biosea

Innovative cost-effective technology for maximizing aquatic biomass-based molecules for food, feed and cosmetic applications

D.1.1 Macro- and microalgae in the BIOSEA project

Contents of priority compounds, their dynamics and main uses

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Summary

Marine and freshwater micro- and macroalgae are among the most promising feedstocks for the production of human and animal food and feed, the extraction of bulk and fine chemicals for a range of applications (pharmaceuticals, neutraceuticals, cosmetics, food supplements, etc.), and production of biofuel. Nevertheless, the algae feedstock market is still facing immature technologies for production and technologies not specifically designed for algae biorefinery. The BIOSEA project is aimed at validating and scaling-up a complete production process of ingredients from main compounds from four micro- and macroalgae species, using a cascading biorefinery approach. The innovation will be on applying them on algae or in combination with other techniques for the recovery of multiple compounds from the same feedstock. This report presents the aims and objectives of the Biosea project, as well as an analysis of the existing data on algal composition and dynamics, in particular those selected in the project.

Marine algae, especially seaweeds, present the largest volume of all cultivated marine organisms, although its value is considerably lower than that of other organisms. The majority of the algae are cultured for the production of phycocolloids (agar, carrageenans, and alginates) and for direct consumption of humans (mainly known under Japanese commercial names as kombu, wakame and nori). Dominant algal species in cultivation are *Kappaphycus/Euchema*, *Saccharina japonica* and *Gracilaria* spp., together representing about 82% of the total cultivated volume. The main cultivated microalga is *Arthrospira (Spirulina)* spp. 99% of algae are cultivated in Asia, with China and Indonesia together accounting for 85%. Cultivation volume in Europe is negligible compared to this, but showed rapid growth over the past five years.

The overall objective of BIOSEA is the development and validation of innovative, competitive and cost-effective upstream and downstream processes for the cultivation of two microalgae (*Spirulina platensis*, official name *Arthrospira platensis*) and *Isochrysis galbana*, and two macroalgae (*Ulva* sp. and *Saccharina latissima*). The aim is to produce and extract high value active principles at low cost to be used in food, feed and cosmetic/personal care high-added value products. The major compound groups the project focuses upon are proteins, carbohydrates, fatty acids, general lipids, phycobilliproteins and carotenoids, but some minor compounds, such as polyphenols will be considered as well. Main applications are food and feed (supplements) and cosmetics and personal care products.

Chapter four presents a detailed overview of the presence and dynamics of the major compound groups and potentially interesting minor compounds in the selected algae species. A thorough analysis of published data indicates high variation of internal levels. The effect and influence of different extraction and analysis methods is briefly discussed. A detailed biochemical profile for each of the four selected species based on current literature is presented. In the second part of the chapter, the main variables affecting compound concentrations in the algae and their interaction are reviewed. Main variables influencing algal composition are temperature, light, pH/CO_2, salinity and nutrients. Interactions are found to be crucial and determining the extent of the effects of the single parameters.

The report finishes with some concluding remarks. With respect to cultivation it is concluded that Europe is lagging far behind compare to Asia, but that the cultivation sector is growing rapidly, with the focus on high quality cultures. Considering the amino acid and fatty acid profiles of the four algae, it is concluded that they are fairly complementary and together present a very interesting feedstock for human and animal food and feed and have various highly valuable compounds for the cosmetics and personal care industry.

1. Introduction

By the end of this century, human world population will have reached between 9 and 10 billion people according to the model predictions of the United Nations (World Population Prospects 2017). Feeding this ever expanding population and providing it with prime matter for energy, transport and other basic needs is one of the largest challenges of society today (Pretty et al. 2010). To cover this demand and claims by other sectors than food and feed, such as biofuels, there is a strong need for new and sustainable bio-based feedstocks.

Considering the European Union specifically, there is a large dependence on imported feedstock. For protein for example, 68% of the demand is covered by imported supplies (FAOSTAT 2013). The total market for bulk chemicals, including biopolymers, biolubricants, biosolvents and surfactants is substantial, estimated at 2.4 million tonnes/year in 2010 (EUROSTAT 2010). At the same time, traditional crops and harvests are under large pressure due to the lack of freshwater resources and climate change (Vörösmarty et al. 2000) and the collapse of global fisheries resources (Jackson et al. 2001, Lotze et al. 2006). Hence to minimize imports, reduce the dependence on traditional resources and to open new markets, the EU should find a new, carbon-neutral source of bio-based feedstock.

Aquatic plant biomass, more specifically micro- and macroalgae have the potential to form such an alternative feedstock with many applications (food, feed, biomedicine, cosmetics, biofuels, etc.) (Wells et al. 2017). Marine algae have the additional advantage that they do not rely on freshwater resources, whereas many freshwater algae can be grown in combination with, or even as a part of wastewater purification plants. At this moment however, its production volume, especially in Europe, is far away from competing with land feedstock as wheat, corn, rice and soybean. Concretely, algae feedstock market is still facing immature technologies for production and technologies not specifically designed for algae biorefinery (Draaisma et al. 2013).

The progress in cultivation is particularly important since improvements in productivity, quality and composition of the biomass are not only relevant to the upstream processing, but have pronounced effects on the downstream and market price as well in terms of process productivity and efficiency (Hafting et al. 2012, 2015, Ruiz et al. 2016). On the other hand, biorefinery of algal biomass is the next step for the commercialization of products from algae. Nowadays the process is at an early stage, and most commercial facilities focus on one single product. Commercial products are currently simply based on harvesting and drying the biomass or on extracting and purifying specific compounds such as omega-3 fatty acids, pigments as β-carotene and others or phycocolloids as agar and alginates all the other fractions obtained from algae are missed, losing high potential.

The BIOSEA project is aimed at validating and scaling-up a complete production process of ingredients from the lipid, protein, carbohydrates and minority compounds fractions from 4 algae species, including upstream and downstream steps using a cascading biorefinery approach that include pre-treatment (including cell disruption), fractionation and conversion technologies. The innovation will be on applying them on algae or in combination with other techniques for the recovery of multiple compounds from the same feedstock, which will require technological adjustments and optimizations.

This report, which together with the Project Management Handbook forms the first deliverable of the project, presents an analysis of the existing data and state of the art in relation to the abovementioned objectives of the BIOSEA project. Chapter two briefly analyses the state of the art of micro- and macroalgae harvest cultivation at a global and European level and their principal uses and application. More details about the Biosea project, the selected algae species and their compounds and applications will be presented in chapter three. Chapter four focuses on the content and dynamics of BIOSEA target compounds in the selected algae. The report will end with some concluding remarks and an extensive bibliography.

2. Micro- and macroalgal cultivation and principal applications

2.1 Global cultivation volume and main species

Cultivation of aquatic plants (freshwater and marine, incl. plants, micro- and macroalgae) comprises an important part of total aquaculture production. Total volume of aquatic plants produced in 2014 was 27.3 million tonnes, 25% of total aquaculture production and hence the second largest culture in terms of volume (Fig. 2.1A, FAO 2016). When considering marine aquaculture only, seaweed culture is even the largest culture in volume. Its value however is considerably lower, at 5.6 million USD representing merely 6% of the total value of aquaculture Fig. 2.1B, FAO 2016).

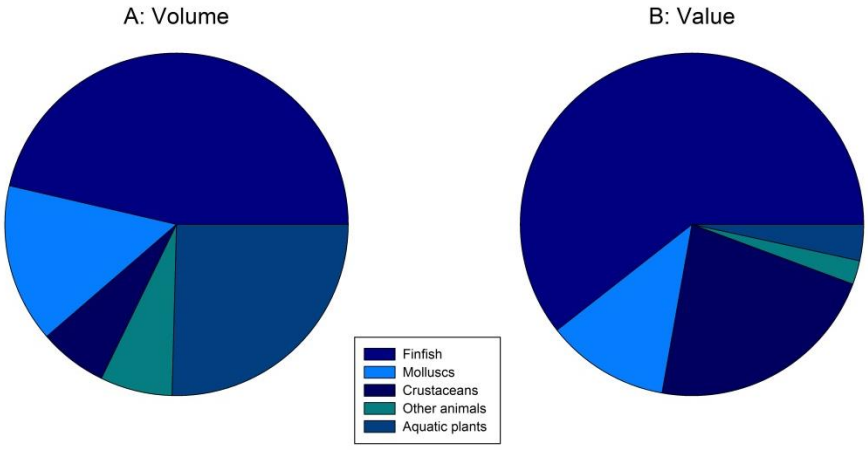

Fig. 2.1A, B: Proportion of total aquaculture volume (A) and value (B) of the different cultivated organism groups. Data: FAO 2016

To the cultured production it has to be added roughly one million tonnes of annual wild harvested that is mainly used for phycocolloids (*Gelidium* and *Gracilaria* species for agar production) and direct human consumption (Nayar & Bott 2014). Apart from that there is local small-scale collection for cattle feed and for use as fertilizer. While production from wild harvest has been declining for years (Nayar & Bott 2014), aquatic plant cultivation has been growing from 6.2 to 8% per year in the last two decades, resulting in a more than doubling of volumes in the last 10 years. This increase is mainly due to the spectacular tenfold increase of seaweed cultivation in Indonesia, basically for the phycocolloid market, although it seems to be topping off now (FAO 2016).

Although generally not counted as aquatic plants, there is also an increase in the recollection and cultivation and use of saltwater depending saltmarsh plants, mainly *Salicornia* and *Sarcocornia* species. The plants are used directly for human food and for the high oil content of their seeds. Exact figures are difficult to localize, however export data from Israel and other exporting countries suggest that it is in the order of hundreds to several thousands of tonnes – far below the volume of other aquatic micro- and macroalgae, however with the increase in knowledge of cultivation methods this sector is growing fast in countries as Israel, Mexico and India, whereas in Europe production the Netherlands are the forerunner, rapidly expanding their *Salicornia* production (Ventura et al. 2013, Gunning 2016).

Aquatic plant biomass cultivation is overwhelmingly dominated by seaweed production, representing 99% of the total volume. In 2014 the production of 37 aquatic plant species (including microalgae) was

registered with the FAO. Carrageenan producers as species from the tropical genera *Kappaphycus* and *Eucheuma*, kelps (*Laminaria japonica* and *Undaria pinnatifida*, commercially known as kombu and wakame respectively), the agarophyte genus *Gracilaria* and species from the genera *Porphyra*/*Pyropia* (the well-known nori) make up the top 5, almost 98% of the total volume of aquatic plant biomass produced in 2014 (FAO 2016).

Despite biofuel-driven incentives and the general agreement on their potential of the last decade, microalgal production is still very modest compared to seaweeds. The lead species is *Spirulina* (usually it is meant with this the species *Arthrospira platensis*), with an estimated production of 86,000 tonnes in 2014 and total microalgal production representing a value of 560 million USD, although FAO advises that microalgae cultivation is poorly reflected in available statistics as data are generally not reported to FAO (Vigani et al. 2015, FAO 2016).

In general, microalgae are cultivated in relatively low volumes for high-value compounds (Milledge et al. 2014). Important species and production volumes in 2000 were *Spirulina* with 2,000 tonnes, *Chlorella* (2,000 tonnes) and *Dunaliela* (1,200 tonnes) (Richmond 2004 chapters 11-13). Compared to the 86,000 tonnes for *Spirulina* alone in 2014, this is a dramatic increase indicating the high growth rates also reported by the FAO (FAO 2016). Apart from the species already mentioned, other promising species for mass cultivation include *Haematococcus*, *Porphyridium*, *Nannochloropsis* and *Nostoc*. Furthermore, many species are used in aquaculture, of shellfish in particular or as accessory cultures (Muller-Feuga 2004).

2.2 Cultivating countries

Large scale seaweed cultivation is almost entirely located in Asia, accounting for 99% of the total global production (Table x, FAO 2016). China is lead producer with close to 50% of total production, directly followed by Indonesia with 35%. The latter country has been the main driver for the growth in production in the past years. It has adopted the maintenance of the growth of mainly carrageenan producing species as a national policy and if this program succeeds, Indonesia is on a fast track of becoming the world's largest producer of seaweeds (FAO 2016). Chile is the largest producing country outside Asia; however the proportion is small in comparison to Asia. Here, high quality *Gracilaria* production might be capable of partly replacing the dwindling *Gelidium* yields for the production of laboratory agar (Callaway 2015). Norway and France are currently the main European producing countries; however, production is negligible on a global scale.

Due to the lack of exact figures it is difficult to make a comparison between microalgae producing countries. Asia still seems to be market leader, although apparently less dominant than in the case of macroalgae as reviewed by Enzing et al. (2014). Important producing countries within Asia include China, Japan, Malaysia, Thailand, Myanmar, India and Taiwan. Large cultivation plants can also be found in the USA (Hawaii in particular), Australia, New-Zealand, Chili, Mexico and Israel. Production in Europe has been expanding during the past 10-15 years, especially in countries as Sweden, The Netherlands, France, Germany, the Czech Republic, Portugal and Spain (Enzing et al. 2014, FAO 2016).

2.3 Micro- and macroalgae cultivation in Europe: outlook

Although local seaweed use in Europe has a long tradition that goes back to prehistoric times, seaweed farming is a new phenomenon (Guiry & Blunden 1991, Anonymous 2012). Nevertheless, considering the currently increasing interest and demand for seaweeds of a guaranteed quality and composition for use directly as human food and as prime matter for food, feed and other products, the economical perspectives for farming are looking much more prosperous than was the case only five to ten years ago (Hafting et al. 2015, Cottier-Cook et al. 2016). First countries to establish farms were France, Norway, the Faroe Islands and Iceland. At this moment, the number of cultivation sites is increasing and (mainly small) sea-based farms can also be found in Ireland, Denmark, Spain (North and South Atlantic coast)

and the Netherlands (https://www.zeewaar.nl/uk/). In North Spain the Asturia-based company Algas de Asturias is culturing algae on small scale at open sea and selling them under the brand name Organic Sea Farmers (http://www.organicseafarmers.com). The company Seaweed Harvest Holland (www. http://seaweedharvestholland.nl) is currently experimenting with a small farm of half a hectare, but is planning a farm of several hectares large that is expected to enter in production in 2019. Commercial land-based farms have been established in Portugal (company ALGAplus, https://www.algaplus.pt/) and Spain (La Huerta Marina, http://lahuertamarina.com/es/), whereas these and other countries (e.g. Germany, UK) also have experimental programs running in various degrees of progress. Concrete figures could not be found but can be estimated in the order of magnitude of several dozen tonnes annually at this moment.

Microalgal cultivation (both freshwater and marine) has a slightly longer history in Europe with first companies appearing in the last decade of the last century. Biofuel-driven incentives have certainly helped in stimulating microalgae start-ups and spin-off companies and nowadays smaller and larger companies can be found all over Europe (Enzing et al. 2014). The scale is still small compared to the large open pond systems that can be found in Asia, Australia, Hawaii and Israel; the largest plants are Roquette in Germany (www.roquette.com) and 4AF in Portugal (www.a4f.pt) and occupy approximately 1 ha. (Enzing et al. 2014, Vigani et al. 2015). Cultivation mainly takes place in photobioreactors in various shapes and sizes; earthen pond farms as can be found elsewhere are rare in Europe.

2.4 Principal uses of macro- and microalgae

Two main types of uses can currently be distinguished for cultivated seaweeds: directly for use in human food and feed for animals and for their use of phycocolloids (McHugh 2003). Table 2.1 shows the main species and produced volumes (data from FAO 2016). Species of the genus *Gracilaria* are used both as food as well as for phycocolloid production, although the latter is by far the most important. *Saccharina japonica*, apart for food is also used for alginate production, however only in case of surplus production (McHugh 2003).

Table 2.1: Cultivated species of seaweeds, their common name (in case of food macroalgae), total volume (thousand tonnes), and percentage of total.

Scientific name	Common name	Cultivated volume	% of total culture
Kappaphycus/Euchema		10,992	40.4
Saccharina japonica (*Laminaria japonica*)	Kombu	7,655	28.1
Gracilaria spp.	Ogonori	3,752	13.8
Undaria pinnatifida	Wakame	2,359	8.7
Porphyra/Porypia	Nori	1,806	6.6
Sargassum fusiforme	Hijiki	175	0.6
Others		482	1.8

Bearing this in mind it can be seen that approximately half of the seaweeds are grown for food production. Minor uses of cultivated seaweeds include the use as food and feed additives and the extraction of fine biochemicals for cosmetics and pharmaceutical uses. Uses as organic fertilizer, soil conditioner or for the production of liquid fertilizer rely almost completely on collected seaweeds from the wild and not on cultivation (McHugh 2013). Cultivated seaweed in Europe is used mainly for human food and to a lesser degree for alginates.

Microalgae are traditionally been sold unprocessed (only dried) as food supplements for the health food market and also as feed, especially in marine aquaculture (Chapters 11-23 in Richmond 2004, Enzing et al. 2014). Besides the use of whole-dried algae for food and feed there is a still increasing market for specific high-value components. Although in general, production of synthetic alternatives is cheaper than algae-based molecules, in some cases the latter have specific advantages, making their use

commercially viable (Spolaore et al. 2006). Examples of this category include the pigment astaxanthin and other carotenoids as β-carotene and lutein (fig. 10 in Enzing et al. 2014).

A very recent compilation of the most important high-value molecules that are currently on the market (pigments and fatty acids), the microalgae they are extracted from, their application and traditional or synthetic alternatives is given in figure 11 in Enzing et al. (2014). The following figure 12 lists the major products and producers and the microalgae source. This list is progressively growing with the discovery and development of new applications of carotenoids and other molecules (polysaccharides), especially in the field of health products and medicine (Raposo et al. 2013, 2014).

2.5 Further bibliographical and online resources

The booming interest in the cultivation and application of macro- and microalgae has also led to a spectacular increase in the publication of books and manuals. Whereas for seaweeds, during many years, notes on applications merely formed limited chapters in more general scholarly books on algae, currently there is a vast volume of books dedicated almost entirely to this topic. For microalgae the flood of publications has been going on longer as their economic potential has been recognised already for a longer time. The following lists a by no means exhaustive overview of most recent books and online available reports on cultivation and application of macro- and microalgae and derived products.

- Seaweed Resources in Europe: Uses and Potential (1991). Guiry MD, Blunden G (Eds.), John Wiley & Sons: Chichester, 432 pp.
- Seaweed Resources of the World (1998). Critchley AT, Ohno M (Eds.), JICA: Yokosuka, 431 pp.

These are the two "classical" books on use and potential of seaweeds. Due to the recent fast development they are outdated as considering the state of the art of cultivation but still provide a good overview of used and potentially valuable species per area.

- A guide to the seaweed industry (2003). McHugh DJ, FAO Fisheries Technical Paper, No. 441, FAO: Rome, 105 pp. *Available online:* http://www.fao.org/3/a-y4765e.pdf

Slightly outdated but still relevant overview of the world seaweed industry and main applications.

- Bioactive Marine Natural Products (2005). Bhakuni DS, Rawat DS, Springer: Dordrecht, 382 pp.

Very comprehensive overview of natural products known at the time of publication that could be obtained from macro- and microalgae.

- Handbook of Microalgal Culture: Biotechnology and Applied Phycology (2003). Richmond A (Ed.), Wiley-Blackwell, 588 pp.
- Algal Cultures, Analogues of Blooms and Applications (2006). Volumes 1 and 2. Subba Rao DV (Ed.), Science Publishers: Enfield, 971 pp.

Extensive overviews of ecology, ecophysiology, culture techniques and applications of microalgae.

- Marine Medicinal Foods, Volume 64. Implications and Applications, Macro and Microalgae (2011). Kim SK (Ed.), Academic Press: Waltham, 466 pp.

The main focus of this book is on macroalgae, but it contains plenty of information on microalgae as well. Information is categorized by species, but also by disease and by compound, furthermore provides information on general nutritional value (for humans) of algae, applications in medicine, nutraceuticals, cosmetics, culinary uses, etc.

- Handbook of Marine Macroalgae: Biotechnology and Applied Phycology (2011). Kim SK (Ed.) Wiley: New York, 592 pages

- Seaweeds. Edible, available and sustainable (2013). Mouritsen OG, University of Chicago Press: Chicago, 304 pp.
- Products from marine algae: Methods and Protocols (2015). Stengel DB, Connan S (Eds.), Springer: Heidelberg, 439 pp.
- Seaweed Sustainability. Food and Non-Food Applications (2015). Tiwari BK, Troy D (Eds.) Elsevier: Amsterdam, 470 pp.
- Seaweed in Health and Disease Prevention (2016). Fleurence J, Levine I (Eds.), Academic Press, Waltham, 476 pp.
- Seaweed Polysaccharides. Isolation, Biological and Biomedical Applications (2017). Venkatesan J, Anil S, Kim SK (Eds.) Elsevier: Amsterdam, 408 pp.
- Biotechnological Applications of Seaweeds (2017). Nabti E (Ed.) Nova Science Publishers: New York.

Series of recent books on seaweed biotechnology and applications and specific book on polysaccharides from seaweeds.

- Microalgae-based products for the food and feed sector: an outlook for Europe (2014). Enzing C, Ploeg M, Barbosa M, Sijtsma L, Vigani M, Parisi C, Rodríguez Cerezo E, European Commission, Joint Research Centre, Institute for Prospective Technological Studies: Seville, 78 pp. *Available online:* *http://publications.jrc.ec.europa.eu/repository/bitstream/JRC85709/final%20version%20online%20ipts%20jrc%2085709.pdf*

Important and recent report for the microalgae production and processing sector in Europe.

- Marine Polysaccharides: Food Applications (2016). Venugopal V (Ed.) CRC Press, 396 pp.

Specific book on marine polysaccharides with chapters on macro- and microalgae.

- *Spirulina platensis* (*Arthrospira*). Physiology, cell-biology and biotechnology (1997). Vonshak A (Ed.) Taylor & Francis, London, 233 pp.

Entirely dedicated to ecophysiology, biochemistry, cultivation and application of *Arthrospira* spp.

- Manual for protoplast preparation from Ulva and their seeding for cultivation (2011). Reddy CRK, Seth A. CSIR-Central Salt and Marine Chemicals Research Institute, Bhavnagar, India, 10 pp. Available online: http://prospettivehitech.com/progetti/b4b/wp-content/uploads/b4b/wp2-submitted/Manual_for_protoplasts_preparation_and_cultivation.pdf

Online practical guide for protoplast preparation of *Ulva* spp.

- New England Seaweed Culture Handbook Nursery Systems (2014). Redmond S, Green L, Yarish C, Kim J, Neefus C, Connecticut Sea Grant CTSG-14-01, 92 pp. *Available online: http://media.ctseagrant.uconn.edu/publications/aquaculture/handbook.pdf, companion YouTube video instruction series on setting up seaweed nurseries: http://s.uconn.edu/seaweedplaylist*

Cultivation manual of several brown (kelp) and red seaweed species, including a series of instruction videos available online.

- EnAlgae project: www.enalgae.eu

The publications area of this project contains a variety of freely downloadable reports on microalgae cultivation and applications, including good practice guides for standard operations, strain maintenance, cultivation and processing as well as policy reports, overviews of rules and regulations, etc.

- Phycomorph COST Action: www.phycomorph.org

Phycomorph is dedicated to unifying a scattered European research landscape on the topic of seaweed reproduction and cultivation to develop aquaculture techniques. Joint publications, many of which freely downloadable on seaweed reproduction and cultivation can be found under “Activities – Joint publications” on the website.

3. The BIOSEA project: species, compounds and applications

3.1 Objectives and expected results of the BIOSEA project

The overall objective of BIOSEA is the development and validation of innovative, competitive and cost-effective upstream and downstream processes for the cultivation of 2 microalgae (*Spirulina platensis*, official name *Arthrospira platensis* and *Isochrysis galbana*), and 2 macroalgae (*Ulva* sp. and *Saccharina latissima*) to produce and extract high value active principles at low cost (up to 55% less than with current processes) to be used in food, feed and cosmetic/personal care high-added value products. By this way, BIOSEA will contribute in reducing Europe's dependence on feedstock imports, diminishing the pressure of land resources and opening new markets and business opportunities. The project will include, on the one hand upstream and downstream processing steps for the acquisition of selected active compounds and the evaluation of their bioactive properties. Special attention will be paid to the separation, extraction and purification of the compounds of interest maximizing yield per mass unit and optimizing the cost (achieving a decrease up to 55% of the production costs) in order to allow for a cost-effective scale-up of the process.

This general objective is further concretized in 6 specific objectives, S1 to S6:

S1. To develop and validate innovative 4 cost-effective and environmental friendly processes (1 for each alga in the objective) addressing upstream and downstream processing steps to obtain the highest concentration of the added value active principles at the lowest process costs, to be used in the production of products for food, feed and cosmetic/personal care market.

S2. To improve the cost effectiveness of the production of ingredients using algae with55% compared to current routes towards the same comparable products.

S3. To improve the yield per mass unit of the desired compounds using BIOSEA processes vs. current routes.

S4. To develop and formulate new ingredients from active principles obtained with BIOSEA processes for obtaining the products for the selected applications with improved properties and high added value complying with the EU legislation.

S5. To develop and validate a microencapsulation process for enhancing the stability of bioactive compounds to incorporate them in industrial formulations when necessary and to obtain a smart system for controlled release of the active compound.

S6. To ensure economic, technical, and environmental sustainability of BIOSEA processes and products.

The BIOSEA hence has a vertical approach, from culture to product, adding value to all steps in the chain (Fig. 3.1)

The main results expected to arise from BIOSEA are:

R1. Specific aquatic biomass suitable for application in food, feed and personal care markets, using both micro- and macroalgae from the aquatic environment. The food subsector to which the products will be focus are the vegans and vegetarian population, ecological awareness population, sensitive to food allergens populations, the specialized distributors of healthy foods and, also, the food ingredients industries because new substitutes for proteins, sugars, vitamins and fats will be obtained.

R2. New growth conditions of micro- and macroalgae for substantially increased yields.

R3. Isolation of the proteic fraction with added value due to its rheological, nutritional, functional properties in food industry (specifically in the substitution/alternative proteins in the food

formulations without meat proteins/meat surrogates for the vegan and vegetarian market) and, in feed industry (specifically in the substitution of soy proteins).

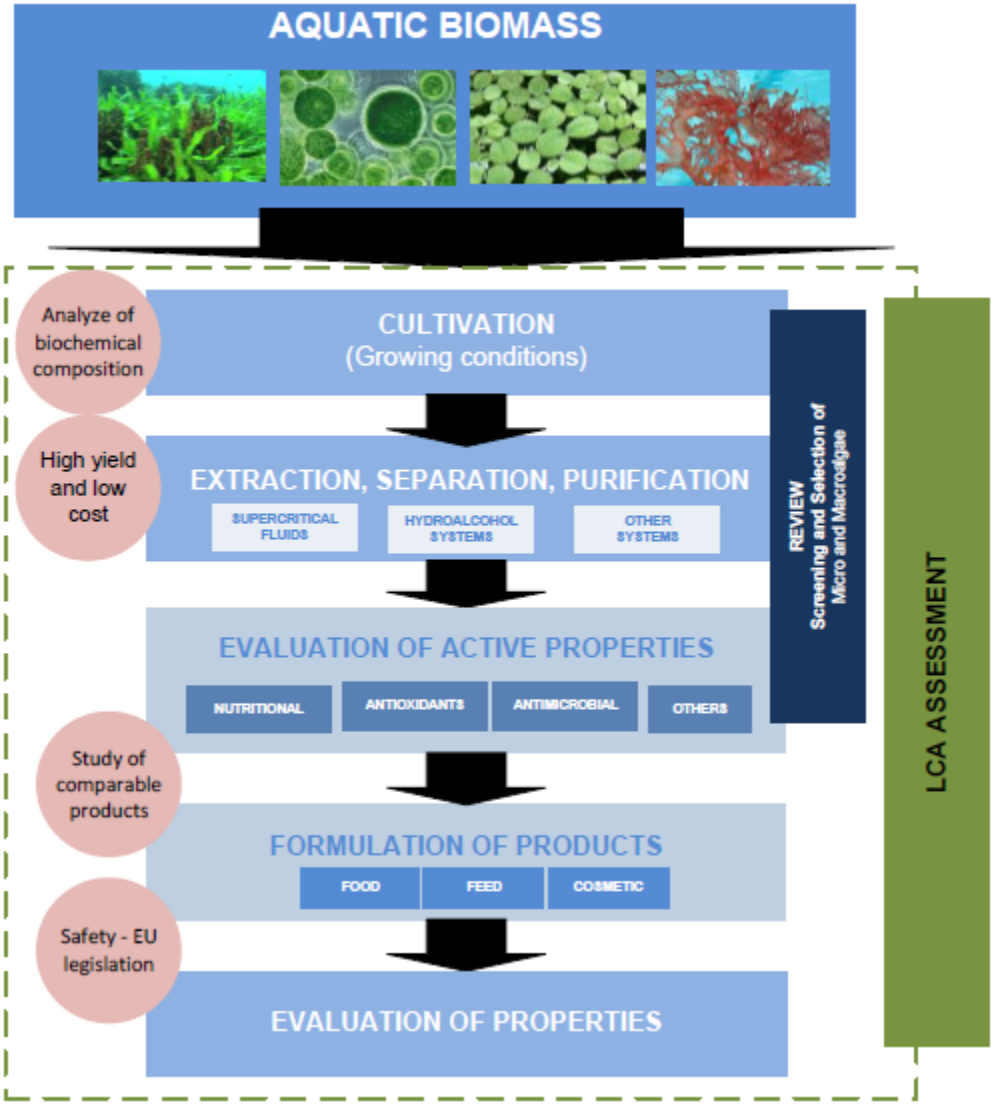

Fig. 3.1: Diagrammatic representation of the BIOSEA approach (from BIOSEA project proposal).

R4. Isolation of the antioxidant fraction with added value for its natural source in feed and cosmetic industry.

R5. New eco-efficient/eco-sustainable separation, purification and extraction methodologies for obtaining the maximum yields of the proteic (up to 39.3%) and functional antioxidative fractions (carotenoids) (up to 34.43%) minimizing the use of organic solvents up to 55%. Optimised cascading conversion including all required steps (cultivation, drying, cell disruption, extraction, purification, drying) of the aquatic biomass and desired compounds achieving a reduction of the process cost up to 55%.

R6. Acquisition of at least 6 compound groups with specific properties for the different final applications

R7. Functional evaluation of the compounds extracted from aquatic biomass using the nematode *Caenorhabditis elegans* (*C. elegans*) as a model organism. This analysis will be conducted with compounds extracted from aquatic biomass or in combination thereof, estimating the optimal dose in order to observe benefit in the nematode for each of the experimental models (obesity, aging, neurodegeneration, inflammation, antimicrobial activity and toxicity). Finally, the metabolic target of the selected compound (s) will analysed by transcriptomics methods. The results of the evaluation of bioactive properties can show that one active compound has

application in pharma sector. In this case, the consortium will contact with different companies of the pharma sector in order to know their interest in the compound and start future research.

R8. Validation of final products developed for final applications in food, feed and cosmetic sectors, with improved properties and high added value, reflected in cost-efficiency, improved sensory characteristics and techno-functional properties, which will be measured and ranked.

3.2 Algae selected in BIOSEA: general information

To achieve these objectives, two species of microalgae and two species of macroalgae had already been pre-selected for the project, based on the variety of the components they are known to possess suitable for their incorporation in food, feed, and cosmetic products, the large knowledge base already available and advanced state of readiness level of technology for cultivation of these four species. The species are the microalgae *Arthrospira platensis* (better known as *Spirulina platensis*) and *Isochrysis galbana* and the macroalgae *Ulva* sp. and *Saccharina latissima*. In the following paragraphs briefly the taxonomy, morphology, and ecology of the species will be discussed as well as the general current status of cultivation and applications of these species.

3.2.1 *Arthrospira platensis* Gomont

Of the four selected species, *A. platensis* is the only prokaryote, the others are all eukaryotes. It belongs to the phylum of the Cyanobacteria, order Oscillatoriales, family of Microcoleaceae. In academic circles it is debated whether cyanobacteria should be called algae or not (Bolton 2016), here the functional definition of algae is used including cyanobacteria. The name *Spirulina platensis* or simply *Spirulina* is a commercial name, generally used for a food supplement that consists either of *A. platensis* and *A. maxima* or a mixture of these (Guiry & Guiry 2017).

Units tend to have a coiled, multicellular form, and are called trichomes; the size of trichomes and their degree of coiling can vary from almost straight to tightly coiled. Filaments are solitary and individual cells range from 2 to 12 or even 16 µm in diameter (Fig. 3.1). The species naturally occurs both in freshwater as well as in alkaline brackish and saline waters from subtropical and tropical regions. *A. platensis* is the most widely distributed *Arthrospira* species and is mainly found in Africa, Asia, and Central America (Guiry & Guiry 2017).

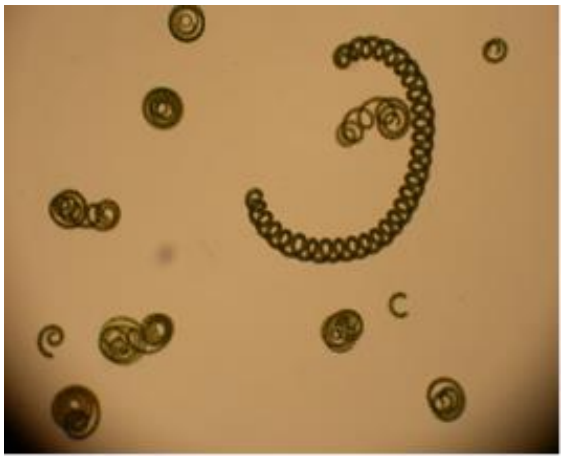

Fig 3.1: *Arthrospira plantensis* (*Spirulina plantensis*) trichomes. Photo: IGV, Germany.

A. platensis has a long history of application and cultivation. In fact it was the first cyanobacterium to be commercially cultivated using modern biotechnology, with cultivation starting in the late 1970s (Hu 2004). Its use for human food however, goes back much further in time, at least 700 years. In Mexico and lake Chad vicinity in Africa, the harvesting and consumption of accumulations of sun dried *Arthrospira* in Mexico and Africa, lake Chad vicinity, was common around the year 1300 and perhaps even earlier. Nowadays, the main volume of the algae is still used in dried form for food, or food supplements to be more exact and to some extent also as feed, especially in the aquaculture sector (Hu 2004, Vigani et al. 2015). In addition, the alga is exploited for its phycocyanin pigments that have various

applications in food, feed, cosmetics and medicine (Enzing et al. 2014). Research in the past decade has revealed the presence of various components in *Arthrospira* that can be classified as pharmaceuticals and nutraceuticals and other fine chemicals that are in the process of development and commercialization.

As said above, cultivation of *Arthrospira* started in the late 1970s in Asia. Commercial cultivation is generally performed outdoors in open raceways or circular ponds (Hu 2004). Most of the cultivation facilities are still localized in the Asian-Pacific region, however plants have also been established in Central America (Mexico, California), Israel and even in Europe (Germany) in greenhouses (Enzing et al. 2014). Production as whole dried microalgae was estimated to be as high as 5,000 tonnes dry weight per year in 2012 (Vigani et al. 2015). In the BIOSEA project, *Athrospira* cultivation will take place in Germany in two types of photobioreactors: classic tubular glass ones and the new scalable mesh-ultra-thin-layer lechnology (MUTL) photobioreactor by the company IGV.

3.2.2 *Isochrysis galbana* Parke/ *Isochrysis* aff. *galbana* (T-ISO)

Isochrysis galbana is a microalga found in the moderate cold waters of the Atlantic Ocean in Europe and North America (Guiry & Guiry 2017). However, when referred to in literature in the context of aquaculture and cultivation, generally it is meant a tropical strain for long known as *Isochrysis* aff. *galbana* or T-ISO (short for Tahitian *Isochrysis,* after its origin), as for sale from algae collections as the Culture Collection of Algae and Protozoa in Oban, Scotland (UK). Based on an analysis of DNA sequences, this species has now been renamed to *Tisochrysis lutea* Bendif et Probert (Bendif et al. 2013). Nevertheless, both genera are eukaryotes of the Chromista kingdom, belonging to the phylum Haptophyta, order Isochrysidales, family of Isochrysidaceae.

Both species are free living, solitary cells of similar size (2.5 – 6 µm length), golden brown in colour and of spherical or ovate to oblong morphology (Fig. 3.2). Both possess two flagellae of 5 – 7 µm length and are covered by a dense layer of thin organic scales (Parke 1949, Bendif et al. 2013). Both are fully marine species, with *I. galbana* being distributed as said in moderate cold waters and *T. lutea* in tropical oceans (Guiry & Guiry 2017).

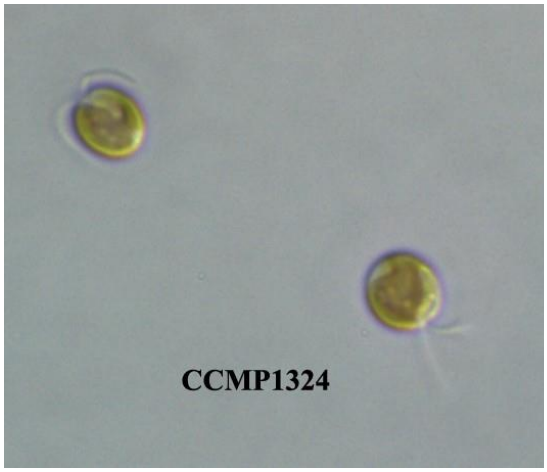

Fig 3.2: *Tisochrysis lutea* (*Isochrysis* aff. *galbana* or T-ISO) cells. Photo: Bigelow National Center for Marine Algae and Microbiota, USA – ncma.bigelow.org

T. lutea (as *Isochrysis* aff. *Galbana* or T-ISO) is one of the species most widely used in the rearing of bivalves in hatcheries or nurseries (Coutteau 1996) as well as for peneid shrimps and accessory cultures (Muller-Feuga 2004). The algae have rich profiles in proteins and especially in lipids and fatty acids (Muller-Feuga 2004).

Isochrysis cultivation takes place worldwide, generally, bearing in mind its application in aquaculture, concentrated in areas where bivalve or peneid shrimp aquaculture. No exact data are known on cultivation volumes, however an idea of the order of magnitude might be obtained from the volume of bivalve cultures. Muller-Feuga (2004) calculated based on data from 1999 that worldwide approximately 5000 tonnes dry weight microalgae would have been required to support the mollusc production, if

hatcheries would have been the sole source for all mollusc production. Most alga production takes place on the site of the aquaculture farm itself or at specialized hatcheries, there are relatively few companies dedicated exclusively to the cultivation of T-ISO (Enzing et al. 2014). Typical T-ISO is mass cultivated outdoors in polyethylene bags or also in open ponds and tanks that even allow for continuous cultivation (Zmora & Richmond 2004); several attempt haven been made for cultivation in flat-panel reactors (Zmora & Richmond 2004) or tubular photobioreactors (van Bergeijk et al. 2010) with changing success. Likewise to *Athrospira* cultivation, *Isochrysis* will be cultured in the BIOSEA project in Germany in two types of photobioreactors.

3.2.3 *Ulva* L.

The genus *Ulva* represents a large group of very closely related species; at present 125 names have been flagged as currently accepted taxonomical entities (Guiry & Guiry 2017). They belong to the phylum of the Chlorophyta (green algae), order Ulvales, family Ulvaceae. There are two basic morphologies: tubular (formerly incorporated in the genus *Enteromorpha*, Hayden et al. 2003) and flat. Depending on conditions, some species that possess a predominantly tubular morphology might appear flat (Spoerner et al. 2012). Apart from being either tubular or flat, there are no macroscopical and only few microscopical characteristics to distinguish the different species; apart from that, species have demonstrated high morphological flexibility causing any identification based solely on these characteristics to be inherently unreliable, therefore obliging the use of molecular methods for identification (Malta et al. 1999, Hayden & Waaland 2004). This taxonomic confusion also complicates the determination of the exact geographical distribution of the different species; it is clear however that there are many species in the genus that have an almost cosmopolitan distribution (Kraft et al. 2010). Moreover, although the genus is predominantly marine, its species are known for their wide tolerance of salinity (Malta et al. 1999); some species are even capable of growing in both fully marine and fully freshwater conditions (Rybak 2016). *Ulva* can be found worldwide from the littoral zone to the upper sublittoral. It is well known for forming nuisance bloom in eutrophic areas all over the world (Morand & Briand 1996), including the well-documented green tides in the Yellow Sea in China, that first appeared in 2008, threatening the sailing competition of the Olympic Games of that year (Leliaert et al. 2008).

Considering their biochemistry, especially with regards to protein and polysaccharide composition, species show very little differences between them (Lahaye et al. 1999, Robic et al. 2009, Angell et al. 2016). The species preselected in the BIOSEA project was *U. intestinalis*, however, bearing in mind the abovementioned remarks on *Ulva* taxonomy and biochemical composition, it seems opportune to not to adhere too strictly to this. Instead a more pragmatic strategy will be adopted by cultivating the species that are commonly found in the region of the project partners responsible for the production of the *Ulva* biomass, selecting both a species of flat and of tubular morphology and to use both of them in the project. Exact identification will then be made a posteriori using molecular techniques.

As said, two basic macroscopic morphologies can be identified, tubular and flat (Fig. 3.3A and B). Tubular species consist of single or branched tubes of one cell layer thickness, whereas flat species consist of a double layer of cells without displaying a central cavity (Koeman 1985). Cells are rounded to square to polygonal and the species has an isomorphic alternation of generations. Form and location of the chloroplast can vary, so that in microscopical view cells may appear entirely green or green and transparent (Koeman 1985).

Fig. 3.3 A and B: *Ulva* sp. with tubular (left) and flat morphology (right). Photo: E. Malta.

Ulva species have a long tradition of use as human food, animal feed and fertilizer in Asia in particular, but also in some European and North- and South-American countries (Critchley and Ohno 1998). Tubular species (formerly called *Enteromorpha*) have a history of cultivation for human consumption in Japan and Korea on nets suspended on floating rafts of poles, however at much lower volumes compared to other seaweed species (McHugh 2003). Over the last decade, commercial cultivation of *Ulva* (flat species) has taken a flight in South Africa. The algae are often grown in integrated (tank or earthen pond) paddle wheel systems in conjunction with abalone aquaculture (Bolton 2009). In 2012, production was estimated to be about 2884 tonnes wet weight, probably turning it in the leading *Ulva*-producing country (Maneveldt & Robertson-Andersson 2016). Interest in *Ulva* cultivation appears to be growing worldwide; tank or earthen pond cultivation can be found in Portugal (company Alga+, https://www.algaplus.pt/), Spain (La Huerta Marina, http://lahuertamarina.com/gb/) and Israel (Seakura, http://www.seakura.net/copy-of-seakura). The Dutch company Zeewaar (https://www.zeewaar.nl/uk/) is growing *Ulva* in a marine inlet in the Netherlands. Production of these latter companies is in the order of magnitude of several dozens of tonnes wet weight per year. A Google search on *Ulva* farming will also deliver news stories from the USA, Canada, Chili and Brazil, although no detailed information on companies could be found.

3.2.4 *Saccharina latissima* (Linnaeus) C.E.Lane, C.Mayes, Druehl & G.W.Saunders

Sugar kelp, *S. latissima*, until 2006 named *Laminaria saccharina*, and together with other Laminariaceae species commercially best known as kombu, is a brown alga. It belongs to the phylum Ochrophyta, class Phaeophyceae (brown algae), order Laminariales and family Laminariaceae. It mainly occurs in the Northern Atlantic in cold to temperate waters, although the species has also been reported from the Pacific and Indian Ocean (Guiry & Guiry 2017). Like other kelps, it is a truly marine species that can be found from the upper subtidal (or even lower intertidal in northern areas) to a depth of about 15 to 20 m (Edwards et al. 2012).

These are large algae that are easily recognizable, of a shiny greenish brown colour. The morphology consists of a holdfast made of a number of branched root-like outgrowths, a relatively short, round stipe (60 cm maximum) and fronds (blades) that are ruffled on the sides and un-branched. The algae can reach considerable lengths, up to 5 m (Fig. 3.4). As many seaweeds it has a bi-stage, anisomorphic life-cycle with the sporophyte being the well-known kelps and a microscopic gametophyte (Edwards et al. 2012). *Saccharina* is an important part of the subtidal ecosystem as it can form dense meadows or subtidal forests that are an important habitat for marine fauna and play a role as nurseries for many fish and other animal species (Christie et al. 2009).

Fig. 3.4: *Saccharina latissima* being harvested and harvested biomass in the drying room. Photo: AT~SEA Technologies, Belgium.

Kelps are the world's second most cultivated seaweeds with a cultivation history that goes back centuries, although this generally concerns the Asian species *Saccharina japonica*. Of this species more than 7 million tonnes was produced in 2015 (FAO 2016) in Japan, the Koreas and China. Cultivation of *S. latissima* is occurring at a much more modest scale and mainly limited to Canada, Europe and the USA, with a history of hardly two decades. Currently, various farmers are active in Europe, both on a commercial and experimental scale, especially in the Scandinavian countries, France, NW Spain and Portugal (Abreu et al. 2013). Summing data that can be found from companies on internet, total production in Europe is estimated to be in the order of dozens of tonnes wet weight per year. Lead producer probably is the company Ocean Rainforest (http://oceanrainforest.com/) in the Faroe Islands. Other companies include Seaweeds from Norway (http://www.seaweedfromnorway.no/) and Seaweed Energy Solutions (http://www.seaweedenergysolutions.com/en). BIOSEA partner At-Sea Technologies is developing innovative textiles for *S. latissima* farming (among others) and the Dutch-Norwegian based company Hortimare (www.hortimare.com) provides sporelings from its hatchery.

3.3 BIOSEA targeted compound groups and relation with BIOSEA applications

Following the general objective, BIOSEA is dedicated to obtain compounds from the algae that can be used for food, feed and cosmetics/personal care products. In order to do this, the project will focus on the following compound groups that can be obtained from the algae: proteins, carbohydrates, fatty acids, carotenoids and phycobiliproteins. However, as BIOSEA strives to maximize utilization of algal biomass, screenings will be performed for other compounds or compound subgroups as well, such as phenolic compounds and other antioxidants, other pigments, dietary and non-dietary fibres, etc. The contents of each of the interest compound groups for all four priority species will be discussed in detail in the next chapter. Here the BIOSEA priority compounds groups will be introduced with some general notes on their function and how algae could contribute (Fig. 3.2).

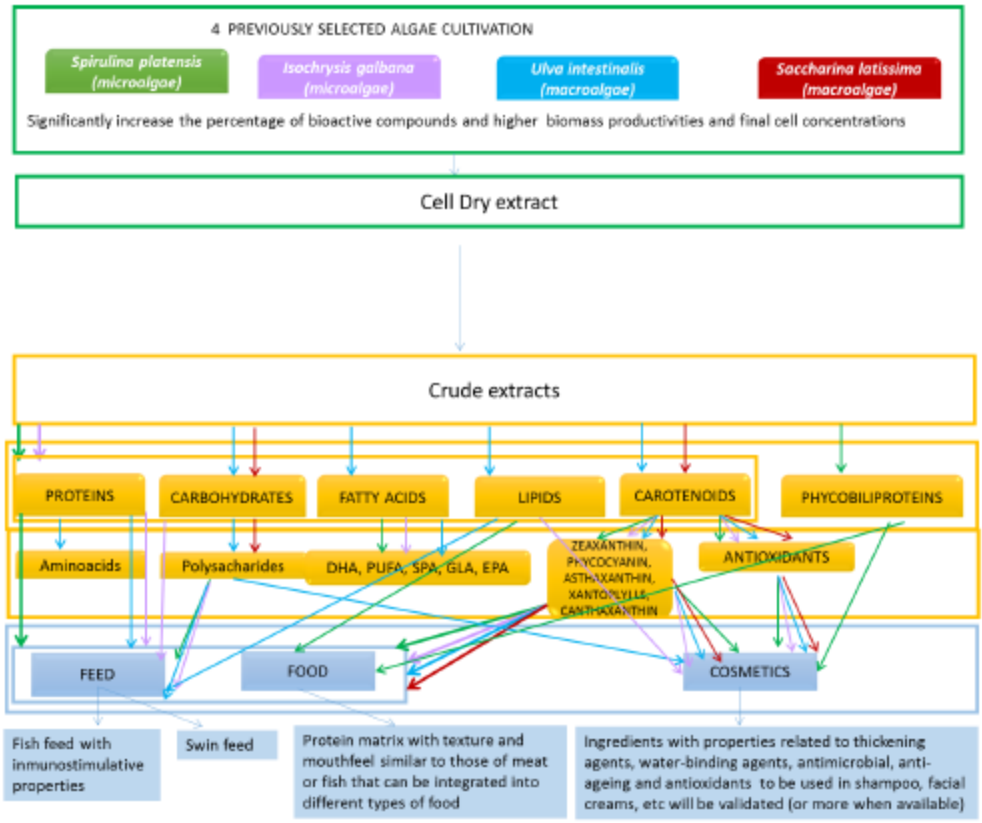

Fig. 3.2: Origin and use of the priority compound groups in the BIOSEA project (from the BIOSEA conceptual approach in the project proposal).

3.3.1 Proteins

The first group of interest is the proteins. Proteins are essential components of the human and animal diet. Currently, more than 65% of the European protein supply is imported (FAOSTAT 2013). Protein production from agriculture (incl. animal husbandry), fisheries and aquaculture is suffering under multiple stressors, such as drought, habitat loss, overfishing and water pollution (FAO 2016). Climate change will exacerbate those stresses. This in turn affects the price of prime matter ingredients used for the production of food and feed for cattle and aquaculture. For instance, prices of soybean meal, one of the main sources of prime matter has more than doubled in the last decade and is subject to strong fluctuations (FAO 2016).

Both micro- and macroalgae can have high protein levels, depending on growth conditions and do not compete for many of the resources required for agriculture. Hence, the prime interest of BIOSEA in algae as a protein source is for the production of food and animal feed. The quality of algae protein differs per species, but is generally high and close to protein sources as egg and soybean (Becker 2007, Bleakley & Hayes 2017). Of the essential amino acids, cysteine is probably least available, or even non-detectable in micro- and macroalgae. Tryptophan and lysine are also often limiting amino acids, although some species have more favourable profiles (Bleakley & Hayes 2017). On the other hand, most algae are particularly rich in essential amino-acids as isoleucine, leucine, methionine and valine and in the conditional non-essentials arginine and glutamine (Becker 2007, Bleakley & Hayes 2017). The main problem with algal protein lies in the digestibility and the extraction, due to the presence of insoluble polysaccharide, a feature they share with many plant proteins (Bleakley & Hayes 2017).

3.3.2 Carbohydrates

The second compound group of interest to BIOSEA is the carbohydrates. These compounds perform a range of essential roles in living organisms, from energy storage and structural components to signal molecules and stimulants of the immune system. In addition, they also have important non-biological

applications, such as the production of bioplastics or fermentation products as ethanol or methane that can be used as biofuels. Main food sources of carbohydrates include the major agricultural crops rice, wheat, potatoes, cane, etc. These face the same stressors as mentioned above for protein and it is questionable if they might be able to support the growing human population on the longer term (FAO 2016b). Therefore, as with proteins, there is an ongoing interest in finding new and more sustainable sources of carbohydrates.

Carbohydrates can be found in important concentrations in all algae species, but are particularly high in most seaweeds, where contents of up to 76% of dry weight have been found (Kraan 2012). Chemical composition of the carbohydrates varies widely, ranging from simple monosaccharides to complex, large sulphated polysaccharides. Many of the latter are used extensively already in the food and feed industry as thickeners, gelling agents and emulsion stabilizers (Holdt & Kraan 2011). Best known are the agar and carrageenans of the red seaweeds and the alginates of several brown seaweeds, but many more are in use (Bhakuni & Rawat 2005). Among more recent applications are their inclusion in neutraceuticals and functional feed, cosmetics and pharmaceuticals (Kraan 2012, Rioux & Turgeon). They also are a promising feedstock for the fermentative production of ethanol and methane (Milledge et al. 2014). BIOSEA intends to explore the use of micro- and macroalgal carbohydrates in all of its three priority applications (food, feed and cosmetic/personal care products).

3.3.3 Fatty acids

The third priority compound group of BIOSEA encompasses the fatty acids. These are vital energy sources for animals as they render large quantities of ATP when metabolized. The main interest of the project is in the so-called essential fatty acids; (poly)unsaturated fatty acids (PUFAs) or omega-3 and -6 fatty acids, specifically docosahexaenoic acid (DHA), gamma linoleic acid (GLA), eicosapentaenoic acid (EPA), but other PUFAs that can be extracted from the algae will be studied as well. Plant oils are rich sources of fatty acids, as is seafood. The aquaculture industry highly depends on feeds rich in PUFAs, which is getting more and more difficult to get by due to overexploitation of marine fish stocks and rising prices of soybean (FAO 2016). Hence the main interest of obtaining these compounds is for the feed and food industry.

Especially microalgae have been mentioned widely as a promising source of fatty acids as many species can contain high levels of PUFAs (Richmond 2004, Adarme-Vega et al. 2014). Concentrations in macroalgae are generally considerably lower, nevertheless due to their potentially lower production cost and easier extraction, seaweeds are considered a potentially interesting source as well (Pereira et al. 2012). The main limit on the large-scale cultivation of algae for the purpose of fatty acid extraction are the relatively high production costs; with increasing prices for prime matter and application of new cascade biorefinery techniques it is however becoming more economically interesting (Vigani et al. 2015).

3.3.4 Lipids

Another compound group covered by BIOSEA is lipids in general (other than fatty acids). Similar to proteins, lipids play a vital role in basically all functions of living organisms, from energy storage, to structural functions, signaling and transport molecules, etc. Major dietary lipid sources are plant and animal fats (for instance dairy products) and oils. Furthermore, lipids form the basis for biofuel (biodiesel) production, which in light of the urgent need for renewable energy resources lays a high strain on plant resources (Ho et al. 2014), explaining the need for new and renewable sources of lipids. Finally, lipids have major applications in cosmetics and personal care products and pharmaceuticals, among others due to their antioxidant capacity and consequent anti-aging effects (Rabasco Álvarez & González Rodríguez 2000, Balboa et al. 2014). In BIOSEA the use of lipids of algal origin will be studied in all three priority applications (food, feed and cosmetic/personal care products).

Both micro- and macroalgae have been suggested as a potentially interesting commercial source of lipids. In particular microalgae, generally having higher lipid contents than macroalgae, have been studied intensively as potential lipid source, in part sparked by the search for renewable fuels (Wijffels & Barbosa 2010, Ho et al. 2014). Currently some companies are producing microalgae and mainly lipid-

based microalgal extracts, in particular for the cosmetics and personal care products market (Enzing 2014). Exploration of the potential of macroalgae lipids has been lagging behind compared to microalgae, although currently lipid composition of an increasing amount of species has been determined, paving the way for applications (Holdt & Kraan 2011, Tiwari & Troy 2015).

3.3.5 Carotenoids

Carotenoids form the fifth compound of interest in the BIOSEA project. Carotenoids are a group of pigments generally present in most plants and algae, which can be split in two main groups: carotenes (for instance β- and α- carotene) and xantophylls (e.g. zeaxanthin, fucoxanthin, lutein, etc.). They have a double function, on the one hand serving as accessory pigments in the absorption of energy from light for photosynthesis and on the other hand they have a photoprotective function as well, protection chlorophyll against photodamage caused by excess light (for instance in the so-called xanthophyll cycle, Vershinin & Kamnev 1996). Carotenoids have various properties for which they are used in the food, feed, neutraceutics and cosmetics industries. They are precursors of vitamins (β-carotene is a vitamin A precursor), have anti-oxidant effects and are in use as colouring agents.

The carotenoid composition of algae differs depending on the taxonomical group to which they belong (Lobban & Harrison 1997, Barsanti & Gualtieri 2014). Microalgae in particular (e.g. *Dunaliela salina*) have a relatively long history of cultivation for the purpose of β-carotene extraction (Jeffrey & Wright 2006) and nowadays there are various companies dedicated to carotenoid production from microalgae (Enzing 2014). Carotenoid composition of macroalgae is well defined but they are generally not exploited as extractable component, due to their low concentrations (Kadam et al. 2015).

3.3.6 Phycobiliproteins

The last priority compound group in BIOSEA is the phycobiliproteins. These are water soluble protein pigments present in cyanobacteria, red algae (rhodophytes), glaucophytes and several cryptophyte species where they are the light harvesting component of the photosynthetic apparatus of these species (Barsanti & Gualtieri 2014). The major phycobiliproteins are r-phycoerythrin, b-phycoerythrin, c-phycocyanin and allophycocyanin. Phycobiliproteins are being applied in cosmetics, for their anti-oxidant and anti-inflammatory properties, as a colorant in cosmetics, food and feed and in immune assays among others (Spolaore et al. 2006, Richa et al. 2011).

Of the selected algae for the BIOSEA project, the cyanobacteria *Arthrospira platensis* is the only one possessing phycobiliproteins. Together with the rhodophyte microalga *Porphyridium*, *A. platensis* is the main commercial producer of phycobiliproteins (Spolaore et al. 2006) Contents can be as high as 17% dry weight, making the species an excellent source (Hu 2004). The widest use of *A. platensis* phycobiliproteins is as fluorescence tags in clinical analyses or assays (Hu 2004). According to Enzing et al. (2014), at least three companies, located in Germany and Japan, are dedicated to the production of phycobiliproteins from *A. platensis*.

4. Content and dynamics of BIOSEA target compounds in the selected algae

4.1 Composition of algae with respect to target compounds

In the first stage of the project extensive literature studies have been performed to gather data on the general composition of the selected algae in the BIOSEA project for the corresponding target compound groups. The target was to find at least 20 data from at least five different studies to provide reliable information on the range of concentrations that has been detected in the algae. By using data from different studies and authors, different extraction and analytical methods have probably been covered. The data are summarized in Table 4.1.

Table 4.1: Levels of BIOSEA target compound groups found in the four selected algae species in the BIOSEA project. Range and median values are % DW, N is number of observations.

Compound	**Content**	**Species**			
		Arthrospira platensis	*Isochrysis galbana*	*Saccharina latissima*	*Ulva intestinalis*
Target compounds identified in Biosea project					
Proteins (incl. free amino acids)	N	31	33	32	392
	Range	36.0-70.0	6.30-32.40	4.90-32.9	0.40-44.30
	Median	61.30	21.18	8.05	13.10
Carbohydrates (incl. alginate, mannitol, laminarian, ulvan, uronic and iduronic acids, etc.)	N	20	23	28	41
	Range	5.0-35.0	5.51-51.7	38.00-77.00	6.00-68.30
	Median	14.80	13.60	61.54	44.60
Fatty acids	N	17	18	24	12
	Range	2.35-9.16	2.05-28.90	0.13-3.90	0.6-5.63
	Median	5.12	14.91	1.50	2.19
Total lipids (incl. fatty acids)	N	14	32	28	60
	Range	4.90-11.01	5.70-35.32	0.62-5.50	0.7-15.75
	Median	7.18	23.50	1.72	2.30
Carotenoids (including fucoxanthin, lutein and other xanthophylls, carotenes)	N	7	21	66	42
	Range	0.02-0.509	0.06-1.82	0.0073-0.40	0.00013-1.45
	Median	0.05	0.32	0.077	0.028
Phycobiliproteins	N	7	-	-	-
	Range	4.7-20.4	-	-	-
	Median	16.7	-	-	-

Sources: Brown (1991), Molina Grima et al. (1994), Fidalgo et al. (1998), Grobe & Murphy (1998), Foster & Hodgson (1998), Ötles & Pire (2001), Figueroa et al. (2003), Malta et al. (2003), de Padua et al. (2004), Xia et al. (2004), Barbarino & Lourenço (2005), Volkman & Brown (2005), Ortiz et al. (2006), Natrah et al. (2007, in Prartono et al. [2010]), Ramadan et al. (2007), Chaiklahan et al. (2008), de Sousa et al. (2008), El-Baky (2008), George et al. (2008, in Prartono et al. [2010]), Carvalho et al. (2009), Diraman et al. (2009), Gómez-Ordoñez et al. (2010), Huerlimann et al. (2010), Prartono et al. (2010), Zhang et al. (2010), Holdt & Kraan (2011), Peña-Rodríguez et al. (2011), van Ginneken et al. (2011), Yaich et al. (2011), Gosch et al. (2012), Kim et al. (2012), Kraan (2012), Pereira et al. (2012),

Ahmed et al. (2014), Dörner et al. (2014), Figueroa et al. (2014), Goiris et al. (2014), Hallerud (2014), Hwang et al. (2014), Lee et al. (2014), Meng et al. (2014), Moustafa & Saeed (2014), Nascimento et al. (2014), Saranya et al. (2014), Schmid et al. (2014), Ak et al. (2015), Angell et al. (2015), Boisvert et al. (2015), El-Kassas et al. (2015), El Maghraby & Fakhry (2015), Kendel et al. (2015), Khairy & El-Sheikh (2015), Marinho et al. (2015), Schiener et al. (2015), Vilg et al. (2015), Bansemer et al. (2016), Bernardi et al. (2016), Boderskov et al. (2016), Bruhn et al. (2016), Carl et al. (2016), Chirapart et al. (2016), Fische (2016), Malta & de Nys (2016), Mata et al. (2016), McCauley et al. (2016), Neveux et al. (2016), Nielsen et al. (2016), Osuna-Ruiz et al. (2016), Tibbetts (2016), Barbosa et al. (2017), Gnouma et al. (2017), Mohamad Basri & Wan Maznah (2017). Concentrations of compounds in *A. patensis* are own data of IGV GmbH, except for data on fatty acids.

Logically, the variation encountered in composition is considerable in all species, bearing in mind that these are results from species grown in different regions or under different environmental or cultivation conditions. Another important factor is methodological differences that can lead to varying outcomes.

To start with proteins, basically there are four major protocols for protein determination. Three of them are based on direct extraction and analyses, namely the well-known procedures following Bradford or Lowry and the quantification of total amino acids, which is considered the most accurate method for protein determination (Angell et al. 2016 and references therein). Furthermore there is the indirect determination based on analysis of total nitrogen content, which is then converted to protein by multiplying with the standard factor of 6.25 (Mariotti et al. 2008 in Angell).

While the Bradford, Lowry and N-content conversion might work fine for many materials, specifically in plants and algae these have proven to lead to inaccuracies. Main reasons for this include generally low protein extraction yields in these organisms due to strong chemical bonds with cell wall polysaccharides and the interference of other compounds, for instance free polyphenols as are present in high levels in brown seaweeds (Fleurence et al. 1995, Angell et al, 2016). In general, Bradford and Lowry determinations tend to underestimate protein level in micro- and macroalgae (Barbarino & Lourenço 2005), whereas the traditional 6.25 conversion factor represents an overestimation (Lourenço et al. 2002, Angell et. al 2016) at least for seaweeds. Based on the analysis of an extensive database, available online (Angell et al. 2015), at least for seaweeds recommend a conversion factor of 5.0, if total amino acid determination is not available or feasible. However, this again is questioned as recently it has been shown this factor can vary considerably in *Laminaria digitate* and *Saccharina latissima* (Manns et al. 2017). This is hence of the aspects that require further attention in the BIOSEA project.

Similar problems exist regarding carbohydrate analyses. First of all, there is a difference in analytical methods; carbohydrates can be analysed directly using the “classical methods” of anthrone coloration (Yemm & Willis 1954) or sulphuric acid and phenol coloration (Dubois et al. 1956) or more advanced methods (Peris-Tortajada 2004). However, many authors also apply an indirect method of estimating carbohydrate “by difference” i.e. assuming that excluding other components resulting from a proximate analysis all that remain are carbohydrates. This typically leads to higher estimates than direct analyses (Peris-Tortajada 2004). On the other hand, direct analyses tend to underestimate real carbohydrate contents, especially in algae, owing to the fact that these are often hard to extract due to the complex chemical bonds they are forming with cell wall proteins and lipids (Lobban & Harrison 1997).

Regarding fatty acids, it was particularly difficult to find literature sources that list total fatty acid contents. Due to the method applied (typically lipid extraction followed by fatty acid methylester [FAME] preparation and GC analysis), most works only list fatty acid composition as percentage of total fatty acid content without listing total FA content (Connan 2015, Guihéneuf et al. 2015). In addition, many authors fail to discriminate between total lipid and total fatty acid content. This confusion may also arise occasionally with total lipids analysis, although and general and as can be seen from Table 4.1 variance is much lower for this compound group.

The problem with carotenoids, in particular in seaweeds lies in their generally low concentrations, however the use of highly sensitive fluorescence HPLC methods greatly resolved this problem (Garrido & Roy 2015). In some cases, data in the table only represent the major carotenoids such as fucoxanthin (*I. galbana* and *S. latissima*), β-carotene (all species) or lutein (*Ulva* spp.), thereby (slightly) underestimating total carotenoid content. Variation in phycobilliprotein levels in *A. platensis* is reduced and most likely main represents variation due to different growth conditions or stages in the mass cell

culture. In the following, the composition of each of the species for each compound will be discussed in detail.

4.1.1 *Arthrospira platensis* (Spirulina) composition

As commented earlier, *A. platensis* already has a long history of application and cultivation. Nevertheless, studies have mainly been focusing on specific components and have largely ignored others. Despite its high protein contents, these are generally not exploited as a food and feed source at a large scale due to the presence of cheaper alternatives and problems with the consistence and sensorial acceptance by potential consumers (Becker 2007). Phycobilliproteins form an important part of total protein content, ≥ 25% following Table 4.1, basically consisting of c-phycocyanin and allophycocyanin (Hu 2004, Borowitzka 2013). Quality of the proteins, in terms of digestibility, protein efficiency, etc. have been poorly studied thus far in microalgae in general (Bleakley & Hayes 2017). The few data available for *A. platensis* however, are promising. Biological value (BV), digestibility coefficient (DC), net protein utilization (NPU) and protein efficiency ratio of *A. platensis* are all close to or even higher than conventional plant proteins and in the same range as other tested microalgae (Becker 2007). Regarding amino acids, *A. platensis* seems to possess an excellent profile, with high contents (similar to or higher than soybean) of the essential amino acids isoleucine, leucine, valine, phenylalanine, methionine, threonine and in the semi-essentials arginine, glycine, histidine and tyrosine (Becker 2007). Concentrations of the essentials lysine and especially tryptophan and the semi-essential cysteine are generally lower than those of conventional sources (Becker 2007).

With respect to carbohydrates, contents on average are low as in most microalgae in comparison to seaweeds (Table 4.1) and literature data on them are scarce. The data below come from the review of Cohen (1997). A common polymer found in *A. platensis* (and other photosynthetic and non-photosynthetic bacteria), often used for bioplastics and other applications, is polyhydroxybutyric acid (PHB), whose contents appears to be highly variable but can rise as high as 6% dry weight. Calcium spirulan is a sulphated polysaccharide which has been found to demonstrate inhibiting effects in various viruses (incl. influenza, avian influenza, HIV, Herpes simplex, etc.) and could thus be interesting for the development of antiviral agents (Hu 2004). The algae are known to excrete polysaccharides in the medium consisting of mainly galactose, glucose and xylose, and to a minor degree fructose rhamnose and mannose (trace levels). Finally, approximately 1.5% DW of the biomass consists of lipopolysaccharides, consisting mainly of rhamnose and galactose and polyols as glycerol and inositol.

As for most cyanobacteria, fatty acid and lipid contents of *Arthrospira* are relatively low compared to other microalgae (Cohen 1997). However, it is a rich source of several polyunsaturated fatty acids, especially shorter chain ones. In fact, some claim it is the richest algal source of gamma linolenic acid that may account for up to 22% of total fatty acids (Hu 2004, Volkman & Brown 2006 in Subba-Rao). Linoleic acid also tends to be high (Ötles & Pire 2001). Examples of complete fatty acid profiles of various *A. platensis* strains and products made of them (tablets) are presented by Ötles & Pire (2001) and Diraman et al. (2009). Major lipids are the galactolipid monogalactosyldiacylglycerol (MGDG), sulfoquinovosyldiacylglycerol (SQDG) and the glycerophopspholipid phosphatidylglycerol (PG), together accounting for approximately 70% of all lipids. The galactolipid digalactosyldiacylglycerol (DGDG) contributes about 7-10%, while triglycerides are generally low, less than 2% (review of Cohen 1997).

Apart from the phycobilliproteins already discussed above, *A. platensis* is also rich in carotenoids (Cohen 1997, Aouir 2017). A more or less complete list is given in the review of Cohen (1997). Main carotenoids are β-carotene and the xanthophylls myxoxanthophyll and zeaxanthin, furthermore it can also contain important amounts of lutein and β-cryptoxanthin (Carcea et al. 2015).

Other potentially valuable compounds that can be found in *A. platensis* are sterols, vitamins and polyphenols. Sterols are present in low concentrations, mainly consisting of clionasterol and Δ^5-avenasterol (see Cohen 1997 for more details). Important vitamins are vitamin B12 (11 mg/kg dry weight, Cohen 1997) and vitamin E (ranging from 2.4 to 75.0 mg/100 g dry weight in “dihé” samples, Carcea et al. 2015). Phenolic contents were found to be highly variable among strains, but could be as high as 67 Gallic Acid Equivalent (GAE)/g dry weight, which is considerable (Aouir et al. 2017). Based on

this, both vitamins and polyphenols might be interesting compounds as by-products of an *A. platensis* culture.

4.1.2 *Isochrysis galbana* Parke/*Isochrysis* aff. *galbana* (T-ISO) composition

As mentioned in Chapter 3, *I. galbana* and T-ISO are widely applied as a feed source in aquaculture, mainly due to their high content in fatty acids and lipids and balanced protein composition (Coutteau 1996, Muller-Feuga 2004). Biochemically, the species are very similar (Brown 1991). Total protein levels are slightly lower than in most microalgae, depending on cultivation conditions (Brown 1991, Fidalgo et al. 1998), however digestibility was found to be high for various shellfish species (Albentosa et al. 1993, 1996). Amino acid profile is very balanced with concentrations for nearly all essential amino acids close to or higher than conventional plant sources and especially high values for proline, an essential amino acid for molluscs (Brown 1991, Becker 2007).

With respect to carbohydrates, concentrations for this species on average are lowest of the four selected species in BIOSEA (Table 4.1). A detailed analysis of composing sugars of *Isochrysis* and other microalgae has been published by Brown (1991). He found that the majority (91 to 96%) of the carbohydrates is present in the form of readily-hydrolysable polysaccharides. The main difference with other microalgae was that this species generally contained more arabinose and less rhamnose in their polysaccharide, than did other classes of algae. Glucose is by far the most dominant sugar, followed by galactose, arabinose, mannose, ribose and xylose (Brown 1991, Batista et al. 2013). Trace levels of fructose have been found. The typical storage carbohydrate of algae from this group is chrysolaminarin, a β-1,3-linked glucan (Becker 2004). A potentially interesting polysaccharide isolated recently from *I. galbana* is a highly branched (1 → 3,1 → 6)- β -D-glucan that showed inhibition of the proliferation of U937 human leukemic monocyte lymphoma cells and therefore has potential anti-tumor activity (Sadovskaya et al. 2014).

The high fatty acid content of *I. galbana* is one of the main reasons of its popularity as a live feed ingredient in aquaculture, a large part (40 – 60%) of which is present in the form of polyunsaturated fatty acids (PUFA) (Becker 2004, Volkman & Brown 2006). It is especially rich in the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), while also containing important levels of other omega-3 PUFAs as stearidonic acid (SDA) and heneicosapentaenoic acid (HPA) (Fidalgo et al. 1998). For complete profiles see for instance Sukenik & Wahnon (1991), Fidalgo et al. (1998), Prartono et al. (2010) and Batista et al. (2013).

Lipid composition can be highly variable, depending on the phase of the culture of the alga, the type of culture, etc. (Pernet et al. 2003, Nalder et al. 2015). Main lipid groups (apart from pigments) of *I. galbana* (T-ISO) include neutral lipids, galactolipids (glycolipids) and phospholipids (Sukenik & Wahnon 1991, Fidalgo et al. 1998); sterols (cholesterol) and triglycerides (triacylglycerols) are important lipid classes (Pernet et al. 2003), although Rcykebosh et al. (2014), did detect various phytosterols but not cholesterol in *Isochrysis* T-ISO. Major identified lipids are sulfoquinovosyldiacylglycerol (SQDG), monogalactosyldiacylglycerol (MGDG), diacylglycerylcarboxyhy-droxymethylcholine (DGCC) and digalactosyldiacylglycerol (DGDG) (Huang et al. 2017).

Next to the high contents in PUFAs, *I. galbana*/T-ISO also presents a rich source of carotenoids (Table 4.1). Some studies have reported contents up to 1.82% dry weight (Kim et al. 2012). The main carotenoid is fucoxanthin (more than 80% of total carotenoid content, Ryckebosch et al. 2014, Gilbert López et al. 2015). Other carotenoids are (β-)carotene, diadinochrome and diatoxanthine, however all in far lesser amounts than fucoxanthin (Ryckebosch et al. 2014, Gilbert López et al. 2015). Fucoxanthin is known and commercially used as a powerful antioxidant (Goiris et al. 2012, Kim et al. 2012). Other pigments, apart from chlorophyll *a*, include pheoporphyrin-MgDVP (magnesium divinyl pheoporphyrin a_5 mono-methylester, MgDVP), chlorophyll c_1, chlorophyll c_2, and, chlorophyll c_2-MGDG (monogalactosyl diacylglyceride ester) (Jeffrey & Wright 2006).

Isochrysis galbana and T-ISO have been found to have high antioxidant capacities, although the ranking among other microalgae highly depends on the method used for determining antioxidative capacity (Goiris et al. 2012, Custodio et al. 2014, Ryckebosch et al. 2014). Most likely this capacity is related to

polyphenol contents. Goiris et al. (2014) report polyphenol levels of 2.67-4.57 Gallic Acid Equivalent (GAE)/g dry weight. Considering vitamins, in comparison with other microalgae *I. galbana*/T-ISO is particularly rich in vitamin C (de Roeck-Holtzhauer 1991, Seguineau et al. 1996) and to a lesser degree in vitamins B_1, B_2 and B_{12}, niacin, folic acid and panthothenic acid (Seguineau et al. 1996). This might be specific for the T-ISO strain however (Fabregas & Herrero 1990). It has been suggested that T-ISO might be used in aquaculture to boost the vitamin C content of other live feeds, such as rotifers (van Bergeijk et al. 2010b). T-ISO can also be a source of iodide and iodate that can possibly serve as precursors for thyroid hormones Van Bergeijk et al. (2013, 2016).

4.1.3 *Ulva* L

Despite being one of the most common and abundant macroalgae genera worldwide, exploitation of *Ulva* has been relatively limited in comparison to other macroalgal species, but has been growing rapidly over the last years (see Ch. 3). Its biochemical profile has however been very well studied from viewpoints varying from basic ecology and eutrophication thematics to mitigation of aquaculture effluents to its uses as food and feed sources. The seaweed protein/amino acid database of Angell et al. (2015) lists close to 400 entries for the *Ulva* genus, of which 73 are based on total amino acids content. A total of 92 complete amino acids profiles are listed, the majority coming from Angell et al. (2014). Protein content shows large variation (Table 4.1) but is one of the highest among seaweeds, which can be explained by their highly nitrophilic nature (Fletcher 1996), although it must be stressed that in particular the highest reported values might be overestimated due to methodological problems (see earlier discussion on this in Ch. 3).

Proteins of *Ulva* have been classified by various authors as a promising ingredient in human food and in animal feed considering high acceptance and digestibility (Tabaoda et al. 2010, 2011, Bikker et al. 2016). Analysing the amino acid profiles listed in Angell et al. (2015), it can be seen that of the essential amino acids, presence of tryptophan could not have been confirmed by all studies and if found, concentrations were very low. Methionine levels also tend to be on the low site. On the other hand, the alga is particularly rich in leucine, lysine, threonine and valine. Of the semi-essential amino acids, histidine and cysteine are present in low levels compared to various microalgae and conventional protein sources, whereas they were relatively rich in glycine (Becker 2007 and Bikker et al. 2016 for comparison with microalgae and conventional sources). Of the non-essentials, the high levels of alanine, aspartic acid and glutamic acid stand out, the latter two forming the main components of the characteristically “umami” taste sensation (Carl et al. 2016).

Median carbohydrate contents of *Ulva* reflect the average found in seaweeds (Rioux & Turgeon 2015 in Tiwari). The main sugars are glucose, rhamnose and xylose, whereas galactose, mannose and arabinose can be found in trace level concentrations (Yaich et al. 2011, Bikker et al. 2016). The main storage sugar is starch that can vary considerably depending on growth conditions (Bikker et al. 2016, Korzen et al. 2016). As all seaweeds *Ulva* is a rich source of polysaccharides, which are mainly located in the cell walls (Rioux & Turgeon 2015), although in contrast to the well-known agars and carrageenans of the red algae and the alginates of the brown algae, green algal polysaccharides have been largely underexploited . Main cell wall polysaccharides of *Ulva* are cellulose, xyloglucan, mannans, glucuronan and (1 → 3)- β – glucan (Lahaye et al. 1994, Tsekos 1999, Lahaye & Robic 2007, Rioux & Turgeon 2015).

Another important and very interesting cell wall component is the sulphated polysaccharide ulvan, typical for members of the order of the Ulvales. Ulvan has been found to make up between 8 and 29% of *Ulva* dry weight (Lahaye & Robic 2007, Coste et al. 2015). Ulvan is a sulphated polysaccharide, mainly composed of the monosugars l-rhamnose, d-xylose, d-glucose, and d-glucuronic acid; in addition the presence of iduronic acid was also demonstrated (Quemener et al. 1997, Lahaye et al. 1999, Robic et al. 2009). Its sulphate contents range between 16% - 23% (Coste et al. 2015). Ulvan consists of two major subunits: ulvanobiuronic acid A (b-d-glucuronosyluronic acid-(1,4)-a-l-rhamnose 3-sulfate) and B (a-l-iduronopyranosic acid – (1,4)-a-l-rhamnose 3-sulfate) (Lahaye & Ray 1996, Quemener et al. 1997). Its molecular weight ranges from 150 kDa to 2000 kDa (Siddhanta et al. 2001). Although first publications on the structure of this polysaccharide already appeared in the 1950s, the term ulvan was only introduced in 1993 by Lahaye (1993 in Tiwari). It was especially the ground-breaking work of Lahaye’s lab and coworkers that unravelled its chemical composition, opening up possibilities for exploitation.

Interest in this polysaccharide has been booming over the last few years, as can be seen from the exponential increase in the number of scientific publications on the topic (Fig. 4.1) – of 116 publications reviewed, 76 are from the last five years. The reason for this interest is no doubt due to its high versatility, as it has been demonstrated to possess a wide range of properties, including antiviral, anticoagulating, antioxidant, immunomodulating in plants and animals among others (Qi et al. 2005, 2007, Mao et al. 2006, Castro et al. 2006, Leiro et al. 2007, Julneau et al. 2011). Furthermore, it can be used in the production of biomaterials such as nanofibers, micro- and nanoparticles, molecular sponges for cell cultures, etc. (Toskas et al. 2001, Alves et al. 2013, Fernández-Díaz et al. 2017). It is also useful in combination with other compounds; for instance, it was found that addition of ulvan and other *Ulva* polysaccharides to ß-carotene emulsions considerably improved their chemical and physical properties compared to that of commercial polysaccharides as Arabic gum and beet pectin (Shao et al. 2017). The discovery of various bacteria, capable of breaking down ulvan in oligosaccharide fractions will no doubt open the door for further innovative applications (Barbeyron et al. 2011, Kopel et al. 2016, Coste et al. 2015).

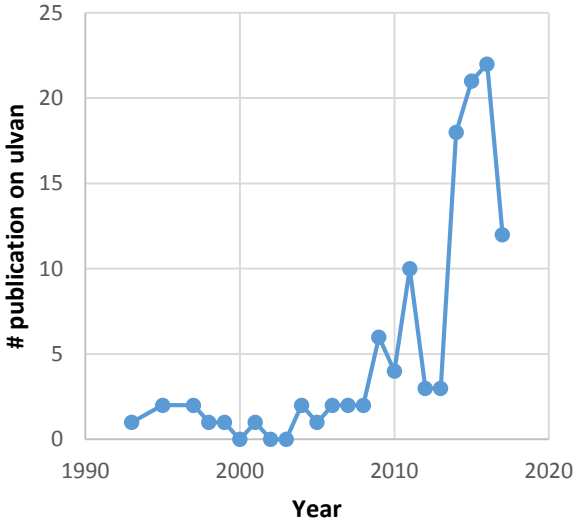

Fig. 4.1: Number of publications on the sulfated polysaccharide ulvan in the period 1993 – 2017 (still ongoing).

As in most seaweeds species, fatty acid and total lipid content for *Ulva* are considerably lower than in eukaryote microalgae or many plant seeds (Table 4.1, Hamid et al. 2015). In general, about 50-60% of the lipids are present in the form of fatty acids; the decrease of this percentage with increasing lipid content is stronger for green than for brown seaweeds (Gosch et al. 2012). Despite this, *Ulva* is considered a potentially rich source of harvestable fatty acids, also due to its ability to grow effectively and rapidly in culture. Interestingly, *Ulva* differs from other green algae by showing significantly higher levels of mono- and polyunsaturated fatty acids (MUFAs and PUFAs) (Pereira et al. 2012). Variation in total FA content and FA profiles encountered in *Ulva* is considerable as shown by a recent review of McCauley et al. (2016). According to this study, FA profile on average consists of 35.3 ± 13.7% saturated fatty acids (SFA), 15.9 ± 6.6% MUFA and 41.8 ± 15.6% PUFA. In total 7-31 FAs are identified in *Ulva* with an average of 16. The most dominant SFAs are palmitic acid (C16:0, around 30% on average), myristic acid (C14:0), behenic acid (C22:0) and stearic acid (C18:0) as is common for macroalgae (Pereira et al. 2012). Interestingly, its dominant PUFA is the, for humans and other animals essential, α-linolenic acid (C18:3 n-3), which is generally present at appreciable levels (15% following McCauley et al. 2016) and can even be as high as 28% (Alamsjah et al. 2008). Other important omega-3 fatty acids (≥ % of total FA content) are stearidonic acid (C18:4 n-3), the C16:4 n-3 that is typical for green algae/*Ulva* (Mata et al. 2016) and C18:2 n-3. In addition, it also contains the also essential linoleic acid (C18:2 n-6); McCauley et al. (2016) report levels of 6-12% of total FA depending on cultivation conditions, but values of up to 21% have been reported (Alamsjah et al. 2008). Information on lipids in general and specific lipid classes is scarce for *Ulva*. For *U. armoricana* neutral lipids have been reported as the main lipid class, followed by glycolipids and phospholipids (Kendel et al. 2015).

Considering pigments, as in all green algae, dominant pigments in *Ulva* spp. are chlorophyll *a* and *b* (Lobban & Harrison 1997). Main carotenoid is lutein of which levels as high as 1.45 % DW have been

reported, although median concentration found in the literature is considerably lower (Table 4.1, Lobban & Harrsison 1997, Malta et al. 2003). ß-carotene has been reported, but only in very low concentrations (Malta et al. 2003, Bernardi et al. 2016). Part of the antioxidant defences in *Ulva* spp. are based on the so-called xanthophyll or violaxanthin cycle for which it contains zeaxanthin, violaxanthin and antheraxanthin, all generally at low levels and other carotenoids that can also be found in higher plants such as siphonaxanthin and its two esters siphonein A and B (Vershinin & Kamnev, 1996).

Being algae that inhabit the highly dynamic (light) environment of the intertidal and upper subtidal zone, *Ulva* spp. have a well-developed defence mechanism against oxidative stress, which is in part based on polyphenolic compounds, although ulvan may also play an important role here (Hardouin et al. 2016). Reported polyphenol levels in the literature range between 0.0035-1.1 % DW, with a median value of 0.425 (Hardouin et al. 2016, Osuna-Ruíz et al 2016). It must be noted however that these compounds are typically strongly bound to membrane compounds and hence currently available data probably underestimate real contents (Hardouin et al. 2016, Coste & Malta unpubl. data).

Other potentially interesting compounds in *Ulva* include phytosterols up to several mg per kg DW (Lopes et al. 2011), mainly (HDL-)cholesterol and isofucosterol (see Lopes et al. 2011, Kendel et al 2015 for profiles). *Ulva* is particularly rich in vitamins C, B3 and B 12 and also contains tocopherols albeit generally in lower amounts than most brown seaweeds (Ortíz et al 2006, MacArtain et al. 2007, García-Casal et al. 2009) and as all marine algae is a rich source of minerals (Cardoso et al. 2014, Carl et al. 2016, Neveux et al. 2016).

4.1.3 *Saccharina latissima* (Linnaeus) C.E.Lane, C.Mayes, Druehl & G.W.Saunders

As noted in Ch. 3, *S. latissima* is currently the main seaweed being cultured in Europe, with a harvest of several dozens of tonnes wet weight per year. It is one of the species commercialized under the name kombu and also known as sugar kelp due to its high mannitol contents. Its composition is less well studied than that of its Asian sister species *Saccharina japonica*, the most cultivated seaweed for (human) food purposes, hence many data might come from this species. Nevertheless, qualitative (not quantitative) composition of the two species is comparable (Holdt & Kraan 2011).

As typical for brown seaweeds, average protein contents are lower than those of green and red seaweeds or microalgae (Fleurence 1999); indeed, of the four selected BIOSEA algae it has the lowest protein content (Table 4.1), although it can be increased considerably when cultivated under nitrogen rich conditions (Gómez-Ordóñez et al. 2010, Marinho et al. 2015). The nutritional value of the alga cultivated in a commercial integrated aquaculture system (nutrient rich compared to “normal” seawater) was reported to be comparable to wheat, in particular with respect to essential amino acid profile and contents (Marinho et al. 2015). All essential amino acids are present, although methionine, lysine and especial tryptophan only in low to very low amounts depending on season (Mai et al. 1994, Marinho et al. 2015, Fissche 2016); on the other hand, the alga is rich in leucine, lysine, phenylalanine and valine (Mai et al. 1994, Fissche 2016). The semi-essentials histidine and particularly cysteine are present in low to very low levels, whereas glycine is relatively high. Of the non-essentials, as in *Ulva* and most other seaweeds, the high levels of alanine, aspartic acid and glutamic acid stand out. It can be concluded that amino acid composition is very close to that of *Ulva*, although total levels in the latter are generally higher.

With respect to carbohydrates, of all four species in BIOSEA, *S. latissima* is the alga that shows the highest levels by far (Table 4.1). Particularly standing out are the high levels of glucose and mannitol (hence the name sugar kelp). Elaborate analyses of sugar profiles have been carried out for *S. latissima* specifically (Schiener et al. 2015, Fissche 2016, Sterner & Edlund 2016). The main storage carbohydrates for *S. latissima* are mannitol and the glucan laminarin, whereas alginate, cellulose and fucoidan starch represent the structural components (Schiener et al. 2015). Main oligosugars/polyols are mannitol (10 – 25% DW), glucose (up to 2.5 – 3 %) and mannuronic acid (levels similar to glucose). Mannitol is a very interesting component for several food applications as an alternative sweetener to glucose as it is low caloric, tooth-friendly and has close to zero glycemic and insulinemic indices (Rioux & Turgeon 2015 and references therein). Minor sugars include xylose, fucose, galactose, mannose, xylose and guluronic acid (all ≤ 1% DW). Alginate levels vary between 15 – 30% DW, cellulose between 12 – 15% DW. Fucoidan

starch varies between 2 – 6% (Bruhn et al. *in press*). Laminarin contents are generally much lower and much more dynamic than mannitol, varying between 1 – 13% DW (Schiener et al. 2015).

As said in 2.4, *Saccharina japonica*, is sometimes also used for alginate extraction, however only in case of surplus production (McHugh 2003). Alginate is an important gelling substance with many applications in feed, food, cosmetics and pharmaceutics. It is composed of mannuronic acid and guluronic acid, whose ratio may change depending on frond age, growth conditions, season, etc. (Rioux & Turgeon 2015 in Tiwari). Guluronic acid is the crucial factor in determining gel strength, a high number of GG dimers in alginates renders a firm and rigid gel. *S. latissima* grown in Norway under different growth conditions showed relatively high mannuronic acid contents, leading to the hypothesis that its alginate might be dominated by this component, roughly in a 3:1 mannuronic acid:guluronic acid ratio (Fissche 2016). Similar results were found for Swedish specimens (Sterner & Edlund 2016). Consequently its alginate might have lower gelling capacity than its Asian sister species, although this needs to be further studied.

Fucoidans, or its more general term fucose-containing sulphated polysaccharides, are the brown algal homologues to the sulphated polysaccharides of red algae (floridean starch, agarans and carrageenans) and the ulvan of the Ulvophyceae. It has also been found in several marine animals, as sea cucumbers and sea urchins (Rioux & Turgeon 2015). Simultaneously to ulvan, it has a high number of potential applications leading to a subsequent exponential growth in the number of scientific publications over the last 30 years (Fig. 1 in Ale et al. 2011). Its general structure consists of a backbone of (1→3)-linked α-l-fucopyranosyl or of alternating (1→3)- and (1→4)-linked α-l-fucopyranosyl residues, but also include sulphated galactofucans with backbones built of (1→6)-β-d-galacto- and/or (1→2)-β-d-mannopyranosyl units with fucose or fuco-oligosaccharide branching, and/or glucuronic acid, xylose or glucose substitutions, varying greatly between species (Ale et al. 2011). Until recently, most studies on fucoidans were from *Fucus* species and several others. Detailed information on fucoidan in *S. latissima* is limited thus far, however in a comparative study it was found that among the fucoidans from nine algal species, those of *S. latissima* showed the potential with respect to anti-inflammatory, anticoagulant, antiadhesive and antiangiogenic activities (Cumashi et al. 2007). First studies indicate that the main component in *S. latissima* is a fucan sulphate containing 3-linked α-l-fucopyranosyl residues backbone, but that composition can even vary within the species (BIlan et al. 2010, Ehrig & Alban 2015). Sulphate content ranges between 14 – 23%, although differs highly between fractions (Ehrig & Alban 2015). Several bacteria have been isolated that possess enzymes capable of fucoidan depolymerisation, although as far as known none for *S. latissima* fucoidans specifically (Rioux & Turgeon 2015 and references therein).

Average and median total lipid and fatty acid content of *S. latissima* are the lowest of the four algae selected in the BIOSEA project (Table 4.1). Nevertheless, as fatty acids constitute 80 – 90% of all lipids, this might still be a valuable by-product in a cascade biorefinery approach. Moreover, even though total fatty acids are low for this species, the proportion of PUFAs can be as high as 50% of total FA although variation can be considerable (Schmid et al. 2014). Various authors have published complete FA profiles of this species (e.g. Schmid et al. 2014, Vilg et al. 2015, Barbosa et al. 2017). Based on the data from these studies, FA profile of *S. latissima* consists on average of 34.7 ± 2.1% saturated fatty acids (SFA), 20.3 ± 3.2% MUFA and 41.1 ± 4.1% PUFA. 17-18 different fatty acids were identified in *S. latissima*. Most dominant SFAs are palmitic acid (C16:0, 20 – 25%) myristic acid (C14:0, 8 – 14%), and stearic acid (C18:0, 1 – 2%). Oleic acid (11 – 19%) and palmitoleic acid, as common for plants and most seaweeds (Pereira et al. 2012) are the main MUFAs.

PUFA profile is interesting, with appreciable levels of the essential fatty acids α-linolenic acid (ALA, C18:3 n-3, 2.5 – 4%) and eicosapentaenoic acid (EPA, C20:5 n3, 6 – 10%). Vilg et al. (2015) report levels of 8% docosahexaenoic acid (DHA, C22:6n3) in some of their samples but less than 1% or non-detectable in others, whereas other authors report absence of low levels of DHA (Schmid et al. 2014, Barbosa et al. 2017). Other dominant PUFAs are the omega-6 FAs linoleic acid (essential, C18:2 n-6, 7 – 10%) and especially arachidonic acid (AA, conditionally essential in humans, 7 – 13%) as is common in many brown algae (McCauley et al. 2016). No specific information on lipid classes could be found. In general, in algae

from the Laminariales order neutral lipids (sterols, pigments and tri-, di- and monoacylglycerols) are dominant, probably followed by glycolipids and polar lipids (phospholipids) (although there is debate on this, Wielgosz-Collin et al. 2016 in Fleurence). Mayor glycolipids in brown algae include monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), trimethyl-beta-alaninediacylglycerol and sulfoquinovosyldiacylglycerol (SQDG) (Hamid et al. 2015 in Tiwari). Predominating polar lipids are phosphatidylglycerol and phosphatidylethanolamine, Laminariales also contain phosphatidylcholine (Wielgosz-Collin et al. 2016 in Fleurence).

Basic pigments of brown algae are chlorophyll *a* and *c* and *fucoxanthin* (Lobban & Harrison 1997). The latter is also the main carotenoid. Total carotenoid level can reach 0.4% DW, but varies orders of magnitude (Table 4.1). Zeaxanthin and ß-carotene have been reported in concentrations of ≤ 0.04 % DW (Vilg et al. 2015, Bruhn et al. 2016, Nielsen et al. 2016). As part of the brown algal xanthophyll cycle, trace levels of violaxanthin and antheraxanthin are also present (Vershinin & Kamnev, 1996).

Kelp algae (Laminariales) as *Saccharina latissima* are well known for their often considerable amount of phlorotannins, polyphenolic compounds as phloroglucinol and its polymers (Lobban & Harrison 1997). These are structural cell wall compounds and have also been shown to play a role in photoprotection, as herbivore repellent and to prevent growth of epiphytes, among others (Lobban & Harrison 1997, Hotchkiss & Murphy 2012, Figueroa et al. 2014). Phlorotannins are strong antioxidants and have been reported to possess anti-inflammatory, antiviral and antibacterial properties among others, for which they are highly interesting compounds for cosmetic and pharmaceutical applications and also for food and feed preservation (Hotchkiss & Murphy 2012, Peng et al. 2015). Although not as high as in most fucoid seaweeds, levels in *S. latissima* are appreciable and could be an interesting by-product. Concentrations range between 0.04 – 1.19 %DW, with a median concentration of 0.4 % DW (Werner 2013, Schiener et al. 2015, Vilg et al. 2015).

Other potentially interesting compounds in *S. latissima* include sterols (Safe et al. 1974). They are a rich source of minerals and trace elements, iodine in particular as is common with brown seaweeds (CEVA 2011, Schiener et al. 2016). Iodine contents are somewhat lower than in many other brown algae, which is an advantage as it reduces the risk of excessive intake when used as human food (Schiener et al. 2016). Oher interesting compounds include vitamins A, C and E (CEVA 2011).

4.2 Dynamics of compounds in micro- and macroalgae

4.2.1 General remarks

As can be seen from Table 4.1, dynamics of the compounds can be highly variable. Most of them are key metabolic compounds that logically vary with changes in growth and growth conditions, life stage (macroalgae in particular), age of the culture or fronds, etc. The timescale of the variation differs per group or type of compound and also per environmental parameter; some compounds can vary on the timescale of seconds to minutes (e.g. enzyme activation/deactivation, pigment degradation and transformation, antioxidant responses), whereas for other compounds variation is on the scale of hours to days (e.g. structural membrane compounds, large polysaccharides, *de novo* protein synthesis). Likewise, certain responses to changes in light intensity for instance might be almost instantaneous (Rubisco activation, changes in short-chain carbon components), whereas changes in growth temperature usually takes longer (e.g. formation of membrane lipids and anti-freezing polysaccharides, etc.). Algae grow in highly dynamical environments; acclimatization of the composition to optimize growth rates on the one hand is balanced by the tendency towards homeostasis that has also been observed in algae (Montechiaro et al. 2006, Persson et al. 2010, Nejrup et al. 2013).

As all species selected in the BIOSEA project are primarily autotrophic photosynthetic organisms, their general responses to environmental conditions basically follow the same patterns, although responses to determined conditions can be species specific. For instance, species will have specific optimum

growth temperatures or salinities. Logically there is also a difference in dynamics of composition between the unicellular microalgae whose cells are dividing various times per day or even hour and the longer living macroalgae that invest more in structural compounds. Among these there are differences as well due to ecological growth strategies that impact composition and composition dynamics, as in the case of the fast growing *Ulva* spp. that display multiple reproductive periods per year and have much stronger and faster responses to variation in growth conditions than the slower growing *S. latissima* that has a very marked seasonal cycle – these can be compared to a fast growing herbaceous land plant vs. a land tree. Nevertheless, as said, general tendencies in biochemical composition of algae responses to growth conditions can be distinguished, which will be discussed in the following paragraphs. Knowledge of these responses will enable us to optimize conditions in the case of controlled cultivation or time of harvest in the case of field cultures in relation to desired compounds or composition.

The main environmental parameters determining algal growth and composition are temperature, light, pH/CO_2 (especially in dense cultures, less fluctuating in the natural environment), salinity and nutrient availability (Lobban & Harrison 1997, Richmond 2004, Subba-Rao 2006). Biological parameters that affect composition are for instance cell cycle in microalgae or life-stage in seaweeds, attacks from viruses, herbivores and parasites, etc.. Furthermore, these variables will interact, thereby complicating the prediction of the outcome of the response on composition. The following sections will discuss the effect of environmental and biological parameters on algal growth and composition separately, as well as in interaction. Where available, species specific data will be given. The main experimental evidence of direct effects of parameters comes from experiments with microalgae. For macroalgae, main data come from analyses of seasonal differences of field harvest algae, which are hence by definition a results of the interaction of all parameters, for instance light covaries in a season with temperature, whereas nutrient levels often show inverse patterns.

4.2.2 Temperature

Due to its fundamental effect on chemical reaction rates and physical processes, both in organisms as well as in the environment, temperature is perhaps one of the most important environmental variables that influence growth rate, cell size, reproduction and chemical composition of algae (Lobban & Harrison 1997, Barsanti & Gualtieri 2014). Under field conditions, aerial temperature is of course highly dynamical on short time-scales, depending on wind conditions, clouding, etc.. However, as the BIOSEA algae are principally subtidal species, a considerable part of this variation is buffered by the water bodies they naturally inhabit, so the timescale of variation to which they are exposed in their natural environment is reduced. Nevertheless, it is noteworthy to remark that even a one to two degree decrease in temperature at night can have significant impact on growth rates, as has been demonstrated for example for *Ulva pertusa* (Wang et al. 2007) and *U. rigida* (Figueroa et al. 2014b). To a lesser degree this is also valid for outdoor semi-controlled cultures (lesser water volume, possibility to influence temperature), whereas in inside cultures we can usually control temperature. It is important to bear in mind these aspects of temperature when revising literature data on effects of this parameter on growth and composition, as these are nearly always coming from experimental laboratory cultures under constant (non-natural) temperature conditions, which cannot be translated directly to field conditions.

Growth rate responses of algae to (cultivation) temperature typically follow a bell-shaped curve, leaving a temperature window in which growth is maximal (Lobban & Harrison 1997). First *Arthrospira (Spirulina) platensis* were collected from water bodies with a relatively high water temperature; the temperature window for optimum growth was determined to range from 35 – 38 °C (Vonshak 1997). Later, it was shown that optima varied considerably between strains, some strains even showing very large windows ranging from 25 – 40 °C (Vonshak 1997, Kumar et al. 2011). Hence, all it can be said is that optimum temperatures are generally ≥ 25 °C, but exact windows are highly strain-specific. For the other microalga in BIOSEA, *Isochrysis galbana*, results also vary greatly. As explained in section 3.2.2, often the tropical strain, now identified as the species *Tisochrysis lutea* (*Isochrysis* aff. *Galbana* or T-iso) is confused with the "official" *Isochrysis galbana* from temperate waters, whose optimum growth

temperatures differ markedly. T-iso is found to display a relatively broad optimum window ranging from 25 – 32 °C (Ewart & Pruder 1981, Kaplan et al. 1986, Marchetti et al. 2012), whereas the temperate *I. galbana* favours a much narrower and much colder window, ranging from 17 – 20 °C (Ewart & Pruder 1981, Durmaz et al. 2008).

Similar problems in indicating optimum growth temperatures exist for *Ulva* spp. Similar morphologies may display different growth optima (Malta et al. 1999). No specific temperature-growth response information could be found for *U. intestinalis*, the *Ulva* species originally selected in the BIOSEA project. Nevertheless, considering that main appearance of this species shifts from summer to winter, going from northern to southern Europe, indicates that maximum growth occurs at water temperatures below 20 °C (Bäck et al. 2000, Lotze et al. 2000, Pérez-Lloréns et al. 2004). On the other hand, broad temperature maxima and strain variation has been shown in various *Ulva* species (Malta et al. 1999, de Casabianca et al. 2002, Cui et al. 2015) and in the last few years a species of tropical origin, *U. ohnoi*, seems to have become dominant in southern Spain and Portugal that has a capacity of fast growth at the high temperature range (25 – 35 °C, Alsufyani et al. 2014, Malta & Coste unpubl. res.). Finally, *S. latissima* is a typical cold to temperate water kelp species, which shows an optimum growth window between 10 – 15 °C and strong stress symptoms at temperatures ≥ 20 °C (Bolton & Lüning 1982, Andersen 2013).

The effects of different growth temperatures or changes in temperature are a result of changes in the underlying physiological processes, which are not only expressed as differences in growth rates, but also in algal composition and (at least to some extent) in morphology. In general, microalgae growing at their optimum temperature, provided no other factors are limiting growth, have minimal cell size (Juneja et al. 2013). For *A. platensis*, it is described that ultrastructure of the colony changes to a more tightly wound helix (van Eykelenburg 1979).

One of the most conspicuous direct effects attributed to different growth temperatures in algae are the alterations in the levels of unsaturation of membrane fatty acids (Vonshak & Torzillo 2004). Saturated fatty acids levels tend to increase with temperature, whereas at lower (suboptimal) temperatures PUFA levels have been reported to be higher (Thompson et al. 1992). The explanation for this is that increasing the amount of unsaturated fatty acids in the cell membrane will compensate for the reduced membrane fluidity as a result of the lower temperature and at the same time it will counter the increased susceptibility of the cells to damage by free radicals resulting from photoinhibition (Raven & Geider 1988). For *A. platensis* total FA increased with temperature but percentage of saturated FA indeed decreased (Cohen 1997). The same was observed for *I. galbana* / T-iso (Renaud et al. 1995, Zhu et al. 1997, Durmaz et al. 2008). On the other hand, in comparing various *I. galbana* and a T-iso strain, Sayegh & Montagnes (2011) found no consistent trend in effects of temperature on composition and even an increase of the PUFA docosahexaenoic acid at high temperatures in *I. galbana* (probably T-iso) was reported by Huang et al. (2017). For the BIOSEA macroalgae, experimental evidence is very limited. In *U. pertusa* total fatty acids levels as well as the percentages of the two major PUFAs increased at a low temperature (Floreto et al. 1993). Seasonal harvests from the Sea of Japan confirmed this pattern for *U. fenestrate* and for Saccharina japonica (Sanina et al. 2008) as well, however a field study of Marinho et al. (2015) seemed to contradict this for *S. latissima*.

The impact of temperature on protein contents is little studied. Field data generally report higher protein contents in winter months, at least for seaweeds (Lobban & Harrison 1997). However, this might not be a consequence of temperature only as many variables interact in the field; nutrient concentrations in particular considerably higher in winter than in summer, which probably more important than temperature (see below). Studies in phytoplankton and *Ulva pertusa* report a decrease in protein synthesis at temperatures beyond the optimum for growth and increases at reduced temperature or during the night in a day/night cycle with temperature decreases at night (Juneja et al. 2013 and references therein). This was also reported for the Tahitian *Isochrysis* (T-iso, Renaud et al. 2002). For a putative *Spirulina* species, maximum protein contents at the optimum growth temperature, with decreases at both lower and higher temperatures (Ogbonda et al. 2007).

With respect to carbohydrates, algae have been shown to display complex responses to changes in temperature, often complicated by interactions with other parameters that themselves also respond to temperature shifts, dissolved CO_2 in particular (see for example Figueroa et al. 2014 and Kang & Kim 2016 for interaction studies in three seaweed species incl. *Ulva*). Therefore direct responses are difficult to assess, which has to be taken in to consideration when analysing literature data. For *A. platensis*, accumulation of carbohydrates concentrations has been reported at higher temperatures (Rafiqul islam et al. 2003, Panyakampol et al. 2015). In contrast, no consistent trends were found in studies on various phytoplankton species incl. T-iso (Renaud et al. 2002, Sayegh & Montagnes 2011), whereas Zhu et al. (1997) found a decrease in carbohydrates with increasing temperature for *I. galbana* (probably T-iso). A similar response was found for *Saccharina latissima*, however only in a temperate water isolate, whereas no response was found in an Arctic isolate (Olischläger et al. 2014).

A very significant effect of temperature has been found in carotenoid levels in different microalgal species (reviewed in Hu 2004, Juneja et al. 2013). Carotenoids play a double role in algae; they absorb light, thereby enhancing photosynthesis, but they also act as protecting agents against photodamage. Hence carotenoids tend to increase with increasing temperature. Indeed this was for *A. platensis* (Kumar et al. 2011). However, the opposite was reported for *I. galbana* (Durmaz et al. 2008). No data were found testing the single effect of temperature on carotenoids in the BIOSEA macroalgae.

4.2.3 Light

Being photoautotrophic organisms, algae depend on light as energy source for the capture and conversion of carbon dioxide into sugars. Light influences algal growth through its effect on photosynthesis. Although many factors interact in determining the exact form of the response curve of growth of a species/strain, there is a maximum light intensity (assuming optimum growth conditions) at which photosynthetic activity no longer increases (saturation) and beyond which it decreases again. Light refers to the part of the light spectre of 400 to 700 nm that algae (and other photosynthetic organisms) are capable of using for photosynthesis, also called photosynthetically active radiation (PAR). PAR is expressed as µmol photons m^{-2} s^{-1}. Actually, only a small part of this range is used, as all light-absorbing pigments have relatively narrow absorption peaks (Fig. 4.2). This is especially relevant to bear in mind in modern cultivation systems that use monochromatic blue and red LED-light; as this is "high quality light", saturation of growth is already reached at a lower PAR than natural sunlight or artificial white light. General overviews of algal growth and photosynthesis can for example be found in Thompson (2006), Ch. 3 and 4 in Barsanti (2014), and Ch. 5 in Hurd et al. (2014).

Saturating light intensity for photosynthesis of different *A. platensis* strains varied between 614 and 645 µmol photons m^{-2} s^{-1} (Vonshak 1997). A modelling study showed a saturating light intensity for *I. galbana* (T-iso) of 500 – 600 µmol photons m^{-2} s^{-1} (Ippoliti et al. 2016). Considering that this is a tropical species adapted to a high light environment, it is likely that the N. Atlantic "true" *I. galbana* saturates at a much lower intensity. For *U. intestinalis*, determinations of saturating light level are between 174 – 245 µmol photons m^{-2} s^{-1} (Arnold & Murray 1980, Rautenberger et al. 2009); values for other *Ulva* species from temperate waters range between 245 – 465 µmol photons m^{-2} s^{-1} (Arnold & Murray 1980, Ramus & Rosenberg 1980, Henley 1992, Rautenberger et al. 2015). Saturating light levels for *S. Latissima* are considerably lower, between 30 – 60 µmol photons m^{-2} s^{-1} (Davison et al. 1991, Iñiguez et al. 2016).

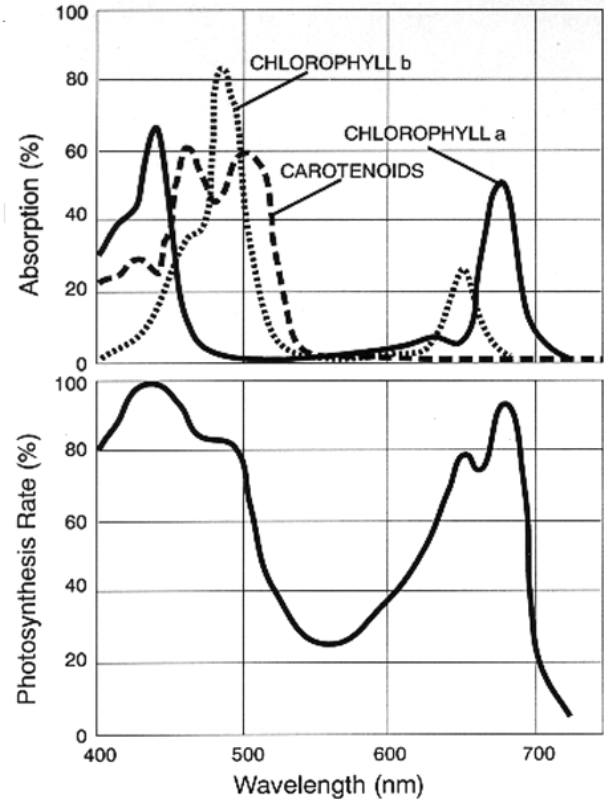

Fig. 4.2: John Whitmarsh and Govindjee. - http://www.life.uiuc.edu/govindjee/paper/gov.html, from "Concepts in Photobiology: Photosynthesis and Photomorphogenesis", Edited by GS Singhal, G Renger, SK Sopory, K-D Irrgang and Govindjee, Narosa Publishers/New Delhi; and Kluwer Academic/Dordrecht, pp. 11-51. From unpublished data., CC BY-SA 2.0, https://commons.wikimedia.org/w/index.php?curid=6958887

Algae are capable of acclimatizing to different light conditions (low or excessive light) by a series of morphological (at cellular and intracellular level) and physiological adaptations that has profound impact on algal composition. Algae growing under limiting light level show increases in pigment content (Chl *a* and accessory pigments) to increase light absorption. This includes the production of phycobiliproteins in *A. platensis* (Bryant 1982, Tomaselli et al. 1997), various xanthophylls in *I. galbana* (Grant & Louda 2010, Obata & Taguchi 2012), chlorophyll *b* and lutein in *Ulva* (Malta et al. 2003, and chlorophyll *c* in *S. latissima* (Boderskov et al. 2016). Under excessive light, algae need to protect themselves against photoinhibition and consequent photodamage. Here pigments also play an important role, especially those of the xanthophyll cycle (zeaxanthin, violaxanthin, etc., Vershinin & Kamnev 1996). B-carotene and other carotenoids and (in *A. platensis*) certain phycobiliproteins also increase at excessive light (beyond saturation, Bryant 1982, Gevaert et al 2002, Vonshak & Torzillo 2004).

Due to changes in production and growth rates, respiration and other mechanisms, changes in light conditions affect cellular composition of the algae. Although patterns are difficult to distinguish from interaction with other parameters, in general protein content decreases with increasing light intensities (up to saturation), whereas lipids and carbohydrates increase (Juneja et al. 2013 and references therein). On the other hand, percentage of unsaturated fatty acids, specifically PUFAs might be higher at low light intensities. Nevertheless, Cohen (1997) reports no effect or even a slight increase at low light of fatty acids and little changes in total % of PUFAs, although PUFA composition did change. Excessive light leads to damage and oxidation of PUFAs. For *U. pertusa* a higher proportion of PUFAs were found at high light, without changes in total lipids (Floreto & Teshima 1998).

4.2.4 pH/CO_2

Water pH is a crucial factor in algal growth because of its great role in water chemistry, in particular in determining the availability of CO_2 for the algae. This is especially relevant in (dense) algal cultures where pH significantly rises due to uptake of inorganic carbon of the algae, even to levels higher than 10 (e.g. Gordon & Sand-Jensen 1990, Grobbelaar 2004), although this phenomenon also occurs in nature,

for example in dense algal mats or in tide pools (Lenzi et al. 2013, Olischläger et al. 2013). Maximum algal growth occurs around neutral pH (Juneja et al. 2013), at high pH growth slows down as it reduces availability of CO2 and bicarbonate (Chen & Durbin 1994). The degree of growth suppression is species-specific, not all species can use bicarbonate as a carbon source (Gordon & Sand-Jensen 1990, Mata et al. 2007). *Arthrospira* for instance is known for its alkalophilic nature, having an optimum pH for growth of 8.5 and is good in coping with high pH (Vonshak 1997). The same is true for *Ulva* spp. and *S. latissima* that are capable of maintaining high growth rates at a pH of more than 9 due to its carbon-concentrating mechanism and resulting high affinity for bicarbonate (Frost-Christensen & Sand-Jensen 1990, Axelsson et al. 1995, Axelsson et al. 2000, Klenell et al. 2004). *I. galbana* (T-iso) on the other hand shows a quite narrow pH optimum between 7.0 – 7.5 with strong declines in growth rates at a pH higher than 8 (Marchetti et al. 2012). For "true" *I. galbana*, the optimum pH seems to be slightly higher, around 8 (Molina Grima et al. 1992).

Direct effects of medium or environment pH on the chemical composition are difficult to determine and not always unequivocal for all algal species (Juneja et al. 2013). Considering its determining role in water carbon chemistry and consequently its effect on carbon acquisition and hence photosynthesis and growth of algae and its crucial role in many cell processes, specifically membrane transport processes, its major impact might be expected on the major carbon compounds of algae, such as carbohydrates and lipids. Indeed, several effects have been reported on lipid contents of changes in pH, either by addition of CO_2 to growth media or otherwise. In some cases an increase of lipids, however a decrease in the portion of unsaturated FAs was found under more acidic conditions. For *I. galbana* specifically, no changes in lipid content were found at more acidic pH, however optimum production of PUFAs occurred at a pH close to the optimum for growth (Molina Grima et al. 1992); likewise no effect of CO2 addition on lipid concentration was found for T-iso (Picardo et al. 2013). For *Ulva rigida* it was found that pH (CO_2 addition) basically mediated the effect of nitrogen on lipids; effects of CO_2 were found on lipid class composition but not total lipids (Gordillo et al. 2001).

Interesting effects of pH on pigments and antioxidants in *A. platensis* were reported by two studies. Pigment (phycobilliprotein and carotenoid contents) followed an optimum curve; however Sharma et al. reported a pH of 7, below the growth optimum, as optimal for production of carotenoids, phycocyanin, allophycocyanin and phycoerythrin (2014), whereas Ismaiel et al. (2016) reported an optimum of 9.0 for phycobiliproteins and slightly lower for carotenoids. This difference might be due to variation between strains. The latter study also reported a strong effect of pH on phenolic compounds and antioxidative power with optimum pH of 8.5 – 9 for both. Gordillo et al. (1998) reported an increase in carbohydrate content and a decrease in proteins and pigments at elevated CO_2 in an *A. platensis* culture.

4.2.5 Salinity

Salinity is another parameter that has a profound impact on algal growth rate and composition. Of the algae selected in the BIOSEA project, *A. platensis* mainly occurs in alkaline or brackish to saline environments, whereas the other algae are truly marine species. Although *A. platensis* does occur in brackish to marine environments and is able to clematises to moderate salinities, it thrives best in zero to low (about 5 psu) salinity environments (Kebede 1997, Vonshak 1997, Ravelonandro et al. 2011). Optimal growth salinity for *I. galbana* / T-iso ranges between 20 – 40 psu (Sen et al. 2005, Abu-Rezq et al. 2009, Alkhamis & Qin 2013). *Ulva* species, in particular the species with tubular morphology have been reported over the entire salinity range, from fresh water (Ichikara et al. 2009, 2013, Rybak et al. 2014) to fully marine conditions (Malta et al. 1999). Part of this flexibility is species-specific, for example *U. flexuosa* thrives best under lower salinities and *U. limnetica* appears exclusively in fresh water, although it proved to be highly viable in seawater (Ichikara et al. 2009, Rybak et al. 2014), but other species are capable of growing at a salinity range from 5 – 40 psu, although most grow best between 15 – 30 psu (Malta et al. 1999, Taylor et al. 2001, Kang et al. 2016). *S. latissima* inhabits estuaries and open sea coasts, to salinities as low as 16 psu although 25 – 35 psu are optimum for growth (Schubert et al. 2011, Nielsen et al. 2016).

The main effects of salinity on composition of *A. platensis*, include strain-specific increases in carbohydrates, mainly of low molecular weight that act as osmotic substances (Vonshak 1997). Reports of effects of salinity on proximate composition contradict each other, in particular with respect to protein levels. According to the results of Sujatha & Nagarajan (2014), small increases in salinity result in increases in phycocyanin and lipids, however at higher salinities they decrease again, whereas proteins decreased at all salinities. A similar result for proteins was reported by Ravelonandro et al. (2011). The results of Sharma et al. (2014) corroborate this for phycobiliproteins; besides they found a decrease of carotenoids with increasing salinity. Yilmaz et al. (2010) likewise found an increase in lipids and carbohydrates with increasing salinity but no decrease at higher salinities and maximum protein levels at the lowest salinity. Almahrouqi et al. (2015) tested the response over a salinity range from 5 – 35 psu and observed the lowest protein at the lowest salinity, increases in lipids and carbohydrates with salinity, and an increase in PUFAs with salinity.

For *I. galbana* (T-iso) a linear increase in lipid contents was found over a 10 – 35 psu salinity range with little effects on carbohydrates and protein and modifications in FA contents and composition (Renaud & Parry 1994). They found highest PUFA levels under both the lowest and highest salinities, however the optimum for the maximum production of lipids and essential FAs was at 25 psu. For "true" *I. galbana*, an increase of FAs with salinity was found as well with highest levels of PUFAs at intermediate and high salinity (Nedbalová et al. 2016). For protein content, an increase was reported in "true" *I. galbana* with increasing salinity (Fabregas et al. 1985).

Varies studies have investigated the effect of salinity on the composition of different *Ulva* spp. For *U. fasciata* and *U. pertusa* both total fatty acids, as the percentage of PUFAs have been found to increase with salinity (Mohsen et al. 1972, Floreto et al. 1994, Floreto & Teshima 1998). Mohsen et al. (1972) also reported an increase in sugar content up to the optimum for growth (35 psu) after which it decreased again. In a detailed study where *U. ohnoi* was grown in a salinity range of 10 – 60 psu, total protein content (as total amino acids) remained more or less constant, but amino acid composition changed drastically, with linear increases in proline, tyrosine, and histidine with salinity, a moderate, non-linear increase of methionine at the optimum for growth (30 – 35 psu) and a decrease in alanine (Angell et al. 2015). On the other hand, for *U. prolifera* Luo & Liu (2011) did found an increase in protein with salinity. Finally, salinity significantly increased antioxidant contents and antioxidant enzyme activity in *U. fasciata* (Lu et al. 2006) and *U. prolifera* (Luo & Liu 2011).

Most studies on the potential effects of environmental parameters on *S. latissima* are field studies, describing changes in biochemical composition during the year on different locations, with different salinity gradients. It is therefore complicated to single out the direct effect of separate parameters (Nielsen et al. 2014). Salinity in an estuary for instance tends to strongly co-vary positively with temperature and light and negatively with nutrients, depending on the specific characteristics of the site. In Denmark, highest biomass production potential and the highest protein content were found at sites with high salinities, as opposed to the highest levels of fermentable sugars (up to 90% DW!) and pigments that were highest in low salinity sites (Nielsen et al. 2014). Another study from Denmark indicated a potential increase of fucoidean levels in cell walls, at least over the lower salinity range (10 – 20 psu), although ecotypical variation between populations may exist (Bruhn et al. *in press*). This is in agreement with an earlier study that reported that the best yield of sulphated polysaccharides would be in periods or sites with high salinity (Ehrig & Alban 2015). A strong irreversible reduction of low salinity on the capacity of CO2-uptake was reported (Mortensen, *in press*), indicating effects on lipid and carbohydrate contents.

4.2.6 Nutrients

Nutrients, and especially nutrient limitations, have profound effects on the chemical composition of algae, depending on the degree of limitation and the limiting nutrient. Macronutrients are carbon, nitrogen and phosphorus. Furthermore there are the micronutrients, mainly consisting of trace metals, iron (Fe) being the most important one. Other main trace metals are manganese (Mn), cobalt (Co), zinc

(Zn), copper (Cu) and nickel (Ni) (Lobban & Harrison 1997, Barsanti 2014). As carbon availability is closely coupled to pH, the effect of carbon was already discussed in 4.2.4. In the following the effect on biochemical composition of the BIOSEA algae of other nutrients will be discussed briefly.

Nitrogen and phosphorous are crucial elements for algal growth and development. Nitrogen is key in the formation and composition of proteins and nucleic acids. Phosphorus is part of essential molecules as ATP, the energy carrier of cells, and of DNA and RNA. It is also an important element of phospholipids. Extensive knowledge is available on the effects of nitrogen and phosphorus deficiency in micro- and macroalgae, see the review of for example Juneja et al. (2013) and the textbooks listed in 2.5 for detailed accounts of effects and mechanisms. As general patterns are similar for all algae, here a summary of the main effects will be given with some specific examples for the BIOSEA algae if available.

As said, nitrogen forms an integral part of amino acids (proteins) and nucleic acids and is hence fundamental for algal growth. Its contents range between 7 – 20% DW in microalgae and between 0.5 – 9% in macroalgae, the difference basically has to be attributed to the much lower amount of structural biomass in microalgae (Lobban & Harrison 1997, Hu 2004). Consequently, nitrogen limitation or starvation generally leads to a decrease in protein content and a surplus of carbohydrate skeletons coming from photosynthesis, part of which are stored as carbohydrates and lipids (Gómez Pinchetti et al. 1998, Gordillo et al. 2001). Finally growth rates will drop and a new equilibrium will be reached. However, it appears that this general conundrum is not applicable to cyanophytes; lipids did not accumulate or even decrease in nitrogen starved *A. platensis* (Cohen 1997, Gordillo et al. 1999). The pattern was confirmed for *I. galbana*, however, although total lipids increased under nitrogen limitation, omega 3 fatty acids and other PUFAs decreased (Flynn et al. 1993, Dörner et al. 2014, Fosse 2016). Similar results, including accumulation of carbohydrates, were reported for *Ulva* spp. (Floreto et al. 1996, Angell et al. 2013, Malta & de Nys 2016) and experimental evidence and seasonal variation of composition suggests a similar effect in *S. latissima* (Wheeler & Weidner 1983, Schiener et al. 2015). Another generally observed effect of nitrogen limitation is the decrease in pigment content, in particular of primary photosynthetic pigments Chl *a* and phycobiliproteins (Jeneja et al. 2013) and other pigments as well, for instance those of the xanthophyll cycle (Stengel et al. 2014, Boderskov et al. 2016).

Although phosphorus content of algae is much lower than nitrogen, it is certainly not of less importance. Concentrations in micro- and macroalgae are in the range of 0.1 – 1% DW (Atkinson & Smith 1983, Juneja et al. 2013). The first direct effect of phosphorous limitation is the decrease of the regeneration of substrates for the Calvin-cycle and consequently a decrease in photosynthetic efficiency of carbon fixation (Barsanti & Gualtieri 2014). In general, effects of phosphorus limitation are quite similar to those of nitrogen limitation. They include an accumulation of lipids, but a decrease in PUFAs and a change in lipid composition with a decrease in phospholipids. Furthermore, carbohydrates tend to increase and protein, chlorophyll and other pigments decrease with phosphorous limitation (see review in Juneja et al. 2013 and textbooks). This pattern was largely confirmed for *A. platensis*, which showed drastic increase of carbohydrates under phosphorus limitation (Markou et al. 2012). Similar results were found for *I. galbana* (Sun & Wang 2009, Roopnarain et al. 2014). The number of studies on the effects of phosphorous limitation on macroalgae is limited as from an ecological point of view, nitrogen has always been considered the main limiting nutrient. For *U. pertusa* little effect was found on total fatty acids and a small decrease in the unsaturated palmitic (16:0) fatty acid in favour of its PUFA 16:4 n-3 (Floreto et al. 1996). Kumari et al. (2014), confirmed the general consequences of phosphorous limitation in algae for *U. lactuca* and specifically found a decrease of phospholipids and an increase in sulpholipids and 1, 2-diacylglyceril-3-O-4'-(N, N-trimethyl)-homoserine (DGTS) under low phosphorous. For *S. latissima* an increase in mannitol was found after phosphorous addition to limited plants (Connolly & Drew 1985).

Trace metals are important components in practically all biochemical processes of living organisms, as co-factors of vitamins, catalysts, in enzymes, electron transfer in photosynthetic organisms, etc. Their role is summarized in the review of Juneja et al. (2013). Deficiencies in trace metal can limit algal growth, whereas excesses or a high concentration hinders growth and damage cells, the cell membrane in particular, and render them inappropriate for human and animal consumption. Presence of excess

trace metals or toxic heavy metals tends to increase antioxidants in algae; see for instance Rijstenbil et al. (1998) and Malea et al (2006) for effects of copper on *Ulva* spp. Generally, in the natural environment and especially in coastal regions, trace metals are not limiting, except from some areas of the open ocean (Kolber et al. 1994). In polluted regions metals are known to accumulate in macroalgae for which they are used as bioindicators of pollution (Sanchiz et al. 2001, Chakraborty & Owens 2014). In cultures, balanced media are used and metal limitation or toxicity is normally not an issue, therefore this will not be further discussed here.

4.2.7 Parameter interactions and other factors

Environmental parameters rarely, if ever, act singularly and tend to positively or negatively co-vary, not only in the natural environment, but even under the most controlled culture conditions. Algal growth leads to nutrient depletion, pH increase, a denser culture and hence self-shadowing, etc. Changes in one parameter, for instance temperature, will increase or decrease growth and metabolism, thereby influencing other parameters, which will have their effects as well. Specific effects of changes in certain parameters on algal biochemical composition will therefore largely depend on the interaction with other parameters. Low nutrient availability for instance will have little effect under low light and/or temperature conditions, as growth is limited by those anyhow. Likewise, low or high temperature will completely change responses of algae to low or high light with subsequent effects on their composition. In their review, Juneja et al. (2013) provide various examples of effect of interactions of environmental parameters on biochemical composition of microalgae.

Some of these interactions can be used to our benefit, for instance if we want to increase levels of specific components. Neveux et al. (2015) and Malta & de Nys (2016) provide examples of pre- and post-harvest treatments in macroalgae that specifically increase the quality of the algal feedstock in terms of carbohydrates and lipids. The effect of interaction of nutrients and environmental factors is crucial for a thorough understanding of the processes in algal cultures, especially in large-scale systems (Juneja et al. 2013).

Apart from these non-biological parameters, there are various biological parameters that can alter biochemical composition of algae. Circadian rhythms and cell-cycle in microalgae are important elements of a culture. Light-dark cycles can induce synchronized growth in various microalgae, but is has not been found in diatoms (Kromkamp & Claquin). Changes in cell-cycle result in changes in metabolism and hence composition, for instance proteins and fatty acids (Kromkamp & Claquin 2006). For the green microalgae a maximum in starch, protein and fatty acid content was obtained just before cell division and in a circadian light-dark cycle, starch and total FA accumulated during the day. Also, fatty acid composition changed during the cell cycle with a build-up of neutral lipids during the day, especially in cells that were arrested in their cell cycle (de Winter et al. 2013, 2014). Cycle disruption in the diatom was found to lead to unbalanced growth and the accumulation of storage products, especially lipids (Kim et al. *in press*). On the other hand, marginal effects were found on composition of *I. galbana* (T-iso) after growth activation (Cho et al. 1999). In macroalgae, sporulation specifically encompasses a whole set of morphological changes at cell level that also affect composition (Wichard et al. 2015). Lipids in general might increase considerably (pers. comment J. Cremades), however little is known on the subject of the effects of cell cycle and sporulation on composition and this hence has to be studied more in depth.

Herbivory, virus attacks, parasites, life-cycle or cell stage drive the defences of algae and induce the production of secondary metabolites (Amade & Leme 1998, Mancini et al. 1998, Pereira et al. 2017). *S. latissima* and other brown algae are well known for grazing-induced production of phlorotannins and other phenolic compounds (Vilg et al. 2015). However, grazing does not occur in indoor cultures and is an inevitable part of cultivation in the natural environment; a detailed discussion on its effects on algal composition falls therefore outside the objectives of BIOSEA and of this report.

5. Concluding remarks

Algae have become more and more important as an alternative feedstock for a number of applications, including food and neutraceuticals, animal feed, cosmetics, pharmaceuticals, biofuels and others. The BIOSEA project aims at developing and validating innovative, competitive and cost-effective upstream and downstream processes for the cultivation of 2 microalgae (*Spirulina platensis*, official name *Arthrospira platensis* and *Isochrysis galbana*), and 2 macroalgae (*Ulva* sp. and *Saccharina latissima*) to produce and extract high value active principles at low cost (up to 55% less than with current processes) to be used in food, feed and cosmetic/personal care high-added value products. These selected algae cover a wide range of growing environments, from freshwater alkaline, to brackish and fully marine conditions.

Considering the overview of chapter 2 of cultures and cultivating countries, it can be concluded that in cultivation of both micro- and macroalgae, Europe is still a minor player. Large-scale microalgal cultivation mainly takes place in Asia, Australia, Hawaii and Israel, whereas for macroalgae, dominance of Asia and China in particular is striking. In contrast to other areas, microalgal cultivation in Europe mainly takes place in photobioreactors and is often aimed at specific components. Nevertheless, growth in cultivation of both micro- and macroalgae is boosting in Europe. New companies are being founded at an increasing rate. For macroalgae, the first large-scale cultures are currently running or being set-up all over Europe.

Considering algal composition, the selected algae for the BIOSEA project prove to be an interesting mix with added value in a cascade biorefinery approach. Comparisons of their essential amino acid and polyunsaturated fatty acid profiles show that composition is fairly complementary for human and animal (incl. finfish and shellfish aquaculture) feed and food production. Of the essential amino acids, only tryptophan is present only in low concentrations, as is normal for algae, all others can be provided for (Table 5.1). This goes for fatty acids as well, all algae contain valuable omega-3 and 6 and other PUFAs. With respect to carbohydrates, the macroalgae in particular prove interesting, containing high amounts of relatively rare oligosugars and high levels of sulphated polysaccharides. However, microalgae can also contribute in this, *A. platensis* in particular as it contains polyhydroxybutyric acid (PHB) often used in bioplastics. A potentially interesting polysaccharide for cosmetics and pharmaceuticals recently isolated from I. galbana is a highly branched β –glucan.

Table 5.1: Comparison of the profile of essential amino acids in the algae selected in the BIOSEA project. * = semi-essential amino acid, ** = essential for molluscs.

Essential amino acid	*A. platensis*	*I. galbana*	*Ulva* spp.	*S. latissima*
Arginine*	X			
Cysteine*			X	X
Glycine*	X		X	X
Histidine*	X		X	X
Isoleucine	X			
Leucine	X		X	X
Lysine			X	
Methionine	X			
Phenylalanine	X		X	X
Proline**		X		
Threonine	X		X	X
Tryptopohan				
Tyrosine*	X		X	X
Valine	X		X	X

Dynamics of compounds in the algae have been described. Contents of many compounds are highly variable and influenced by basic environmental parameters and especially their interaction. In cultures, some of these interactions provide interesting possibilities to influence the contents of components of specific interest. Apart from environmental parameters as temperature, light, nutrients, etc., there are also biological parameters such as cell-cycle and reproduction that can affect biochemical composition of the algae. However, these fall largely outside the BIOSEA project and have not been reviewed extensively. Interacting effects of changes in cultivation parameters on the ecophysiological responses of the algae provide interesting perspectives for the manipulation of composition towards desired compounds in tank cultivated algae.

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