sobral, A.J., and Burrows, H.D. A rapid reversed-phase HPLC method for the simultaneous analysis of olanzapine, simvastatin, ibuprofen and paracetamol in microalgae bioremediation process.

Encarnação T., Palito, C., Pais, A.C., Valente, A., and Burrows, H.D. Removal of pharmaceuticals from water by the microalga *Nannochloropsis* sp.

Encarnação, T. Santos, D., Pais, A.C., Valente, A., Pereira, J.C., and Burrows, H.D. Removal of imidacloprid from water by the microalgae *Nannochloropsis* sp. and development and validation of a RP-HPLC method for the analysis of imidacloprid.

Other published paper not directly related with the work presented in this Thesis:

Figueiredo, F., Encarnação, T. and Campos, M.G. Algae as Functional Foods for the Elderly. Food and Nutrition Sciences, (2016) 7, 1122 -1148.

Preface

Pollution is creating a global emergency, which is affecting the entire planet. Air, water and soil are necessary for the existence and well-being of all living beings. Current production and consumption patterns have been exhausting the natural resources and increasing pollution in all environments. A shift at a global scale of ideas is an urgent necessity. We all, politicians, business leaders, scientists, teachers, citizens and all members of the society, have the responsibility to intervene, to protect the planet and to preserve our future and that of future generations. It was with this responsibility in mind, that in 2014 and 2015, we coordinated two proposals for funding from H2020 research grants (Appendix D). These projects proposed a new integrated system for producing biobased products from processes using microalgae to rehabilitate the environment; a biorefinery concept, based on a circular economy. Although these projects were not funded, the reviewers' positive comments have provided us the encouragement to proceed. The study, presented in this Thesis, includes the proof-of-concept of the proposed projects. The inherent motivation carried out in this work is the need to contribute to the knowledge and solution of this global problem.

Preamble / Thesis organization

The present thesis is the result of the research work on the development of microalgae technology, carried out by me in the Department of Chemistry and in the Faculty of Pharmacy of the University of Coimbra.

The thesis is divided in five parts, A, B, C, D and E. In part A, which is subdivided into three chapters, the state of the art and a brief description of the general methods used are presented. Since different fields of research are considered, the first two chapters provide an overview of the current state of the art, one with special focus on microalgae as renewable sources of valuable compounds, and another, a comprehensive review of endocrine disrupting chemicals. Part B combines microalgae cultivation technology with the removal of selected endocrine disrupting chemicals from water, and includes two chapters, Chapter 4 and Chapter 5. Part C comprises two chapters, Chapters 6 and 7, each covering the research work developed in the search for new products obtained from the biomass resulting from the bioremediation processes. Part D has three chapters. Chapters 8 and 9 concern the development of analytical methods and their validation for the analysis of the selected endocrine disrupting chemicals, during microalgae bioremediation. Chapter 10 describes preliminary evaluation of a new dye as lipid fluorescent probe, used in the developed method for the monitoring of microalgae lipids, presented in Chapter 6. Conclusions and some final remarks are gathered in Part E. Finally, the Appendixes include some preliminary data on the development of a new method to evaluate microalgae cell population, and a study on deuterated species for use as biomarkers in magnetic resonance imaging or bioassays.

Resumo

Os efeitos adversos das actividades humanas têm levado à contaminação da água por uma gama cada vez mais complexa de poluentes emergentes. Estes não são eliminados pelos métodos convencionais de tratamento de água e, através do ciclo hidrológico urbano, podem entrar em águas subterrâneas e superficiais, persistir no meio ambiente, bioacumular-se através da cadeia alimentar e chegar à água potável. Incluem-se nos poluentes emergentes fármacos, pesticidas, plastificantes e vários outros grupos de contaminantes; são revistos nesta tese, alguns dos mais relevantes.

A combinação de diferentes tecnologias de tratamento de água pode reduzir significativamente a ocorrência de poluentes no ambiente aquático, diminuindo a exposição aos contaminantes e consequentes condições médicas associadas, levando a uma melhoria da saúde e bem-estar.

Esta tese aborda a necessidade de uma solução para o problema da poluição da água. Propõe a descontaminação de águas residuais recorrendo a microalgas, com uma abordagem baseada na geração de biomassa e na sua valorização económica, através de potenciais produtos de valor acrescentado. Esses produtos podem atrair investimentos na tecnologia e, portanto, acelerar o seu desenvolvimento.

Em primeiro lugar, avaliou-se a eficácia e eficiência do uso de microalgas na biorremediação de substâncias químicas sintéticas. Foram selecionados para o estudo os fármacos mais frequentemente detectados no ambiente e um representante do principal grupo de pesticidas, caracterizado pela sua toxicidade em insectos benéficos e pela exposição a que está sujeita a população em geral. Subsequentemente, avaliou-se a viabilidade da transformação de biomassa proveniente da biorremediação em produtos ecológicos inovadores. Paralelamente, diferentes métodos analíticos foram optimizados e desenvolvidos para a análise e avaliação de processos e produtos. Dentre estes, destaque-se o desenvolvimento de um método simples e confiável com resposta rápida para detecção e quantificação de lípidos, combinando uma nova sonda fluorescente altamente lipofílica, BODIPY BD-C12, e análise de imagem para determinar o conteúdo lipídico de algas e a produção de lípidos na microalga *Nannocloropsis* sp ..

Os estudos de biorremediação revelaram que a microalga marinha *Nannochloropsis* sp. remove paracetamol, ibuprofeno, olanzapina, sinvastatina e imidacloprida, apresentando diferentes eficiências. A remoção do paracetamol e ibuprofeno foi significativamente maior em células imobilizadas em álcool polivinílico do que em células livres, após as primeiras 24 horas de cultura.

No grupo das células livres, a remoção de olanzapina revelou maior eficiência, sugerindo uma maior afinidade das células à molécula de olanzapina do que ao paracetamol e ibuprofeno. Os estudos de imobilização das células mostraram que, embora o álcool polivinílico seja considerado biocompatível, este inibe a proliferação celular. Experiências com o polímero mostraram a libertação de células das esferas e sua desintegração devido à dissolução do álcool polivinílico.

Após o processo de biorremediação, a produção de produtos específicos foi avaliada e otimizada; com a otimização das condições, foi possível alcançar um elevado rendimento de 74% do teor de lípidos na espécie *Nannochloropsis* sp. e, com as partes restantes das células, foi possível demonstrar a sua aplicação em produtos ópticos avançados. Estes encontram aplicações em lentes oftálmicas, que podem ser usadas em óculos graduados e de sol, tendo potencial para ser a primeira lente verde no mercado.

Este estudo fornece um importante primeiro passo na tecnologia de biorremediação recorrendo a microalgas, um entendimento sobre a viabilidade da sustentabilidade num conceito de biorrefinaria de microalgas, com base na economia circular.

Palavras-chave microalgas, immobilização, biorremediação, disruptores endócrinos, biopolímeros, lípidos

Abstract

Adverse effects of human activity are leading to water contamination by an increasingly complex range of emerging pollutants, which are not readily treated by conventional means, and, through the urban water cycle, can enter ground and surface waters, can persist in the environment, bioaccumulate through the food chain and reach drinking water. These include pharmaceuticals, pesticides, plasticisers and several other groups of contaminants. Some of these compounds are reviewed in this thesis.

The combination of different water treatment technologies could significantly reduce the occurrence of pollutants in the aquatic environment, which leads to an improvement of health and well-being by preventing exposure to chemicals and the related diseases arising from contaminated water.

This thesis addresses the need for a solution to the problem of treatment of emerging pollutants, and proposes the decontamination of wastewaters using microalgae, with an approach based on the generation of biomass, and on the respective economic valorization. It aims to study the potential of obtaining high value-added products that could attract investment in this field and therefore, accelerate the development of microalgae technology.

In the first part of the Thesis, the effectiveness and efficiency of using microalgae in the bioremediation of synthetic chemicals were evaluated. The most frequent pharmaceuticals detected in the environment, and a representative of the major group of pesticides, were selected for the study, based on their respective toxicity to beneficial insects and ubiquitous exposure of the general population. The second stage assessed the feasibility of the transformation of the biomass from bioremediation into innovative bio-based products. In parallel, various methods were optimised and developed for the analysis and evaluation of processes and products. Of these, the most significant one is a simple reliable method with fast response for lipid detection and quantification, combining a new highly lipophilic fluorescent probe BODIPY BD-C12 and image analysis to determine the algal lipid content and the lipid production in the microalga *Nannochloropsis* sp..

The bioremediation experiments revealed that the marine microalga *Nannochloropsis* sp. removes the pollutants paracetamol, ibuprofen, olanzapine, simvastatin and imidacloprid with different efficiencies. The removal of paracetamol and ibuprofen after the first 24 hours of culture was significantly higher in polyvinyl alcohol immobilised cells than in free cells. In the group of free cells, the removal of olanzapine revealed a higher efficiency, suggesting more affinity of cells to the molecule of olanzapine than to paracetamol and ibuprofen.

The immobilisation experiments showed that, although polyvinyl alcohol is considered to be biocompatible, it inhibits cell proliferation. Also, experiments with the polymer showed the leakage of cells from the beads, and its disintegration due to dissolution of polyvinyl alcohol.

After the bioremediation process, the formation of specific products was assessed, and with the optimisation of the conditions it was possible to achieve a high yield of 74 % of lipid content in *Nannochloropsis* sp. and, with the remaining parts of the cells, it was possible to demonstrate the application of products from microalgae bioremediation in advanced optical products. These find applications in ophthalmic lenses, which can be used on prescription glasses and sunglasses, having potential to be the first green lens in the market.

This study provides an important first step in bioremediation using microalgae, and an understanding toward the feasibility and sustainability of the microalgae biorefinery concept, based on the circular economy.

Keywords microalgae, immobilisation, bioremediation, endocrine disrupting chemicals, biopolymers, lipids

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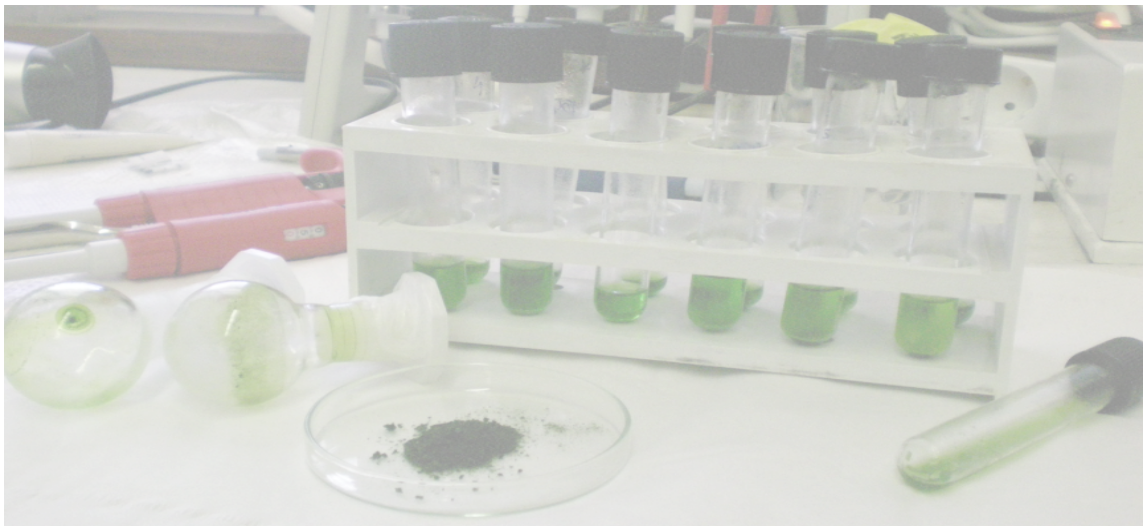
Part A

Introduction and methods

Chapter 1

Cyanobacteria and microalgae



Microalgae and cyanobacteria are rich sources of many valuable compounds, including important bioactive and biotechnologically relevant chemicals. Their enormous biodiversity, and the consequent variability in the respective biochemical composition, make microalgae cultivations a promising resource for many novel chemically and biologically active molecules and compounds of high commercial value such as lipids and dyes. The nature of the chemicals produced can be manipulated by changing the cultivation media and conditions. Algae are extremely versatile because they can be adapted to a variety of cell culture conditions. They do not require arable land, can be cultivated on saline water and wastewaters, and require much less water than plants. They possess an extremely high growth rate making these microorganisms very attractive for use in biofuel production - some species of algae can achieve around 100 times more oil than oil seeds. In addition, microalgae and cyanobacteria can accumulate various biotoxins and can contribute for mitigation of greenhouse gases since they produce biomass through carbon dioxide fixation.

The content of the present chapter was fully or partially published in paper form in Encarnação, T., Pais, A.C., Campos, M.G. and Burrows, H.D. Cyanobacteria and microalgae: A renewable source of bioactive compounds and other chemicals; Science Progress, 2015, 98, 145-68.

1.1 Microalgae as a renewable source of bioactive compounds and other chemicals

Microalgae belong to a wide group of microorganisms, primary producers, present in almost all ecosystems around the globe. It has been estimated that between 200 000 and several million species of microalgae exist[1]. This remarkable diversity is reflected in their biochemical composition. As a consequence, microalgae possess great biotechnological potential for applications in the pharmaceutical, food, cosmetic, and other industries.

The phytoplankton biomass present in the ocean is less than 1% of the total carbon contained in all photosynthetic biomass. However, it is estimated that phytoplankton store between 30 and $50x10^{15}$g of carbon annually[2]. This gives to these photosynthetic microorganisms a crucial role in the most critical natural cycles of the planet, as well as being the base of the food chain. One of the major consequences of the activity of these life forms is their influence on the climate, and the reduction of the greenhouse effect to capture CO_2 from the atmosphere and deposit it in the bottom of the oceans[3]. Phytoplankton and plants, that evolved about 500 million years ago, use the energy of the sun to split water molecules into hydrogen and oxygen. The oxygen is released as waste, allowing the survival of all animal life on earth, while the carbon cycle depends on the use of the water hydrogen atoms by the photosynthetic organisms for primary conversion of inorganic carbon as CO_2 into organic matter, such as sugars, amino acids and other biological molecules within their cells. Factors such as light, pH, temperature, salinity and nutrient availability affect both photosynthesis and biomass productivity, and influence the activity and mechanisms of cellular metabolism, thus influencing the chemical and biochemical composition of cells[4].

The biodiversity of microalgae represents a tremendous resource for production of a variety of interesting chemicals, many of them with considerable commercial value. However, despite this diversity, only a dozen species of microalgae are cultivated on a large scale. Table 1.1 indicates some of the companies that are producers of microalgae. The major obstacle to large scale production is that the cost of production is still too high. Many studies have been focused on the technology of the production. Most are about cultivation reactors, while others have been conducted to evaluate variations of growth parameters in the cellular composition to optimize production.

Table 1.1 *Companies producers of microalgae and microalgal products.*

Microalgae species	Application/product	Company	Country
Nannochloropsis *Tetraselmis* *Phaeodactylum*	Cosmetics and aquaculture	Astaxa	Germany
Nannochloropsis *Haematococcus pluvialis* *Spirulina* *Chlorella*	PUFA, omega 3, EPA astaxanthin	Bluebiotech	Germany
Haematococcus pluvialis	Astaxanthin/human, fish and animal consumption	Alga Technologies	Israel
Nannochloropsis sp. *Phaeodactylum tricornutum* *Amphora* sp. *Navicula* sp. *Dunaliella* sp. *Chlorococcum* sp. *Tetraselmis* sp. *Nannochloris* sp.	Food additives, animal and fish feed, biofuel	Seambiotic	Israel
Haematococcus pluvialis	Astaxanthin	Biogenic Co., Ltd.	Japan
Spirulina *Haematococcus pluvialis*	Astaxanthin	Cyanotech	USA
Chlorella	Biofuels, nutritional, health sciences, chemicals	Solazyme	USA
Spirulina *Chlorella* *Crypthecodinium Cohnii*	DHA/dietary supplements, aquaculture feeds, cosmetics	Femico	Taiwan

The most common biotechnological applications of microalgae have been in aquaculture for feeding fish and other organisms of economic interest, such as mollusks and crustaceans. Pigments, anti-oxidants and food supplements can also be derived from microalgal extracts. Microalgae can synthesize toxins, but can also produce a wide range of other bioactive molecules with antibiotic, anticancer, anti-inflammatory and antiviral, or other pharmacological properties, see below. In addition, they can be used in wastewater treatment, in numerous industrial processes for the biological detoxification and removal of contaminants from the environment, in the synthesis of biopolymers and cosmetics, and in the production of biofuels. In agriculture, the biomass can be used as a soil biofertilizer. The algal biomass consists of about 50% carbon which means that approximately 1.8 kg of CO_2 is required to produce 1 kg of algae[5]. Since pure CO_2 is expensive, cost effective alternatives of this gas, such as gases emitted from industrial combustion processes, fermentation processes or engines have been tried. Furthermore, microalgae can be used in the reduction of greenhouse gas emissions from industrial plants, such as thermal power plants and cement factories, through the CO_2 flue gas assimilation.

1.2 The early History of Microalgae

The first living organisms, bacteria and unicellular prokaryotic primitive organisms appeared on Earth at least 3.5 billion years (Gyr) ago, during the Archean period (Figure 1.1). Although controversial, geological evidence suggests that, in southwest Greenland, 3.8 Gyr old Isua rocks present what may be the oldest sediment record of life on Earth[6].

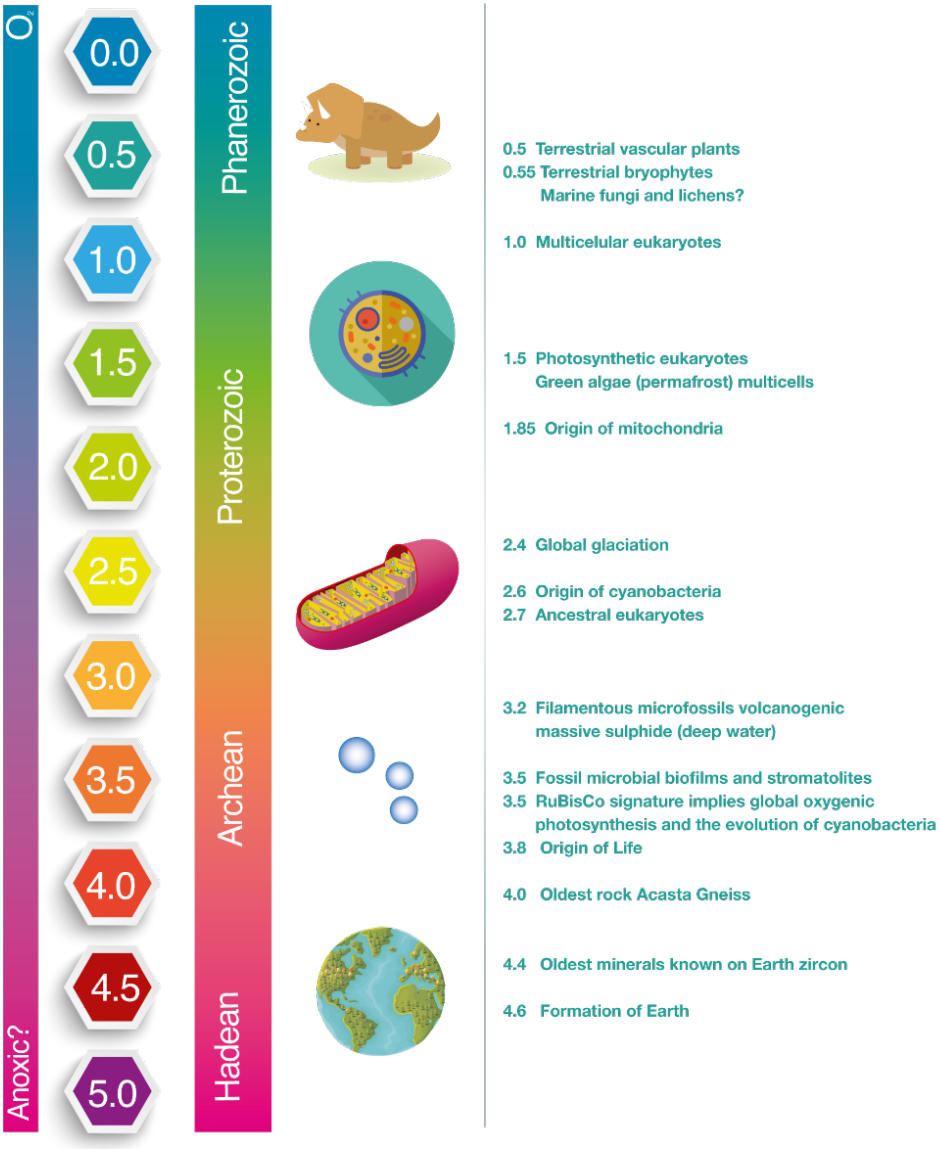


Figure 1.1 *Timeline with important biological events. In discussing geological time, 1 Gyr corresponds to 10^9 years.*

If confirmed, this life has existed possibly by anoxygenic photosynthesis. In the later Archean period, the Earth's atmosphere was rich in carbon dioxide and, prior to 2.5 Gyr, was free of oxygen. The current values of oxygen concentration would be found only from 600 million years (Mya) ago[7]. Around 3.5 Gyr, data from the signature RuBisCo (Ribulose-1,5-bisphosphate carboxylase) suggest a global oxygenic photosynthesis and evolution of cyanobacteria. According to geological and paleontological evidence, cyanobacteria are the most ancient photosynthetic organisms, and producers of oxygen on Earth. Fossil populations of cyanobacteria, known as stromatolites, and carbon isotope ratios confirm that autotrophs may have existed 3.5 Gyr ago, which fixed carbon through the Calvin cycle. Oxygen production by these organisms and their possible accumulation in the atmosphere caused the first mass extinction of anaerobic organisms (ca. 2000 MY ago)[8]. The eukaryotic cell arose during the Proterozoic, dating back 2.5 billion years and extending to 542 million years ago. There is a view that green algae may have emerged through the symbiosis between cyanobacteria and eukaryotic non-photosynthetic ancestors, leading to photosynthetic eukaryotes that would give rise to the first algae 1.5 billion years ago[9]. Fossil records suggest the appearance of seaweed eukaryotes around 1.2 Gyr with the possible colonization of freshwater lakes around 1.1 Gyr. These organisms, and most of photoautotrophs that evolved from it, used the enzyme Rubisco to catalyze the photosynthetic reduction of carbon[10]. Terrestrial Bryophytes started appearing from around 550 MY followed by vascular land plants 440 MY ago during the Devonian period. Other species such as lichens and fungi are believed to have appeared around the earliest appearance of the first bryophyte[8].

1.3 Biochemical Composition of microalgae and cyanobacteria

As with other food sources (Table 1.2), microalgae are composed primarily of proteins, lipids, carbohydrates and nucleic acids in different proportions, that vary both from species to species and with the growth conditions. They also contain polysaccharides, minerals, vitamins and pigments. For example, cells of *Nannochloropsis* (Figure 1.2), a marine microalgae, contain the pigments *β*-carotene, violaxanthin, vaucheraxanthin, zeaxanthin and anteraxanthin, in addition to the amino acids aspartate, glutamate, proline, methionine, tryptophan, cystine, histidine, hydroxyproline and several fatty acids, such as eicosapentaenoic acid[11].

Table 1.3 presents the chemical composition of some microalgae species. It should be kept in mind that the values presented correspond to specific growth conditions and for these microalgae species, and have not necessarily been optimized for each compound.

Table 1.2 *Chemical composition of some food sources.*

Food	Carbohydrates Total Sugar(%)	Proteins (g/100g)	Lipids (% dry weight biomass)	Ref.
Milk	4.9	3.6	3.5	[12,13]
Rice (white, raw)	25-26.6	6.7	1.4	[12]
Soybean (dry, raw)	-	34.1	17	[12]
Meat (beef)	-	16.5	10-30	[12]
Fish (haddock)	-	18.3	0.1	[12]

Table 1.1.3 *Chemical composition of some species of algae.*

Microalgae species	Carbohydrates (% dry weight)	Proteins (% dry weight)	Lipids (% dry weight)	Pigments	Ref.
Amphora sp.	-	-	26.4-81.5	-	[14]
Aphanothece microscopica Nägeli	13.4-17.6	41.3-49.3	7.1-7.9	-	[15]
Arthrospira (spirulina) Platensis	15.0-26.97	45.0-62.2	8.04-13.79	-	[16,17]
Botryococcus braunii	2.38	39.61	33	13.05	[18]
Chlamydomonas rheinhardii	17	48	21	-	[13]
Chlorella vulgaris	16.74	40.95	9.95	12.41	[18]
Dunaliella tertiolecta	13.95	29.41	11.44	7.61	[18]
Nannochloropsis sp.	8-14	33-44	22-31	-	[19]
Porphyridium cruentum	22.8-39.3	27.7-40.8	5.78-7.55	-	[20]
Scenedesmus obliquus	10-17	50-56	12-14	-	[13]
Spirulina platensis	11	42.33	11	16.12	[18]
Synechococcus sp.	15	63	11	-	[13]

1.4 Microalgae and cyanobacteria as sources of bioactive compounds

Many of the compounds used for therapeutic purposes have their origin in nature, more specifically, in plants. Studies of natural products based on biological diversity have continuously unveiled novel chemical structures that, in many cases, lead to new drugs. Many molecules of natural origin also serve as structural models for creating synthetic analogues. Cyanobacteria, commonly called blue-green algae, comprise a vast and varied morphological group of oxygenic phototrophic bacteria, and represent one of the largest phyla of Bacteria[21], as it was highlighted above. Falkowski and Raven[22] estimated that there are approximately 10^{25} cells of cyanobacteria in the oceans, so that the fixation of photosynthetic carbon and nitrogen in modern oceans is carried out by cyanobacteria. These phototrophic prokaryotes produce oxygen as a waste product, which has made possible the evolution of complex multicellular organisms. These simple prokaryotes have drawn considerable attention due to secondary metabolites and their fascinating structures. Cyanobacteria are known to contain a huge variety of toxic and bioactive substances. These metabolites have been explored as potential antibiotics anticancer, anti-inflammatory and antiviral drugs (Figure 1.3). This remarkable chemical diversity has been particularly evident in studies with the marine cyanobacteria *Lyngbya majuscula* (Figure 1.2). This unique species is known to cause the dermatitis knowns "swimmers itch", and has been shown to produce a wide range of metabolites which include polyketides, lipopeptides, potent toxins and many others[23].

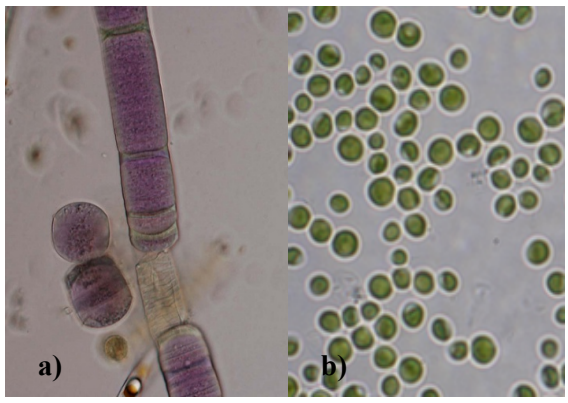


Figure 1.2 *(a) Cyanobacteria Lyngbya majuscula. (Figure downloaded from Florida Fish and Wildlife Conservation Commission)*[24]. *(b) Marine microalgae Nannochloropsis* sp. *cells.*

Analysis of 424 marine cyanobacterial natural products showed that the content of them are lipopeptides (40.2 %), are pure amino acids (5.6%), fatty acids (4.2%), macrolides (4.2%) and amides (9%)[25] (Figure 3). And yet, Cyanobacteria remain a largely unexplored resource of new bioactive compounds.

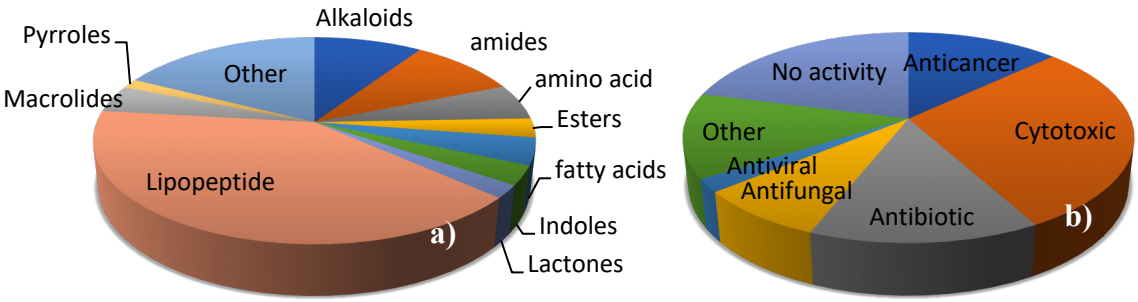


Figure 1.3 *(a) Compounds isolated from cyanobacteria. (b) Reported biological activity of marine cyanobacterial compounds (Adapted from Burja et al. [26])*

Dinoflagellates, which are eukaryotes, also demonstrate an extraordinary chemical diversity. However, apart from producing bioactive molecules of interest, such as macrolides and polyethers, they also yield potent toxins. For example, the species dinoflagellate *Gambierdiscus toxicus*, which is very common near reefs, produces the gambiertoxin. This toxin enters the food chain through fish that have ingested toxic microalgae[26]. The dinoflagellate marine genus *Amphidinium,* in turn, produce a wide range of polyketides and cytotoxic macrolides that may lead to new anticancer drugs. Table 4 shows the biological activity of the compounds obtained from several species of microalgae and cyanobacteria.

1.5 Biofuels and microalgae

Biofuel production has generated intensive global debate due to the global energy, environmental and food supply crisis. Biofuels are usually produced from plant oils, such as corn, soybean, sugar-cane, sugar-beet, sunflower and palm. Rises in global food prices have been attributed to the use of arable land to produce biofuels. The decrease in rice and wheat production and a significant reduction in the market stockpile of corn has contributed to a potential food-crisis[27]. Photosynthetic microorganisms, such as cyanobacteria and microalgae, are renewable energy sources that also help reducing greenhouse gases, have low pollutant emission, the highest CO_2 fixation and O_2 production efficiencies, can be used to treat water waste and do not compete with the food industry since they do not require arable land, and have fairly modest water requirements. Microalgae cultures can be grown in photobioreactors that can be installed in regions ranging from the desert to snow soils. They have growth rates higher than traditional crops, with a complete life cycle that lasts a few days, and they can be easily genetically manipulated. In addition to extract lipid for producing biodiesel, other fractions of cell can be used to produce pigments, nutraceuticals, etc.. In terms of yield, there are some algae that yield around 50% of their own

weight of oil and it has been estimated that they can produce around 125 tonnes ha^{-1} of biodiesel[27]. Table 5 presents some indicatives values of oil production for different crops.

Table 5 *Yield of various plant oils*[27].

Crop	Oil (L ha^{-1})
Soy	446
Safflower	779
Sunflower	952
Conola/Rapeseed	1000
Castor	1413
Coconut	2689
Palm	5950
Algae	80000

The lipid content can vary greatly between the species and with different growth conditions. In general, microalgae possess a high content of lipid under depleted nitrogen conditions. Neutral lipids, in particular, triacylglycerols (TAG) are the dominant type. TAGs are considered to be preferred to phospholipids or glycolipids for biodiesel because of their higher percentage of fatty acids and lack of phosphate[28]. For engine applications, biodiesel must comply with some requirements such as cetane number, viscosity, flash and solidifying points[28]. The properties of the triglyceride and the biodiesel fuel are determined by the amount of each fatty acid that is present in the molecules. Physical characteristics of both, fatty acids and triglycerides, are determined essentially by chain length and number of double bonds[29].

Microalgae can produce different types of biofuels such as biodiesel, ethanol, hydrogen, etc. Production of hydrogen by cyanobacteria is a viable alternative energy source. It is renewable, ecofriendly, carbon neutral and efficient[30].

Although the use of microalgae can be the most efficient biological producer of oil on the planet and a versatile biomass source and may soon be one of the Earth´s most important renewable fuel crops[31], at the present, the microalgae and cyanobacteria based biofuels are still costly and therefore, unable to compete with petroleum-based fuels.

1.6 Microalgae and their application as biofertilizers

Nitrogen is an essential macronutrient upon which agricultural and global food supply is dependent. This macronutrient is vital in the formation of biomolecules such as DNA, proteins and amino acids. Although there is plenty of nitrogen in the atmosphere, water and soil, most plants and microalgae are unable to assimilate the available nitrogen form, dinitrogen ($N_{2)}$. In general, the microalgae and plants assimilate nitrates, ammonia or organic nitrogen sources such as urea. Besides being costly, synthetic chemical fertilizers, pesticides and herbicides, may have adverse effects on the environment, soil fertility and productivity. Biofertilizers may help overcome these

problems. Diazotrophic heterocystous cyanobacteria are capable of assimilating N_2 through the process of nitrogen fixation, and have long been used as biofertilizers, for instance, for rice. *Anabaena azollae* is a heterocystous nitrogen-fixing cyanobacteria which lives in symbiotic association with the water fern *Azolla* capable of fixing atmospheric nitrogen. The use of *Azolla-Anabaena azollae* in agriculture has a long history in China and other countries in the Far East. The earliest mention of the plant seems to be 2000 years ago in an ancient dictionary, and in some Chinese poems in which the water plant is described[32].

The dinitrogen fixation in cyanobacteria is carried out by the enzyme nitrogenase found in specialized non-dividing cells known as heterocystis. In addition to enriching the soil with nitrogen, the cyanobacteria improve soil texture, plant growth and crop yield since they provide biologically active substances to plants, such as gibberelin, auxin, cytokinins, vitamins, amino acids, polypeptides, substances with antibacterial and antifungal activities and exopolysaccharides[33]. There have been a number of studies of the effect of cyanobacterial biofertilization. In a literature review of 634 field experiments, inoculation of rice with cyanobacteria biofertizer was found to have induced an increase of grain yield of 267 kg ha^{-1} [34].

Despite the success of rice production with diazotrophic heterocystous cyanobacteria, its use is restricted to a very limited hectarage. This is mostly due to technological obstacles of inoculums quality, establishment of protocols for use under field situations and poor shelf-life[34]. However, potentially biofertilization with cyanobacteria provides a renewable non-costly, easily manageable biological nitrogen source that can contribute to a sustainable agricultural development. In addition to biologically fixing nitrogen, another very interesting application of cyanobacteria in agriculture is their use in the degradation of pesticides and the release of inhibitors of plant pathogen.

1.7 Microalgae for removal of pollutants

Due to anthropogenic activities, the lakes, the rivers and streams, the seas and oceans of the earth are heavily polluted. Water pollution is a serious environmental threat, mostly caused by pollutants released from industrial and sewage discharges and runoffs from agriculture. Not only it affects habitats and entire ecosystems, but also, specifically, human health. Many of the pollutants found in water bodies are considered disruptors to the endocrine system of humans and wildlife; they are called endocrine disrupting chemicals (EDCs)[35]. Through the urban cycle of water, pollutants such as bisphenols, organochlorine pesticides, heavy metals and so many others, are found in ground, surface and drinking waters [35,36]. The conventional drinking and wastewater treatment, which include aeration, sedimentation, flocculation, coagulation, flotation, activated sludge, ozonation, chloronation and oxidation processes, do not completely remove most of these pollutants [35]. Also, the excessive concentration of nitrates and phosphates in the water bodies lead to the eutrophication with the inevitable consequences of having microalgal blooms, sometimes

with toxic and lethal species, that reduce the oxygen present in the water causing the death of the native flora and fauna.

One potential contribution to solve the water pollution problem consists on the use of biological agents, together with existing methods. The biological treatment processes include bacteria, fungi, protozoa, enzymes and microalgae. The bioremediation using microalgae has several advantages over the conventional methods. The potential solution to the concerns regarding all aspects of the environment and the economic valorization of the resulted biomass are among the most important advantages of this treatment.

In conclusion, microalgae and cyanobacteria are a promising natural resource. The possibilities of commercial products derived from them are immense. However, at present, the products offered in the marketplace are very few. The cost for obtaining some of the microalgae products, and the difficulty to scale up others are barriers to the commercial exploitation. However, the possibility to produce culture of them in wastewater treatment plants, the growing shortage of resources, coupled with improving extraction and production techniques are turning microalgae and cyanobacteria into an attractive renewable source of bioactive compounds and other fine chemicals. In the next chapter, focus is given to a group of emerging pollutants that will be the object of bioremediation in later chapters.

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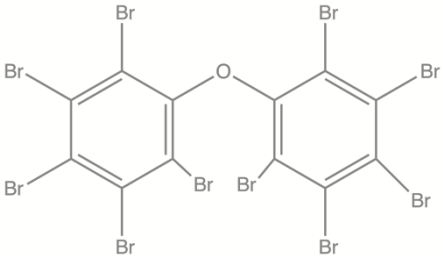
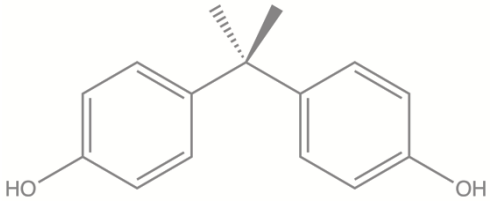
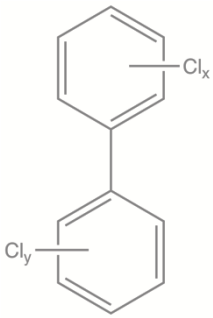
Chapter 2

Endocrine-disrupting chemicals

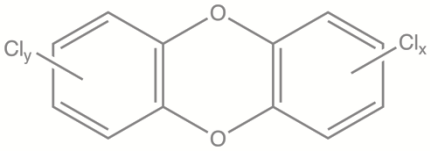






Endocrine disrupting chemicals are a group of pollutants that can affect the endocrine system and lead to diseases and dysfunctions across the lifespan of organisms. They are omnipresent. They are in the air we breathe, in the food we eat and in the water we drink. They can be found through personal care products, household cleaning products, furniture and in children´s toys. Every year, hundreds of new chemicals are produced and released onto the market without being tested, and they reach our bodies through everyday products. Permanent exposure to those chemicals may intensify or even become the main cause for the development of diseases such as type 2 diabetes, obesity, cardiovascular diseases and certain types of cancer. In recent years, legislation and regulations have been implemented, that aim to control the release of potentially adverse endocrine disrupting chemicals, often invoking the precautionary principle.

The following sections will provide an overview of research on environmental aspects of endocrine disrupting chemicals and their effects on human health, based on evidence from animal and human studies. Emphasis is given to three ubiquitous and persistent groups of chemicals, polychlorinated biphenyls, polybrominated diphenyl ethers, and organochlorine pesticides, and on two non-persistent, but ubiquitous ones, bisphenol-A and phthalates. Some selected historical examples are also presented, and successful cases of regulation and legislation described. These led to a decrease in exposure and consequent minimization of the effects of these compounds.



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2.1 Endocrine-disrupting chemicals (EDCs)

Some of the most serious problems that humankind faces are climate change and global warming, biodiversity loss, ocean dead zones, achieving energy sustainability, increasing population, which is estimated to exceed nine billion in 2050[1], overconsumption of resources, waste management, air, soil and water pollution. These are posing dramatic challenges that can jeopardize our future. A myriad of synthetic chemicals has been released into the environment, especially since World War II, and some in large quantities. In 2015, the total production of chemicals within the 28 member states of European Union (EU) was 323 million tonnes, 205 million tonnes of which were considered hazardous to health[2]. Figure 2.1 presents the production of chemicals hazardous to health, over the last 12 years, according to five toxicity classes. The production rate does not necessarily translate into the release into the environment and human exposure, but it is, nevertheless, an indicator of the potential impact on human health and on the environment.

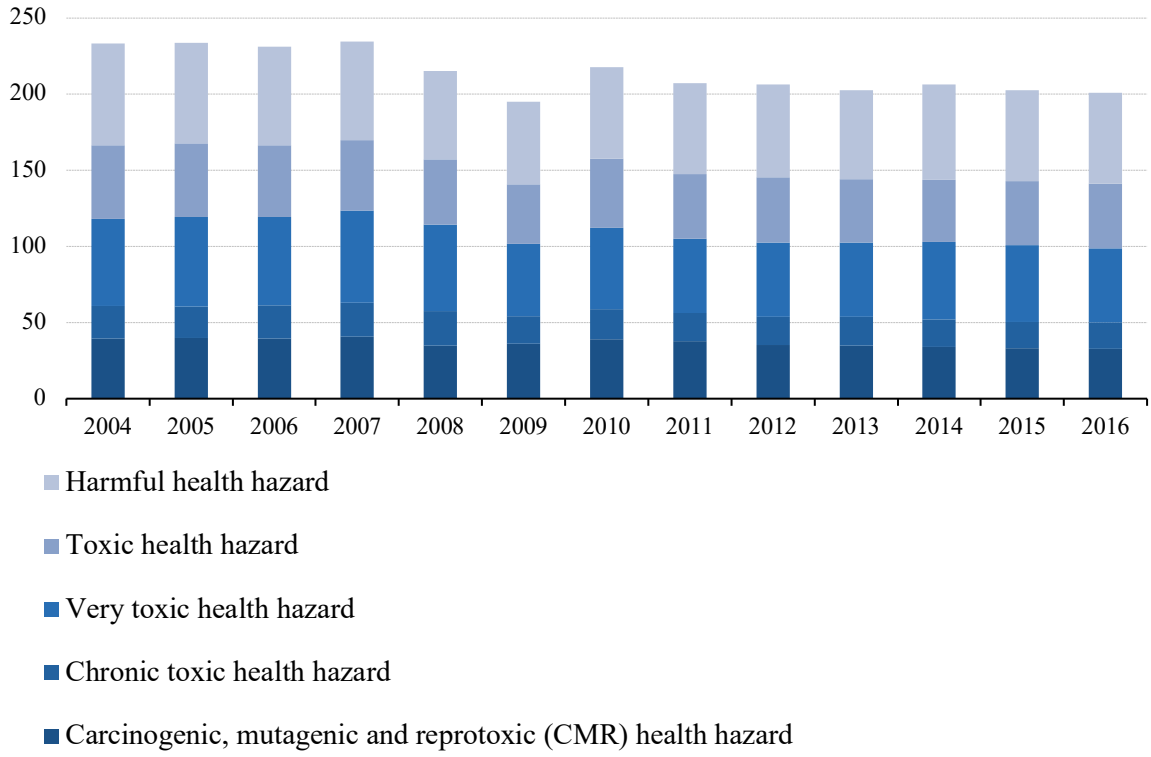


Figure 2.1 *Production of chemicals considered hazardous to health in EU-28, between 2004 and 2016 (million tonnes). Consumption does not differ significantly from production. However, consumption is generally higher than production, due to importation (source:Eurostat).*

Concerns regarding exposure to these chemicals and potential adverse effects are due, in part, to the increasing number of reports of their role as endocrine disruptors. In the last twenty years, a large number of research papers have been published, which address various aspects of endocrine disrupting chemicals (EDCs), including environmental occurrence, ecological effects and consequences of human exposure.

In 1996, with support from the European Commission, the World Health Organization (WHO), Organisation for Economic Co-operation and Development (OECD), and other national authorities, the international meeting, "The impact on the endocrine disruptors on human health and wildlife", appealed for a better understanding of epidemiological aspects, and to the needs of identification, monitoring and the development of methods to test and screen endocrine disrupting chemicals (The Weybridge Report)[3]. This meeting considered that measures to reduce exposure to these chemicals should be made according to the Precautionary Principle (1992 Rio Declaration)[1]. The Precautionary Principle arose from environmental considerations, and can be viewed as an ethical ideal. Another outcome of this international meeting was the definition of EDCs: an endocrine disrupting chemical was defined as an "exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations". Also, "a potential endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub)populations" [4] (WHO, International Programme on Chemical Safety) [5].

In 2009, The Endocrine Society published the first Scientific Statement on EDCs addressing the concerns to public health, based on evidence from animal models, clinical observations and epidemiological studies[6]. The statement includes evidence of the effects of endocrine disruptors on male and female reproduction, breast development, prostate and breast cancer, neuroendocrinology, thyroid, metabolism and obesity, and cardiovascular endocrinology, and calls for an increased understanding of the effects of EDCs, with the involvement of individual and scientific society stakeholders in communicating and implementing changes in public policy and awareness[6]. In 2013, the WHO released The State of the Science of Endocrine Disrupting Chemicals - 2012[7] expressing concern on the impact of endocrine disrupting chemicals; this was supported by extensive research that improved the understanding of the mechanisms involved in endocrine disruption, and stated that there is ample evidence attesting to the impacts of EDCs on human and

[1] Rio Declaration, 1992 Principle 15: "In order to protect the environment the Precautionary Approach shall be widely applied by States according to their capabilities. Where there are threats of serious or irreversible damage, lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation". In a Judgement (C180/96, point 99), on May 5, 1998, the Court of Justice, has said that "where there is uncertainty as to the existence or extent of risks to human health, the Commission may take protective measures without having to wait until the reality and seriousness of those risks become apparent"

wildlife health[7]. Even after decades of research on EDCs, and the two decades since the Weybridge Report, there is still no consensus on EDCs among scientists, regulators and activists, either within the EU or at the international level. On 16 December 2015, a judgment of the General Court of the Court of Justice of the EU declared that the European Commission "unlawfully failed to adopt delegate acts concerning the scientific criteria for the determination of endocrine properties" (Case T-521/14)[8]. The European Commission was expected to deliver, before December 2013, scientific criteria for the definition and identification of chemicals that may possess endocrine disrupting properties, and to adopt and delegate acts according to such criteria[9]. In October 2017, the member states' representatives rejected the Commission´s definition on EDCs, and, one year later, there is still no defined strategy that addresses the challenges they pose. The regulation of chemicals includes carcinogens, mutagens, teratogens and substances that disrupt reproduction. EDCs represent a relatively new classification, involving different chemical classes that are able to mimic or antagonize endogenous hormones. Although there is no consensus on their regulation, EDCs are addressed in various cases in EU law, such as the Water Framework Directive, REACH, PPPR, and Cosmetic Regulation. These will be described briefly below.

EDCs are found in many of our everyday products, for example, those that we use for personal or domestic care, in the air we breathe, in the food we eat, and in the water we drink. The challenges that arise from the field of endocrine disruption are the immense diversity of chemicals produced, but not tested, the mixtures and the unknown interactions between them, together with their consequent effects. The lack of an effective and strong legislation and regulation poses a significant threat to humans, animals and plants, and contributes to the exposure to chemicals that may disrupt the endocrine system.

Since World War II, more than 140,000 synthetic chemical compounds have been produced; about 1000 to 2000 new compounds are synthesised each year[10], and approximately 800 chemicals are known, or suspected, to interfere with the endocrine system[11]. The increasing incidence rate of some medical conditions, such as breast and prostate cancer, types 2 diabetes, cardiovascular diseases, in the last decades, and the higher rates observed in the industrialized world, cannot be explained only by genetic factors (see Figures 2.2 and 2.3). Environmental factors, nutrition and lifestyle, viral diseases, are some of the other variables in such a complex system.

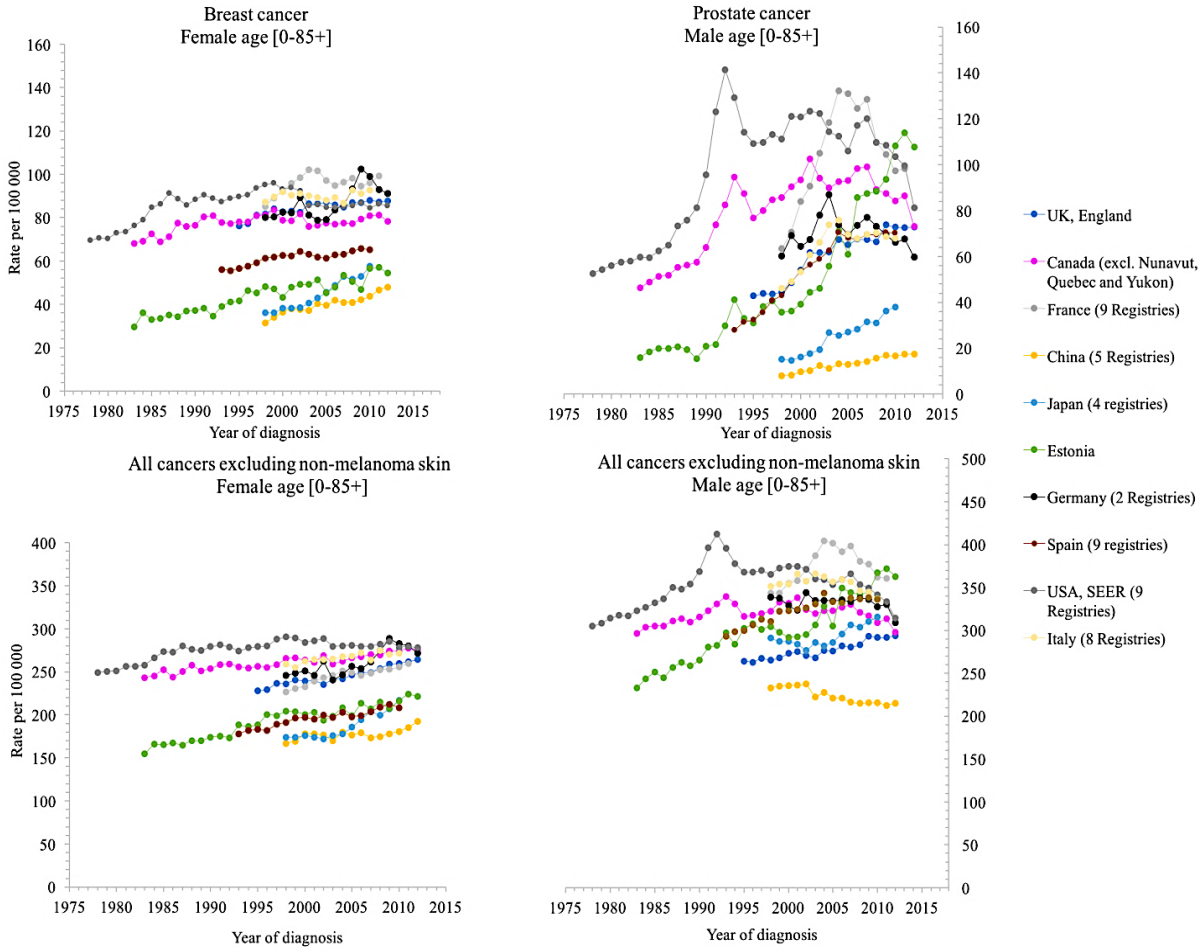


Figure 2.2 *Trends in cancer incidence, in the selected countries, by year of diagnosis. Data on trends in incidence for breast, prostate, and all cancers excluding non-melanoma skin, for the selected countries, were extracted from CI5plus-Cancer Incidence in Five Continents Time Trends (http://ci5.iarc.fr/CI5plus/Default.aspx).*

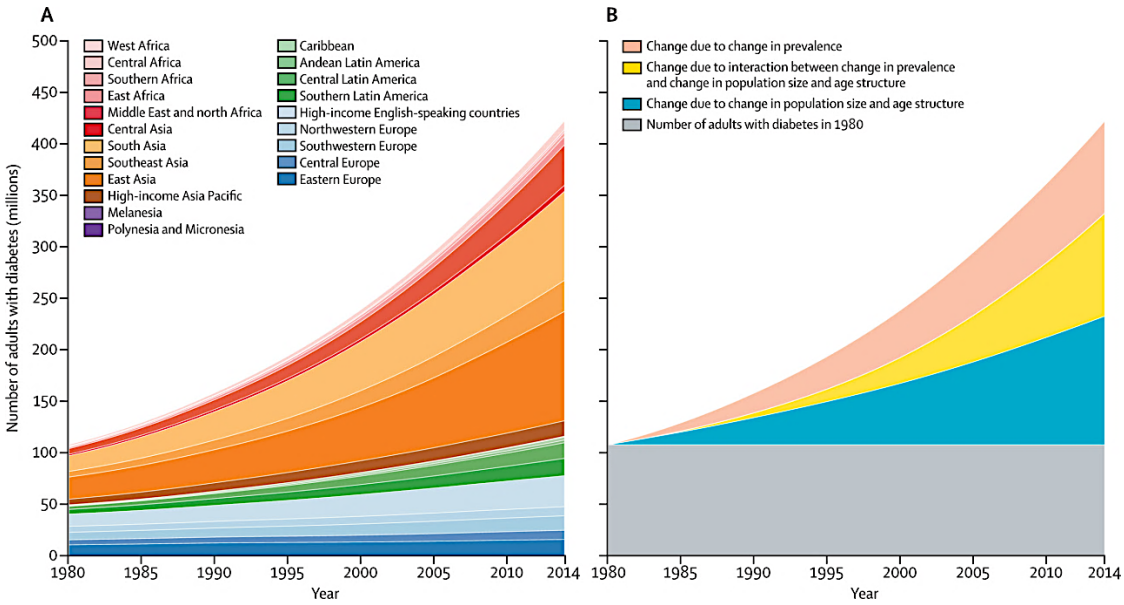


Figure 2.3 *Worldwide trends in the number of adults with diabetes by region (A) and decomposed into the contributions of population growth and ageing, rise in prevalence, and interaction between the two (B)*[12]*. Source: DOI:https://doi.org/10.1016/S0140-6736(16)00618-8 and Creative Commons Attribution License (CC BY).*

In recent decades, several chemicals, such as polychlorinated biphenyl (PCBs), polybrominated diphenyl ethers (PBDEs), dioxins, furans, pesticide, etc., have been shown to interfere with various metabolic pathways, leading to alterations in development, growth and reproduction, and causing medical conditions which may not become evident until many years after exposure (see below). In 2005, the Environmental Working Group (EWG) found an average of 200 industrial chemicals and pollutants present in the umbilical cord blood, and tests revealing a total of 287 chemicals[13]. Among these, some were compounds banned by the Stockholm Convention. Of the total 287 chemicals detected in umbilical cord blood, 180 are reported to cause cancer in humans or animals, 217 are toxic to the brain and nervous system, and 208 cause birth defects or abnormal development[14]. Chemical exposure begins in the womb, even, potentially, in pre-conception stages, and can have a dramatic effect later in life. Certain environmental chemical pollutants can cause such effects weeks, months or even years after exposure (see below). Available data from these studies clearly indicate the exposure of the general population to at least, some of these pollutants. Many chemicals present in the environment originate in municipal wastewater. Several environmentally relevant organic chemicals, such as pharmaceuticals, pesticides, plasticizers, persistent organic pollutants (POPs), are not eliminated by conventional water treatment methods,

and, through the urban water cycle, can enter ground and surface waters (Table 2.1). In addition, these pollutants can persist in the environment, bioaccumulate through the food chain and reach drinking water.

Table 2.1 *Endocrine disrupting chemicals found in consumption and surface waters.*

Compound	Adverse effects	Occurrence	Concentration ($ng L^{-1}$)	Source
Perfluorooctanoic acid (PFOA)	Carcinogenic, endocrine disrupter, hepatotoxic	Consumption waters (Germany)	43–519	15
Carbamazepine epoxide	Enzyme inhibition in fish	Consumption waters (Spain)	2	16
17α-ethinyloestradiol	Endocrine disrupter	Europe	0.3	17
Ibuprofen		Surface waters (River Minho, Portugal)	204.0	18
Bisphenol A	Endocrine disrupter, cardiovascular disorders, diabetes, infertility	Surface waters (Rivers Ave, Portugal)	7.9–521.8	19
17α-ethinyloestradiol	Endocrine disrupter	Surface water (Ria Formosa Portugal)	14.4–25.0	20
Nonylphenol	Oestrogenic properties, endocrine disrupter	Surface water (Ria Formosa Portugal)	12.2–547	20

This chapter provides an overview of research on environmental aspects of endocrine disrupters. Emphasis is given to three ubiquitous and persistent group of chemicals, e.g. polychlorinated biphenyls, polybrominated diphenyl ethers, and organochlorine pesticides, together with two non-persistent, but ubiquitous groups, bisphenol-A and phthalates, whose occurrence and effects on human health and wildlife have been widely reported. Selected historical cases are also presented to highlight the fact that, generally, human corrective actions tend to appear only following catastrophes, and in turn, the importance of the precautionary principle, a science-based approach that accepts uncertainty and requires action in order to avoid and prevent harm. Some successful cases are described of regulation and legislation that have prevented or decreased the exposure, and consequent effects, of these compounds.

2.2 Understanding the processes involved

Our current knowledge of the mechanisms involved in the disruption of the normal functions of cells by EDCs is still limited. Nevertheless, it is known that EDCs can have multiple mechanisms of action, interact with different receptors, and affect the entire endocrine system. The majority of studies focus on the estrogen, androgen and thyroid receptors and their mechanistic pathways. Accordingly, this section will give only a very brief description of the normal endogenous estrogen pathway and their disruption by estrogenic EDCs. There are other mechanisms of action of EDCs (Table 2.2), especially those involving the aryl hydrocarbon receptor (AhR) which regulates the expression of several genes, including the cytochrome P450 (CYP)-1 gene family members and glutathione S-transferase M, which mediates many of the responses to environmental toxic chemicals[21]. An overview of some of the mechanisms involved can provide insight for more comprehensive understanding of the impact on human health. For additional background information, or detailed description of the endocrine events and their disruption by EDCs, several excellent reviews are available in the literature that cover this broad and continually evolving research field[22, 23, 24-26].

The endocrine system is a complex network of glands that release specific hormones into the blood stream. It controls growth, development, metabolism, circadian cycles, glucose levels, sex hormones, T-cell development, calcium levels, and many other functions[27]. The endocrine glands, located at various sites in the body, release certain chemicals, the hormones, which affect specific cells, target cells and receptors. Other chemicals can interfere with this hormone action. When they enter the body, EDCs mimic or antagonize natural hormones, interacting with hormone receptors, and can potentially disrupt the body´s normal functions.

Nuclear receptors (NRs) are a class of proteins, transcription factors, that regulate gene expression, and are activated by steroid hormones, such as estrogen and progesterone, in addition to other lipid-soluble signal molecules such as retinoic acid, oxysterols and thyroid hormones[27]. Some of these NRs are considered to be orphans, as their specific ligand or physiological functions remain unknown. So far, 48 NRs have been discovered in humans through sequencing of the human genome[28]. A review on the mechanism of action of the NRs can be found in the paper of Gronemeyer et al. [29]. Steroid hormone receptors, such as the estrogen receptors (ERα and ERβ), progesterone receptors and androgen receptors, are included in this NRs superfamily. Many EDCs exhibit estrogenic activity, targeting estrogen receptors (ERs), in particular, ERα and ERβ. EDCs that interfere with estrogen receptor signalling, generate biological responses via both genomic (nuclear) and nongenomic (extranuclear) pathways[30].

Estrogen is not only a reproductive hormone, but also exerts effects on almost all tissues of the body[22], and has been linked to the development of conditions such as cancer, endometriosis, obesity, insulin resistance as well as cardiovascular, autoimmune and neurodegenerative diseases[22]. Estrogens act through three types of receptors, the classical ERs, the ERα and ERβ, and the recently discovered, non-classical, G protein-coupled membrane receptor 30, GPR30. The GPR30 receptor is believed to react through non-genomic mechanisms, as illustrated in Figure 2.4.

According to the proposed classical model of estrogen action, following the binding of estrogen to the receptor in the cytoplasm, this complex dimerizes and translocates to the nucleus where it binds to a specific estrogen responsive element (ERE), located within the promoter region of the target gene, to regulate gene transcription (Figure 2.4)[22, 31]. The steroid hormone receptors are bound to protein chaperones, such as Hsp 70 and Hsp 90, which regulate their functions[32]. Once the ligand binds to the receptor in the cytoplasm, the receptor is freed from the protein chaperone and the co-chaperone p23, allowing homodimerization and movement towards the nucleus. The co-chaperone p23 is a small protein, with a relatively simple structure, and is found in all eukaryotes, from yeast to humans; it is best known as a co-chaperone of Hsp90. The p23 molecule is involved in various cellular processes[33]. Once in the nucleus, the estrogen-ER complex recruits transcriptional coactivator proteins and components of the RNA polymerase complex that induce the transcription of target genes in response to the particular ligand[22].

Some EDCs, such as alkylphenols, BPA, dioxins, furans, heavy metals, and halogenated hydrocarbons[34], can cross the cell membrane and bind directly to these receptors, either activating them by acting as agonists, or inhibiting them and acting as antagonists to the NRs. Thus, either a new protein is synthesized or mRNA transcription is terminated. This nuclear receptor activation can occur with picogram quantities and nanomolar concentrations[28] of EDCs and, as with the steroid hormones, these can have an impact at similar very low doses[28, 35, 36].

Table 2.2 *Human nuclear receptors, their function, some major target genes and important ligands.*

NR	Function	Examples of target genes	Examples of endogenous ligand	Examples of endocrine disrupting chemicals	References
Androgen (AR)	Male sexual development	Probasin, KGF, p21, maspin, PSA	Testosterone	p, p'-DDE, pesticides, plasticizers, phthalates, alkylphenols	21–24
Oestrogen (ER)	Female sexual development	IGFBP4 GREB1 CTSD	Oestradiol	BPA, benzophenone derivatives, parabens, pesticides, dioxins, furans, heavy metals	23,25,26
Thyroid hormone (TR)	Metabolism heart rate	THRB	Thyroid hormones	PCBs, BPA, dioxins, furans, PBDEs, phthalates pesticides, perchlorates, phytoestrogens	23,27–29
Progesterone receptor (PR)	Female sexual development		Progesterone	Musk compounds, BPA, herbicides, insecticides, fungicides	23,24
Glucocordicoid receptor (GR)	Development metabolism stress response		Cortisol	BPA, phthalates, PCBs, DPCBs, methyl sulphones, tolylfluanid	23,24
Aryl hydrocarbon receptor (AhR)	Proliferation and differentiation Neurogenesis Circadian rhythm	Cytochrome P450s (CYPs)	Indol and tryptophan derivatives	PCBs, dioxins, herbicides, indoles, pesticides, flavonoids	23,24
Peroxisome proliferator-activated receptors (PPARs)	Gluconeogenesis Lipid, fatty acid and cholesterol metabolism	Cytochrome P450s (CYPs), glutathione S-transferase (GST)	Lipids Arachidonic acid derivatives Linoleic acid derivatives	BPA, organotins, phthalates	23,24

Table 2.2 *(continued)*

NR	Function	Examples of target genes	Examples of endogenous ligand	Examples of endocrine disrupting chemicals	References
Pregnane X receptor (PXR)	Xenobiotic detoxification Glucose tolerance and energy balance	Cytochrome P450s (CYPs) CYP3A CYP2B CYP2Cs OATP2 MRP2 MDR1 GST-A2 BSEP CYP7A	5 β-cholestane-3α, 7α, 12α-triol steroids	PCB, BPA	24,30–32
Retinoid X receptors (RXRs)	Cellular proliferation Development, growth and homeostasis		Retinoic acid	Organochlorine pesticides, styrene dimers, alkylphenols parabens, organotins	22,33
Constitutive androstane receptor (CAR)	Xenobiotic detoxification	CYP2B CYP3A CYP2Cs OATP2 MRP2 UGT1A1	Androstane and derivatives	PCB	24,32

DDE: dichlorodiphenyldichloroethylene.

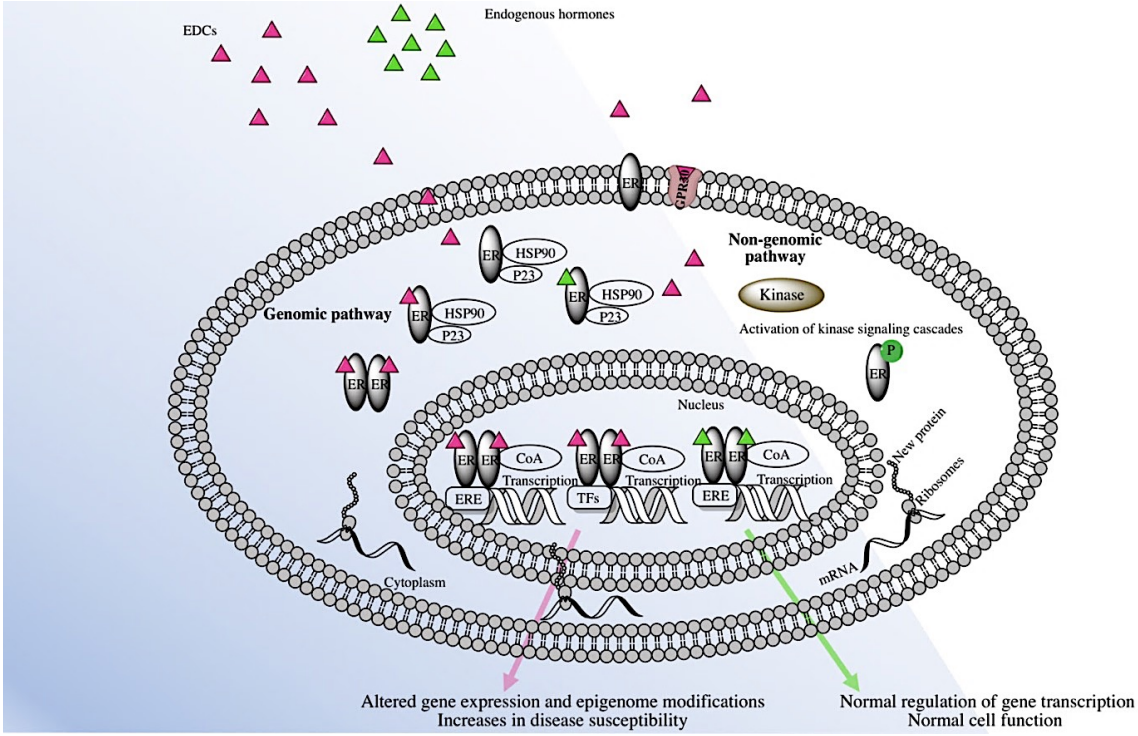


Figure 2.4 *Representation of endocrine disruption by EDCs. In the genomic pathway, EDCs bind to estrogen receptors and, in the nucleus, bind to the ERE affecting the transcription of target genes in the nucleus. The non-genomic pathway of EDCs may occur through the binding of GPR30, located in the cytoplasmic membrane, and consequently triggering subsequent stimulation of protein kinase activation and phosphorylation, affecting the transcription of target genes, by the ERE or by interaction between ERs with other transcriptional factors. The interaction between ERs and GPR30, in gene expression and intracellular signalling, can result in a cellular response, which may lead to adverse effects on organs, e.g., carcinogenesis. Abbreviation: ER, estrogen receptor; ERE, estrogen response element; CoA, coactivators; P, phosphorylation; TFs, transcriptional factors.*

2.3 Persistent Endocrine Disrupting Chemicals

Persistent Organic Pollutants (POPs)

Persistent Organic Pollutants, POPs, constitute a class of chemicals that share similar properties, such as persistence in the environment, high toxicity, and half-life times of years or even decades before their degradation into less toxic forms (Table 2.3). They bioaccumulate through the food chain, and their lipophilicity means they will accumulate in fatty tissues, and thus pose a threat to human health and wildlife. Bioaccumulation leads to biomagnification, as POPs are absorbed by

lower trophic organisms, such as phytoplankton, that are consumed by zooplankton, and accumulate in the fatty tissues of the organisms that are then eaten by higher organisms. This may magnify their effect up the food chain. This concentration effect reaches its maximum level in top predator species, such as humans and other mammals. POPs have moderate volatility, and are chemically and environmentally stable. Because of these characteristics, they may travel long distances and may be found everywhere, including the Arctic, Antarctica, and even remote Pacific Islands[47]. POPs that are deposited and accumulate in the soil and water, can evaporate or sublimate to the atmosphere and travel long distances, to condensate again, for example, in these remote regions[48, 49]. Through this cycle, these pollutants can contaminate indigenous people and wildlife in such regions, by entering in the food web or through inhalation (Table 2.4)[48]. Because of their genetics and lipid accumulation, indigenous people often have the highest levels of contamination with POPs, even though they did not produce or directly consume the products. POPs include polyhalogenated industrial chemicals, such as PCBs, polybrominated biphenyls (PBBs), organochlorine pesticides such as dichlorodiphenyltrichloroethane (DDT), aldrin and heptachlor, and industrial byproducts such as polyaromatic hydrocarbons (PAHs) (Figure 2.4 and Table 2.5). They were banned by the Stockholm Convention, which became international law in 2004. However, some are still used. Mosquitoes that spread malaria are responsible for significant morbidity and the deaths of one million people each year, mostly children, and the solution for this problem is not clear. DDT is effective in killing and repelling the mosquitoes that transmit malaria. Although DDT is highly toxic to health and to the environment, in some countries, mainly in Africa, the benefit of using this pollutant to tackle malaria may compensate for the risk. Some pollutants are granted exemptions for use, such as DDT in certain countries where malaria poses a major health threat, or some PCBs used in old electric transformers and capacitors in developing countries, where the alternatives are too expensive or too complicated to produce.

Polychlorinated biphenyls (PCBs)

Polychlorinated biphenyls, PCBs, constitute a class of chemically stable aromatic compounds in which 1 to 10 chlorine atoms are attached to a biphenyl backbone, allowing for, theoretically, 209 different congeners. PCBs were widely used in agriculture and in a number of industrial applications during the past decades. Their applications included dielectric fluids in capacitors and transformers, hydraulic fluids, their use in building materials, such as additives and plasticisers in paints, plastics, and rubber sealants, copying paper, adhesives, pesticides, etc. Due to their widespread use and careless disposal over several decades, PCBs have become widely distributed in the environment; although bans and restrictions exist on them at the global level, they are still found in the atmosphere, rivers, fish, other wildlife, human breast milk, and other biological samples and in ecosystems. The toxic impact of PCBs on human health and on the environment

was first recognized in the 1960s, in particular with the Yusho incident in Japan, and later, with the Yu-Cheng accident in Taiwan. Both will be briefly explained bellow in the item "Some historical cases". PCBs exhibit endocrine disrupting effects, such as ERs Transcriptional activity and MCF-7 proliferation, and affect enzymes mediated by thyroid hormone[50]. In addition to the bioaccumulation and biomagnification characteristics of PCBs, these chemicals have also been passed to the future generations through pregnancy and breastfeeding, thus exposing the most vulnerable in the womb and infancy during critical stages of development, where the disruption can have a dramatic effect later in life. Because of the strong evidence for them inducing cancer in humans and in animals, PCBs were classified in 2013, by the International Agency for Research on Cancer (IARC), as carcinogenic to humans (Group 1)[51-53].

Polybrominated diphenyl ethers (PBDEs)

Polybrominated diphenyl ethers, PBDEs, constitute a class of polyhalogenated aromatic compounds with a basic structure consisting of two phenyl rings linked by an oxygen atom, with a variable number from 1 to 10 bromide atoms, allowing for 209 different possible congeners, which depend on the number and position of the bromine atoms (Figure 2.5). Only a few of those combinations are commercially available, and their toxicity depends on the specific bromine substitution. The congener deca-BDE is poorly absorbed and rapidly eliminated[54]. In contrast, the congeners tri- to hexa-BDEs are strongly absorbed, slowly eliminated and strongly bioaccumulative[54].

PBDEs are widely used as brominated flame retardants, and since the 1970s have been present in a number of consumer goods, plastics, electronics, furniture and textiles. Although PBDEs have been gradually phased-out worldwide (in the EU they were banned in 2004 and 2008), biomonitoring studies indicate that they are still ubiquitous in human blood and breast milk worlwide[55]. A number of older products, particularly e-wastes, such as obsolete electronic and electrical items, contain significant amounts of these pollutants that are still being released into the environment, leading to contamination of ecosystems. PBDEs have been found in fish, eggs, human breast milk and in infants and toddlers, probably as a result of contamination via house dust. The highest concentrations are found at the top of the food chain, indicating biomagnification[54]. The exposure routes include diet, ingestion, inhalation of indoor dust, and dermal contact.

Certain PBDEs and their mixtures have been shown to disrupt the thyroid hormone thyroxine and have been associated to neurodevelopment deficits in rats and humans. Mixtures of PBDEs have increased cell proliferation in human breast cancer cell lines, and also carcinogenic effects in humans[56,54,57, 58].

Organochlorine pesticides (OCPs)

Organochlorine pesticides, OCPs, are a ubiquitous, diverse group of persistent organic pollutants. Representative members of this group include DDT, mirex, dieldrin, chlordane, heptachlor, endrin, hexachlorobenzene (HCB), aldrin, and toxaphene, which are listed in the so-called "dirty dozen", banned by the Stockholm Convention. These pesticides were extensively used in agriculture and as insecticides for mosquito control between the 1940s and 1970s. Although banned, as a result of their strong persistence in the environment and bioaccumulation, the principal metabolites of DDT are detectable in more than 25% of the general population[59, 60]. The adverse health effects of organochlorine pesticides on animals and human health have been extensively reported in the literature, and linked to diabetes, cancer, neurodevelopment problems in children, miscarriages, and many other health outcomes. Due to agricultural activities, traces of OCPs and their metabolites have been found in both surface and groundwater. The presence of OCPs in ground water has been associated with increased risk of cancer among people consuming the contaminated supplies. The presence of these pollutants in several shallow groundwater samples and the corresponding potential health hazards were recently discussed[61]. In this study, HCB, HCHs, resulting from the degradation of lindane and p,p´´-DDE, a DDT metabolite, were detected, several years after their use. When the OCPs reach groundwater, they may remain there for several years. Human exposure towards OCPs includes the consumption of food and water contaminated with residues and their degradation products, dermal contact and inhalation.

As with the other POPs, OCPs are characterized by their persistence, bioaccumulation and biomagnification, lipophilicity, and they may be transported over long distances by winds and ocean currents.

2.4. Non-persistent Endocrine Disrupting Chemicals

Bisphenol-A

Bisphenol-A, BPA, with its full IUPAC name, 4-[2-(4-hydroxyphenyl) propan-2-yl]phenol, is an important monomer, used for the production of polycarbonates, epoxy resins, polyesters, polysulfones and polyether ketones, and is also applied as polymer additive in plasticizers and halogenated flame retardants. It is present in a wide range of applications including baby bottles and linings for metal-based food and beverage cans, ophthalmic lenses, medical equipment, consumer electronics and electric equipment.

BPA was first synthesized by the Russian chemist Aleksandr P. Dianin in 1891[62] who reacted phenol with acetone in the presence of an acid catalyst. In 1905, Theodor Zincke also reported its synthesis[63]. In the 1930s, BPA was found to have estrogenic activity but, because of the development in 1938 of the more powerful synthetic estrogen, diethylstilbestrol (DES), which

stronger than natural estrogen, BPA was ignored for this application. In 1953, the Bayer chemist Hermann Schnell synthesized polycarbonates by reacting BPA with phosgene. The new material produced was clear, strong and stable, and could be heated in microwave ovens without deformation. The production of new polycarbonates reached industrial levels in the 1960s and BPA production has increased greatly since then. In 2015, the production of BPA was estimated at more than 4.5 million tonnes[64,65]. Each year, more than 100 tonnes is released into the atmosphere and is found in air, drinking water, house dust, food, lakes, sea, and soil. BPA is ubiquitous. Human exposure occurs through food, drinking water, household products, cosmetics, medical equipment, dental materials, and occupational sources[66]. The polymerization of polycarbonate plastics is not 100% complete, and, as consequence, the unbound monomer, or the additives, can easily leach out of the products into the environment[67]. Prompted by the broad applications of BPA, several studies have demonstrated continuing exposure of the general population to this chemical.

Although the estrogenic activity of BPA is lower than that of natural estrogens[68, 69], its prevalence at high concentrations in the environment, including surface, ground and drinking waters, poses a potential risk to human health. In humans, after exposure, almost 100% of BPA is eliminated in the urine, excreted mainly as BPA-glucuronide[70]. In contrast to polychlorinated biphenyls (PCBs), which can persist in the human body for decades[71], BPA has an average half-life of approximately six hours in humans[69]. However, in spite of this short half-life, BPA can be considered as persistent due to its widespread occurrence and the continuous exposure that the population receives. There are several studies reporting the presence of BPA in urine samples of children and adults[69, 70, 72]. BPA is found in the amniotic fluid, providing evidence for its passage through the placenta[73]. Its presence has been reported in maternal and fetal serum and amniotic fluid, indicating significant exposure during the prenatal stage[73]. In a biomonitoring study, 100% of the 81 children examined revealed the presence of BPA in their urine samples. The median intakes estimated for dietary ingestion, nondietary ingestion and inhalation were 109, 0.06, and 0.27 ng/kg/day, respectively[70].

A European level project on biomonitoring measured the BPA levels of 653/639 child-mother pairs and determined the mean values of 2.04 μg/L for children and 1.88 μg/L for mothers. Environmental, geographical, and life style and dietary habits were considered factors that could predict exposure to BPA[72].

Numerous studies of human biomonitoring of BPA exposure in the global population have been reported. These studies reveal the exposure of more than 90% of the world´s population and demonstrate that there are no differences in the BPA levels between countries or continents, and that the urinary levels among the youngest groups tend to be higher than in the older ones[72].

Following from the restrictions imposed on the use of BPA in many countries, BPA analogues, such as the most common substitutes bisphenols F and S, have been synthesized and have replaced BPA in numerous consumer products.[74] Sixteen bisphenol analogues have been documented, and are

used commercially in thermal papers, food containers, toys lacquers, dental sealants, personal care products, and in many other applications[74]. These analogues are now frequently detected in biomonitoring studies and in the environment[75], and also exhibit endocrine disrupting activity, cytotoxicity, genotoxicity, reproductive toxicity and neurotoxicity[74, 75].

Phthalates

Phthalates are a group of multifunctional industrial chemicals, and are used in a wide range of consumer products. They are used as plasticisers, to improve flexibility in plastics, such as polyvinyl chloride (PVC), or as additives in the textile industry, and as carriers in pesticide formulations. They are added to pharmaceuticals as coatings in time-release pharmaceuticals, and are also used as industrial solvents and lubricants. Phthalates are frequently added to consumer and personal care products, such as cosmetics, perfume, deodorants, hair sprays, skin cleansers, etc., to retain colour or fragrance, and are used in medical devices, toys and fuels to enhance performance. They are diesters of phthalic acid (1,2-benzenedicarboxylic acid) and an alcohol moiety. The phthalate products display different properties which depend on the length and degree of the branching of the side chain. The branched chain di-(2-ethylhexyl)-phthalate (DEHP), with 8 carbon atoms in the alkyl side chain, is one of the most commonly used phthalates worldwide. The global consumption of DEHP during 2012-2018 exceeds 3000 thousand metric tons[76].

The polymer cellulose nitrate was synthesized in 1833 by Pelouze, while preparation of cellulose acetate was reported by Schutzenberger in 1865 and that of polyvinyl chloride by Baumann in 1872[77]. However, these polymers were too intractable to be processed and needed addition of substances, plasticisers, to soften them. Gum copal, natural rubber, linseed oil, castor oil and camphor were some of the substances tested to overcome the inherent intractability of these polymers[77]. The high volatility, flammability and unpleasant odour of the most commonly used plasticiser, camphor, triggered the search for alternatives, and, in the 1900s, diphenyl and dicresyl phthalates, and phthalic esters in general, were patented. Phthalates became commercial during the 1920s and 1930s, along with a number of new thermoplastic polymers.

The first reports of the adverse effects of phthalates on animals date from 1945[78]. Since then, several reports have been published associating phthalates with a wide range of health outcomes (Table 3).

Phthalates are not covalently bonded to the polymeric matrix and, as such, may be easily and continuously released from the polymer chains to the surrounding environment, during their life cycle. Consequently, human exposure is widespread. Exposure is primarily through ingestion, inhalation, and skin contact. As with bisphenol-A, phthalates are ubiquitous and the continuous exposure of general population to them has been demonstrated in various biomonitoring studies[79, 80]. The Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) NOAEL (No-

Observed-Adverse-Effect-Level) values were identified for four phthalates (DINP, DNOP, DEHP, DIDP), which settled concerns with the so-called phthalate syndrome. The phthalate syndrome is the variety of effects observed in rat experiments and is characterised by malformations of their reproductive organs, reduced anogenital distances and nipple retention[81, 82]. Evidence of the human analogue of the "phthalate syndrome" has been reported in recent years, and has demonstrated the disruption of the androgen signalling pathway[83-85]. Phthalates have been implicated in various male malformations of external genitalia, such as cryptorchidism and hypospadias. Reports on the effects of prenatal exposure to phthalates in humans showed that birth outcomes and reduced anogenital distance were associated with high levels of phthalate esters, notably di(2-ethylhexyl) phthalate (DEHP), in the urine of the mothers during pregnancy [86, 87]. The incidence of these genital malformations is increasing in male new born babies[87]. In a recent study, the levels of phthalates in urine from 1170 peripubertal girls were measured. Concentrations of phthalates ranged from <1 to 10 000 μg/L and associations between phthalates exposure and puberty age were estimated[85].

Phthalates have been found in several consumer products, and in food, milk, and drinking water. Although the exposure to a single phthalate below a certain threshold does not produce any evident effect, the mixture of several phthalates could exhibit toxicity in the exposed individual [82].

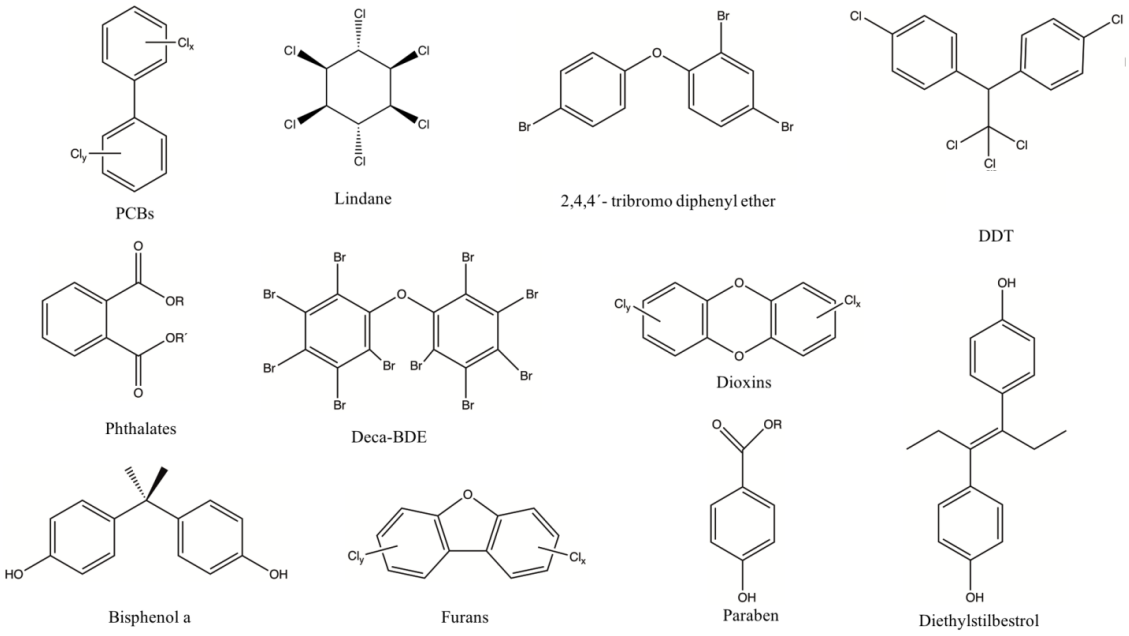


Figure 2.5 *Molecular structure of common endocrine disruptors.*

2.5 Impact on human health

Although strong and robust evidence is mounting on the adverse effects of EDCs on human health, there is still considerable controversy surrounding this area[7, 88-94]. The complexities in

understanding of the multiple causes and effects that lie behind the mode of action of EDCs, coupled with scepticism, denial machines, and industrial interests, contrarian scientists with their *argumentum ad hominem*, have all fuelled this controversy. In addition, some concepts can be difficult to accept or assimilate.

There is considerable evidence of adverse effects of EDCs in animal models. However, the evidence of their effects on human health is more controversial and challenging. The outcomes of exposure to certain environmental pollutants may only be evident years or decades later. Another difficulty, when assessing the consequences of EDCs on human health, is that humans are exposed to a large number of chemicals; each has specific modes of action, while interactions between them may lead to different results than with the individual compounds itselfs[95].

The phrase "the dose makes the poison" is a traditional toxicological assumption first proposed by Paracelsus, where larger doses have a greater impact than lower ones. However, with EDCs, the dose no longer makes the poison, and understanding their toxicity is not a trivial process. Very low doses of some chemicals can have a greater impact on health than much higher ones. At low doses, they can bind to the receptors and induce a biological response. In contrast, higher doses can saturate these receptors, and inhibit the correspondent pathways[94, 96]. Long term exposure of some chemicals at extremely low doses, can have adverse health effects. The timing of exposure can also be a crucial factor. The toxicity of EDCs is a time-dependent process, and exposure to them during critical stages of development can have dramatic effects later in life[97].

Some historical cases

The historical case of diethylstilbestrol (DES) is an example of how exposure of a fetus to synthetic estrogen can adversely impact health later in life. DES was synthesised in 1938 by the biochemist Charles Dodds[98, 99]. Between the 1940s until the early 1970s, DES was prescribed to millions of American and European women, initially to treat the symptoms of the menopause, and later prescribed to pregnant women to prevent miscarriage, although there is no clinical evidence or adequate testing that supported such a claim. In 1971, Herbst et al.[97] demonstrated that DES causes a rare vaginal cancer in young woman, who were the daughters of mothers who had been exposed to the synthetic estrogen. Since then, various other reports have been published demonstrating adverse outcomes associated with DES[100-102].

Thalidomide, α-phthalimidoglutarimide, was synthesised by Chemie Grunenthal in West Germany in 1954. This company was searching for low cost production of antibiotics from peptides and, when they failed to demonstrate any antibiotic activity, they decided to explore the potential of this new molecule as a sedative in humans[103]. After the tests in mice, rats, guinea pigs, rabbits, cats and dogs have not apparently revealed any toxicity or side effects, Chemie Grunenthal distributed free samples to doctors in West Germany and Switzerland. In 1957, and after the

company created its own tests, the "jiggle cage", to demonstrate to the licensing authorities the efficacy of thalidomide as a sedative, it was commercialized, first in Germany and later in other European countries. The drug rapidly became a success and, soon after, was sold in 46 countries. Although studies of the effects on foetus were never performed, the company wrote in 1958 to all German physicians advising the prescription of thalidomide to pregnant women for morning sickness and nausea[103]. In 1961, reports started to appear, linking thalidomide with a variety of birth defects, including the rare malformation phocomelia. Between 1957 and 1961, thousands of babies were born worldwide with severe congenital malformations. Widukind Lenz, a German paediatrician, found that the administration of the drug between the 20th and 36th day of gestation could lead to the development of malformations in the foetus. Although the link with these birth defects precludes its prescription to pregnant women, currently, the drug thalidomide is used with an enormous success in various medical conditions, including erythema nodosum leprosum, immune system disorders, multiple myeloma, cancer and in many other situations[103, 104, 105].

Another important historical case occurred in Japan in 1968; a rice cooking oil produced by Kanemi Company was accidentally contaminated with polychlorinated biphenyls (PCBs), polychlorinated dibenzofurans (PCDFs), polychlorinated quaterphenyls (PCQs) and other compounds from heat exchangers[106, 107]. Over 1800 people presented a range of symptoms, including dermal lesions, pigmentation of the skin, nails and conjunctiva, numbness of the limbs, irregular menstrual cycles; more than 500 have died from exposure to this contaminated oil. The incident is known as the "Yusho oil disease", and clinical and epidemiological studies from it are extensively documented[106-108]. From 2001 to 2003, measured mean blood levels of total dioxins and 2,3,4,7,8-penta-chlorodibenzofuran (PeCDF) in Yusho patients revealed that, 36 years after the exposure, those levels were 3.4-4.8 and 11.6-16.8 times higher than in a control, providing evidence that PCBs and dioxins persist for many years in the human body[71].

In 1979, a similar case occurred in Taiwan, the "Yu-Cheng" incident, in which 2000 people had consumed PCB- and PCDF- contaminated rice cooking oil. The symptoms were similar to those in the Yusho victims. High mortality from liver diseases was reported within three years after the incident [109] and children born to exposed mothers were found to have ectodermal defects, development delay, behavioural problems and poor cognitive development up to 7 years[110,111]. A higher prevalence in type-2 diabetes among the Yu-Cheng cohort was also reported [112].

These historical cases drew attention to the possible dramatic consequences to human health and the environment, arising from the production and use of untested and unregulated synthetic chemicals, particularly in critical stages of development.

2.6 Exposure and growth, development and health of children

Foetuses, infants and children are particularly vulnerable to contaminants. Various environmental chemicals, including pesticides, polychlorinated biphenyls (PCBs), methyl-mercury, are known to be neurotoxic, and may alter brain development. Neurodevelopment is a complex process that begins at conception with the formation of a primitive neural tube, the first stage of brain development, followed by a differentiation of the cells and the formation of the central nervous system. During the first two trimesters of foetal development, the basic structure of the brain is formed. Changes in neural connectivity and function occur in the last trimester and the first few postnatal years. Synaptogenesis and myelination, the most prolonged changes, occur in the last trimester and continue postnatally into adulthood[113,114]. The disruption of these complex processes could lead to neurodevelopmental disorders, such as attention-deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD), schizophrenia, intellectual disabilities, and neurological disorders, such as Rett syndrome (Table 2.3). The prenatal or early life exposure to EDCs can have repercussions on brain functions later in life. The persistent organic pollutants (POPs) are of particular concern, due to their chemical structure, persistence, and lipophilicity. They are widely dispersed into the environment, accumulate in organisms, and can lead to biomagnification in the food chain. PCBs are among the most extensively studied environmental contaminants in terms of their effects on neurodevelopment and cognitive functions. Several reports have revealed PCBs adverse effects of these compounds, which include immunotoxicity, endocrine disruption, neurotoxicity and cancer. The parental exposure to certain EDCs has been linked with a higher risk of congenital malformations in future progeny and changes in the sex ratio of the offspring. For example, the exposure of men to the dioxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), is linked to the decrease of the sex ratio in their offspring. The Seveso disaster, occurred in 1976, with the release of the dioxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and resulted in the exposure of the population to this dioxin. It was observed that the children born from parents with the highest exposure to TCDD, in the first decade after the exposure, were all female[115, 116]. Many epidemiological studies are underway to study the long term adverse health effect of this dioxin. Another example of the effects of pre-conception stages is related with occupational exposure; the pre-conception exposure of parents to pesticides is linked to the development of congenital malformations of their offspring[117-119]. Since 1970, childhood cancer has been rising in Europe and USA by 1% per year, and the data show evidence for an increase in this trend[120,121]. The causes of most childhood cancer are largely unknown. A few causes have been established, such as exposure to radiation and carcinogens, timing of exposures, and genetic susceptibility to development of cancer, but only a limited number of chemical compounds have been directly linked to childhood cancer.

Table 2.3 *Examples of EDCs with widespread distribution in the environment and their reported health effects.*

Endocrine disrupting compound	Used for	Found in	Biological half life (in humans)	EDCs	Reported health impact	References
Bisphenol A	Plasticizer Improvement of physical, thermal and mechanical properties	Weak bond polycarbonate Epoxy resins Food and drink packaging	6 h	2.04 μg L^{-1} for children (n = 653) 1.88 μg/L for adults (n = 639)	Oestrogenic effect Increase prostate, child behaviour problems, breast cancer, metabolism and breast cancer risk Anti-androgen Child behaviour problems	46–50
17-α ethynyloestradiol	Synthetic hormone used as contraceptive pills	Rivers, groundwaters and superficial waters	17 h		Mimic the endogenous oestrogen	17,51
Polybrominated diphenyl ethers (PBDEs)	Flame retardants in furniture, textiles, plastics, electronics, etc.	Environment, human blood, placenta, foetal cord blood, urine and breast milk, children, fish, marine mammals and eggs	2–16 years	1.80–16.5 ng g^{-1} in human biological samples	Increase cell proliferation, carcinogenic, neurodevelopment deficit	52–57
PCBs	Dielectric fluids in capacitors and transformers, additives and plasticisers in paints, plastics and rubber sealants, pesticides, etc.	Environment and wildlife, human breast milk, human blood	Few years to 35 years	140 ng L^{-1} of PCB-153 in cord serum	Intellectual impairment in young children Immunotoxicity, ectodermal defects, development delay, behavioural problems and poor cognitive development, neurotoxicity, cancer	58–63

Table 2.3 *(continued)*

Endocrine disrupting compound	Used for	Found in	Biological half life (in humans)	EDCs	Reported health impact	References
Phthalates	Plasticisers in plastic, PVC baby toys, flooring, perfume, etc.	Environment, human biological fluids	36 h	2×10^{-9}–8×10^{-6} M	Low sperm counts, metabolism, birth defects, asthma, neurobehaviour problems, cryptorchidism, hypospadias	48,64–67
TCDD	Pesticides, unwanted by-products of thermal and industrial processes	Environment, wildlife, food chain	5–10 years	2 ρg g^{-1} body fat	Changes in the sex ratio, chloracne, porphyria, transient hepatotoxicity, and peripheral and central neurotoxicity	56,66–69
Triclosan	Disinfectant and antiseptic used in soaps, toothpastes, kitchen utensils, toys, bedding, clothes	Environment, wildlife, human biological fluids	21–96 h in human plasma	1.65 μg L^{-1} estimate of urinary concentration per unit dose of triclosan	Biocidal effects and contributes to bacterial resistance, dermatitis, skin irritation. Triclosan react with chlorinated water producing chloroform	48,70–72

EDC: endocrine disrupting chemical; TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin.

2.7 Presence in indoor air and dust

New sources of human exposure to endocrine-disrupting chemicals have been identified in indoor environments, such as homes and the workplace. Over the last decades, there has been a marked increase in new materials for building construction, furnishings, carpets, textiles, electrical and electronic appliances, and many other consumer products that are present in indoor environments. These lead to common classes of chemicals, such as formaldehyde, pesticides, phthalates, polychlorinated biphenyls, brominated flame retardants, alkylphenols (e.g. nonylphenol, alkylphenol ethoxylates), and parabens, being found in indoor air and dust. Advances in technologies to improve thermal comfort have decreased the ventilation rates of indoor spaces, and have, consequently, led to a decline in the quality of air inside the buildings. This has repercussions on health. Respiratory illness, sick building syndrome, allergies, and loss of productivity are some the health implications reported[135,136,137].

We spend about 90% of our life in enclosed spaces, particularly the home, workplace and in cars [138]; indoor air quality (IAQ), therefore, has a considerable impact on our exposure to contaminants. A number of major studies have been conducted on air and dust pollution of indoor environments (Table 2.4). The samples collected in these studies were found to have high levels of contaminants, and, in general, their concentrations in indoor air exceed those in outdoor air, and they are considered as "one of the most serious environmental risks to human health" [139,140,141] .

Table 2.4 *Organic chemicals detected in indoor air and dust.*

Samples (homes)	Number of organic chemicals identified		Number detected per home		Most abundant organic chemicals identified	Concentrations		References
	Air	Dust	Air	Dust		Air	Dust	
120	52	66	13–28	6–42	Phthalates, alkylphenols, brominated flame retardants, pesticides	50–1500 ng m^{-3}		139
2	PCBs	PCBs (52, 105 and 153)	–	–	PCBs (52, 105 and 153)	8–35 ng m^{-3}	21–190 μg g^{-1}	140
34	–	65 PCBs congeners	–	65 PCBs congeners	–	–	260–23,000 ng g^{-1}	141
50	63	–	–	–	Phthalates, alkylphenols, PAHs	>1 μg m^{-3}	–	142

PCB: polychlorinated biphenyl; PAH: polyaromatic hydrocarbon.

Children are at greater risk than adults since their normal behaviour, such as playing close to the ground, and considerable hand-to mouth and object-to-mouth contacts, increases the exposure through inhalation and ingestion routes. Physiological factors, such as the small body mass, their weaker detoxification capabilities, and the rapid growth and development, also contribute to the increased risk of health outcomes[144]. The process of ventilation dilutes and removes the chemicals produced by the daily activities and released from indoor sources. However, the removal of the sources of these chemicals would be a more effective way to decrease the exposure to pollutants.

2.8 Government policies

As mentioned above, the phrase "the dose makes the poison" has been the basis for implementation of public health policies and imposition of thresholds regulations. However, when dealing with endocrine disrupting chemicals, the adage should be "the dose no longer makes the poison". For example, studies of the effect of phthalates in animal models have shown that, although the doses of each phthalate individually were below the "adverse effect threshold", the mixtures of phthalates exhibited testicular toxicity[95,129], which raises concerns when humans are exposed to a panoply of chemicals every day.

As in many other fields of science, one reason for scepticism among the public on EDCs comes from the influence of predominantly industry backed lobbies. Although there are notable exceptions of very positive participation of certain individuals in industry, in general, this negative influence can weaken policies, and delay, or even put at risk their implementation protecting human health and the planet. The current legislation and regulations are ineffective to safeguard human population, nature and ecosystems. The tendency has been for the majority of industrial chemicals to go to the market without being extensively tested, and it is only when any adverse effects are dramatically evident, or when major accidents happen, that they are banned or regulated. In contrast, pharmaceuticals are subject to an intense, and very expensive, testing procedure before they can be released on the market. Even when they have been proven to be safe, there may always be some secondary effects that could arise, and in such cases, the risk/benefit must be debated. Shouldn´t the release of industrial chemicals to the market be subject of the same rigorous procedures? The regulatory process must not be just at the national level, but, instead, a global agreement must be the goal, since some EDCs do not respect barriers or frontiers. POPs, for example, can evaporate from hotter regions and travel thousands of miles around the planet to condensate again in the polar and/or mountainous regions in the so-called "grasshopper effect". As stated, the inhabitants in these places, such as the indigenous people from the Arctic regions, have some of the highest values of POPs, and they are far away from where those chemicals were produced or used[145].

Several European and International environmental treaties already exist on EDCs. In 1999, the European Commission´s Scientific Committee for Toxicity and Ecotoxicity and the Environment (SCTEE) and the European Parliament presented the Community Strategy for Endocrine Disrupting, whose objective was to identify the problem of endocrine disruption, its causes and consequences, and to define appropriate policy action to be taken by adopting a strategy in line with the precautionary principle "fulfilling the Commissions obligation to protect the health of the people and the environment" [146].

On 22 May 2001, in what can be considered a major achievement, governments of the whole world met in Sweden to sign and adopt an international treaty concerning POPs, and consider the view that they can pose significant threats to health and the environment. This is the Stockholm Convention on Persistent Organic Pollutants. The Convention is a global treaty, signed by 152 Nations and administrated by the United Nations Environment Programme, with the purpose of protecting human health and the environment from persistent organic pollutants. It comprised five essential aims: "eliminate dangerous POPs, starting with the 21 listed in the Convention, support the transition to safer alternatives, target additional POPs for action, clean-up old stockpiles and equipment containing POPs, and work together for a POPs-free future"[47]. The treaty requires Parties to take measure to eliminate or reduce the release of POPs into the environment. It started with 12 substances, "the dirty dozen", which included eight chlorinated pesticides (aldrin, dieldrin, endrin, mirex, chlordane, heptachlor, DDT, and toxaphene), two industrial chemicals (polychlorinated biphenyls and hexachlorobenzene) and two byproducts (polychlorinated dibenzofurans and polychlorinated debenzo-p-dioxins). The convention entered in force and became international law on 17 May 2004. In May 2009, nine more substances were added. Table 2.5 lists the POPs banned or restricted under the Stockholm Convention. As of March 2018, the Convention has 182 Parties as signatories[147].

Other important international agreements include the Basel International Convention and the Rotterdam Convention, that regulate chemicals, pesticides and hazardous wastes at the global level[148]. The Plant Protection Products Regulation (No 1107/2009 – PPPR) and the Biocidal Products Regulation (No528/2012– BPR) both banned substances with endocrine-disrupting properties, and established that endocrine disrupting chemicals should be regulated on the basis of hazard and without a specific risk assessment, in addition to providing scientific criteria to identify endocrine disruptors.

EDCs are considered to be of similar regulatory concern as substances of very high concern in the Registration, Evaluation and Authorization of Chemicals (REACH) regulation (No 1907/2006), which should be regulated on a specific case, case-by-case basis.

Other relevant EU legislation on EDCs include the Water Framework Directive (2000/60/EC), Regulation 1272/2008 on the classification, labelling and packaging of substances and mixtures, the Toy Safety Directive (2009/48/EC) and the Cosmetics Regulation 1223/2009.

The European Commission Directive 2011/8/EU banned, on the basis of the precautionary principle, the production and sale of baby bottles and food-related products for children containing BPA (for more detailed information on European laws and regulations, readers are referred to the EUR-Lex[2]). BPA has been restricted since 2011, in the European Union, United States and other countries, because of its endocrine disrupting properties. As a consequence of these bans, the total exposure to BPA has effectively decreased, and BPA levels in the population of children showed a marked decrease from 2000 to 2008 [69].

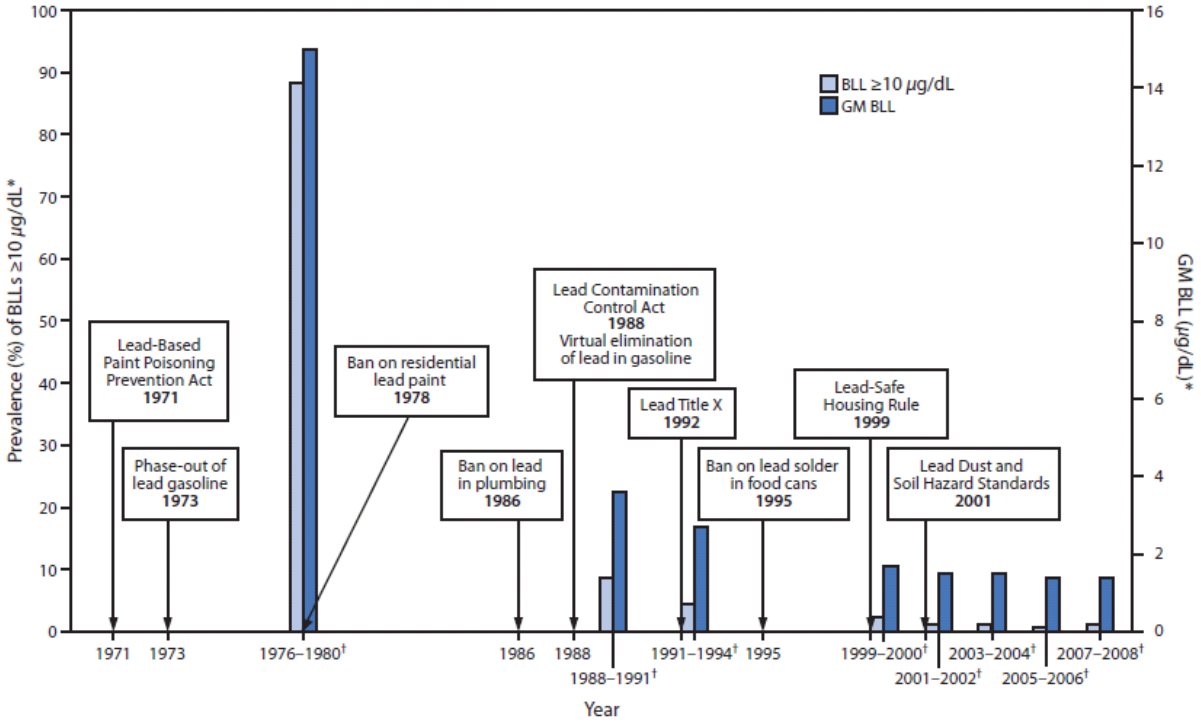


Figure 2.6 *Timeline of lead poisoning prevention policies and blood lead levels in children aged 1-5 years, by year – National Health and Nutrition Examination Survey, United States, 1971-2008* [149,150,151] *Available at https://phil.cdc.gov. Accessed November 19, 2018.*

** National estimates for GM BLLs and prevalence of BLLs ≥10 µg/dL, by NHANES survey period and sample size of children aged 1–5 years: 1976–1980: N = 2,372; 1988–1991: N = 2,232; 1991–1994: N = 2,392; 1999–2000: N = 723; 2001–2002: N = 898; 2003–2004: N = 911; 2005–2006: N = 968; 2007–2008: N = 817.*
† NHANES survey period.

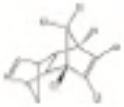
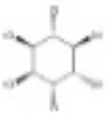
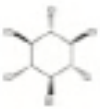
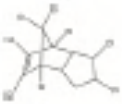
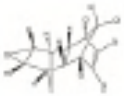
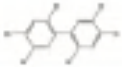
In Figure 2.6, the lead blood levels in young children are shown, and are linked to the timeline of policies on lead poisoning prevention in USA. Before the implementation and intervention of policies, millions of children suffered from neurological effects and diminished intelligence

[2] https://eur-lex.europa.eu

capacity due to lead exposure from gasoline, paints and other consumer products[149,152,153]. There is no threshold or safe level of lead in blood. Strong and decisive evidence revealed that the cognitive deficits and behavioural problems can occur at blood levels below 5μL/dL[154,155]. After the first policies were introduced, from 1976 to 1994, the lead blood levels dropped from 13.7 μL/dL to 3.2 μL/dL and continued to decrease as policies were implemented.

These, and the historical cases presented previously, indicate the limitations of science, policies and the actions of the common citizens to avoid and prevent irreversible damages but, at the same time, demonstrate that concerted international effort, coupled with scientifically informed political decisions can have a tremendous impact and effectiveness, through well-conceived policies and regulations, on the environment and human health.

Table 2.5 *Persistent organic pollutants listed in Annexes A, B and C in the Stockholm Convention.*

Chemical	Use		Molecular formula	Chemical structure
Annex A (Elimination) Aldrin	Pesticide	Organochlorine insecticide	$C_{12}H_8Cl_6$	
α-hexachlorocyclohexane	Pesticide/by-product	Organochloride insecticide By-product of lindane (for each ton of lindane produced, 6–10 ton of α-hexachlorocyclohexane are also obtained)	$C_6H_6Cl_6$	
β-hexachlorocyclohexane	Pesticide/by-product	Organochloride insecticide Same properties as α-hexachlorocyclohexane	$C_6H_6Cl_6$	
Chlordane	Pesticide	Used extensively to control termites pesticide for corn and citrus crops, environmental half-life of 10–30 years	$C_{10}H_6Cl_8$	
Chlordecone	Pesticide	Chlorinated pesticide and by-product of Mirex	$C_{10}Cl_{10}O$	
Dieldrin	Pesticide	Organochloride pesticide	$C_{12}H_8Cl_6O$	
Endrin	Pesticide	Insecticide	$C_{12}H_8Cl_6O$	
Heptachlor	Pesticide	Insecticide. Can persist in the environment for decades	$C_{10}H_5Cl_7$	
Hexabromobiphenyl	Industrial chemical	Flame retardant	$C_{12}H_4Br_6$	

(Continued)

Table 2.5 *(continued).*

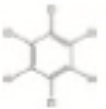
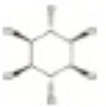
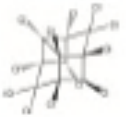
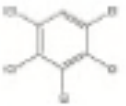
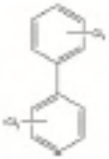
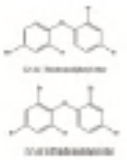
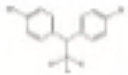
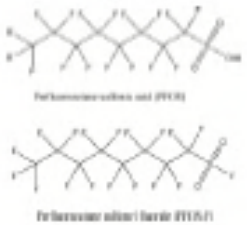
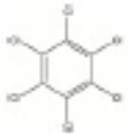
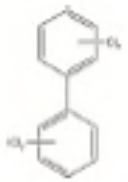
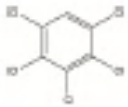
Chemical	Use		Molecular formula	Chemical structure
Hexabromodiphenyl ether and heptabromodiphenyl ether (commercial octabromodiphenyl ether)	Industrial chemical	Flame retardant Theoretical number of possible congeners is 209[147]	$C_{12}H_{(0\text{-}9)}Br_{(1\text{-}10)}O$	
Hexachlorobenzene (HCB)	Pesticide/industrial chemical	Used as fungicide in food crops	C_6Cl_6	
Lindane	Pesticide	Organochlorine pesticide	$C_6H_6Cl_6$	
Mirex	Pesticide	Organochloride pesticide	$C_{10}Cl_{12}$	
Pentachlorobenzene	Pesticide/industrial chemical	By-product of the manufacture of carbon tetrachloride and benzene, intermediated in the production of pesticides	C_6HCl_5	
Polychlorinated biphenyls (PCBs)	Industrial chemical	Used in agriculture and in a number of industrial applications electric transformers and capacitors	$C_{12}H_{10\text{-}n}Cl_n$	
Tetrabromodiphenyl ether and pentabromodiphenyl ether	Industrial chemical	Additive flame retardant	$C_{12}H_6Br_4O$ $C_{12}H_5Br_5O$	
Toxaphene	Pesticide	Pesticide used on cotton, crops and vegetables. Half-life of up to 12 years	$C_{10}H_8Cl_8$	

Table 2.5 *(continued).*

Chemical	Use		Molecular formula	Chemical structure
Annex B (restriction)				
DDT	Pesticide	Pesticide used in agriculture and to control malaria	$C_{14}H_9Cl_5$	
Perfluorooctane sulphonic acid (PFOS), its salts and perfluorooctane sulphonyl fluoride (PFOS-F)	Industrial chemical	Man-made fluorosurfactant, used in impregnation formulations for textiles, leather, paints, varnishes, carpets, etc.	$C_8HF_{17}O_3S$ $C_8F_{18}O_2S$	
Annex C (unintentional production)				
Dioxins PCDDs	By-product	75 congeners	$C_{12}H_{9-n}Cl_nO_2$	
Furans PCDFs	By-product	135 congeners	$C_{12}H_{9-n}Cl_nO$	
HCB	By-product	By-product from the manufacture of certain chemicals that can give rise to dioxins and furans	C_6Cl_6	
PCBs	By-product	Besides being industrial chemicals, are also by-products	$C_{12}H_{10-n}Cl_n$	
Pentachlorobenzene	By-product	Unintentionally produced during combustion, thermal and industrial processes	C_6HCl_5	

2.9 Final remarks and future perspectives

The exposure to EDCs begins before we are born, and even before conception. The extent of our chemical burden is unknown; every year, hundreds of new chemicals are produced and released onto the market without being tested, and reach our bodies through everyday products. The omnipresence of this exposure may have affected us all; we are not the same today as we were three centuries ago, before the invention of synthetic chemicals. We have opened Pandora´s box, and because of the overwhelming consequences of our actions, may be destroying our planet and our future. But the situation is not hopeless. A shift in lifestyles and consumption patterns is needed. We want to live our lives in the comfort that the modern age has brought to us, but we can aim for a more sustainable and "green" future, one that provides health and wellbeing and preserves the only home we have, Earth. By changing habits of consumption, we influence commerce, industry and national and international policies. In this context, it is noteworthy that there are some significant shifts in policies and citizen´s thinking and choices that may have, if the trend persists, an important impact on our planet. This proenvironmental behaviour has increased considerably in recent years; the global organic food and beverages industry has grown at a Compound Annual Growth Rate around 14.56% between 2017 and 2024[166]. The worldwide number of electric vehicles increased from 2012 to 2017 from 110 thousands to 1.9 million [167]. The global renewable power capacity has grown from around 850 GW in 2000, to 1829 GW in 2014[168], just to mention a few examples. International Politics must also be influenced. However, this it is not a trivial process, since each country has different declared and other interests. For example, countries which have significant heavy chemicals industry are more hostile to changes towards a greener chemicals production. Hope also lies in the cooperation between countries, such as the Basel, Rotterdam and Stockholm Conventions. These have raised awareness of the problem, and have led to worldwide efforts to enforce laws and regulations to protect the planet, to protect us. There is, therefore, some hope that it might be possible to see all countries in the world working together on a solution to this global problem.

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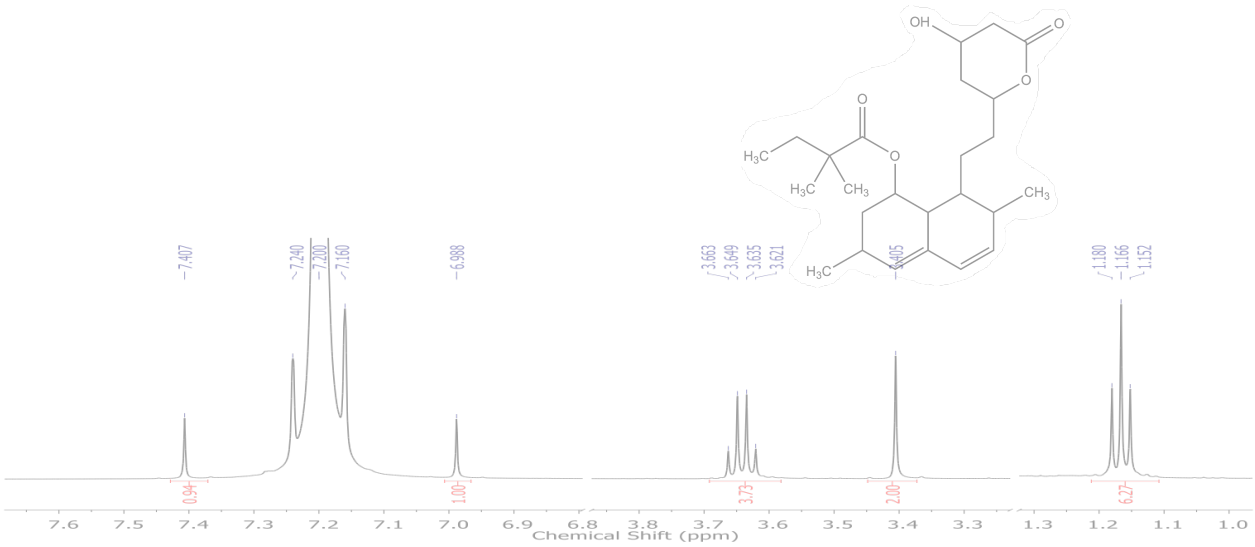
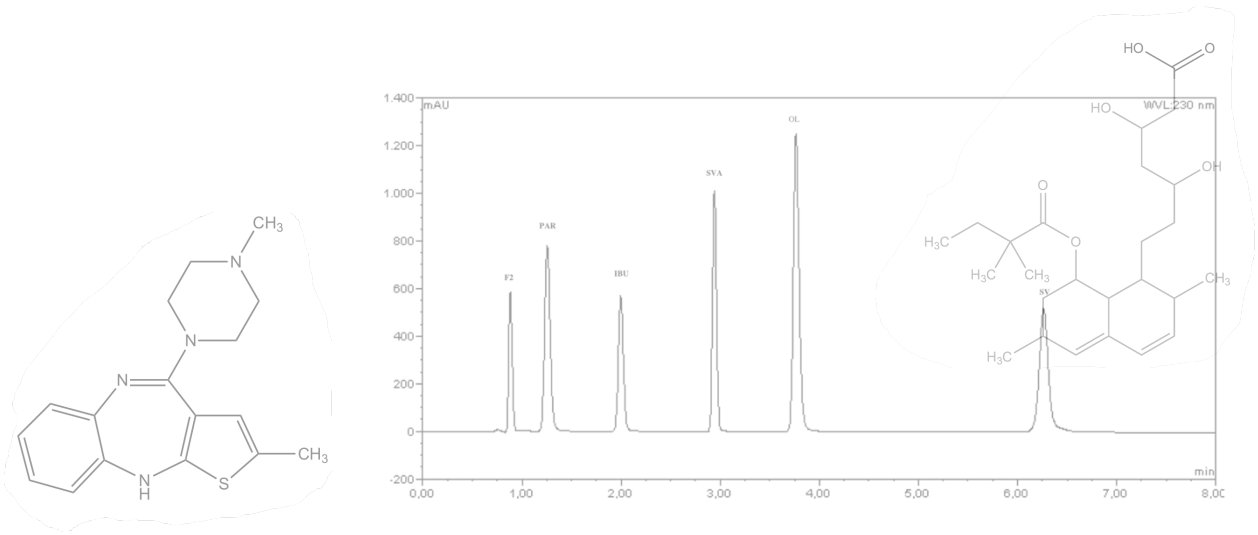
Chapter 3

Materials and methods



This chapter provides an overall description of the methods employed to carry out relevant tasks in this work. These include extraction techniques and chromatographic, spectroscopic, and hyphenated techniques.



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3.1 Sampling and preconditioning

In the screening for new metabolites and products, or in their production, cultures from microalgae collections, commercial suppliers or the isolation of indigenous wild type microalgae may be considered. Some of the databases for culture collections worldwide include the CCALA[7] in Trebon, Czech Republic, the SAG[8] in Göttingen, Germany, the ACOI[9] in Coimbra, Portugal, the CCAP[10] in Windermere, U.K., the Chlamydomonas Resource Center[11] in Minneapolis, U.S.A. and the UTEX[12] in Austin TX, U.S.A..

Isolation of microalgae strains into pure cultures include traditional isolation techniques, such as single-cell isolation by a micropipette, isolation of cells on agar plates, dilution method, gravity separation, phototaxis, and automated isolation techniques, such as flow cytometry cell sorting and optical trapping. Single-cell isolation by micropipetting is the most common method for isolation of microalgae strains. Basically, the isolation is performed using a Pasteur pipette or a glass capillary to collect a single cell from a sample and transfer it to a sterile droplet. A detailed description can be found in Andersen, 2005 [13]. Single cells can be identified and separated from contaminants and other cells, and sorted into multiwell plates for establishing new microalgae cultures[13].

3.2 Microalgae cell- counting

The cell-counting may be conveniently determined using microalgae cell suspension in a standard hemocytometer or Neubauer chamber. The glass chamber is divided into sections of a well-defined and calibrated area and depth. The chamber is loaded with a sample of cell suspension by using a micropipette. After the cells settle, for high density cell population, the four corners and the middle square of each side are counted under a microscope (Figure 3.1). The number of cells is determined by

Cell per mL of suspension = $X/5 \times 25 \times 10^4$

This method presents several advantages, such as low cost and being easily performed, and the disadvantages of being time-consuming and labor intensive.

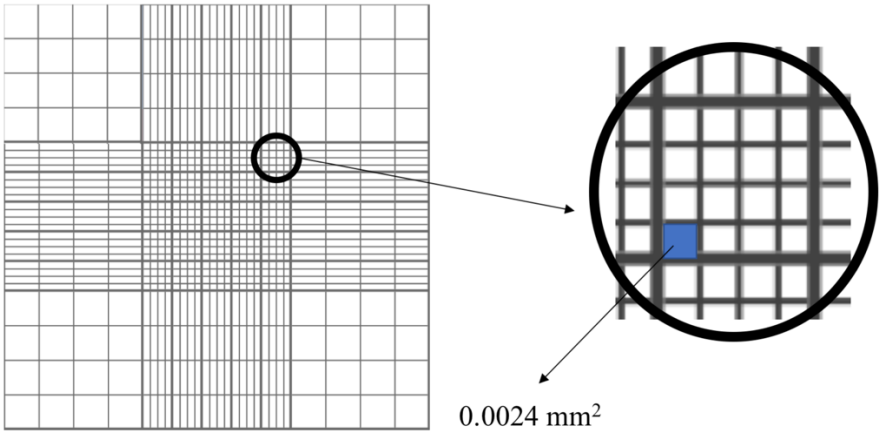

Figure 3.1 *Line drawing of ruling of the Neaubauer chamber. The central area of the grid is formed by a 5x5 grid of 25 squares with a total volume of 0.1 mm^3 (1x1x0.1mm, the latter dimension corresponding to the depth). Each of these squares possesses an area of 0.04 mm^2 and a total volume of 0.004 mm^3. Each is further divided into a 4x4 grid of 16 squares, each with an area of 0.0024 mm^2 and a volume of 0.00025 mm^3.*

3.3 Extraction techniques

The solvent extraction process is a crucial step in isolation and purification of individual distinct types of metabolites. The method chosen can directly affect the biological activity of the extracted compounds,[15] as well as their reproducibility, efficiency and effectiveness. Therefore, when choosing the most appropriate method from amongst the several available options, certain important considerations must be addressed. First, microalgae and cyanobacteria present a remarkable biodiversity, which is reflected in the chemistry of their unique secondary metabolites, and associated cell wall characteristics and structure. Thick-walled, silicified membranes, multilayered walls, wall-bound exopolysaccharides and armored walls present barriers to permeation by the solvents used in extraction, and must be disrupted prior to this to allow the release of compounds of interest[16].

Labile compounds may be subject to degradation upon solvent extraction under elevated temperatures, and light, air and pH are among the factors that should be controlled to prevent the decomposition of these compounds.

3.3.1 Cell disruption

Cell disruption techniques include soaking, pestle and mortar (tissue grinders), maceration, cryogrinding, bead-beating, homogenization, planetary micro milling, sonification[17], mixer milling[16], high-pressure homogenization[18], microwave, autoclaving, and addition of hydrochloric

acid, sodium hydroxide or alkaline lysis. Maceration is a cost-effective method and is commonly used in algal cell disruption. Bead beating has been demonstrated to be very effective in disrupting the cell of *Botryococcus braunii*[19] and can be suitable for industrial scale up[17]. Sonification is one of the most commonly used laboratory method to disrupt the cell wall in microalgae, and has been shown to give reliable results in several reports[17, 20-21]. However, thermolabile compounds may be degraded due to the heat generated by this method; therefore, when low temperatures are required, cells should be placed in an ice bath during the entire sonification process to prevent the formation of artefacts or degradation products, and disruption of the cells may be confirmed by optical microscopy.

3.2.2 Metabolites extraction

The microalgal cell is a complex biological system, with many cellular and sub-cellular structures where several different processes occur and a panoply of chemically distinct metabolites are produced. Because different classes of metabolites have different chemical characteristics, there is currently no standard established extraction procedure. Procedures described in the literature include conventional extraction techniques, supercritical fluid extraction, enzyme extraction, etc.

The presence of thermolabile compounds requires the use of techniques working at low temperature, to prevent thermal degradation, hydrolysis and hydrosolubilization. The cell wall characteristics, the nature of molecules to be extracted from the matrix, extraction times and coextraction of different compounds are some of the factors that have to be considered.

Conventional solvent extraction

Solvent extraction In conventional solvent extraction, the appropriate solvent or mixture of solvents is added to the microalgal biomass from which the desired metabolite is to be extracted. Once the crude extract is separated from the cell residue and filtered, the solvents, if volatile, can be evaporated.

Soxhlet extraction The procedure described by Soxhlet[22] has commonly been used for the extraction of primary compounds as lipids from biological samples. Secondary metabolites are also extracted with this methodology from the microalgal material, placed in a thimble, by repeatedly washing and leaching (percloration) with the appropriate organic solvent under reflux in the Soxhlet apparatus. The solvent in the flask is heated to boiling. The steam solvent is condensed in the condenser, flows through the sample and moves back down into the distillation flask. The resulting crude extract should be filtered, to remove any remaining unwanted matter, and can then be concentrated on a rotary evaporator or dried under a nitrogen steam. In the Soxhlet procedure, heat is required to drive the extraction, and this may cause decomposition of thermolabile compounds.

In these cases, high temperatures must be avoided by choice of appropriate solvents in order to prevent the development of decomposition products.
The extraction process using organic solvents has come under increasing criticism due to its reliance on solvents that are known to be toxic, carcinogenic or environmental pollutants. Chloroform, dimethyl acetamide, dimethyl formamide, dimethyl sulfoxide and methanol are some of the solvents that are becoming considered unsuitable for such extractions, and there is increasing interest in the development of green solvents or other techniques for this[16].
Regardless of the choice, the selection of a suitable solvent or solvent system should address some aspects, such as grade and purity, solubility of the compounds of interest, safety, and environmental issues.

3.4 Chemical characterization

The separation and detailed identification of microalgal metabolites has only really become possible with the development of advanced instrumental methods of analysis, particularly those involving hyphenated techniques (e.g. HPLC-MS). The traditional methods of screening for secondary metabolites have lead to the successful identification and development of many drug candidates[23]. For the structural elucidation of metabolites in algae and in other natural products, a repertoire of analytical techniques is currently available including, chromatographic, spectroscopic, hyphenated and genome mining techniques. However, due to the diversity and complexity of microalgal and cyanobacterial metabolites, it is unlikely that one single analytical technique will provide enough information about the metabolites that are present in these organisms and, generally, it is necessary to use various complementary techniques that reveal different facets which enable the complete chemical structure characterisation.

3.4.1 Chromatographic Techniques

High Performance Liquid Chromatography (HPLC)

HPLC is a chromatographic technique that can separate complex mixture of compounds and can be used to identify, quantify and purify individual analytes of a mixture[33]. Generally, equipped with ultraviolet (UV) and fluorescence (FLD) detectors, is a widely used method for the analysis of metabolites because of its sensitivity and ease of use. The separation of different molecules and the retention of analytes in HPLC involve different aspects of molecular behaviour and molecule surface interactions. Chromatographic separations are based on the transport of the mobile phase which carries the sample along the column containing the stationary phase. The differences in the

interactions of the analytes with the mobile and stationary phases result in different migration times for a mixture of compounds.

One of the most common techniques of chromatography is reverse-phase HPLC (RP-HPLC). The process involves mainly dispersive forces, such as hydrophobic or van der Waals interactions. The surface of the stationary phase is hydrophobic, and the mobile phase is polar. RP-HPLC has the advantage of a high analytical sensitivity and has the ability to discriminate between molecules which are structurally related.

Specific examples of application of this technique include the analysis of microalgae secondary metabolic peptides[34] and alkaloids[35] and the analysis of pesticides and pharmaceuticals in bioremediation using microalgae. Although UV detectors are limited to molecules having suitable chromophores, HPLC-UV is suitable to the detection and quantification of several metabolites.

3.4.2. Spectroscopic Techniques

Fluorescence spectroscopy

Fluorescence is the emission of light when electronically excited states relax back to the ground state. Its theoretical basis is closely related to, and involves the same electronic states, as UV-VIS absorption spectroscopy. However, the nature of measurement technique means that it may have a sensitivity several orders of magnitude greater than absorption spectroscopy. This is a great advantage in analytical applications, such as the study of microalgal metabolites, which may be present at very low concentrations. Not all molecules have significant fluorescence. Analytically interesting fluorescent organic molecules typically have rigid conjugated structures, such as derivatives of aromatic hydrocarbons. Experimentally, its study requires an exciting light source, the sample, appropriate dispersive elements, such as monochromators, and a detector. It is possible to scan the emission spectrum using a constant excitation wavelength, or to observe the emission at a constant wavelength and study the effect of changing the excitation wavelength. These give the emission and excitation spectra respectively. A number of excellent descriptions of the technique are available[40,41]. Typical examples of applications include analysis of chlorophylls[42], carotenoids, and alkaloids, such as β-carbolines[43]. It is also possible to add fluorescent probes which are sensitive to particular environments, such as hydrophobic ones, which can provide detailed information on lipid content[44,45]. The high sensitivity of fluorescence means that it is valuable for combining with chromatographic methods, such as HPLC, in hyphenated techniques. It can also be combined with microscopy in fluorescent imaging and can furnish more detailed information through time-resolved measurements.

3.4.3 Nuclear Magnetic Resonance (NMR)

NMR is a powerful technique that provides both qualitative and quantitative information. It allows the simultaneous detection of diverse groups of microalgae metabolites (lipids, aminoglycosides, alkaloids, terpenoids and so on). NMR exploits the magnetic properties of certain nuclei having a nuclear spin (such as ^{1}H, ^{13}C, ^{31}P), which, when placed in a magnetic field, absorb electromagnetic radiation in the radio-frequency region of the spectrum (frequency of resonance). The resonance frequency depends on the chemical environment of the nucleus, and thus, each nucleus in a molecule gives a specific and characteristic signal (given as its chemical shift), allowing structural elucidation. In addition, the technique provides information on the nearest neighbour atoms through "coupling" of the nuclear spins. There are many excellent books on NMR spectroscopy, describing the fundamental theory, applications and the typical values for chemical shifts and coupling constants, in addition to several online databases that provide valuable data resources[46], and useful information regarding organic structures and corresponding spectra are available[46, 47].

Although NMR is a reliable and very robust technique that provides unambiguous information, it has some disadvantages that can limit its application. These include the expensive equipment needed, the time-consuming measurements, the high concentrations of samples required, and the overlapping signals that may be present when applied to complex bio-organic compounds, making the interpretation of the exact structure of the compound difficult.

In general, the NMR technique does not require elaborate sample preparation, and liquid samples, solvent extracts and dried or live microalgal cells can directly be analysed. It is a straightforward and non-destructive technique, such that samples can be further analysed by other techniques. NMR spectroscopy has been extensively used in biological studies and significant improvements are continuously being made in the use this high-throughput technique.

Since a large number of NMR experiments are possible in metabolomics, different approaches can be considered when using NMR spectroscopy. One dimensional ^{1}H NMR is one of the most widely used in metabolomics analysis. However, when analysing complex mixtures, ^{1}H NMR spectra can be very challenging due to overlapping of signals, which can hinder the identification of metabolites.

A relatively new methodology in the field of Metabolomics studies is *in vivo* High Resolution Magic Angle Spinning Nuclear Magnetic Resonance (^{1}H HR-MAS NMR) that can be applied to live microalgal cells[48].

In ^{13}C NMR spectroscopy, where chemical shifts cover at least 200 ppm, compared typically with 10 ppm for ^{1}H NMR, the signals are better resolved. However, the major limitation of ^{13}C NMR spectroscopy is the lower sensitivity arising from the low natural abundance of ^{13}C.
A customized metabolomics NMR database[49], $^{1}H(^{13}C)$-TOCCATA, contains a complete set of ^{1}H and ^{13}C chemical shift information on individual spin systems and isomeric states of common metabolites, and allows the identification of metabolites in complex mixtures[50].

3.4.4 Hyphenated techniques

The combination of individual separation techniques, in particular various types of chromatography, with NMR and MS has led to the development of hyphenated techniques which have greatly increased the analytical capabilities in metabolomics research. Examples include gas chromatography–mass spectrometry (GC–MS)[52], liquid chromatography–nuclear magnetic resonance spectroscopy LC-NMR[53], high performance liquid chromatography-mass spectrometry (HPLC-MS)[54]. HPLC-MS is a technique that combines the separation of mixture of compounds of liquid chromatography (HPLC) with the mass analysis of mass spectrometry (MS). HPLC-MS, for example, would provide direct and very useful information on the structure of deuterated lipids eluting from the column.

3.4.5 Differential scanning calorimetry

Differential scanning calorimetry (DSC) is a powerful technique to study thermal transitions. It detects the temperatures and the heat flows when the heat capacity suffers alteration, or when recording endothermic and exothermic processes, as a function of temperature (or time). DSC can measure and determine melting temperature, glass transition temperature, crystallization temperature, specific heat, heat capacity, heat of fusion, denaturization of macromolecules, and may also provide information of temperatures at which chemical changes, such as oxidation, occur. The reference material must be inert and present a well-defined heat capacity over the range of temperature studied. This technique provides qualitative and quantitative information on heat absorption (endothermic processes) or evolution (exothermic processes).

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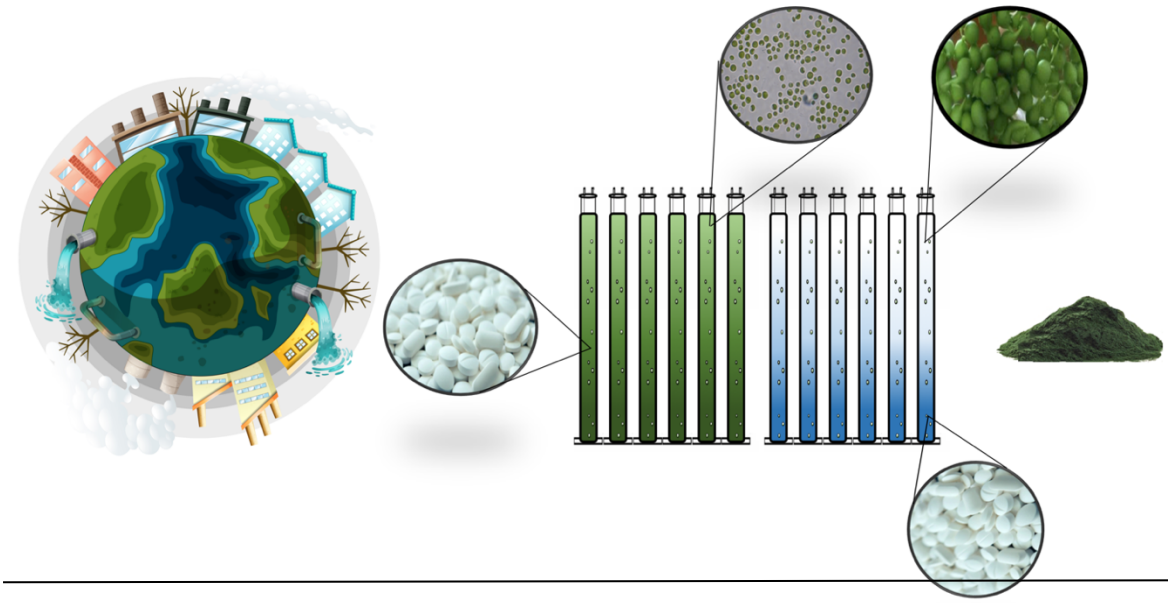
Part B

Bioremediation

Chapter 4

Removal of pharmaceuticals from water by microalgae *Nannochloropsis* sp.



Pharmaceuticals and their metabolites are released to the environment by domestic, hospital, and pharmaceutical industry wastewaters. The concentrations of these compounds that are innocuous to humans could be deadly to non-target organisms. Conventional wastewater treatment technology does not guarantee effluents of high quality; the apparently clean water may be loaded with pollutants. In this study, we assess the performance and efficiency of free and immobilised cells of microalgae Nannochloropsis sp. *for the removal of four pharmaceuticals, chosen for their occurrence or persistence in the environment. These are paracetamol (PAR), ibuprofen (IBU), olanzapine (OLA) and simvastatin (SIM). The results showed that free microalgae cells remain alive for longer time than the immobilised ones, suggesting the inhibition of cell proliferation by PVA. Both cells, free and immobilised, respond differently to each pharmaceutical. The removal of PAR and IBU by Nannochloropsis* sp., *after 24 hours of culture, was significantly higher in immobilised cells. Free cells removed a significantly higher concentration of OLA than immobilised ones, suggesting more affinity to this molecule than to PAR and IBU. The results demonstrate the effectiveness of Nannochloropsis* sp. *free cells for removing OLA and Nannochloropsis* sp. immobilised cells *for removing PAR and IBU.*

The content of the present chapter was fully or partially submitted in paper form in Encarnação T., Palito, C., Pais, A.C., Valente, A., and Burrows, H.D. Removal of pharmaceuticals from water by the microalga *Nannochloropsis* sp.

4.1 Introduction

Recent effects of human civilisation have changed the face of the Earth in a way without precedents in our history. We are now living the Anthropocene epoch. Human activities have had significant impact on the Earth´s ecosystems, and we have all been living in a way beyond the capacity of our planet, such that we are facing dramatic environmental challenges related to global warming and climate change, plastic pollution, biodiversity loss, air, water and land pollution. All of these changes deeply affect us; us, humans, and other species. Biologists and palaeontologists suggest that the Earth´s sixth mass extinction is already underway[1,2], as a result of our impact on the Earth. "We humans are big eaters and garbage producers"[3].

These activities lead to global pollution. In addition to air pollution, water pollution is one of the most important environmental problems that the world urgently needs to address, since clean water is a vital resource for every living organism on planet. The water of oceans, seas, rivers and lakes have natural mechanisms of self-cleaning, that rely on plankton, which are also the base of food-chains and food-webs. They remove the waste and contaminants produced by other organisms in aquatic ecosystems, such as carbon dioxide, nitrates and phosphates. They also remove the non-natural anthropogenic contaminants, produced by humans, such as pesticides, industrial chemicals and pharmaceuticals. However, there are certain threshold levels, above which these mechanisms start to fail. Anthropogenic contaminants reach water bodies through different pathways. Direct sewage effluent discharges, via rivers, urban and agricultural run-offs, dumpings, atmospheric transport, and various other means. According to the United Nations Environment Programme, UNEP, 80% of the global wastewater are released untreated into water bodies[4]. Pharmaceuticals and their metabolites are released to the environment by domestic and hospital wastewaters and by pharmaceutical industry wastewater[5]. In general, these are reported at the range of nanogram to microgram per litre, while some have been detected up to the range of milligrams per litre. Increasing evidence is emerging on the implications of endocrine disrupting chemicals in the environment and wildlife, and on human health, at very low doses. Effects on biota have been reported with concentration in the range of ng per litre[6,7]. Pharmaceuticals may be excreted in their original form, or/and as their metabolites, through urine and faeces. The metabolic path of some pharmaceuticals, containing up to 90% of the active ingredient, may involve excretion in the original form. Pharmaceuticals are designed to be biologically active, and to trigger a specific therapeutic response in humans or animals, based on biology and size. The concentrations that are innocuous to humans could be deadly to non-target organisms; that share certain homologous receptors with humans. The dimension of the problem requires a global action. On March 2019, the

European Commission released the European Union Strategic Approach to Pharmaceuticals in the Environment, contributing to achieving the Sustainable Development Goal 6, on clean water and sanitation[8]. This Communication defines a strategic approach to addressing the global problem of pharmaceuticals in the environment. It calls for more advanced wastewater treatment technologies and more research, and encourages innovation "where it can help to address the risks, and promote the circular economy by facilitating the recycling of resources such as water, sewage sludge and manure" maintaining "the access to safe and effective pharmaceutical treatments for human patients and animals".

Conventional wastewater treatment technology does not guarantee effluents of high quality; the apparently clean water may be loaded with pollutants, while the advanced wastewater treatments, which include physicochemical treatments, such as chemical precipitation, ozonation, UV light, reverse osmosis, may not remove the thousands of chemicals present in wastewater. One potential methodology, which could be combined with the currently used wastewater treatment plants, is microalgae technology. It is well known that microalgae remove nitrates and phosphates from wastewaters. They need nitrates and phosphates to growth. There are already companies successfully using successfully microalgae technology to remove heavy metals or recover nutrients from wastewater[9,10].

In line with the European Commission´s strategy, and with Sustainable Development Goal 6, in this chapter we assess here the performance and efficiency of free and immobilised cells of microalgae *Nannochloropsis* sp. for the removal of four pharmaceuticals, chosen for their occurrence or persistence in the environment. These are paracetamol (PAR), ibuprofen (IBU), olanzapine (OLA) and simvastatin (SIM). Acetaminophen, commonly named as paracetamol (PAR), is a widely used analgesic and antipyretic drug, and is known to be heavily present in the aquatic environment. Ibuprofen is a non-steroidal anti-inflammatory drug, commonly used for the relief of pain, fever and inflammation. Both are generally classified as harmful to aquatic organisms[11]. Olanzapine, an antipsychotic drug, used for the treatment of schizophrenia, is resistant to photodegradation by sunlight, and is found in surface waters[12,13]. Simvastatin is a lipid lowering drug, known for anti-hyperlipidemic activity. This substance is one of the most commonly used prescription worldwide and is, therefore, of environmental concern. This pharmaceutical compound is a lactone that is hydrolysed in water to the corresponding β-hydroxyacid, denoted simvastatin acid (SIMA) (Figure 4.1).

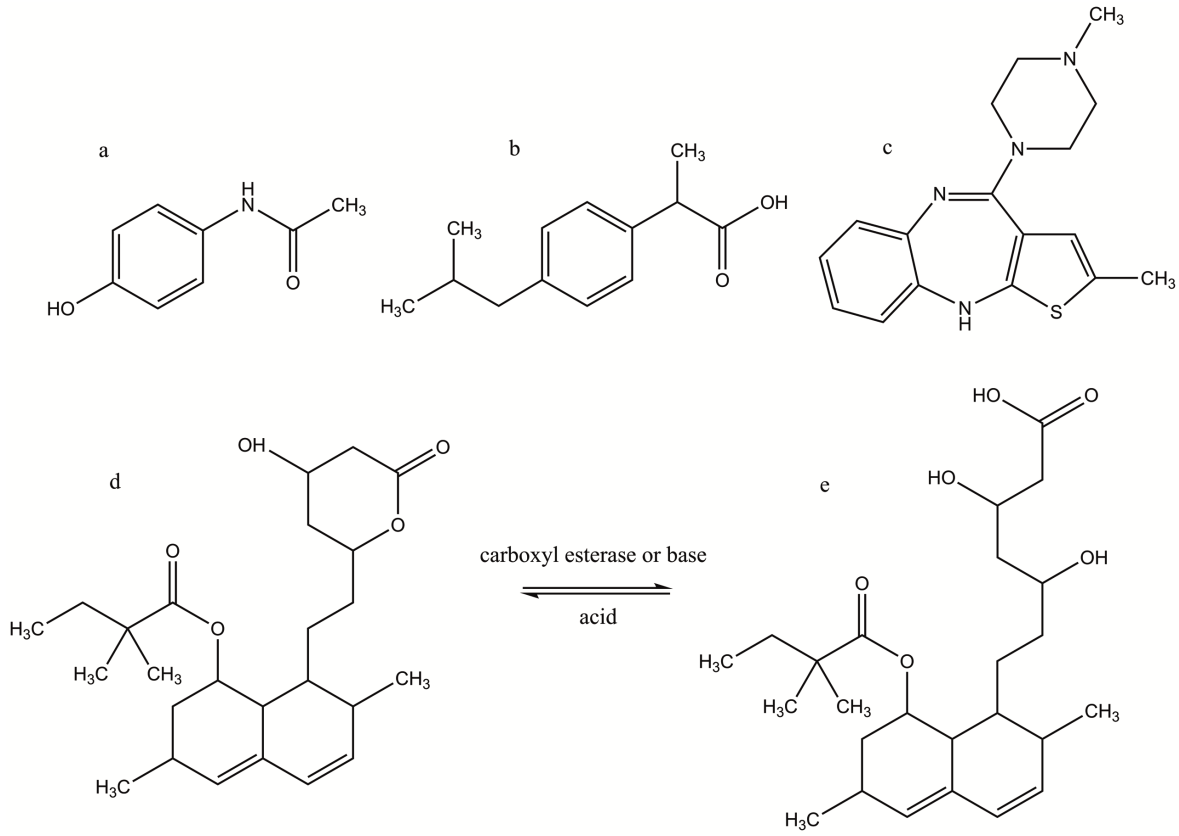


Figure 4.1 *Chemical structures of (a) paracetamol, (b) ibuprofen, (c) olanzapine, (d) simvastatin and (e) simvastatin acid.*

4.2 Materials and methods

4.2.1 Reagents and chemicals

Paracetamol was purchased from Fagron Iberica (Spain) and Ibuprofen supplied by Laboratórios Medinfar (Lisboa, Portugal). Olanzapine was acquired from Zhejiang MYOY Import & Export Co., Ltd (Hangzhou, China). Simvastatin was kindly provided by Labesfal, Laboratórios Almiro, S.A. (Santiago de Besteiros, Portugal). Microalgae medium f/2 was obtained from Varicon Aqua Solution (Malvern, UK). Polyvinyl alcohol (PVA), having a molecular weight of 61.000, commercial name Mowiol® 10-98, was obtained from Aldrich Chemistry. Boric acid (H_3BO_3) with an average molecular weight of 61.83 was purchased from Sigma-Aldrich (India). Calcium chloride ($CaCl_2$), with a molecular weight of 110.99, was acquired from Merck. All other reagents and solvents were of analytical or HPLC grade.

4.2.2 Organism and culture conditions

Nannochloropsis sp. was obtained from Varicon Aqua Solution, Malvern, UK, and was cultivated for 6 days in 2 L f/2 medium. 100 cm^3 of a *Nannochloropsis* sp. culture were filtered and washed. The cells with a concentration of about 2 x 10^7 cell cm^{-3} were subsequently transferred to a closed photobioreactor, to which were added 100 cm^3 of culture medium Cell-hi TEViT (Varicon Aqua Solution, Malvern, UK), based upon the f/2 medium, deprived of nitrates. Nitrate concentration was 0.30 g L^{-1} and salinity 25 g L^{-1}. The culture was aerated by bubbling atmospheric air, at a rate of 300 cm^3 min^{-1}, were grown at 25 ± 2 ºC under light with an irradiance level of ± 100 µmol $m^{-2}s^{-1}$ with 16:8 photoperiod and kept for 60 hours. Milipore water was added when needed to ensure the same constant volume, compensating for water loss by evaporation. Samples of 5 ml were replaced with ultrapure water. Each experiment was carried out in triplicate. The cellular density was determined using the Neubauer chamber (hematocytometer) in an optical microscope. The supernatant (filtrate culture medium) was collected, filtered, and stored at -20 ºC until analysis. Microalgae from genus *Chlorella* are the most commonly used ones for bioremediation studies. The specie used in this study was *Nannochloropsis,* which is not so common in bioremediation studies. This specie was chosen for the uptake studies because it has high growth rate, is resilient, adapts to a wide range of cultivation condition, and can accumulate large amounts of lipids, which is an advantage as it provides added value to biomass produced during the bioremediation process.

4.2.3 Fluorescence microscopy observation

Fluorescence images of *Nannochloropsis* sp. cells were obtained using an Olympus BX51 M microscope,equipped with a UplanFL N 100x/1.30 oil immersed objective lens (∞/0.17/FN/26.5), a filter set type U-MBF3, U-MWV2, and an UV-mercury lamp (100W Ushio Olympus). Images were digitized on a computer through a video camera (Olympus digital camera DP70) and were analysed with an image processor (Olympus DP Controller 2.1.1.176, Olympus DP Manager 2.1.1.158). The observations were carried out at room temperature (≈25ºC).

4.2.4 Immobilization procedures

The immobilization of the microalgae was achieved through the formation of beads of PVA gel. A 24% PVA solution was first prepared, dissolved with continuous stirring at about 50 ° C for two hours. In parallel, the microalgae were centrifuged and washed to remove media salt and nutrients, and then added to the PVA solution in a 1:2 portion (cells: PVA). This mix was dripped with a sterile syringe into a saturated solution of boronic acid and 2% of calcium chloride in which the beads formed remained for one hour. As a control, beads were prepared consisting only of PVA. In parallel, the solutions with the pharmaceuticals were prepared.

4.2.5 Instrumentation and chromatographic conditions

The chromatographic analysis of PAR, IBU, OL, SV and SVA was carried out using a Dionex Ultimate 3000 system equipped with an auto injector and four variable UV/visible dual wavelength detectors. The column used for the analysis was a Luna Phenyl-Hexyl, Phenomenex® (Torrance, USA), with 5 μm particle size, 3 mm internal diameter and 150 mm length, supported with a SecurityGuard™ cartridge Phenomenex® (Torrance, USA), with 3.0 mm internal diameter, in an oven at a temperature of 35ºC. The results were acquired and processed using Chromeleon software. The mobile phase consisted of a mixture acetronitrile (eluente A) and 0.01M dipotassium hydrogen phosphate aqueous solution (eluent B), at a pH 7.3 ± 0.1 and constant flow rate of 0.8 mL/min. Chromatographic analysis was conducted in multistep gradient mode (0 min 70 % Eluent B, 1 min 60 % Eluent B, 2 min 40 % Eluent B, 5 min 35 % Eluent B, 7 min 30 % Eluent B, 8 min 70 % Eluent B).

4.2.6 Method validation

The method for PAR, IBU, OLA, SIM, and SVA quantification was validated according to the US Food and Drug Administration (FDA), the International Conference on Harmonization (ICH) guidelines, and the Eurachem, with respect to system suitability, linearity, accuracy, precision, recovery, limits of detection and quantification, selectivity and specificity. The optimization and validation details and results are presented in Chapter 8.

4.2.7 Experimental design

Different, simultaneous sets of experiments were conducted at 25 ± 2 ºC, under light with an irradiance level of ± 100 μmol (Figure 4.2). To the 100 cm^3 free cells *Nannochloropsis* sp. cultures, a concentration of 50 μg mL^{-1} of each pharmaceutical, PAR, IBU, OLA, SIM and SIMA, was added. Similarly, a blank with 50 μg mL^{-1} of each pharmaceutical, PAR, IBU, OLA, SIM and SIMA, was added to 100 cm^3 of culture medium f/2, without cells. The experiments with cells in beads were performed in the same way; the immobilised cells *Nannochloropsis* sp. cultures, beads, were transferred to the 100 cm^3 of culture medium f/2. The control experiment was carried out with beads in 100 cm^3 of culture medium f/2, but in the absence of cells. All the experiments had a 16:8 photoperiod, and were run for 60 hours. Millipore water was added when needed, to ensure constant volume in the presence of water loss by evaporation. Samples of 5 mL were replaced with Millipore water. The cultures were aerated by bubbling atmospheric air, at a rate of 300 cm^3 min^{-1}, and kept for 60 hours. The samples were collected after 12, 24, 36 and 60 hours, filtered and analysed by RP-HPLC. Each experiment was carried out in triplicate.

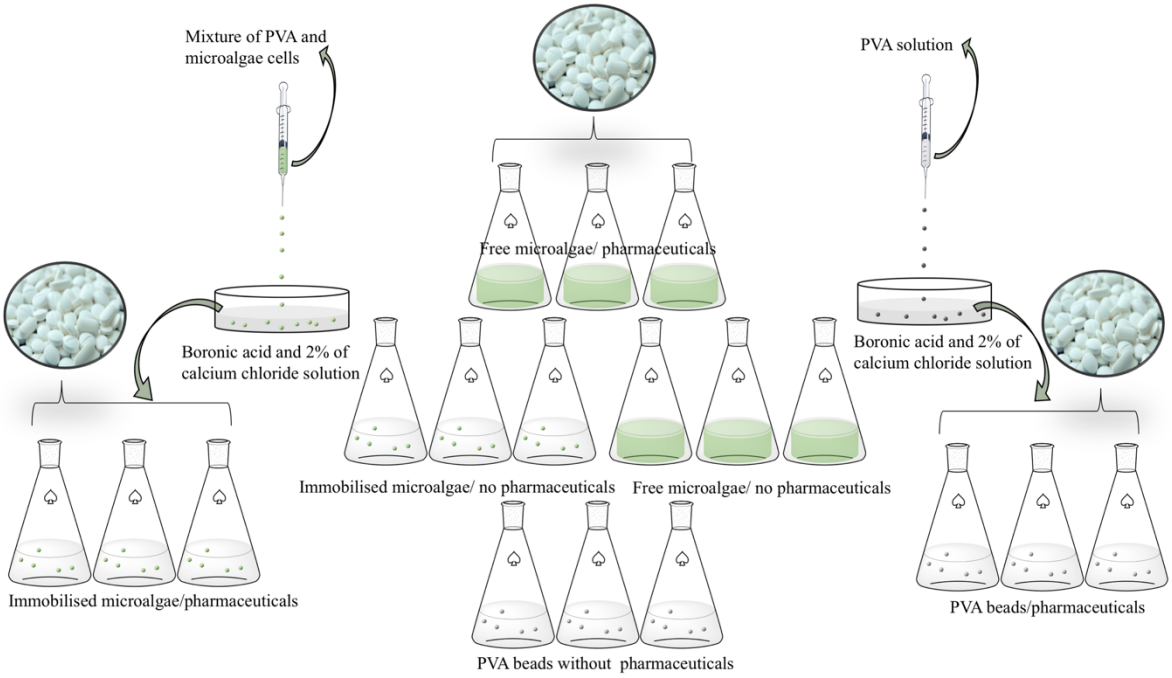


Figure 4.2 *Schematic Representation of the procedure used in the bioremediation experiments.*

4.2.8 Statistics

Single factor ANOVA analysis was conducted to establish the statistical significance within each treatment. A probability level of <0.05 was used as the criterion for null hypotheses. This analysis was performed using Microsoft Excel® (Microsoft Corp., Redmond, WA).

4.3 Results and discussion

4.3.1 Evaluation of resistance toward pharmaceuticals in *Nannochloropis* sp.

In the preparation of the beads, 2mL of PVA and 1mL of microalgae cells yielded, approximately, 25 unities of beads, each with 4mm of diameter (Figure 4.3). Preliminary studies were carried out with free and immobilised cells in a f/2 medium solution where 100 μg mL^{-1} of each pharmaceutical had been added (Figure 4.4), and with each pharmaceutical separately (Figure 4.5). From Figure 4.4 it can be seen that the free cells, in the presence of the four pharmaceuticals, remained as a viable population for, at least, 12 days. In contrast, immobilised cells started to fade from the 6th day on, and no viable cells are observed at the end of the experiment.

Figure 4.3 *Nannochloropsis* sp. *cells immobilised in PVA beads.*

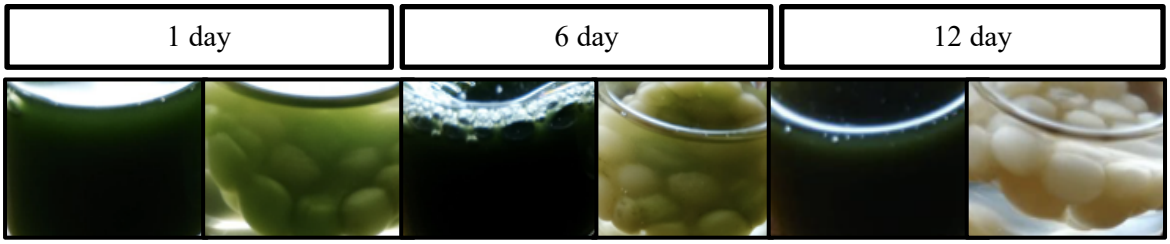


Figure 4.4 *Free (left) and immobilised Nannochloropsis* sp. *cells (right) grown in f/2 medium and PAR, IBU, SIM and OLA, for the indicated periods.*

In the evaluation of each individual pharmaceutical, Figure 4.5 shows that the most harmful pharmaceutical to the immobilised cells was ibuprofen. After 4 days, cells were completely faded, indicating the breakdown of the culture. The control group (no pharmaceuticals added to the medium) showed some fading after 4 days. The immobilised cells grown in the presence of olanzapine, showed a higher resistance.

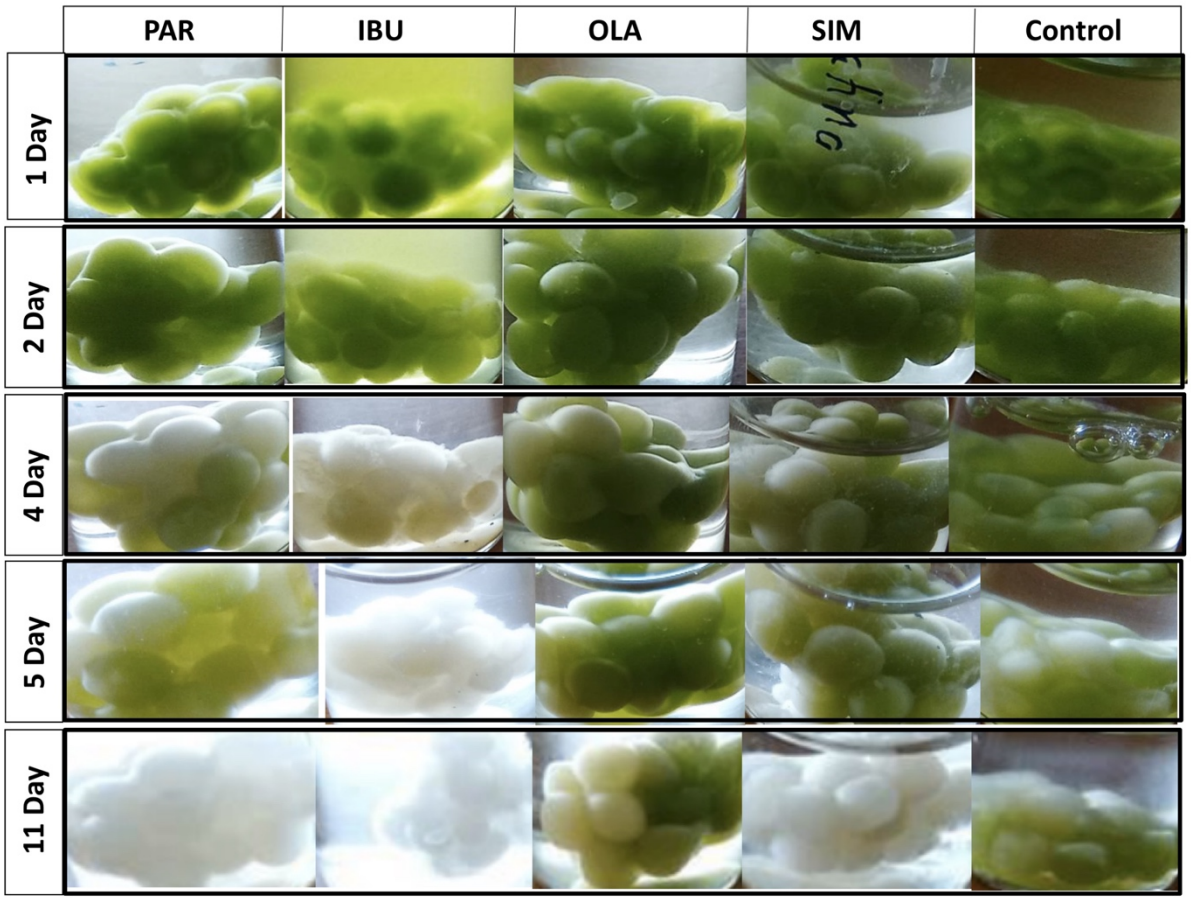


Figure 4.5 *Immobilised Nannochloropsis* sp. *cells grown in f/2 medium and PAR, IBU, SIM and OLA, during 11 days of culture.*

4.3.2 Fluorescence microscopy observation

The marine specie *Nannochloropsis* sp. possesses a characteristic cell structure; the cell relies on a big chloroplast which occupies more than half of the cell (see schematic representation of the cell in Figure B.2), when grown in nitrogen replete medium. Observed in Figure 4.6 are the chloroplasts of the cells emitting the red fluorescence of chlorophyll. Fluorescence microscope observations (Figure 4.7) showed fluorescence of olanzapine located inside the cells. Differences can be discerned between cells grown autotrophically, and cells grown in the dark.

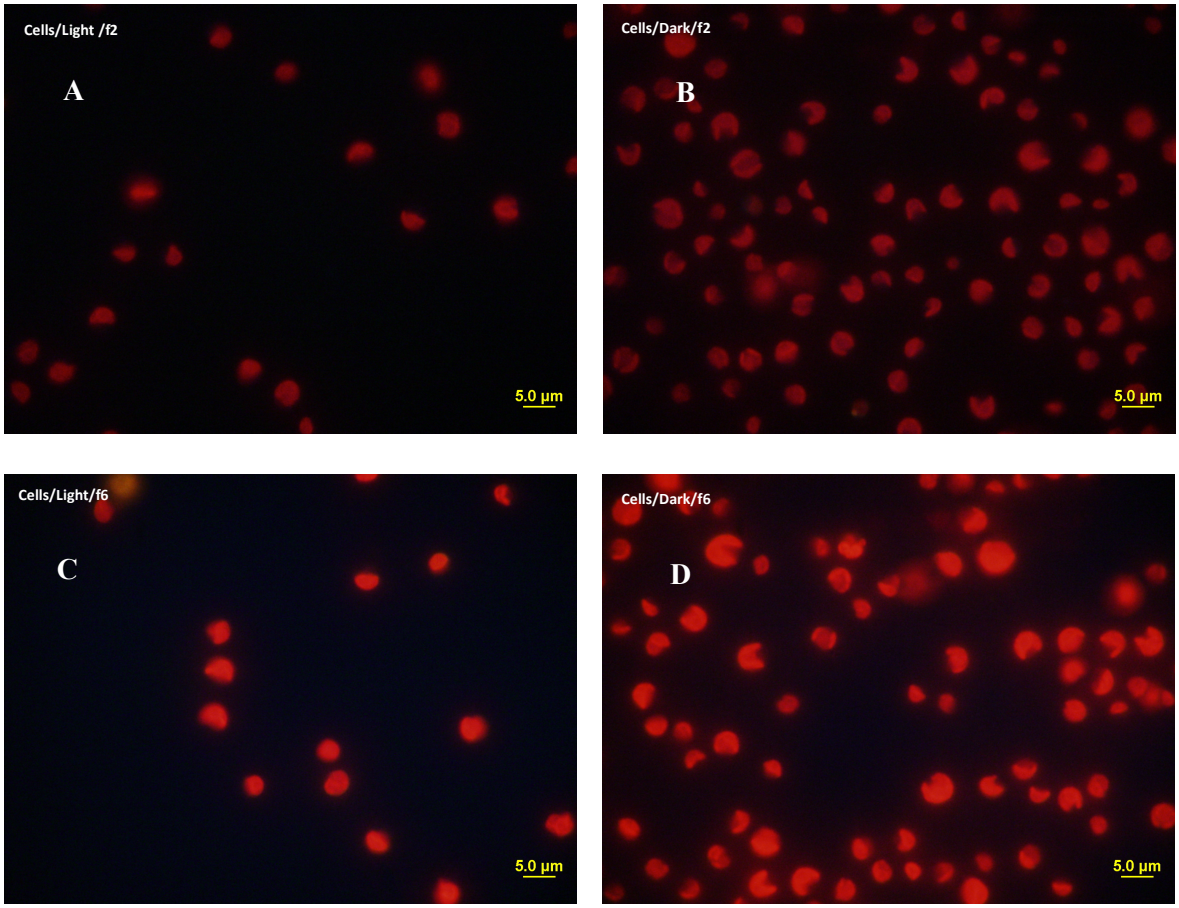


Figure 4.6 *Epifluorescence microscopy images of Nannochloropsis* sp. *cells showing the red autofluorescence of chlorophyll from chloroplasts. Cells grown in f/2 medium (no pharmaceuticals added to the medium). (A) and (C) cells grown autotrophically (microscope filters f2 and f6). (B) and (D) cells grown in the dark (different microscope filters, f2 and f6).*

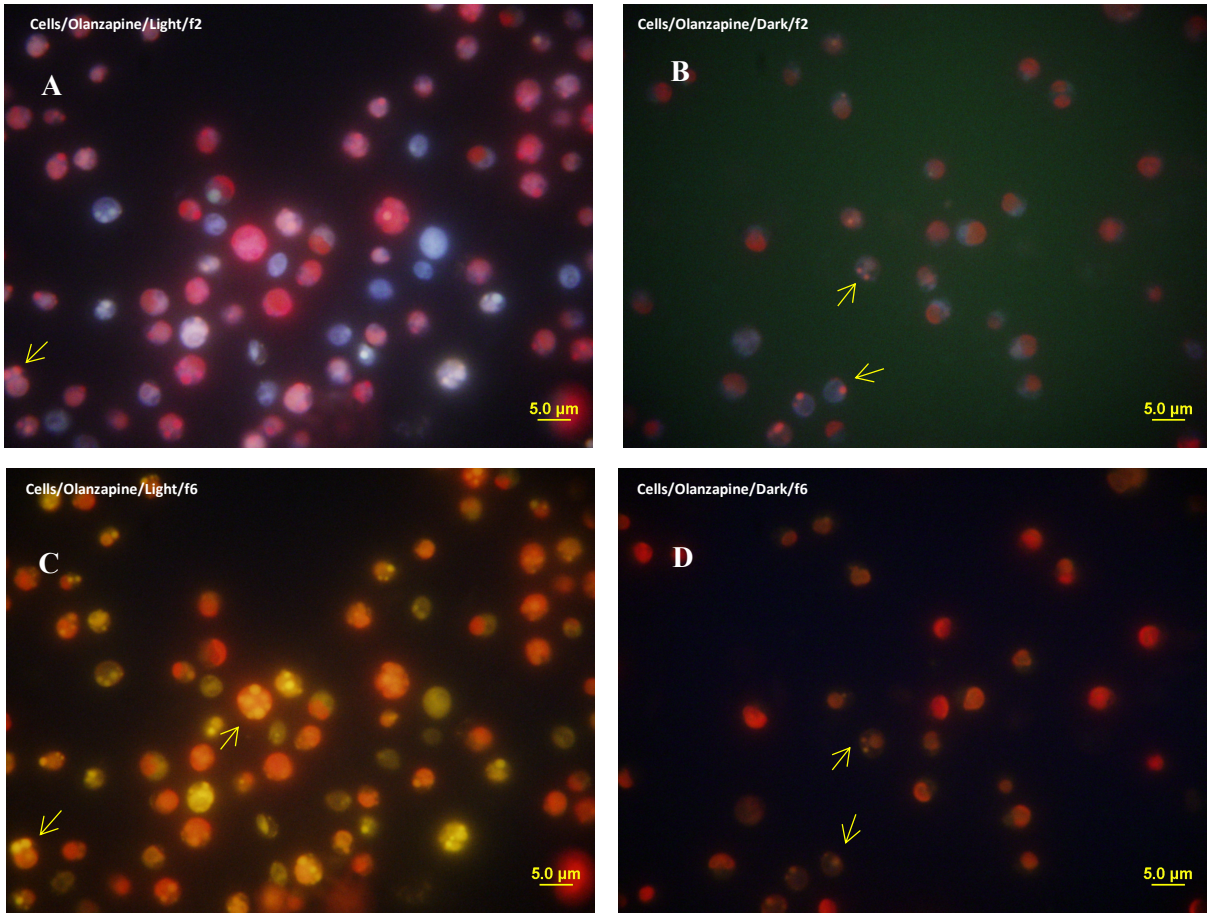


Figure 4.7 *Epifluorescence microscopy images of Nannochloropsis* sp. *cells showing the red autofluorescence of chlorophyll from chloroplasts and fluorescence of the olanzapine. Cells grown in f/2 medium with OLA added to the medium. (A) and (C) cells grown autotrophically (microscopy filters f2 and f6). (B) and (D) cells grown in the dark (microscopy filters f2 and f6).*

Cultures of *Nannochloropsis* sp. immobilized in PVA beads grew from 1.9 x 10^7 cells mL^{-1} to 2.4 x 10^7 cells mL^{-1} in the first 12 hours and remained stable, with a slight decrease after 60 hours of culture, 2.2 x 10^7 cells mL^{-1} (Figure 4.8). Cultures of *Nannochloropsis* sp. immobilized in PVA and grown in the presence of the pharmaceuticals had a similar growth in the first 12 hours and then decreased continuously until the end of the experiment.

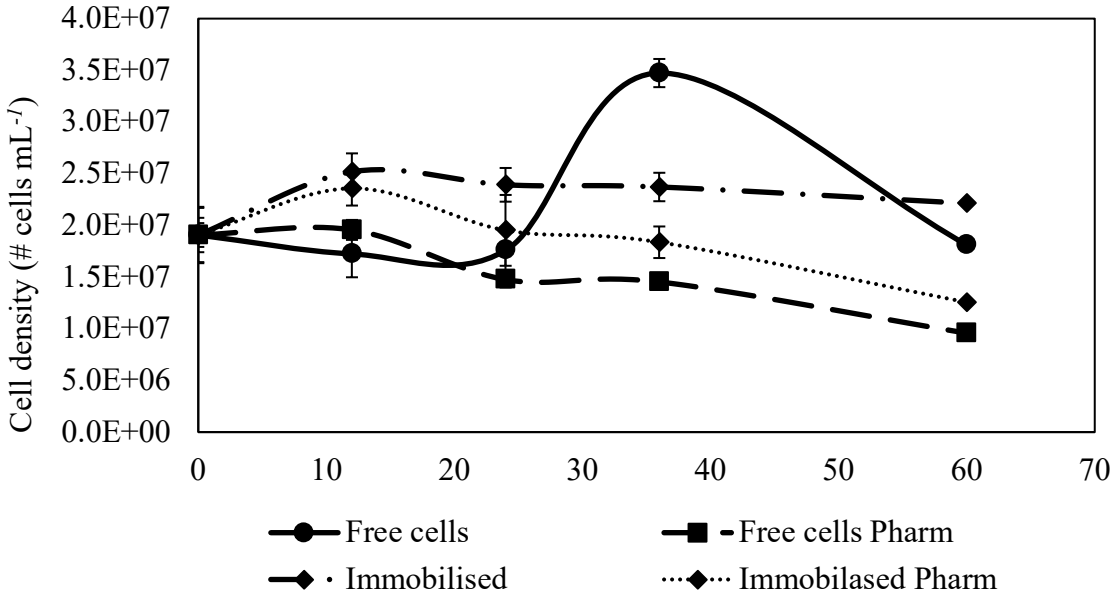


Figure 4.8 *Growth curve of Nannochloropsis* sp. *in continuous cultures for 60 hours. Error bars represent SD.*

The highest growth was obtained in free cells without the presence of pharmaceuticals during which, after 36 hours of culture, the population reached 3.5 x 10^7 cells mL^{-1}. The lowest growth, a cell density of 9.6 x 10^7 cells mL^{-1}after 60 hours, was observed with the free cells grown in the presence of pharmaceuticals.

Although PVA is considered to be biocompatible, the experiments showed that it inhibits cell proliferation (see immobilised cells without pharmaceuticals). The lower growth rates could be due to limitations in the diffusion of nutrients through the bead structure. However, it is interesting to note that the population remained stable over time in contrast with the free cells without pharmaceuticals. In this culture the free cells have all the nutrients available for consumption. After depletion of all the nutrients, the population decreases.

Upon analysis by RP-HPLC, the chromatograms revealed the presence of the PAR, IBU and OLA; simvanstatin was not detected. To check the possibility of precipitation of the compound, a filtrate was extracted with acetonitrile and analysed by RP-HPLC. The chromatogram revealed the presence of SIM in its original concentration, confirming precipitation. For this reason, this study focuses only on PAR, IBU and OLA.

Figure 4.9 shows that the removal of PAR by *Nannochloropsis* sp. after 24 hours of culture was significantly higher in immobilised cells, from 50,5 to 44.4 μg mL^{-1}, with a p value = 0.05. f/2 medium, beads and free cells have not influenced the concentration of PAR. However, the concentration of PAR in the group with the immobilised cells apparently shows an increase from 44.4 to 48.4 μg mL^{-1} after 60 hours of culture.

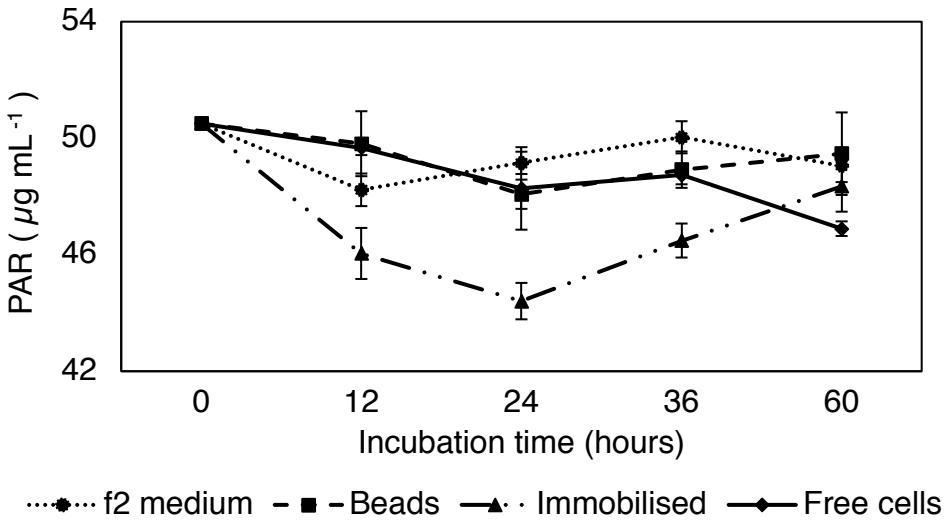


Figure 4.9 *PAR removal by Nannochloropsis* sp. *in continuous cultures for 60 hours. Bars represent the SD.*

However, upon closer examination (Figure 4.10), it appears that some fraction of the PVA beads have dissolved, with the probable release of the PAR into the medium. Also, leakage of cells from the beads was also observed.

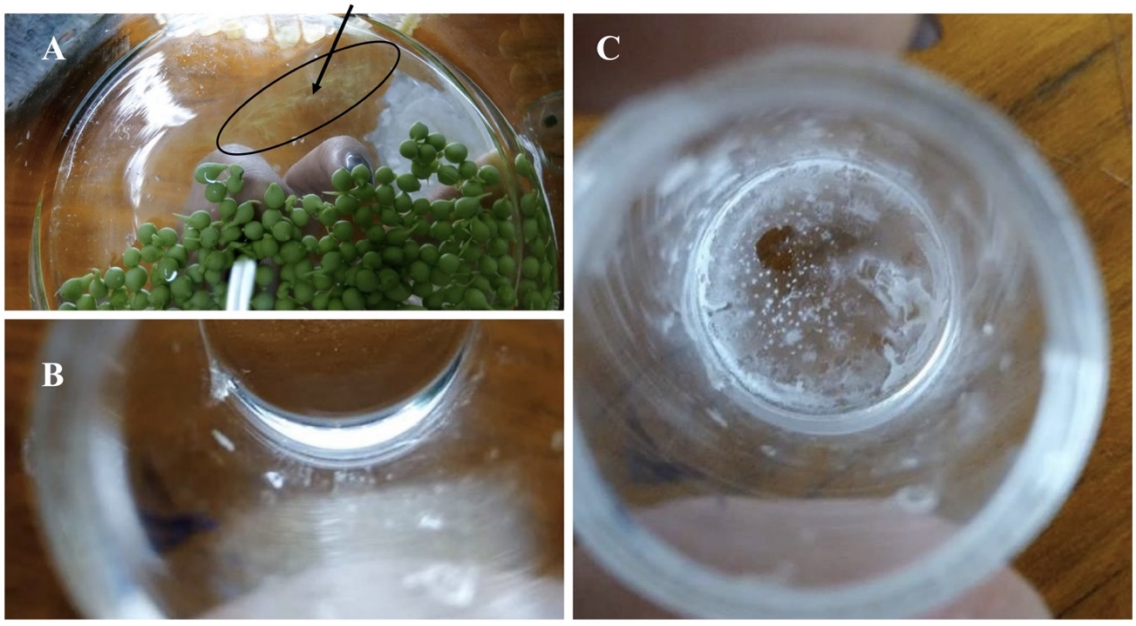


Figure 4.10 *Evaluation of the integrity of the PVA beads. (A) Leakage of cells from the beads, after 7 days of being prepared. (B) Filtrated solution (f/2 medium and addition of pharmaceuticals) of the control group, after the removal of the beads of PVA without cells. (C) Evaporation of the water, a film of PVA settled at the bottom of the glass beaker.*

Figure 4.11 shows the same behaviour with ibuprofen. Immobilised cells removed IBU, from 50.4 to 44.3 µg mL^{-1} after 24 hours of culture ($p=0.002$ and $F>F_{crit}$) and, after 60 hours of culture, IBU concentration apparently increased from 44.3 to 45.8 µg mL^{-1}. The free cells group showed a slight removal of IBU, from 50.4 to 48.4 µg mL^{-1}, after 60 hours of culture.

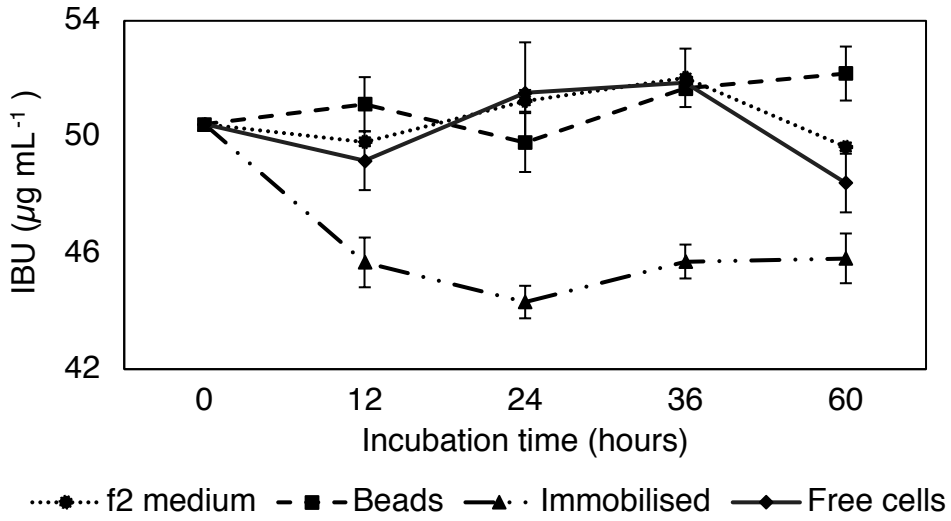


Figure 4.11 *IBU removal by Nannochloropsis sp. in continuous cultures for 60 hours. Bars represent the SD.*

Results of Figure 4.12 display a different scenario. In particular, with the control group, f/2 medium; there was a drastic reduction from 49.0 to 33.1 μg mL^{-1} for the concentration of OLA, after 12 hours. All groups were aerated, and it has previously been reported that this could affect the removal of some pollutants in the growth medium[14]. In the following chapter, Chapter 5, this aspect will be discussed further. The same trend occurred in all groups, a drastic reduction in the first 12 hours, with a more pronounced reduction in the group of the immobilised cells, from 49.0 to 29.9 μg mL^{-1}, and in the free cells group, from 49.0 to 19.8 μg mL^{-1}. Interestingly, after 12 hours of culture, in the f/2 medium, beads and immobilised groups, the concentration of OLA remained unchanged. In the group of free cells, the concentration of OLA reached 12.2 μg mL^{-1}after 60 hours of culture, suggesting more affinity to the molecule of OLA than to PAR and IBU, presumably reflecting the electrostatic interactions and other forces between them and the components of the membrane.

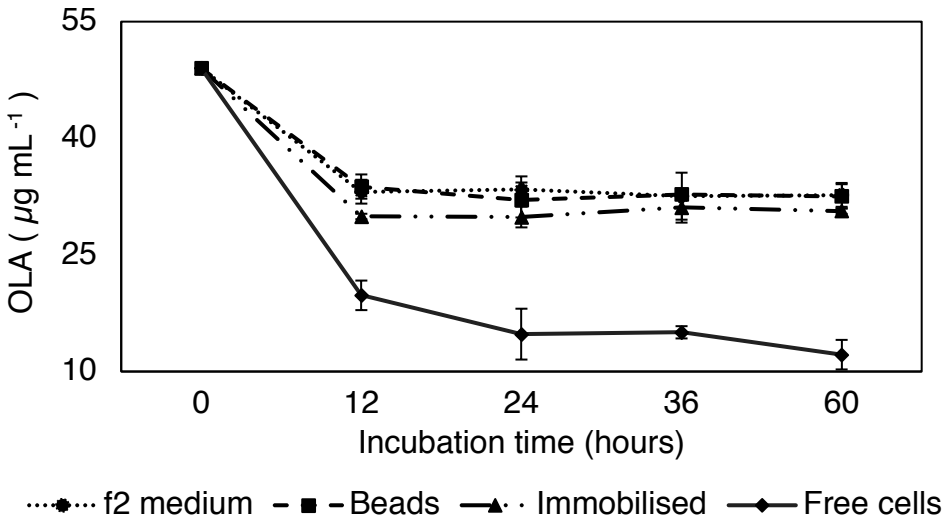


Figure 4.12 *OLA removal by Nannochloropsis sp. in continuous cultures for 60 hours. Bars represent the SD.*

4.4 Conclusions

The microalga *Nannochloropsis* sp. was found to remain alive in the presence of the four pharmaceuticals, and to remove them from water, although with different efficiencies. The immobilisation studies indicated that PVA beads have limited the growth of the cells; however, comparing with the free cells, the immobilised ones showed a higher resilience in the presence of the pharmaceuticals. In what pertains to the integrity of the beads, the experiments revealed the dissolution of the PVA beads. This could cause other environmental issues, such as the release of

PVA to the environment, together with the removed pollutants. Therefore, more research is required to improve the stability and integrity of the beads.

From the results obtained in this study, it became apparent that the microalga *Nannochloropsis* sp. could be considered as a promising specie in the removal of pharmaceuticals from effluents.

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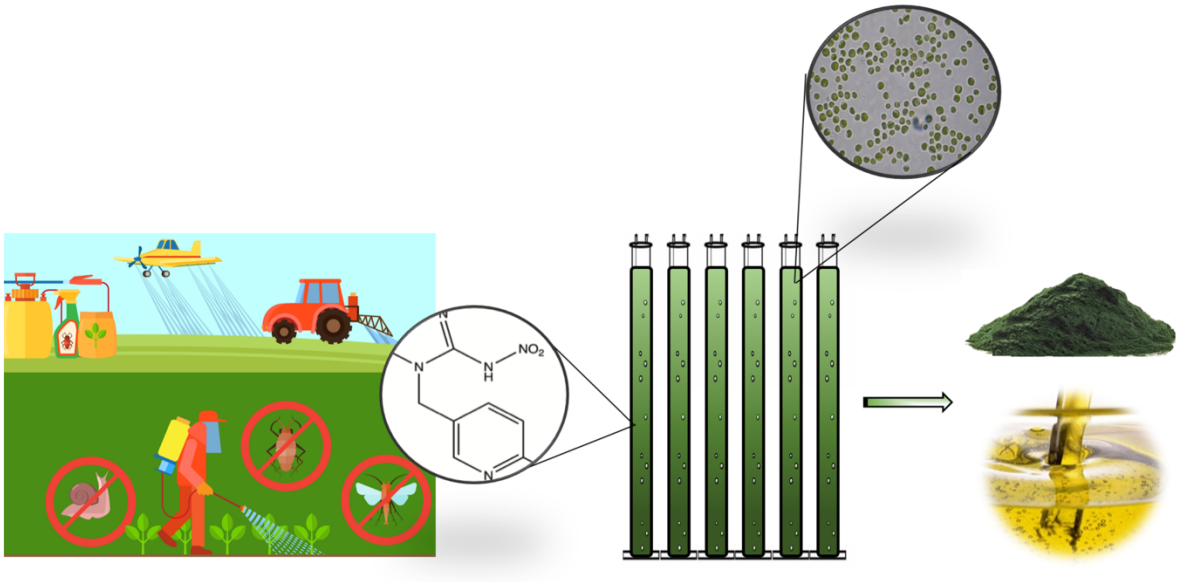
Chapter 5

Microalgae *Nannochloropsis* sp. removal of imidacloprid from water



The large-scale use of pesticides has been one of the major causes of the dramatic disruptions of our environment. Pesticides, such as imidacloprid (IMID), are imposing increasing pressure on hydric resources and ecosystems. This neonicotinoid insecticide has been implicated in bee health decline and in the toxicity to other beneficial insects, and, due to its persistence in the environment and its accumulation in the food chain, poses a threat to human health. Therefore, it is important to test possible environmentally-friendly solutions for their elimination, such as bioremediation.

The present study evaluates the efficiency of the marine microalgae Nannochloropsis sp. *for the removal of the neonicotinoid insecticide imidacloprid from synthetic wastewater. A cell density of 5.5* x *10^7 removed 4.39 µg mL^{-1} from an original content of 9.59 µg mL^{-1} of IMID in the first 20 hours, grown autotrophically. This study showed that the removal of the pesticide by the marine microalgae Nannochloropsis* sp. *is both effective and light dependent.*

The content of the present chapter was fully or parttialy submited in paper form in Encarnação, T., Santos, D., Valente, A., J.C. Pereira, M.G. Campos and Burrows, H.D., Pais, A.C. Removal of imidacloprid from water by the microalgae *Nannochloropsis* sp. and development and validation of a RP-HPLC method for the analysis of imidacloprid

5.1 Introduction

The neonicotinoid insecticide imidacloprid, N-{1-[(6-Chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl}nitramide, is a systemic contact insecticide, that acts on the insect central nervous system. Chemically, neonicotinoids are similar to nicotine, and the name, literally, means "new nicotine-like insecticides". The term "systemic" refers to chemicals that are absorbed by plants and transported to all tissues, resulting in making the whole plant toxic. Thus, systemic neonicotinoids are present in nectar and pollen of the affected plant, representing a risk to the global pollinators' population. It was found that the LD_{50} of imidacloprid was about 2.5 to 5 ng per bee[1,2,3]. Neonicotinoids are the most widely used insecticides in the world[1], and imidacloprid is the most common one. It is often used in agriculture for seed treatment, and to control insect pests in urban and residential areas. Imidacloprid posseses high toxicity, high stability, a high water solubility, is persistent in the environment, it bioaccumulates and could be transported by winds and dust accumulation[4]. It can easily contaminate water resources through streams and shallow groundwater[5], and it has been found in soil, groundwater, wetlands, dust, nontarget plants, vertebrate prey and food[6]. Reports from nine countries studied have found that about 80% of surface waters were contaminated with neonicotinoids[1]. Since neonicotinoids are persistent in the environment, for years[7], the affected organisms and ecosystems are continuously exposed to them. When in sublethal concentrations, chronic toxicity leads to delayed and cumulative lethal effects over time, resulting in significant decrease of populations[1]. Bees, midges, ostracods and mayflies are among the reported affected populations[1]. Thus, the reduction of invertebrate populations may impact populations of birds, fish, bats, frogs and other animals that rely on insects for food. Based on the evidence on the sub-lethal impact of neonicotinoids (clothianidin, thiamethoxam and imidacloprid) on bees, the European Commission, in 2013, implemented the regulation (EU) No 485/2013[8] were three neonicotinoids were severely restricted and prohibited for outdoor use. With the exception of this restricted use in the European Union, the widespread use of imidacloprid and its systemic profile represent environmental and human health threats. Few studies have been conducted to evaluate the effects of the neonicotinoids on human health, even though the existing literature has raised alerts for potential health effects. China is the largest producer of imidacloprid in the world, and the general population are ubiquitously exposed to it. In rural and urban Chinese population, imidacloprid was detected in 95 to100% of urine samples[9,10]. It has been suggested that imidacloprid may be genotoxic[9,11,12], and may cause adverse neurodevelopmental effects such as teratogenicity and autism spectrum disorder[13,14].

Imidacloprid, like all pesticides, is not easily removed from aquatic environments. These compounds reach seas and rivers via municipal wastewater, agricultural effluents, and agricultural industry, such as postharvest treatment of fruits and vegetables and their washing.

Bioremediation, activated carbon adsorption, reverse osmosis, nanofiltration, ozonation and chemical oxidation are some of the technologies which may be used for treatment. The combination of technologies could significantly reduce the occurrence of pesticides and other pollutants in the aquatic environment.

Within the context of bioremediation of imidacloprid, it is important to understand to what extent the microalgae *Nannochloropsis* sp. is able to remove it from water. This study aims to answer that question.

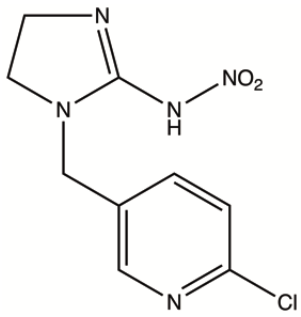


Figure 5.1 *Chemical structure of the insecticide imidacloprid.*

5.2 Materials and methods

5.2.1 Reagents and chemicals

Imidacloprid (IMID) was purchased from Sigma-Aldrich. Microalgae medium f/2 Cell-hi TEViT was obtained from Varicon Aqua Solution, Malvern, UK. All other reagents and solvents were of analytical or HPLC grade.

5.2.2 Growth of *Nannochloropsis* sp. and composition of synthetic wastewater

Microalgae *Nannochloropsis* sp. was purchased from Varicon Aqua Solution (Malvern, UK) and was grown in f/2 medium. Nitrate concentration was 0.30 g L^{-1} and salinity 25 g L^{-1}. The culture was grown autotrophically with an irradiance level of ± 100 µmol photons m^{-2} s^{-1}, with 24:0 photoperiod, temperature of 25 ± 2 °C and pH of 8.

5.2.3 Removal of imidacloprid from water by *Nannochloropsis* sp.

A volume of 100 cm^3 of a *Nannochloropsis* sp. culture were centrifuged at 2000 rpm, then washed, and cells were subsequently transferred to Erlenmeyers flasks, to which 100 cm^3 of f/2 culture medium Cell-hi TEViT (Varicon Aqua Solution, Malvern, UK) were added. To the 100 cm^3 *Nannochloropsis* sp. culture, a concentration of 10 μg mL^{-1} of IMID was added. Similarly, a blank with 10 μg mL^{-1} of IMID was added to 100 cm^3 of culture medium f/2, without cells. The cultures and the f/2 media were aerated by bubbling atmospheric air, at a rate of 300 cm^3 min^{-1}, and grown at 25±2 ºC under light with an irradiance level of ± 100 μmol $m^{-2}s^{-1}$ with 24:0 photoperiod and kept for 7 days. Millipore water were added when needed to ensure the same volume due to water loss by evaporation. Samples of 15 mL were replaced with fresh f/2 culture medium. Each experiment was carried out in triplicate.

5.2.4 Microalgae cell density

The cellular density was determined using the Neubauer chamber (hematocytometer) in an optical microscope. The specific growth rate (μ) was calculated according to the exponential equation[15],

$\mu = (\ln N_1 - \ln N_0)/(t_1 - t_0)$

where N_0 and N_1 are the cell density at times t_1 and t_0, respectively.

5.2.5 Fluorescence microscopy observation

Epifluorescence microscopy images were obtained using an Olympus BX51 M microscope, equipped with a UplanFL N 100x/1.30 oil immersed objective lens (∞/0.17/FN/26.5), a filter set type U-MBF3, U-MWV2 and an UV-mercury lamp (100W Ushio Olympus). Images were digitized on a computer through a video camera (Olympus digital camera DP70) and were analyzed with an image processor (Olympus DP Controller 2.1.1.176, Olympus DP Manager 2.1.1.158). The observations were carried out at a temperature of 25 ± 2 °C.

5.2.6 Instrumentation and RP-HPLC analysis

The RP-HPLC analysis of imidacloprid was carried out using a Dionex Ultimate 3000 system equipped with an auto injector and four variable UV/VIS dual wavelength detectors. The column used for the analysis was a Luna Phenyl-Hexyl, Phenomenex® (Torrance, USA), with 5 μm particle size, 3 mm internal diameter and 150 mm length, supported with a SecurityGuard™ cartridge Phenomenex® (Torrance, USA), with 3.0 mm internal diameter in an oven temperature of 35 ˚C.

The data were recorded using Chromeleon software. Chromatographic analysis was conducted in isocratic mode. The mobile phase consisted of a mixture of acetonitrile and ultrapure water of 50:50 (v/v), at a constant flow rate of 0.8 mL min^{-1}. pH was 7.0 ± 0.2. Preferential UV detector was set at 275 nm for the detection of imidacloprid. The injection volume was 10 µL for standard and samples. A run time of 3 min was found adequate for the analysis of the analyte. Before analysis, every standard and sample was filtered through 0.22 µm filters.

5.2.7 Method validation

The method for IMID quantification in water was validated according to the guidelines of the US Food and Drug Administration (FDA)[16], SANTE/ 11813 2017: Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed[17], and Water quality standards for imidacloprid proposal for an update according to the Water Framework Directive[18], and Eurachem[19], with respect to system suitability, linearity, accuracy, precision, recovery, limits of detection and quantification, selectivity and specificity. The method validation is described in detail in the Chapter 9.

5.3 Results and discussion

The removal efficiency of IMID by *Nannochloropsis* sp. was first accessed with 1 µg mL^{-1} of the insecticide. From analysis of Figure 5.2, it is possible to note that, control and sample, showed a similar behaviour, and that the concentration of IMID decreased by exactly the same amount. No immediate explanation could be provided for these results; however, the aeration, the possible presence of UV radiation and the degradation of IMID were factors considered and hypothesised as most likely to affect the concentration of IMID. After confirmation that no significant UV radiation was present, the experiment was repeated with a higher concentration under dark conditions. The same reduction in the concentration of IMID, both in control and sample, was obtained (Figure 5.3). Since the various reports regarding stability of IMID found in the literature are contradictory[21,22], an analysis using UV/Vis spectrophotometry of IMID in the f/2 culture medium, without cells, was carried out, in order to evaluate the stability. The results showed that IMID remains stable over 12 days, under room temperature and lighting.

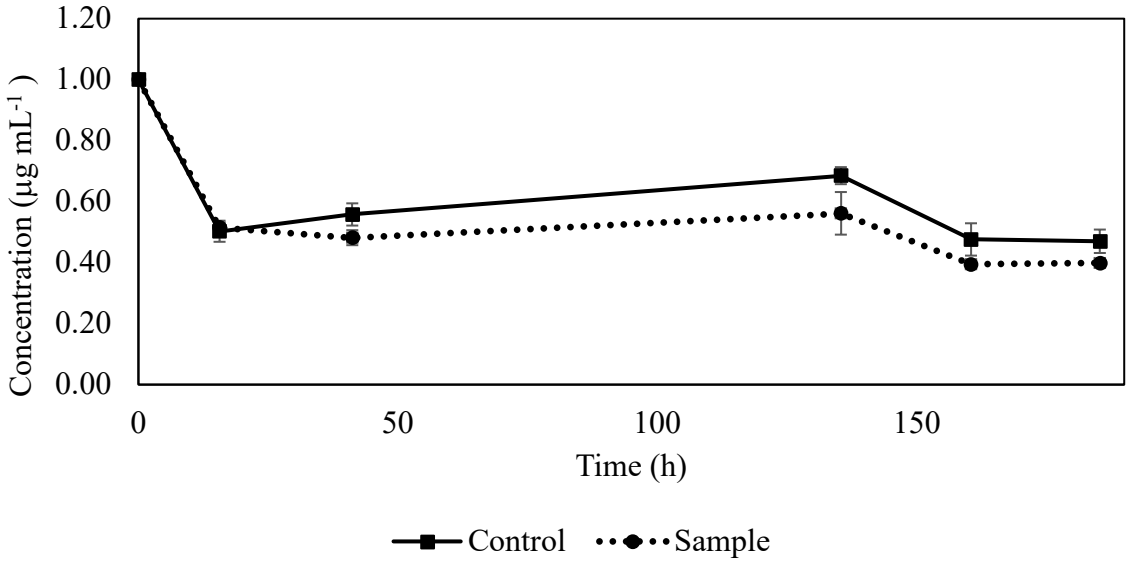


Figure 5.2 *Temporal variation of the imidacloprid concentration present in water during bioremediation process. The cultures and the f/2 media were aerated by bubbling atmospheric air, at a rate of 300 cm^3 min^{-1}, and grown at 25±2 ºC under light with an irradiance level of ± 100 µmol $m^{-2}s^{-1}$ with 24:0 photoperiod and kept for 7 days.The initial concentration was 1 µg mL^{-1}. The error bars represent the standard deviation of the three independent replicates.*

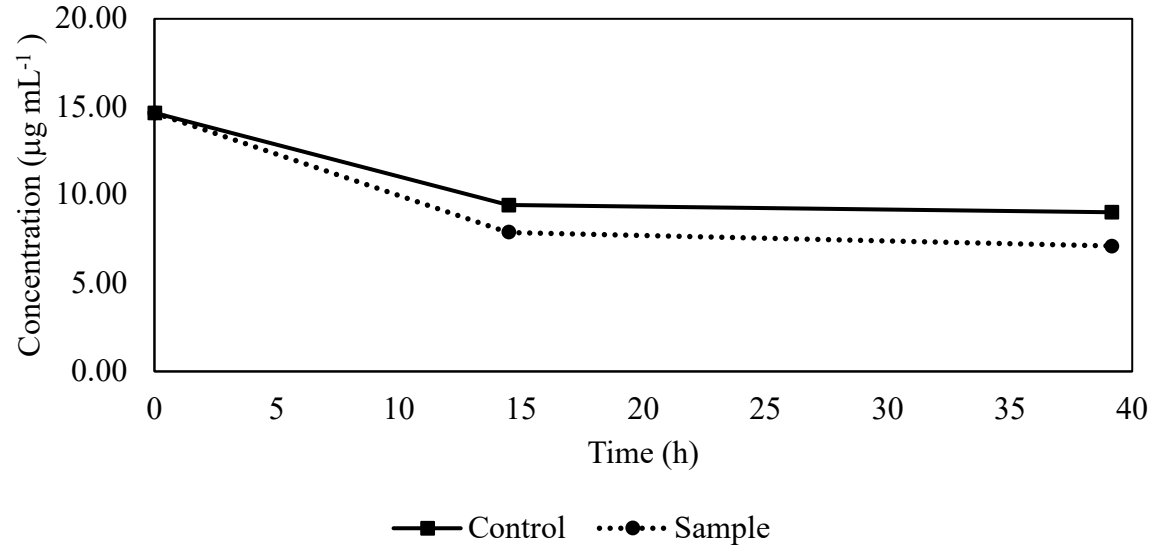


Figure 5.3 *Temporal variation of the imidacloprid concentration present in water during bioremediation process. The cultures and the f/2 media were aerated by bubbling atmospheric air, at a rate of 300 cm^3 min^{-1}, and grown at 25±2 ºC in the dark and kept for 40 hours. The initial concentration was 15 µg mL^{-1}. The error bars represent the standard deviation of the three independent replicates.*

After confirming that the IMID was stable under room temperature and light conditions, the effect of aeration was evaluated, by studying not aerated the cultures (Figure 5.4). The experiment was conducted in the dark. No significant reduction of IMID occurred. Figure 5.5 show the temporal evolution of the IMID concentration under light and no aeration of the cultures. It can be seen that microalgae cells removed 4.39 from 9.59 μg mL^{-1} of IMID in the first 20 hours, grown autotrophically. After the first 48 hours until the 7 days of cultivation, the cells removed only 0.64 μg mL^{-1} of IMID from water. The results show that IMID was not completely removed from water. Also, from Figure 5.6, it can be seen that the culture entered a decline stage, after 48 hours of culture. This decline in both trials could be related with the interruption of the CO_2 supply, since the cultures were not aerated. It has been reported that aeration could affect the removal of pollutants in the growth medium[23]. In experiments where the air pumps were turned on, the aeration promoted the projection of volume, but this projection and the evaporation and replacement of the corresponding volume is not sufficient to explain the reduction of concentration of IMID. The removal of IMID from the water is, to some extent, enhanced by aeration.

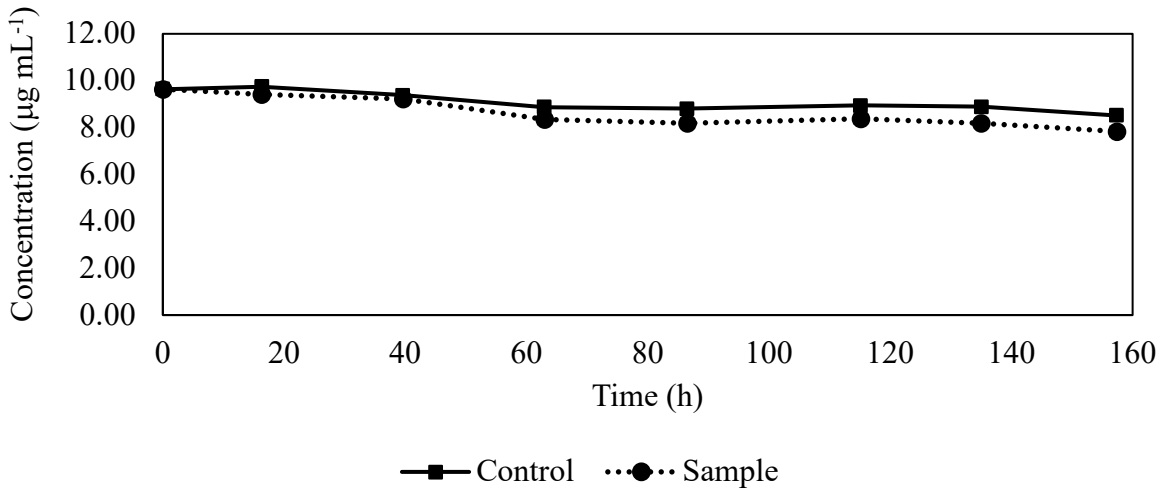


Figure 5.4 *Temporal variation of the imidacloprid concentration present in water during bioremediation process. The initial concentration was 10 μg mL^{-1}. The cultures and the f/2 media were not aerated and were grown at 25±2 ºC in the dark and kept for 7 days. Error bars represent the standard deviation of the three independent replicates.*

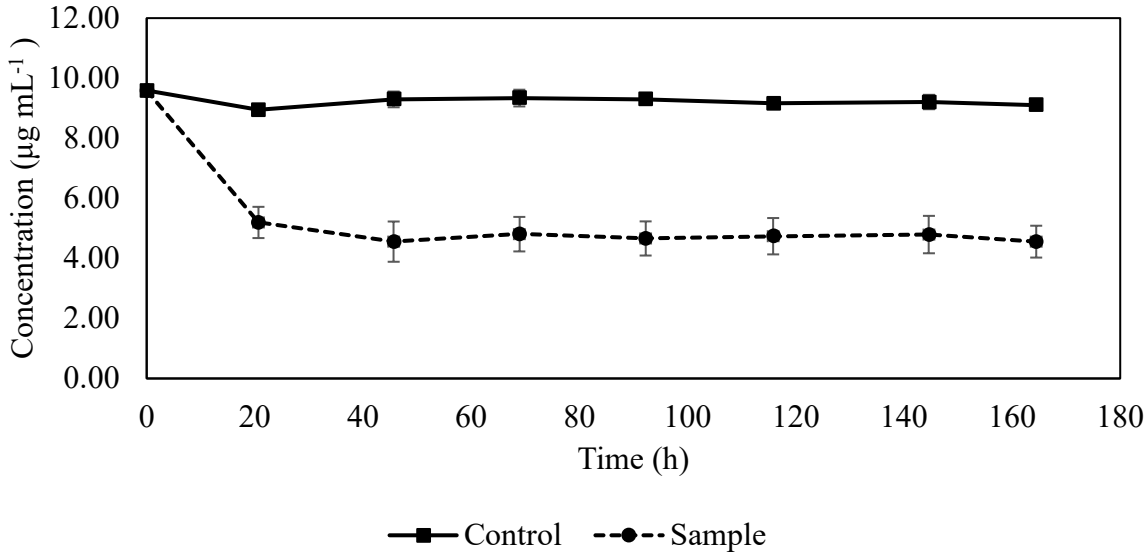


Figure 5.5 *Temporal variation of imidacloprid concentration present in water during bioremediation process. The initial concentration was 10 µg mL^{-1}. The cultures and the f/2 media were not aerated and grown at 25 ± 2 ºC under light with an irradiance level of ± 100 µmol m^{-2}s^{-1} with 24:0 photoperiod and kept for 7 days. Error bars represent the standard deviation of the three independent replicates.*

In the chromatogram, the appearance of additional and unidentified peaks suggests that biotransformation of IMID might occurs. The effect of IMID on the microalgae population was also evaluated. The microalgae cell density is presented in Figure 5.6. Control cultures were carried out to establish the effect of the IMID on microalgae population. The IMID removal experiments has started in the stationary phase of growth, with the high initial cell density of 5.5 x 10^7 cell mL^{-1}. The drastic decrease in population in both, control and sample, could be attributed to cell disruption due to the centrifugation treatment. A decrease in the population (death phase) is observed after 2 days of cultivation. Both control and sample produced negative specific growth rates (Table 5.1).

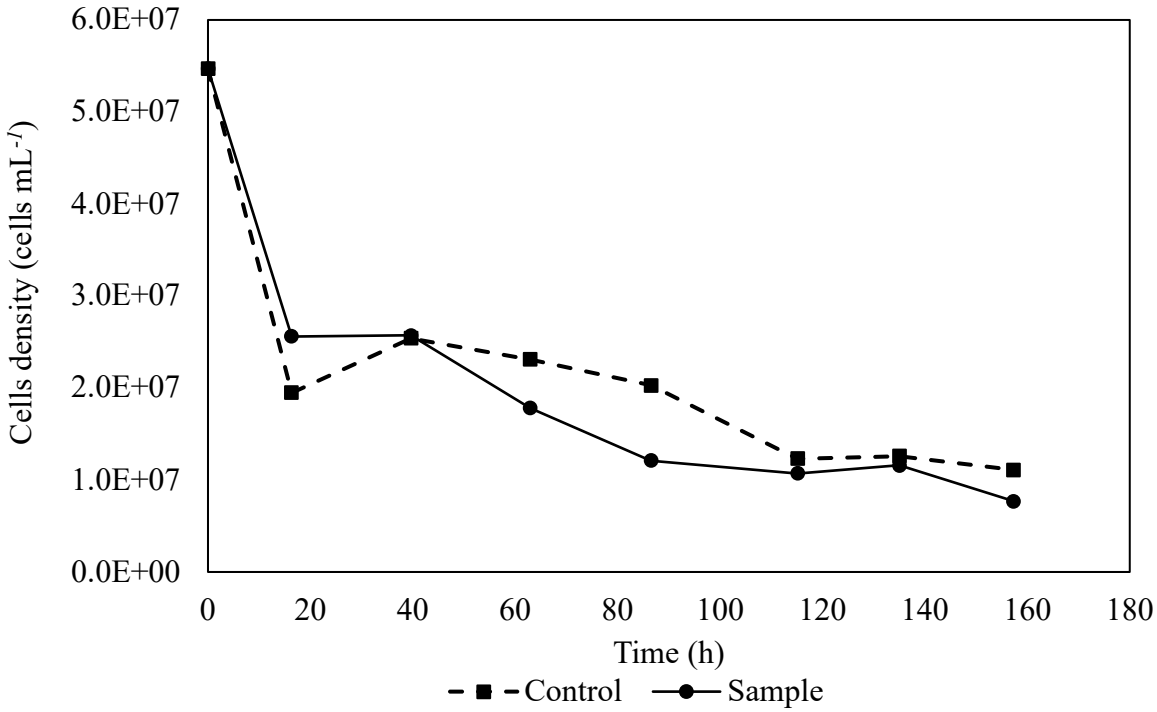


Figure 5.6 *Cell density evolution of cells grown in f/2 medium without IMID (control), and cells grown with 10 µg mL^{-1}of imidacloprid (sample). The cultures and the f/2 media were not aerated and were grown at 25 ± 2 ºC under light with an irradiance level of ± 100 µmol m^{-2}s^{-1} with 24:0 photoperiod and kept for 7 days.*

Table 5.1 *Specific growth rates and removal of IMID from water Nannochloropsis* sp.

Time (hours)	*Specific growth rate (µ)*		IMID removal (%)
	Control	*Sample*	
16	-1.03	-0.76	45.8
40	-0.38	-0.38	52.5
60	-0.29	-0.37	49.9
90	-0.25	-0.38	51.4
115	-0.30	-0.33	50.6
135	-0.24	-0.26	50.0

Epifluorescence images of control cells without any exposure to IMID, and cells incubated with IMID are shown in Figure 5.7. In addition to the presence of dead cells (in blue), bright red fluorescent spots can be observed inside the cells, confirming the cell internalisation of the insecticide, Figures 5.7 B and C. With respect to the size of the chloroplasts, the observed differences between the two images, Figures 5.7 (A) and 5.7 (B), indicated that cells exposed to imidacloprid have their chloroplasts reduced.

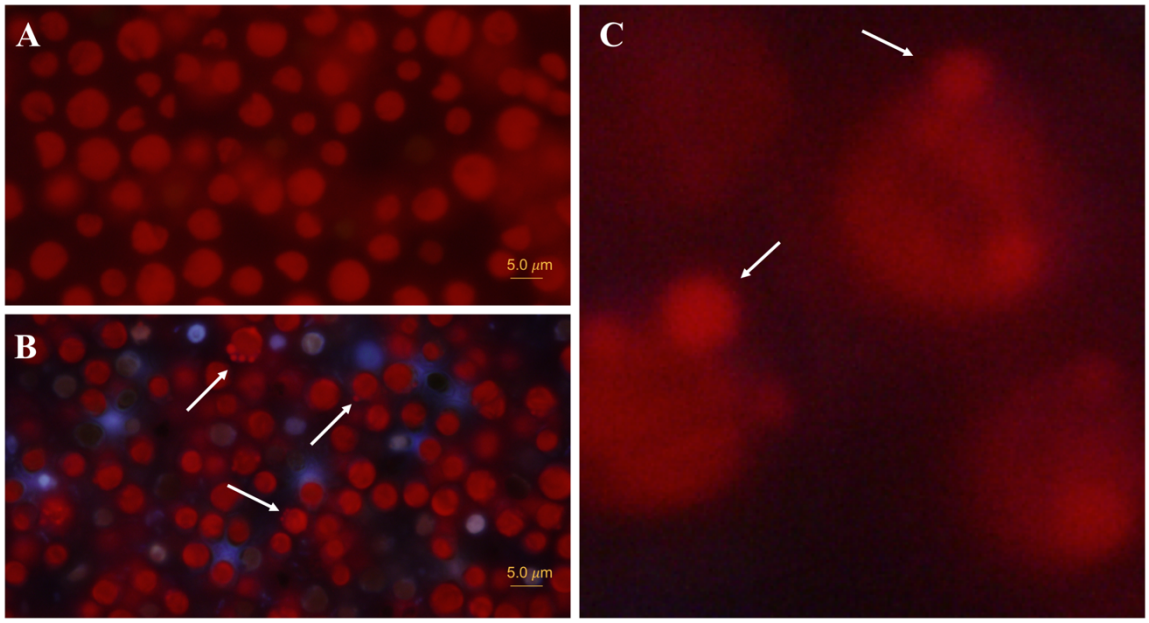


Figure 5.7 *(A) Epifluorescence microscopy images of microalgae cell showing the red autofluorescence of chlorophyll from Nannochloropsis* sp. *chloroplast, (B) Nannochloropsis* sp. *cells grown in medium with 10 µg mL^{-1} of imidacloprid, after 7 days of cultivation, and (C) detail of Nannochloropsis* sp. *cells showing specific staining (red) associated to the cellular internalisation of the imidacloprid.*

Chloroplasts are structural and functional organelles, whose structure changes when the cell is exposed to environmental stress, natural or anthropogenic factors. It has been reported that IMID affects the photosynthesis of the treated plant and may lead to the disruption of chloroplasts[20].

5.4 Conclusions

The main conclusion that can be drawn from the results of the present study is that *Nannochloropsis* sp. microalgae remove, to some extent, imidacloprid from water very rapidly, attaining ca. 50% in the first 20 hours. Another conclusion is that they are efficiently, when a source of light is present, while aeration is another factor that might contribute to the removal. Several factors can influence the removal efficiency, but this is a vastly and unexplored field, calling for more research. However, this study and the one from the previous Chapter, also allow to infer on the impact of the propagation of anthropogenic pollutants to higher trophic organisms and, eventually, to humans, due to the biomagnification of pollutants within the food chains. Microalgae are natural filters, and, in their natural medium, they clean and absorb the nutrients and wastes of other forms of life, and they are the feed to other organisms. They are at the bottom of the food chains in a circular life system. In this anthropocene era, humans are contaminating the planet with their waste, and these organisms absorb the anthropogenic pollutants, and they are the feed for the

fish that humans consume. Through the food-webs biomagnification of pollutants, entire ecosystems and all forms of life are exposed to such pollutants that can, potentially, produce harmful effects on living organisms.

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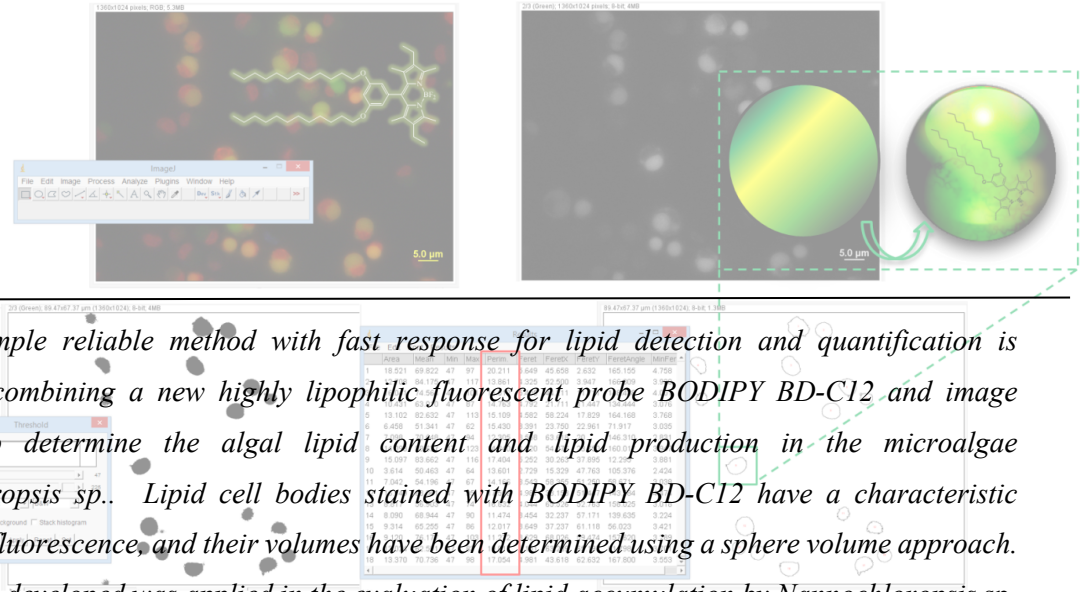
Part C

Products and applications

Chapter 6

Monitoring oil production for biobased feedstock with the BODIPY BD C-12



A simple reliable method with fast response for lipid detection and quantification is proposed, combining a new highly lipophilic fluorescent probe BODIPY BD-C12 and image analysis to determine the algal lipid content and lipid production in the microalgae Nannochloropsis sp.. Lipid cell bodies stained with BODIPY BD-C12 have a characteristic multicolor fluorescence, and their volumes have been determined using a sphere volume approach. The method developed was applied in the evaluation of lipid accumulation by Nannochloropsis sp. *under different cultivation conditions (varying nitrate and salinity concentrations and the combined effect of these two variables). The results show an increase of lipid content in Nannochloropsis* sp. *cultivated in nitrogen replete and depleted conditions, from 9.4 to 40.8 μm^3 cell^{-1} and 35.5 to 73.5%, respectively. The findings are compared with conventional methods for determination of neutral lipids, and with results obtained from the dyes Nile Red and BODIPY 505/515. A reasonable agreement between neutral lipid production measured by BODIPY BD-C12 and gravimetric methods (correlation coefficient of 0.98) was obtained. The neutral lipids production decreased from 964.6 to 244.8 mg L^{-1} and from 809.1 to 396.7 mg L^{-1}, as the nitrate concentration increased from 0 to 0.3 g L^{-1}. It is observed that, with the two commercially available dyes, lipid quantification using Nile Red lead to an overestimation of lipid content, while the use of BODIPY 505/515 led to unreliable results due to rapid bleaching of the chromophore.*

The method proposed shows excellent potential to become a standard, yet advanced, strategy for rapid evaluation and quantification of intracellular lipids in microalgae, a crucial step of the scaling-up process involved in the production of biobased products.

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6.1 Introduction

The limited availability of fossil fuels, and the environmental impact resulting from their respective use have been the subject of massive attention in the last decades, stressing the urgency required to find renewable and efficient alternatives [1,2]. One such alternative consists of producing biofuels from microalgal biomass [2-5]. The potential of this technology has been recognized and explored to preclude issues of agricultural land exploration and food supply issues present with other biofuels[2].

Microalgae are an attractive and renewable energy source, providing a sustainable alternative for lipid production, with reduced land and water requirements[6]. These neutral lipid collectors have growth rates higher than traditional crops, with a complete life cycle of only a few days, and can produce more than 50% of their own weight in oil content, with an estimated biodiesel production of ca. 125 tonnes/ha/year[2,7]. However, the optimization and full control of the steps of biodiesel production from microalgae still face many challenges[8], and are expected to require some long-term efforts before full commercialization. These are due in part to the high cost of production, the selection and maintenance of algal strains in the cultivation systems, the productivity of biomass with a high content of lipids, and the downstream processes, such as harvesting, drying and lipid extraction. Isolation and screening of intracellular lipid content, with optimization of staining conditions and rapid quantification, are crucial tasks that have been addressed and discussed in the literature[8,9,10,11,12]. Major challenges, among those previously indicated, are selecting lipid-rich strains[2] and efficient probes for lipids in the cells. The high lipid content in *Nannochloropsis* sp.[2,13,14,15] is a key factor that supports the suitability of this alga as an alternative species for production of microalgal oil and renewable biodiesel. *Nannochloropsis* is a marine eustigmatophyte, widely used in aquacultures for the initial steps of an artificial food chain, and its other attractive features, including resistance to environmental stress and halotolerance, have been explored for cultivation in fresh waters[2,13,14,15]. All these characteristics have motivated the selection of *Nannochloropsis* sp. as the strain selected for the present study.

Intracellular lipid bodies can be defined as dynamic organelles, encircled by a monolayer of amphipathic phospholipids, glycolipids and sterols[17]. Neutral lipids are contained in these lipid bodies, primarily triacylglycerols (TAG), diacylglycerols and sterol esters. In microalgae, the accumulation of lipid bodies is enhanced in response to environmental stresses, such as nitrogen depletion and osmotic stress[17,2].

Fluorescent lipid probes, such as Nile Red and BODIPY 505/515[2,18-22] have been extensively used to quantify lipids in microalgae species, displaying different labeling and permeation abilities, specificity, conservation conditions and photostability. The fluorescent staining ability for lipids provides a rapid and inexpensive tool to measure neutral lipid content, overcoming several issues

related to other conventional gravimetric methods[2,19]. However, in spite of some concerns associated with the gravimetric determination of lipids, it is still a reference method, indispensable for comparison purpose[2]. As such, there is substantial information on the correlation between fluorescence and gravimetric data.

Several studies have listed the difficulties occurring when using Nile Red with microalgae, and these include limitation of labeling due to ineffective permeation in cells with thick and rigid walls, impaired uptake induced by the cell wall composition and structure, fluorescence quenching, low photostability and interference with chlorophyll[2,23]. BODIPY dyes have shown improved photostability and effectiveness in staining microalgae[2]. The enhanced selectivity of BODIPY 505/515 to stain lipid droplets in live algal cells was first reported by Cooper and co-workers[9]. Since then, BODIPY dyes have been used in similar studies[2,24-28] but much less frequently compared to Nile Red dyes[2]. The improved ability of BODIPY 505/515 for permeation has been recognized for a variety of microalgae species, such as *Ophiocytiummaius Naegeli*, *Chrysochromulina* sp. *Mallomonas splendens*, *Chlorella vulgaris*, *Dunaliella primolecta* and *Chaetoceros calcitrans*. This probe penetrates the microalgae cells, even those with a thick and rigid cell wall. BODIPY 505/515 has a higher logarithmic octanol/water partition coefficient (logP) of ca. 4.3, compared to Nile Red and other commercially available BODIPY probes, which allows the crossing of cell and organelle membranes. However, a recent study has highlighted the low stain permeation rate across the thick and rigid cell wall of *Nannochloropsis oculata* cells, displaying low fluorescence and differentiation between fully and partially labeled cells. The new dye used in this work, 2,6-diethyl-4,4-difluoro-1,3,5,7-tetramethyl-8-[3,5(bis-dodecyloxy)phenyl]-4-bora-(3a,4a)-diaza-s-indacene (BODIPY BD-C12), possesses long alkyl chains attached to the BODIPY core (Fig.1), which confer an increased lipophilicity (with an estimated logP in excess of 10) in comparison to other lipid dyes, such as BODIPY 505/515, leading to an improved staining ability. This proposed amphiphilic fluorescent dye (Figure 6.1) was synthesized and fully characterized by some of the authors[29] and possesses good photochemical stability, high molar extinction coefficients and high fluorescent quantum yields.

Additionally, it promotes a more efficient staining, in contrast to the other dyes previously employed for this purpose. Lipid cell bodies stained with BODIPY BD-C12 have a characteristic multicolor fluorescence, suggesting some specificity towards different lipid types that should be further exploited.

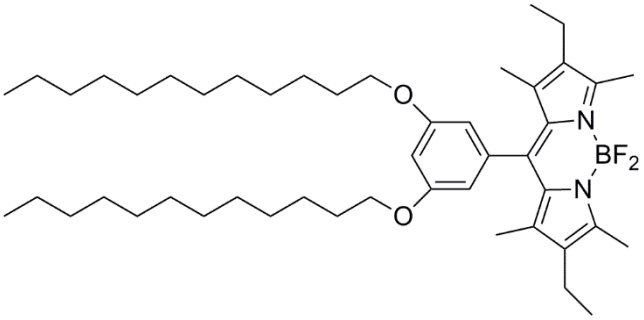


Figure 6.1 *Chemical structure of the synthesised 2,6-diethyl-4,4-difluoro-1,3,5,7-tetramethyl-8-[3,5(bis-dodecyloxy)phenyl]-4-bora-(3a,4a)-diaza-s-indacene (BODIPY BD-C12).*

To the best of the authors' knowledge, fluorescence methods used for determination of microalgae lipid content have not resorted to image processing. This provides an attractive tool that can be used with microalgae cells, and a new addition to the repertoire of options available, in both R&D and routine procedures, for rapid lipid quantification.

6.2 Materials and Methods

6.2.1 Organism and culture conditions

Nannochloropsis sp. was obtained from Varicon Aqua Solution, Malvern, UK. The culture is supplied as disks containing ca. 3 billion cells. One disk was cultivated for 6 days in 2 L f/2 medium. 300 ml of a *Nannochloropsis* sp. culture were filtered and washed. The cells with a concentration of $2.5x10^7$ cell ml^{-1} were subsequently transferred to a sterilized closed photobioreactor, to which were added 300 ml of culture medium Cell-hi TEViT (Varicon Aqua Solution, Malvern, UK), based upon the f/2 medium deprived of nitrates. Nitrate levels were set at 0, 0.15 and 0.30 g L^{-1} and salinity 0, 20 and 40 g L^{-1}. Table 6.1 depicts the coding used for nitrates and salinity content.

The cultures were aerated by bubbling atmospheric air, at a rate of 300 cm^3 min^{-1}, were grown at 15±2 ºC under light with an irradiance level of ± 100 μmol photons $m^{-2}s^{-1}$ with 24:0 photoperiod and kept for 13 days. The culture media was previously sterilized by microwave irradiation. Each experiment was carried out in triplicate.

Table 6.1 *Notation for nitrates and salinity content of culture media (g L^{-1}).*

	N1S1	N1S2	N1S3	N2S1	N2S2	N2S3	N3S1	N3S2	N3S3
Nitrates	0	0	0	0.15	0.15	0.15	0.30	0.30	0.30
Salinity	0	20	40	0	20	40	0	20	40

6.2.2 Staining of algal cells using BODIPY BD-C12, Nile Red and BODIPY 505/515

BODIPY BD-C12 was prepared in 50:50 (v:v) ethanol:DMSO to give a stock solution of 2.96 x 10^{-4} M and stored in the dark. 2 mL samples of algal cells were stained with 2.5, 5, 10, 15 and 20 μL of the stock solution. After incubation in the dark at room temperature for 20 min, the 2 mL of stained cells were filtered through Nylon 66 membrane filters with a pore diameter of 0.2 μm, washed with ultrapure water to discard the fluorescence in the culture medium and resuspended in 2 mL of ultrapure water.

A similar procedure was used for both the Nile Red and BODIPY 505/515 dyes.

6.2.3 Spectrofluorometric determinations

Fluorescence spectra were recorded on a Horiba-Jobin-Yvon Spex Fluorolog 3-2.2 spectrophotometer. Emission spectra were measured from BODIPY BD-C12, *Nannochloropsis* sp., and *Nannochloropsis* sp. cells stained with BODIPY BD-C12, Nile Red and BODIPY 505/515. The excitation wavelengths used for BODIPY BD-C12, Nile Red and BODIPY 505/515 were 524, 480 and 500 nm, respectively.

6.2.4 Fluorescence microscopy observation and lipid determination with BD-C12

Immediately after staining, fluorescence images of lipid bodies were obtained using an Olympus BX51 M microscope, equipped with a UplanFL N 100x/1.30 oil immersed objective lens (∞/0.17/FN/26.5), a filter set type U-MBF3, U-MWV2 and an UV-mercury lamp (100W Ushio Olympus). Images were digitized on a computer through a video camera (Olympus digital camera DP70) and were analyzed with an image processor (Olympus DP Controller 2.1.1.176, Olympus DP Manager 2.1.1.158). The observations were carried out at room temperature (≈25ºC).

6.2.5 Image processing and analysis

The analysis of fluorescence images and the volume determination of lipid bodies were performed resorting to the public domain software, ImageJ (version 1.46, available at http://rsb.info.nih.gov/ij/). ImageJ is an open source software for image processing and analysis, designed for scientific multidimensional images. Based on the assumption that lipid bodies in

stained microalgal cells are perfect spheres[11], the contours and the respective area and volume per cell were determined. The procedure used consists of six main steps:

(i) acquisition of fluorescence microscopy images of the cells;
(ii) calibration of the scale by converting pixels to physical dimensions, drawing a straight line over the scale bar of the microscope image;
(iii) RGB decomposition and green channel selection;
(iv) processing using the appropriate thresholds for defining the contours of the fluorescent lipid bodies;
(v) measurement of the area of these bodies, using the particle analyzer tool;
(vi) calculation of volume using the value of the area, calculation of the radius of the corresponding circle and use of this radius for the calculation of the volume of a sphere.

Note that lipid droplets are individualized and do not substantially deviate from a spherical shape, which validates this spherical approximation. For other shapes, different approximations have been used[31].

The output of each particle analysis corresponds to the total area of the lipid body per cell. At least 100 cells per replica were assessed.

6.2.6 Gravimetric determination of neutral lipids and comparison with the BD-C12 method

After cultivation, the biomass was decanted and filtered through Nylon 66 membrane filters with a pore diameter of 0.2 μm, and washed with a solution of ammonium bicarbonate, followed by ultrapure water, using a method adapted from Zhu and Lee[30] to remove remnants of the culture medium. The biomass was then frozen at -20 °C in test tubes and dried in a Labconco, Freeze Dry System/Freezone 4.5 apparatus. Total lipids were extracted according to the method of Bligh and Dyer in chloroform:methanol:water (1:2:0.8 and 2:2:1.8 v/v). Briefly, 20 mg of lyophilized microalgae biomass were mixed with 2 mL of chloroform, 4 mL of methanol and 1.6 ml of water. The mixture was homogenized in a vortex for 2 minutes, and then another 2 ml of chloroform and 2 ml of 5% NaCl solution were added. The addition of NaCl solution is to enhance the equilibrium of lipids through the chloroform phase. After vortexing for 2 min, the homogenate was sonicated in an ice bath for 10 min and centrifuged at 1200 g for 5 min. The chloroform layer was collected, after which 1 mL chloroform was added to the remaining portion. This extraction procedure was repeated three times. The lipid extracts were combined, and the chloroform was evaporated under a nitrogen gas stream. The weight of the crude lipids extracts was measured using an electronic balance. Samples were stored at -20 ºC under a nitrogen gas atmosphere until further analysis. The

conventional gravimetric method was compared with the BODIPY BD-C12 method. For that, 100 replicates of stained cells were used, while for the gravimetric determination 3 replicates were considered. A correlation curve was obtained between the gravimetric and the BODIPY BD-C12 method. The microalgal oil densities can be explained by differences in fatty acid composition of the samples, which in turn is affected by culture conditions, such as nitrates and salinity. Assuming that an algal oil density could range from 0.857 to 0.96 g cm^{-3}, as reported in references[32,33], a density value equal to 0.857 g cm^{-3} was used in the calculations.

6.2.7 Statistical analysis

Statistical analysis was performed using Microsoft Excel® (Microsoft Corp., Redmond, WA). Results are given as mean ± SEM (n=100 and n=3 BODIPY BD-C12 and gravimetry replicate assays respectively). Two factor ANOVA with replications analysis was conducted to establish the statistical significance within each treatment.

6.2 Results

6.3.1 Spectrofluorometric characteristics of BODIPY BD-C12 stained cells, fluorescence microscopy observation and lipid quantification with ImageJ

BODIPY BD-C12 fluorescence intensity was measured at 538~541 nm in *Nannochloropsis* sp. cells using a spectrofluorometer with excitation at 524 nm. The fluorescence spectra of BODIPY BD-C12 and of unstained cells exhibit no fluorescence emission in this wavelength range. In the red region, the spectrum of *Nannochloropsis* sp. shows the typical fluorescence peak of chlorophyll at 680 nm (Figure 6.2 A). As seen from the spectra of the three dyes, Figure 6.2 A, the maximum peak wavelengths of BODIPY BD-C12, Nile Red and BODIPY 505/515, emission is located at 558, 644 and 512 nm, respectively.

The addition of BODIPY BD-C12 to microalgae cells produces a very strong fluorescence. The dye permeates the cell membrane and accumulates in intracellular lipid vesicles. Figures 6.2 A and 6.2 C show very bright fluorescence emissions of lipid bodies, which differ distinctly from the red autofluorescence of chlorophyll from chloroplasts, Figure 6.2B.

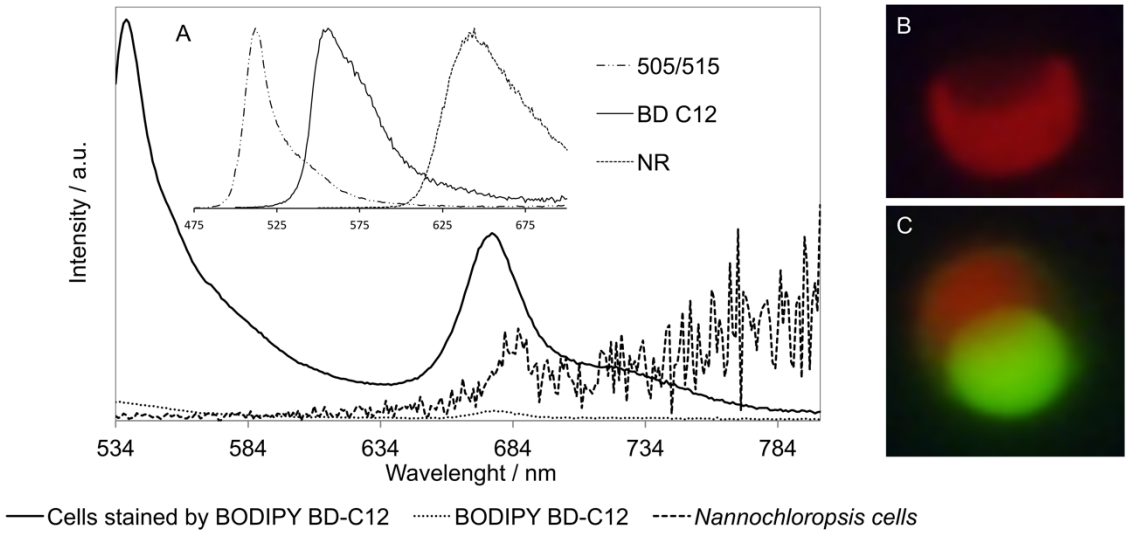


Figure 6.2 *(A) Emission spectra of BODIPY BD-C12, BODIPY 505/515, Nile Red, Nannochloropsis sp. cells, and Nannochloropsis sp. cells stained with BODIPY BD-C12. (B) Epifluorescence microscopy images of microalgae cell showing the red autofluorescence of chlorophyll from Nannochloropsis sp. chloroplast and (C) Nannochloropsis sp. cells stained with BODIPY BD-C12.*

Different concentrations of BODIPY BD-C12 were tested, showing that above 5 µl of the dye there is no variation of the fluorescent intensity, as can be seen in Figure 6.3. The effect of the staining time was also investigated in order to determine the photobleaching and the fading characteristics of the dye. BODIPY BD-C12 showed resistance to fading and gave a continuous fluorescence emission during 60 min after staining.

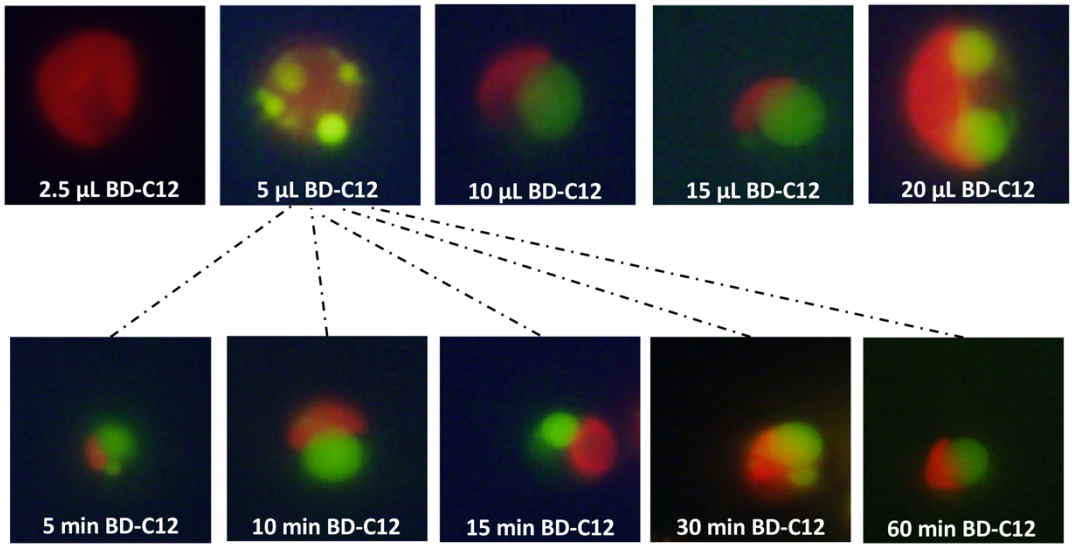


Figure 6.3 *Epifluorescence microscopy images of Nannochloropsis* sp. *cells stained with different concentrations of BODIPY BD-C12, showing the effect of the staining time, in different cells.*

A preliminary study was performed to evaluate the practical aspects of staining the microalgae cells with BD C12 in routinely basis (Figures 6.4 and 6.5). The method was revealed to be practical under the established conditions and could be used in the fast determination of lipid accumulation over time.

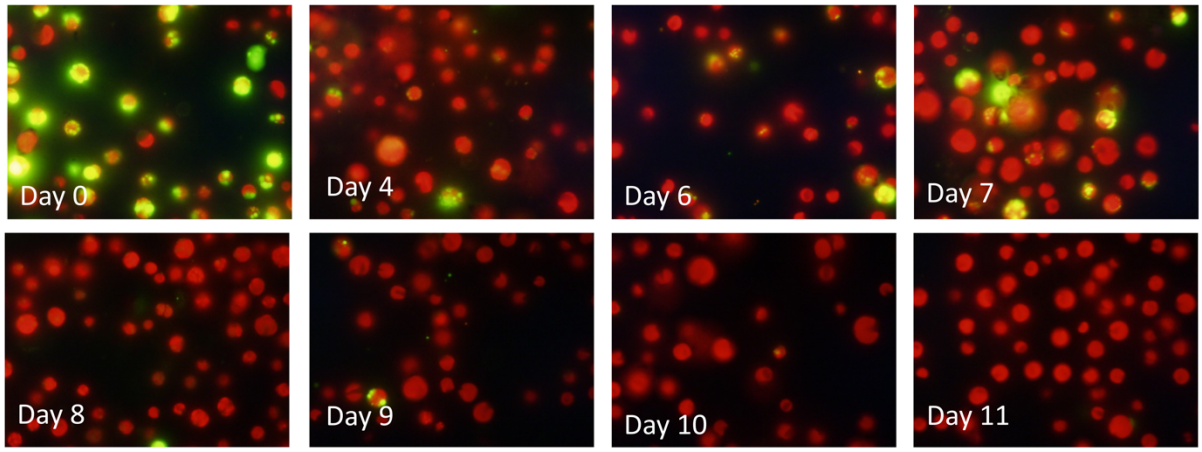


Figure 6.4 *Fluorescence images of Nannochloropsis* sp. *cells stained with BD C12 (BD - C12 stock solution of 2.96 x 10^{-4}M). The green fluorescence of stained lipid bodies in the cell decreases after the addition of NaNO3.Cells cultivated under f/2 medium, 25 g/L NaCl, 0.15g/L NaNO3, 25±2ºC, 100 µmol.m-2s-1, photoperiod 24:0 during 11 days. 10% of the culture were harvest every day and replaced with 10% of fresh medium daily.*

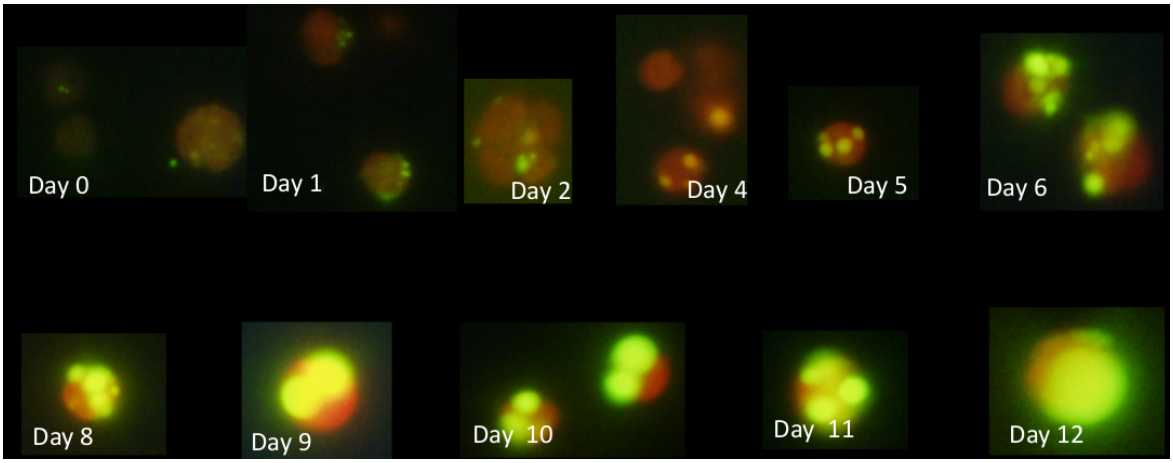


Figure 6.5 *Fluorescence images of Nannochloropsis* sp. *cells stained with BD C12 (BD - C12 stock solution of 2.96x10^{-4}M). The green fluorescence of stained lipid bodies in the cell increases with time. Cells cultivated under f/2 medium, 25 g/L NaCl, 0.15g/L $NaNO_3$, 25±2 ºC, 100 µmol.m^{-2}s^{-1}, photoperiod 24:0 during 12 days. Dry weight biomass 0.45g/L.*

The lipid content of *Nannochloropsis* sp., determined independently by the BODIPY BD-C12 and conventional gravimetric methods, demonstrate that they produce similar results, as can be seen in Figure 6.6A. An increase of nitrate concentration in the growth medium from 0 to 0.3 g L^{-1}, produces a decrease in the neutral lipids from 964.6 to 244.8 mg L^{-1} for BODIPY BD-C12, and from 809.1 to 396.7 mg L^{-1} in case of the gravimetric method. The correlation analysis, Figure 6.6 B, indicates no significant difference, and a positive correlation coefficient of R^2= 0.98 was obtained between the two methods.

In addition, in Figure 6.6 A, it can be seen that the difference between these two methods in culture with depleted nitrogen (N=0 g L^{-1}) is higher than in the culture with medium nitrogen (N=0.15 g L^{-1}). Correlation coefficients of 0.98, 0.80 and 0.93 are obtained for methods with BODIPY BD-C12, BODIPY 505/515 and Nile Red, respectively, relative to the gravimetric approach.

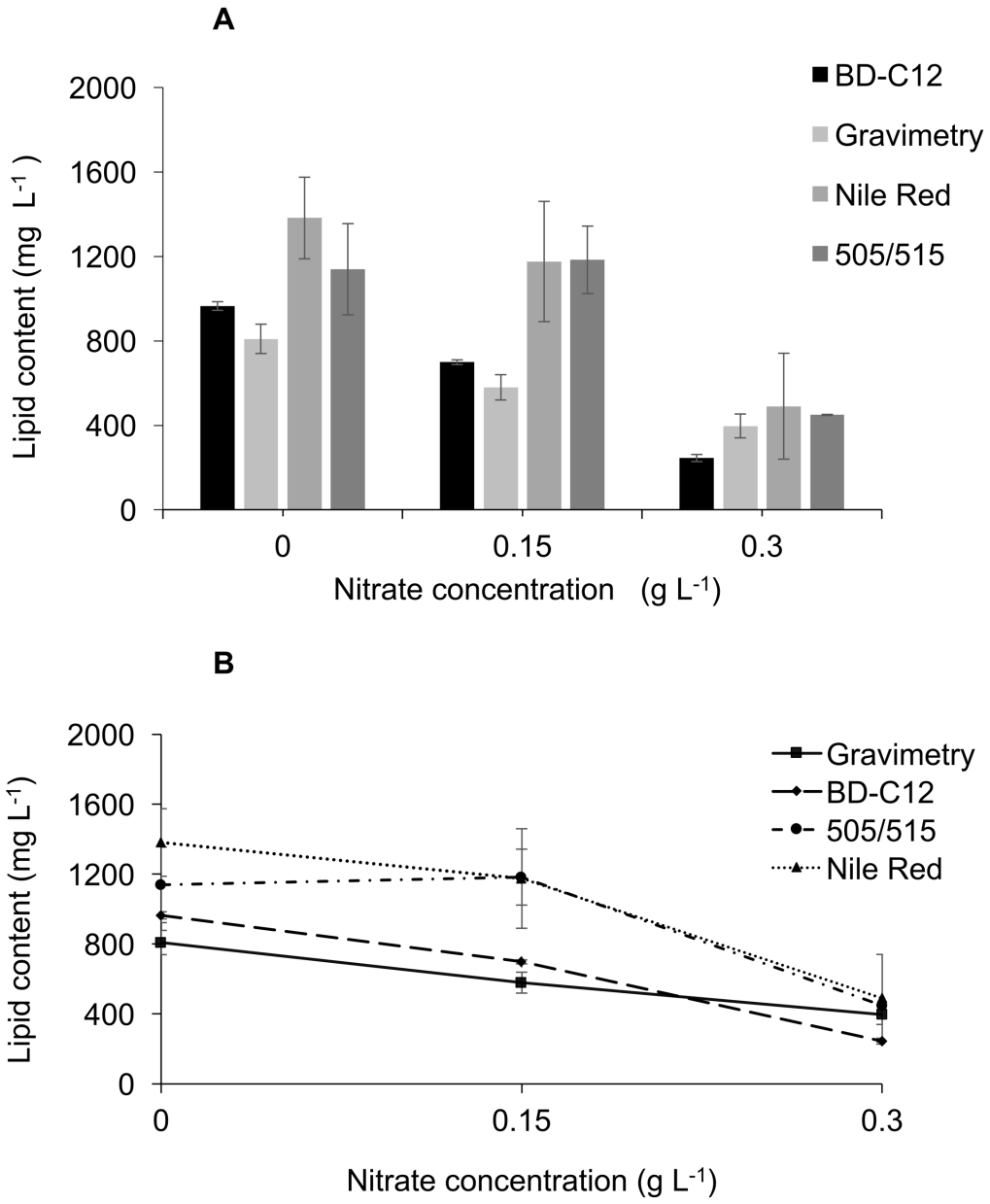


Figure 6.6 *Comparison of lipid content determined by BODIPY BD-C12, BODIPY 505/515 and Nile Red methods (in µm^3 cell^{-1}) and the conventional gravimetric method (in % of dry weight) ($p<0.05$). Data are given as mean ± SEM (n=100 and n=3 BODIPY BD-C12 and gravimetry replicate assays respectively) (A). Lipid content of Nannochloropsis* sp. *determined by gravimetric and BODIPY BD-C12, BODIPY 505/515 and Nile Red methods under different nitrogen concentrations. (B) Linear representation of the above data.*

From the epifluorescence imaging, it can be seen that the fluorescence emission of the new probe was still stable for a considerable time (see video in supplementary material in reference Encarnação et al.[41]). In contrast, with imaging results using BODIPY 505/515, there was rapid photobleaching of the chromophore by the mercury lamp, which led to a decrease in the mean fluorescence (see video in supplementary material in reference Encarnação et al.[41]).

In the fluorescence microscope images of *Nannochloropsis* sp. cells stained with Nile Red (Figure 6.7), some disadvantages of Nile Red staining, such as blurring, staining of entire cell and undefined shapes are observed.

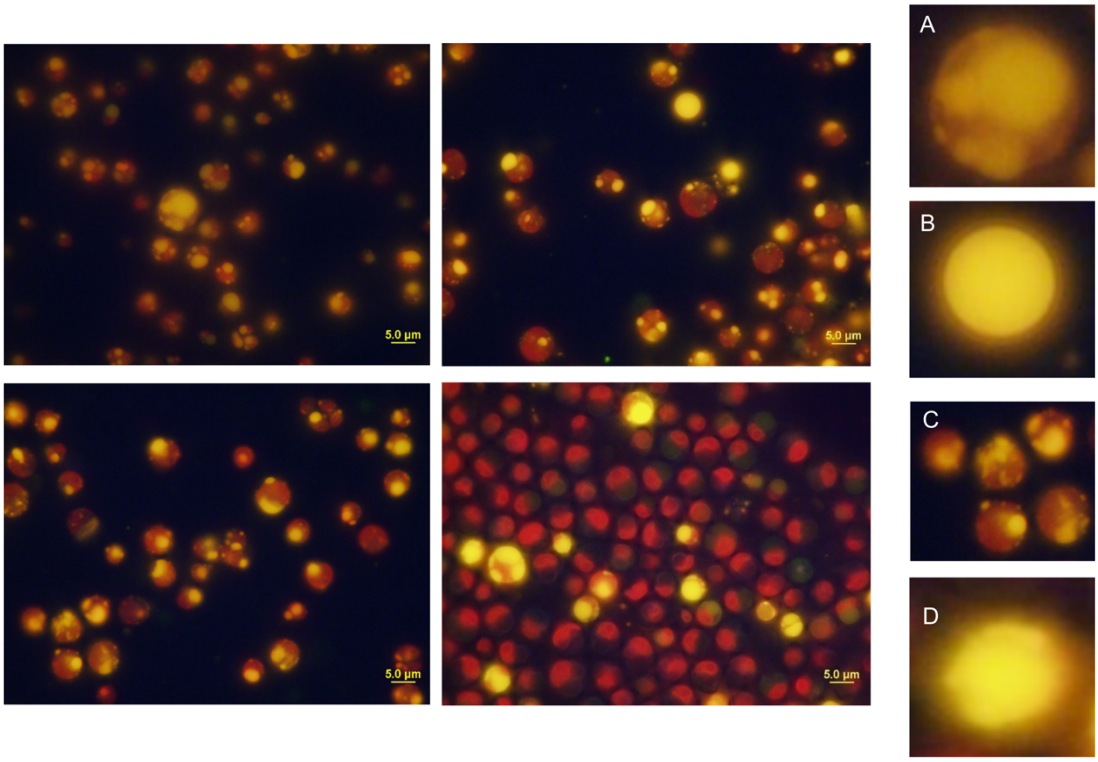


Figure 6.7 *Epifluorescence microscopy images of Nannochloropsis* sp. *cells stained with Nile Red showing some drawbacks of staining with this dye, such as blurring (B,D), staining of the entire cell (B) and undefined shape (A,C).*

6.3.2 Effect of nitrogen and salinity on lipid accumulation

The effect of nitrogen concentration on lipid accumulation was also investigated using the BODIPY BD-C12 method. Different levels of nitrate were used in the cultures after a good cell density was achieved. Figures 6.8 and 6.9 show lipid distribution and accumulation over time in replete and depleted media. These figures also show that the lipid droplets are, in general, approximately spherical and well defined.

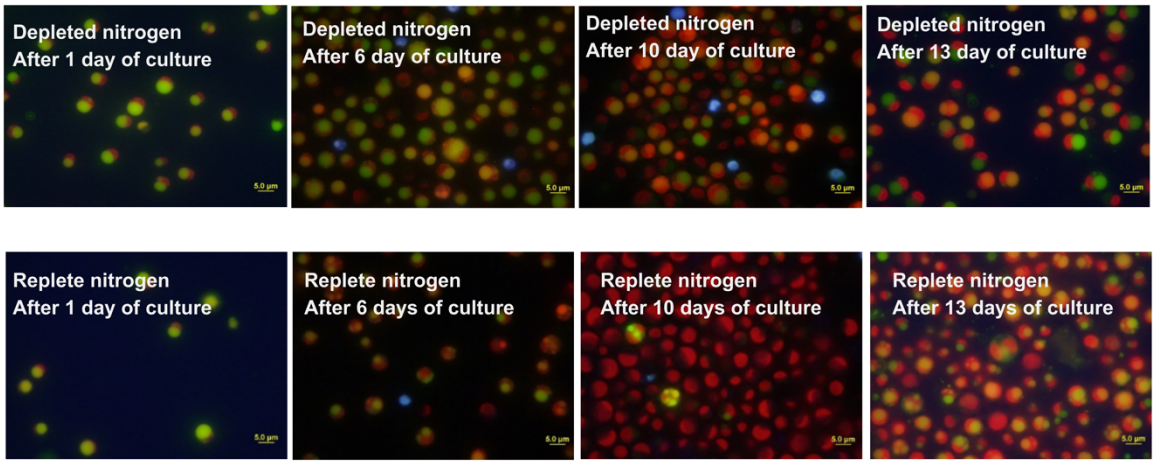
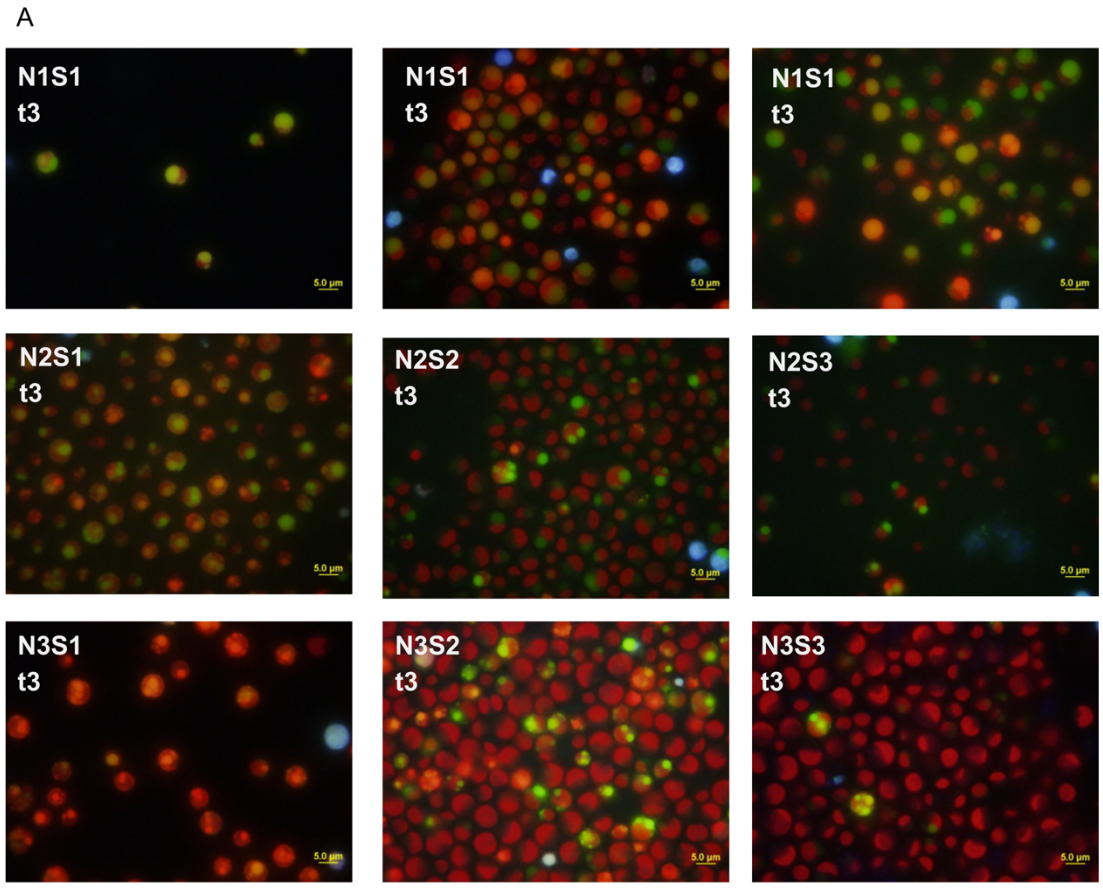
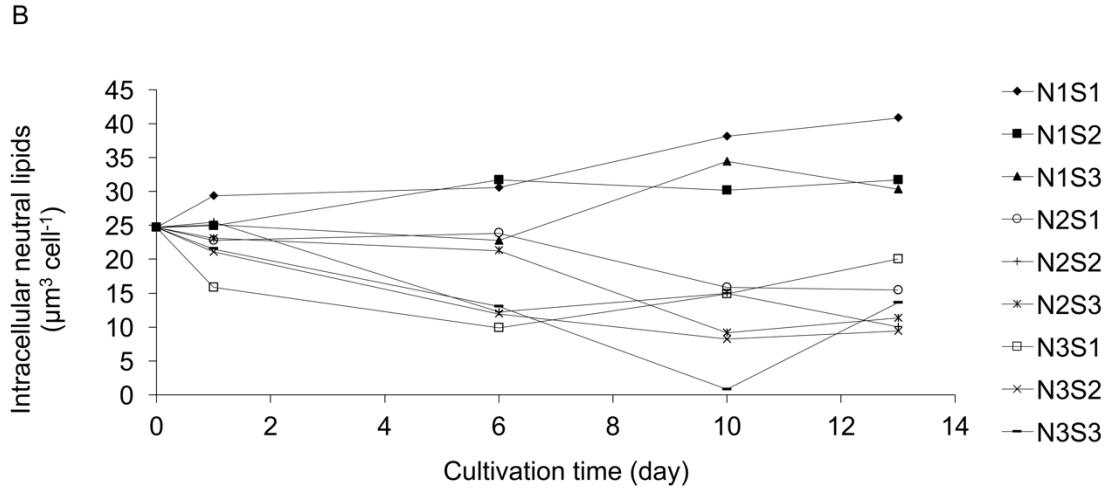


Figure 6.8 *Epifluorescence microscopy images of Nannochloropsis sp. cells stained with BODIPY BD-C12 with 0.15 and 0.30 g L^{-1} of NO_3 and lipid accumulation over time.*

The highest lipid content was achieved after 13 days of culture in depleted nitrogen medium, reaching 40.8 μm^3 $cell^{-1}$ (Figure 6.10 B). The addition of 0.30 g L^{-1} of $NaNO_3$, replete nitrogen, led to visible changes in lipid accumulation over time. The intracellular neutral lipids progressively decreased from 24.7 to 0.82 μm^3 $cell^{-1}$, in treatment N3S3, after 10 days of culture to increasing again at day 13. The lipid content of *Nannochloropsis* sp. cells decrease with increasing salinity at nitrogen depletion and sufficient levels, and increase occurs with salinity at replete nitrogen levels (Figure 6.10 B). The lipid content in cells cultivated in high salinity medium (40 g L^{-1}) was lower in nitrogen depleted medium than in cells cultivated in replete nitrogen medium, Figure 6.10 A and B. In Figure 6.10 B, it can be seen that the lipid content had the lowest yield in a medium of replete nitrogen and the absence of NaCl.

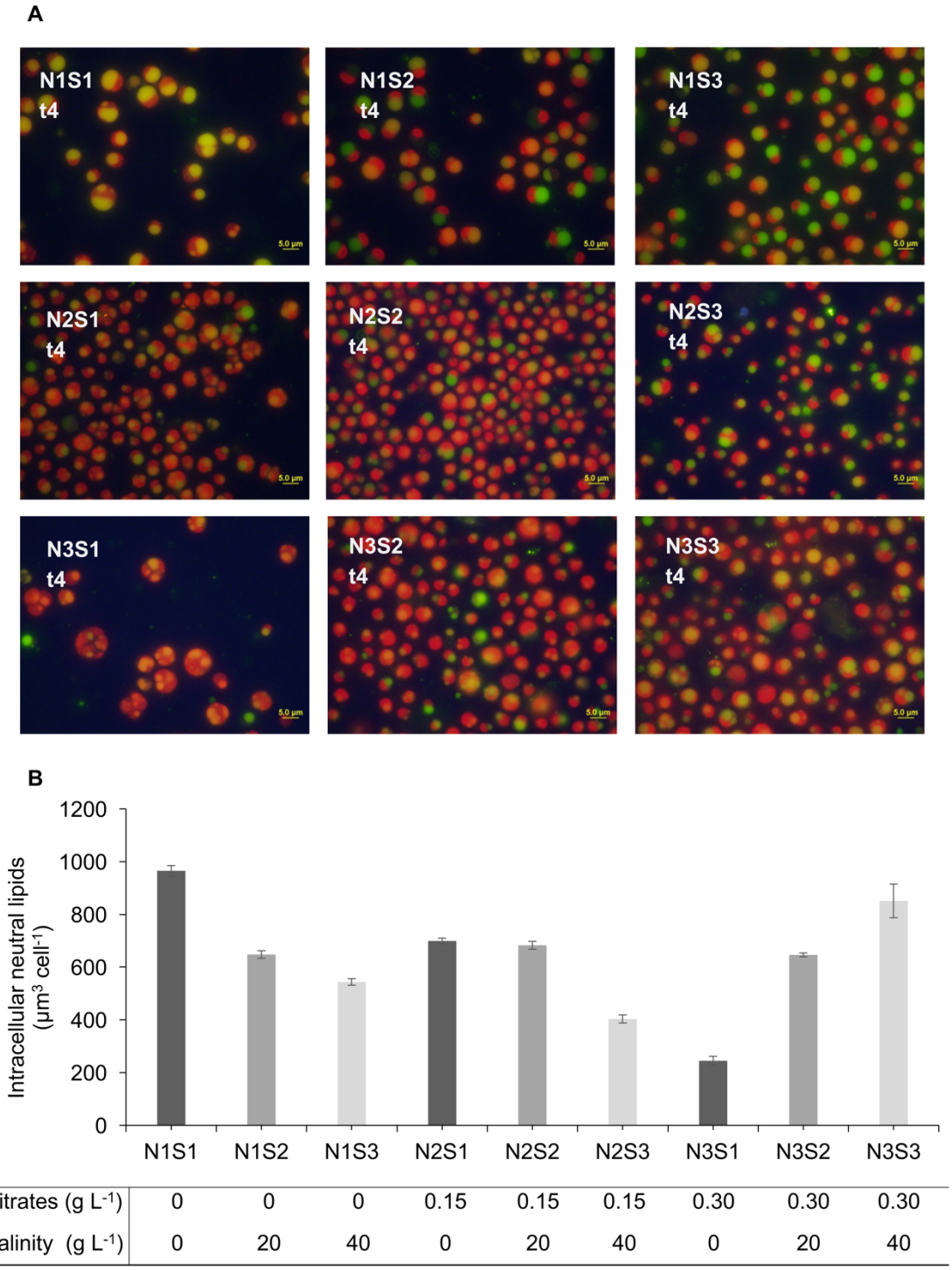




	N1S1	N1S2	N1S3	N2S1	N2S2	N2S3	N3S1	N3S2	N3S3
Nitrates (g L^{-1})	0	0	0	0.15	0.15	0.15	0.30	0.30	0.30
Salinity (g L^{-1})	0	20	40	0	20	40	0	20	40

Figure 6.9 (A) *Epifluorescence microscopy images of Nannochloropsis sp. cells stained with BODIPY BD-C12 after 10 days of culture. Images are presented for day 10, in which the changes in lipid accumulation are more pronounced. (B) Temporal evolution of lipid production by Nannochloropsis sp. cells, stained with BODIPY BD-C12, under different nitrogen conditions.*

Label t3 corresponds to day 10. See also Table for the coding of culture medium, in what pertains to nitrates and salinity.



Nitrates (g L^{-1})	0	0	0	0.15	0.15	0.15	0.30	0.30	0.30
Salinity (g L^{-1})	0	20	40	0	20	40	0	20	40

Figure 6.10 *(A) Epifluorescence microscopy images of Nannochloropsis sp. cells stained with BODIPY BD-C12 after 13 days of culture. (B) Lipid content of Nannochloropsis sp. after 13 days of culture determined by BODIPY BD-C12 method under different nitrogen and salinity concentrations. Data are given as mean ± SEM (n=100). Notation as in Figure 7, with t4 denoting day 13.*

The measured diameters of the lipid bodies range from 0.24 to 5.1 µm. It can be observed that the lipid bodies are not always present and are highly variable in number and size; this variation seems to be associated to the culture conditions and culture stage. Cells with a higher lipid content (those cultivated in depleted nitrogen, N1) possess a smaller number of lipid bodies, tendentially one, of large size. Cells cultivated in sufficient and replete nitrogen (N2 and N3), possess multiple smaller lipid bodies after 6 days of culture. After 10 days, the occurrence of multiple lipid bodies decreases in cells cultivated in sufficient nitrogen (N2), and reach minimal values (less than 30 cells with lipid bodies in 100 cells) in cells cultivated in replete nitrogen (N3). After 13 days, the size of individual lipid bodies in cells cultivated in sufficient nitrogen (N2) increases, with a tendency to one lipid body. At this stage, all the cells cultivated in sufficient and replete nitrogen (N2 and N3) possess one or more lipid bodies.

In Figure 6.11, another interesting characteristic of the dye BODIPY BD-C12 can be noted, which is that the color of fluorescence of lipid bodies varies from yellow to green and from yellowish green to red yellowish.

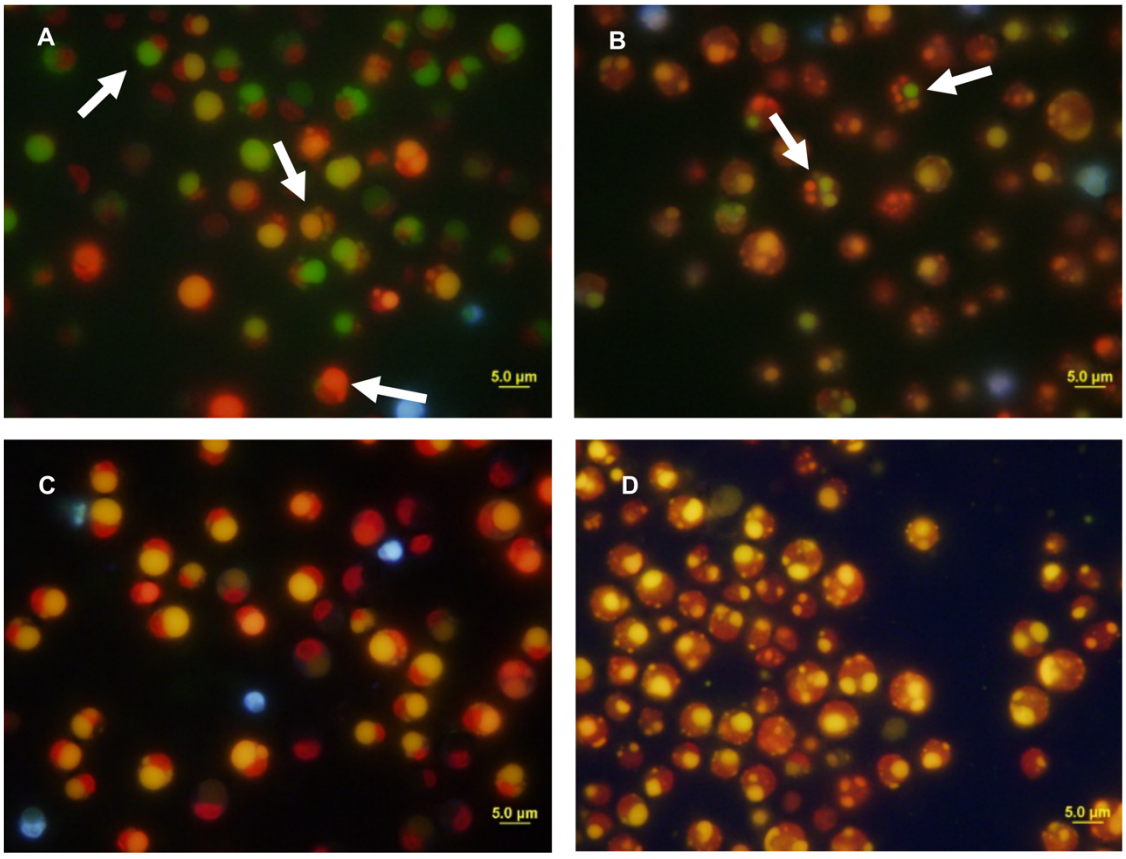


Figure 6.11 *Morphological variations of lipid droplets in algal cells of Nannochloropsis* sp.. *It is possible to distinguish different fluorescent colours within the same cell. Cells stained with BODIPY BD-C12 (A and B) and Nile Red (C and D). (Epifluorescence microscopy images of Nannochloropsis sp. cells stained with BODIPY BD-C12 and Nile Red under the same conditions).*

It is possible to distinguish different fluorescent colors within the same cell (Figures 6.11 A and B and Figure 6.12). These can be compared with cells stained with Nile Red, Figures 6.11 C and D, where there is no evidence of such color changes. Also, it is possible to see the differences in colors between lipid extracts of N1S2 and N1S3, stained with BODIPY BD-C12 (Figure 6.12).

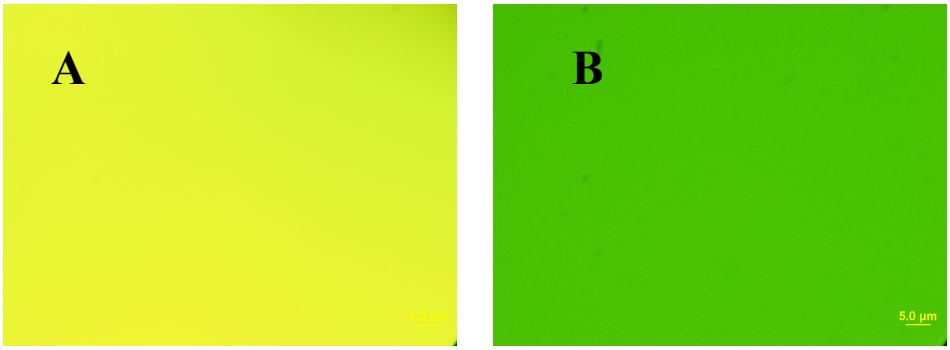


Figure 6.12 *Lipid extracts of N1S2 (A) and N1S3 (B), stained with BODIPY BD-C12.*

6.4 Discussion

6.4.1 Spectrofluorometric characteristics of BODIPY BD-C12 stained cells, fluorescence microscopy observation and lipid quantification with ImageJ

The epifluorescent microscopy images of microalgae cells, Figure 6.2, show that BODIPY BD-C12 has an intrinsically high affinity for intracellular lipid bodies, which can be seen without visualization of the cytoplasmic compartments.

Different concentrations of BODIPY BD-C12 and the effect of the staining time were also investigated. As can be seen in Figure 6.3., above 5 µL of the dye there is no variation of the fluorescent intensity, and therefore, the optimal dye concentration is the minimum required, 5 µL, to stain with an efficiency of 100%. The resistance to fading and the continuous fluorescence emission during 60 min after staining, revealed an excellent photostability of the chromophore.

The lipid content of *Nannochloropsis* sp., determinations demonstrate that BODIPY BD-C12 and conventional gravimetric methods produce similar results, as can be seen in Figure 6.6 A. An increase of nitrate concentration in the growth medium from 0 to 0.3 g L^{-1}, produces a decrease in the neutral lipids from 964.6 to 244.8 mg L^{-1} for BODIPY BD-C12, and from 809.1 to 396.7 mg L^{-1} in case of the gravimetric method providing evidence of a good agreement between the two techniques. The correlation analysis, Figure 6.6 B, indicates no significant difference, and a positive correlation coefficient of R^2= 0.98 was obtained between the two methods. The same conclusion has been reached by other authors [11,19] using BODIPY 505/515 and Nile Red dyes to quantify neutral lipids in different species of microalgae with correlation coefficients of 0.93 and 0.998,

respectively, indicating the usefulness of these fluorescent dye techniques for intracellular lipid quantification in microalgae. The two methods differ, however, in quantitative terms, with p=0.0065, as obtained in ANOVA. This may be due to an inefficient extraction of the lipids using the Bligh and Dyer method. It was shown previously[34] that, when samples of known lipid content were compared with results from the Bligh and Dyer and Folch methods, values were greatly underestimated (ca. 45% for the former). The same study also showed that for samples containing more than 2% in lipid, the Bligh and Dyer method produces significantly lower estimates of lipid content and that this tendency increases with increasing lipid content of the sample. This underestimation may be due to a limited solubility of the nonpolar lipids, such as TAGs, in the polar solvent solution used in Bligh-Dyer method, which was developed to extract phospholipids efficiently[34]. The samples used in the present study range, in lipid content, from 20 to 80% of dry weight, depending on nutrient availability. Upon limiting nutrients, the cell division in microalgae cultures decreases, and the increases of total lipid are due largely to the accumulation of TAGs. During the growth cycle, the lipid class composition changes due to differential partitioning of photosynthate between polar and neutral lipid class biosynthesis[35]. In the first stages of microalgae growth, the cells involved in rapid division show low proportions of TAG and high proportions of structural lipids, such as galactolipids and phospholipids. As the culture ages, this proportion changes and the neutral lipids, TAGs, commonly associated with lipid storage during stationary phase and nitrogen limitation, increase. The difference of values between BODIPY BD-C12 and gravimetric methods could reflect a limited solubility of TAGs and differences in the lipid classes. Again, in Figure 6.4 A, it can be seen that the difference between these two methods in culture with depleted nitrogen (N=0 g L^{-1}) is higher than in the culture with medium nitrogen (N=0.15 g L^{-1}), in agreement with the tendency of underestimation of lipid content of the samples by the Bligh and Dyer method. The discrepancy observed between the methods in culture with replete nitrogen (N=0.3 g L^{-1}) may be due the fact that the BODIPY BD-C12 is a dye that permeates the membrane and stains the lipid bodies, but not the structural lipids, while the Bligh and Dyer method allows to extraction of all lipids, including the structural ones[36-38]. It resorts to a binary mixture of chloroform and methanol. Methanol provides the disruption of the hydrogen bonding and electrostatic forces between membrane-associated lipids and proteins. It has been suggested that lipid bodies are surrounded by a monolayer of phospholipids and proteins, with phospholipids polar heads directed towards aqueous cytoplasm and non-polar tails directed towards TAG core [39,17]. Moreover, phospholipids are the major structural lipids of membranes (generally, lipids in the membranes are composed mostly of polar phospholipids, ca. 70%, neutral steroids, glycolipids, and small amounts of triglycerides)[40]. They present an amphiphilic character, with a phosphate-containing polar head group and a nonpolar hydrocarbon tail. Chloroform can easily dissolve the highly polar phospholipids, after the disruption of the phospholipid layers by methanol[36-38].

In culture with replete nitrogen (N=0.3 g L^{-1}), there is less lipid bodies in the cells, since TAG is used to synthetize structural lipids to produce new membranes. Therefore, the dye BODIPY BD-C12 stains the few lipid bodies in the cells and gives a low content in lipid. Inversely, the Bligh and Dyer method, being more efficient to extract phospholipids from membranes, will yields a higher content in lipid.

In what pertains to differences promoted by nitrogen treatments, ANOVA highlighted a substantial impact of the nitrogen concentration of the culture media. Correlation coefficients of 0.98, 0.80 and 0.93 are obtained for methods with BODIPY BD-C12, BODIPY 505/515 and Nile Red, respectively, relative to the gravimetric approach.

As seen from the spectra of the three dyes, BODIPY BD-C12, Nile Red and BODIPY 505/515, Figure 6.2 A, each emits in different regions of the fluorescent spectrum. These differences have implications in terms of the epifluorescence microscopy technique. The maximum peak wavelength of Nile Red emission is located at 644 nm. Fluorescence response of microalgal pigments, such as carotenoids and chlorophyll *a*, lies in the same peak area, 600-700 nm, which interfere with Nile Red staining measurement and may lead to an overestimation of lipids (Figures 6.6 A and 6.7). Some disadvantages of Nile Red staining (Figure 6.7), such as blurring, staining of entire cell and undefined shapes, could be observed.

The maximum peak wavelength of BODIPY BD-C12 emission is located at 558 nm, a wavelength region more suitable for the quantification of lipid by image processing.

From the epifluorescence imaging, it can be seen that the fluorescence emission of the BODIPY BD-C12 was still stable for a considerable time (see video in supplementary material in ref Encarnação et al.[41] displaying an excellent photo-stability. In contrast, the rapid photobleaching of the chromophore BODIPY 505/515 by the mercury lamp, led to a decrease in the mean fluorescence, interfering with the determinations. Reliable measurements may be thus affected by the lifetime of the fluorophore.

6.4.2 Effect of nitrogen and salinity on lipid accumulation

The effect of nitrogen concentration on lipid accumulation was investigated using the BODIPY BD-C12 method. According to the literature, depletion of nitrogen enhances lipid accumulation. An increase from 32% to 60% lipid content was reported[42] in *Nannochloropsis* sp. cultivated in nitrogen sufficient and deficient conditions. In another interesting study[42], the abrupt and progressive nitrogen limitation on the production of lipids in *Nannochloropsis oculata* was compared. The progressive nitrogen limitation had the highest lipid production, reaching 49.7% of dry weight, and the highest biomass concentration was achieved under conditions for lipid accumulation. Thus, although nitrogen deprivation stimulates production of the maximal volume of intracellular neutral lipid, nitrate replete conditions increase the biomass yield rate whereby nitrogen starvation can be induced after the maximum cell density is achieved. In the present study,

different levels of nitrate were used in the cultures after a good cell density was achieved. The lipid distribution and accumulation over time in replete and depleted media (Figures 6.8 and 6.9) show that the lipid droplets are, in general, approximately spherical and well defined, in agreement with the assumption that lipid bodies in stained microalgal cells are perfect spheres.

The decrease of intracellular neutral lipids from 24.7 to 0.82 μm^3 $cell^{-1}$, in treatment N3S3, after 10 days of culture and the increase at day 13 could be related to a possible full consumption of nitrates from the medium. A nitrogen depleted medium has a high impact on cell metabolism. TAGs are increased under nitrogen depletion conditions. Previous work has suggested that increasing TAGs contributes to a degradation of preexisting membrane lipids during nitrogen starvation. Simionato et al.[43] found that levels of the galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), both lipids of the thylakoid membranes, were lower in N depletion conditions compared to those in N replete medium. Also, the authors have suggested the existence of a galactolipid-to-TAG route through the occurrence of recycling of 20:5 fatty acids from polar lipids, galactolipids, to TAGs together with a significant *de novo* fatty acid synthesis. Nitrogen starvation also has a high impact on the photosynthetic apparatus. Our previous results, Encarnação et al. in a nitrogen depleted medium, where the chlorophyll and carotenoid content decreased and the concentrations of all the major complexes involved in photosynthetic electron flow were reduced are in line with [43]Simionato et al.; in fact, it is interesting to note in our work that chloroplasts are larger and have a more intense red autofluorescence of the chlorophyll in treatments with nitrogen replete than in nitrogen starvation conditions.

The lipid content of *Nannochloropsis* sp. cells decrease with increasing salinity at nitrogen depletion and sufficient levels, and increase with salinity at replete nitrogen levels (Figure 6.10 B). The lipid content in cells cultivated in high salinity medium (40 g L^{-1}) was lower in nitrogen depleted medium than in cells cultivated in replete nitrogen medium, Figure 6.10 A and B. This increase in lipid content, in treatment N3S3, may be related with a consumption of nitrogen that stimulates lipid storage. According to the literature[44,45] *Nannochloropsis* sp. cells accumulate larger amounts of lipids at high salinities; the total lipid content, cultivated in f/2 medium[46], increases with increasing salinity over the range 10-15 ppt.

Pal et al.[47] suggested that, in the absence of NaCl in N sufficient media, the enhanced growth of *Nannochloropsis oceanica* is related to adjustments of the pigment apparatus and chloroplast lipid composition, leading to a photosynthetic carbon fixation and photoassimilation allocation to simple carbohydrates, proteins, and structural lipid biosynthesis, rather than to the production of lipid reserves; in fact, in Figure 6.10 B, it can be seen that the lipid content had the lowest yield in a medium of replete nitrogen and the absence of NaCl.

In Figure 6.11, with cells stained with BODIPY BD-C12, it is possible to distinguish different fluorescent colors within the same cell (Figures 6.11 A and B). In Figures 6.11 C and D, cells stained with Nile Red, there is no evidence of such color changes. Although further work will be

required to draw definitive conclusions about these color differences and the processes involved, it is possible to speculate on the possible causes of these variations. BODIPY BD-C12 permeates into the cells to enhance fluorescence of the lipid bodies and, according to the class of lipids stained, will emit a corresponding color. Brennan et al.[20], and other authors cited within this paper, stated that cells permeated with Red Nile emit a yellow-gold fluorescence in the presence of neutral lipids, and a red fluorescence for phospholipids. Bartley et al.[45] observed that at high salinity treatments, the non-polar lipid content of *Nannochloropsis salina* has a general tendency to increase and the membrane lipid content to decrease. Transposing these statements to our fluorescence images, in Figure 6.10 8, it can be noted that, in treatments N1S1, N2S1 and N3S1, with no salinity in the cultivation medium, the predominant emitting color of BODIPY BD-C12 is yellow, and in N1S2 and N1S3 treatments, the predominant emitting color is green. In turn, for the lipid extracts of N1S2 and N1S3, stained with BD C12, yellow greenish and green colors are displayed, respectively (Supplementary material).

HPLC and GC-MS studies are in progress to evaluate the possibility of this correspondence. The variation in occurrence, number and size of lipid bodies is a consequence of the metabolic response to environmental changes, and seems to be related to the culture conditions and with the culture stage. During the lipid accumulation stage, two possible processes can occur: small lipid bodies in the cell merge to form one large lipid body, filling a considerable area of the cell or each lipid body gradually increases trough time. It was reported that nitrogen depletion induces the process of autophagy of the structural lipids of thylakoid membranes[48-51]. Autophagy is a catabolic process that involves the degradation of a variety of cellular components, allowing the recycling of cytoplasmic macromolecules and organelles into basic components, providing a bioenergetically efficient alternative to *de novo* synthesis[52]. It could play an important role as a physiological response of *Nannochloropsis* sp. cells to nitrogen depletion during nitrogen starvation and lipid accumulation[48]. In the present study, from day 6 to 10 day of culture, the occurrence of lipid bodies reaches minimal values in cells cultivated in replete nitrogen and high salinity (N3S3) (less than 5 cells with lipid body in 100 cells in day 10) suggesting the synthesis of new membranes from the TAG acyl groups[48]. Structural lipids are produced while lipids bodies disappear. After 13 days of culture, the occurrence and the size of individual lipid bodies increase, each cell possessing a tendency to one lipid body. It can be suggested that, from day 10 to day 13, medium became depleted in nitrogen and triggered lipid accumulation contributes to the degradation of the thylakoid membranes lipids.

6.5 Conclusions

This work suggests an effective approach, that combines the new BODIPY derivative (BD-C12) and simple image processing, for screening lipid-rich microalgae species such as *Nannochloropsis* sp, with rapid quantification of intracellular lipid content. We believe that this constitutes an important contribution for the cost-effective and sustainable development of algal-based biofuels and biomaterials. The procedure was successfully used to measure volume fractions of lipid bodies within the microalgae cells, showing a great potential as a standardized procedure with statistically significant results.

Biomass and oil yield rates are important parameters that must be considered in conjunction with the best harvest time. BODIPY BD-C12 staining is a viable and promising technique for rapid lipid determination, which will help optimize cultivation conditions to maximize the lipid production and to choose the best harvest time. The present study also demonstrates that oil produced from *Nannochloropsis* sp. is a promising biobased feedstock, since optimized culture conditions can result in a very substantial increase of lipid biosynthesis.

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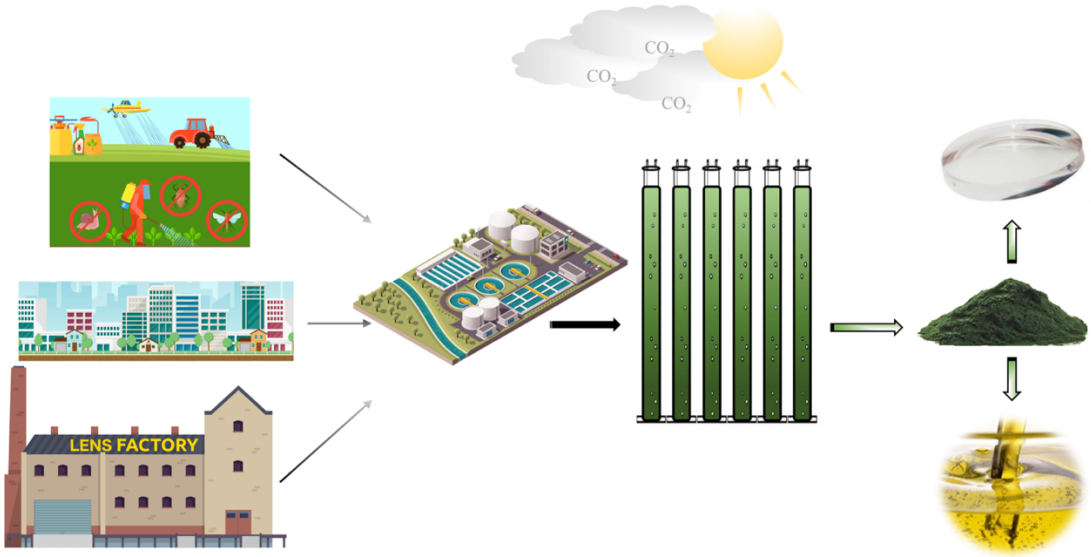
Chapter 7

On the use of poly(lactic acid) in ophthalmic and advanced optical applications



More than 1260 million ophthalmic lenses are produced annually, together with an equal number of solar lenses are produced, and more than 600 million corrective reading lenses, totalling about 3000 million units/year. The polymers used for ophthalmic lens production come from the petrochemical industry. During the manufacturing process, various wastes are produced, which are not adequately removed by normal treatment and, thus, the manufacturing process of these advanced optical products contributes significantly to wastewater contamination by nano and microplastics. It is, therefore, urgent to find alternatives to the materials used for their production. The solution proposed in this chapter is the application of a biopolymer, polylactic acid, for the manufacture of optical amplifiers or corrective lenses, since this bioplastic comes from natural and biodegradable sources. After the bioremediation using algae, the maximisation of lipids and their extraction, it is possible to obtain feedstock for producing PLA. Therefore, the feasibility of polylactic acid for application in advanced optical products was assessed through thermal and optical analysis.

The content of the present chapter was partially published in patent form in Encarnação, T. and Burrows, H.D. Provisional Patent nº 110536 PT, Optical or corrective lens.

7.1 Introduction

It is estimated that more than 8 million tons of plastic are thrown into the sea every year, the equivalent of dumping a garbage truck into the sea, every minute. Such an extension of pollution implies a tremendous impact on ecosystems and, consequently, on human health. Ingestion of microplastics by invertebrate organisms has been extensively demonstrated; this has implications at the level of the food chain and poses a risk to human health. Human exposure to microplastics is made through the consumption of fish, bivalves, salt, water and meat. In February 2017, the UN launched an unprecedented global campaign to eliminate major sources of marine litter, including microplastics used in cosmetics, and disposable, single-use plastics[1]. Microplastics are, therefore, one of the major environmental and public health challenges that humankind faces. Alongside global warming, water pollution represents a dramatic global threat to animal and human life. Wastewater effluents contain pollutants that may persist in the environment, accumulate through food webs and contaminate drinking water through the urban water cycle. Numerous environmental pollutants (parabens, phthalates, flame retardants, bisphenol a, etc.) are identified as endocrine disrupting chemicals that, even in reduced doses of exposure, may exert hormonal effects and cause adverse health effects. For example, bisphenol A is a diphenol used in the production of polycarbonate. It has a structure similar to estrogen, is considered an endocrine disruptor, and has been associated with several adverse effects on human health[2]. The polymeric materials currently used in the production of advanced optical products, such as ophthalmic lenses, include polycarbonate, dicarbonate resins ethylene glycol di-allyl, polyureas and polyurethanes.

The number of people who use ophthalmic lens users has been increasing significantly in recent years, because of the natural aging of the population, and an increase of myopia due to new lifestyle habits (presbyopia growth rate 2.5%, myopia growth rate 3.3%). More than 1260 million ophthalmic lenses are produced annually, together with an equal number of solar lenses are produced, and more than 600 million corrective reading lenses, totalling about 3000 million units/year[3]. This is, therefore, an industry that has a strong environmental impact. The lenses are not recycled, and the manufacturing and thinning process produce a large amount of wet waste. In addition to contributing to increased landfill waste, all the processing produces a considerable amount of untreated nano and microplastics that is dumped into the watercourses and, consequently, into the ocean, inevitably finally ending up in the animal and human food chain. The amount of waste produced by the ophthalmic lens industry tends to increase; currently, of the 4.4 billion people who need optical correction, only 1.9 billion people wear glasses. In addition, there is a widespread trend towards increased use of sunglasses. In 2015, 1.4 billion people used sunglasses, leading to a considerable increase in waste, with a strong environmental impact. If we add to this the increase

in the world population, as well as accessibility of these optical devices, the amount of waste produced in this industry is worrying.

The evolution of the advanced optical materials for these application has been directed towards decreasing the lens' thickness and increasing the respective impact resistance, without considering their environmental impact[4].

The polymers used for ophthalmic lens production come from the petrochemical industry. The largest manufacturers are in Asia and America. Polymers are produced in blocks that are later worked in lens factories around the world, which turn them into ophthalmic lenses to be sold to the end users, by optical stores. During the manufacturing process, various wastes are produced, which are not adequately removed by normal treatment. It is important to note that, even in closed circuits, the filters of the cutting and grinding machines are periodically washed, which leads to water contamination. During the lens manufacturing process, plastic waste is generated, first from cutting the initial block to produce a patella, and then producing the lens with the desired grade. Throughout this process, there is a need for water cooling, generating moist wastes which may, in some cases, be subjected to a primary centrifugal treatment to reduce their amount of water before being driven to final destination. Also, in the commercialization phase, these lenses continue to produce waste, as they require thinning in order to fit the customer's frames. Thus, once again, the lens is cut off, generating wet waste in the form of small polymer particles. In this case, the waste of organic lenses is mixed with those of mineral lenses, since the cutting machine is usually the same for the various types of materials, making it even more difficult to properly treat these wastes. During all stages of the process, from fabrication, cutting and mounting on a frame to final disposal, many are non-recycled lenses, so-called scrap lenses, lenses that do not pass through quality control, at one or another stage of the process, and old carrier lenses that are sent to landfill. The recovery processes of these wastes are compromised due to the additives, that are incorporated into the polymeric structure, and treatments that ophthalmic lenses contain, although many can be removed by washing and refining. Also, the large number of different polymers used makes this recycling process difficult.

From the above, we have shown that the manufacturing process of these advanced optical products contributes significantly to wastewater contamination by nano and microplastics. It is, therefore, urgent to find alternatives to the materials used for their production. The solution proposed in this chapter is the application of a biopolymer, polylactic acid, for the manufacture of optical amplifiers or corrective lenses, since this bioplastic comes from natural and biodegradable sources. Polylactic acid (2-hydroxypropionic acid), or polylactide, PLA, is a thermoplastic aliphatic polyester consisting of repeating units of lactic acid (2-hydroxypropionic acid), obtained from two monomers: lactic acid and lactide. Both have stereoisomers. Lactic acid has two stereoisomers, L-lactic acid and D-lactic acid, while lactide has four different isomers: L-lactide, D-lactide, a racemic mixture of D-lactide and L-lactide and meso-lactide. Being an optically active molecule, it can existing in both L and D stereo forms. The different proportions of the L and D

stereoisomers are directly related to the crystallinity of the polymer and thus determine the physical, mechanical and optical properties of PLA. Polymerization of L-lactide acid (greater than 90%) yields a semicrystalline polymer with a melting temperature of about 180 ° C and a glass transition temperature of around 67 ° C. The polymer derived from a racemic mixture of D- and L-lactic acid gives amorphous polymers with glass transitions near room temperature.

Renewable sources such as sugar, corn, food, agricultural, forestry and microalgal biomass can be processed to yield D-glucose, which is then fermented by optimized Lactobacillus strains, resulting in lactic acid. PLA is a biodegradable polymer, compostable, biocompatible, used as an alternative to conventional petroleum-derived plastics, has low toxicity, and is found in many industries, including food packaging, biomedical devices, agriculture, and many others[5]. Biotic lactic acid production offers several advantages compared to chemical synthesis, such as low environmental impact, low substrate cost, low energy consumption and high product specificity.

PLA is a raw material that allows various green additives to shape its characteristics, such as refractive index, melting temperature, transparency, color, among others. Synthetic polymers most commonly used in the manufacture of ophthalmic lenses include ethylene glycol di-allyl di-carbonate resins, better known by their trade name CR-39®, polycarbonate, Trivex® and high refractive index polymers. Of the various polymers applied in ophthalmic lens manufacturing, CR-39®, created by Columbia Laboratories in Ohio, USA in 1940, remains the most widely applied in this industry, despite having a Normal Refractive Index $1.48 \leq nd < 1.54$, it has an excellent Abbe number, of around 60. CR-39® resin corresponds to 80.5% of lenses sold today. This polymer was originally invented to help create glass-reinforced plastic fuel tanks for the B-17 bomber in World War II, reducing weight and increasing bomber range. In 1947, Armorlite Lens Company realizing the excellent optical characteristics of the CR-39®, manufactured the first ophthalmic lenses with this material. Orma 15 resin has a refractive index similar to CR-39® (1.502), but with a higher scratch resistance and UV protection, up to 370 nm. High-index polymers, refractive index 1.54 to 1.67, have a large chromatic dispersion, as their Abbe number is quite low. Also, polycarbonate, with a refractive index of 1.59, has the same low Abbe number but with the advantage of being more impact resistant.

The advantages of an ophthalmic lens made from PLA over those on the market include:

• significant reduction of waste caused by ophthalmic optics industry;

• less dependence on fossil fuels;

• use of material from renewable raw materials including plants, food, agricultural, forestry and microalgal biomass from water and/or air cleaning processes, industrial and urban waste and CO2 capture;

• high number of Abbe;

• transparency at the wavelengths of the spectrum in the UV region and, consequently, low UV and visible degradation, not acquiring, over time, the characteristic yellowish hue of conventional plastic ophthalmic lenses[6].

The present study consists on the development of an optical amplifier or corrective lenses made from a thermoplastic aliphatic polyester, polylactic acid, thereby contributing to the solution of the exponential increase of nano and microplastics in the environment and reducing the enormous environmental impact caused by lens made of synthetic polymers.

In a perfect scenario, the feedstock used for the production of the lactic acid would come from air and water cleaning processes using microalgae, in a concept of biorefinery, combining several aspects as sustainability, energy efficiency, and circular economy, creating value from waste.

7.2 Materials and methods

7.2.1 Reagents and chemicals

In preliminary tests, blends of PLA and CR-39®, polycarbonate, Trivex® and high refractive index polymers were tested. The molding processes used for the preliminary evaluation were injection and casting with organic solvents. PLA is a polymer with intrinsic brittleness, which could be a limitation for several application. However, it has been blended with other polymers that act as plasticizers to improve the physical and mechanical properties. Also, PLA from different sources, such as corn and potato, were evaluated. Films from the different PLAs were prepared, for simple visual inspection. The samples which have not presented the transparency required were discarded, and only the suitable ones were further evaluated. In this chapter, only the most promising results are presented.

Two different commercial grades of PLA, PLA 3052D, with molecular weight of ca. 6×10^4 g mol^{-1}, and PLA 3251D, with molecular weight of ca. 9×10^4 g mol^{-1}, were supplied by NatureWorks®. CR39® lens sample was kindly provided by Optimed®. Some physical and mechanical properties of PLA 3052D and CR39, with relevance for the production of ophthalmic lenses, are presented in Table 7.1.

Table 7.1 *Physical and Mechanical properties of PLA 3052D and CR39. (Natureworks data).*

Property	PLA 3052D	CR-39®
Specific weight	1.24	
Notched Izod Impact [ft-lb/in (J/m)]	16	0.2-0.4 (15)
Flexural Modulus [kpsi (GPa)]	3.6	2.7
Flexural Strength [psi (MPa)]	108	91
Tensile Modulus [kpsi (GPa)]	3.7	1.6
Tensile Strength at Break [psi (MPa)]	57	51

7.2.2 Thermal Analysis Study-Differential Scanning Calorimetry (DSC)

The studies were performed on a Pyris1 power compensate ion calorimeter from Perkin Elmer with an intracooler cooling unit at 10ºC/min scanning rate on PLA 3052D and PLA 3251D, in order to characterize their thermal behaviour from ambient temperature until fusion. Representative DSC curves of heating runs performed at a 10°C/min scanning rate are presented in Figures 7.1 and 7.22 for the PLA 3052D and PLA 3251D.

7.2.3 Thermal Analysis Study-Polarized light thermo-microscopy (PLTM)

The PLA 3251D were characterized by PLTM using a hot stage Linkam system, model DSC600, with a Leica DMRB microscope and a Sony CCD-IRIS/RGB video camera. Real Time Video Measurement System software by Linkam was used for image analysis. The images were obtained by the combined use of polarized light and wave compensators, using a 200x magnification.

7.2.4 Refractive index and Abbe number

A sample of a 2 mm thick PLA 3052 D polymer, obtained using rapid microwave heating and following injection to a mould, was measured for a refractive index using an Abbe's refractometer Ura 325, and an Abbe number was found from a dispersion table. For comparative purposes, a CR39® lens sample was used and measured using the same equipment.

7.3 Results

7.3.1 Thermal Analysis Study-Differential Scanning Calorimetry (DSC)

Representative DSC curves of heating runs, between 25 ºC and 190 ºC, performed at a 10°C/min scanning rate, are presented in Figure 8.1, for the PLA 3052D and PLA 3251D. The glass transition temperatures are 63.6 and 59.0°C, and the degradation temperatures are observed between 160 and 180ºC, and 140 and 160ºC for PLA 3051D and PLA 3252D, respectively.

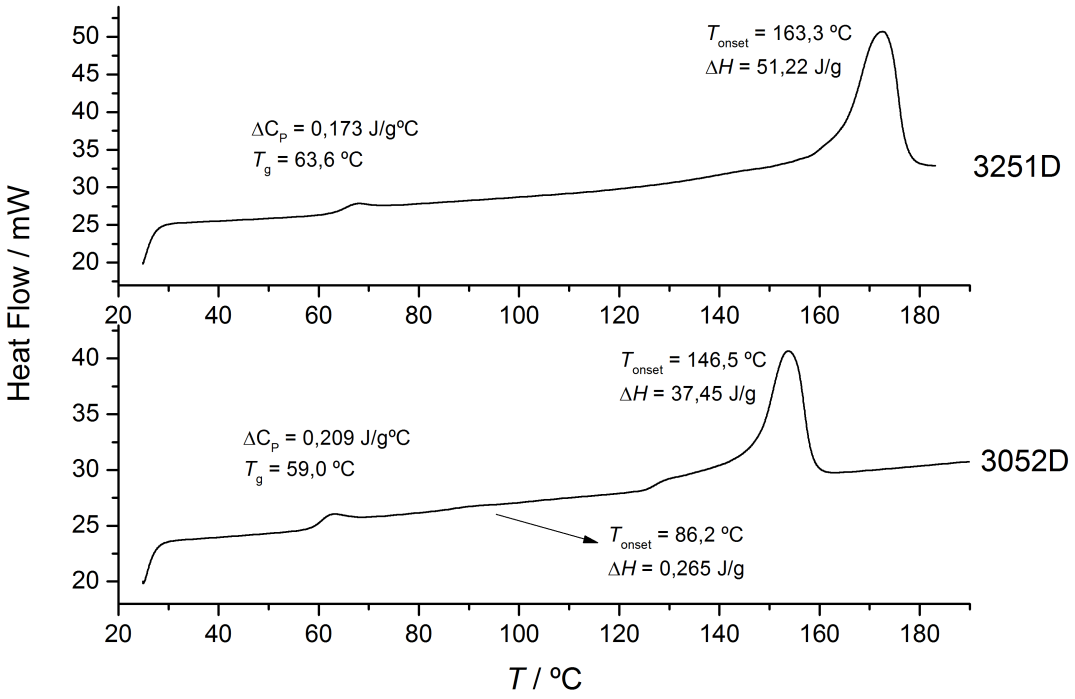


Figure 7.1 *DSC heating curves of PLA 3051D and PLA 3252D. Scanning rate 10ºC/min.*

7.3.1 Thermal Analysis Study-Polarized Light Thermal Microscopy (PLTM)

Polarized light thermomicroscopy allowed the correct interpretation of the DSC thermogram. The PLTM experiments show devitrification at 63.6ºC, followed by slow crystallization and finally the melting at 163.3 ºC.

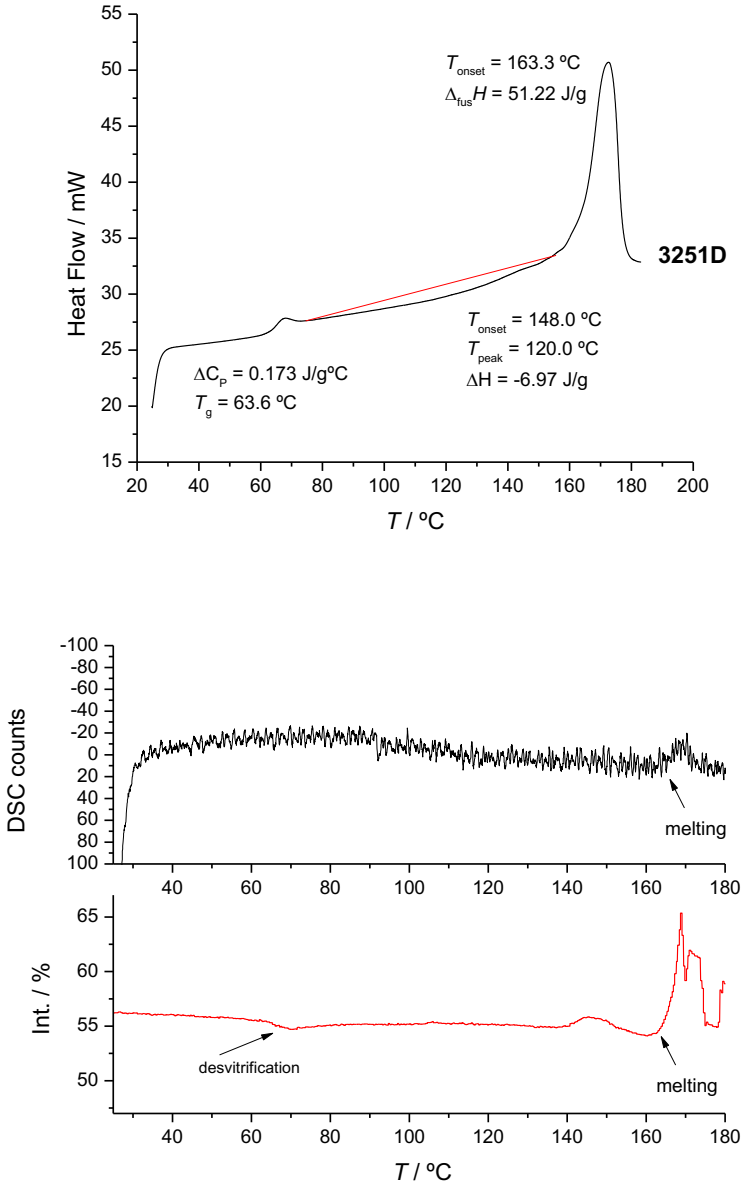


Figure 7.2 *DSC heating curves of PLA 3251D between 25 ºC and 190 ºC. Scanning rate 10ºC/min.*

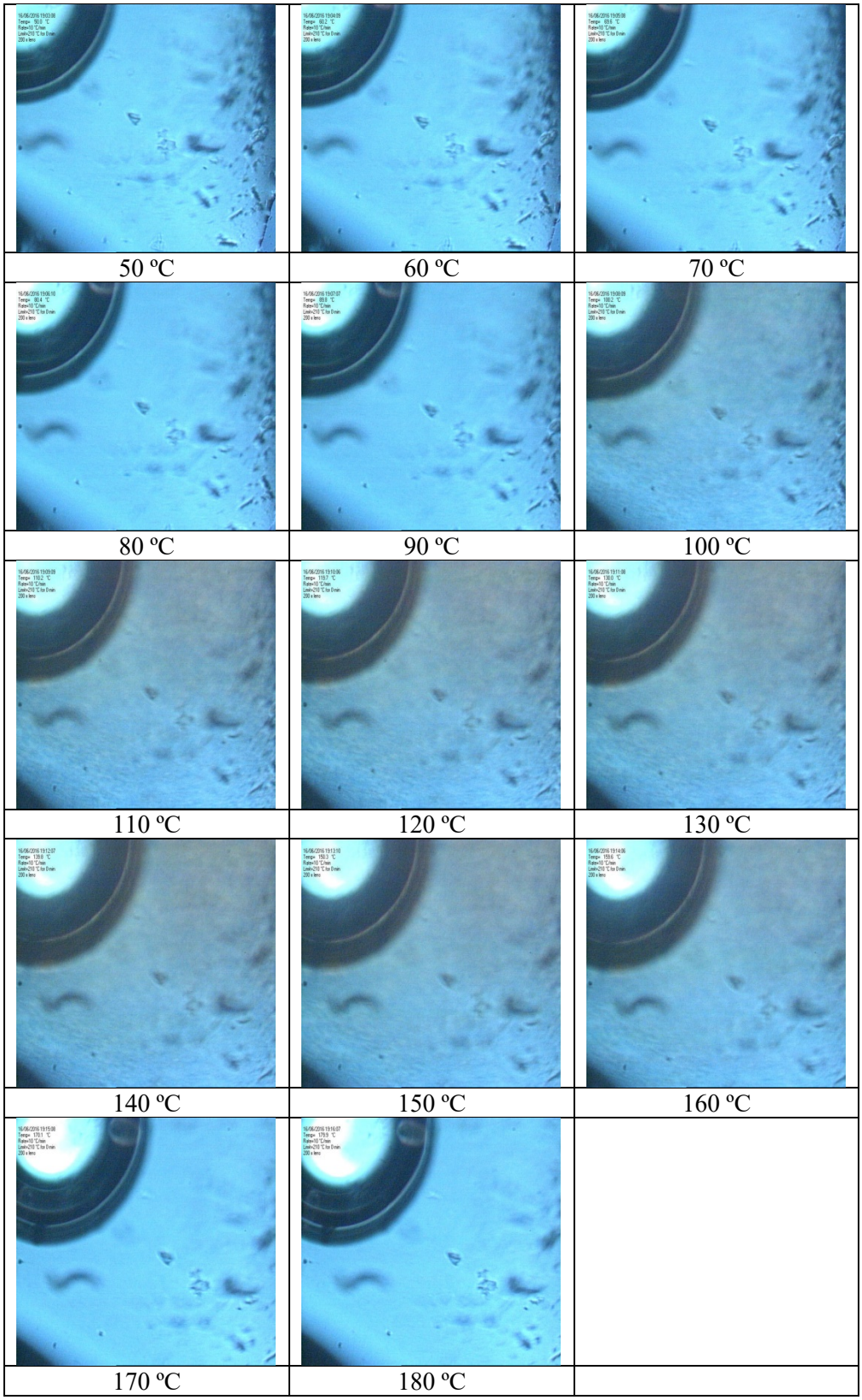


Figure 7.3 *PLTM images of the crystallization of PLA 3251D.*

7.3.2 Optical properties

PLA is colorless and fully transparent to the longest wavelengths of the UV spectrum. This characteristic makes this biopolymer exhibit low degradation by UV and visible radiation and does not, over time, acquire the characteristic yellowish coloration of conventional plastic ophthalmic lenses. The Abbe number of a PLA lens is very close to the Abbe number of CR-39®, the most used polymer in ophthalmic lens manufacturing, which has the best Abbe value in the market. In Table 7.2, the refractive indices and Abbe numbers of the sample and CR-39® lenses can be compared.

Table 7.2 *Refractive Indices and Abbe numbers of PLA 3052D and CR39.*

Polymer	Refractive index	Abbe number
PLA 3052D	1.46	55.24
CR39	1.50	55.88

7.4 Discussion

Feasibility studies on the use of PLA in Ophthalmic Lenses were performed. For comparative purposes, a CR39 polymer lens was used. CR39 is a commercial material that presents the best optical quality on the market. In Table 8.1, we compare the mechanical properties of PLA 3052D with CR-39®. With these, PLA presents better performance when compared with CR-39® and issuitable for its application in ophthalmic lenses. The thermal behavior of PLA 3052D was also studied and showed that the glass transition temperatures are around 59.0 °C, while the degradation temperatures are observed between 140 and 160ºC. PLA 3052D has suitable thermal and mechanical properties that allow it to be processed by various production methods, such as injection molding, casting or 3D printing. Refractive index and Abbe number were also measured and, in Table 8.2, show similar values for the two lenses. The Abbe number of a transparent material is as dimensionless quantity that arises when comparing the refractive index of the material at its different frequencies. In this comparison, it is observed that the white light incident on the transparent material, which is made up of several wavelengths, suffers a larger deviation in the shorter wavelengths. The higher the Abbe number, the better the optical quality of a lens in chromatic aberration (decomposition of white light into various colors, the coloration observed when looking through the lens). PLA present similar optical performance when compared with CR-39®.

Additives and catalysts may be added which increase the performance of the ophthalmic lens with respect to refractive indices, and temperatures. These include lactic acid oligomers and citrate esters, which are non-toxic and substitute the traditional plasticizers, such as phthalates. Lactic acid oligomers and citrate esters improve the thermal stability and mechanical properties of the material. Long term stability is another factor to

consider. Low molecular weight citrate esters increase the enzymatic degradation of PLA, while high molecular weight citrate esters decrease it when compared to PLA without plasticizers. In this context, a compromise between persistence and ephemerality must be established; while requiring a material that is biodegradable, which does not remain in the environment for a long time, it is essential that the material maintains the same stability with respect to the mechanical and optical properties over the life of the product. Thus, in the composition of an ophthalmic lens in PLA, "green" additives could be added, which are biodegradable, have passed the toxicity, sensitization, mutagenicity and estrogenicity tests, and have the same or better performance than conventional additives. Included in this class of plasticizers are isosorbide esters, which have properties similar to 2-ethylhexyl phthalate (DEHP), citrate esters and glycerol citrates (acylated glycerol derivatives). PLA also has a high surface energy, which makes it suitable for receiving the various optical treatments commonly used in ophthalmic lenses, namely anti-reflective, photochromic, UV and scratch protections.

7.5 Conclusions

Nannochloropsis sp. is rich in lipids, carbohydrates and proteins. After the extraction of lipids, the lipid free residues can be neutralised and concentrated to sugars, which can be fermented by a wide range of microorganisms, such as bacteria, yeasts and fungi. The product obtained from the fermentation is the lactic acid, which is an important biobased block for producing PLA. In this chapter, we focus on the final stage of whole value chain and circular economy; after the bioremediation using algae, the maximisation of lipids and their extraction, and the processing of residues for producing PLA, a new application is developed. The results suggest that PLA polymers are potential candidates for advanced optical applications and finds applications in ophthalmic lenses, which can be used on prescription glasses and sunglasses, having potential to be the first green lens reaching the market.

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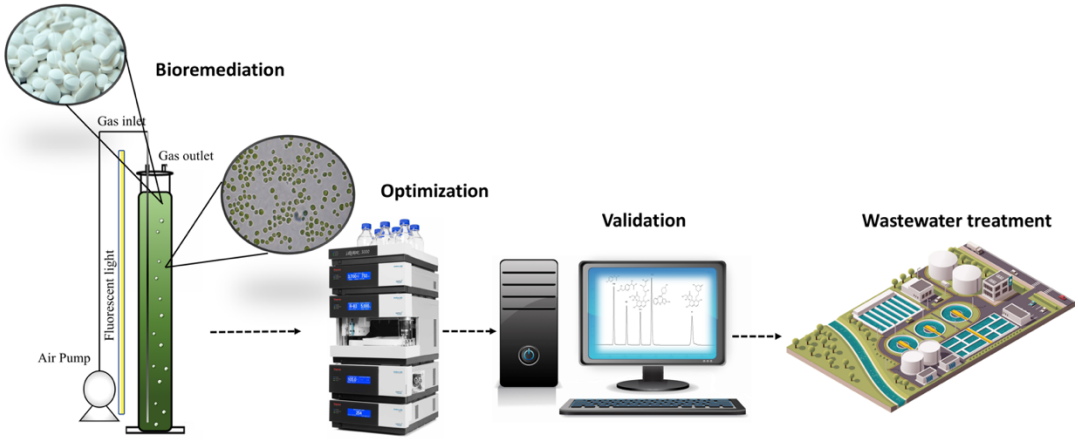
Part D

Methodologies development and optimisation

Chapter 8

Development and validation of a RP-HPLC method for the simultaneous analysis of paracetamol, ibuprofen, olanzapine, and simvastatin in microalgal bioremediation



A rapid reverse phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the simultaneous quantification of paracetamol, ibuprofen, olanzapine, simvastatin and simvastatin acid in the context of microalgae bioremediation. The method was validated according to the guidelines of the US Food and Drug Administration (FDA), the International Conference on Harmonization (ICH), and Eurachem with respect to system suitability, linearity, accuracy, precision, recovery, limits of detection and quantification, ruggedness, selectivity and specificity. The estimated limits of detection and quantification were, respectively, 0.03 and 0.10 μ mL^{-1} *for paracetamol, 0.03 and 0.09* μ mL^{-1} *for ibuprofen, 0.04 and 0.13* μ mL^{-1} *for olanzapine, 0.27 and 0.83* μ mL^{-1} *for simvastantin, and 0.05 and 0.14* μ mL^{-1} *for simvastantin acid. The inter-day and intra-day precision results were within the acceptance limit of relative standard deviation (%RSD) of less than 2, and the percentage recovery was found to be within the required limits of 80-110 %. The developed method is rapid, linear, precise, robust and accurate, and has been successfully applied to the determination of the above comon pharmaceutical products during microalgae bioremediation.*

The content of the present chapter was fully or partially submitted in paper form in Encarnação, T., Palito, C., Aguiar, A., Pais, A.C., Campos, M.G., Sobral, A.J., and Burrows, H.D. A rapid reversed-phase HPLC method for the simultaneous analysis of olanzapine, simvastatin, ibuprofen and paracetamol in microalgae bioremediation process

8.1 Introduction

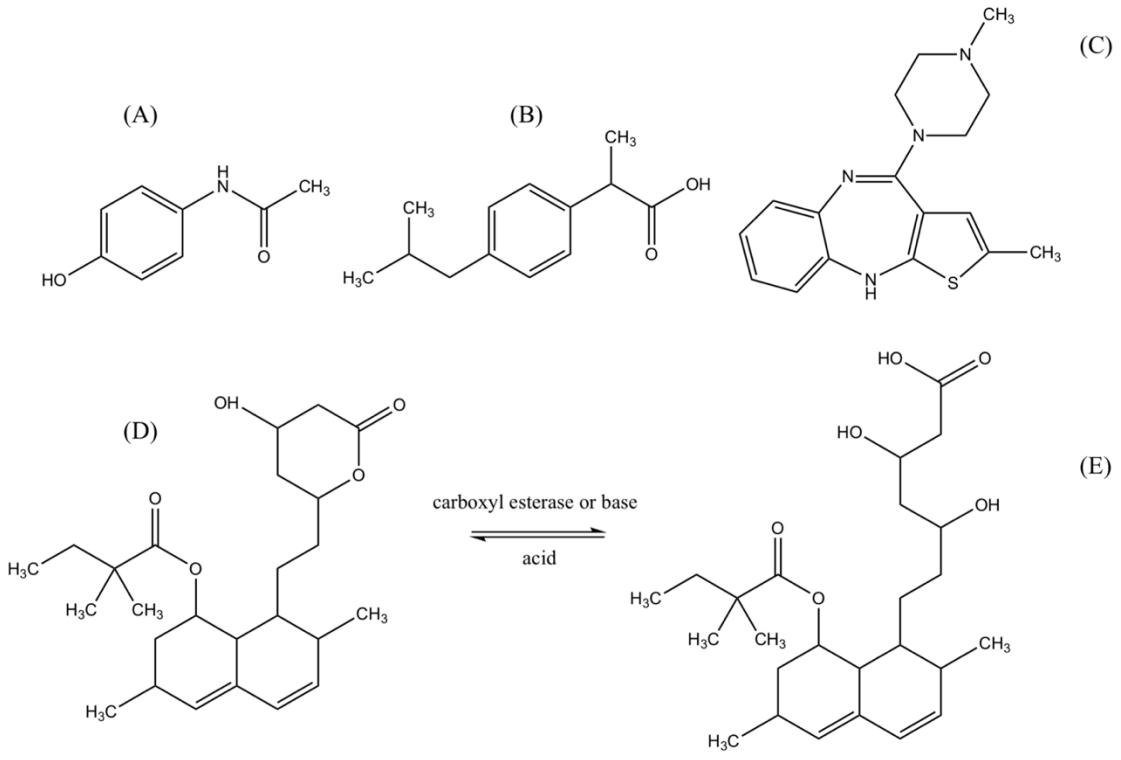
Water pollution is a severe global threat to human health and wildlife. As a result of domestic, agricultural and industrial water usage, effluents from wastewater frequently contain pollutants that can persist in the environment, bioaccumulate through the food web and reach drinking water. Several environmentally ubiquitous organic chemicals, e.g. pharmaceuticals, plasticizers, persistent organic pollutants (POPs) are not eliminated by conventional treatment methods, and may be found in drinking water. The U.S. EPA estimates that 20% of the total dietary exposure to emerging pollutants comes from drinking water [1,2] .Conventional wastewater treatment technology includes preliminary, primary and secondary treatments [3], which remove the majority of the biochemical oxygen demand (BOD) and suspended solids, found in wastewaters. However, the conventional treatment cannot produce effluents of high quality, since the apparently clean water is loaded with emerging pollutants, together with nitrogen and phosphorous compounds which cause eutrophication. Advanced wastewater treatments include tertiary treatment, using physicochemical techniques, such as chemical precipitation, ozonation, UV light, reverse osmosis, together with quaternary treatment. The quaternary treatment is designed for the removal of heavy metals, organic pollutants and soluble mineral ions (also denoted as quinternary). The use of microalgae for wastewater treatment is not new and has been exploited for decades. The removal of certain pollutants from wastewater has been reported in the treatment of industrial textiles [4], dairy effluents [5] and urban wastewater [6]. Currently, the use of microalgae has been implemented in several stations for the treatment of diverse wastewater sources, including municipal and industrial ones[7,8]. Wastewater treatment is an expensive process but microalgae are a source of multiple products such as pigments, bioplastics and secondary metabolites [7], which may help lessen the respective economic burden.

In order to reduce the effects of discharges of pollutants and comply with present and future regulations for disposal of wastewaters, more advanced systems are needed. Also, more and better methods for monitoring and validation of emerging pollutants are required.

The pharmaceuticals used in this study include paracetamol (or acetominophen) (PAR), Ibuprofen (IBU), olanzapine (OLA) and simvastatin (SIM). They were chosen for their occurrence or persistence in the environment; it should be noted that paracetamol and ibuprofen are generally classified as harmful to aquatic organisms [8]. Paracetamol is the most commonly used analgesic drug and is heavily present in effluents. Ibuprofen is one of the most frequently used nonsteroidal anti-inflammatory drug. Olanzapine, an antipsychotic drug, used for the treatment of schizophrenia, is resistant to photodegradation by sunlight, and is found in surface waters [9,10] . Simvastatin is a lipid lowering drug and also often found in effluents. This pharmaceutical is a lactone that is hydrolysed

in water to the corresponding β-hydroxyacid, the simvastatin acid (SIMA) (Fig. 8.1). In influents, concentrations of 492340-6924 ng L^{-1} of PAR, 1681-33764 ng L^{-1} of IBU, 7-115 ng L^{-1} of OLA and 1230 ng L^{-1} of SIM have been reported [8,11].

HPLC is widely used by the pharmaceutical industry to quantify drugs, with particular focus on its quantification in tablets [12,13]. The quantification of emerging pollutants, such as pharmaceuticals in wastewaters is a field needing further exploration. In this context, the objective of this study was to develop and validate a RP-HPLC method for four common pharmaceuticals, with relevance for bioremediation purposes.



8.1 *Chemical structures of (a) paracetamol, (b) ibuprofen, (c) olanzapine, (d) simvastatin and (e) simvastatin acid.*

8.2 Materials and methods

8.2.1 Reagents and chemicals

Paracetamol was purchased from Fagron Iberica (Spain) and Ibuprofen supplied by Laboratórios Medinfar (Lisboa, Portugal). Olanzapine was acquired from Zhejiang MYOY Import & Export Co., Ltd (Hangzhou, China). Simvastatin was kindly provided by Labesfal, Laboratórios Almiro, S.A. (Santiago de Besteiros, Portugal). Microalgae medium f/2 was obtained from Varicon Aqua Solution (Malvern, UK). All other reagents and solvents were of analytical or HPLC grade.

8.2.2 Instrumentation

The RP-HPLC analysis of PAR, IBU, OLA, SIM, and SVA was carried out using a Dionex Ultimate 3000 system equipped with an auto injector and four variable UV/VIS dual wavelength detectors. The column used for the analysis was a Luna Phenyl-Hexyl, Phenomenex® (Torrance, USA), with 5 μm particle size, 3 mm internal diameter and 150 mm length, supported with a SecurityGuard™ cartridge Phenomenex® (Torrance, USA), with 3.0 mm internal diameter, which was in an oven at a temperature of 35 ˚C. The data were recorded using Chromeleon software. Chromatographic analysis was conducted in multistep gradient mode, as indicated in Table 8.1. Preferentially, one UV detector was set at 230 nm for the simultaneous detection of PAR, IBU, OLA, SIM, and SVA. The injection volume was 10 μL for standard and samples. Before analysis, every standard and sample was filtered through 0.22 μm filters. A run time of 8 min was found adequate for the separation of the five analytes, followed by a washing step of 3 min with buffer between runs.

Table 8.1 *Chromatographic conditions of the gradient RP-HPLC method.*

Time (min)	Eluent A (%)	Eluent B (%)	Flow rate (ml min^{-1})
0	30	70	0.8
1	40	60	0.8
2	60	40	0.8
5	65	35	0.8
7	70	30	0.8
8	30	70	0.8

Eluent A: acetonitrile; Eluent B: phosphate buffer at pH 7.3

8.2.3 Preparation of mobile phase, stock and standard solutions and quality controls

The mobile phase consisted of a mixture of a buffer and acetonitrile according to the gradient mode, Table 1. The buffer was prepared by dissolving 1.15 g of dipotassium hydrogen phosphate salt in 650 mL of ultrapure water. The value of the pH was adjusted to 7.3 ± 0.1 with phosphoric acid. All solutions were filtered through a 0.22 μm membrane filter and sonicated to degas. Five acetonitrile stock solutions at 1mg mL^{-1} of PAR, IBU, OLA, SIM and SIMA were prepared. SIMA was obtained by alkaline hydrolysis of SIM; a SIM methanol solution of 2mg mL^{-1} was prepared and mixed to an equal volume of 0.04 M NaOH solution. This mixture was heated to 60 °C for 45 min and then neutralized with 1 M HCl. A SIMA solution of ca. 1 mg mL^{-1} is obtained and confirmed by the absence of the SIM peak in RP-HPLC analysis [14]. Two working standard solutions containing the five analytes were prepared at the concentrations of 100 and 10 μg mL^{-1} by dilution of each stock solution with the mobile phase (acetronitrile:buffer, 50:50). For the determination of the limits of detection and quantification, six standard solutions (0.1, 0.25, 0.5, 0.75, 1 and 1.25 μg mL^{-1}) were prepared from the 10 μg mL^{-1} working solution. Eight standard solutions (0.5, 1, 5, 10, 25, 50, 75, 100 μg mL^{-1}) were prepared by dilution of the working standard solution with the mobile phase (acetronitrile:buffer, 50:50). For the quality control solutions, six replicates of 0.5, 1.5, 50 and 100 μg mL^{-1} standards containing the five analytes were considered. All stock solutions were stored at -20 °C and working solutions were freshly prepared as needed.

8.2.4 Method Validation

The method for PAR, IBU, OLA, SIM, and SVA quantification was validated according to the US Food and Drug Administration (FDA)[15], the International Conference on Harmonization (ICH) guidelines[16] in addition to Eurachem[17], with respect to system suitability, linearity, accuracy, precision, recovery, limits of detection and quantification, selectivity and specificity.

System suitability The system suitability test ensures that the complete testing system, including instrument, reagents, column and analyst, is suitable for the intended application. For that purpose, six consecutives injections were made with the standard solution of PAR, IBU, OLA, SIM and SIMA at a concentration of 75 μ mL^{-1}. The parameters theoretical plate number (*N*), capacity factor (*k´*), resolution (*R*), tailing factor (*T*) were analysed. *N* is indicative of column efficiency, *k´* is a measure of where the peak of interest is located with respect to the void volume, *R* is a measure of how well two peaks are separated, and *T* is a measure of peak symmetry.

Limits of detection (LOD) and quantification (LOQ) LOD is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value, and LOQ is the lowest amount of analyte in a sample which can be quantitatively determined with acceptable

accuracy and precision. The LOD and LOQ values were determined by using regression parameters from a calibration curve from six standard solutions (0.1, 0.25, 0.5, 0.75, 1 and 1.25 μg mL^{-1}) containing the five analytes (3.3σ/S and 10σ/S, respectively, where σ is the standard deviation of the residues and S is the slope).

Linearity Linearity is a critical criterion for quantitative analysis and constitutes a measure of accuracy over the range of the method. The linearity of the proposed method was evaluated through calibration curves, constructed with eight standard solutions, containing the five analytes, ranging from 0.5 to 100 μg mL^{-1}, to calculate coefficient of correlation, slope, and intercept values. Data were analysed using the Analysis ToolPak of Microsoft Excel® (Microsoft Corp., Redmond, WA) with linear regression by the least squares method.

Accuracy and precision The accuracy of an analytical method expresses the closeness between the reference value and the value that was actually found. The mean value should be within 15% of the theoretical value, except at the LLOQ, where it should not deviate by more than 20%[15]. The accuracy of the method was established by analysing six replicates of the four quality controls and by calculating the trueness for each analyte. Trueness is expressed in terms of bias, and represents the systematic deviation from a true central value and was calculated as

% accuracy = (observed concentration/nominal concentration) x 100.

Precision represents the degree of concordance between the measured value and the reference value[17]. Precision is generally dependent on analyte concentration, and must therefore be determined within the range of concentrations of interest. Evaluation of precision was determined by repeatability (intra-day) and intermediate precision (inter-day) for three consecutive days. Six replicates of four quality control solutions (0.5, 1.5, 50 and 100 μg mL^{-1}) were prepared and analysed according to intra-day and inter-day precision. The relative standard deviation (RSD) of the results should be lower than 15%, according with the requirements, except for the LLOQ, where it should be lower than 20%.

Selectivity and specificity Analytical selectivity could be interpreted as "*the extent to which the method can be used to determine particular analytes in mixtures or matrices without interferences from other components of similar behaviour*"[18]. IUPAC recommends the term selectivity, while other areas, e.g. pharmaceutical field, use the term specificity, yet agreement exists on the interpretation. In the developed method, the response of the solution containing only PAR, IBU, OLA, SIM and SIMA was compared with microalgae culture medium f/2.

Recovery Recovery studies may be used to address the level of bias. The recovery of PAR, IBU, OLA, SIM and SIMA from the microalgae culture medium was determined by comparison of the respective concentrations with those of standard solutions in the mobile phase at three different concentrations (1, 50 and 100 μg mL^{-1}).

Method applicability-*Nannochloropsis* sp. culture conditions *Nannochloropsis* sp. was obtained from Varicon Aqua Solution, Malvern, UK, and was cultivated for 6 days in 2 L f/2 medium. 100 cm^3 of a *Nannochloropsis* sp. culture were filtered and washed, and cells were subsequently transferred to a sterilized closed photobioreactor, to which 100 cm^3 of culture medium Cell-hi TEViT (Varicon Aqua Solution, Malvern, UK) were added. This is based upon the f/2 medium deprived of nitrates. Nitrate concentration was 0.30 g L^{-1} and salinity 25 g L^{-1}. The culture media was previously sterilized by microwave irradiation.

Method applicability-Removal of pharmaceuticals from water by *Nannochloropsis* sp. To the 100 cm^3 *Nannochloropsis* sp. culture, a concentration of 50 μg mL^{-1} of each pharmaceutical, PAR, IBU, OLA, SIM and SIMA, was added. Similarly, a blank with 50 μg mL^{-1} of each pharmaceutical, PAR, IBU, OLA, SIM and SIMA, was added to 100 cm^3 of culture medium f/2, without cells. The cultures and the f/2 media were $m^{-2}s^{-1}$ with 16:8 photoperiod and kept for 60 hours. Millipore water was added when needed to ensure the same volume due to water loss by evaporation. Samples of 30 mL were replaced with Millipore water. The cultures were aerated by bubbling atmospheric air, at a rate of 300 cm^3 min^{-1}, and grown at 25±2 ºC under light with an irradiance level of ± 100 μmol. Each experiment was carried out in triplicate.

8.3 Results and discussion

8.3.1 Method development and optimization

A RP-HPLC method for the simultaneous analysis of PAR, IBU, OLA, SIM and SIMA was developed. According to the published literature, an earlier RP-HPLC method for the simultaneous determination of OLA, SIM and SIMA, used a Phenyl-Hexyl column for the analysis of the three analytes with good separation and short retention times, which motivated the choice for this column. Regarding the analysis of PAR and IBU, there are a number of reported methods in literature[19].
The pKa of the analytes was also considered; pKa´s from PAR, IBU, OLA, SIM are 9.46, 4.85, 7.24 and 14.91, respectively. Following the usual rule of thumb, the pH of the mobile phase should be selected two units above or below the pKa of the analyte. Attaining pH values near the higher and lower values of pH, 12.91 and 2.85, is, however, detrimental to the column. For strongly acidic values of pH, IBU is not dissociated and shows a strong hydrophobic attraction with the silica bed, resulting in retention times near 4 min. However, at pH 3, the retention times of PAR and OLA are 1.103 and 1.047 resulting in a poor separation of the peaks and low resolution. Therefore, a pH value around 7 was chosen for the separation of the five analytes under analysis (Figure 8.2). For choice of mobile phase, methanol was discarded since, in the presence of small amounts of acid, the transesterification of IBU to the corresponding methyl ester may occur. Thus, acetronitrile was the choice for eluent A and phosphate buffer for eluent B. Different ratios of acetronitrile and buffer were tested until the best separation was found in gradient mode, as described in Table 8.1. Under the described conditions, PAR, IBU, OLA, SIM and SIMA eluted at 1.26, 2.10, 3.72, 6.35 and 2.99 min, respectively. The method was validated over the range of 05-100 μg mL^{-1}.

8.3.2 Method validation

System suitability. The system suitability parameters, summarised in Table 8.2, including %RSD, theoretical plates, tailing factor and resolution, met the required criteria. Peaks show symmetry and high resolution. Capacity factor values for PAR and IBU are below 2, however, in a gradient chromatogram, the values from the capacity factor have no theoretical meaning. The results obtained with the system suitability indicate that the selected chromatographic parameters are suitable to identify PAR, IBU, OLA, SIM and SIMA.

Table 8.2 *System suitability test parameters*

Chromatographic parameters	PAR (75 µg mL^{-1})		IBU (75 µg mL^{-1})		SIMA (75 µg mL^{-1})		OLA (75 µg mL^{-1})		SIM (75 µg mL^{-1})		
	Retention time (min)	Peak area	Retention time (min.)	Peak area	Retention time (min)	Peak area	Retention time (min)	Peak area	Retention time (min)	Peak area	Acceptance criteria
Mean (n=6)	1.26	37.15	2.10	25.0	2.99	17.6	3.72	51.0	6.35	31.3	-
S.D.	0.002	0.51	0.002	0.50	0.004	0.25	0.004	0.67	0.006	0.50	-
%RSD	0.13	1.38	0.11	2.00	0.13	1.42	0.11	1.30	0.09	1.58	≤ 2.0% [a]
Theoretical plates (N)	2816		8670		23708		20120		64570		> 1000 [a]
Capacity factor (k')	0.52		1.52		2.59		3.47		6.62		> 2.0 [b]
Tailing factor (T)	1.08		1.06		1.14		1.23		0.98		≤ 2.0 [b]
Resolution (r_s)	9.00		10.6		8.03		20.0		14.4		> 2.0 [b] (> 1.5 [ac])

[a] [20], [b] [21], [c] [22].

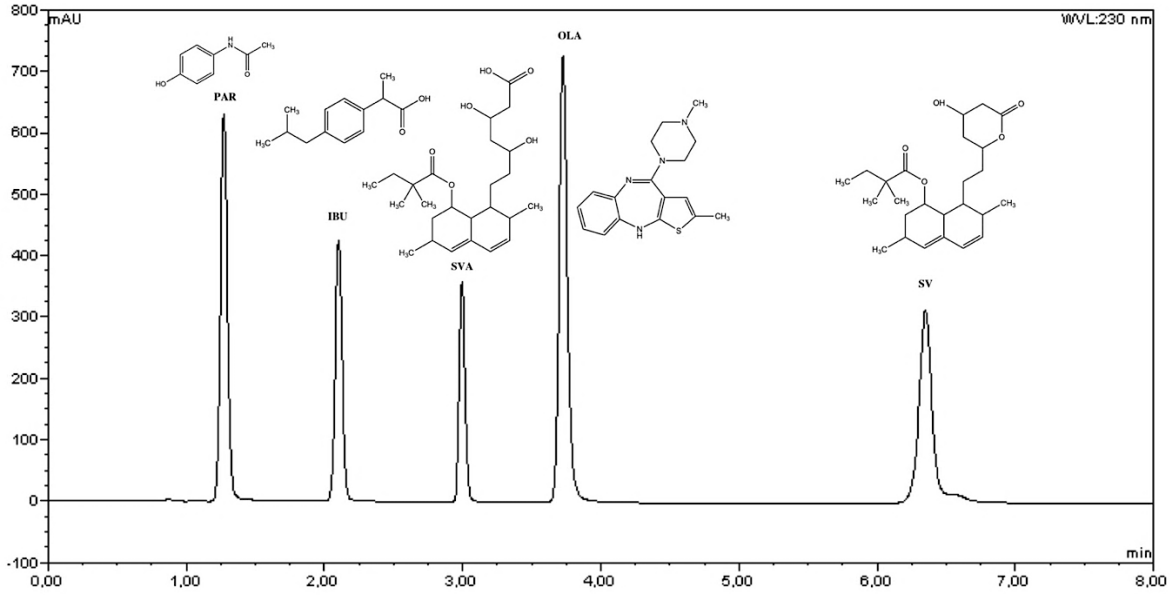


Figure 8.2 *Chromatogram of the standard 75 µg mL^{-1} solution of PAR, IBU, SIMA and SIM considered for the evaluation of the system suitability.*

Limits of detection (LOD) and quantification (LOQ). The estimated values for LOD and LOQ are summarized in Table 8.3. The LOD and LOQ values show the sensitivity of the method for the detection and quantification of PAR, IBU, OLA, SIM and SIMA.

Table 8.3. *Estimated limits of detection LD and quantification LQ for PAR, IBU, SIMA, OLA and SIM.*

Analyte	LD (µg mL^{-1})	LQ (µg mL^{-1})
PAR	0.03	0.10
IBU	0.03	0.09
SIMA	0.05	0.14
OLA	0.04	0.13
SIM	0.27	0.83

Linearity. For the assessment of the linearity of the method, solutions with concentrations in the range of 0.5-100 µg mL^{-1} were evaluated. The correlation coefficient was found to be greater than 0.9999 for the five analytes, indicating good linearity of the calibration curve (Table 8.4). The residual standard deviations close to zero, 0.17, 0.15, 0.11, 0.25, 0.5 for PAR, IBU, OLA, SIM and SIMA respectively, confirm linearity. However, by visual inspection of the plot response against concentration, a systematic trend in the distribution reflects a change in variance with level. The residuals plot for simvastatin indicates that the model is good fit for high concentrations, and a good predictor of lower concentration values (Table 8.4).

Table 8.4 *Results obtained from the regression analysis by the weighted least squares method for PAR, IBU, SIMA, OLA and SIM (n=6).*

Analyte	Mean R^2	Mean slope ± S.E.	Mean intercept ± S.E.
PAR	0.99994	0.50 ± 0.070	-0.004 ± 0.07
IBU	0.99991	0.34 ± 0.001	-0.060 ± 0.07
SIMA	0.99997	0.44 ± 0.001	0.010 ± 0.05
OLA	0.99994	0.80 ± 0.003	0.230 ± 0.12
SIM	0.99940	0.50 ± 0.005	0.367 ± 0.24

Accuracy and precision. The data obtained with the evaluation of the accuracy and precision are shown in Table 8.5. All the results met the acceptance criteria. The intra-day and inter-day %RSD and bias values fall within the acceptance criteria of 15% of the theoretical value demonstrating that the developed method is accurate, reliable and reproducible[15].

Selectivity and specificity. The selectivity of the method was evaluated by analysing standard solutions in the presence of the components of the culture medium (Fig. 8.3). The absence of any signal at the same elution time as the five analytes suggests that there were no matrix interferences.

Recovery. As can be seen from Table 8.6, all calculated mean recovery (trueness) are in the range of 81-109% being within in the acceptable recovery percentage of 80-110% for the analytes concentration of 1 ppm indicating the adequacy of simultaneous quantification of the five analytes.

Method applicability. The method applicability was assessed by evaluating the efficiency of bioremediation, using the microalgae *Nannochloropsis* sp. for removal of the five pharmaceuticals from contaminated water. To quantify PAR, IBU, OLA, SIM and SIMA in contaminated water, microalgae cells were filtered, and the supernatant was analysed by RP-HPLC by the method developed in this work and explained above. *Nannochloropsis* sp. was able to remove 11.33, 7.98 and 35.64 μg mL^{-1} of PAR, IBU and OLA, respectively. The results for SIM and SIMA were inconclusive; due to some precipitation of SIM and SIMA, it could not be established clearly whether the disappearance of both compounds was due to bioremediation or precipitation. However, some additional peaks appeared at different retention times but with the same wavelength of SIM. This could suggest metabolization of the pharmaceutical with the correspondenting excreted metabolite. The present study demonstrates that the specie used for the removal of the pharmaceuticals is suitable for the bioremediation of PAR, IBU, and OLA in highly concentrated wastewaters.

Table 8.5 *Intraday and interday precision and accuracy for PAR, IBU, SIMA, OLA and SIM (n=6).*

Nominal concentration (μg mL^{-1})	Intraday (*n*=6)			Interday (*n*=18)		
	Measured concentration (μg mL^{-1}) mean ± SD	Precision %RSD	Accuracy %bias	Measured concentration (μg mL^{-1}) mean ± SD	Precision %RSD	Accuracy %bias
PAR (0.5)	0.49 ± 0.01	3.80	-2.82	0.50 ± 0.01	4.75	0.28
PAR (1.5)	1.53 ± 0.02	2.29	2.12	1.52 ± 0.01	1.61	1.23
PAR (50)	51.22 ± 0.55	2.14	2.44	50.74 ± 0.41	1.61	1.47
PAR (100)	100.56 ± 0.92	1.82	0.56	102.52 ± 1.04	2.02	2.52
IBU (0.5)	0.52 ± 0.01	5.20	4.69	0.52 ± 0.01	4.27	3.59
IBU (1.5)	1.60 ± 0.01	2.66	6.61	1.57 ± 0.02	3.34	4.52
IBU (50)	50.50 ±0.54	3.10	1.01	49.78 ± 0.38	2.21	-0.43
IBU (100)	98.90 ± 0.73	0.73	-1.10	102.73 ± 1.18	3.36	2.73
SIMA (0.5)	0.50 ± 0.01	5.54	0.95	0.50 ± 0.01	5.44	-0.03
SIMA (1.5)	1.49 ± 0.03	4.29	-0.60	1.50 ± 0.02	2.60	-0.29
SIMA (50)	53.24 ± 0.93	3.96	6.48	51.68 ±0.80	3.51	3.36
SIMA (100)	100.44 ±0.89	2.01	0.44	102.42 ± 2.40	5.31	2.41
OLA (0.5)	0.51 ± 0.02	3.98	2.89	0.51 ± 0.02	4.84	2.65
OLA (1.5)	1.49 ± 0.04	2.98	-0.59	1.49 ± 0.03	2.70	-0.29
OLA (50)	49.51 ± 1.26	3.10	-0.97	48.99 ±0.85	2.10	-2.03
OLA (100)	96.87 ± 1.13	1.41	-3.13	97.08 ±1.65	2.06	-2.92
SIM (0.5)	0.45 ± 0.01	5.25	-9.98	0.44 ± 0.01	5.00	-11.55
SIM (1.5)	1.52 ±0.02	2.81	1.57	1.52 ± 0.02	2.60	1.08
SIM (50)	45.05 ± 0.96	0.96	3.97	46.07 ±0.76	3.07	-7.86
SIM (100)	91.48 ± 0.77	1.57	-8.52	92.76 ± 0.88	1.76	7.24

Table 8.6 *Percentage of recovery of PAR, IBU, SIMA, OLA and SIM from the microalgae culture medium (n=6).*

	PAR			IBU			SIMA		
μgmL^{-1}	1	50	100	1	50	100	1	50	100
%	102.5	98.0	98.7	99.8	96.9	95.7	99.6	108.7	108.9

	OLA			SIM		
μgmL^{-1}	1	50	100	1	50	100
%	80.8	93.0	101.3	104.7	93.0	101.3

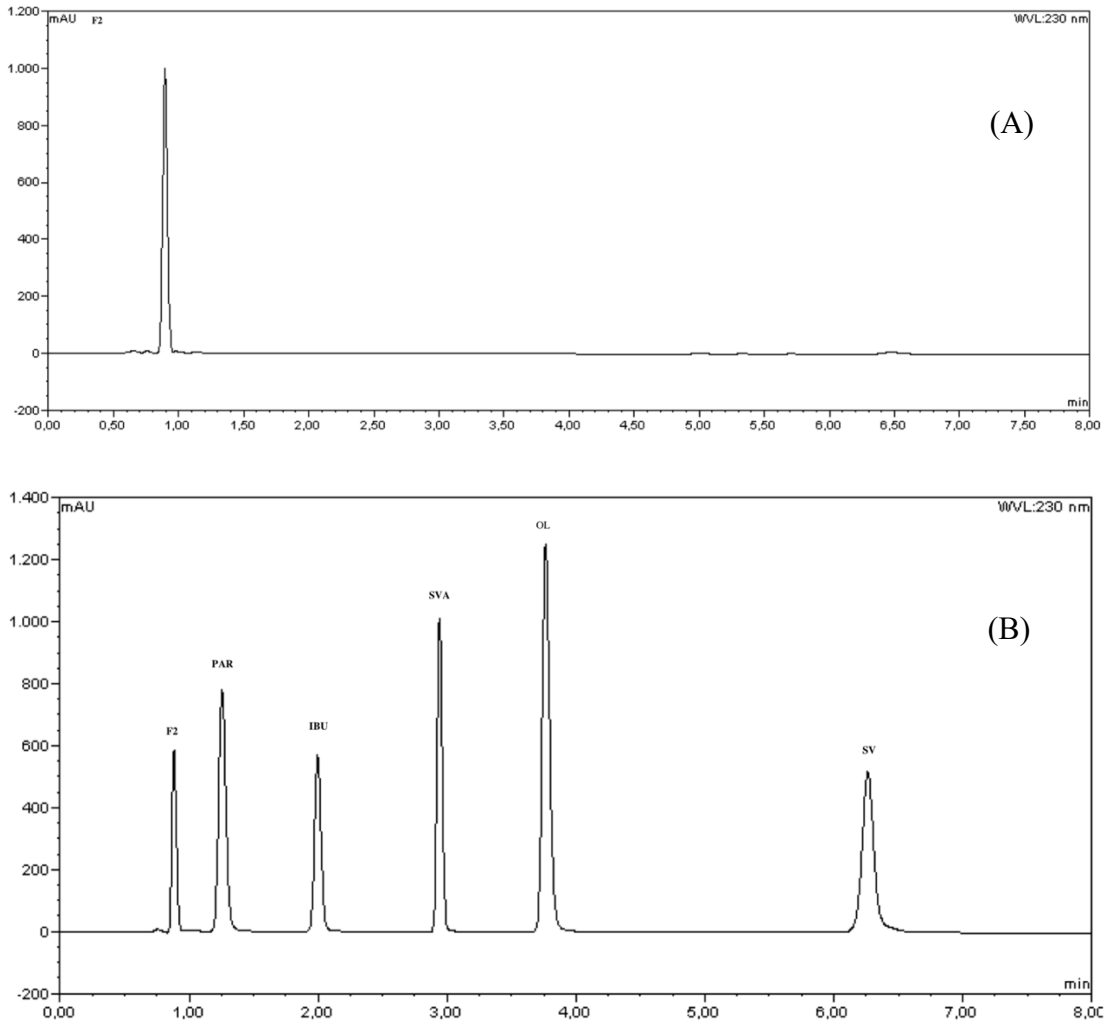


Figure 8.3 *Chromatograms (A) of f/2 medium and (B) of the standards PAR, IBU, SIMA and SIM considered for the evaluation of the matrix effect.*

8.4 Conclusions

An RP-HPLC method was developed and optimized for the determination and quantification of PAR, IBU, OLA, SIM and SIMA during bioremediation using microalgae. The developed method was shown to be rapid, linear, precise, robust and accurate, and is suitable for the evaluation of microalgae bioremediation efficiency. It is the is the first time that these five drugs were evaluated in simulated wastewater, with biodegradation and further bioremediation using *Nannochloropsis* sp. with some success. This can be reproduced at a larger scale in the near future.

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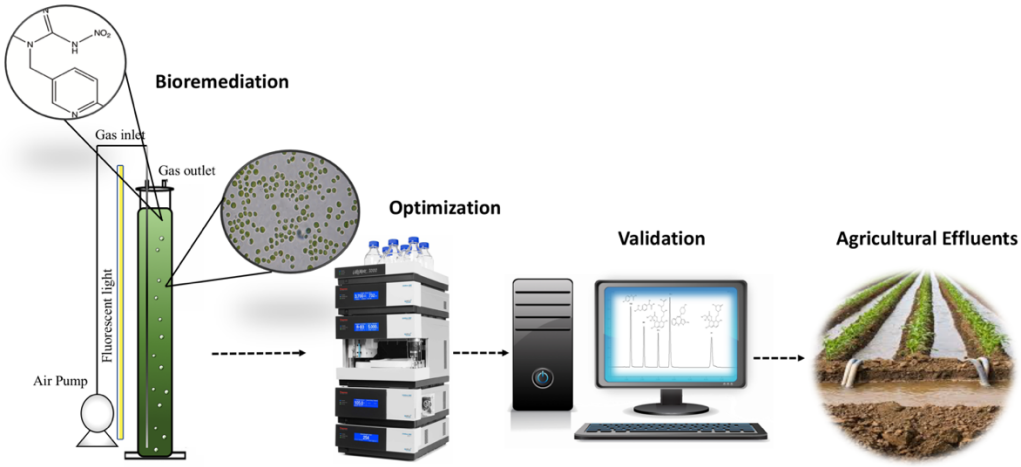
Chapter 9

Development and validation of a RP-HPLC method for the analysis of imidacloprid



A rapid reverse phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the analysis and quantification of imidacloprid in the context of microalgae bioremediation. The method was validated according to the guidelines of the US Food and Drug Administration (FDA), SANTE/ 11813 2017: Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed, and Water quality standards for imidacloprid Proposal for an update according to the Water Framework Directive, and Eurachem, with respect to system suitability, linearity, accuracy, precision, recovery, limits of detection and quantification, selectivity and specificity. The estimated limits of detection and quantification were, respectively, 0.030 and 0.091 µ mL^{-1} *for imidacloprid. The inter-day and intra-day precision results were within the acceptance limit of relative standard deviation (%RSD) of less than 5%, and the percentage recovery was found to be within the required limits of 80-115%. The developed method is rapid, linear, precise, robust and accurate, and has been successfully applied to the determination of the imidacloprid in water, during microalgae bioremediation.*

The content of the present chapter was fully or parttialy submited in paper form in Encarnação, T. Santos, D., Pais, A.C., Valente, A., Pereira, J.C., and Burrows, H.D. Removal of imidacloprid from water by the microalgae Nannochloropsis sp. and development and validation of a RP-HPLC method for the analysis of imidacloprid

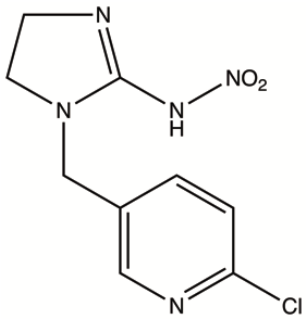
9.1 Introduction

The neonicotinoid insecticide imidacloprid, N-{1-[(6-Chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl}nitramide, is a systemic and contact insecticide, that act on the insect central nervous system (some of the physical and chemical characteristics of imidacloprid are presented in Table 9.1). It is often used in agriculture for seed treatment and to control insect pests in urban and residential areas. Imidacloprid has high toxicity and high stability, is persistent in the environment, it bioaccumulates and could be transported by winds and dust accumulation[1]. Can easily contaminate water resources trough streams and shallow groundwater[2], and it has been found in soil, groundwater, wetlands, dust, nontarget plants, vertebrate prey and food[3]. Imidacloprid, like all pesticides, are not easily removed from aquatic environments, which raise environmental and human health concerns. Bioremediation, activated carbon adsorption, reverse osmosis, nanofiltration, ozonation and chemical oxidation are some of the technologies which may be used. But none alone can remove or degrade them completely, instead, a combination of technologies could significantly reduce the occurrence of pesticides in the aquatic environment.

In the context of the bioremediation of imidacloprid, it is useful to understand in what extend the microalgae *Nannochloropsis* sp. is able to remove it from water. For that, a rapid and validated analytical method for the determination of imidacloprid is required. Therefore, this Chapter describes the development and validation of an RP-HPLC method for the detection and quantification of the neonicotinoid insecticide imidacloprid, applied in the bioremediation studies, presented in Chapter 5.

Table 9.1 *Physical and chemical characteristics of imidacloprid.*

Properties	**Chemical structure**
Chemical name	N-{1-[(6-Chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl}nitramide
#CAS	105827-78-9
Molecular weight	255.661 g mol^{-1}
Solubility (20 ºC)	610 mg L^{-1}
Vapor pressure	4.0 x 10^{-10} Pa
Melting point	136.4-141.8 ºC
Density	1.54 g cm^{-3}
pKa´s	5.28 and 9.39



9.2 Materials and methods

9.2.1 Reagents and chemicals

Imidacloprid was purchased from Sigma-Aldrich. Microalgae medium f/2 was obtained from Varicon Aqua Solution, Malvern, UK. All other reagents and solvents were of analytical or HPLC grade.

9.2.2 Instrumentation

The RP-HPLC analysis of imidacloprid was carried out in a Dionex Ultimate 3000 system equipped with an auto injector and four variable UV/VIS dual wavelenght detectors. The column used for the analysis was a Luna Phenyl-Hexyl, Phenomenex® (Torrance, USA), with 5 µm particle size, 3 mm internal diameter and 150 mm lenght, supported with a SecurityGuard™ cartridge Phenomenex® (Torrance, USA), with 3.0 mm internal diameter in an oven temperature of 35 ˚C. The data were recorded using Chromeleon software. Chromatographic analysis was conducted in isocratic mode. Preferential UV detector was set at 275 nm for the detection of imidacloprid. The injection volume was 10 µL for standard and samples. Before analysis, every standard and sample was filtered through 0.22 µm filters. A run time of 3 min was found adequate for the analysis of the analyte.

9.2.3 Preparation of mobile phase, stock and standard solutions and quality controls

The mobile phase consisted of a mixture of acetonitrile and ultrapure water of 50:50 (v/v), at a constant flow rate of 0.8 mL min^{-1}. pH was 7.0 ± 0.2. All solutions were filtered through a 0.22 µm membrane filter, and sonicated to degas. A stock solution at 1 mg mL^{-1} of imidacloprid was prepared. Two working standard solutions containing the analyte were prepared at concentrations of 100 and 10 µg mL^{-1} by dilution of the stock solution with the mobile phase. For the determination of the limits of detection and quantification, six standard solutions (0.1, 0.25, 0.5, 0.75, 1 and 1.25 µg mL^{-1}) were prepared from the 10 µg mL^{-1} working solution. Six replicates of eight standard solutions (0.5, 1, 5, 10, 25, 50, 75, 100 µg mL^{-1}) were prepared by dilution of the working standard solution with the mobile phase. For the quality control solutions, six replicates of 0.5, 1.5, 10, 50 and 100 µg mL^{-1} standards containing the analyte were considered. All stock solutions were stored at -20 ºC and working solutions were freshly prepared as needed.

9.2.4 Method Validation

The method for imidacloprid quantification in water was validated according to the guidelines of the US Food and Drug Administration (FDA)[4], SANTE/ 11813 2017: Guidance document on analytical quality control and method validation procedures for pesticide residues and

analysis in food and feed[5], and Water quality standards for imidacloprid Proposal for an update according to the Water Framework Directive[6], and Eurachem[7], with respect to system suitability, linearity, accuracy, precision, recovery, limits of detection and quantification, selectivity and specificity.

System suitability. System suitability test ensures that the complete testing system, including instrument, reagents, column and analyst, is suitable for the intended application. For that purpose, six consecutives injections were made with the standard solution of imidacloprid at a concentration of 75 μg mL^{-1}. The parameters theoretical plate number (N), capacity factor (k′), resolution (R), tailing factor(T) were analysed. N is indicative of column efficiency, k′ is a measure of where the peak of interest is located with respect to the void volume, R is a measure of how well two peaks are separated, and T is a measure of peak symmetry.

Limits of detection (LOD) and quantification (LOQ) LOD is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value, and LOQ is the lowest amount of analyte in a sample which can be quantitatively determined with acceptable accuracy and precision. The LOD and LOQ values were determined by using regression parameters from a calibration curve from six standard solutions (0.1, 0.25, 0.5, 0.75, 1 and 1.25 μg mL^{-1}) containing the analyte (3.3 σ/S and 10σ/S, respectively; where σ is the standard deviation of the residues and S is the slope).

Linearity Linearity is a critical criterion for quantitative analysis and is a measure of accuracy over the range of the method. The linearity of the proposed method was evaluated through calibration curves, constructed with eight standard solutions, containing the analyte, ranging from 0.5 to 100 μg mL^{-1}, to calculate coefficient of correlation, slope, and intercept values. Data were analysed using the Analysis ToolPak of Microsoft Excel® (Microsoft Corp., Redmond, WA) with linear regression by the least squares method.

Accuracy and precision. The accuracy of an analytical method expresses closeness between the reference value and the value that was actually found. The mean value should be within 15% of the theoretical value, except at the LLOQ, where it should not deviate by more than 20% [8]. The accuracy of the method was established by analysing six replicates of the four quality controls and by calculating the trueness for each analyte. Trueness is expressed in terms of bias, and represents the systematic deviation from a true central value and was calculated as

% accuracy = (observed concentration/nominal concentration) x 100.

Precision represents the degree of concordance between the measured value and the reference value [9]. Precision is generally dependent on analyte concentration and must therefore be determined within the range of concentrations of interest. Evaluation of precision was determined by repeatability (intra-day) and intermediate precision (inter-day) for three consecutive days. Six

replicates of four quality control solutions (0.5, 1.5, 10, 50 and 100 μg mL^{-1}) were prepared and analysed according to intra-day and inter-day precision. The relative standard deviation (RSD) of the results should be lower than 15%, according with the requirements, except for the LLOQ, where it should be lower than 20%.

Selectivity and specificity Analytical selectivity could be interpreted as "*the extent to which the method can be used to determine particular analytes in mixtures or matrices without interferences from other components of similar behaviour*"[10]. IUPAC recommends the term selectivity, while other areas, e.g. pharmaceutical field, use the term specificity, yet agreement exists on the interpretation. In the developed method, the response of the solution containing only IMID was compared with microalgae culture medium f/2.

Recovery. Recovery studies may be used to address the level of bias. The recovery of IMID from the microalgae culture medium f/2 was determined by comparison of the respective concentrations with those of standard solutions in the mobile phase at three different concentrations (1, 50 and 100 μg mL^{-1}).

Method applicability: removal of imidacloprid from water by *Nannochloropsis* sp. *Nannochloropsis* sp. was obtained from Varicon Aqua Solution, Malvern, UK, and was cultivated for 6 days in 2 L f/2 medium. 100 cm^3 of a *Nannochloropsis* sp. culture were filtered and washed, and cells were subsequently transferred to a closed photobioreactor, to which 100 cm^3 of f/2 culture medium Cell-hi TEViT (Varicon Aqua Solution, Malvern, UK) were added. Nitrate concentration was 0.30 g L^{-1} and salinity 25 g L^{-1}. To the 100 cm^3 *Nannochloropsis* sp. culture, a concentration of 10 μg mL^{-1} of IMID was added. Similarly, a blank with 10 μg mL^{-1} of IMID was added to 100 cm^3 of culture medium f/2, without cells. The cultures and the f/2 media were aerated by bubbling atmospheric air, at a rate of 300 cm^3 min^{-1}, and grown at 25±2 ºC under light with an irradiance level of ± 100 μmol $m^{-2}s^{-1}$ with 24:0 photoperiod and kept for 7 days. Millipore water were added when needed to ensure the same volume due to water loss by evaporation. Samples of 15 mL were replaced with fresh f/2 culture medium. Each experiment was carried out in triplicate.

9.3 Results and discussion

9.3.1 Method development and optimization

A RP-HPLC method for the rapid analysis of IMID was developed. The Phenyl-Hexyl column was chosen because of the excellent resolution, selectivity and good sensitivity, and the increased retention of aromatic compounds. The pKa´s of the analyte was also considered; pKa´s from IMID are 5.28 and 9.39. Following the usual rule of thumb, the pH of the mobile phase should be selected two units above or below the pKa of the analyte, therefore, a pH value around 7 was chosen. Regarding mobile phase, acetonitrile was the choice for eluent A and water for eluent B. Different ratios of acetonitrile and water were tested and the ratio 50:50 (v/v) was used to elute the analyte. Under the described conditions, IMID eluted at 1.45 min. The method was validated over the range of 0.5-100 μg mL^{-1}.

9.3.2 Method validation

System suitability The system suitability parameters, summarised in Table 9.2, including %RSD, theoretical plates, tailing factor and resolution, met the required criteria. Peak show symmetry and high resolution. Capacity factor value for IMID is below 2. The results obtained with the system suitability indicate that the selected chromatographic parameters are suitable to identify IMID (Figure 9.1).

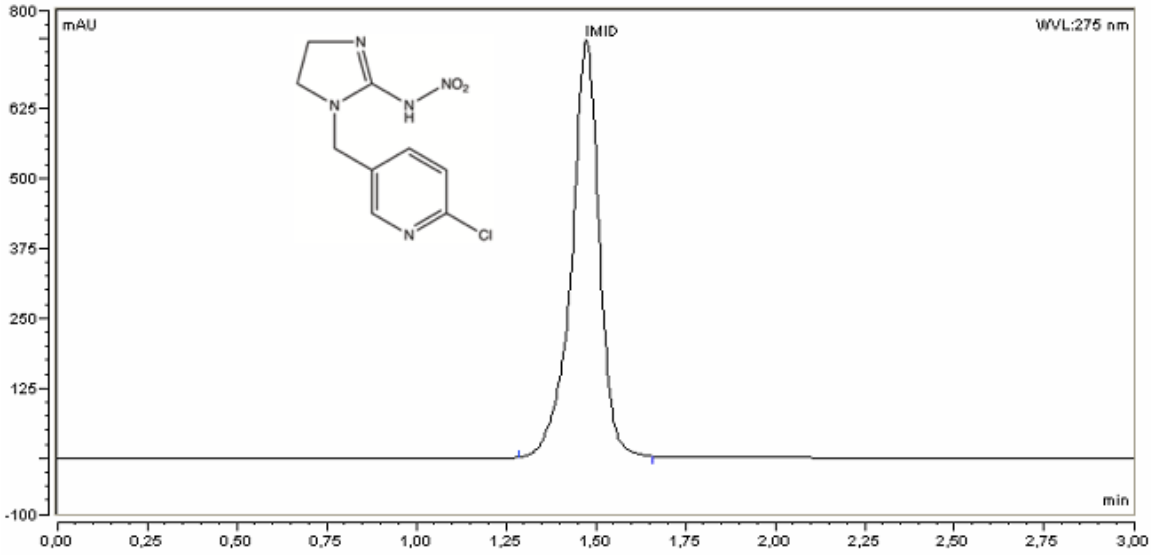


Figure 9.1 *Chromatogram of the standard 75 μg mL^{-1} solution of IMID, considered for the evaluation of the system suitability.*

Limits of detection (LOD) and quantification (LOQ) The estimated values for LOD and LOQ are 0.030 and 0.091 μ mL^{-1} respectively.

Table 9.2 *System suitability test parameters.*

Chromatographic parameters	IMID (75 μg mL^{-1})		
	Retention time (min.)	Peak area	Acceptance criteria
Mean (*n*=6)	1.4525	78.4	-
S.D.	0.0012	1.3	-
%RSD	0.08	1.7	≤ 2.0%
Theoretical plates (*N*)	2598		> 1000
Capacity factor (*k´*)	1.33		> 2.0
Tailing factor (*T*)	0.82		≤ 2.0
Resolution (r_s)	3.96		> 2.0 (> 1.5 [ac])

Linearity For the assessment of the linearity of the method, solutions with concentrations in the range of 0.5-100 μg mL^{-1} were evaluated. The mean slope of 0.90 ± 0.004 and the mean intercept of 0.22 ± 0.20 The correlation coefficient was found to be more than 0.9999 for the analyte, indicating good linearity of the calibration curve. The residual standard deviations close to zero, 0.41, for IMID, confirm linearity. The residuals plot for IMID indicates that the model is good fit for high concentrations, and a good predictor of lower concentration values.

Accuracy and precision The data obtained with the evaluation of the accuracy and precision are shown in Table 9.3. All the results met the acceptance criteria. The intra-day and inter-day %RSD and bias values fall within the acceptance criteria of 15% of the theoretical value demonstrating that the developed method is accurate, reliable and reproducible.

Table 9.3 *Intraday and interday precision and accuracy for IMID (n=6).*

Nominal concentration (μg mL^{-1})	Intraday (*n*=6)			Interday (*n*=18)		
	Measured concentration (μg mL^{-1}) mean ± SD	Precision %RSD	Accuracy %bias	Measured concentration (μg mL^{-1}) mean ± SD	Precision %RSD	Accuracy %bias
0.50	0.483 ± 0.010	2.1	3.4	0.498 ± 0.012	2.4	0.4
1.5	1.418 ± 0.032	2.2	5.5	1.423 ± 0.028	1.9	5.1
10	9.05 ± 0.21	2.3	9.5	9.14 ± 0.19	2.1	8.6
50	46.4 ± 1.0	2.2	7.2	46.1 ± 1.4	3.0	7.8
100	92.6 ± 1.3	1.4	7.4	93.3 ± 1.0	1.1	6.7

Selectivity and specificity The selectivity of the method was evaluated by analysing standard solutions in the presence of the components of the culture medium (Figure 9.2). The absence of any signal at the same elution time as the analyte suggests that there were no matrix interferences.

Recovery All calculated mean recovery (trueness) are in the range of 108-112% being within in the acceptable recovery percentage of 70-120% for the analytes concentration of 1 ppm indicating the adequacy of simultaneous quantify the analyte.

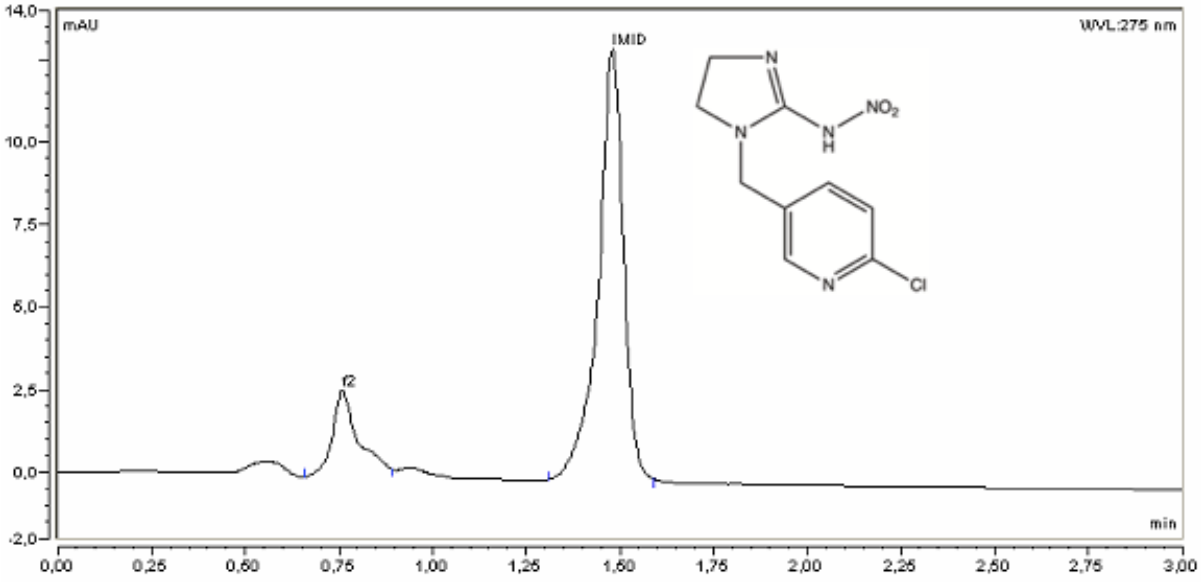


Figure 9.2 *Chromatogram of the standard 1 µg mL^{-1} solution of IMID considered for the evaluation of matrix interferences.*

Method applicability: removal of imidacloprid from water by *Nannochloropsis* sp. The method applicability was assessed by evaluating the efficiency of bioremediation, using the microalgae *Nannochloropsis* sp. for the removal of the pesticide IMID from contaminated water. To quantify IMID in contaminated water, microalgae cells were filtered, and the supernatant was analysed by RP-HPLC by the method developed in this work. *Nannochloropsis* sp. removed 4.39 from 9.59 µg mL^{-1} of IMID in the first 20 hours. The present study demonstrates that this specie used is suitable for the removal of the neonicotinoid pesticide IMID in highly concentrated wastewaters.

9.4 Conclusions

An RP-HPLC method was developed and optimized for the determination and quantification of IMID during bioremediation using microalgae. The developed method was shown to be rapid, linear, precise, robust and accurate, and is suitable for the evaluation of IMID microalgae bioremediation efficiency. Despite the previous results obtained, further studies should be carried on in industrial scale to optimize the use of *Nannochloropsis* species.

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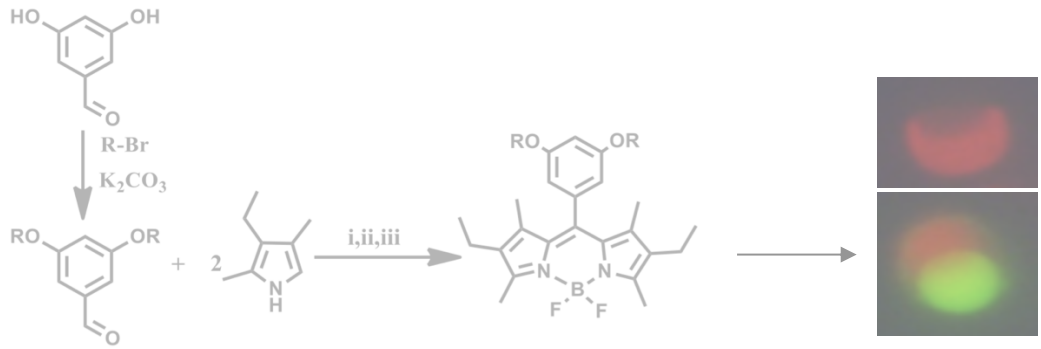
Chapter 10

New amphiphilic BODIPYs as lipid probes in microalgae

Among the numerous classes of highly fluorescent probes, the family of compounds based on the 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene, commonly known as BODIPY, are of special interest, due to the excellent physical chemical properties and low toxicities. The combination of these highly lipophilic fluorescent probes and image analysis software, permit to visualize and quantify the lipid content, and to monitoring lipid production in microalgae. Therefore, BODIPYs are among the most promising candidates for the next generation of in vivo fluorescent labels and probes.



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10.1 Introduction

Fluorescence spectroscopy and imaging are important tools in the fields of clinical diagnostics, biotechnology, biochemistry, materials science and environmental chemistry[1-5]. Among the numerous classes of highly fluorescent probes, the family of compounds based on the 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (commonly known as BODIPY) shows great potential and therefore have become very popular as fluorescent probes within the last decade.

The synthesis of the first member of this group was reported as early as 1968,[6] and the uses of these pyrrolic compounds have been growing thenceforth. Since the first reports, the BODIPYs have been studied in a wide variety of possible applications, such as fluorescent[7], ion/molecule[5, 8] and pH probes[9], sensors for redox active molecules[8,] metal chelators[8, 10, 11], agents for cellular imaging[12, 13] photodynamic therapy[14, 15], drug-delivery materials [8 16] and organic dyes for photovoltaic applications[17, 18].

BODIPY's properties such as photochemical and chemical stability, high molar absorption coefficients and high fluorescence quantum yields, negligible triplet-state formation, narrow emission bandwidths with high peak intensities, good solubility, resistance to self-aggregation in solution, excitation/emission wavelengths in the visible spectral region and fluorescence lifetimes in the nanosecond range make these compounds very appropriate for the applications referred above[7, 19, 20]. As a direct consequence of their excellent physical chemical properties and low toxicities, BODIPYs are among the most promising candidates for the next generation of *in vivo* fluorescent labels and probes. Several examples have been reported in the literature proving their applicability as fluorescent sensors of biomolecules such as proteins[21, 22], toxins[23] , monosaccharides[24] or lipids [25, 26].
The direct *in vivo* quantitative determination of lipids is a matter of great industrial importance. Actual techniques to measure *in vivo* or *ex vivo* the lipid content in specific organisms require expensive equipment and involve long working times[27-29]. In opposition, fluorescent dyes offer an indirect but fast measurement for lipids. Some dyes, namely Nile Red have been used to label lipid droplets within microalgae cells, and recently the BODIPY 505/515 was presented as a good potential marker for visualizing neutral lipid content by fluorescence microscopy[30]. The BODIPY 505/515, which is commercially available [31], usually provides a clear and distinct lipid fluorescence imaging but has some disadvantages, such as sensitivity to acid and basic treatments, a fluorescence spectrum influenced by the solvent polarity and a tendency to form aggregates[30], leaving room for further improvements in the class of BODIPY fluorescent sensors.

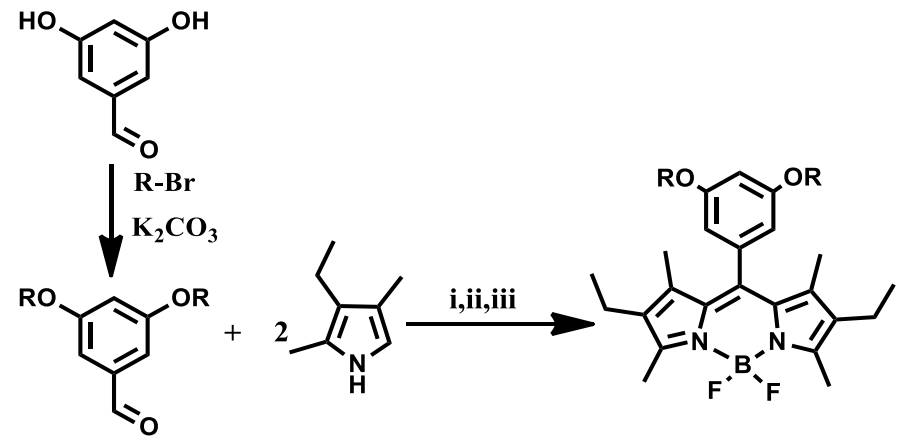
BODIPY derivatives with long linear alkyl chains on the periphery can minimize the aggregation problem associated with the π-π stacking, which is common with this kind of compounds. In fact, BODIPYs using a single long alkyl chain have already been reported, and are also valuable fluorescent probes for applications in the study of fluidity changes in cell membranes [32] or sol-to-gel transitions[33].

To the best of our knowledge there are no reports using a BODIPY having two long alkyl chains as fluorescence probes. These systems would allow, in principle, a better staining activity due to increased lipophilicity in cell membranes, when compared with BODIPY 505/515, in addition to decreased aggregation processes due to the bulky effect of those double chains. Therefore, we report here, the evaluation of a new family of lipophilic BODIPYs bearing at the meso phenyl group, two alkoxy groups with long linear alkyl chains (C10, C12, C14, C16) in biological systems, looking for their potential as sensors for lipids, produced by microalgae of the genus *Nannochloropsis* sp..

10.2 Materials and methods

10.2.1 General procedure for the preparation of the BODIPYs

The 3-Ethyl-2,5-dimethyl-pyrrole and the alkylated 3,5-dihydroxybenzaldehyde were added to dried, freshly distilled dichloromethane (150 mL) and the solution was stirred for 1 h under a flux of N_2. Then, trifluoroacetic acid was added (3 drops) and the mixture was stirred overnight under N_2 atmosphere, at room temperature. After that, 2,3-dichloro-5,6-dicyanobenzoquinone was added to the reaction mixture and this was stirred overnight, under N_2 atmosphere, at room temperature. Then N, N-diisopropylethylamine and BF_3EtO_2 were added to the reaction mixture and the reaction was left under slow stirring overnight, under N_2 atmosphere, at room temperature. The solvent was evaporated under reduced pressure. After solvent evaporation, 150mL of dichloromethane were added and the washing was made with water and brine. Drying of the organic phase was accomplished with anhydrous Na_2SO_4. General procedure for the preparation of the BODIPYs is schematised in Figure 10.1.



Ald-C10 R: $CH_2(CH_2)_8CH_3$
Ald-C12 R: $CH_2(CH_2)_{10}CH_3$
Ald-C14 R:$CH_2(CH_2)_{12}CH_3$
Ald-C16 R:$CH_2(CH_2)_{14}CH_3$

BD-C10 R: $CH_2(CH_2)_8CH_3$
BD-C12 R: $CH_2(CH_2)_{10}CH_3$
BD-C14 R:$CH_2(CH_2)_{12}CH_3$
BD-C16 R:$CH_2(CH_2)_{14}CH_3$

Figure 10.1 *General procedure for the preparation of the BODIPYs. i) TFA; ii) DDQ; iii) N,N-diisopropylethylamine and $BF_3.Et_2O$.*

10.2.2 Preliminary evaluation of double alkyl chain BODIPYs as lipid fluorescent probes

This new series of BODIPYs, designed to possess high lipid solubility and membrane permeability to behave as fluorescent probes in living biological systems, was tested *in vivo*, choosing as targets the lipids produced by microalgae of the genus *Nannochloropsis* sp.

Stock solutions of the BODIPYs were prepared in 50:50 (v:v) ethanol:DMSO to give a stock solution of about $3x10^{-4}$ M and stored in the dark. 2 mL samples of algal cells were stained with 5µL of the stock solution. After incubation in the dark at room temperature for 20 min, 2 ml of stained cells were filtered through Nylon 66 membrane filters with a pore diameter of 0.2 µm, washed with ultrapure water to discard the fluorescence in the culture medium and resuspended in 2 ml of ultrapure water. The maximum algal cell concentration used was $8.3x10^7$ cell/ml.

Steady state fluorescence spectral measurements were then performed on the cells and emission features compared. All fluorescent spectra were made by using an excitation wavelength of 524 nm (or 522 nm for BD-C10).

10.3 Results and discussion

Figure 10.1 shows the fluorescence spectra of the cell suspension after staining with the new BODIPYs. The spectra were taken using a front-face configuration in the fluorimeter, to avoid errors caused by scattering and self-absorption by the microalgae solutions.

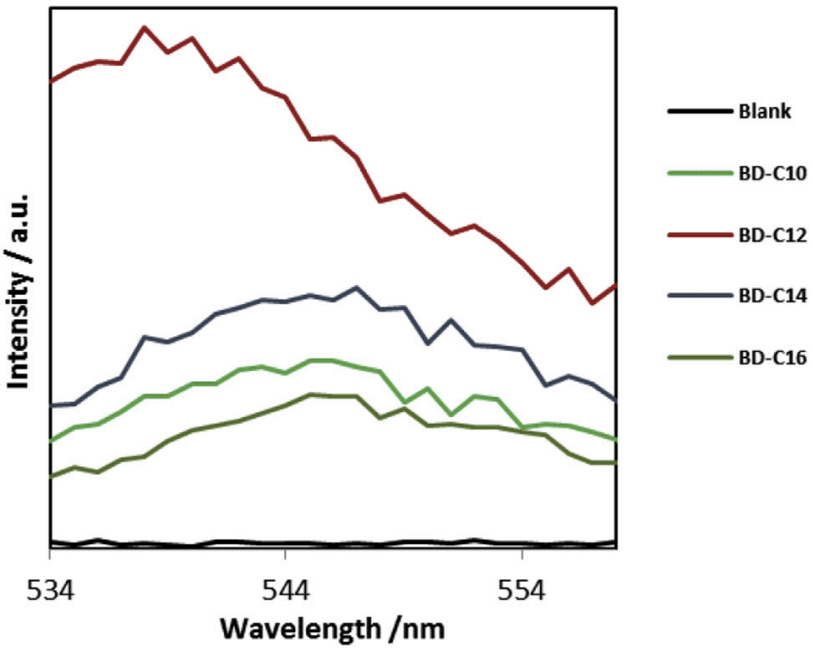


Figure 10.2 *Fluorescence spectra for microalgae solutions stained with the new BODIPY dyes.*

From the analysis of the fluorescence spectra, we see differences in the efficiency of staining cellular lipid bodies with the different BODIPYs. The emission maxima are the same for all the dyes except for BD-C12. In fact, for samples stained with the new BODIPY derivative bearing twelve carbon atom alkyl chains, the maximum is around 538 nm, while for all the other samples the maximum is 546 nm, corresponding to a bathochromic shift of 7 nm. In addition, the fluorescence intensity of the samples stained with that dye is higher than for all the others. From these preliminary results it seems that BD-C12 interact with the cell membrane and may be a good lipid probe.

In Figures 10.2 and 10.3 we can see the cells stained with the various BODIPYs tested. It is worth noting that the dyes were easily taken up, and no signs were observed of morphological damage to the cells upon treatment with these new chromophores, in

agreement with other studies on related BODIPYs[35]. In the fluorescence microscopy images corresponding to BD-C12, fluorescent oil droplets can be seen in all cells. In contrast, cells stained with others BODIPYs show fewer fluorescent bodies and in fact, most of the cells do not exhibit any fluorescence due to the BODIPYs. We may confirm from these observations that BD-C12 is the dye with the best capacity for probing the lipid content of the cell of *Nannochloropsis* sp. Although the reason for this is not fully explained at present, it must be associated with the balance between hydrophobic and hydrophilic interactions that allows for a better cell uptake of the BD-C12.

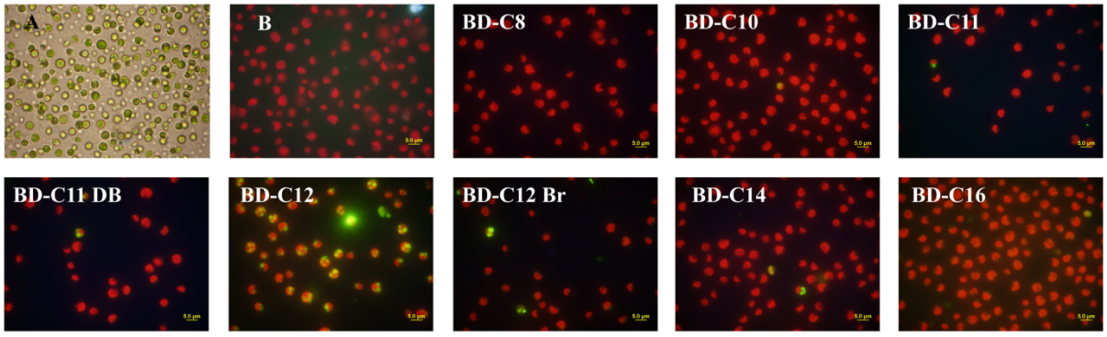


Figure 10.3 *Optical microscopy images of Nannochloropsis sp. cells (A), fluorescence microscopy images of Nannochloropsis* sp. *cells showing red chlorophyll autofluorescence emitted by chloroplasts (B) and the green fluorescence due to the staining with the new BODIPYs BD-C8/C10/C11/C11DB/C12/C12Br/C14 and C16.*

In order to confirm that the interaction of BODIPY BD-C12 actually involve the lipids and no other biomolecules, we checked for the interaction between that BODIPY fluorophore and the surfactant dodecyltrimethylammonium chloride (DTAC), known to mimic lipid properties through micelle formation. The assays were made by placing 2 cm^3 of ultra-pure water in a quartz 1x1 cm cuvette, and adding to this $5x10^{-3}$ cm^3 of a solution of BD-C12 in ethanol/DMSO (1:1, v/v), giving a final BODIPY concentration of $6.63x10^{-7}$ M. Then, aliquots ($10x10^{-3}$ cm^3) of the surfactant solutions, $1.86x10^{-1}$ M for DTAC, in ultra-pure water, were added and the fluorescence spectra taken after each addition. This initial concentration was chosen to give data below and above the critical micelle concentration of the surfactant. Excitation spectra were taken from the initial solution of BD-C12 and at the end of the experiment. From the spectra we can see that, upon addition of the surfactant, the maximum of absorption shifted from 526 nm to 529 nm and the shape of the spectrum becomes slightly sharper. In addition, the intensity of the fluorescence excitation spectrum increased upon the addition of the surfactants, meaning that the surfactants are inhibiting radiationless deactivation of the excited state of the BODIPY BD-C12, probably due to increased hydrophobicity interactions. In Figure 10.3 we see a clear enhancement of the fluorescence intensity

upon addition of the surfactants to the BD-C12 solution. The intensity increases until the concentration of surfactant reaches the critical micellar concentration (cmc). From the moment that micelles of surfactant start to form, the interactions between the BODIPY and the surfactant molecules are constant and the dye is solubilized within the micelles. In addition, we observe a small bathochromic shift in the emission maximum wavelength, confirming changes in the BODIPY environment as a consequence of incorporation within micelles of the surfactant molecules.

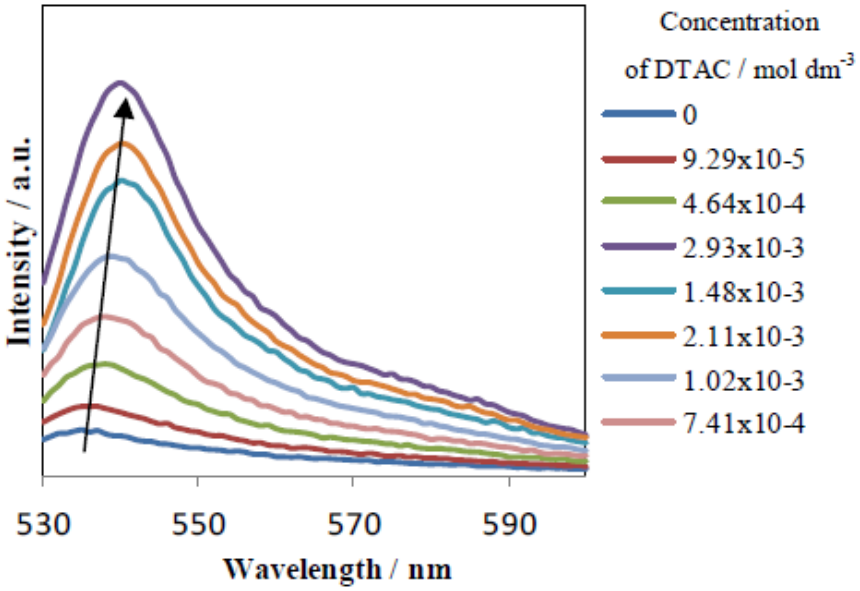


Figure 10.3 *Influence of the addition of a DTAC solution in the emission spectra of a BD-C12 solution in ultra-pure water.*

10.4 Conclusions

BODIPYs present a class of compounds with increasing scientific and technological interest that are suitable for fluorescence sensing and imaging of biomolecules. We synthesised a new series of BODIPYs bearing double long alkyl linear chains, designed to possess high lipid solubility and therefore be able to act as lipid fluorescent probes. The synthesis was planned to introduce hydrophobic motifs without disturbing the original excellent fluorescent properties. Different double alkyl chain BODIPY derivatives as fluorescent probes in living biological systems was tested, choosing as targets the lipids produced by microalgae of the genus *Nannochloropsis*, and the results were very positives for the new BODIPY BD-C12 with twin n-dodecyl alkyl groups.

From the experiments described in this chapter, both in fluorescence spectroscopy and in cell imaging through fluorescence microscopy, it may be concluded that from all the synthesized BODIPYs the new compound BD-C12 stood out as the best candidate for a lipid sensor. The experiments with surfactants, taken as lipid models, strongly support the idea that this behavior results from an interaction between the BODIPY BD-C12 and the lipid organelles within the microalgae cells.

In conclusion, it can be seen that the new BODIPY BD-C12 has very good properties as lipid fluorescent sensor, being very promising for lipid determination with microalga cultures, since it allows lipid content determination necessary to optimize cultivation conditions, to maximize the lipid production and to choose the best harvest time, in lipid based biotechnology.

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Part E

Final Remarks

Chapter 11

Conclusions and future work

The work developed in this thesis contributes to bioremediation of emerging pollutants from water, using microalgae. We present a viable approach based on the generation of biomass, and on its respective economic valorization. This aims at obtaining high value-added products that may attract investment in this field and, therefore, accelerate the development of microalgae technology. This is focused on a biorefinery concept; a concept based on sustainability and in circular economy.

Various important conclusions can be drawn from all the work developed in this Thesis. The first concerns the bioremediation process. The microalga *Nannochloropsis* sp. can be considered to be a promising species for the removal of emerging pollutants from effluents. This species remained alive in the presence of the pollutants studied, and removed them from water although with varying efficiencies. Immobilisation studies indicated that PVA beads have limited the growth of the cells; however, comparing the behaviour with free cells, the immobilised ones show a higher resilience in the presence of the pollutants. Considering the integrity of the beads, the experiments indicated the dissolution of the PVA beads, as has previously been described in the literature with related polymers and materials. This could cause additional environmental issues, such as the release of PVA to the environment, together with the removed pollutants. More research is required to improve the stability and integrity of the beads, possibly through crosslinking. The bioremediation studies also allow us to infer the potential impact of the propagation of anthropogenic pollutants to higher trophic organisms and, eventually, to humans, due to the biomagnification of pollutants within the food chains. Through this food-web biomagnification of pollutants, entire ecosystems and all forms of life are exposed to such pollutants that can, potentially, produce harmful effects on living organisms.

More questions than answers where found considering the bioremediation process: what metabolites are produced? Are they converted into innocuous or less toxic products? What factors influence the removal efficiency? These questions require further research and development.

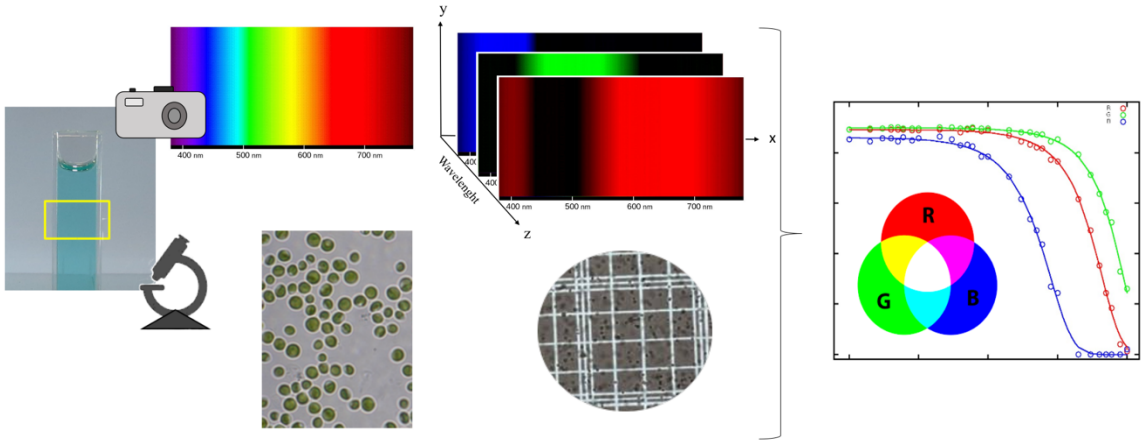
Considering the case of high-value products, a novel method, combining the BODIPY BD-C12 fluorescent probe and simple image processing, was successfully developed, and could help to optimize cultivation conditions and maximized lipid production. This also demonstrates that oil produced from *Nannochloropsis* sp. is a promising biobased feedstock, since optimized culture conditions can result in a very substantial increase of lipid biosynthesis. *Nannochloropsis* sp. is rich in lipids, carbohydrates and proteins. After the extraction of lipids, the lipid free residues can be neutralised and concentrated to produce sugars, which can be fermented by a wide range of microorganisms. One product obtained from fermentation is lactic acid, which is an important biobased block for producing PLA. We have focused on the final stage of whole value chain and circular economy; after the bioremediation using algae, the maximisation of lipids and their extraction, and the processing of residues for producing PLA, a new application was developed.

The results demonstrated that PLA polymers are potential candidates for advanced optical applications and finds applications in ophthalmic lenses, which can be used in prescription glasses and sunglasses, with the potential to be the first green lens in the market. For this, more research is required; additives are needed to influence the properties of the biopolymeric materials. Therefore, a new class of effective, "green" and environmentally friendly additives needs to be found.

As a final note, during the course of my work, I came across with the opportunity to work with schools and comunities, to raise awareness to the global pollution. There, I found a fertile ground for change. Events, and changes in lifestyles, took place. Understanding the behavior of the societies, what drives them to change lifestyles and what can influence and implement policies is a multidisciplinary field that urgently needs to be developed.

Appendix A

Microalgal cell density estimation with digital image-based colorimetry



Using colour index information of RGB digital images, it is possible to relate the instrumental responses with several features of the photographed objects in a quantifying approach. In this work, we used a high quality digital camera and controlled image acquisition conditions to evaluate cell density in microalgae culture suspensions. Results showed that it is possible to accurately assess cell density (less than 15 % of relative standard error) over about 2 orders of magnitude in cell concentration in terms of cell number. The proposed method is coherent and robust and does not requires constant recalibration. This method allows us to obtain a very fast and accurate estimation of the cell population, and finds application in large-scale production of microalgae.

This section concerns the development of a new method to evaluate microalgae cell population. The study will be submitted in a scientific journal after conclusion of the experimental work. The method described can improve efficiency and reduce time consumption, providing an alternative to the Neubauer chamber and the UV photospectroscopy methods.

A.1 Introduction

Microalgae are considered one of the most promising renewable resource for high value biobased products and its use can be extended in a number of ways including the production of hydrogen, biodiesel and biopolymers, or even bioremediation of both organic and inorganic pollutants[1,2]. The microalgal biomass is both environmentally and economically relevant, and microalgae can be commercially cultivated in open ponds and closed photo-bioreactors. The main advantage of microalgae is related to the high oil to biomass ratio and in their rapid cycle of growth. The microalgae population varies very rapidly with culture age, and several metabolic pathways are activated at different growth stages, whereby, it is possible to promote changes in the lipids and in polyunsaturated fatty acids (PUFA´s) profile, and in many other compounds, by manipulating the cultivation method and harvesting at a specific growth phase for commercial purposes[3]. In this sense, it is important to monitoring the growth phases of microalgae cultures. The standard cell density evaluation methods include hemocytometers, nannoplankton chambers, spectrophotometry, fluorometry and Secchi depth[4]. However, these methods present inconveniences, particularly with regard to the time consuming and accuracy. Spectrophotometry is a quality assessment technique which may provide an alternative. It uses essentially analyte UV-visible and IR response to evaluate cell concentration. The analytical advantages are related with the fact of using monocromatic radiation light sources, well controlled experimental conditions and a very sensitive detector/transducer for quantification purposes. Due to its very sensible electronics, these instruments require a controlled laboratorial environment and face some analytical problems when dealing with real samples and non-homogeneous solutions-dispersed media and interfering agents are some of these problems. Flow cytometry is another common method for measuring cellular culture density and it is also used to analyse different bioprocesses in microalgae, such as the production of lipids and pigments[5]. The disadvantages of this technique include the fact that is an expensive technique, with high operating costs, requiring highly trained personnel to operate and maintain, and standardisation of methods and measurements.

As alternative, colour evaluation is an interesting technological tool for the analysis of cellular culture density, providing direct, rapid and consistent measures.

Usually, colour evaluation is used in terms of comparison with established reference standards, stored and preserved in controlled conditions which may be, side-by-side compared. Digital imaging consists thus of a non-expensive and high-quality technology, with applications in industrial quality control.

Some of the work based on digital imaging processing have focused on applications specific to microalgal growth and respective biomass assessment. Pioneer work is found in reference 6, focusing on the analysis of *Ajuga pyramidalis* cells to monitor biomass productivity and

anthocyanin pigment accumulation, without interrupting the culture cycle. In the same context, a simple method to assess cell growth of *Rhodococcus erythropolis* DCL14 in the presence of alcohols and hydrocarbons was proposed using standard software[7]. More recently, a method was developed[8] to determine cell biomass concentration and distribution of *Rhodobacter capsulatus* in photobioreactors. Also, relevant aspects such as the light intensity distribution inside the photobioreactors and the effect of light tubes arrangement[9] have been assessed. A smartphone camera was used to capture images of leafs from soybean plants, for the estimation of total chlorophyll *a* and *b* and carotenoids, giving accurate estimations[10]. In that work, RGB, and also other color models, were extracted from the smartphone images and correlated to the values obtained spectrophotometrically.

In the present work, we explore the possibility of using a non-destructive method based on digital imaging and numerical treatment in order to more accurately evaluate colour evolution and quantify microalgae cell colony growth. A mathematical model was developed to estimate culture cell density as a function of color indexes in the red-green-blue (RGB) (Figure A.1) and a one-year time lapse between experiments allowed the verification of the robustness of the model.

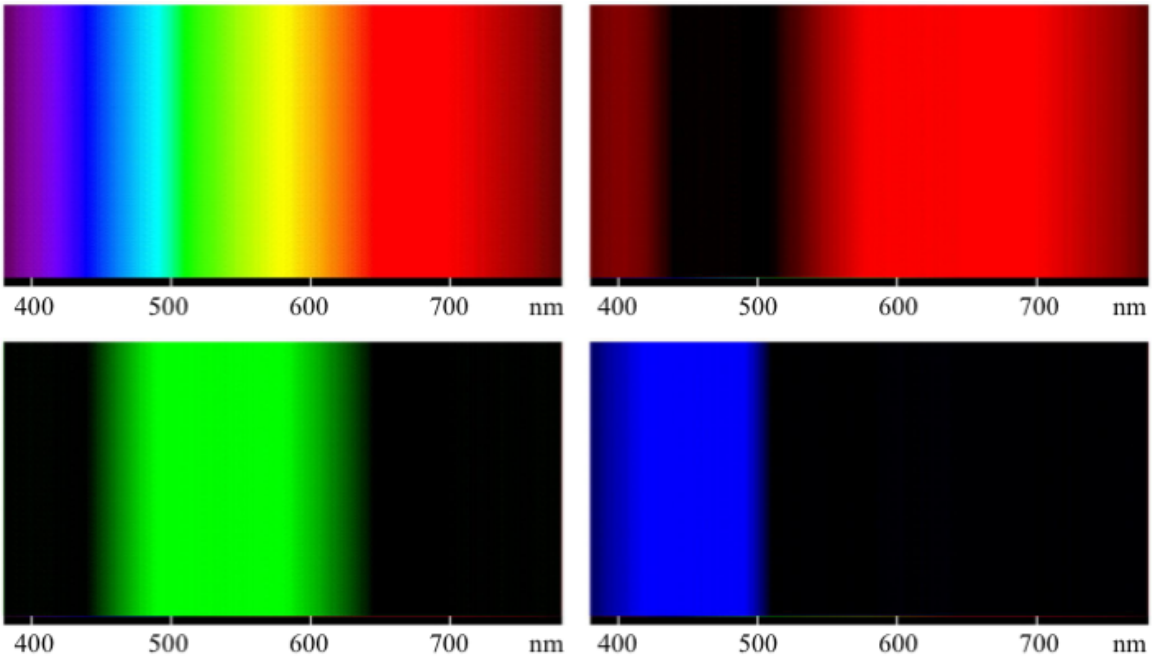


Figure A.1 *Image processing example using the continuos visible spectrum (≅ 380 - 800 nm). On the top left panel resides the original JPEG image. The other images are related to the respective colour components. By combining all layers, the initial image is recovered.*

A.1.1 Colour quantication: some aspects

In the RGB model, a two dimensional (2D) image is represented by points (pixels). Each pixel located in XY image coordinates is coded into 3 colour indexes (R, G and B) using an unsigned 8-bit integer format, with values ranging from 0 to 255. Image XY matrix dimensions depend upon digital camera characteristics and picture acquisition conditions. Using a soft data processing language such as Octave it is possible to convert digital fotos (JPG files) into treatable numerical information. A simple example is depicted in Figure A.2, in which an RGB image is decomposed. Each digital image can be treated in a global perspective, using all available surface 2D information, or may be segmented in partial areas or lines. It can be further analysed in terms of grayscale, after transformation, or separately analysed in each colour plane information, or in any other possible combination (RG, RB, GB). In Figure A.2, we present R, G and B colour indexes by averaging with respect to X coordinates of the original image presented in Figure A.1.

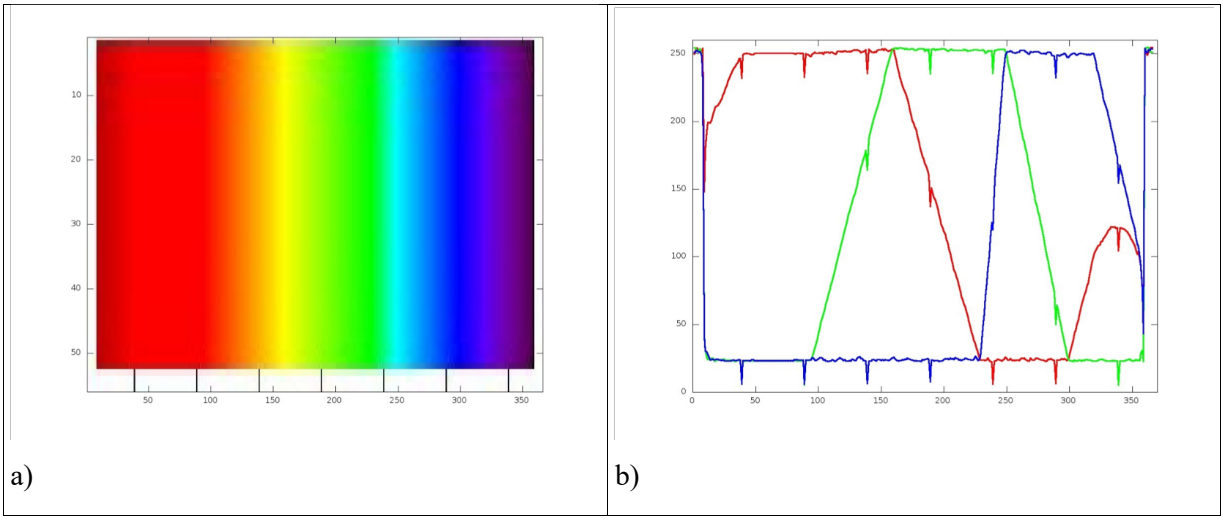


Figure A.2 *Demonstration of the ability of image processing in working with images in terms of numeric approaches: using same x coordinates of original image (in the left) we represent the average R, G and B colour indexes (in the right).*

A.2 Materials and Methods

A.2.1 Microorganism Source and Culture Conditions

The marine microalgae *Nannochloropsis* sp., obtained from Varicon Aqua Solution, Malvern, UK, was cultivated for 6 days in sterile 2L commercial media Cell-hiF2P (Varicon Aqua Solution, Malvern, UK), based upon the f/2 medium (Guilliard, 1975). The culture was aerated by bubbling atmospheric air, at a rate of 300 cm^3 min^{-1}, and were grown at 15±2 ºC under partial illumination (with a 18:6 photoperiod) using fluorescent lamps at an irradiance level of 100 µmolm^{-}

$^{2}s^{-1}$. A very concentrated cell culture suspension was obtained via centrifugation/decantation cycles. In order to calibrate the measurements, initial concentrated cell culture, here defined as 100% relative concentration, was successively diluted to obtain other sample solutions (till 0.01 % of initial solution). Three independent cell cultures, grown under the same conditions, were used in order to establish the robustness of digital image processing to access cell culture density.

A.2.2 Cell culture density evaluation

An improved Neubauer chamber (Marienfeld, Germany) was used in order to obtain experimental cell culture density values, using their respective standard procedures.

It was validated on the basis of the linearity of the response as a function of the cell density. The latter was varied following a rigorous dilution plan over four orders of magnitude, from the most concentrated (100 %) to the less concentrated (0.01 %).

The use of digital image treatment, based on colour index estimates (R,G,B) resorts to cell culture images of specific concentrations, producing what can be seen as the instrumental response. This will be related with cell counting using appropriate functions, in what are, in fact, calibration curves, to be discussed on the results section. Note, however, that this method intends to provide an alternative to direct counting and, as such, the calibration procedure must be simple, not time-consuming and stable over time. This implies a "one-time" calibration that has prompted the use of cell culture replicates after 1 year elapsed time after last calibration to check for method robustness and accuracy.

Whenever possible, central estimates are indicated with respective uncertainty (in parenthesis) or as 95 % confidence interval (in brackets) associated.

A.2.3 Digital image acquisition and processing for cell density evaluation

Three high density fluorescent lamp (125 W) were used in order to produce a well-controlled and homogeneously illuminated environment for samples image acquisition with illumination produced by one, two or three lamps tested during calibration.

A standard digital camera (Canon SX240 HS, Power Shot) was used and controlled image acquisition was imposed. Cell solutions were photographed with digital images collected in JPEG (Joint Photographic Experts Group) format file, and processed using Gnu Octave programming language [8,14]. (https://www.gnu.org/software/octave/)

A.3. Results and discussion

A.3.1. Solution image acquisition

Digital image acquisition for quantification seems deceivingly simple but, in order to obtain consistent and reliable results, several factors have to be systematically controlled. The process is influenced by object characteristics such as its dimensions, e.g. depth in the case of translucid

media, surface finishing, homogeneity and opacity to light. Also, the camera specifications and setting conditions must be kept constant in order to obtain consistent results. Fluids, e.g. solutions and gases, dispersions and suspensions, present in general homogeneous media, which is very important to evaluate correctly and consistently their colour. However, some caution is required in order to preserve this property, and mixing may be used to enhance homogeneity. Fluids possess other advantages for this kind of analysis: their dimensions and shape are defined by the respective container. In Figure A.3, the effect of the recipient shape, illustrated by the use of a test tube and a spectrophotometric cell cuvette, and its effects upon the data evaluation of a coloured solution are highlighted.

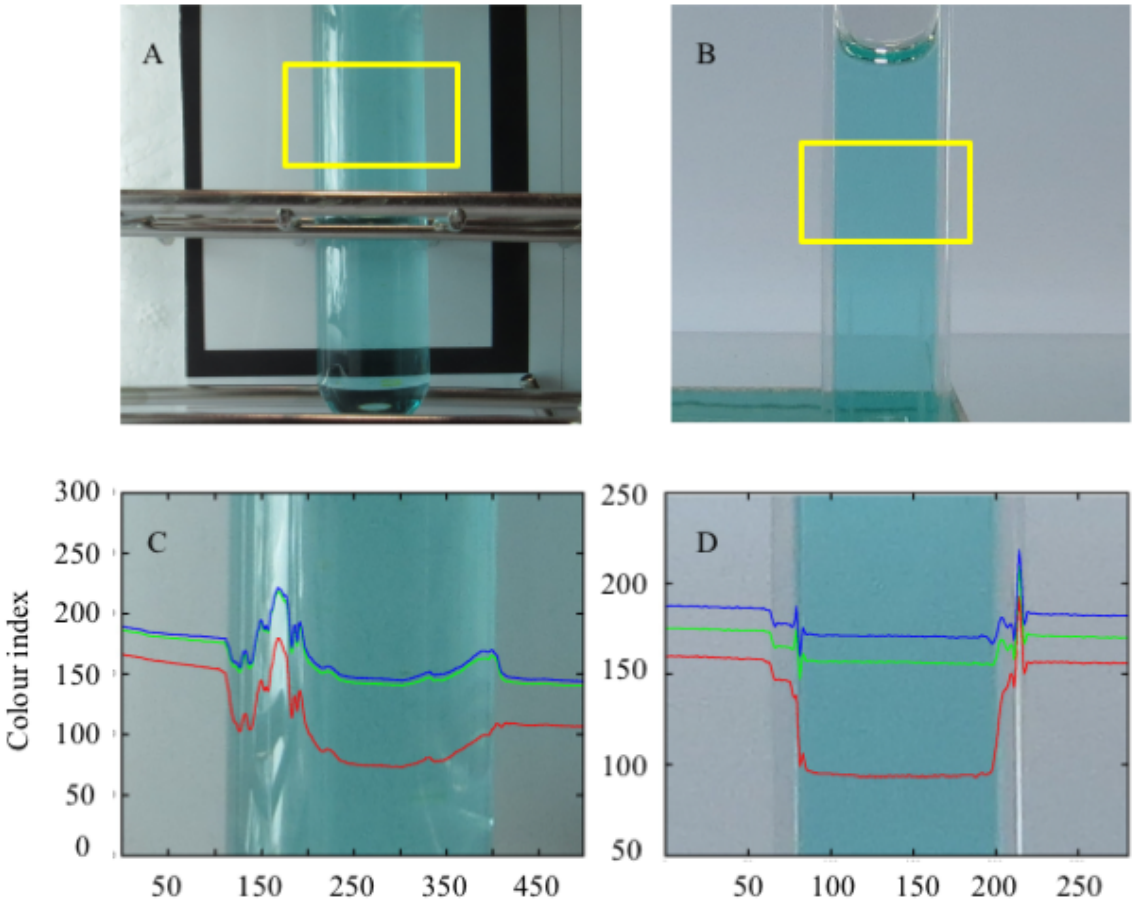


Figure A.3 *Colour index quantication for (A) a test tube exhibiting light refection and pronounced asymmetric light exposure, (B) spectrophotometric cell, with an uniform rectangular section, exposed to a more isotropic lighting; and close-ups (C) of the test tube and (D) spectrophotometric cell with respective colour indexes (RGB) superimposed. These colour indexes are averaged by pixel column.*

From Figure A.3 it can be seen that light reflection affects dramatically the colour index values. In the case of the test tube, Figure A.3(C), light reflection is more pronounced in the left side but also affects colour indexes on the right side, where a residual light reflection occurs. The asymmetric illumination is evidenced comparing colour index levels outside the actual test tube, where higher values on the left side are related to a higher illumination relative to the right side. It is also possible to see that inside the test tube, there is a central region were colour indexes are more homogeneous and present minimal variability. In what concerns the image obtained with the spectrophotometric cell, Figure A.3(D), it is seen that the image was obtained with light homogeneity, and the solution inside displays a homogeneous media.

Colour quantification depends on the ability to estimate respective colour index characteristics. In order to quantify colour indexes, the sampling area shown as the rectangular image section, Figure 3(B), was converted into colour index histograms, Figure A.4. These are shown for both test tube and spectrophotometric cell images from Figure A.3.

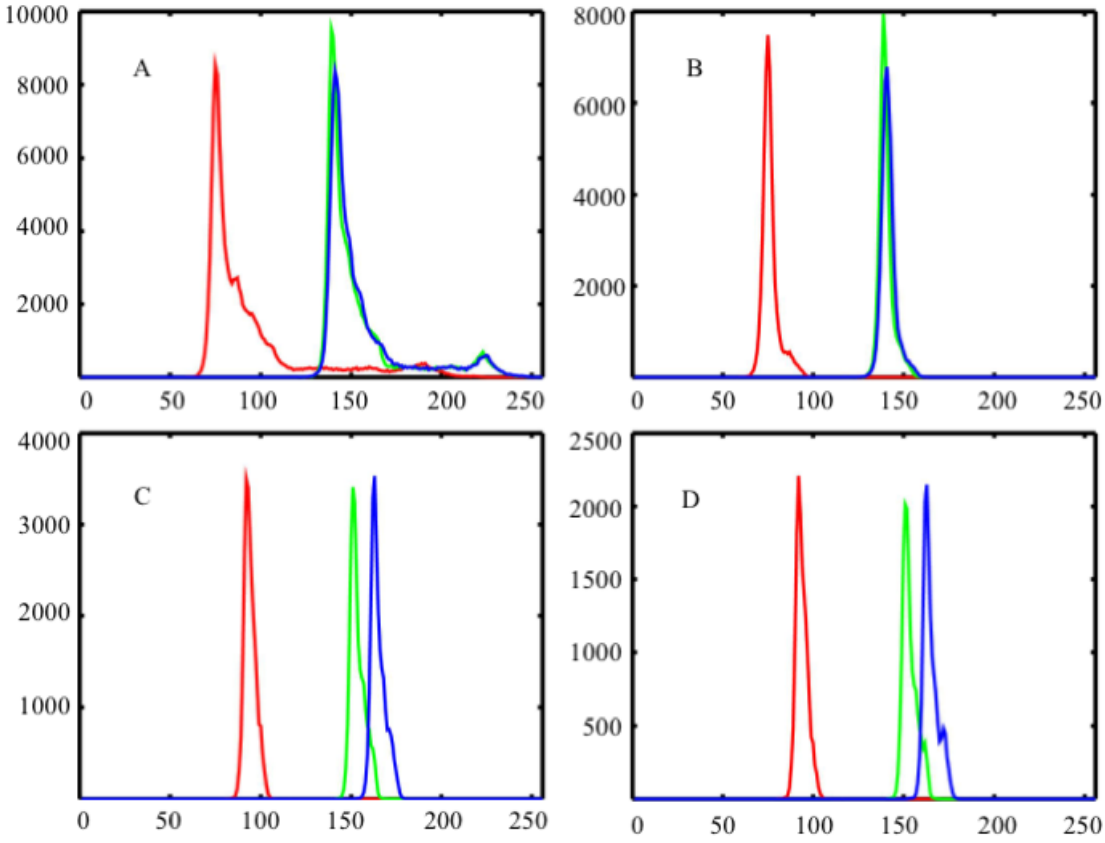


Figure A.4 *Colour index histograms of test tube (A,B) and spectrophotometric cell (C,D) from Figure A.3: A) and C) refers to the full path length of solution inside each container, while B) and D) refer a more central (≅ 50%) inside region.*

From Figure A.4(A), it is clear that reflections, light heterogeneities and other entropic effects distort the colour index histograms, causing the broadening of the respective distribution and thus

contributing for uncertainty on colour index estimates. Restricting image processing sampling area to the central area of test tube have the consequence of avoiding light reflections and select a more homogeneous light path length, and thus these histograms are sharper, cf. Figure A.4(B), allowing to obtain a more reliable colour index estimate. In cases in which the image is relatively homogeneous, cf. Figure A.4(C), reducing further the image sampling area may increase noise effects and introduce some extra defects, as depicted in Figure A.4(D). From the colour index histogram, we also see that there are maxima frequency values that may be responsible for respective overall colour index perception.

In order to obtain reliable values with homogeneous light conditions, reflections minimization and constant solution thickness we choose to preform algae solution's digital image acquisition under controlled constant conditions, using homogeneous light field and using spectrophotometric cells.

Robust unbiased estimates of each chromatic colour index (R,G,B) were used; median for central distribution estimate and central percentile 95% confidence interval to define respective confidence limits.

A.3.2. Cytometry cell culture evaluation

The determination of cell culture density with the Neubauer chamber was started with a dense cell culture (100% reference concentration solution), followed by a dilution procedure in order to obtain a concentration sequence down to 0.01% of the initial concentrated solution. Results are presented in Table A.1.

In order to validate Neubauer chamber for culture algae cell density response it was expected to obtain a linear response between obtained cell density values and respective relative concentration amount in terms of a first degree polynomial function such as

$$y_i = b_0 + b_1 x_i \quad (1)$$

Since the intercept presents a huge uncertainty associated (198.8%) and thus, not having statistical significance, Equation 1 was then simplified to obtain Equation 2.

$$y_i = b_1 . X_i \quad (2)$$

Removing the intercept from initial model, Equation 1 residual sum of squares (SS) increased from 7.385 to 7.453 but respective error model variance decreased from 0.274 (n = 29, p = 2) to 0.266 (n = 29, p = 1). Using incremental *F-test*, the obtained test value (TV = 0.25) conduces to *a p-value* of 0.624 evidencing that both models, Equations 1 and 2, fit data in a similar way and thus, Equation 2 corresponds to best fitting model, also known as the parsimonious model.

In Figure A.5 we present Neubauer chamber linear response given by Equation 2. It can be seen that there is a very good linear dependency between relative cell amount (cell content, %) and cell density obtained with the Neubauer chamber across a wide concentration range of 4 orders of magnitude. Single parameter linear dependency, Equation 2, is also an evidence for insignificant constant bias in response, and thus, we conclude that Neubauer chamber is able to give accurate values in microalgae cell culture density evaluation.

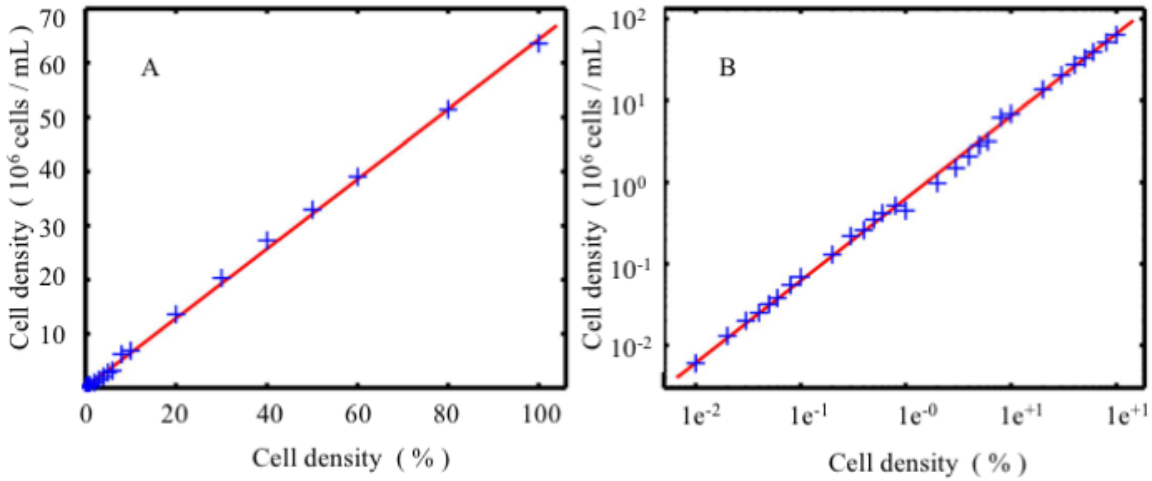


Figure A.5 *(A) Neubauer chamber hemocytometer calibration for microalgae cell culture density evaluation, from data in Table A.1. In (B), the representation resorts to a LogLog plot to allow for response linearity assessment over all tested range.*

A.3.3. Digital image calibration

In table A.1 are given colour index central estimates (median) for R, G and B layers.

Table A.1 *Evaluation of cell density for a series of diluted algae cultures and respective image colour indexes (R, G, B), given as median values. The most enriched culture is assigned 100 % content.*

Relative amount (% Dilution)	Cell density (x10 Cells/mL^6)	Predicted values (x10 Cells/mL^6)
100	63.58	64.718
80	51.43	51.774
60	39.05	38.831
50	32.97	32.359
40	27.28	25.887
30	20.32	19.415
20	13.60	12.944
10	6.85	6.472
8	6.20	5.177
6	3.17	3.883
5	2.85	3.236
4	2.08	2.589
3	1.50	1.942
2	0.98	1.294
1	0.45	0.647
0.8	0.52	0.518
0.6	0.42	0.388
0.5	0.35	0.324
0.4	0.26	0.259
0.3	0.22	0.194
0.2	0.13	0.129
0.1	0.069	0.0647
0.08	0.055	0.0518
0.06	0.038	0.0388
0.05	0.032	0.0324
0.04	0.025	0.0259
0.03	0.020	0.0194
0.02	0.013	0.0129
0.01	0.006	0.0065

R, G and B indexes are represented in Figure A.6 (A) and the combined colour indexes (RG, RB, GB and RGB), obtained by averaging respective initial estimates are presented in Figure A.6 (B). In order to describe colour index dependency upon microalgae cell concentration (calibration curve), several models were tested but the simplest and parsimonious only involves the estimation of 3 parameters (p = 3) according to,

$$z_i = \frac{\theta_1}{1 + \theta_2 exp(\theta_3 . x_i)} \quad (3)$$

were z_i corresponds to colour index estimate of x_i solution concentration, given by cell density or simply given by relative concentration index.

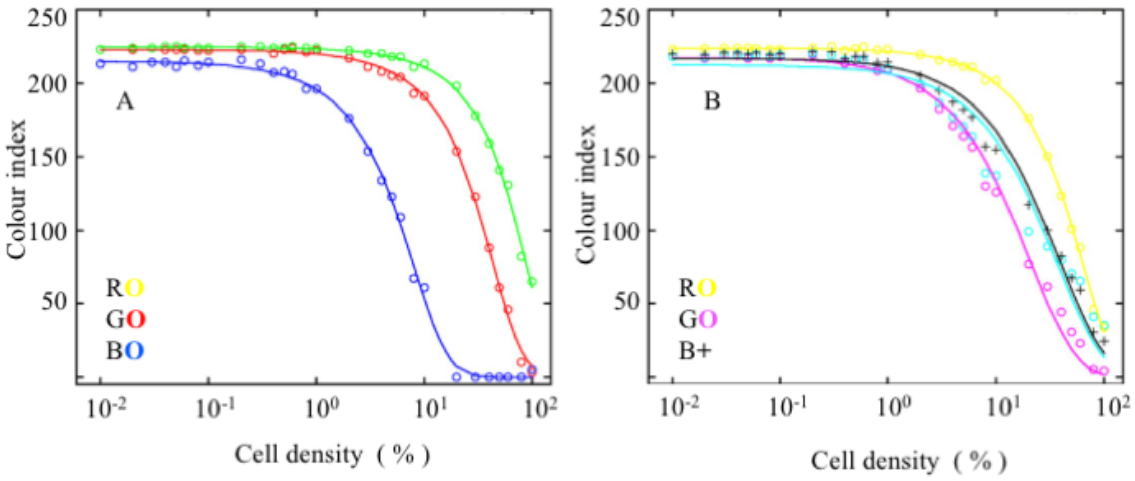


Figure A.6 *Semi-log representation of colour indexes with respect to the relative cell concentration values obtained with the Neubauer chamber hemocytometer: (A) direct colour indexes (R, G and B) medians and (B) combined (averaged) colour indexes (RG, RB, GB and RGB).*

Table A.2 *Colour layer model estimates for each tested response using Equation 3 in respect to culture cell density obtained with Neubauer chamber hemocytometer.*

Colour layer	R	G	B	RG	RB	GB	RGB
RMSE	4.10	2.59	4.53	2.74	8.36	13.67	7.61
R^2	0.9969	0.9967	0.9976	0.9978	0.9897	0.9591	0.9874
θ_1	270.2	262.8	1.56e4	288.2	8.48e5	3.79e6	6.98e5
θ_2	0.220	0.172	718.7	0.2918	3946	1.79e4	3228
θ_1	0.081	0.047	0.204	0.0537	0.068	0.039	0.037

RMSE - root mean squared error (residual standard error); R2 - coecient of determination.

In this model, Equation 3, θ_1 is related to maximum signal plateau in the lowest part of concentration domain; the other two parameters, θ_2 and θ_3, are related with the positioning of 50% signal response in terms of solution's cell concentration (inflection point of the response curve is obtained at x_i= - ln ((θ_2)/ θ3). Standard unweighted least squares approach was used in order to obtain parameters estimates of colour index calibration curve, Equation 3 and results are presented in Table 2. Calibration curve models are overlaid with respective plotted data in Figure A.6.

From Table A.2 it is apparent that single layer colour indexes (R, G and B) are well fitted using the proposed calibration model, Equation 3, and able to justify 99.7-99.8 of experimental data variability. In what concerns to the use of colour layer combinations, Table A.2 and Figure A.6 b,

there are two drastic situations: only RG colour combined response seems to behave like a single colour index while other combinations fail in general to follow proposed calibration model, Equation 3. This fact is related to the great similarity of R and G dependencies upon the same independent variable that the averaged value preserve a similar curve shape and may be fitted with this simple model, see Figure A.6.

A.3.4. Digital image cell culture density evaluation

The quantification function for cell culture density (x_A) was driven from inverse transformation of proposed model, Equation 4. For a given solution presenting a signal value of z_A, sample cell culture density can be estimated using Equation 4,

$$x_A = \frac{1}{\theta_3}.\ln\left(\frac{\theta_1 - y_A}{\theta_2.z_A}\right) \qquad (4)$$

which allows to estimate cell culture density accordingly to the same amount unities used in the calibration process, here expressed as (cells mL^{-1}).

In order to obtain rough estimates for confidence interval on cell culture density, it was used chromatic colour index 95% confidence interval, previously established with percentiles $P_{0.025}$ and $P_{0.975}$, as signal limits and estimate correspondent cell amounts. Assuming this concentration amplitude ΔX_A as expanded uncertainty for a symmetrical confidence interval, it was estimated standard uncertainty (as standard error, SE), assuming k=2 in respect to 95% coverage, using uncertainty measurement approach, Equation 5.

$$SE \approx \frac{\Delta X_A}{2} \qquad (5)$$

The best calibration curves, obtained with digital responses of R, G, B and RG, Table A.2, were further studied in order to evaluate respective analytical range. Performing a *t-test* to evaluate analytical accuracy in cell density estimate, it was defined the analytical range based on *p-values* higher than 0.05 for result coherence with cell density values given by Neubauer chamber. The results are summarized in Table A.3. Imposing also the limit of 15 % for systematic errors (RE≤15%) and for uncertainty (RE≤15%), the analytical ranges for R and RG of about 1 magnitude order lies in region 6-60 x 10^6 cells mL^{-1}; for G colour index analytical response is a little expanded over the range 2-60 x 10^6 cells mL^{-1}; the B colour index is the most sensitive analytical range corresponding to 0.3-6 x 10^6 cells mL^{-1}.

Table A.3 *Evaluation of analytical range based on accuracy t-test for cell density estimates obtained by colour index R, G, B and RG in respect to values obtained with Neubauer chamber hemocytometer.*

cell density* ($\times 10^6$ counts/mL)	Estim. ($\times 10^6$ counts/mL)	RE (%)	RSE (%)	TV	$p-value$	Estim. (10^6 counts/mL)	RE (%)	RSE (%)	TV	$p-value$
	R					G				
63.58	74.26	16.8	15.9	1.80	0.213	61.16	-3.8	0.8	4.83	0.040
51.43	59.03	14.8	3.2	4.47	0.047	52.50	2.1	0.8	2.45	0.134
39.05	38.32	-1.9	1.0	1.96	0.190	37.60	-3.7	1.0	3.88	0.061
32.97	33.97	3.0	0.9	3.30	0.081	34.36	4.2	1.1	3.69	0.066
28.28	27.72	-2.0	0.9	2.35	0.144	28.39	0.4	1.4	0.28	0.803
22.32	20.93	-6.2	1.0	6.48	0.023	21.69	-2.8	2.0	1.47	0.279
15.60	15.23	-2.4	1.4	1.73	0.226	14.69	-5.8	3.4	1.80	0.213
6.85	6.82	-0.5	3.7	3.79	0.063	6.53	-4.7	9.2	0.53	0.650
6.20	6.37	2.8	3.9	4.52	0.046	6.57	5.9	7.8	0.62	0.597
3.17						3.28	3.5	18.3	0.17	0.881
2.85						3.08	8.0	19.5	0.35	0.763
2.08						2.21	6.1	30.5	0.19	0.870
	B					RG				
63.58						62.4	-1.8	4.0	2.29	0.149
51.43						53.9	4.8	2.8	1.64	0.244
39.05						38.1	-2.4	6.6	0.38	0.741
32.97						34.4	4.5	7.3	0.42	0.716
28.28						28.3	0.1	8.8	0.00	0.997
22.32						21.3	-4.6	11.7	0.19	0.868
15.60						14.6	-6.7	15.8	0.16	0.887
6.85	6.23	-9.1	11.2	0.82	0.498	7.1	3.3	21.2	0.03	0.979
6.20	5.77	-7.0	13.9	0.67	0.570	7.1	14.1	18.4	0.10	0.927
3.17	3.37	6.5	11.6	0.35	0.759					
2.85	2.78	-2.5	5.4	0.26	0.816					
2.08	2.04	-2.3	5.6	0.18	0.876					
1.50	1.42	-5.6	8.5	0.11	0.920					
0.98	1.02	3.5	12.5	0.08	0.947					
0.45	0.49	8.4	14.3	0.03	0.976					
0.52	0.49	-5.4	11.6	0.03	0.978					
0.42	0.43	3.3	12.1	0.03	0.981					
0.35	0.33	-4.7	16.0	0.02	0.987					
0.26	0.22	-14.2	18.5	0.01	0.992					

* - experimental counts obtained with Neubauer Chamber hemocytometer; Estim. - estimates obtained via calibration curve; RE - relative bias; RSE - relative standard error.

All analytical ranges are about same amplitude (1 order of magnitude), corresponding to colour index transition, Figure A.6. It is also patent that green (G) colour index transition is more intense for cell densities higher than 10 x 10^6 cells mL^{-1}, while the blue (B) colour index transition is more intense for cell densities of 1-10 x 10^6 cells mL^{-1}. These complementary transition ranges allow us, with two calibration curves, to obtain accurate estimates in cell culture density from 0.3 to about 60 x 10^6 cells mL^{-1}. In order to evaluate systematic errors, recovery plots for G and B colour index responses to cell culture density were represented, Figure A.7.

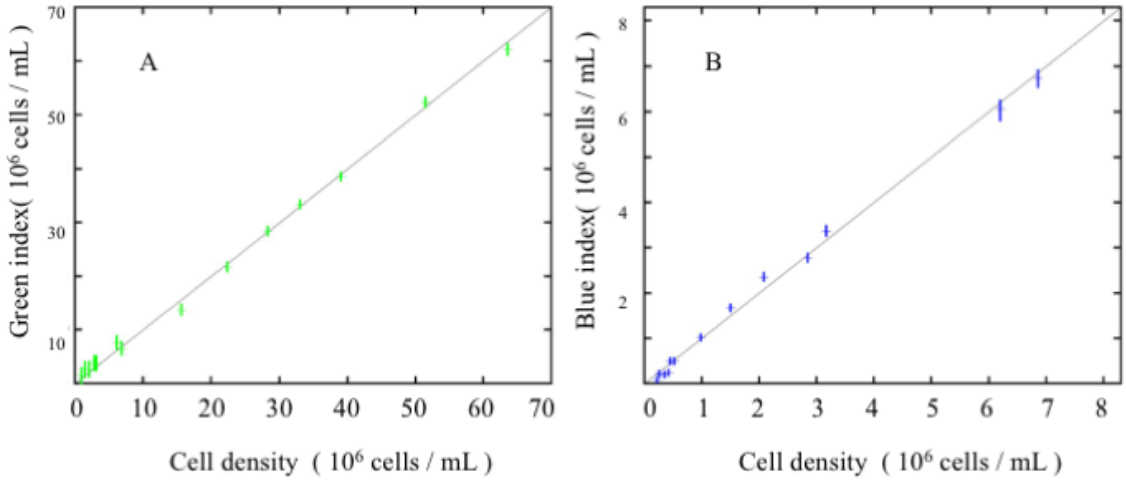


Figure A.7 *Recovery plots for a) G and b) B colour index cell culture density estimates in respect to values obtained with Neubauer chamber hemocytometer.*

For the purpose of verifying the robustness and coherence of the proposed method, after approximately one year of interval, the same quantification model was used to access cell density on new cultures of same microalgae in a 13 days period. Results are presented in Table A.4.

Table A.4 *Evaluation of method robustness and coherence accessing new cell culture density using previous calibration estimates after a 1 year elapsed time.*

time (days)	cell density* ($\times 10^6$ counts/mL)	Estim. ($\times 10^6$ counts/mL)	RE (%)	RSE (%)	p-value
1	1.1	0.4	-63.1	12.4	0.021
2	1.2	0.6	-50.4	13.5	0.026
3	1.3	0.8	-40.1	15.0	0.047
4	1.2	1.3	4.1	9.1	0.720
5	2.0	1.6	-18.9	8.7	0.090
6	2.3	1.9	-18.7	6.9	0.085
7	2.9	2.2	-24.6	7.3	0.034
8	2.8	2.3	-19.0	5.0	0.061
9	4.3	3.6	-15.3	7.5	0.045
10	4.5	4.6	4.4	6.6	0.470
11	5.6	5.5	-1.2	6.6	0.814
12	7.1	7.2	1.0	6.4	0.848
13	8.1	8.8	7.7	5.9	0.215

* - experimental counts obtained with Neubauer Chamber hemocytometer; Estim. - estimates obtained via calibration curve; RE - relative bias; RSE - relative standard error.

From Table A.4 it is apparent the method robustness and coherence, while for cell densities in 1.3-8.1 x 10^6 cells mL^{-1} the proposed method is exact. Contrary to previous evaluation for a new calibration curve, Table A.3, we expected to be able to correctly quantify lower amounts down to near 0.2 x 10^6 cells mL^{-1}. However, considering a 1 year time span this can be in fact a very good result showing that proposed method is in fact robust and coherent. These results are very important since they show that it is not necessary to perform systematic recalibrations using cell counting methods. The basis for consistent results in this work is to keep maximal controlled constant conditions. The proposed method depends on colour properties, which are assumed to be constant. When dealing with microalgal cell culture, colour is a property that tends to change due to several factors, such as nutrients, light regime, sun exposure, among others. Our future goals are to evaluate the applicability of the proposed method in the presence of coloured contaminants and in the presence of colour evolution phenomena.

A.4. Conclusions

The proposed method allows us to circumvent inconsistent spectrophotometric difficulties due to light scattering and lack of homogeneity. In this work we proved that, under controlled constant conditions, image acquisition and subsequent data treatment in order to quantify colour characteristics may be used to estimate cell culture density. If cell cultures maintain their colour properties it is possible to accurately estimate (RE<15%) microalgae cell density over approximately 2 orders of magnitude.

The proposed method may be used to follow cell culture proliferation in a conservative, non-expensive and fast way.

References

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Appendix B

Bioremediation

In preliminary studies of the bioremediation potential of *Nannochloropsis* sp., the drug alprazolam was used. Analysis of UV–Vis spectroscopy revealed a high percentage of alprazolam removal from water. Epifluorescence microscopy images of *Nannochloropsis* sp. cells (Figure B.1) showed the fluorescence of alprazolam in whole cell indicating the diffusion of the drug. The initial results suggested the potential use of the specie *Nannochloropsis* for bioremediation.

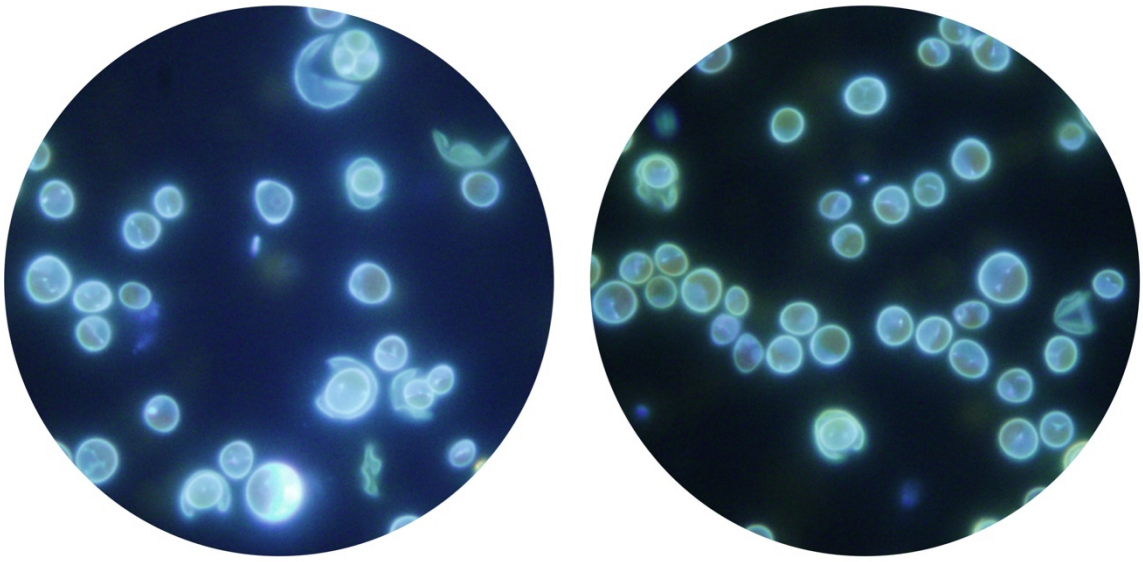


Figure B.2 *Epifluorescence microscopy images of Nannochloropsis* sp*. cells cultivated in the presence of alprazolam.*

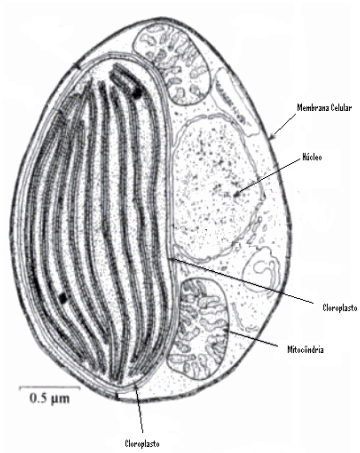


Figure B.2 *Schematic representation* of *Nannochloropsis* sp.. Source: Van den Hoek, C.; Mann, D. G.; Jahns, H. M.; Algae: An Introduction to Phycology. Cambridge University Press, New York 1995.

Appendix C

Deuterated lipids

In addition to experiments presented in this thesis, initial studies were performed using deuterium oxide, or D_2O/water mixtures for preparation of the culture medium with the aim of developing cheap and simple routes to specifically deuterated species for use as biomarkers in magnetic resonance imaging or bioassays. The identification of the compounds was performed using a combination of HPLC-MS and NMR spectroscopy. The results indicated the deuteration of *Nannochloropsis* total lipids extract. Although the initial results were favourable in the evaluation of the deuteration of lipids, the study was limited by sample size. Further evaluation with a large sample size would be required. Illustrative examples are presented in Figure C.2 and Figure C.3.

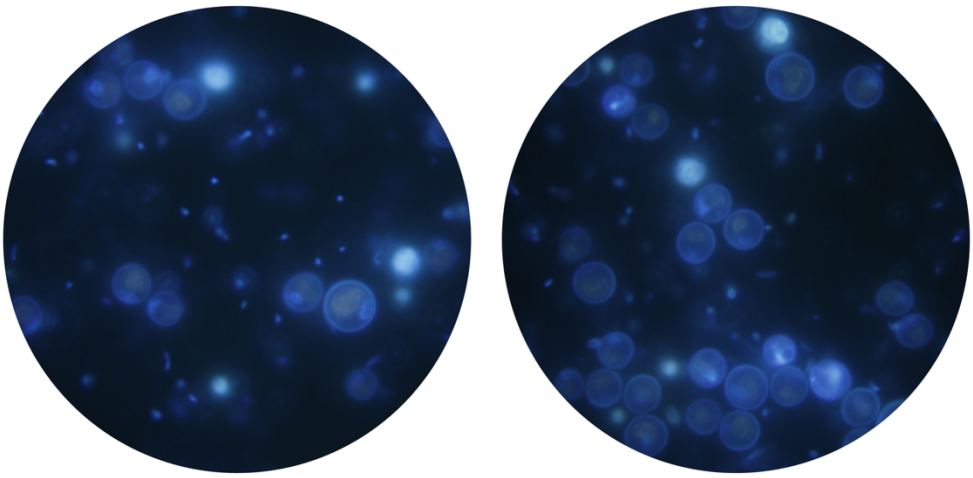


Figure C.1 *Epifluorescence microscopy images of deuterated Nannochloropsis* sp. *cells. showing changes in the appearance of the chloroplasts.*

C.1 1H-NMR spectra of undeuterated and deuterated *Nannochloropsis* total lipids extracts

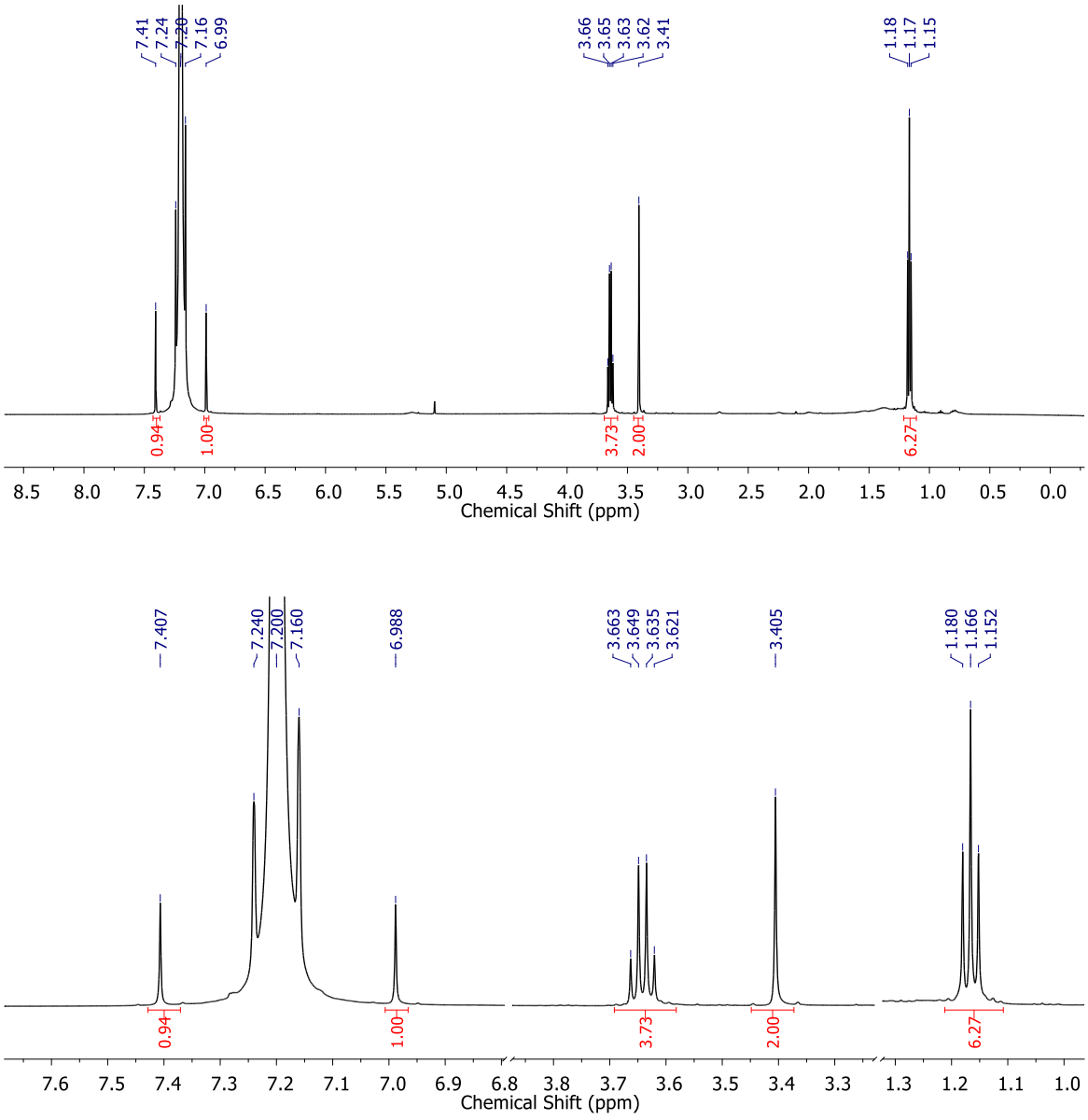


Figure C.2 *^{1}H-NMR spectra of undeuterated Nannochloropsis total lipids extract.*

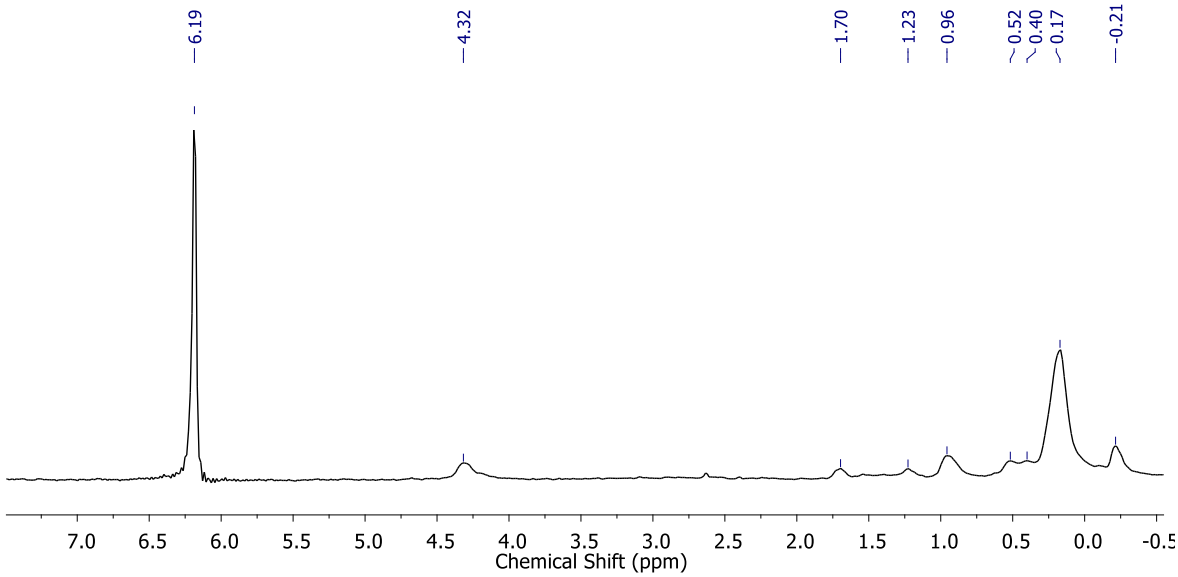
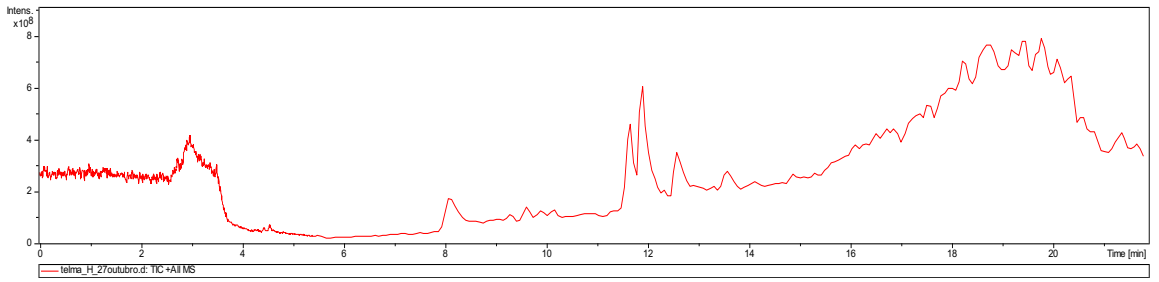
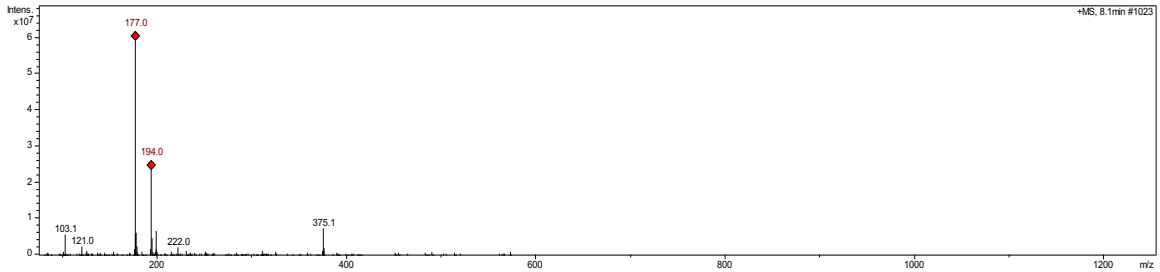
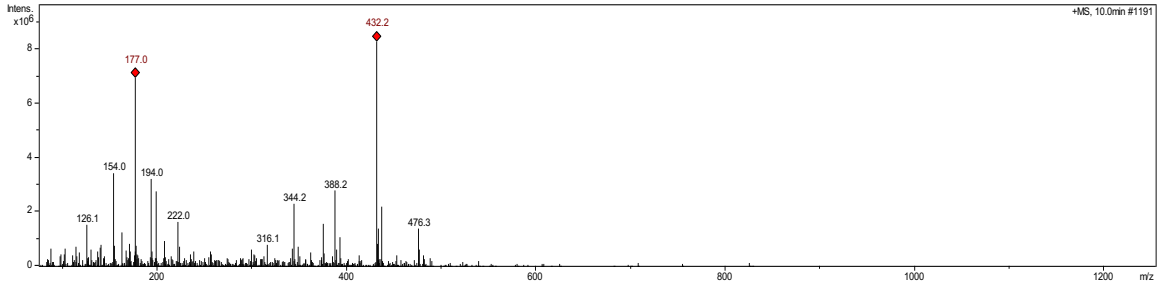


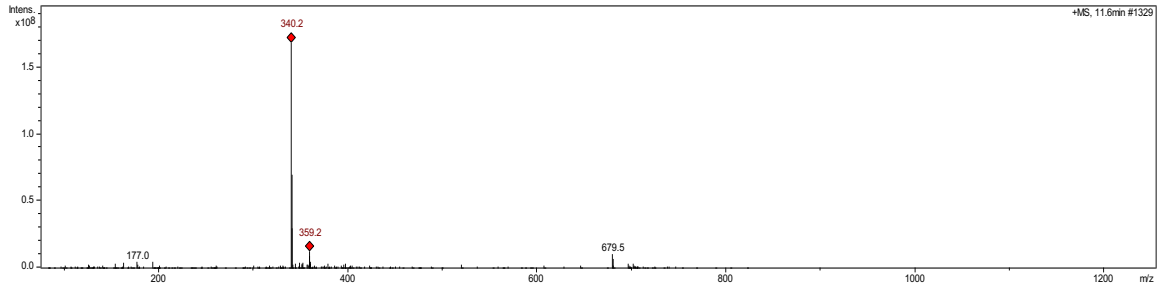
Figure C.3 *^{1}H-NMR spectra of deuterated Nannochloropsis total lipids extract.*

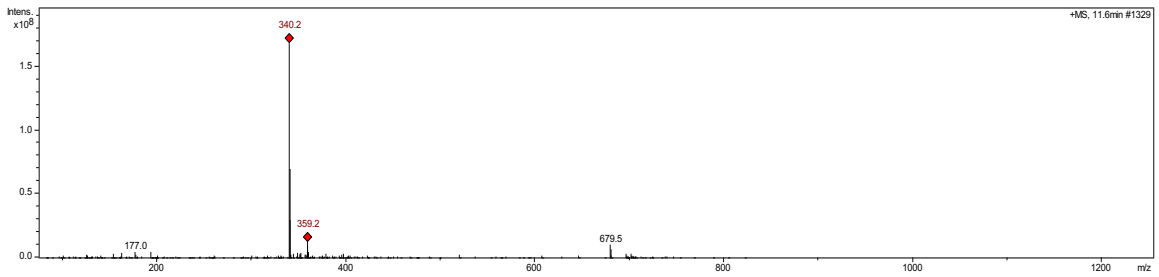
C.2 Mass spectrometric analysis of undeuterated sample

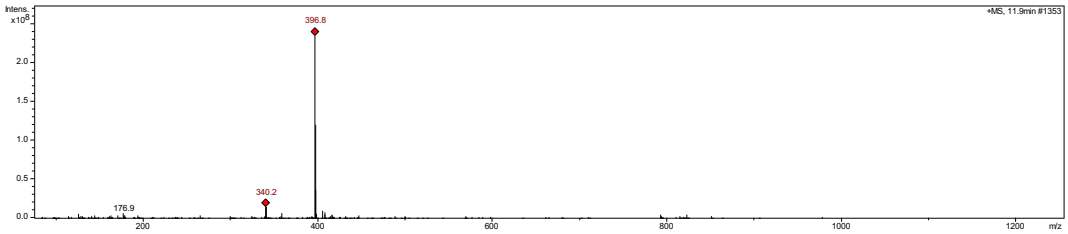








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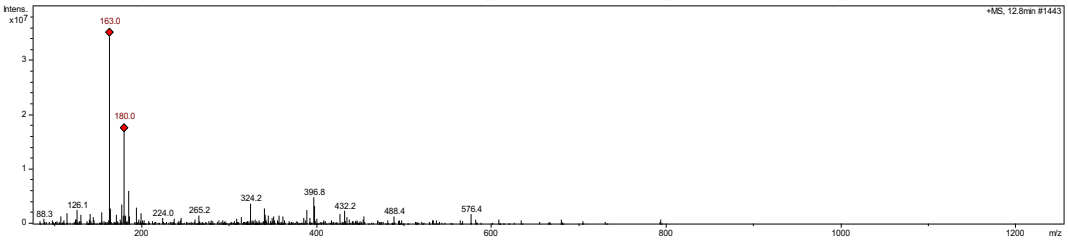
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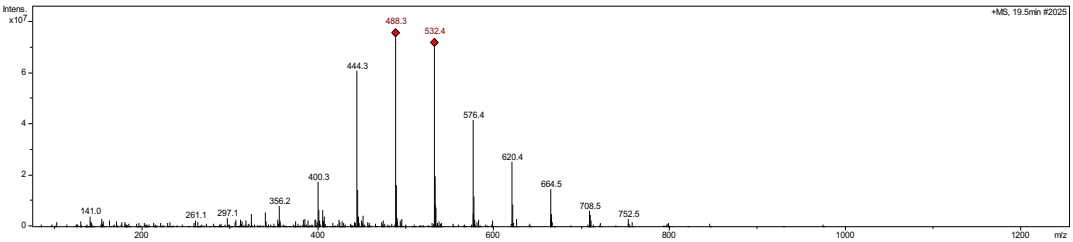
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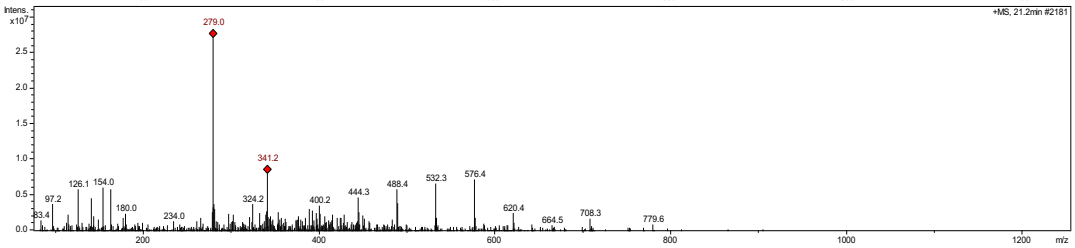
297.1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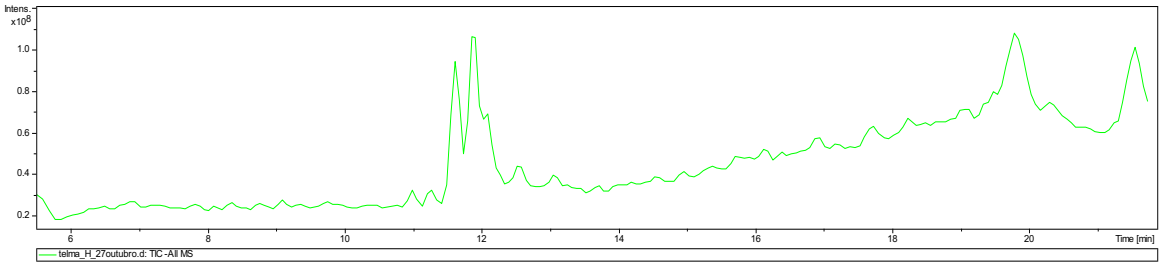
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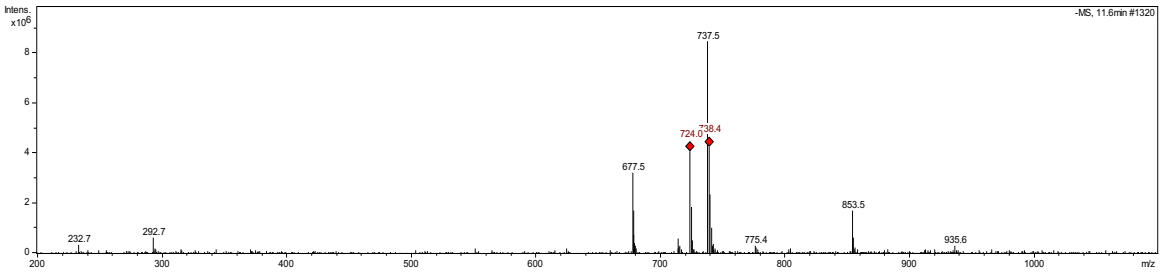
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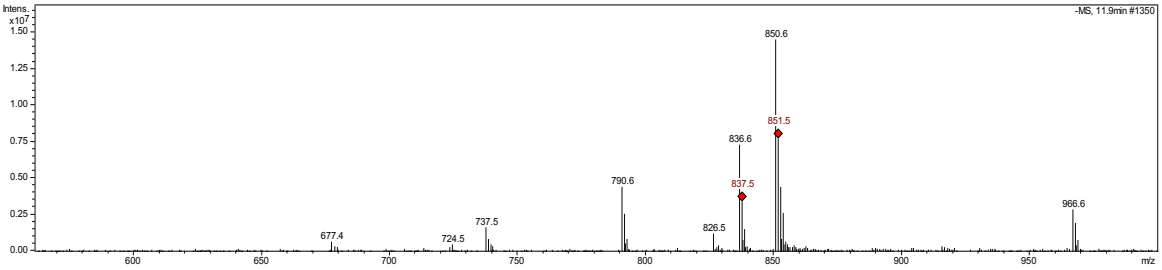
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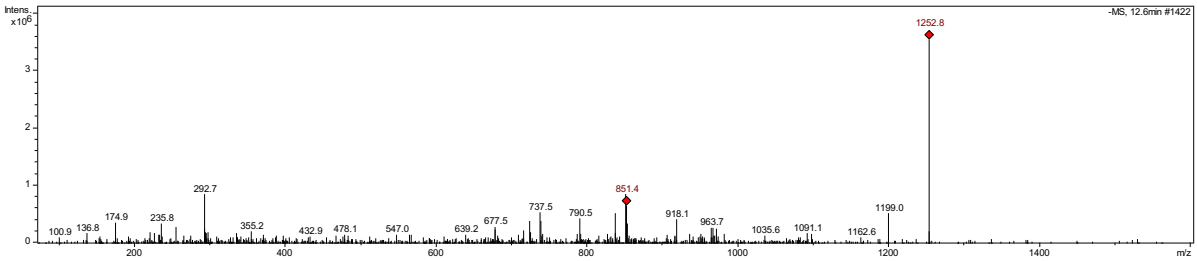
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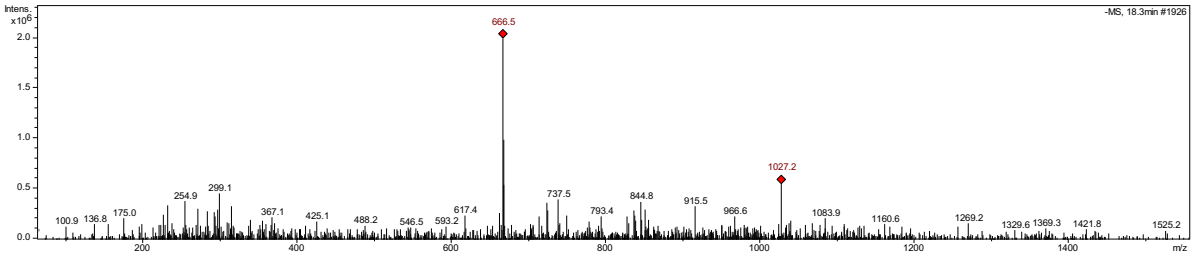
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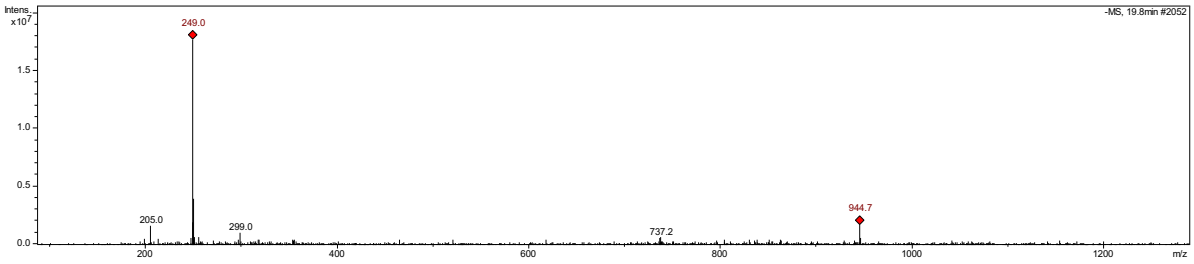
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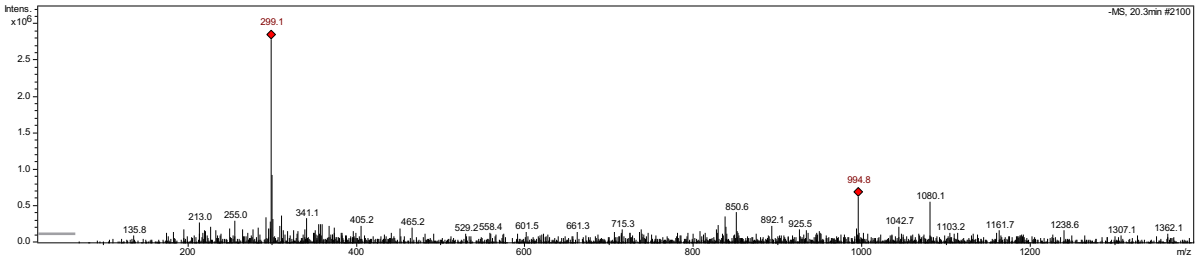
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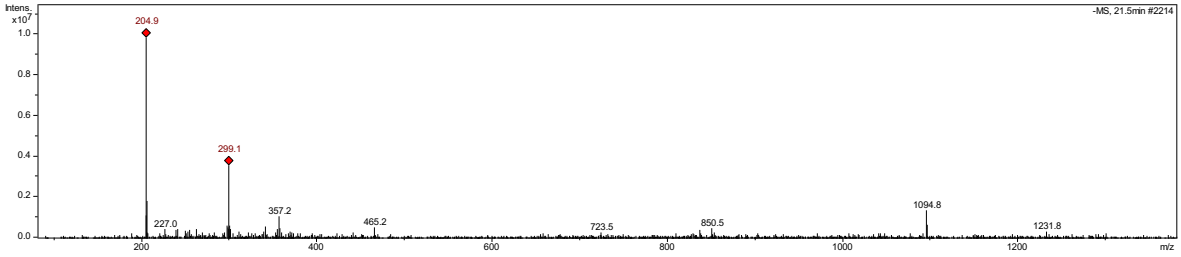
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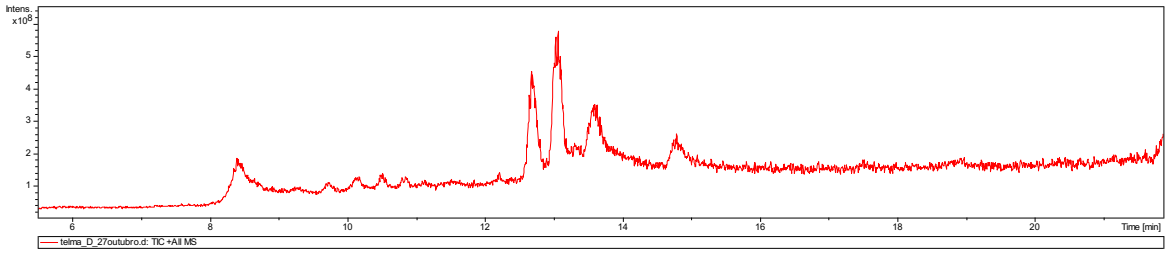
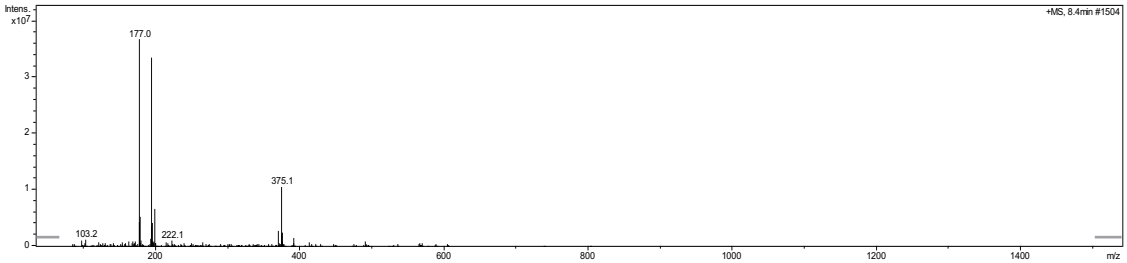
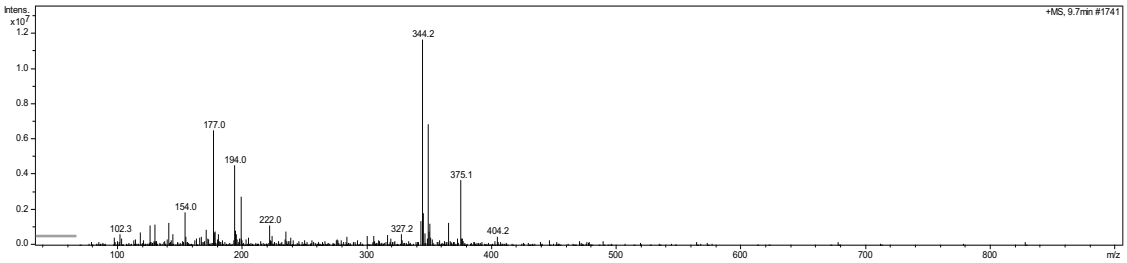
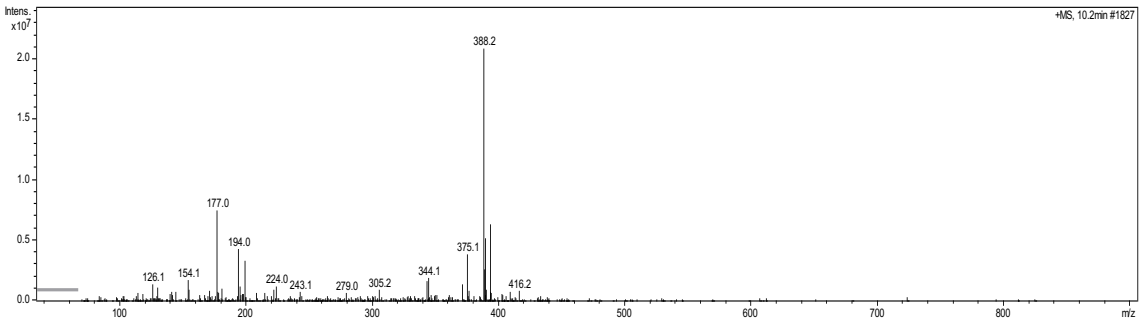
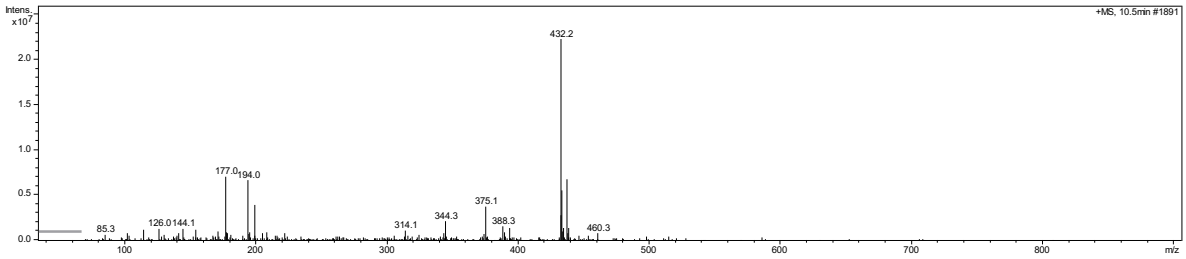
C.3 Mass spectrometric analysis of deuterated sample







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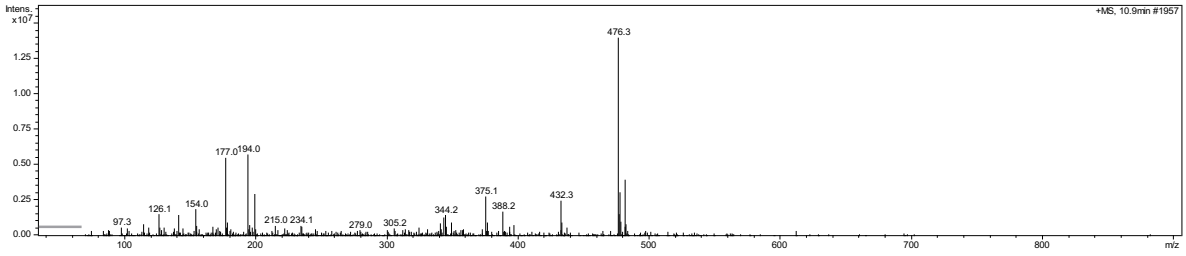
194.0
375.1
344.3
388.3
85.3
126.0
144.1
314.1
460.3
m/z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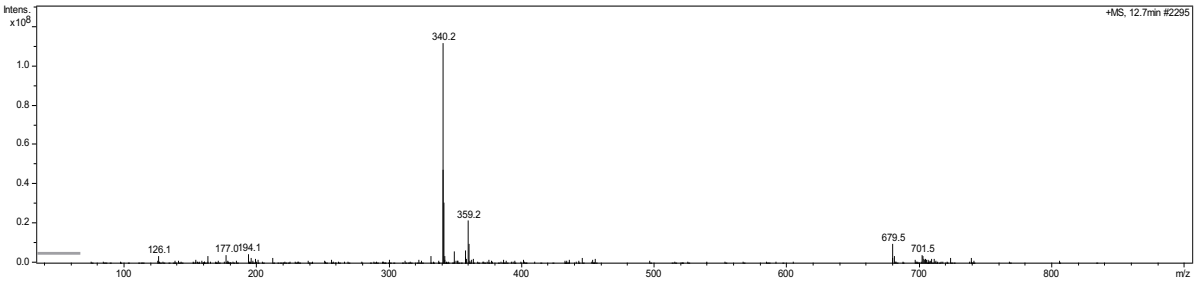
234.1
279.0
305.2
344.2
m/z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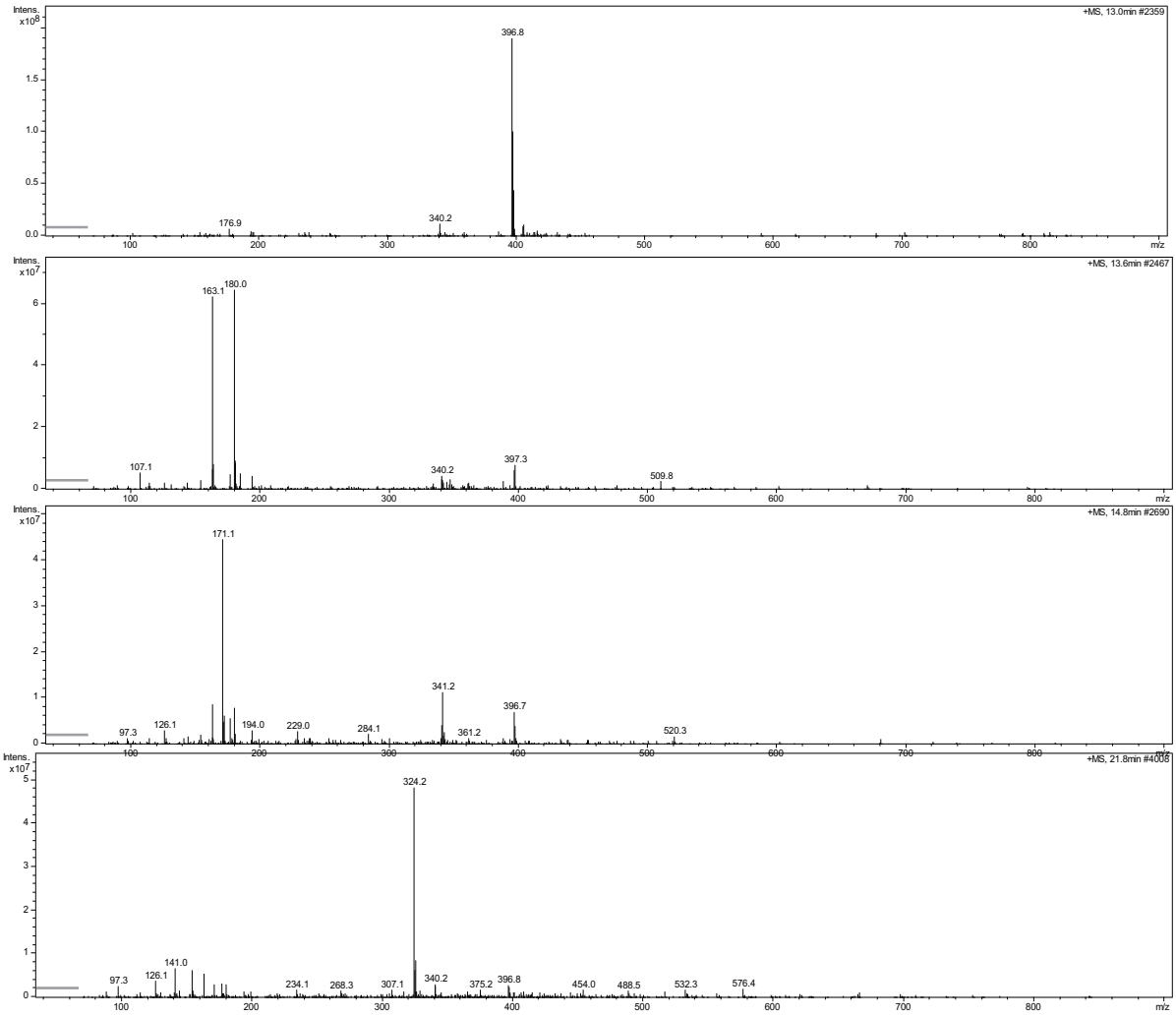
194.1
679.5
701.5
m/z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176.9
340.2
m/z
Intens.
x10^7
+MS, 13.6min #2467
163.1
180.0
107.1
340.2
397.3
509.8
m/z
Intens.
x10^7
+MS, 14.8min #2690
171.1
341.2
396.7
97.3
126.1
194.0
229.0
284.1
361.2
520.3
m/z
Intens.
x10^7
+MS, 21.8min #4008
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141.0
126.1
97.3
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396.8
454.0
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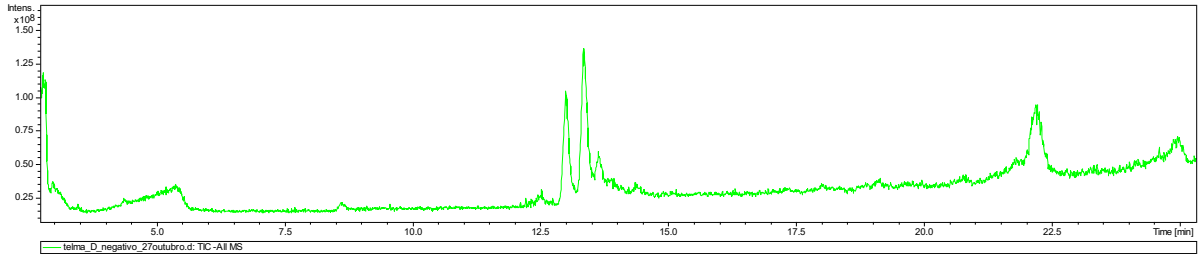
532.3
576.4
m/z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0.50
0.25
5.0
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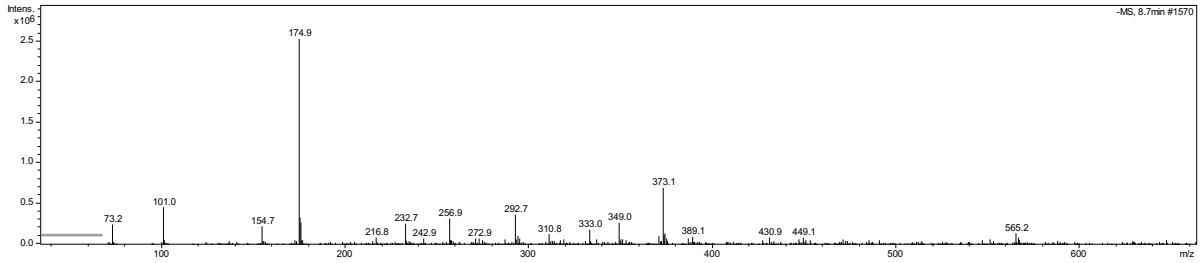
15.0
17.5
20.0
22.5
Time [min]
telma_D_negativo_27outubro.d: TIC -All MS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373.1
101.0
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154.7
232.7
256.9
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272.9
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389.1
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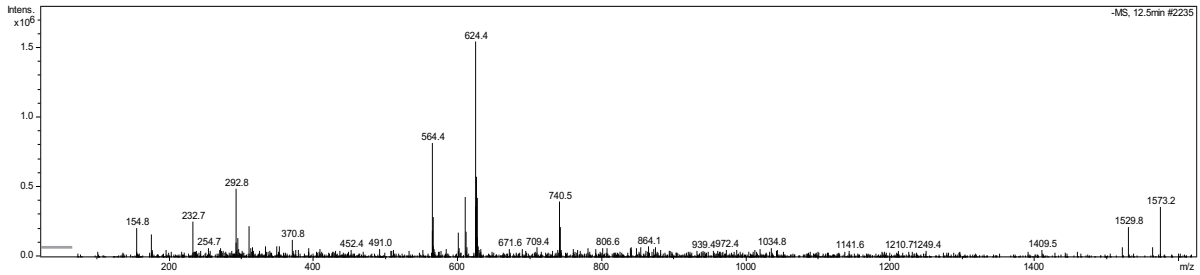
449.1
565.2
m/z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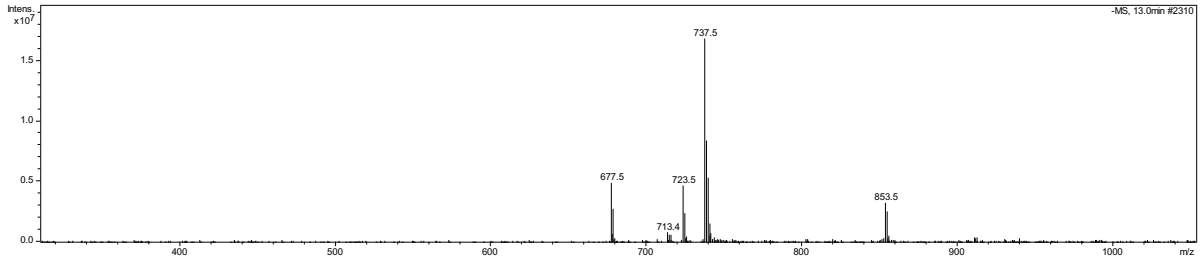
564.4
292.8
740.5
154.8
232.7
254.7
370.8
452.4
491.0
671.6
709.4
806.6
864.1
939.4
972.4
1034.8
1141.6
1210.7
1249.4
1409.5
1529.8
1573.2
m/z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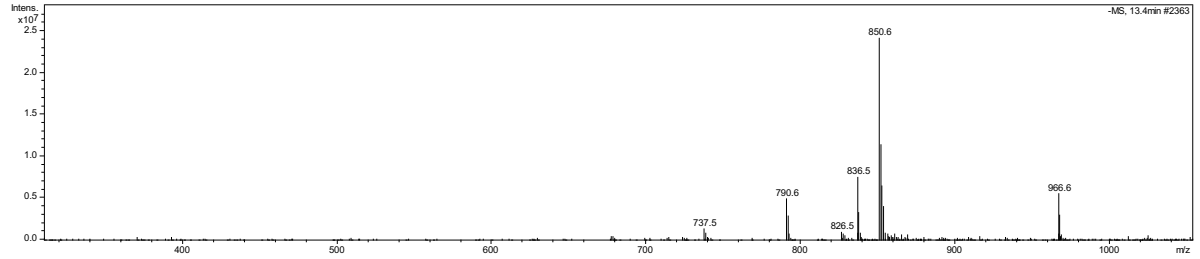
677.5
723.5
713.4
853.5
m/z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836.5
790.6
966.6
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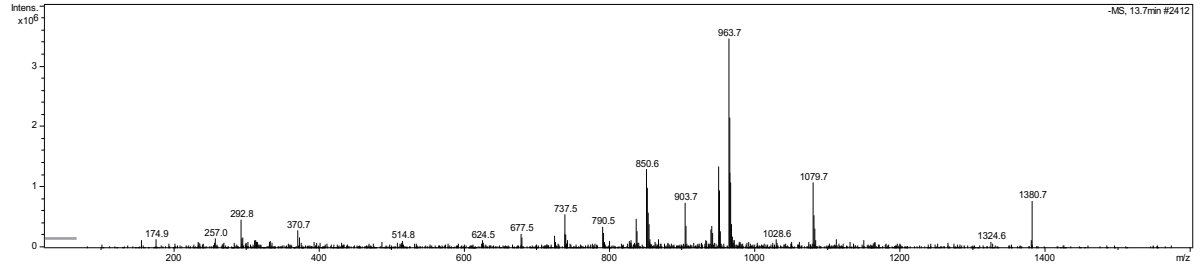
826.5
m/z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-MS, 13.7min #2412
963.7
850.6
1079.7
903.7
1380.7
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790.5
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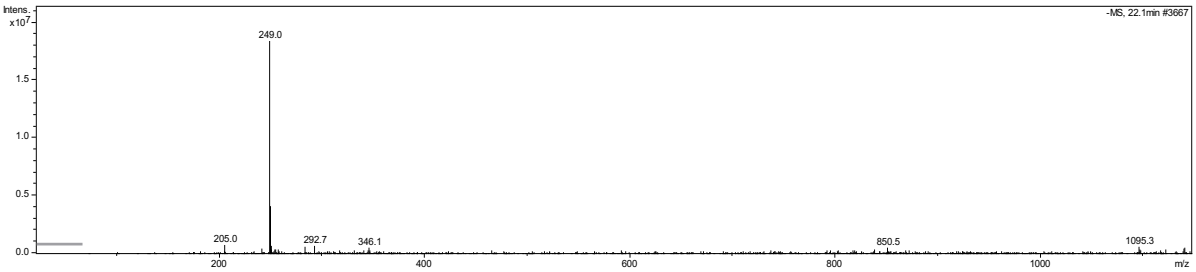
624.5
677.5
1028.6
1324.6
m/z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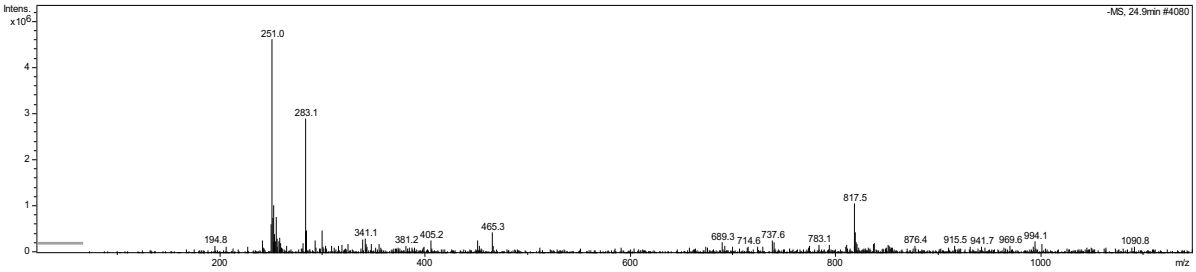
292.7
346.1
850.5
1095.3
m/z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341.1
381.2
405.2
465.3
689.3
714.6
737.6
783.1
876.4
915.5
941.7
969.6
994.1
1090.8
m/z

Appendix D

NABIA

NABIA

New Approach to Bioremediation using Algae

Enhancing health and wellbeing through sustainable transformation of emerging pollutants

Abstract

Hormone related diseases are increasing globally and, in EU alone the total costs associated are about €636 to €637 billion per year and, according to the Health and Environmental Alliance (HEAL) report, by reducing the endocrine disrupting chemicals (EDCs) exposure health savings of up to €31 billion per year are possible; it is estimated that 20% of total dietary exposure to chemicals comes from drinking water.

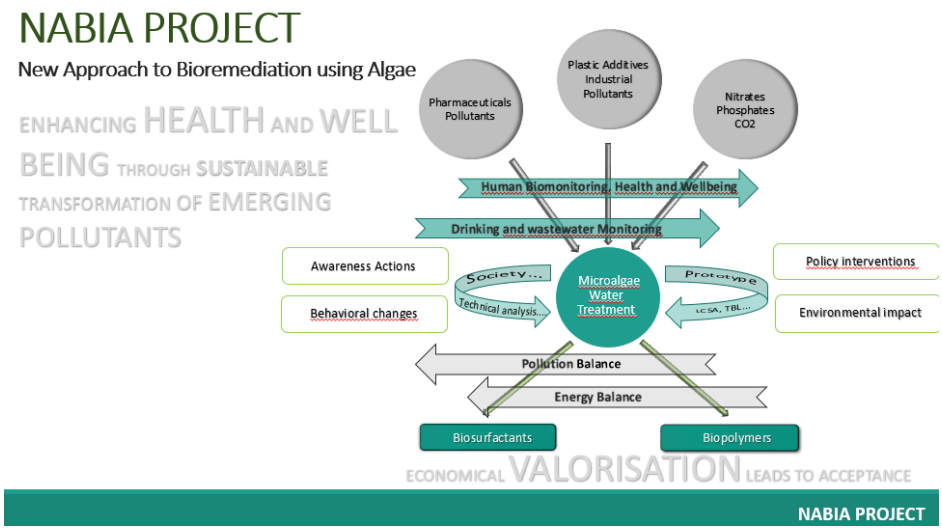
The NABIA project proposes and develops solutions to decontaminate waste and drinking waters using microalgae to improve health and wellbeing, and promote health care savings by preventing exposure to chemicals and the related diseases arising from contaminated water, thus contributing to the sustainability on health and care systems

Using quantitative and qualitative indicators from water monitoring and Human biomonitoring NABIA will identify contributions of environmental chemicals from water usage on the human body burden and link these with aetiological outcomes. Also, it promotes social and political awareness of the adverse effects of water contamination on human health;

NABIA will provide tools to inspire changes in policies and empower policy makers and implementers to apply them by providing robust evidence, data and information. With this approach, the project contributes to preserving, protecting and improving the quality of the environment and to prevent disease and promote health and wellbeing.

NABIA is strongly committed to the transfer of research into policy and practice; to achieve these goals, an international consortium of experts (researchers, public, government, policy makers and implementers, industry, ONGs, private, and civil society actors) will combine efforts and will mobilize support of Health Promotion and Disease Prevention.

Keywords: Environmental Health, Children´s Health, Policy-Making, Microalgae, Bioremediation, Health care systems, Sustainability



***Nabia** was a deity of the Galician-Lusitanian pantheon, considered a goddess of nature, water and renewal, whose cult has parallels in the Celtic pantheon.*

NABIA

Table D.1 *Nabia Consortium.*

Participant No	Participant organisation name	Country
1 (Coordinator)	University of Coimbra	Portugal
2	Hospitais da Universidade de Coimbra	Portugal
3	Institute for the Development of Water Resources	Serbia
4	Serviços Municipalizados de Alcobaça (SMAS)	Portugal
5	Varicon Aqua	United Kingdom
6	University of Patras	Greece
7	Green Technologies	Greece
8	IORIDOmedia	Netherlands
9	Leibniz Institute for Agricultural Engineering Postsdam-Bornim E.V.(ATB)	Germany
10	University of Alicante	Spain
11	University of Perugia	Italy
12	Condensia Química S.A.	Spain
13	University of Aveiro/CESAM	Portugal
14	Maturus Optimi	Netherlands
15	Ministry of Health Directorate - Disease Prevention and Health Promotion	Portugal
16	Orineo	Belgium
18	EUPHA-European Public Health Association	Netherlands
19	Sparks & Co	France
20	University of Manchester	United Kingdom
21	Shamir	Israel
22	ICRA . Institut Català de Recerca de l'Aigua	Spain
23	IMIM Consorci Mar Parc de Salut de Barcelona	Spain
24	University of Amsterdam	Netherlands
25	Different Policy makers, ONGs, SME´s	EU, USA

NABIA

New Approach to Bioremediation using Algae crops
An integrated system for producing bio-based products

Abstract

Microalgae are renewable energy sources that provide a sustainable alternative to oil production and biomaterials for many applications. They are the fastest-growing crops in the world with a complete life cycle of a few days. Their oil yield is over 200 times the yield of the best producer of terrestrial oil seed crops. They reduce greenhouse gases, clean waste water, do not compete with applications in the food and agricultural sphere since they do not require arable land, and have lower water requirements than typical crops. Microalgae systems require relatively small areas, being able to be developed vertically in non-agricultural regions and in water treatment stations.

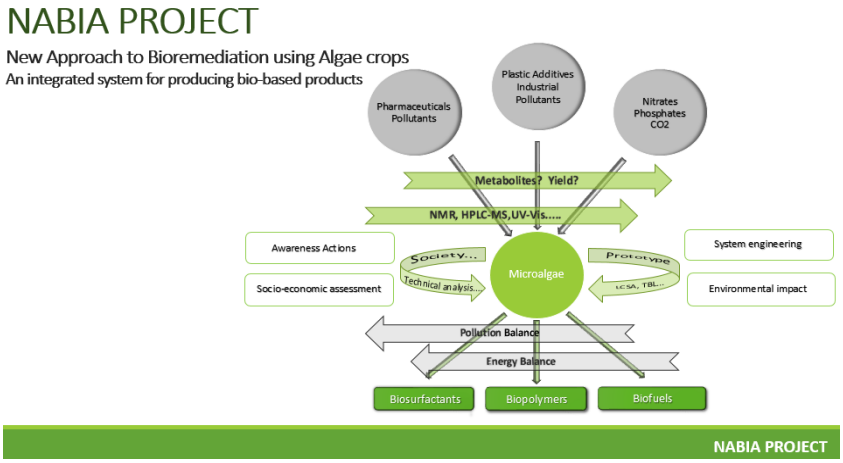
The NABIA project proposes and develops solutions for the increasing demand for oil crops. Its main objective is to transform biomass from bioremediation into innovative bio-based products, such as biosurfactants, biopolymers and biofuels, providing a full use of the biomass in a cascade approach. To achieve these goals, an international consortium of experts will combine extraction, synthesis, characterisation and stability studies, all key factors to understanding and exploiting these novel bio-based products. The action is directed towards developing these materials, which have excellent potential in applications ranging from ophthalmic lenses and optical components, bioplastics, detergents, adhesives and biobinders. The resulting products will be tested and evaluated in industrial environments by consortium SME partners.

An economic, social and technical evaluation will be undertaken, giving recommendations and risk assessment.

NABIA will carry out a Life Cycle Sustainability Assessment as well as an integrated Triple Bottom Line impact evaluation throughout the value chain.

The proposed project will benefit the EU by providing the required knowledge on sustainable and new products in order to meet the increasing demand for oil crops and promote the development of novel bio-based products.

Keywords: Microalgae, Biosurfactants, Biopolymers, Biofuels, Alternative oil crop, Bioremediation, Waste valorisation



Nabia was a deity of the Galician-Lusitanian pantheon, considered a goddess of nature, water and renewal, whose cult has parallels in the Celtic pantheon.

NABIA