North Sea Seaweeds

DIP and DIN uptake kinetics and management strategies

Alexander Lubsch

2019

North Sea seaweeds:

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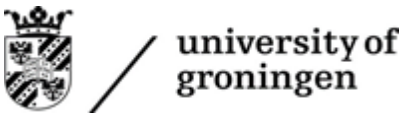

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the decision by the College of Deans.

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Preface

The term 'seaweeds' is used here to describe multi-cellular marine macro-algae, although at some stage in their life-cycle seaweeds can be unicellular (spores, zygotes). One key component for survival and growth in seaweeds is the availability of (dissolved inorganic) nutrients. In this study the term 'nutrients' refers to the biologically available inorganic dissolved forms of nitrogen and phosphorus, two major constituents of living organisms. The focus in this thesis is on investigation of nutrient uptake kinetics, nutrient management, effects of nutrient limitation, growth, and composition in relation to nutrient availability in 4 ecologically and economically relevant (green, brown, and red) native North Sea seaweed species: *Ulva lactuca* (Chlorophyta), *Saccharina latissima*, *Laminaria digitata* (Phaeophyta) and *Palmaria palmata* (Rhodophyta). Insight in these major stimuli of production in seaweed biomass will allow better understanding of the ecophysiology of seaweeds, both under natural, as well as (large scale) production conditions.

It can be envisioned that nutrient availability will not only determine growth, but through the effects on composition it may also affect colour and texture. Hence, in addition to the analysis of the nutrient uptake kinetics and nutrient management strategies, 2 novel approaches with ecological and economical relevance on seaweeds with regard to nutrient availability are presented. Firstly, a combination of spectrophotometric measurements and colorimetric techniques was applied to determine the colour appearance of *U. lactuca*, resulting in a smartphone application 'EyeOnUlva'. This application evaluates the nutritional value (total dissolvable protein content) of this opportunistic seaweed by digital imaging. Secondly, an approach was introduced that allows for standardised methods to infer to effects of nutrient availability and varying hydrodynamic forces on the texture of seaweed, using *L. digitata* individuals.

Contents

Chapter 1 ... 1

1. General introduction ... 1

1.1 Seaweed ... 1

1.2 The North Sea ... 2

1.3 Seaweed farming in Europe ... 5

1.4. Thesis outline ... 8

Chapter 2 ... 26

Uptake kinetics and storage capacity of dissolved inorganic phosphorus and corresponding N:P dynamics in *Ulva lactuca* (Chlorophyta) ... 26

2.1 Abstract ... 26

2.2 Introduction ... 27

2.3 Material and methods ... 29

2.4 Results ... 35

2.5 Discussion ... 41

2.6 Acknowledgements ... 46

Chapter 3 ... 47

Using a smartphone app for the estimation of total dissolvable protein concentration in *Ulva lactuca* Linnaeus (Chlorophyceae) ... 47

3.1 Abstract ... 47

3.2 Introduction ... 48

3.3 Material and methods ... 51

3.4 Results ... 57

3.5 Discussion ... 63

3.6 Acknowledgements ... 67

Chapter 4 ... 68

Uptake kinetics and storage capacity of dissolved inorganic phosphorus and corresponding dissolved inorganic nitrate uptake in *Saccharina latissima* and L*aminaria digitata* (Phaeophyceae) 68

4.1 Abstract ... 69

4.2 Introduction ... 69

4.3 Material and Methods ... 73

4.4 Results ... 80

4.5 Discussion ... 90

4.6 Acknowledgements ... 97

Chapter 5 ... 98

Texture analysis of *Laminaria digitata* (Phaeophyceae) thallus reveals toughness gradient along lamina ... 98

5.1 Abstract ... 98

5.2 Introduction ... 99

5.3 Material and methods ... 101

5.4 Results ... 107

5.5 Discussion ... 110

5.6 Acknowledgements ... 115

Chapter 6 ... 116

Dissolved inorganic phosphate uptake and corresponding dissolved inorganic nitrate uptake in the seaweed *Palmaria palmata* (Rhodophyceae): ecological and physiological aspects of nutrient availability ... 116

6.1 Abstract ... 116

6.2 Introduction ... 117

6.3 Material and methods ... 120

6.4 Results ... 125

6.5 Discussion ... 134

6.6 Acknowledgements ... 141

Chapter 7 ... 142

'Manual for nutrient uptake kinetics in seaweed cultivation' ... 142

7.1 Offshore cultivation ... 142

7.2 Integrated multi-trophic aquaculture (IMTA) ... 145

7.3 Tank cultivation ... 148

7.4 Biofiltration ... 150

Chapter 8 ... 153

Synthesis ... 153

8.1 General findings ... 154

8.2 Innovative aspects / highlights of the thesis ... 159

8.3 Conclusions ... 160

8.4 Research outlook ... 161

References ... 164

Summary ... 191

Samenvatting ... 195

Acknowledgements ... 200

Chapter 1

1. General introduction

1.1 Seaweed

Seaweeds are found throughout the world's oceans and seas. They generally grow attached to rock or other hard substrate (with a few exceptions, i.e. *Sargassum natans* (Linnaeus) Gaillon, *Avrainvillea erecta* (Berkeley) A. Gepp & E.S. Gepp and *Halimeda macroloba* Decaisne) in the intertidal and subtidal zones of coastal areas, down to depths where light levels attenuate to 0.05% of the surface irradiation (Lüning 1990). They differ substantially in many microstructural and biochemical features, including the photosynthetic pigmentation. Based on their pigmentation, seaweeds are classified into three main groups: green (Chlorophyta), brown (Phaeophyta), and red (Rhodophyta). Typically, a seaweed consists of holdfast, stipe, and frond forming the thallus, but more complex structures can develop (Figure 1-1). Many seaweeds have specialized tissues, for example *Fucus vesiculosus* Linnaeus (bladderwrack), which develops air-filled vesicles (air bladders) along the frond for its buoyancy and thus optimizes the surface position for photosynthesis.

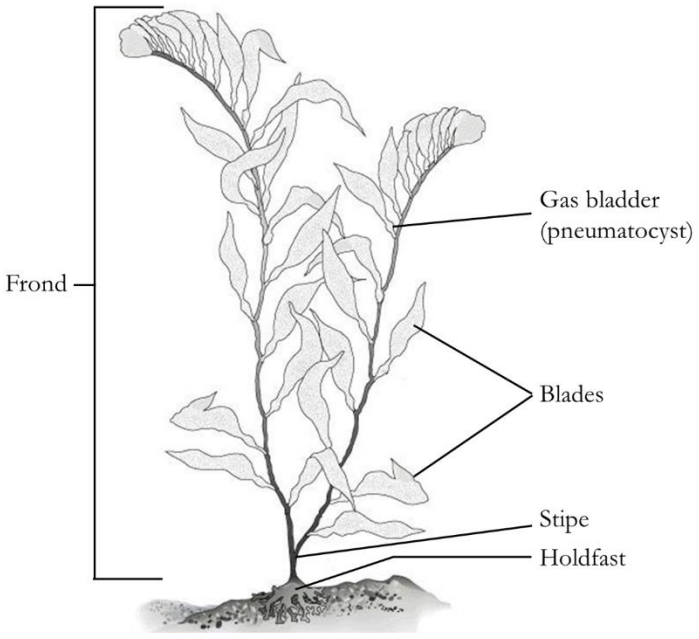

Figure 1-1. Diagram of a typical seaweed with a frond consisting of holdfast, stipe and blades. Some species also develop gas filled bladders (pneumatocysts) for float.

Seaweeds serve many important functions in ecosystems. As so-called ecosystem engineers, they can influence the availability of resources by influencing sedimentation and erosion, thus shaping their habitat and barren rocky sites can be transformed into areas of high structural complexity and diversity (Jones et al. 1994, Bouma et al. 2005). Seaweeds also provide a food source for primary consumers, offer protection (i.e. shelter) from predators and can serve as a nursery for many animal species (Lalli & Parson 1997, McClelland & Valiela 1998). Furthermore, seaweeds play a considerable role in the world's carbon cycle (global primary production). Approximately 6 % of the global net primary production (NPP) of 104.9 Pg (104.9×10^{15} g) carbon per year is ascribed to seaweeds, although seaweeds only colonize 0.1 % of the seafloor (Lieth 1974, Smith 1981). Seaweeds not only grow on saltwater, which covers 70 % of the surface of the planet, they also take up excess nutrients from coastal waters, thus can also reduce eutrophication. Like all photo-autotrophic organisms, seaweeds fix CO_2, a major greenhouse gas.

There is a growing interest in seaweed cultivation in Western Europe (including The Netherlands), as seaweeds are an attractive marine source of biomass and its cultivation offers great possibilities. Unlike terrestrial crops, seaweeds do not require agricultural land for cultivation and many species grow in saltwater (or brackish waters) avoiding competition for land and freshwater. Furthermore, they grow on nutrients available from the sea and do not need pesticides for crop-protection during growth. This thesis adds fundamental knowledge on sustainable seaweed cultivation of ecologically and economically relevant representatives of the marine flora in North-West Europe, all native to the North Sea area.

1.2 The North Sea

The North Sea, a biologically productive sea on the north-western European continental shelf, belongs to one of the world's most productive marine areas (Figure 1-2). Its topography ranges from muddy lowland coastline in the south towards rocky upland coasts in the north.

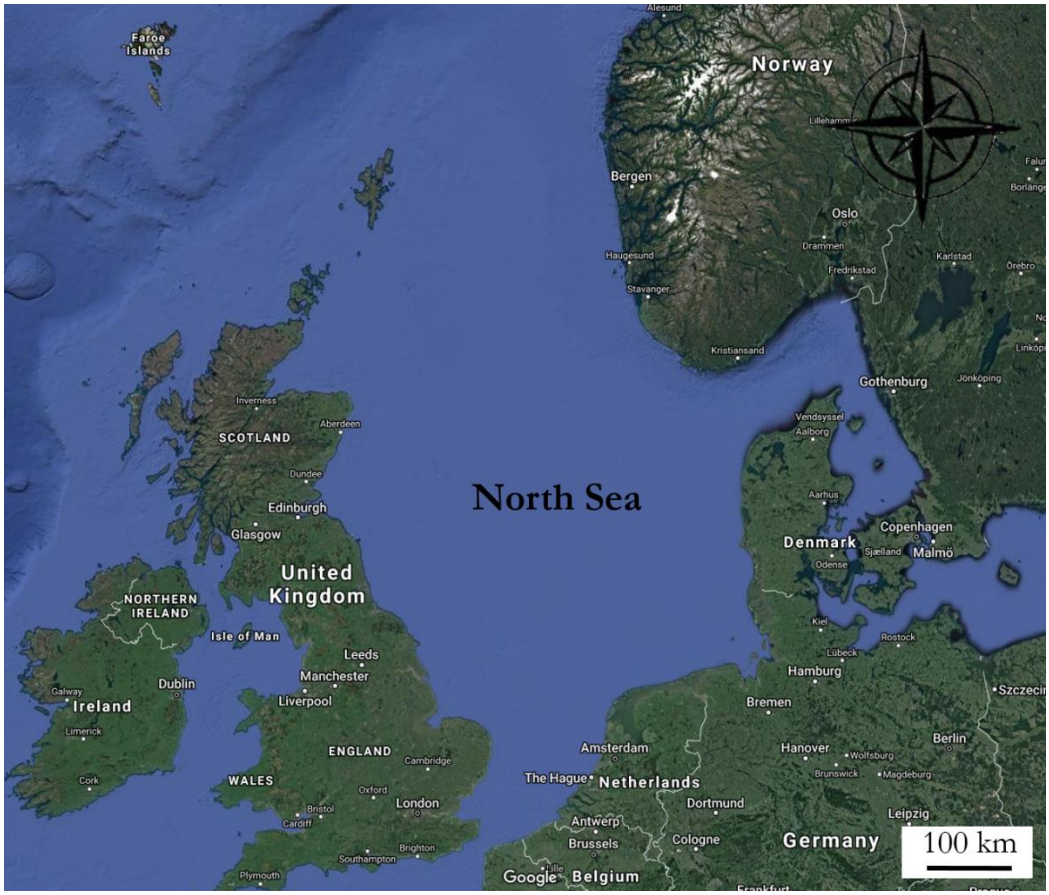

Figure 1-2. The North Sea area with neighbouring states (source: Google Maps. 2018. Retrieved from https://www.google.com/maps/place/NorthSea).

A dominant feature of the North Sea is the tidal motion, which contributes to horizontal and vertical mixing of the water properties (Otto et al. 1990). The overall nutrient budget of the North Sea ecosystem is widely influenced by oceanic inflow from the north-east Atlantic Ocean (Reid & Edwards 2001, Edwards et al. 2002) and nutrient concentrations show a seasonal highly heterogeneous distribution, with nutrient accumulation during the winter (Figure 1-3) and depletion in the summer. Spatial differences in nutrient concentrations exist in coastal areas, largely affected by the run-off waters of several rivers, including Rhine, Elbe, and Thames. These run-off waters often contain considerable amounts of inorganic phosphorus (P) and nitrogen (N) from anthropogenic land-based activities (Sharpley et al. 1992, Rabalais et al. 2009). Besides natural fluctuations, the anthropogenic discharge of nutrients can generate concentration gradients and limitations, which are often observed along coastal zones of the North

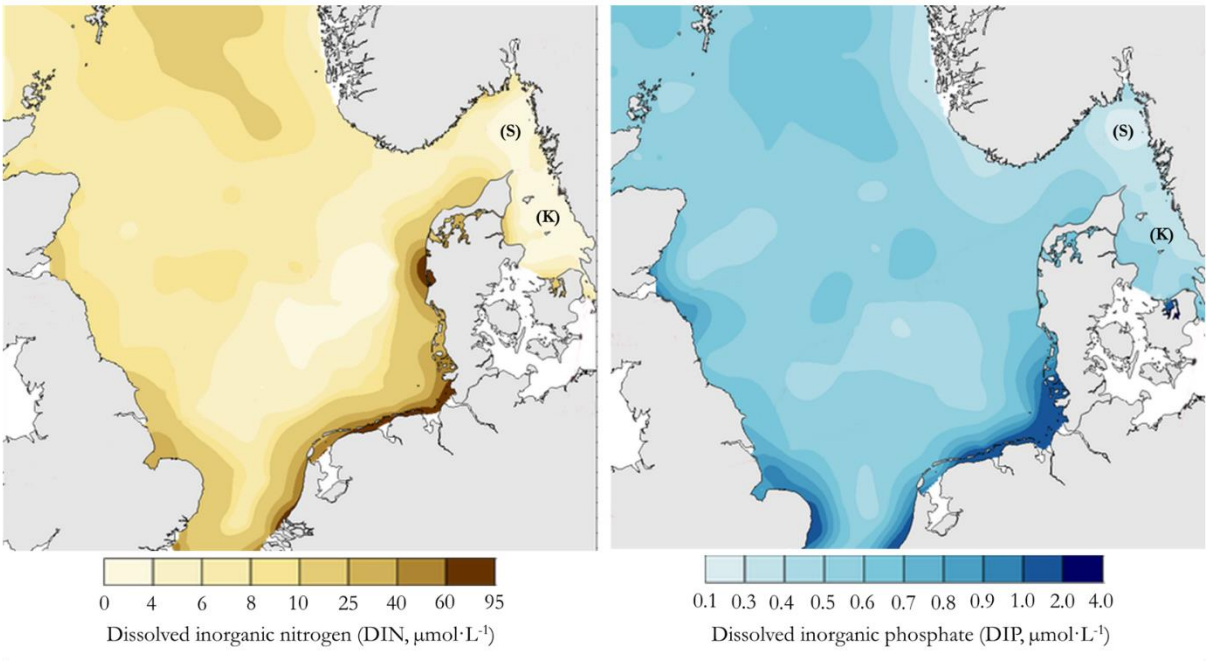

Figure 1-3. Distribution of average concentrations (2006-2014) of (A) winter DIN and (B) winter DIP in the North Sea, the Skagerrak (S) and the Kattegat (K). White areas are not categorized (source: https://www.oap.ospar.org/en/ospar-assessment/intermediate-assessment-2017/pressures-human-activities/eutrophication/nutrient-concentration).

Sea (Brockmann et al. 1990), sometimes causing eutrophication. Eutrophication can be a problem, for example when opportunistic seaweeds like species belonging to the genus *Ulva* (Chlorophyta), can vastly increase their growth and can build up a large biomass in extensive blooms (Teichberg et al. 2008, 2010). These massive blooms are known as 'green tides', when beached and rotting piles of biomass hinder shore-based activities. Green tides were reported along the coastline of southern North Sea since the 1970s, including The Netherlands (Malta & Verschuure 1997), The United Kingdom (Scanlan et al. 2007), and Denmark (Lyngby et al. 1999). Moreover, the sinking and degrading seaweed biomass can cause hypoxia in the water and the development of hydrogen sulphide (H_2S) by microbial decomposition processes, as reported for the German Bight and Danish waters in August 1982 with dramatic consequences for the pelagic and the benthic communities (Westernhagen & Dethlefsen 1983). Measures against eutrophication were installed, when these dramatic effects became evident. Recently it was shown, that the de-eutrophication

efforts have led to a large imbalance in the N:P stoichiometry of coastal waters of the North Sea in north-western Europe (Burson et al. 2016). Increasing N:P ratios, which outpace the Redfield ratio of 16:1 were observed (Radach & Pätsch 2007, Grizzetti et al. 2012) and a pronounced P-limitation can be effective in coastal regions of the southern North Sea.

1.3 Seaweed farming in Europe

A wide variety of seaweeds have been harvested for domestic use, for example as food, feed, and fertilizer by coastal populations in Europe for millennia (Hallsson 1964, Grall & Hall-Spencer 2003). The commercial utilization of seaweed started as early as the 17th century around Europe, with France, Ireland, and Norway being the biggest contributors. Harvested seaweeds were burned to produce potash, which was used in the glass and soap industry. While historically seaweeds were harvested for their crude biomass, the on-going research and development of refinement techniques allowed the extraction of valuable biochemical components, and in the 19th century the main utilization of seaweeds shifted towards the production of iodine. Nowadays, seaweeds in Europe are mainly exploited for their hydrocolloids and especially alginic acid (Porse & Rudolph 2017). Efforts to establish a sustainable seaweed farming in Europe are rising, as seaweeds are increasingly seen as an alternative to land-grown products for food, feed and energy, as well as others (extracts, clothes) and new seaweed product innovation in Europe is at a record level. Seaweeds are known to be an excellent source of polysaccharides, proteins, lipids (including high amounts of poly-unsaturated fatty acids), anti-bacterial compounds, minerals, fibers, photo-pigments and vitamins (Lüning 1990, Lobban & Harrison, 1994, Holdt & Kraan 2011). Their biomass is also recognized as a sustainable source for the utilization to biofuels (Wei et al. 2013, Bikker et al. 2016, Fernand et al. 2016) and bioplastics (Rajendran et al. 2012, Gade et al. 2013) amongst other applications, for example biofiltration purposes (Cahill et al. 2010). While cultivation and use of seaweeds is very common in Asia, it is developing in Europe, but it is still at its infancy. The European seaweed industry is dominantly based on the harvesting of wild seaweed stocks (FAO, 2014). This has raised concern regarding an over-harvesting of natural resources and

the deterioration of ecosystems by harvesting-techniques, as documented for some regions in North- and South America (Ugarte & Sharp 2001, Buschmann et al. 2014). Cultivation of seaweed is the only solution to adequately meet the increasing commercial demand. The high productivity of the North Sea, linked to nutrient availability, is promising for the economical (large scale) cultivation of seaweeds (van der Molen et al. 2018). Currently, seaweed is not farmed at large scale in the North Sea and wild harvests are limited to the very northern region of the North Sea area, around the coastlines of Norway (Vea & Ask 2011), Ireland and Scotland (Kenicer et al. 2000). A considerable number of investigations have been conducted to investigate parameters of seaweed production in the North Sea under environmental and laboratory conditions (i.e. Buck & Buchholz 2004, 2005, Sanderson et al. 2012, Tørring & Oddershede-Nielsen 2014). Available environmental and physiological parameters were used to establish models to calculate yields of seaweed production at different locations in the North Sea, for example for *Saccharina latissima* (Linnaeus) C.E. Lane, C. Mayes, Druehl & G.W. Saunders (Broch & Slagstad 2012). The overall productivity of the open North Sea related to seaweed biomass was estimated at approx. 20 tons drymatter per hectare and year without the addition of nutrients (Reith et al. 2005). It had been concluded that the productivity could substantially be increased through the addition of nutrients and/or layered cultivation (Reith et al. 2005). A recent study investigated the potential production and ecological impacts of seaweed farms by including seven experimental farms and one hypothetical farm site in Dutch and United Kingdom coastal waters into a 3-D numerical model of hydrodynamics and biogeochemistry (van der Molen et al. 2018). This model could not detect significant changes in biogeochemistry and plankton dynamics at any of the farm sites averaged over the farming season. Results also showed that seaweed production depended on prevailing nutrient concentrations and light conditions, with higher levels of both resulting in higher production.

When nutrients are added to a cultivation site, a precise dosage is essential for optimal growth and to prevent eutrophication. On the other hand, large scale cultivation of seaweed can lead to spatial nutrient limitations and depletions, which can mitigate, shift, or change composition

of phytoplankton blooms, thus affecting the whole food web of an ecosystem (Burson et al. 2016). The uptake of nutrients by seaweeds is not always a negative or undesired effect. Seaweed farms could act as a last resort/recycling place, before a scarce nutrient as phosphate is diluted into the deep sea. Phosphate is deemed the first compound to limit agricultural production, hence essential to recycle (Ashley et al. 2011). In a similar context, seaweed cultures can conceivably be used for bioremediation purposes and, for example, minimize the impact of nutrients released by fish farms and areas of eutrophication, as well as in land-based bio-filtration facilities (i.e. Neori et al. 2003, 2004, Cahill et al. 2010). In order to assess the efficieny for bioremediation purposes and to avoid unacceptable damage to the ecosystem by large scale operations, it is necessary to understand the nutrient uptake kinetics and (management) dynamics of the seaweed used. This thesis can, based on its ecophysiological data on different species of seaweeds, help in finding the balance between preserving the marine ecosystem and unlocking its potential for sustainable production of food, feed and fuel.

Each seaweed species has its own growth characteristics and internal composition related to nutrient availability. Some species need large amounts of nitrogen and can handle low concentration of phosphorus, while others require larger quantities of phosphorus, and can cope with relatively low nitrogen concentration. Therefore knowledge on the nutrient management of the different seaweed species is essential, whether it concerns mono-cultures, layered cultures, integrated aquaculture systems, or the integration of multiple approaches. In addition, knowledge on the internal storage capacity in respect to growth rates and the time before nutrient limitations cause significant losses of yield are important physiological factors for a sustainable mariculture. The North Sea seaweed species with the most potential for cultivation were identified to be *Ulva lactuca* Linnaeus, *Saccharina latissima* Linnaeus J.V. Lamouroux, *Laminaria digitata* Hudson J.V. Lamouroux, and *Palmaria palmata* Linnaeus F. Weber & D. Mohr (Reith et al. 2005) (Figure 1-4). These 4 seaweeds are the protagonists in this thesis.

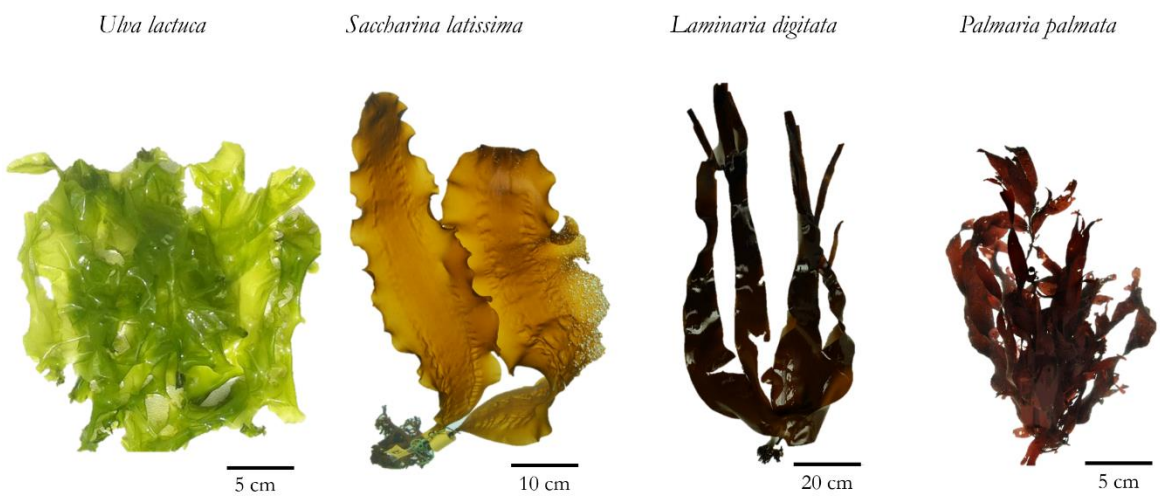

Figure 1-4. The 4 seaweed species under investigation: *Ulva lactuca* (Chlorophyceae), *Saccharina latissima* (with yellow label around the stipe), *Laminaria digitata* (Phaeophyceae) and *Palmaria palmata* (Rhodophyceae).

1.4. Thesis outline

Seaweeds offer interesting options for fundamental research. The North Sea seaweeds identified as the ones with the most potential for cultivation (see above) were the species that I worked with during my PhD at NIOZ (Royal Netherlands Institute for Sea Research). An important infrastructure for seaweed research in Europe is the NIOZ Seaweed Research Centre (https://www.nioz.nl/en/expertise/seaweed-research-centre) consisting of 26 fully insulated, heated/cooled, and aerated cultivation tanks (1600 L each) specially designed for seaweed research. Here, seaweed fundamental scientific work on physiology and ecology can be combined with clear applied options. For example, uptake of nutrients, growth, and their role as crop in a seaweed farm can be studied. The fundamental scientific knowledge gained can be used for sustainable production for food, feed, and chemical extraction for energy in the future, and be a guide how to preserve (coastal) marine ecosystem services.

In my experimental work at the NIOZ Seaweed Research Centre and in the laboratory, I studied the eco-physiology of *U. lactuca*, *S. latissima*, *L. digitata*, and *P. palmata* related to nutrient availability with a focus on long term (i.e. up to several weeks). nutrient uptake kinetics. A

considerable amount of scientific literature is available on uptake kinetics and growth of several seaweed species in relation to dissolved inorganic nitrogen (DIN) (e.g. Thomas & Harrison 1987, Ahn et al. 1998, Naldi & Viaroli 2002, Pérez-Mayorga et al. 2011, Benes & Bracken 2016). Fewer studies refer to uptake kinetics of dissolved inorganic phosphorus (DIP) in seaweeds (e.g. Hurd & Dring 1990, Chopin et al. 1997, Gordillo et al. 2002, Pederson et al. 2010). Often DIN and DIP uptake kinetics were tested independently in short term experiments, in the range of hours (e.g. Runcie et al. 2003, Martínez & Rico 2004, Luo et al. 2012) or provide a momentary insight under field conditions (e.g. Neori et al. 2003, Naldi & Viaroli 2002). Knowledge of long term (weeks) effects on uptake kinetics under DIN- and DIP- replete, limited, or deplete conditions is essential for a proper ecological understanding and a sustainable production and is a key factor in this PhD thesis.

A full factorial design was used to determine DIN and DIP uptake kinetics and management strategies of *U. lactuca*, *S. latissima*, *L. digitata*, and *P. palmata* under laboratory conditions, controlling for temperature, light and hydrodynamics. Prior the experiments the specimen were gently cleaned (potential epiphytes removed and detritus rinsed off) and kept under laboratory conditions for adaptation. They were maintained in filtered, nutrient depleted seawater to ensure nutrient starvation. All experimental conditions were tested on individual specimen in a range of 5 to 7 replicates. During the experiments it was made sure that ample nutrients were available by daily refreshment of the seawater medium, so that constant nutrient levels were pulsed on a daily basis. The removal of nutrients from the seawater medium was followed (chased) in time, assuming the nutrients had been removed by the seaweed (Figure 1-5).

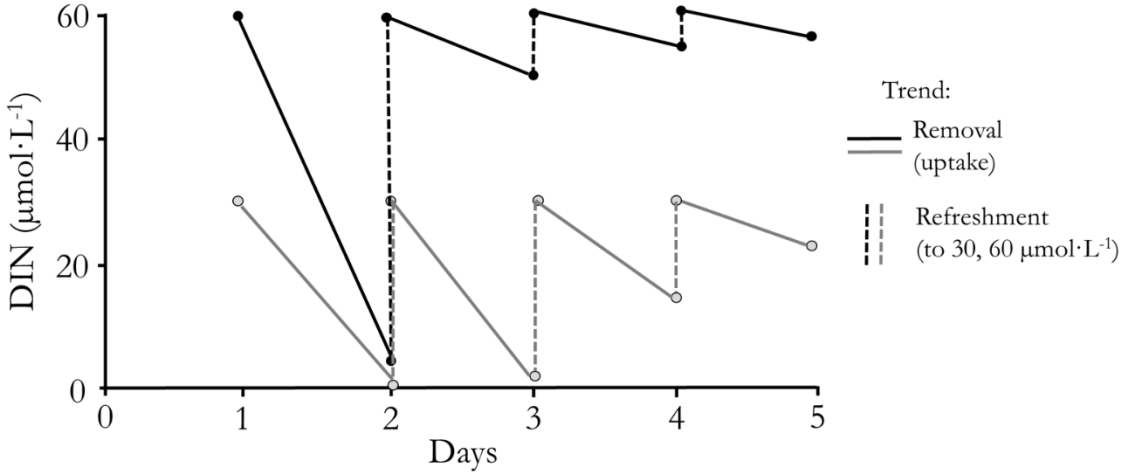

Figure 1-5. Example of daily removal (referred to uptake) of DIN (µmol·L^{-1}) from the seawater medium by nutrient-starved *Ulva lactuca* in a 'pulse-and-chase' approach with daily refreshment of the seawater medium (30 and 60 µmol·L^{-1}) over 5 days.

This 'pulse-and-chase' approach ensured sporophytes to be exposed to nutrient concentrations as intended during the experiment up to several weeks, and as neither random nor large shifts in DIP and DIN concentrations were observed between days during the experimental period, I am confident about the validity of values for DIP and DIN concentrations, respectively uptake rates. By this daily "pulse-and-chase" of nutrient concentration in the seawater medium, surge uptake rates (V_S), maintenance uptake rates (V_M), correlations and ratios of DIN/DIP uptake rates, and internal storage capacity (ISC) for DIN and DIP of the 4 seaweeds were calculated, all standardized for surface area (SA). A standardization of uptake kinetics to SA was chosen, as it enables an intra- and interspecific comparison of the seaweeds over time, while uptake kinetics expressed as a function of dry weight (DW) define the end of the living biomass. The non-destructive method of standardized determination of fresh weight (FW) was not regarded as a useful parameter of biomass, as little variations in the amount of water attached to the living (and growing) seaweed can lead to huge differences in its weight, not only between different samples and over time, but also amongst different experimentators.

It can be envisioned that nutrient limitations/depletion will not only determine growth, but also affect internal composition (e.g. carbohydrate and protein concentration), colour appearance and texture. The combined effects of DIN/DIP availability on growth, photosynthetic efficiency (F_v/F_m), and total dissolvable carbohydrate- and protein concentration were assessed for *P. palmata* and *U. lactuca*. This assessment included the introduction of a novel method to evaluate the total dissolvable protein concentration (nutritional value) in *U. lactuca* by digital imaging of its frond colour. This colorimetric method was integrated into the free smartphone application 'EyeOnUlva' for Android and IOS systems (www.eyeonwater.org/ulva) and enables citizen scientist to participate in ecological studies (Figure 1-6).

Figure 1-6. The smartphone application 'EyeOnUlva' for Android and IOS systems (www.eyeonwater.org/ulva) records the frond colour of the green seaweed *Ulva lactuca* and provides a fast quantification of its total dissolvable protein concentration in % dry weight. The data can be send to a data base, which is part of the CITCLOPS project (www.citclops.eu).

Furthermore, a new approach that allows for standardised methods to study morphological effects on seaweed individuals in response to varying hydrodynamic forces based on the example of *L. digitata*, is proposed. This methodology allows the determination of the total strain deformation (ε) and breaking points by means of applying tensile and compression forces, using

an industrial texture analyser mounted with customized clamps for attaching seaweed samples (Figure 1-7).

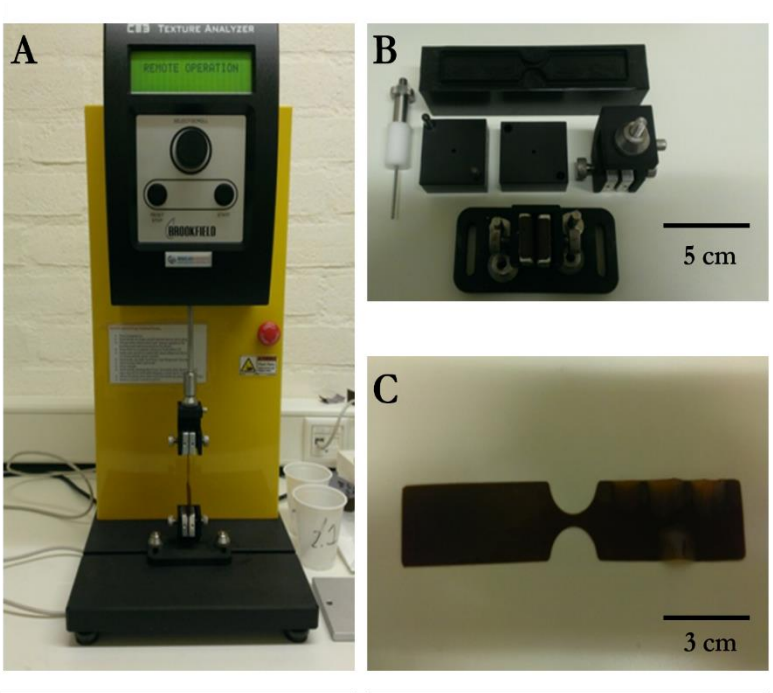

Figure 1-7. A) Front view on fittings of the texture analyser (CT3, Brookfield Engineering, USA) with attached seaweed sample, B) customized clamps, sample attachments and sample stamp for toughness measurements on seaweed (bottom to top) and C) punched out sample of a *Laminaria digitata* frond, ready for toughness analysis.

The studies presented on the 4 native North Sea seaweed species are divided into the following chapters:

Chapter 2 – Lubsch, A. & Timmermans, K.R. 2018. Uptake kinetics and storage capacity of dissolved inorganic phosphorus and corresponding N:P dynamics in *Ulva lactuca* (Chlorophyta). *J. Phycol.* 54: 215-223. DOI: https://doi.org/10.1111/jpy.12612.

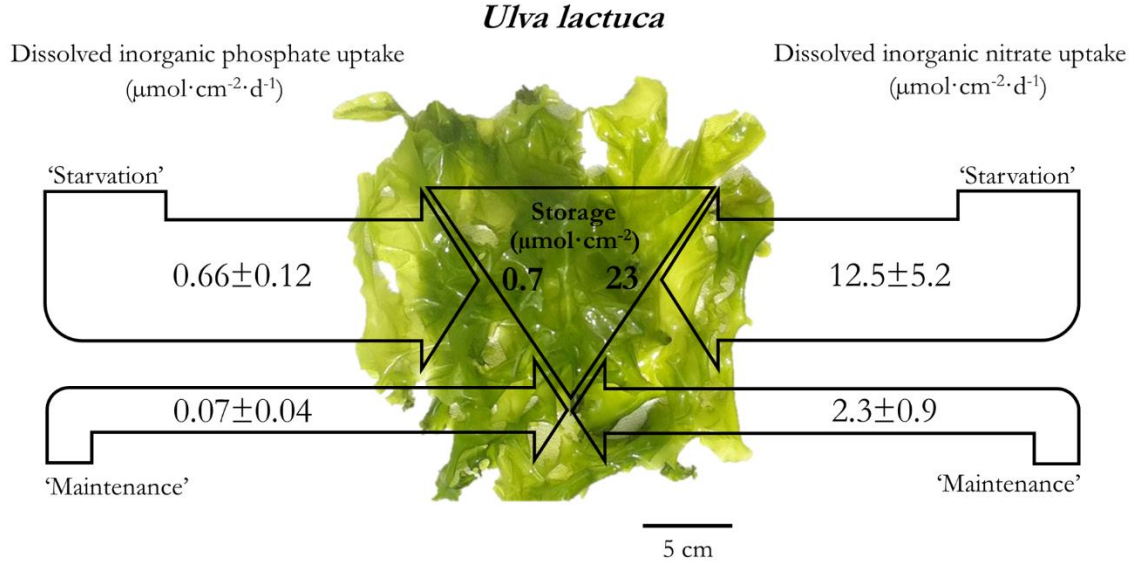

Figure 1-8. Infographics of results on dissolved inorganic phosphate- and dissolved inorganic nitrate uptake dynamics (V_S under starvation, V_M for maintenance, and storage capacity) in *Ulva lactuca* (Chlorophyta).

Chapter 2 is devoted to uptake kinetics and internal storage capacity (ISC) of DIP and corresponding N:P dynamics in *U. lactuca* (Figure 1-8). DIP-uptake kinetics of *U. lactuca* exposed to a range of nominal DIP concentrations (1 – 50 µmol·L^{-1}) and a non-limiting DIN concentration (5000 µmol·L^{-1}) under fully controlled laboratory conditions in a 'pulse-and-chase' assay over 10 days are presented. DIP-uptake kinetics and storage capacity were quantified, as well as N:P-uptake dynamics, and all were standardized for surface area (SA). In order to make comparisons possible with other standardizations, factors for conversion to fresh weight (FW) and dry weight (DW) were presented. The results contribute to the understanding of ecological aspects of nutrient uptake kinetics in *U. lactuca* and quantitatively evaluates its potential for bioremediation and/or biomass production for food, feed and energy. High DIP- and DIN uptake under V_S in saturating

concentrations quickly filled the ISC (within 1 day) and demonstrated the fast response of *U. lactuca* to nutrient pulses, typical for an opportunistic species. After ISC had been filled, uptake rates rapidly declined by approximately 90 % for DIP and 80 % for DIN and reached V_M. In turn, the ISC for DIP and DIN in relation to V_M was depleted after 10 days of external nutrient depletion. This information is indispensable in order to predict the efficiency and sustainability of *U. lactuca* in bio-filtration systems and can help to efficiently clean control effluent streams, as well as monitor productivity. Furthermore, the data allows an estimation of ecological effects on nutrient availability and can contribute to development, modification and preservation of marine ecosystem services at cultivation sites. The conversion factor can assist to assess the total SA from its biomass (FW) and by that can help to select a precise dosage of nutrient additions to (large scale) production sites and/or estimate the efficiency of bio-filtration facilities, as FW is a more practical measure for procedures on a large scale.

Chapter 3 - Lubsch, A., Wernand, M.R., van der Woerd, H.J. & Timmermans, K.R. *In prep*. Using a smartphone app for the estimation of total dissolvable protein concentration in *Ulva lactuca* Linnaeus (Chlorophyceae).

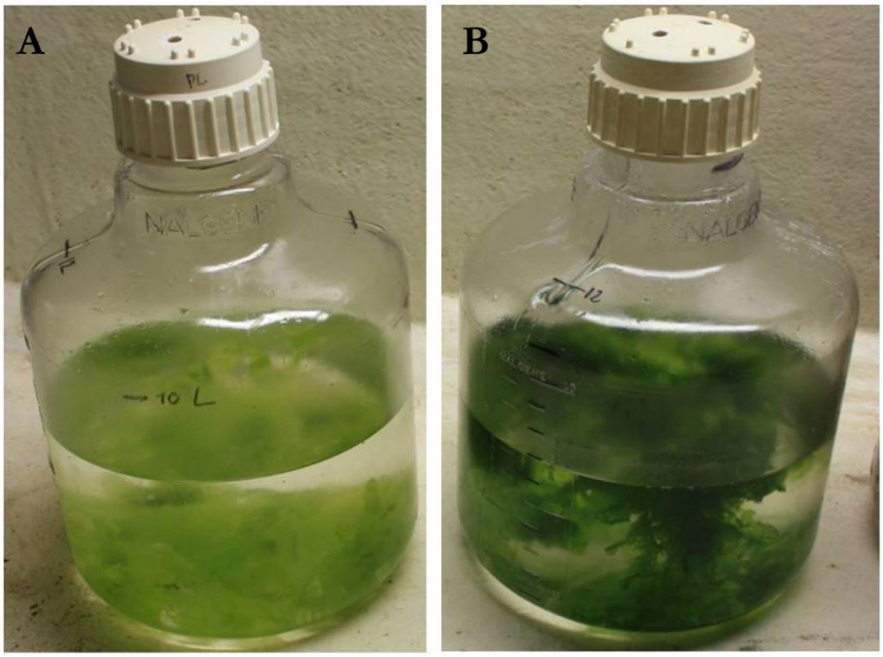

Figure 1-9. Two shades of green. *Ulva lactuca* cultivated under A) nutrient depletion conditions for 2 weeks and B) with addition of non-limiting nutrient concentration for 5 days.

Chapter 3 is dedicated to evaluate the total dissolvable protein content in the seaweed *Ulva lactuca* (Chlorophyta) by digital imaging of its frond colour, applying a combination of spectro-radiometry and colorimetric techniques. In my eco-physiological work with *U. lactuca* (Chapter 2), I observed remarkable (green) colour differences in their fronds (Figure 1-9). These colour differences appeared to be related to their total dissolvable protein concentration. This led to this study, as described in chapter 3, in which we examined the possibility to deploy spectro-radiometry and colorimetric techniques to evaluate the total dissolvable protein concentration in the green seaweed *U. lactuca* based on its frond colour with the clear objective to integrate our results into a smartphone app. The fundamental work and feasibility of a new analytical method to evaluate the nutritional value of a seaweed by colorimetric analysis, on the example of *U. lactuca* is presented.

Furthermore, the sensitivity of *U. lactuca* to nutrient (nitrate, phosphate) availability by means of frond colour is demonstrated. The apparent optical property (frond colour) provided information on the nutritional status of the seaweed, as a correlation between frond colour and the total dissolvable protein concentration was found. The nutritional status of seaweed can give information on the levels of nutrients available in a certain habitat, integrating this over prolonged periods of time (which cannot be monitored with discrete samples taken every now and then). On the other hand, detailed information on nutrient concentrations in the seawater can be used to infer to the nutritional history of a seaweed and hence allow an approximate estimate on its nutritional status in relation to the ISC. This can be very useful for tank cultivations, where effluents are fully controlled.

Based on the data, we developed a smartphone application (EyeOnUlva) for Android and IOS systems, which records the frond colour and provides an inexpensive, reliable, safe and easy-to-use method to give a fast evaluation on the total dissolvable protein concentrations in *U. lactuca*. 'EyeOnUlva' has been tested successfully by a selected group of international university students to verify performance, reliability and ease of use of the application. The 'EyeOnUlva' application not only represents a useful tool to the aquaculture industry to assess the nutritional value of their seaweed crop and determine its feeding quality in a cost-effective way, but also may function as a bio-indicator, giving insight in the nutritional background of a coastal habitat or cultivation site. This makes it also applicable in environmental surveys, including citizen science programs.

Chapter 4 - Lubsch, A. & Timmermans, K.R. (2019) Uptake kinetics and storage capacity of dissolved inorganic phosphorus and corresponding dissolved inorganic nitrate uptake in *Saccharina latissima* and *Laminaria digitata* (Phaeophyceae). *J. Phycol.* DOI: https://doi.org/: 10.1111/jpy.12844.

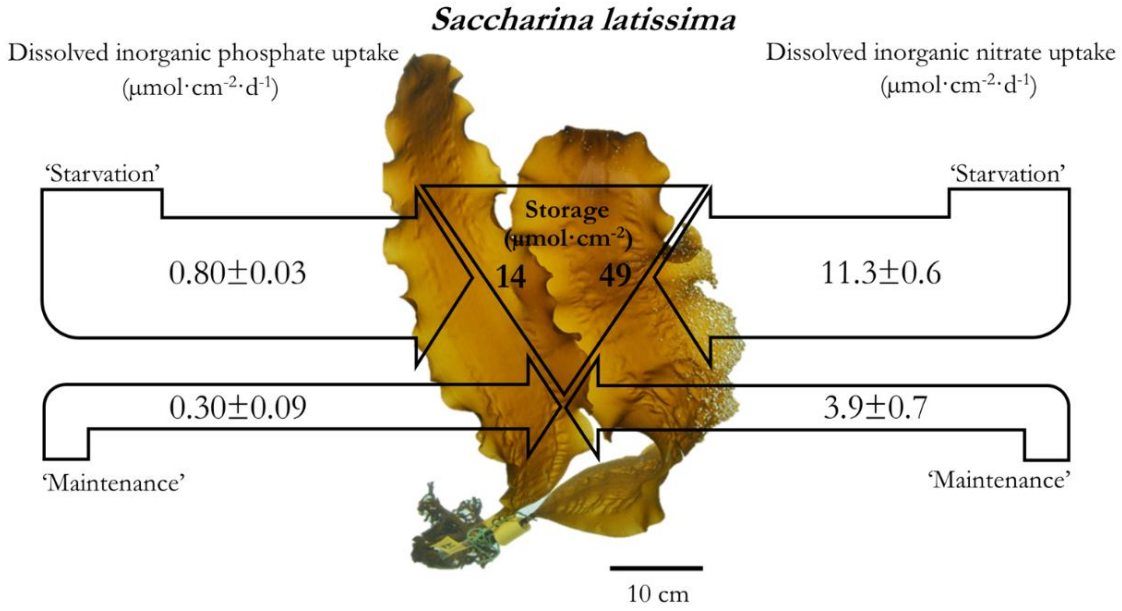

Figure 1-10. Infographics of results on dissolved inorganic phosphate- and dissolved inorganic nitrate uptake dynamics (V_S under starvation, V_M for maintenance, and storage capacity) in *Saccharina latissima* (Phaeophyta).

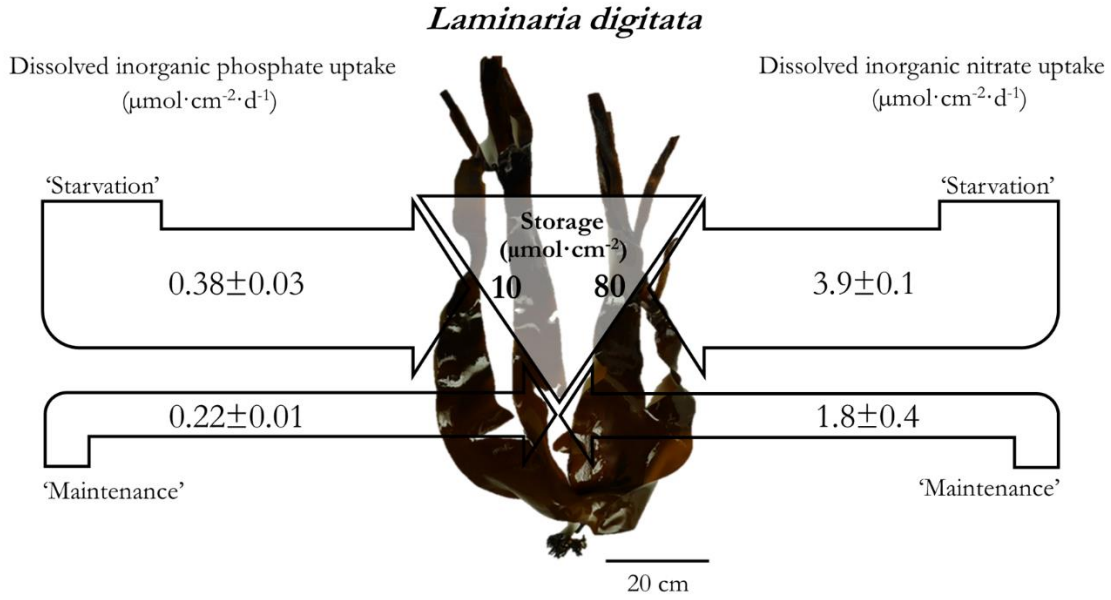

Figure 1-11. Infographics of results on dissolved inorganic phosphate- and dissolved inorganic nitrate uptake dynamics (V_S under starvation, V_M for maintenance, and storage capacity) in *Laminaria digitata* (Phaeophyta).

Chapter 4 is devoted to the uptake kinetics and internal storage capacity of DIP and corresponding DIN uptake in *S. latissima* and *L. digitata* (Figure 1-10 and 1-11). In this study young sporophytes of both species were exposed to a range of nominal DIP concentrations (0 - 6 µmol·L^{-1}) and non-limiting DIN concentration (50 µmol·L^{-1}) under laboratory conditions in a 'pulse-and-chase' approach over 3 weeks. In an additional 'pulse-and-chase' approach, sporophytes of both species were exposed to DIP-depleted, DIN-depleted, DIP and DIN-depleted, as well as DIP and DIN-enriched seawater and the photosynthetic efficiency F_v/F_m as a measure of plant stress was followed over 9 weeks. Based on the data, the DIP and DIN-uptake kinetics, as well as the internal storage capacity of DIP and DIN in *S. latissima* and *L. digitata* were quantified and standardized to SA (Figure 1-10 and 1-11).

The results open opportunities to project impacts of nutrient limitation and shifts in limitation from one element to another, and shed light on possible competitive advantages of *S. latissima* versus *L. digitata* in relation to nutrient availability. Uptake kinetics as a function of SA

allow for a direct comparison of the net nutrient removal from the seawater. *S. latissma* in comparison to *L. digitata* exhibited higher growth rates, as well as a higher V_S and V_M for DIP and DIN and would outcompete *L. digitata* in terms of nutrients. Similarly, *S. latissima* has a larger ISC for DIP than *L. digitata* and can survive longer in limiting DIP conditions. Both species are efficient users of P, as the N:P uptake ratio during V_M indicates. Conclusively, the results support *S. latissima* to be superior competitor for nutrients (as compared to *L. digitata*) and an effective candidate for bioremediation with similar uptake kinetics as *U. lactuca* (Chapter 2). *S. latissima* (Phaeophyceae) typically can be regarded a winter species, while *U. lactuca* (Chlorophyceae) flourishes at relative high temperatures and light intensities. This allows for crop rotation in seagriculture. Analogue to the physiological data on *U. lactuca* (Chapter 2), the information on DIP and DIN uptake kinetics of *S. latissima* and *L. digitata* can help to identify potential locations and modification for commercial cultivation, allows modelling studies to project yields of seaweed biomass at different locations, evaluate the efficiency for bioremediation, estimate ecological effects on nutrient availability, and select nutrient concentration in correspondence to SA and duration of the experimental time related to seaweed research.

Chapter 5 - Lubsch, A. & Timmermans, K.R. 2017. Texture analysis of *Laminaria digitata* (Phaeophyceae) thallus reveals trade-off between tissue tensile strength and toughness along lamina. *Bot. Mar.* 60: 229-237. DOI: https://www.doi.org/10.1515/bot-2016-0075.

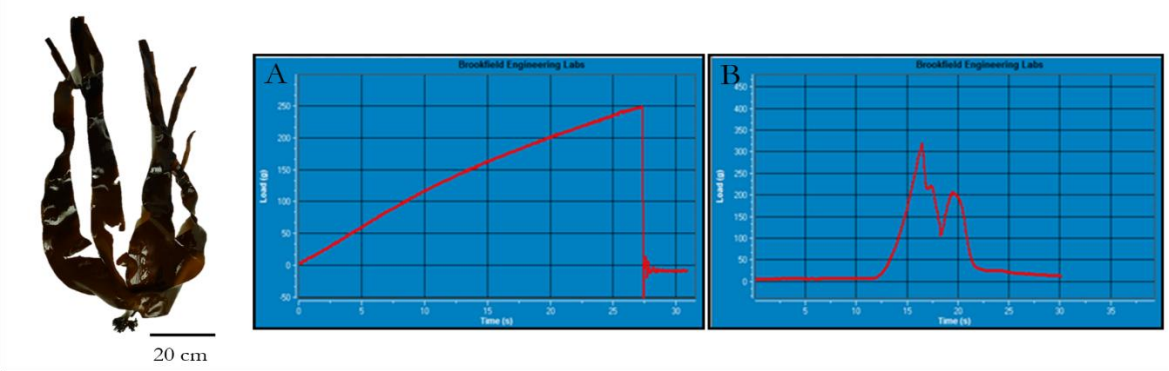

Figure 1-12. Exemplary graphs of (A) ultimate tensile strength (UTS) and (B) ultimate piercing load (UPL) during texture analysis (analyzer: CT3, Brookfield Engineering, USA) of a *Laminaria divitata* frond.

Chapter 5 describes the first standardized data on physical properties in *L. digitata* (Phaeophyceae) thalli by texture analysis. Texture analysis is a method to test physical properties of a material by compression and tension (Figure 1-12), which are important parameters for the selection and survival of stationary organisms, exposed to steady turbulent flow and its varying drag-forces. Breaking points by means of tensile and compression forces, as well as total elongation and thickness of the tissue were evaluated and discussed in an ecological, physiological and morphological context.

Although the method described in this chapter was not aimed for marketing reasons in the first place, it can be transferred to the perception and acceptance of consumers, and also to marine herbivores that graze on seaweeds. The phenotypic plasticity not only serves as a mechanical defence against herbivores and other consumers as tissue toughness is the first physical barrier to overcome (Mauricio 1998), but it is also a key factor to endure mechanical stress caused by hydrodynamic forces (e.g. Koehl 1984, Denny 1994, Harder et al. 2006). Furthermore, knowledge

on the phenotypic plasticity and physical trade interaction, also on cellular level, is essential to understand morphological, ecological and physiological responses of seaweed and seaweed communities to changing environmental terms (Berg & Ellers 2010, Young et al. 2011, Coumou & Rahmsdorf 2012). *Laminaria digitata* is known to be a leathery and tough seaweed and is among the largest seaweeds that thrive in the wave-dominated intertidal zone of the NE Atlantic, including the North Sea area. The occurrence of *L. digitata* in wave-exposed areas can stimulate the settlement of other seaweeds and organisms, and by that opens opportunities to enhance diversity in its habitat. For example, the spatial distribution of epiphytic *P. palmata* population attached to the stipes of *Laminaria* populations (Whittick 1983). A destruction and reduction of 'habitat-engineers' like *L. digitata* for example by an increase of extreme weather conditions, such as intense hydrodynamic forces caused by rough storms, would result in the loss of associated marine flora and fauna. In the North Sea, for example, rough weather conditions can prevail during grow season in the winter months (Grabemann & Weisse 2008) and in cultivation an adequate selection of seaweeds with regard to phenotypic plasticity seems useful, especially in future offshore procedures. In addition, data on physical properties allow to develop and modify mechanical structures for manipulation in seaweed cultivation. Information on physical properties can be very relevant for the design of seaweed supporting structures. In a seaweed cultivation set-up, optimisation should be achieved in ensuring optimal nutrient availability, also in large cultivation farms in combination with structural support elements. When, for example, seaweed is cultivated for carbohydrates, a flexible cultivation set-up should allow for multiple hydrodynamic forcing on a flexible seaweed, in order for the seaweed to invest in structural elements of the cell wall. *Laminaria digitata* and other brown seaweeds are known for their high content in alginates (e.g. Kloareg & Quatrano 1988, Fertah et al. 2017). Alginates, a family of complex polysaccharides, occupy major sectors of global commerce and are widely used in various fields of industry, for example, food and feed, paper, textiles, cosmetics, and pharmaceuticals (McLachlan 1985, Pérez et al. 1992).

The data on the textural properties of seaweed, as gained by the methodology presented, can help to develop adequate seaweed supporting structures for cultivation, as well as help to select and adjust adequate pre-treatment to reduce size of raw material prior bio-refining processes in an energy- and cost efficient way (Zhu and Pan 2010). Many seaweeds have a tough and strong physical structure, including high contents of cellulose, hemicellulose, and lignin (e.g. Martone et al. 2009) that make them very defiant to microbial destruction, similar to woody biomass (Zhu and Pan 2010). An adequate size reduction (in turn increase of SA) of seaweed biomass is an important aspect for pre-treatment in bio-refinery activities.

In this study, results on *L. digitata* showed a positive toughness gradient of 75 % from young to old tissue by means of tensile strength. Reciprocal responses to compression and tension along the lamina and along the gradient from old to newly formed biomass indicated a twined structural alignment to optimise constituent tissue toughness and flexibility. Similar principles of a twined alignment can be found in e.g. historical manufacturing processes of ropes, but also in modern nanotechnology. This experimental approach similarly allows for standardised methods of inferring the effects on nutrient availability and varying hydrodynamic forces on seaweed individuals.

Chapter 6 – Lubsch, A. & Timmermans, K.R. *in revision.* Dissolved inorganic phosphate uptake and corresponding dissolved inorganic nitrate uptake in the seaweed *Palmaria palmata* (Rhodophyceae): ecological and physiological aspects of nutrient availability.

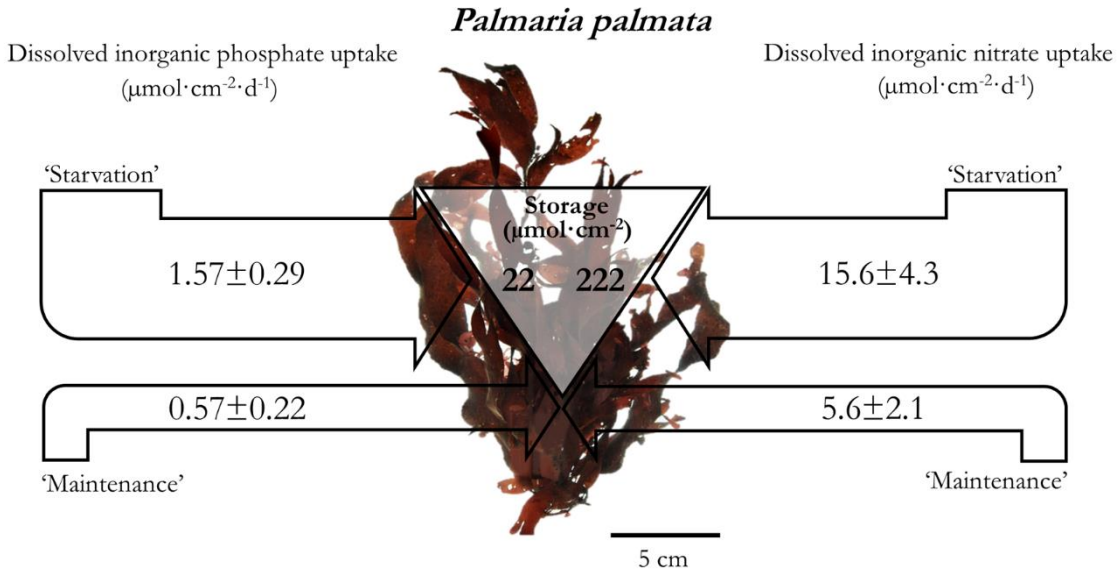

Figure 1-13. Infographics of results on dissolved inorganic phosphate- and dissolved inorganic nitrate uptake dynamics (V_S under starvation, V_M for maintenance, and storage capacity) in *Palmaria palmata* (Rhodophyta).

Chapter 6 gives insight into the DIP and DIN uptake dynamics, as well as internal storage capacities of DIP and DIN in the red seaweed *P. palmata* (Figure 1-13). Young sporophytes were exposed to a range of nominal DIP concentrations (0 - 6 µmol·L^{-1}) and non-limiting DIN concentration (50 µmol·L^{-1}) in a 'pulse-and-chase' approach over 20 days to quantify DIP and DIN uptake kinetics, all described as a function of SA for comparability. The photosynthetic efficiency F_v/F_m was followed for an additional 2 weeks, and based on the response in F_v/F_m to nutritional stress, the internal storage capacity for DIP and DIN was estimated. Finally, the total

dissolved protein- and total dissolved carbohydrate concentration in the sporophytes, exposed to different DIP concentrations were determined after 5 weeks exposure.

The results add to the physiological and ecological understanding of the red seaweed *P. palmata* and opens further insight into ecological aspects of nutrient availability and interspecific competition. In addition, ecological effects on nutrient availability and shifts in limitations from one element to another can be estimated. The observed nutrient management strategies by *P. palmata* differed substantially from those of *U. lactuca* (Chapter 2), *S. latissima* and *L. digitata* (Chapter 4). An elevated DIN uptake in *P. palmata* was coupled to the availability of DIP, which consequently was mirrored by the total dissolved protein concentration, thus the nutritional value of the seaweed. Furthermore, the study showed an oscillating or rhythmic DIP and DIN uptake in weekly intervals, when sporophytes were exposed to saturating nutrient concentration. This rhythmic DIP and DIN uptake management can be related to a niche separation, which transferred to the interspecific competition for nutrients, can secure the coexistence of different seaweed populations competing for the same resources and by that species diversity in an ecosystem can be enhanced. To our knowledge, this nutrient uptake strategy was only been described for microalgae so far. Our findings on uptake dynamics and growth rates support *P. palmata* to be a potent species for bioremediation purposes in layered poly-cultures. Although there is a strong dependency on the availability of DIP for an efficient DIN uptake, the oscillating uptake of *P. palmata* can be used to complement the DIP and DIN removal from the seawater by other seaweeds in bioremediation activities, for example, when integrated into *S. latissima* cultivation in close proximity to fish farms, where high concentrations of N and P compounds can be found. DIP uptake rates in *P. palmata* under V_M outcompete those of the brown seaweed *S. latissima*, while DIN uptake rates are comparable in both species (Chapter 4). Thus our results on uptake kinetics in *P. palmata* not only allow an optimal modification and manipulation for a viable mariculture, but also help to evaluate the efficiency for bioremediation. Analogue to the physiological data on *U. lactuca* (Chapter 2), *S. latissima*, and *L. digitata* (Chapter 4) and related to fundamental seaweed

research, our data on uptake kinetics can serve as a measure to select nutrient concentration in correspondence to SA and duration of the experimental time.

Chapter 7 is devoted to practical implications of the main findings in this thesis. Examples on the implementation of results on DIP and DIN uptake kinetics and strategies in *U. lactuca*, *S. latissima*, *L. digitata*, and *P. palmata* into seaweed related operations, such as offshore cultivation, integrated multi-trophic aquaculture (IMTA), tank cultivation, and bio-filtration are given in the form of a 'manual for nutrient uptake kinetics in seaweed cultivation'. The examples given in this chapter include eco-physiological data on DIP and DIN uptake kinetics, uptake ratios, uptake strategy, as well as DIP and DIN management related to ISC.

Chapter 8 gives a synthesis of general findings and the innovative aspects/highlights of this thesis and includes an overview of the main results (Table 8-1). Conclusions from results and experimental work are provided and an outlook for future research on seaweed is proposed.

Chapter 2

Uptake kinetics and storage capacity of dissolved inorganic phosphorus and corresponding N:P dynamics in *Ulva lactuca* (Chlorophyta)

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2.1 Abstract

Dissolved inorganic phosphorus (DIP) is an essential macronutrient for maintaining metabolism and growth in autotrophs. Little is known about DIP-uptake kinetics and internal P-storage capacity in seaweeds, such as *Ulva lactuca* (Chlorophyta). *U. lactuca* is a promising candidate for biofiltration purposes and mass commercial cultivation. We exposed *U. lactuca* to a wide range of DIP concentrations (1 – 50 μmol·L^{-1}) and a non-limiting concentration of dissolved inorganic nitrogen (DIN) (5000 μmol·L^{-1}) under fully controlled laboratory conditions in a 'pulse-and-chase' assay over 10 days. Uptake kinetics were standardized per surface area of *U. lactuca* fronds. Two phases of responses to DIP-pulses were measured: (1) a surge uptake (V_S) of 0.67±0.10

μmol·cm^2·d^{-1} and (2) a steady state uptake (V_M) of 0.07±0.03 μmol·cm^2·d^{-1}. Mean internal storage capacity (ISC_P) of 0.73±0.13 μmol·cm^2 was calculated for DIP. DIP uptake did not affect DIN uptake. Parameters of DIN uptake were also calculated: V_S=12.54±5.22 μmol·cm^2·d^{-1}, V_M=2.26±0.86 μmol·cm^2·d^{-1}, and ISC_N=22.90±6.99 μmol·cm^2. Combining ISC and V_M values of P and N, nutrient storage capacity of *U. lactuca* was estimated to be sufficient for approximately 10 days. Both P and N storage capacities were filled within two days when exposed to saturating nutrient concentrations, and uptake rates declined thereafter at 90 % for DIP and at 80 % for DIN. Our results contribute to understanding the ecological aspects of nutrient uptake kinetics in *U. lactuca* and quantitatively evaluates its potential for bioremediation and/or biomass production for food, feed and energy.

2.2 Introduction

Seaweeds are important primary producers. An essential macronutrient for maintaining the metabolism and growth of these autotrophs is dissolved inorganic phosphorus (DIP), along with nitrogen (N). Understanding the demand and management strategy for nutrients by seaweeds is economically and ecologically of central importance as it allows for optimal manipulation in cultivation and biofiltration facilities, as well as it opens opportunities to forecast ecological impacts of nutrient limitation and shifts in limitation from one element to another, which can significantly affect the internal composition, physiology and growth of seaweeds (Pederson & Borum 1996, Gevaert et al. 2001).

Nutrient uptake by seaweed can be split into three distinct phases, referred to as surge uptake (V_S), metabolic or internally controlled uptake (V_M), and externally controlled uptake (V_e) (Conway et al. 1976, Harrison et al. 1989). V_S refers to the filling of internal nutrient pools, uncoupled from growth (Conway et al. 1976), and has often been described for nutrient-starved seaweeds (e.g. Fujita 1985, Harrison et al. 1989, Dy & Yap 2001). The uptake rates gradually decrease as internal nutrient pools in cytoplasm and vacuoles are filled (Rosenberg et al. 1984,

Fujita 1985). When internal nutrient concentrations are constant and relative uptake rates of nutrients remain relatively stable over time, $V_{M,}$ which is considered equal to the rate of assimilation, is attained (Taylor & Rees 1999, Barr et al. 2004). The previously filled nutrient pools can be utilized at times of low external nutrient availability (Probyn & Chapman 1982, Pederson & Borum 1996).

Ulva lactuca (Linnaeus), a seaweed in the division Chlorophyta, is found worldwide and is prolifically abundant where nutrients are readily available (Morand & Merceron 2005). *Ulva lactuca* has been identified as a promising species in water treatment facilities (biofilters) and in integrated multi-trophic aquaculture (IMTA) systems (e.g. Cohen & Neori 1991, Neori et al. 2003). *U. lactuca* is also recognized as a promising species for commercial mass cultivation and subsequent production of food, animal feed and fertilizer (Critchley & Ohno 1998, Sahoo 2000, Thangaraju 2008, Holdt & Kraan 2011). Only a few studies have examined DIP-uptake kinetics and internal DIP-storage capacity in seaweeds in general (e.g. Gordon et al. 1981, Chopin et al. 1997, Gordillo et al. 2002, Pederson et al. 2010) and in *U. lactuca*, in particular (Runcie et al. 2004, Tsagkamilis et al. 2010). The majority of studies related to the efficiency of N and P removal from seawater by *U. lactuca* have been conducted under field conditions (Neori et al. 1991, Neori et al. 2003, Naldi & Viaroli 2002). For example, Tsagkamilis et al. (2010) indicated finding an optimal combination of biomass and water flow rates for satisfactory nutrient uptake by *U. lactuca*, by measuring DIP removal from the effluent in a small-scale water treatment facility. Quantification of DIP uptake kinetics over time, however, and the saturating storage capacity of DIP in *U. lactuca* has not yet been studied. In addition, uptake kinetics are usually expressed as functions of either fresh weight (FW), dry weight (DW) or surface area to volume (SA:Vol), which makes it difficult to compare data accurately without conversion.

In this study, we present the DIP-uptake kinetics of *U. lactuca* exposed to a range of nominal PO_4^{3-} concentrations (1 – 50 $\mu mol \cdot L^{-1}$). This range of concentrations is equivalent to exposing *U. lactuca* to phosphate concentrations of 0.02 – 0.67 $\mu mol \cdot cm^{-2}$, which is within the range

of natural concentrations. The experiments were performed under laboratory conditions, controlling for temperature, light and hydrodynamics in a "pulse-and-chase" (i.e. add a pulse of nutrients and follow their removal from the water over time) approach over 10 days. DIP-uptake kinetics and storage capacity were quantified, as well as N:P-uptake dynamics, and all were standardized for SA. In order to make comparisons possible with other standardizations, we calculated factors for conversion to fresh weight (FW) and dry weight (DW).

2.3 Material and methods

All experiments and analyses were conducted at the Royal Netherlands Institute for Sea Research (NIOZ), Texel, The Netherlands. Clean and healthy fronds of *U. lactuca* (after Stegenga and Mol 1983), originally collected from the coastline of the island of Texel in the summer of 2013, were obtained from the NIOZ Seaweed Centre (www.nioz.nl/seaweedcentre) cultivation tanks in September of 2014 and transferred to a temperature-controlled (12.0±0.6 °C) room for a 10-day adaptation phase under fully controlled laboratory conditions in nutrient-depleted seawater (PO_4^{3-} = 0.008 μmol·L^{-1}, NH_4^+ = 0.022 μmol·$L^{-1}$and NO_3^- = 0.003 μmol·L^{-1}). This ensured that the *U. lactuca* were nutrient starved after 10 days (after Fujita et al. 1985).

Following the adaptation/starvation phase, *U. lactuca* fronds of comparable sizes (76.4±11.5 cm^2) were individually transferred into 200 ml glass flasks filled with 100 ml seawater medium and enriched with a range of nominal PO_4^{3-} concentrations (1 – 50 μmol·L^{-1} added) with three replicates for each of the PO_4^{3-} concentrations. The relation between nominal PO_4^{3-} concentration of the seawater medium and comparable SA of *U. lactuca* resulted in a mean DIP availability ranging from 0.02±0.01 to 0.67±0.12 μmol·cm^{-2}·d^{-1}, resembling a concentration range within the scope of natural phosphate concentration fluxes. The seawater medium was refreshed ("pulsed") to its intended nominal concentration on a daily basis, and samples for dissolved nutrient analysis were taken ("chased"). Each day, after the seawater medium had been refreshed, all flasks were randomly distributed to minimize differences in light availability on a rotating table

providing moderate water movement at a speed of 100 rpm. A constant water movement was maintained for optimal mixing and, hence, availability of nutrients by decreasing diffusion boundary layers between tissue and medium (e.g. Gonen et al. 1995, Hurd 2000), assuming that uptake rates become limited by factors such as enzyme activity (Wheeler et al. 1988). Two tubular fluorescent lamps (OSRAM L18 Watt 965, Deluxe cool daylight), attached 50 cm above the flasks, provided a PAR light intensity of 80± 8 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (light meter ULM- 500, Walz, Germany) inside the glass flasks. A light/dark period of 16/8 h was maintained throughout the experiments.

Seawater medium

As a base for the seawater medium, we used filtered (cellulose acetate filter 0.2 µm, Sartorius, Germany) nutrient-poor seawater from the North Atlantic Ocean (salinity 34.5) with low phosphate (PO_4^{3-}; 0.008 $\mu mol \cdot L^{-1}$), ammonium (NH_4^+; 0.022 $\mu mol \cdot L^{-1}$) and nitrate (NO_3^-; 0.003 $\mu mol \cdot L^{-1}$) concentrations. After pasteurization of the seawater (80 °C for 2 h), the salinity was adjusted to 29.5, as measured at the NIOZ seaweed centre and around the island of Texel, by mixing with ultrapure water (Milli-Q, Merck KGaA, Massachusetts, USA), followed by adding mono-ammonium-dihydrogen-phosphate ($(NH_4)H_2PO_4$) and potassium nitrate (KNO_3) as sources for PO_4^{3-}, NH_4^+ and NO_3^- until reaching the desired nominal concentrations (treatments) of 1.0, 1.5, 2.5, 4.0, 7.0, 13.0, 25.0 and 50.0 $\mu mol \cdot L^{-1}$ of PO_4^{3-} and NH_4^+. The NO_3^- concentration was set to 5000 $\mu mol \cdot L^{-1}$ (Table 2-1). The pH of the medium, measured using a pH-Meter (GHM-3511, Greisinger, Germany), was 8.1±0.1 (n=8) after pasteurization and adding nutrients.

Table 2-1. Daily 'pulsed' DIP and DIN concentration (μmol·L^{-1}) to *Ulva lactuca* in a 10-day 'pulse-and-chase' experiment.

Treatment	Phosphate	Nitrate	Ammonium
A	1.0	5000	1.0
B	1.5	5000	1.5
C	2.5	5000	2.5
D	4.0	5000	4.0
E	7.0	5000	7.0
F	13.0	5000	13.0
G	25.0	5000	25.0
H	50.0	5000	50.0

in μmol·L^{-1}

Nutrient analysis

Nutrients (DIP, DIN=nitrate and ammonium) were measured with colorimetric analysis using a Technicon TRAACS 800 auto-analyzer (Seal Analytical, Germany) in the NIOZ Texel nutrient laboratory. DIP was measured as ortho-phosphate (PO_4^{3-}) at 880 nm after the formation of molybdophosphate complexes (Murphy & Riley, 1962). DIN (nitrate and nitrite) was calculated after nitrate reduction to nitrite through a copperized cadmium coil and measured at 550 nm after complexation with sulphanylamide and naphtylethylenediamine (Grasshoff et al. 1983). Ammonium (NH_4^+) was measured at 630 nm after the formation of an indophenol blue complex with phenol and sodium hypochlorite at pH 10.5. Citrate was used as a buffer and complexant for

calcium and magnesium at this pH (Koroleff 1969 and optimized by Helder & de Vries 1979). Precision for all the measured channels within the automated nutrient analyzer was better than 0.25% (personal communication K. Bakker, NIOZ).

Nutrient uptake kinetics

Nutrient uptake is referred to as the removal of dissolved inorganic phosphate (DIP), nitrate and nitrite (DIN), and ammonium from the medium by *U. lactuca*. Daily uptake rates (V) were derived from changes in the nutrient concentrations of the seawater medium during each day, normalized for SA (cm^2) and time (d), and calculated using the following equation:

$V = (T_1 - T_2) \times SA^{-1} \times t^{-1}$,

with T_1 as the initial nutrient concentration, T_2 as the nutrient concentration before water exchange after 24 h, SA as surface area (cm^2) and t as the incubation time (hours).

Two different uptake rates over time were categorized: surge uptake (V_S, S for surge) after starvation and maintenance uptake with filled nutrient pools (V_M, M for maintenance). The intervals over which Vs and V_M were calculated are indicated in Figure 2-1. V_S was calculated from uptake rates in a non-limiting nutrient concentration using the following equation:

$V_S = (V_2 - V_1) \times (d_2 - d_1)^{-1} = \Delta V \times \Delta d^{-1}$,

where V_1 and V_2 are daily uptake rates on days before a significant decline in uptake rates occurs and no significant variations in nutrient uptake follow. The difference operator between the two days is represented by d_1 and d_2.

Internal storage capacity (ISC) is the maximum filling capacity of internal nutrient pools, which was calculated using the following equation:

$ISC_{N,P} = \Sigma(i \epsilon V_S) - n \times V_M$,

where i represents the daily nutrient uptake from initial exposure and is an element of V_S, n accounts for the number of days from initial exposure to when V_S significantly declined and V_M is the daily uptake when nutrient pools are full. A saturation of these pools is indicated by a significant decline in uptake rates (Figure 2-1).

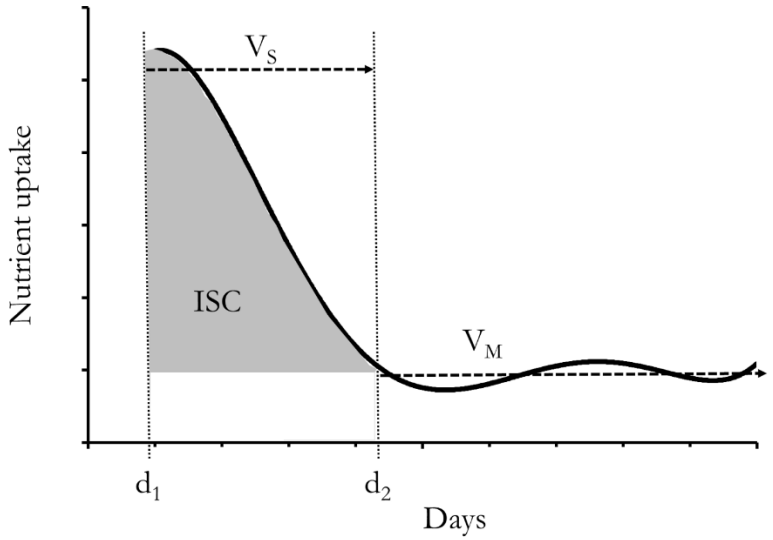

Figure 2-1. Example graph of nutrient uptake over time (days) illustrated with surge uptake (V_S), maintenance uptake (V_M), internal storage capacity (ISC), and d_1 and d_2 as difference operator between days, after a significant decrease in nutrient uptake occurs.

Surface area analysis

Ulva lactuca fronds were spread flat on a white background and covered with a transparent Plexiglas sheet to avoid folding and wrinkling of the frond. A ruler was placed next to the Plexiglas for scale comparison. Photographs (Panasonic Lumix DMC-FT5) were taken on days 1, 3, 5, 7 and 10, enabling an analysis of surface area (SA) by using the open source software ImageJ (ImageJ, U. S. National Institutes of Health, Maryland, USA). For analysis of only pigmented areas in a frond and to exclude potential non-pigmented areas and holes using ImageJ, the scanned colored photograph was converted into grayscale (type 8-bit) and further processed into a binary image before 'particles' (pixels) of the pigmented SA could be analyzed. The software's automated threshold displayed the pigmented SA as dark areas within the grayscale. To analyze the SA, including accidentlly overlapping tissue (darker), the threshold routine was set to manual mode, which allowed for adjustment of the contrast according to the level of overlapping portions of an individual for a refined analysis. The obtained SA represents one side of the two-cell thick lamina

of *U. lactuca*. Differences in SA over time were indicated as growth. Relative growth rates (μ) were calculated according to Kain (1987) using the following equation:

$\mu = (\ln SA_1 - \ln SA_2) \times t^{-1}$,

where SA_1 represents the initial surface area, and SA_2 represents the final surface area after incubation time t.

Relation of SA to fresh weight (FW) and dry weight (DW)

In order to make comparisons possible with our uptake kinetics standardized for SA, conversions to fresh weight (FW) and dry weight (DW) were made. Sixty individuals of *U. lactuca* were centrifuged in a top-loading laundry spinner (BOSCH, 2800 $U \cdot min^{-1}$, 350 W) for 1 minute to dispose of excess water and measured for FW. After this, photographs were taken for SA analysis. Subsequently, to determine DW, the same individuals were quickly rinsed in MilliQ™ to prevent salt residue from forming on the samples after the drying process, and dried for 72 h at 60°C. Both FW and DW were determined using a Mettler Toledo balance (accuracy: 0.01g).

Statistics

All data were tested for normality with the Kolmogorov-Smirnoff test (KS test) for cumulative probability distribution. A two-sided ANOVA was performed to test whether growth rates and nutrient uptake rates varied significantly within and between different nutrient concentrations over time.

2.4 Results

Growth

The mean initial surface area of *U. lactuca* (n=24) in all experimental treatments was 76.4±11.5 cm^2 (SA±SD) and increased to a mean SA of 84.2±14.9 cm^2 after 10 days, which represents significant growth (ANOVA, *df*=23, F=6.20, $p \leq 0.001$). Mean growth between days 1 and 3 was moderate (4.4 %) and gradually decreased to very low (0.6 %) between days 7 and 10 (Figure 2-2). No significant differences in growth between the different DIP treatments were observed (ANOVA, *df*=46, F=4.12, *p*=0.087).

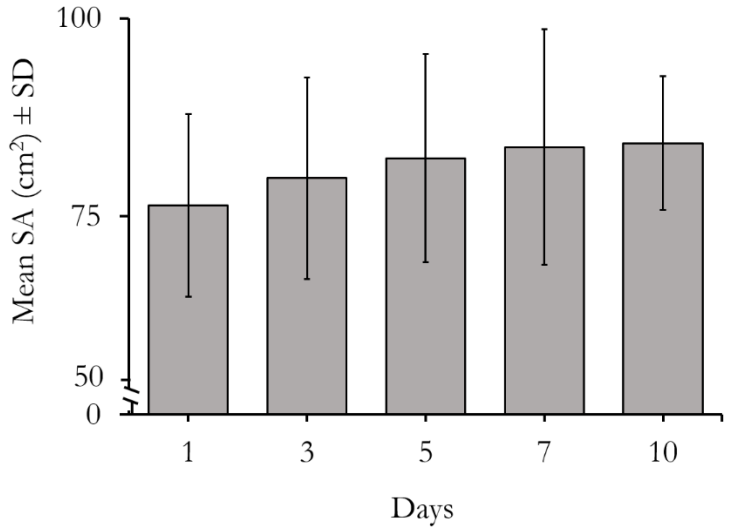

Figure 2-2. Mean surface area (SA) ± SD (n=24) of *Ulva lactuca* on day 1, 3, 5, 7, and 10 of all treatments. No significant differences in growth between treatments with different DIP concentrations were found (ANOVA, df=23, F=1.67, p=0.113).

Relation of Surface Area to FW and to DW

In order to facilitate conversion of the values determined in our study to other standardizations, for example FW or DW, the SA to FW and to DW relations were determined experimentally for *U. lactuca*. Sixty individuals of *U. lactuca* with SA ranging from 5 to 660 cm^2 were analyzed for FW and DW. SA was highly correlated to both FW (*R*=0.991) and DW (*R*=0.988), and showed linearly increasing trends: for FW, y = 0.013x; for DW, y = 0.0026x (Figure 2-3). This

implies, for example, that an *Ulva* frond of 100 cm^2 would have a FW of 1.30 g and a DW of 0.26 g. DW was 20 % of corresponding FW.

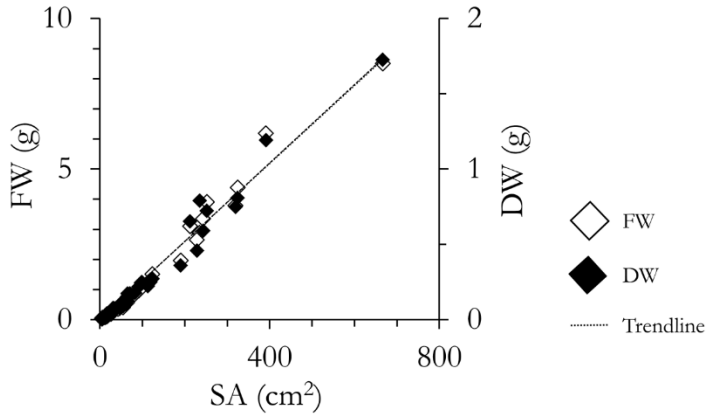

Figure 2-3. Relation of freshweight (FW), dryweight (DW) and surface area (SA) of *Ulva lactuca* (n=60). Trendlines (FW: y = 0.013x, R^2 = 0.978; DW: y = 0.0026x, R^2 = 0.974) are illustrated.

Nutrient uptake kinetics

DIP uptake

When exposed to DIP concentrations of <7 μmol·L^{-1}, *U. lactuca* depleted all the DIP within 24 h, which was faster than the DIP refreshment rate of the medium and indicates non-saturating DIP concentrations (Figure 2-4). When exposed to concentrations >7 μmol·L^{-1} (13, 25 and 50 μmol·L^{-1}), DIP uptake was initially equal to available DIP but eventually decreased to become lower than DIP availability, indicating saturating concentrations. There was a strong correlation between residual DIP concentration and time of exposure (R=0.84). This time lag before a significant reduction in uptake was longer for lower concentrations of DIP availability, occurring on day 5 for 13 μmol·L^{-1}, day 3 for 25 μmol·L^{-1} and day 2 for 50 μmol·L^{-1} (Figure 2-4). DIP uptake at concentrations of 13 and 25 μmol·L^{-1} converged after day 4. For the DIP availability level of 50 μmol·L^{-1}, however, uptake increased again between days 5 and 7 (Figure 2-4) before significantly decreasing between days 7 and 9 (Table 2-2). After day 9, DIP uptake rates at 50 μmol·L^{-1} were

similar to those that had been reached by the 13 and 25 μmol·L^{-1} treatments after day 4 (Figure 2-4). The maximum DIP surge uptake rate for *U. lactuca* was calculated to be 0.7±0.1 μmol·cm^{-2}·d^{-1} (average ± SD, n=3) in the 50 μmol·L^{-1} treatment on day 1. The DIP maintenance uptake rate with filled storage, V_M of DIP, was 0.07±0.03 μmol·cm^{-2}·d^{-1}.

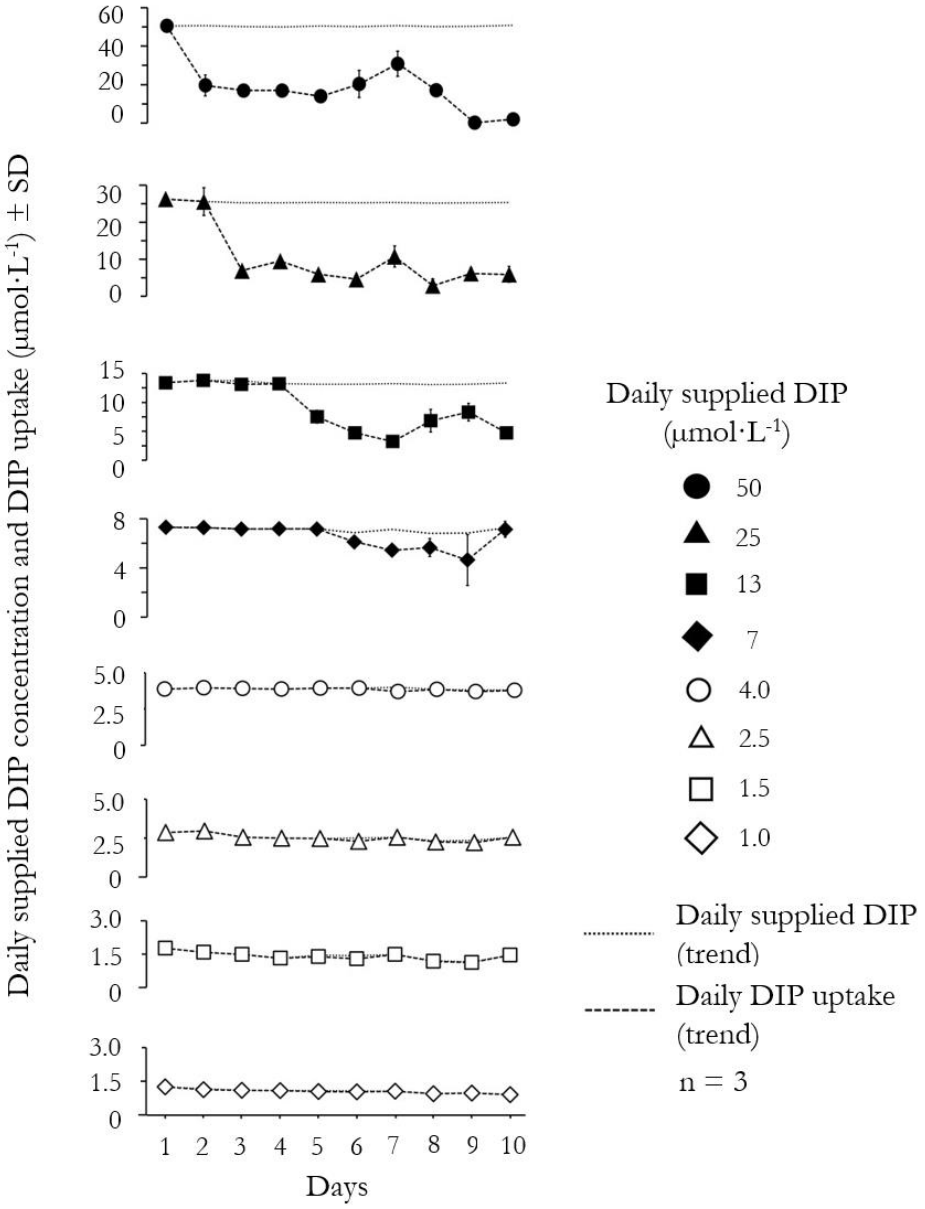

Figure 2-4. Mean DIP uptake (μmol·L^{-1}) ± SD (n=3) by *Ulva lactuca* in treatments with not-saturating (<7 μmol·L^{-1}) and saturating DIP concentrations (>7 μmol·L^{-1}) and daily supplied (pulsed) DIP.

DIN uptake

Similar to DIP uptake, the variations in DIN uptake were strongly correlated with time of exposure (R=0.987) and highly significant over time (ANOVA, *df*=9, F=44.59, p≤0.001), but not between treatments with varying DIP and NH_4^+ concentrations (ANOVA, *df*=23 F=0.57 p=0.944). DIN uptake showed no correlation with DIP uptake (R=0.223) or NH_4^+ availability (R=-0.027). Mean DIN surge uptake was 12.5±5.2 μmol·cm^{-2}·d^{-1} (Figure 2-5). This surge uptake was followed by a highly significant decrease of DIN uptake on days 2 and 3, after which uptake continued without significant differences between time steps (Table 2-2). Mean initial DIN uptake rates with empty DIN-storage (V_S) dropped by 80.7 % within the first 4 days, indicating DIN-storage had been filled and uptake rates only served to maintain metabolism (V_M). The $V_{M\,(DIN)}$ was calculated to be 2.3±0.9 μmol·cm^{-2}·d^{-1}.

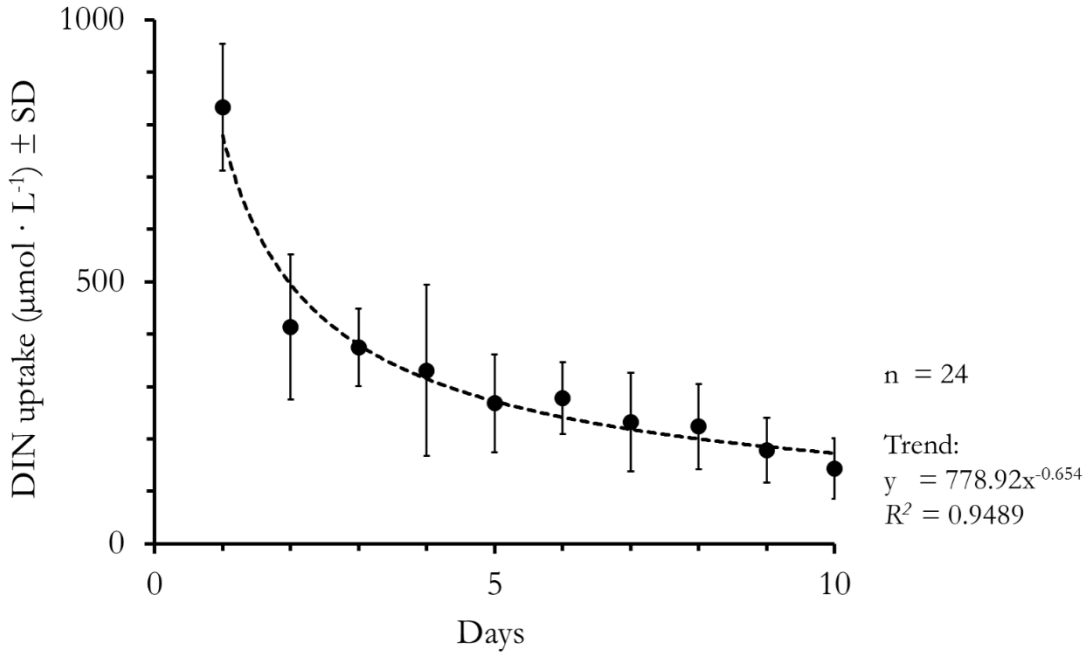

Figure 2-5. Mean DIN uptake (μmol·L^{-1}) ± SD (n=24) of *Ulva lactuca* in saturating DIN concentration (5000 μmol·L^{-1}). No significant variances in DIN uptake between DIP treatments (A-H) were found (ANOVA, df=23 F=0.57 p= 0.944).

Table 2-2. Significances of differences (paired T-test) in DIP and DIN uptake (μmol·cm^{-2}·d^{-1}) of *Ulva lactuca* in treatments with not-saturating (<7 μmol·L^{-1}) and saturating DIP concentrations (>7 μmol·L^{-1}) on consecutive days in a 10-day 'pulse-and-chase' experiment.

Day	Pulsed DIP conc. (μmol·L^{-1})				Pulsed DIN conc. (μmol·L^{-1})
	7.0	13.0	25.0	50.0	5000
1 to 2	0.476	0.448	0.305	**0.005**	**<0.001**
2 to 3	0.442	0.121	**0.006**	0.317	**0.048**
3 to 4	0.414	0.302	0.061	0.007	0.109
4 to 5	0.389	**0.001**	0.010	0.090	0.083
5 to 6	0.115	0.025	0.075	0.302	0.248
6 to 7	0.267	0.065	0.061	0.146	0.317
7 to 8	0.418	0.115	0.045	0.045	0.272
8 to 9	0.272	0.339	0.161	0.024	0.092
9 to 10	0.139	0.090	0.495	0.424	0.335

for DIP n=3; for DIN n=24

Storage capacity

DIP storage

Based on DIP uptake dynamics corresponding to the decline of uptake rates over time when exposed to nominal DIP concentration of 13–50 μmol·L^{-1} (Figure 2-4), we calculated an internal DIP storage capacity of 0.7±0.1 μmol·cm^{-2}. The significant declines in DIP uptake found on days 5, 3, and 2 when exposed to DIP concentrations of 13, 25 and 50 μmol·L^{-1}, respectively (Table 2-2), indicate a time shift in DIP saturation from accumulation of DIP from the seawater medium on days 4, 2 and 1 (Figure 2-4). This occurred after a mean DIP concentration of 0.7±0.1 μmol·cm^{-2} had been removed from the flasks (Figure 2-6).

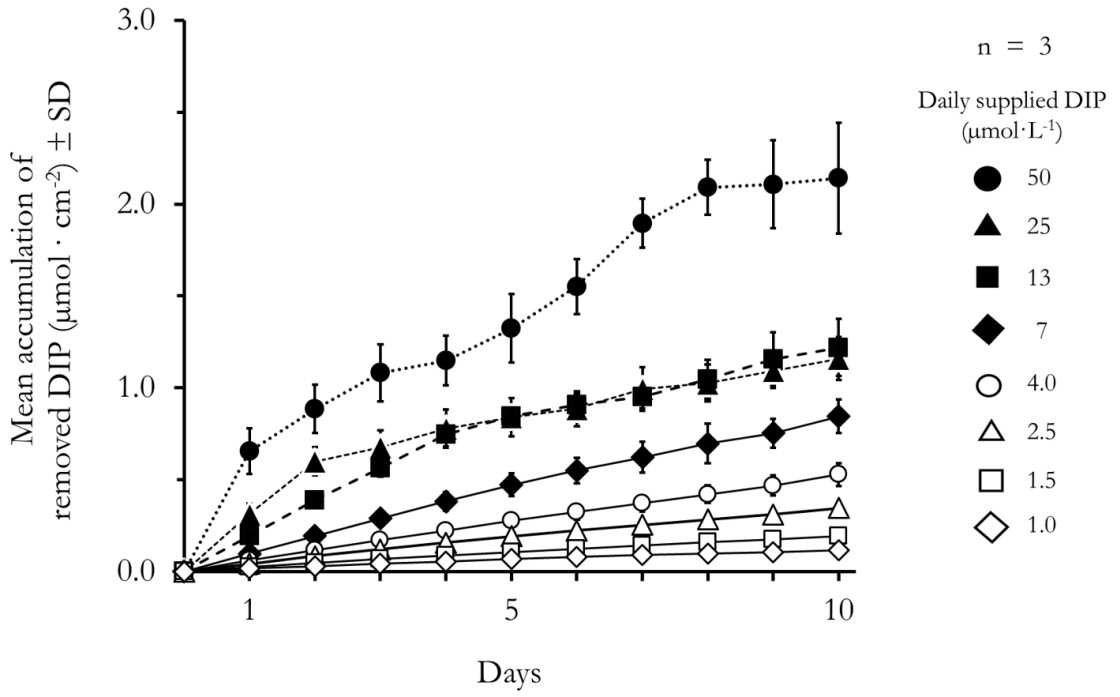

Figure 2-6. Mean accumulation of daily removed DIP (µmol·cm^{-2}) ± SD (n=3) by *Ulva lactuca* in not-saturating (<7 µmol·L^{-1}) and saturating (>7 µmol·L^{-1}) treatments.

DIN storage

A total mean of 43.3±5.0 µmol·cm^{-2} DIN was removed from all flasks by *U. lactuca* within 10 days. 29 % of all removed DIN were taken up on day 1 during maximum surge uptake with a mean DIN accumulation of 12.5±1.9 µmol·cm^{-2} (Figure 2-7). After no significant variations in daily DIN uptake occurred after day 3 (Table 2-2), we concluded that internal DIN storage had been filled. Accordingly, a DIN storage capacity of 22.9±7.0 µmol·cm^{-2} was calculated.

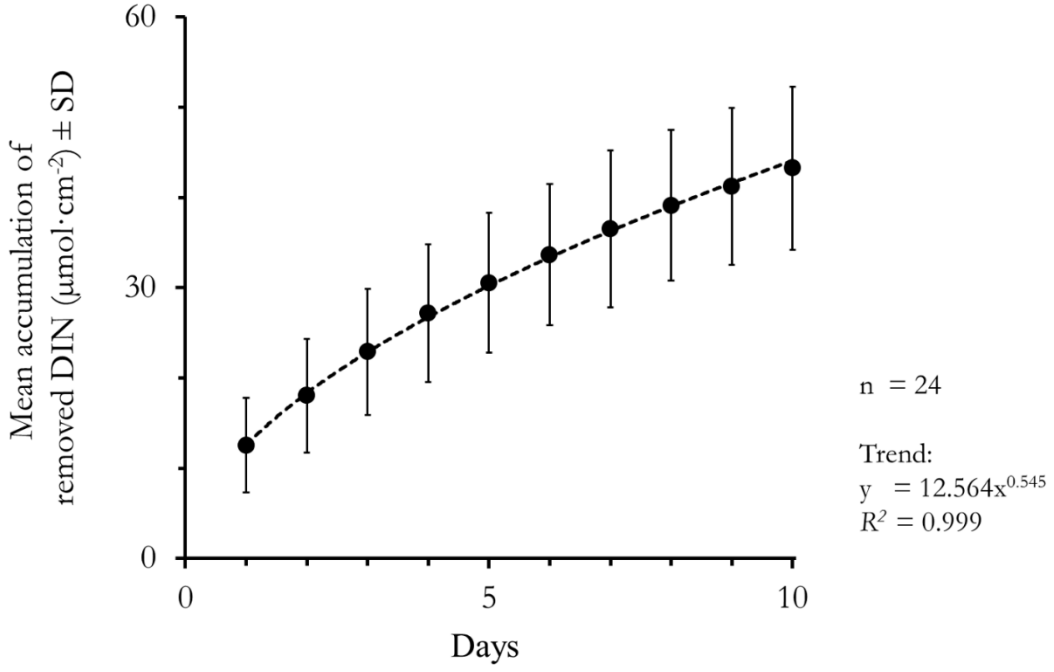

Figure 2-7. Mean accumulation of daily removed DIN (µmol·cm^{-2}) ± SD (n=24) by *Ulva lactuca* in all treatments with DIP concentrations ranging from 1 to 50 µmol·L^{-1}.

N:P dynamics

DIP uptake showed no correlation (R=0.223) to DIN uptake, and the initial filling of the internal nutrient pools during V_S indicated an N:P ratio of 20:1. After internal storage cells had been filled and uptake proceeded after reaching V_M, the N:P ratio levelled off to 30:1.

2.5 Discussion

Ulva lactuca has a maximum thickness of two cell layers; consequently, every cell is in contact with its environment, which makes it an ideal candidate to analyze nutrient uptake kinetics and apply standardized functions of SA for an accurate analysis of nutrient uptake. DIP uptake kinetics and saturating DIP storage capacity, as well as N:P uptake dynamics, were determined under fully controlled laboratory conditions. Growth and nutrient uptake rates in starved *U. lactuca* were not linear over time, and DIP uptake dynamics were clearly different between non-saturating

(<7 μmol·L^{-1}) and saturating (>7 μmol·L^{-1}) DIP concentrations. As growth was not significantly different in treatments with different DIP concentrations, the range of offered nominal DIP concentration (1-50 μmol·L^{-1}) was not the decisive factor for increasing surface area (SA). The increase of total SA is in agreement with reported growth rates for *U. lactuca* (Fortes & Lüning 1980, Fujita 1985). Determination of SA, as a non-destructive method to infer growth, showed a gradual decrease in growth (Figure 2-2).

Two phases of transient responses to nutrient pulses were measured: (1) an initial surge uptake (sensu Conway et al. 1976) after starvation and (2) maintenance (steady state) uptake rates, as measured in continuous cultures (Probyn & Chapman 1982).

In agreement with the total DIP availability in different treatments, V_S was maintained until the ISC had been filled and this is supported by the significant decrease of DIP uptake found in all saturating DIP concentrations (Figure 2-4, Table 2-2) This initial filling of internal nutrient pools under V_S has often been described for nutrient-starved seaweeds (e.g. Fujita 1985, Harrison et al. 1989, Dy & Yap 2001). Although maximum V_S for DIP could not be determined accurately, since all offered DIP was depleted in all the treatments on day 1 (Figure 2-4), an approximation of 0.66±0.12 μmol·cm^{-2}·d^{-1} appears realistic. The $V_{M\ (DIP)}$ for maintenance DIP requirements in *U. lactuca* was calculated as 0.07±0.04 μmol·cm^{-2}·d^{-1}, supported by the DIP uptake rates found in *U. lactuca* exposed to nominal concentrations of 7 and 50 μmol·L^{-1} (Figure 2-6).

U. lactuca exposed to 7 μmol·L^{-1} did not show any significant variations in DIP uptake rates over time (Table 2-2) and removal of DIP from the flasks remained approximately 100 %. The average DIP uptake relative to SA in this treatment was 0.07±0.03 μmol·cm^{-2} on day 10, which is equivalent to V_M and would approximately account for 100 % of the offered DIP over the 10-day assay (Figure 2-4). *Ulva lactuca* exposed to the DIP concentration of 50 μmol·L^{-1} showed similar uptake rates on days 3 to 5, after the surge uptake, and on days 9 to 10, after increased DIP uptake had peaked on day 7 (Figure 2-4). This oscillation in the uptake of DIP over a five-day interval in *U. lactuca* exposed to 50 μmol·L^{-1} DIP could have been caused by various interacting mechanisms,

such as luxury uptake, over-compensation or stress-related responses. In general, luxury uptake describes the ability of plants to store extra nutrients (for seaweeds, e.g. Harrison & Hurd 2001, Naldi & Viaroli 2002) without prior starvation (Eixler et al. 2006). Factors that influence luxury uptake are poorly understood, but external phosphorus concentration is correlated with accumulation and utilization of acid-soluble polyphosphates (ASP) and acid-insoluble polyphosphates (AISP) in microalgae (Powell et al. 2009). Some of these polyphosphates, which are normally involved in metabolic processes, are considered to also form part of the internal short-term phosphorus storage with turnover times of approximately five days (Powell et al. 2009). This 5-day period perfectly matches our finding of re-occurring enhanced DIP uptake rates (Figure 3) when *U. lactuca* was exposed to DIP concentrations of 50 $\mu mol \cdot L^{-1}$. Alternatively, over-compensation can be considered as an explanation for oscillating DIP uptake (Cembella et al. 1984). Over-compensation of internally stored phosphorus can occur when phosphorus-starved algae are re-introduced to high concentrations of external DIP (Aitchison & Butt 1973, Chopin et al. 1997). Finally, oscillating uptake can also reflect a stress reaction to high external nutrient concentration (e.g. Fourcroy 1999, Jiang & Yu-Feng 2008), allowing for mobilization and uptake of sufficient DIP to provide temporary relief.

The time-shifting of DIP saturation found in *U. lactuca* exposed to residual concentrations of 13, 25 and 50 $\mu mol \cdot L^{-1}$ among days 5, 3 and 2, respectively, clearly suggests that internal DIP storages had been filled before V_S turned to V_M (Figure 2-4). The calculated ISC for DIP in *U. lactuca* was 0.73 ± 0.13 $\mu mol \cdot cm^{-2}$. This storage can be utilized during times of low external DIP availability (Chapman & Craigie 1977, Pederson & Borum 1996) and considering the V_M value (0.07 ± 0.04 $\mu mol \cdot cm^{-2} \cdot d^{-1}$), a fully filled internal DIP storage system can fuel metabolic processes for 10 days. This corresponds with results from Fujita (1985), which showed inhibited growth of *U. lactuca* after 10 days of exposure to nutrient-depleted seawater.

Similar to DIP uptake, the mean DIN uptake gradually decreased until day 3, and no significant variations in DIN uptake rates were observed afterwards (Table 2-2), indicating a steady

state uptake for DIN. The calculated value of the V_M for DIN in *U. lactuca* (2.3±0.9 μmol·cm^{-2}·d^{-1}) was approximately 20 % of the V_S. It should be noted that the presence of ammonium (NH_4^+) can influence the uptake of nitrate in *U. lactuca* (Holdt & Kraan 2011, Ale et al. 2011). In our study, daily DIN uptake was not significantly affected (R=-0.027) by the presence of ammonium (NH_4^+). This, in combination with the low NH_4^+: DIN ratios and the full removal of NH_4^+ in all treatments throughout the experiment (not depicted), give us full confidence that the presence of ammonium had no significant effects on DIP uptake kinetics.

Ulva lactuca showed effects of DIN saturation in all treatments on day 3, represented by a significant decline in DIN uptake rates, which indicated that internal N pools had been filled. Thus, a mean DIN storage capacity of 22.9±7.0 μmol·cm^{-2} was calculated. DIN-ISC was a 10-fold higher than DIN-V_M, which is also in agreement with findings of inhibited growth in *U. lactuca* after exposure to nutrient-depleted seawater for 10 days (Fujita 1985).

Uptake rates between starved (V_S) to saturated state (V_M) differed by a magnitude of 10 for DIP and 5 for DIN. This aspect can reflect the ecological competitiveness for DIN (pulses) in opportunistic seaweed (after Littler & Littler 1980), such as *U. lactuca*. Alternatively, we can conclude that *U. lactuca* was successfully starved of nutrients in the precondition phase of our experiment, independent of its nutritional history. There was no correlation between rates of uptake of DIP and DIN (R=0.223), which is contrary to the strong evidence of co-limitation in DIP and DIN in the brown macroalga *Fucus vesiculosus* (Perini & Bracken 2014) and the red macroalgae (Rhodophyta) *Palmaria palmata* (Chapter 6 in this thesis).

Based on V_M, an optimal N:P ratio for *U. lactuca* was estimated to be 30:1, consistent with a mean N:P ratio estimated for marine macrophytes (Atkinson & Smith, 1983). Consequently, *U. lactuca* is twice as likely to suffer from N-limitation as P-limitation when considering the Redfield ratio, the relatively consistent stoichiometric atomic ratio of N and P (16:1) found in coastal regions to open ocean. Yet, *U. lactuca* most commonly inhabits coastal zones, which can receive considerable nutrient pulses with high N:P ratios from land-based anthropogenic activities through

rivers (Jickells 1998) or near-shore fish aquaculture (Pearson & Black 2001). Burson et al. (2016) reported an offshore gradient from DIP to DIN limitation in the North Sea during spring, with a nearshore N:P ratio of 375:1 and a 1:1 ratio in the central North Sea. Exactly such a nearshore nutrient stoichiometry can allow *U. lactuca* to thrive, given its low DIP requirements.

A set-up with comparable initial physiological conditions for all organisms is a key element for representative laboratory experiments. *Ulva lactuca* has been reported to be able to grow for 9 days under external nitrogen depletion (Fujita 1985). Accordingly, we assumed that 10 days of nutrient starvation (P and N) would result in *U. lactuca* individuals with similar physiological status with respect to depletion of internal P and N pools, which would lead to representative and comparable responses by all individuals to varying DIP treatments. This assumption is supported by the reproducible DIP and DIN uptake kinetics found in our experiments. Our experimental results moreover confirm the period of time that *U. lactuca* is able to grow under nutrient starvation: using the experimentally determined V_M values, ISC depletion is calculated to take exactly 10 days.

In this study we offer correlation factors for SA with FW and DW in *U. lactuca*, which enables conversions between these standardization units and allows for accurate comparison of data to other studies. Moreover, our standardized data adds to the physiological understanding of *U. lactuca*, enables estimation of ecological effects on nutrient availability and can contribute to development and modification of applications in a bio-based economy. In order to predict the efficiency of *U. lactuca* as efficient biofilter, for example in land-based tank systems (e.g. Robertson-Andersson et al. 2008, Copertino et al. 2009) or in *situ* applied biofilters at inlets of cooling water for power plants, information about uptake kinetics are indispensable and can help to control effluent and productivity for environmentally responsible practices. Despite the quickly filled ISC and the corresponding declines in nutrient uptake rates of approximately 90 % for DIP and 80 % for DIN in saturating concentrations, saturated state uptake rates in *U. lactuca* can significantly contribute to excess nutrient uptake, leading to less eutrophic waters and production of valuable biomass for food, feed and energy.

2.6 Acknowledgements

We thank the NIOZ nutrient laboratory, especially Karel Bakker, Sharyn Ossebaar and Jan van Ooijen for their precise nutrient analyses and we are grateful to Wouter Visch and Vera Visser for their skilled assistance in the laboratory and around the NIOZ Seaweed Centre. We also thank the anonymous reviewers for comments on an earlier version of this paper and their friendly support.

Chapter 3

Using a smartphone app for the estimation of total dissolvable protein concentration in *Ulva lactuca* Linnaeus (Chlorophyceae)

In preparation

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3.1 Abstract

Visual inspection is the first apparent approach for assessing the well-being of a plant, animal or even environment. Smartphones, all equipped with digital cameras can be used to produce realistic images that can basically be used as three-band radiometers analysing Red, Green, Blue (RGB). Given the omnipresence of smartphones, observations from the public can help to complement observational gaps enabling a wide spatio-temporal coverage of environmental monitoring within ecological research (e.g. landscape ecology, macro-ecology) and biological studies (e.g. species documentation, eutrophication, ecophysiological status). Here, we introduce

the smartphone application 'EyeOnUlva' (Android, IOS), which can record the frond colour of the green seaweed *Ulva lactuca* and which provides an estimation of its total dissolvable protein concentration. 'EyeOnUlva' is an economically and ecologically relevant application, as the frond colour of this pre-eminent primary producer not only indicates its protein concentration, but can also function as a bio-indicator giving insight in the nutritional status of a coastal habitat or cultivation site. 'EyeOnUlva' represents a novel, inexpensive and simple-to-use tool and allows citizens and scientists, using smartphones in the context of participatory science, to support environmental monitoring. In the 'EyeOnUlva' App, a combination of spectrophotometric measurements and colorimetric techniques were applied to determine the colour appearance of the fronds of *U. lactuca*, cultivated in different nutrient concentrations and light conditions under laboratory conditions. Subsequent colorimetric analysis of randomly collected *U. lactuca* in the field and quantification of their total dissolvable protein and carbohydrate concentrations showed a correlation in colour appearance to dissolvable protein concentrations (R^2=0.72). Considering the geographical distribution of *U. lactuca*, 'EyeOnUlva' has a worldwide application.

3.2 Introduction

Visual inspection is the first apparent approach for assessing the well-being of a person, object or the environment. For example, the colour (optical properties) of natural waters provides information on presence of phytoplankton and dissolved organic matter content, and changes in water colour allow to predict effects on aquatic ecosystems (Haines et al. 1995, Bissel et al. 2003, Peperzak et al. 2011, Wernand et al. 2013a). Scientists have been measuring the transparency of natural waters with a Secchi-disc and classified water colour via comparison using the Forel-Ule colour comparator scale for more than 100 years (Wernand & Gieskes 2011), forming a dataset with one of the largest spatial and temporal coverage.

Lottig et al. (2014) showed that citizen-collected data, consisting of more than 140,000 individual Secchi-disc measurements between 1938 and 2012, revealed geographical patterns and temporal trends in lake water clarity across eight states in the upper Midwest of the USA. Another well-known example of citizen-based science is represented by the assessment of waterbird population dynamics concerning the winter distribution of the migratory Western Grebes along the west coast of the USA (Wilson et al. 2013).

New analytical tools can increase the data collection by the general public and digital networks enable to collect, combine and compile observations and large datasets in centralized databases (Dickinson et al. 2010). Smartphones, respectively mobile devices, can represent such an analytical tool, also given their omnipresence in our society. Many mobile devices contain various sensors, such as global positioning system (GPS), device orientation sensors, which measure the inclination angels perpendicular to the ground, motion detection sensors (accelerator), and digital cameras. Novel smartphone applications based on standard remote sensing principles were developed within the European funded Citclops project (Citizens' Observatory for Coast and Ocean Optical Monitoring), encouraging the integration and contribution of citizen scientist to complement existing marine datasets and enhance environmental awareness (www.citclops.eu). The freely available smartphone app 'EyeOnWater' (www.eyeonwater.org), for example, integrates the concept of the Forel-Ule colour scale to assess the colour of natural surface waters, both fresh and saline water (Wernand et al. 2013b). Another novel application is 'SmartFluo', an affordable DIY (do–it-yourself) smartphone adapter to measure micro-algal concentration by Chlorophyll *a* (Chl *a*) fluorescence in water (www.citclops.eu). Fluorescence measurements can also be extended to detect nutrient limitations in phytoplankton, macro-algae and seagrasses *in situ* by Nutrient-Induced Fluorescence Transient (NIFT) experiments within minutes (Den Haan et al. 2013), where the NIFT analysis is based on changes in Chl *a* fluorescence induced by the addition of limiting nutrients.

In our ecophysiological work with *Ulva lactuca* (Linnaeus), we observed remarkable (green) colour differences in their fronds (Chapter 2). These colour differences appeared to be related to their total dissolvable protein concentration. This led us to this study, in which we examined the possibility to deploy spectro-radiometry and colorimetric techniques to evaluate the total dissolvable protein concentration in the green seaweed *U. lactuca* based on its frond colour.

The pre-eminent primary producer *U. lactuca* in the division Chlorophyta, commonly known as sea lettuce, can be found worldwide in estuarine and coastal ecosystems (Van den Hoek et la. 1995) and is mostly abundant where nutrients are readily available (Valiela et al. 1997, Morand & Merceron 2005). Moreover, species of the genera *Ulva*, including *U. lactuca*, can vastly increase their growth in response to nutrient pulses and can build up a large biomass in extensive blooms (Teichberg et al. 2008, 2010). These massive blooms became known as 'green tides', when beached and rotting piles of biomass hindered shore-based activities and caused harmful ecological and economic consequences (Westernhagen & Dethlefsen 1983, Smetacek & Zingone 2013, Wan et al. 2017). Eventually, a vast abundance of opportunistic *Ulva* species can be an indication of eutrophication.

Ulva lactuca is not only an ecologically important species, but also a promising seaweed for application in food, animal feed, as fertilizer, or for bioremediation purposes in a bio-based economy (Sahoo 2000, Neori et al. 2003, Holdt & Kraan 2011, Bruhn et al. 2011, Lawton et al 2013). Wild-harvested and cultured *U. lactuca* have been implemented as feed in aquaculture, for instance abalone (Gastropoda) farms with success (Shpigel & Neori 1996, Shpigel et al. 1999, Robertson-Andersson et al. 2008) and can be used as a dietary supplement for fish, goat, poultry and other farm animals (Chapman & Chapman 1980, Ventura & Castañón 1998, Angell et al. 2016a). Hence, the availability of sufficient quantity and quality of dietary protein can be considered economically crucial. The protein concentrations in *U. lactuca* can widely vary depending on environmental conditions and the availability of inorganic nitrogen (DeBusk et al.

1986, Vandermeulen & Gordin 1990, Cohen & Neori 1991), one of the essential macro-nutrients for seaweeds. A broad relationship between nitrogen content and thallus colour in *U. lactuca* was observed by Robertson-Andersson (2003), with darker colour indicating more nitrogen rich material than paler colours. This colorimetric feature can, for example, help to select suitable *Ulva* as a feed source.

Based on the concept of colorimetric techniques, we developed the smartphone application 'EyeOnUlva' for Android and IOS systems, which records the frond colour and provides an inexpensive, reliable, safe and easy-to-use method to give a fast evaluation on the total dissolvable protein concentration in *U. lactuca*.

In this study, a description of laboratory experiments on the colour and total dissolvable protein and total dissolvable carbohydrate concentration in *U. lactuca* is given. Results of relation between colour appearance within the RGB colour scale and total dissolvable protein concentration of 83 samples of *U. lactuca* randomly collected in the field and cultivation sites on the island of Texel, The Netherlands, are presented. These Ulva samples cover a broad range of growing conditions and act as a proof of concept for the application 'EyeOnUlva'.

3.3 Material and methods

All experiments and analyses were conducted at the Royal Netherlands Institute for Sea Research (NIOZ), Texel, The Netherlands. In a laboratory approach, a spectro-radiometric analysis of the reflected wavelengths within the visible light of 20 fronds of *U. lactuca* Linnaeus (after Stegenga & Mol 1983) cultivated in different light and nutrient regimes was conducted over 5 days. Subsequently, 83 *U. lactuca* individuals were collected from outdoor cultivation sites and on beaches surrounding the island of Texel between February 2015 and May 2016. Photographs of

the randomly collected samples were taken with a simple digital camera (Panasonic Lumix DMC-FT5) under daylight conditions, followed by a colorimetric analysis of the photographs.

The total dissolvable protein and total dissolvable carbohydrate concentration in the photographed individuals was quantified and results inspected for a correlation to the colour appearance of the frond. Based on our results we developed the smartphone application 'EyeOnUlva'.

Experimental design

Twenty fronds of *U. lactuca* (freshweight = 5.7±0.2 g; Mettler Toledo balance, accuracy: 0.01 g), originated from the coastline of the island of Texel, were taken from cultivation tanks at the NIOZ Seaweed Centre (https://www.nioz.nl/en/expertise/seaweed-research-centre). These fronds were transferred into a cultivation flask (20 L) in a temperature controlled room (15.7±0.3 °C) for a two week adaptation phase in nutrient depleted seawater (NO_3^- = 0.003 μmol·L^{-1} and PO_4^{3-} = 0.008 μmol·L^{-1}). After the adaptation phase, the fronds were individually transferred into Erlenmeyer-flasks (1000 ml) filled with 500 ml filtered (0.2 μm) seawater-medium (salinity: 29.9±0.1).

Two levels of nitrate (Dissolved Inorganic Nitrogen: DIN - equals here NO_3^-) and phosphate (Dissolved Inorganic Phosphate: DIP - equals here PO_4^{3-}) concentrations were prepared: ambient concentrations (NO_3^- = 25 μmol·L^{-1} and PO_4^{3-} = 1 μmol·L^{-1}) provided by natural seawater tapped from the NIOZ seawater supply system in late October, and enriched concentrations after the Redfield-ratio (NO_3^- = 1600 μmol·L^{-1} and PO_4^{3-} = 100 μmol·L^{-1}). The flasks were placed on a rotating table (100 rpm) inside a two-compartment cultivation cabinet with one compartment providing a light intensity of 70±7 μmol photons m^{-2}·s^{-1} (light meter ULM- 500, Walz, Germany) for optimal light conditions (Fortes & Lüning 1980) and the other compartment providing a low light intensity of 7±2 μmol photons m^{-2}·s^{-1}, emitted by two tubular fluorescence lamps (OSRAM L18 Watt 965, Deluxe cool daylight) installed 60 cm above the flasks. A

photoperiod of 16/8 h (light/dark) was maintained throughout the end of the 5-day experiment. Altogether four treatments with (A) optimal light intensity and high DIN and DIP availability, (B) low light intensity and high DIN and DIP availability, (C) optimal light intensity and low DIN and DIP availability, and (D) low light intensity and low DIN and DIP availability were arranged with five replicates for each treatment (A – D). Sampling of the seawater-medium for actual dissolved N and P was conducted in duplicates for each treatment at the beginning and the end of the experiment.

In the subsequent field approach, an additional 83 individuals of *U. lactuca* were randomly collected from the coastline of the island of Texel, cultivation tanks and the bio-filtration system at the NIOZ Seaweed Centre between February 2015 and May 2016. These samples were, just as the *U. lactuca* photographed in the laboratory assays, individually placed flat and without overlapping parts on a white plastic sheet and were gently dried with a paper towel to remove excess water, in order to minimize light reflection and misrepresentation of the images taken. Photographs (Panasonic Lumix DMC-FT5) were taken from a 90° angle under day-light conditions. After photographs were taken, the samples were prepared for total dissolved protein and total dissolved carbohydrate analysis.

Spectro-radiometric analysis

The spectro-radiometric measurements of reflection by *U. lactuca* were conducted in a colour assessment cabinet with a grey coating inside (VeriVide Ltd. Enderby, Leicester, United Kingdom). All samples were illuminated with a D65 daylight simulating lamps (VeriVide, width: 600 mm, 18 W) on top of a diffuser that homogenized the illumination conditions in the cabinet. The measurements were performed with a spectro-radiometer (Hyperspectral PR655 Photo Research; www.photoresearch.com) installed in front of the light cabinet in a 45° degree angle in respect to the sample on the white plate (Figure 3-1). The remote sensing reflectance (Rrs),

independent on the illumination, was measured with the spectro-radiometer modified with a cosine collector (www.photoresearch.com).

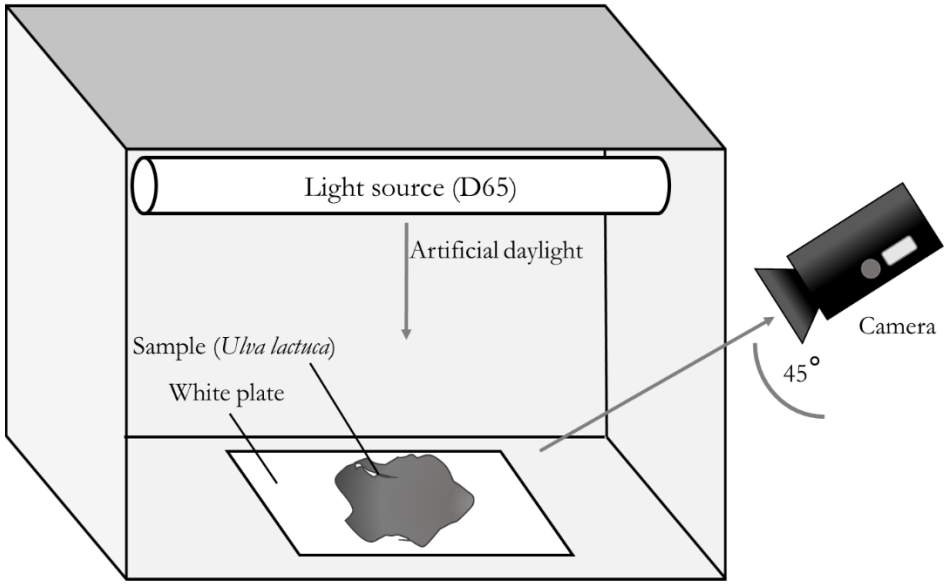

Figure 3-1. Light cabinet set up with a light source (D65) emitting artificial daylight. The sample (*Ulva lactuca*) was placed flat and in a single layer on top of a white plate, at the central bottom of the cabinet, 60 cm underneath the light source. Analysis of the reflected light was carried out with a spectro-radiometer (camera) in a 45° angle in respect to the sample (set up after Novoa et al. 2015).

The difference in Rrs of the sample over 5 days was normalized to day 1 to account for the difference in colour appearance at a wavelength of 556 nm (green) in percentage (Table 3-1) and was calculated as

$$Rrs(556)\% = (Rrs_n * 100)\ Rrs_1^{-1}$$

with Rrs_n and Rrs_1 representing the Rrs values measured on day n and day 1.

RGB analysis

The RGB colour system constructs all the colours from the combination of the red, green and blue colours, each defined as a pixel value from 0 to 255 (Goddijn & White 2006, Goddijn-

Murphy et al. 2009). The standard RGB (referred as sRGB) reduces the light spectra to the physical correlates of human colour perception (CIE 1931 XYZ colour space tri-stimulus), as well as standardizes the illumination correction and is widely used in industrial applications Novoa et al. 2015). All images of *U. lactuca* fronds were analyzed for the RGB values with the software IrfanView (Version 4.44; www.irfanview.com). Prior the RGB analysis of the *Ulva*-images, the background was eliminated (cropped) to exclude potential disturbances affecting the software's analysis.

DIN and DIP analysis

The determination of DIN and DIP was performed by colorimetric analysis using a Technicon TRAACS 800 auto-analyzer (Seal Analytical, Germany) in the NIOZ Texel nutrient laboratory. DIP was measured as ortho-phosphate (PO_4^{3-}) at 880 nm after the formation of molybdophosphate complexes (Murphey & Riley 1962), while DIN (nitrate and nitrite) was calculated after nitrate reduction to nitrite through a copperized cadmium coil and after complexation with sulphanylamide and naphtylethylenediamine measured at 550 nm (Grasshoff & Hansen 1983). The precision for all measured channels within the automated nutrient analyzer was better than 0.25 % (personal communication K. Bakker, NIOZ).

Total dissolvable protein and total dissolvable carbohydrate analysis

The photographed samples were separately frozen (-40 °C), freeze-dried (24 h) and homogenized for the determination of total dissolvable protein concentrations (Lowry et al. 1951), as well as total dissolvable carbohydrate concentrations (Trevelyan et al. 1952). Homogenization of *U. lactuca* was accomplished by transferring each freeze-dried sample into a stainless steel tube (2 ml), including grinding sphere (Ø 2 mm, stainless steel) and inserting the tube in a mixing mill (MM400, Retsch, Germany) set to a frequency of 30 Hz for three times 1 minute. Short pauses between the homogenization intervals were taken to avoid a potential temperature rise inside the tube and overheating of the dried sample. To determine the total dissolvable protein and total

dissolvable carbohydrate concentration in *U. lactuca*, 5-10 mg of the homogenized seaweed sample were added to 5 ml MilliQ™ water and mixed for 30 seconds, using a Turrax® mixer. Another 5 ml MilliQ™ water were added, and the suspension was mixed for another 30 seconds on a vortex mixer, before a refined homogenisation by using a Potter-Elvejhem was performed to finalize the assays' starting mixture.

The total dissolvable protein concentration of each sample was measured in duplicates with different concentrations: 0.25 ml and 0.50 ml of the starting mixture were transferred into test tubes and filled up with MilliQ™ water to a volume of 0.5 ml, after which 1.0 ml Lowry reagent was added. After 10 minutes of incubation at room temperature, 1.0 ml of Folin/Cioccalteus reagent was added and the solution was incubated for another 30 minutes to let a blue colour develop. Its absorbance was measured at 660 nm with a photometer (SpectraMax M2, Molecular Devices, LLC, CA, USA) and the total dissolvable protein concentration was calculated using a calibration curve based on a bovine serum albumin (BSA) stock solution with known protein concentration.

The total dissolvable carbohydrate concentration was determined in triplicates of different concentrations: MilliQ™ water was added to 0.1 ml, 0.2 ml, and 0.3 ml of the starting mixture to a final volume of 1.0 ml. Afterwards 4.0 ml of the Anthrone reagent were added to the prepared starting mixtures and placed in a heating chamber at 95 °C for 6 minutes. After cooling of the solution to room temperature, the absorbance at 620 nm was measured with the photometer and the concentration of total dissolvable carbohydrates was calculated, using a calibration curve based on glucose stock solution with known concentration. Both, the total dissolvable protein- and total dissolvable carbohydrate concentrations ($\mu g \cdot mg^{-1}$) were determined and described as percentages of dryweight (% DW).

3.4 Results

Spectro-radiometric analysis

Ulva lactuca (n=5) showed a significant difference in visual and instrumentally measureable colour appearance in treatments over 5 days (ANOVA, df=4, F=10.59, p<0.001). Visual colour appearance of the fronds changed from pale green to a 'more saturated', darker green (Figure 3-2).

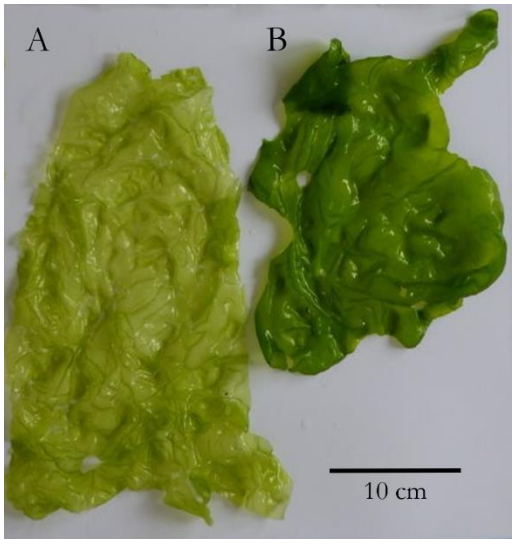

Figure 3-2. Fronds of *Ulva lactuca* (A) cultivated in deplete nutrient concentration (pale greenish) and (B) cultivated in replete nutrient concentrations (saturated green).

The instrumentally detected maximum difference in the remote sensing reflectance (Rrs) was detected within the green colour scale, approximately at a wavelength of 556 nm (Figure 3-3). No significant difference in the Rrs of *U. lactuca* fronds in treatments with different levels of illumination (ANOVA, df=1, F=0.03, p=0.869), but a highly significant difference in treatments with saturating DIN and DIP additions (ANOVA, df=1, F=51.58, p<0.001) was found after 5 days. The interaction between illumination and nutrient availability showed no significant difference between these treatments (ANOVA, df=1, F=0.26, p=0.613), thus the availability of nutrients was the decisive factor for the change in Rrs of the frond. A detected decrease of the Rrs

equals a higher absorption by the frond, resulting in a 'more saturated' or darker green in visual appearance.

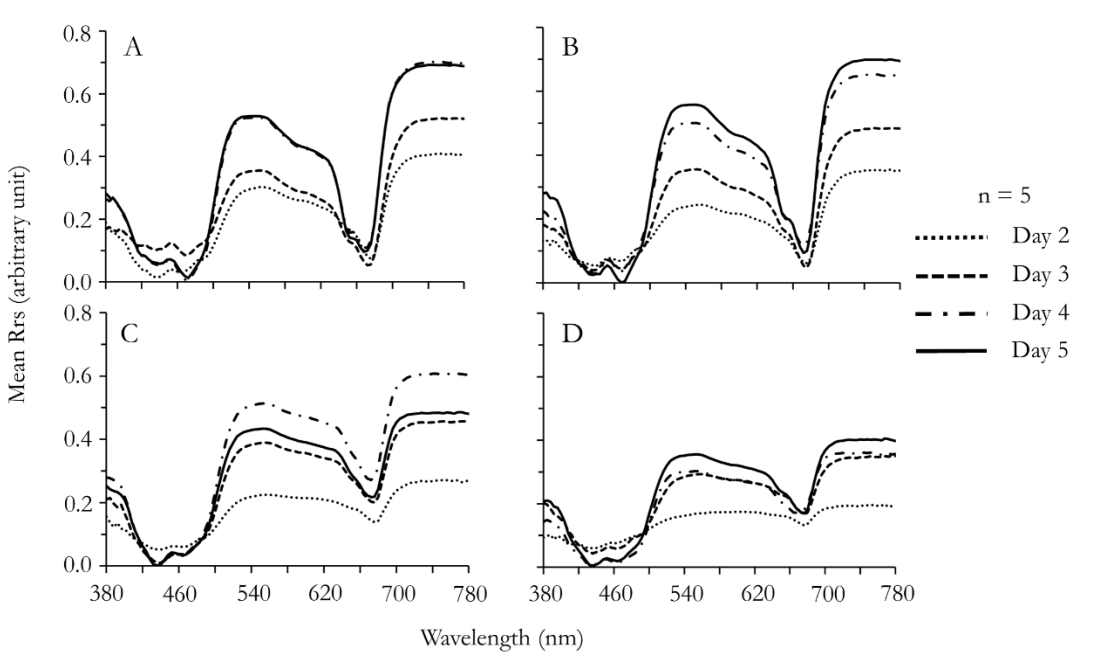

Figure 3-3. Relative change of the mean remote sensing reflectance (Rrs) per steradian (sr^{-1}) of the visible light (wavelengths 380-780 nm) by *Ulva lactuca* (n=5) cultivated in treatments of (A) optimal light intensity (70 µmol photons $m^{-2}\cdot s^{-1}$) and high nutrient supply (NO_3^-: 1600 µmol·L^{-1}; PO_4^{3-}: 100 µmol·L^{-1}), (B) low light intensity (7 µmol photons $m^{-2}\cdot s^{-1}$) and high nutrient supply, (C) optimal light intensity and low nutrient supply (NO_3^- : 25 µmol·L^{-1}; PO_4^{3-}: 1 µmol·L^{-1}), and (D) low light intensity and low nutrient supply in 4 consecutive days, compared to day 1.

In treatments with saturating DIN and DIP availability, the fronds showed no significant difference in Rrs between optimal and low light conditions after 5 days (ANOVA, df=1, F=0.15, p=0.713) and the mean Rrs decreased 53±6 % under optimal light, respectively 56±8 % under low light conditions (Figure 3-3 A & B, Table 3-1). Fronds cultivated in treatments without saturating DIN and DIP availability, showed a significantly higher Rrs, than the ones exposed to enriched seawater medium after 5 days (ANOVA, df=1, F= 56.9, p<0.001). Here, the mean Rrs decreased 43±14 % under optimal light conditions, respectively 36±12 % under low light conditions on day 5, when compared to day 1 (Figure 3-3 C & D, Table 3-1). The maximum

decrease of Rrs of all fronds in all treatments was measured on day 1, after starved *U. lactuca* had been introduced to fresh seawater medium (Table 3-1). No significant difference in Rrs between all four treatments was found (ANOVA, df=3, F=2.51, p=0.108) and the mean Rrs decreased by 24±5 %. Rrs of the fronds continued to decrease in all treatments during the experiment (Table 3-1). In treatments without extra DIN and DIP additions and under optimal light conditions the Rrs of the fronds showed a significant increase between day 4 and 5 (ANOVA, df=1, F=9.39, p=0.015) and the mean Rrs gained 9±4 % (Figure 3-3 C, Table 3-1).

Table 3-1. Relative change (in %) in the remote sensing reflectance (Rrs) ± SD within the green colour spectrum at a wavelength of 556 nm of starved *Ulva lactuca* fronds (n=5), exposed to (A) optimal light intensity (70 µmol photons $m^{-2} \cdot s^{-1}$) and high nutrient supply (NO_3^-: 1600 $\mu mol \cdot L^{-1}$; PO_4^{3-}: 100 $\mu mol \cdot L^{-1}$), (B) low light intensity (7 µmol photons $m^{-2} \cdot s^{-1}$) and high nutrient supply, (C) optimal light intensity and low nutrient supply (NO_3^-: 25 $\mu mol \cdot L^{-1}$; PO_4^{3-}: 1 $\mu mol \cdot L^{-1}$), and (D) low light intensity and low nutrient supply on day 2, 3, 4, and 5, compared to day 1.

	Relative change of Rrs ± SD at 556 nm (in %)			
	Treatment			
Day 1	A	B	C	D
Day 2	30 ± 15	25 ± 14	23 ± 19	17 ± 10
Day 3	35 ± 10	35 ± 11	39 ± 21	29 ± 23
Day 4	52 ± 3	50 ± 9	51 ± 10	30 ± 7
Day 5	53 ± 6	56 ± 8	43 ± 14	36 ± 12

n=5

Total dissolvable protein- and total dissolvable carbohydrate concentration

The total dissolvable protein- and total dissolvable carbohydrate concentration in 83 randomly collected *U. lactuca* samples with varying colour appearances were determined. The total dissolvable protein concentration ranged between percentages of 3.0 % and 26.6 % DW, while the total dissolvable carbohydrate concentration was found within the range of 17 % to 70 % DW

(Figure 3-4). No correlation between protein and carbohydrate concentrations was found (R^2=0.03). Nevertheless, a clear threshold for carbohydrate percentage in the *U. lactuca* samples was detected: when protein content exceeded 15 % DW, carbohydrate content did not rise above 32±3 % DW (Figure 3-5).

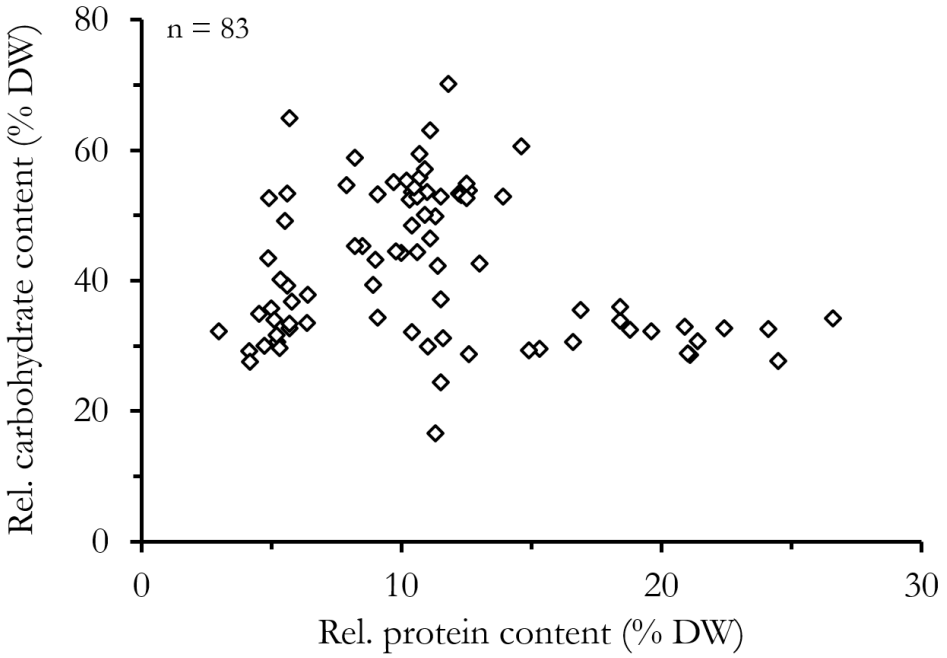

Figure 3-4. Carbohydrate versus protein concentration in % dryweight (% DW) in *Ulva lactuca* (n=83) of varying colour appearance (green), randomly collected from cultivation tanks and natural sites on the island of Texel, The Netherlands, between 2015 and 2016.

Red Green Blue (RGB) analysis

The RGB analysis of the 83 images of *U. lactuca* fronds (collected in the field) showed a clear distribution of detected R, G and B with increasing protein content (Figure 3-5). RGB values decreased with increasing protein content, and measured values for the green colours, represented by G ranged from 139 with a protein percentage of 5.6 % DW to 61 with a protein percentage of 26.6 % DW. R-values within the same protein levels ranged from 125 to 28, and B-values ranged

from 51 to 0 for the blue colour scale. When percentages of mean protein concentration of the fronds surpassed 11.4±1.0 % DW, reflectance of the blue-band (B) was not detected anymore (Figure 3-5). Hence, a best fit correlation between colour appearance and total dissolvable protein concentration was exhibited by the ratio of R and G values, showing a decreasing trend of $y=0.0006x^2 - 0.035x + 1.0168$ with $R^2=0.72$ (Figure 3-6). No correlation between the carbohydrate concentration and R/G-ratio was found ($R^2=0.03$).

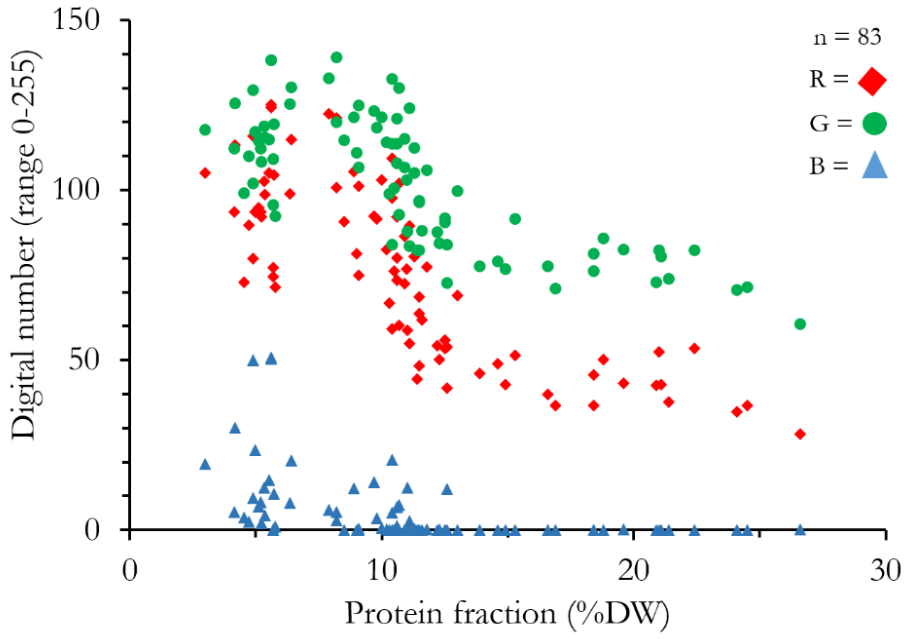

Figure 3-5. Red, green and blue (RGB) colours as measured by digital imaging of *Ulva lactuca* (n=83) versus its protein concentration in percentage dryweight (% DW).

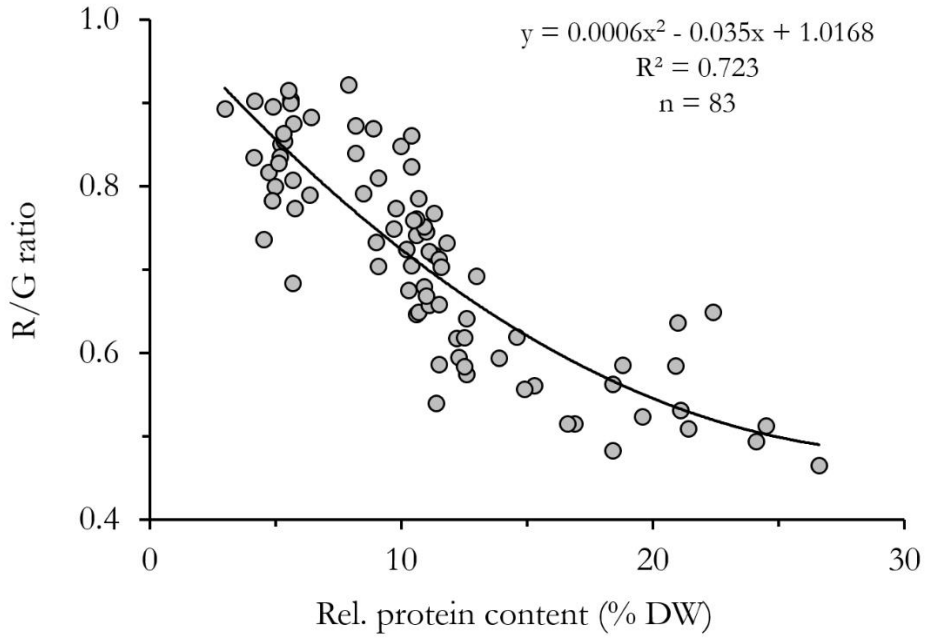

Figure 3-6. Ratio of red (R) and green (G) colours, as measured by digital imaging of *Ulva lactuca* fronds (n=83) versus its protein concentration in percentage dryweight (% DW).

EyeOnUlva

The smartphone application 'EyeOnUlva' (Figure 3-7) is based on the three-band (Red, Green, Blue - RGB) colorimetric analysis and evaluates the percentages of total dissolvable protein concentrations of *U. lactuca* fronds within 5 % intervals, between 0 and 25 % DW. 'EyeOnUlva' has been tested successfully by a selected group of international university students to verify performance, reliability and ease of use of the application, which is now freely available in public domain in the app-store. Compatibility-tests of 'EyeOnUlva' to other representatives of the family *Ulvaceae* are also pending.

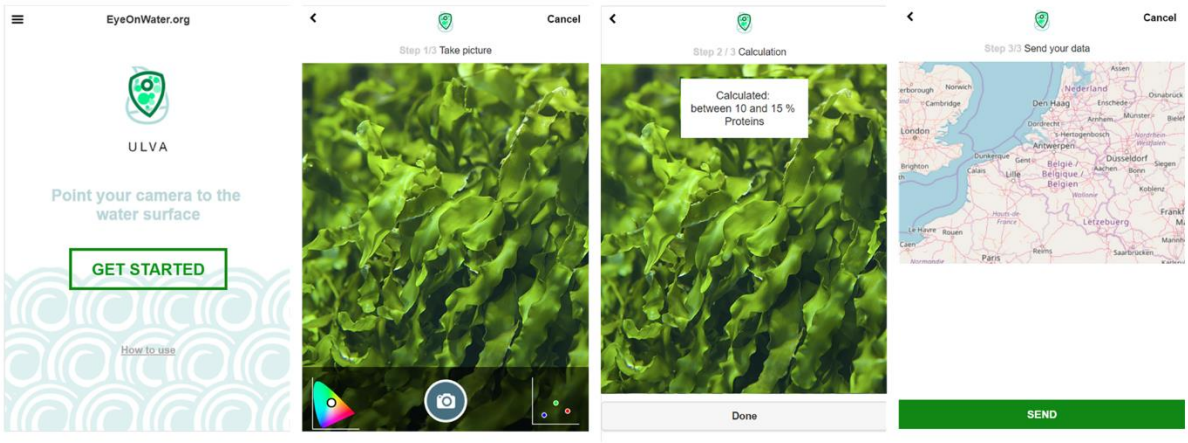

Figure 3-7. Outlook of the smartphone application 'EyeOnUlva' for Android and IOS systems (www.eyeonwater.org/ulva). EyeOnUlva records the frond colour of *Ulva lactuca* (step 1/3) and provides a fast quantification of its total dissolvable protein concentration in 5 % intervals, between 0 and 25 % dry weight (step 2/3). The data can be send (step 3/3) to a data base, part of the CITCLOPS project (www.citclops.eu).

3.5 Discussion

The green colour of *U. lactuca* covers a wide range, from pale to saturated green. Many (co-) factors influence the colour of plants, including seaweed. This led us to study the main environmental factors influencing the green colour of *U. lactuca*, such as the availability of nutrients and light conditions, and investigated a possible relationship between the colour green and cellular composition. A change in frond colour of green seaweed can in theory be related to nutrient availability and/or to varying amounts of light harvesting pigments, such as chlorophylls and their breakdown products. It has been documented that the amount of these pigment proteins is strongly related to the internal nitrogen content (Hegazi et al. 1998) and therefore can alter the colour appearance. Robertson-Andersson (2003) demonstrated that nitrogen starved *U. lactuca* had a green-yellow colour appearance and when cultivated in nitrogen-enriched seawater the colour appearance changed to green and vice versa. This is supported by our results on the interaction between illumination and nutrient availability to frond colour, which exhibited nutrient availability,

most notably the nitrate concentration as the decisive factor for the change of the frond colour in *U. lactuca*. However, illumination levels and especially UV-radiation in the field drastically vary from those applied during in the laboratory experiments. Yet, colorimetric analysis of 83 randomly collected *U. lactuca* in the field under daylight conditions fully supported our laboratory results, hence the proof of concept of the 'EyeOnUlva' smartphone app. These were significant relations between the availability of nutrients, frond colour of *U. lactuca*, and the internal total dissolvable protein concentration. This relationship was transferred into the 'EyeOnUlva' app, enabling an estimation of the nutritional value of *U. lactuca* for food or feed, as well as give insight into eutrophication status of the brackish and marine environments, where *U. lactuca* is found.

Spectro-radiometric analysis

The spectro-radiometric analysis exactly aligns with documented observations and additionally quantifies the change in frond colour, expressed by the remote sensing reflectance (Rrs). Analysis showed a maximum Rrs within the green spectra, which can be related to the synthesis of chlorophylls. This is supported by a change of Rrs within the red and far red regions around 700-780 nm, which corresponds to the change of Rrs within the green spectra around 556 nm (Figure 3-3). This case is widely used in ratio fluorescence measurements, which measures re-emitted light of a plant in the red and far red regions to determine the relative chlorophyll content of a leaf (Maxwell & Johnson 2000).

Our spectro-radiometric measurements of *U. lactuca* fronds over 5 days, cultured at saturating and non-saturating levels of DIN and DIP under low and optimal light conditions, perfectly aligns with documented variations in frond colour of seaweeds contributed to changes in the tissue nitrogen content, and resembles physiological patterns of *U. lactuca.*

Total dissolvable protein- and total dissolved carbohydrate concentration

Both the extraction of the protein and the quantification of the extracted dissolvable protein in seaweeds are prone to discussion: direct extraction procedures to determine the total dissolvable protein concentration are likely influenced by the extraction procedure and the dissolvable proteins are "just" their dissolved fraction (Angell et al. 2016b).The analyses of individual amino acids also has it problems: not all amino acids can be analysed reliably (Angell et al. 2014). The determination of protein concentration in seaweed by analyses of total N and subsequent conversion to protein is hampered by varying conversion factors (Bjarnadóttir et al. 2018), as the protein concentration strongly depends on species, location and time of the year (Fleurence 1999, Gaillard et al. 2018).

Here we made the choice to determine the total dissolvable protein concentration in one species of seaweed, strictly performed in the same manner regardless of sampling time and location. We encourage an open discussion on absolute numbers and (in the meantime) have confidence in relative differences of results derived from different experimental treatments and samples of *U. lactuca* collected in the field and analysed in the laboratory. Our results on total dissolvable protein concentration of 3.0 % to 26.6 % DW in *U. lactuca* are within the reported range found by other authors: Fujiwara-Arasaki et al. (1984) found a maximum protein content of 20 % to 26 % DW in *Ulva pertusa* and Fleurence (1999) reviewed the protein contents for species of the genus *Ulva*, which reportedly ranged from 10 % to 26 % DW and specifically for *U. lactuca* between 10 % and 21 % DW. Similar to proteins in seaweed, carbohydrates have received increased attention as a sustainable resource for biofuels and the manufacture of high valuable carbohydrate products (Adams et al. 2011, Ashok et al. 2013, Saqib et al. 2013). Likewise to the procedure of protein extraction, several extraction and determination methods can be applied to the total dissolvable carbohydrate analysis in seaweed and yet no standardized methodology has prevailed (Manns et al. 2014). The total dissolvable carbohydrate concentration (% DW) in *U.*

lactuca had been described as high as 62 % by Ortiz et al. (2006), which is similar to our results in fronds with less than 15 % DW protein content (Figure 3-4). The clear threshold for carbohydrate content (% DW), when protein content exceeded 15 % DW, can be referred to the role of carbohydrates as internal energy storage, which is utilized in a wide variety of metabolic pathways. Given this threshold in total dissolvable carbohydrate concentration the 'EyeOnUlva' app can also be indirectly used for selecting suitable biomass sources of *U. lactuca* for the biorefinery of carbohydrates.

RGB analysis

Chlorophyll in the photosystems allows *U. lactuca* to absorb energy from light. Its strongest absorbance is within the blue portion of the visible light, followed by the red portion, while green and near-green portions are poorly absorbed and consequently reflected, which produces the green colour of the chlorophyll containing frond. This was clearly mirrored by the spectro-radiometric measurements of living specimen (Figure 3-3) and the analysis of the RGB values in images of *U. lactuca* fronds (Figure 3-5). The digitally created images capture the radiometric characteristics of the scene as realistic as possible, which is physical information about the light intensity and colour of the scene. However, we realize that a number of corrections, such as for 'gamma' and 'illumination', must be applied in the RGB format to maintain reliable and comparable results (Novoa et al. 2015). It can be argued that the relatively high correlation between protein content (% DW) and colour appearance can be contributed to chlorophyll concentrations, the main pigment protein in green seaweeds, embedded in a protein structure (Thomas & Perkins 2003), but still it leaves the applicability of our newly developed 'EyeOnUlva' as proof of concept intact, i.e. enabling to estimate protein concentration based on colour of the fronds.

Implications and applications

In this study, we quantified the relationship between frond colour and protein content (% DW) of *U. lactuca* and developed an inexpensive, fast, easy and safe to use test method to estimate

the protein content (% DW) of *U. lactuca* by digital imaging, accessible to everyone with a smartphone. A similar approach based on a print-out version of a colour comparison scale to determine total tissue nitrogen in *U. lactuca* and *Gracilaria gracilis* (Rhodophyta) was introduced by Robertson-Andersson et al. (2009). With the application EyeOnUlva we will bring the analysis a step forward and eliminate the observer's bias and standardize measurements, as well as offer a convenient and timely solution in a citizen science approach. Moreover, this study shows the fast response in colour change by *U. lactuca* to environmental changes in nutrient availability. This response gives indirect clues for changes in water quality and can support existing water monitoring techniques, such as the Forel-Ule colour comparator scale or Secchi-disk measurements, which has been applied since the 19th century to estimate the water quality of natural waters by its colour. A modern Forel-Ule 'do it yourself' colour comparator for environmental monitoring has been developed by Novoa et al. (2014), with its digital version 'EyeOnWater' successfully in use (eyeonwater.org/colour). The 'EyeOnUlva' application joins this conceptual idea of colorimetric techniques and not only represents a useful tool to the aquaculture industry to assess the nutritional value of their seaweed crop and determine its feeding quality in a cost-effective way, but is also applicable in environmental surveys, including citizen science programs. The 'EyeOnUlva' application can be freely downloaded from the website www.eyeonwater.org. The data collection is part of the CITCLOPS project (www.citclops.eu).

3.6 Acknowledgements

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Chapter 4

Uptake kinetics and storage capacity of dissolved inorganic phosphorus and corresponding dissolved inorganic nitrate uptake in *Saccharina latissima* and L*aminaria digitata* (Phaeophyceae)

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4.1 Abstract

Uptake rates of dissolved inorganic phosphorus (DIP) and dissolved inorganic nitrogen (DIN) under unsaturated (V_S) and saturated conditions (V_M) were studied in young sporophytes of the seaweeds *Saccharina latissima* and *Laminaria digitata* (Phaeophyceae) using a 'pulse-and-chase' assay under fully controlled laboratory conditions. In a subsequent second 'pulse-and-chase' assay, internal storage capacity (ISC) was calculated based on V_M and the parameter for photosynthetic efficiency F_v/F_m. Sporophytes of *S. latissima* showed a V_S of 0.80±0.03 μmol·cm^{-2}·d^{-1} and a V_M of 0.30±0.09 μmol·cm^{-2}·d^{-1} for DIP, while V_S for DIN was 11.26±0.56 μmol·cm^{-2}·d^{-1} and V_M was 3.94±0.67 μmol·cm^{-2}·d^{-1}. In *L. digitata*, uptake kinetics for DIP and DIN were substantially lower: V_S for DIP did not exceed 0.38±0.03 μmol·cm^{-2}·d^{-1} while V_M for DIP was 0.22±0.01 μmol·cm^{-2}·d^{-1}. V_S for DIN was 3.92±0.08 μmol·cm^{-2}·d^{-1} and the V_M for DIN was 1.81±0.38 μmol·cm^{-2}·d^{-1}. Accordingly, *S. latissima* exhibited a larger ISC for DIP (27 μmol·cm^{-2}) than *L. digitata* (10 μmol·cm^{-2}), and was able to maintain high growth rates for a longer period under limiting DIP conditions. Our standardized data add to the physiological understanding of *S. latissima* and *L. digitata*, thus helping to identify potential locations for their cultivation. This could further contribute to the development and modification of applications in a bio-based economy, for example in evaluating the potential for bioremediation in integrated multi-trophic aquacultures (IMTA) that produce biomass simultaneously for use in the food, feed and energy industries.

4.2 Introduction

Dissolved inorganic phosphorus (DIP) and dissolved inorganic nitrogen (DIN) are essential macronutrients for maintaining the metabolism and growth of seaweeds. P and N are a key components of nucleic acids, phospholipids, adenosine triphosphate (ATP) and are also involved in controlling enzyme reactions and in the regulation of metabolic pathways. After N, P

is the second most frequently limiting macronutrient in seaweed growth. Nutrient limitation and shifts in limitation from one element to another can significantly affect the internal composition, physiology and growth of seaweeds (Pederson & Borum 1996, Gevaert et al. 2001). These processes can reflect natural fluctuations, but can also be driven by anthropogenic emissions. For example, agricultural run-off waters contain considerable amounts of inorganic phosphate (PO_4^{3-}) and nitrogenous compounds, like nitrate (NO_3^-) and ammonium (NH_4^+) (Sharpley et al. 1992, Rabalais et al. 2009). Anthropogenic discharge can also generate nutrient concentration gradients, which are often observed along coastal zones due to the proximity of nutrient sources. This can not only lead to alterations in the type and magnitude of nutrient limitations, but may also cause effects of eutrophication. In the North Sea, measures against eutrophication were first installed in the mid 1980's, when its dramatic effects on marine flora and fauna became evident (Westernhagen & Dethlefsen 1983, Malta & Verschuure 1997, Lyngby et al. 1999). Recently it showed, that the de-eutrophication efforts have led to a large imbalance in the N:P stoichiometry of coastal waters of the North Sea in north-western Europe (Burson et al. 2016). Increasing N:P ratios, which outpace the Redfield ratio of 16:1 were observed (Radach & Pätsch 2007, Grizzetti et al. 2012) and a pronounced P-limitation can be effective in coastal regions of the southern North Sea. This can have notable effects on the ecosystem communities and growth and functioning of primary producers. It has been reported that N availability mediates the ability of primary producers to access P, as shown for the brown seaweed *Fucus vesiculosus* Linnaeus (Perini & Bracken 2014).

The perennial brown seaweeds (Phaeophyceae) *Saccharina latissima* (Linnaeus) C.E.Lane, C.Mayes, Druehl & G.W.Saunders and *Laminaria digitata* (Hudson) J.V. Lamouroux are commonly found on the lower shores of the north Atlantic around the northern North American and European coastlines, including the North Sea. *Saccharina latissima* is also distributed along the shores of the north Pacific. As ecosystem engineers, *S. latissima* and *L. digitata* can affect sedimentation and erosion by reducing water currents (Jones et al. 1994, Bouma et al. 2005) and offer shelter, feedstock and nursery habitats to various fauna, thus enhancing the diversity of their

habitat (Jørgensen & Christie 2003). Both seaweeds are rich sources of nutrients and contain large amounts of carbohydrates in the form of structural, storage, and functional polysaccharides, as well as considerable amounts of proteins (Holdt & Kraan, 2011). Aside from the direct use of *S. latissima* and *L. digitata* for culinary and medicinal purposes, there is great interest in the refinement, extraction and application of carbohydrates and proteins in the energy and animal feed industries, as well as the extraction of important food hydrocolloids, including carrageenan and alginates (McHugh 2003, Troell et al. 2006, Holdt & Kraan 2011). However, the content of these compounds varies, depending on nutrient availability, temperature, light and hydrodynamics, alternating in accordance to season and area of cultivation (Murata & Nakazoe 2001, Connan et al. 2004).

The vast range of possibilities for using seaweed, especially *S. latissima* and *L. digitata*, has resulted in an enormous surge in interest over the last decades (McLachlan 1985), hence stimulating the efforts towards large-scale cultivation as a supplement to wild harvests (Neori 2008, Bixler & Porse 2011, Holdt & Kraan 2011, Kraan 2013). Although there is much known about the growth requirements of *S. latissima* (Bartsch et al. 2008, Reid et al. 2013, Marinho et al. 2015) and *L. digitata* (Bolton & Lüning 1982, Schaffelke & Lüning 1994, Harrison & Hurd 2001, Gordillo et al. 2002, Pederson et al. 2010), there is relatively little information available about the DIP uptake kinetics, as well as DIP and DIN management in relation to the internal storage capacity (ISC), the maximal internal duration for growth under external limiting conditions (Pederson et al. 2010). This is important information, as it allows an estimation of ecological effects on nutrient availability and can contribute to development and modification of cultivation sites. A lot of studies related to uptake kinetics for DIN and DIP in *S. latissima* and *L. digitata* have been conducted under field conditions with weekly to monthly sampling intervals (Bolton & Lüning 1982, Schaffelke & Lüning 1994, Reid et al. 2013, Marinho et al. 2015) and the majority of studies under laboratory conditions have focused on uptake of nitrogenous compounds, as NO_3^- and NH_4^+, in *S. latissima* and *L. digitata* (Chapman et al. 1978, Conolly & Drew 1985, Harrison et al. 1986). Often DIN and DIP uptake is

tested independently in short term experiments, usually ranging from minutes to hours (e.g. Runcie et al. 2003, Martínez & Rico 2004, Luo et al. 2012). Long term responses to DIN and DIP availability remain unknown.

Nutrient uptake by seaweed can be split into three distinct phases, referred to as surge uptake (V_S), metabolic or internally controlled uptake (V_M), and externally controlled uptake (V_e) (Conway et al. 1976, Harrison et al. 1989). V_S refers to the filling of internal nutrient pools, uncoupled from growth (Conway et al. 1976), and has often been described for nutrient-starved seaweeds (e.g. Fujita 1985, Harrison et al. 1989, Dy & Yap 2001). The uptake rates gradually decrease as internal nutrient pools in cytoplasm and vacuoles are filled (Rosenberg et al. 1984, Fujita 1985). When internal nutrient concentrations are constant and relative uptake rates of nutrients remain relatively stable over time, $V_{M,}$ which is considered equal to the rate of assimilation, is attained (Taylor & Rees 1999, Barr et al. 2004). The previously filled nutrient pools can be utilized at times of low external nutrient availability (Probyn & Chapman 1982, Pederson & Borum 1996). The internal storage capacity (ISC) and temporal duration of the filled nutrient pools under external nutrient depletion conditions has hardly been focused on in seaweeds (Fujita et al. 1985).

Experimental studies under controlled conditions are critical to further understand the role of nutrients and shifts in nutrient ratios, and will strengthen the understanding of nutrient demand and strategies by seaweeds. This is of great ecological and economic importance, as it will open up opportunities to forecast the impacts of nutrient limitation and will shed light on possible competitive advantages of one species versus the other under shifts in limitation from one element to another. It will also facilitate to identify potential locations for seaweed mariculture and provide insight into optimal cultivation practices in regard to nutrient additions.

4.3 Material and Methods

In this study, we present the DIP- and DIN-uptake kinetics of young *S. latissima* and *L. digitata* sporophytes exposed to a range of nominal DIP concentrations (0 - 6 $\mu mol \cdot L^{-1}$) and non-limiting DIN concentration (50 $\mu mol \cdot L^{-1}$) under laboratory conditions, controlling for temperature, light and hydrodynamics in a 'pulse-and-chase' assay (i.e. adding a pulse of nutrients and following their removal from the water over time). In a second 'pulse-and-chase' experiment under the same laboratory conditions for light, temperature, and hydrodynamics, sporophytes of both species were exposed to DIP-depleted, DIN-depleted, DIP and DIN-depleted, and DIP- and DIN-enriched seawater. Thereafter, the fluorescence signal F_v/F_m, which is a measure of plant stress/photosynthetic efficiency, was measured over 9 weeks. Based on this data, the DIP- and DIN-uptake kinetics as well as the internal storage capacity of DIP and DIN in *S. latissima* and *L. digitata* were quantified and standardized for surface area (SA).

All experiments and analyses were conducted at the Royal Netherlands Institute for Sea Research (NIOZ) located on Texel, The Netherlands. Cultured sporophytes of *S. latissima* and *L. digitata*, offspring from plants originated and collected from the coastline of Den Helder, The Netherlands, were transferred from incubation tanks at the NIOZ Seaweed Centre (www.nioz.nl/en/expertise/seaweed-research-centre) into 4 separate (2 for each species) transparent 20L Nalgene™ bottles (Nalge Nunc International Corporation, Rochester, NY, USA), filled with 15L seawater medium, inside a temperature-controlled room (12.0±0.6 °C, measured hourly by HOBO temperature loggers (Onset, Bourne, MA, USA)), for an adaptation phase under fully controlled laboratory conditions. Two tubular fluorescent lamps (OSRAM L18 Watt 965, Deluxe cool daylight), attached 50 cm above the flasks and covered by two layers of black mosquito netting, provided a PAR light intensity of 18±3 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (n=9; light meter ULM- 500, Walz, Germany) inside the glass flasks in a set light/dark period of 16/8 h. The low light concentration was installed to avoid light induced stress on the sporophytes, as previous cultivation of young

individuals in light concentrations of 70-80 μmol·m^{-2}·s^{-1} (after Lüning 1979, Andersen et al. 2011) in a light/dark period of 16/8 h led to frond-bleaching within 2 days (not depicted). A moderate water movement inside the 20L bottles was provided by aeration, produced by a common air pump (AquaForte V-20, Hailea Group Co., Ltd., China) outside the bottles, which was connected to a PVC hose, connected to a glass pipette (25 ml) inside a bottle.

We used 2 experimental approaches: (1) analysing DIP and DIN uptake kinetics under unsaturated (V_S) and saturated states (V_M) of the two seaweeds, and (2) estimation of their internal storage capacity (ISC) for DIP and DIN, based on V_M and the fluorescence protocol of F_v/F_m, as an indicator for (nutritional) stress. Based on the different 2 experimental approaches to determine DIP and DIN uptake kinetics and ISC for DIP and DIN, the nutrient concentration in the seawater medium inside the 20L bottles differed during adaptation phase, while other parameters like light, temperature, and hydrodynamics were kept constant.

Experimental approach 1

Sporophytes of both species were maintained in nutrient-depleted seawater (PO_4^{3-} = 0.008 μmol·L^{-1}, NH_4^+ = 0.022 μmol·L^{-1} and NO_3^- = 0.003 μmol·L^{-1}) for a 15-day adaptation phase in experimental approach 1, which is similar to the experimental set-up used for determining uptake kinetics in *U. lactuca* (Chapter 2). Exposing the sporophytes to nutrient-depleted seawater ensured nutrient starvation, as data for their nutritional history was not available. After this starvation phase, 49 randomly picked sporophytes of *S. latissima* and *L. digitata* with a frond size range of 1.5 to 6.5 cm^2, respectively 5.5 to 29.9 cm^2 (Figure 4-1) were individually transferred into 200 ml glass jars filled with 100 ml of seawater medium enriched with a range of dissolved inorganic phosphate levels (DIP: 0.0 μmol·L^{-1}, 0.2 μmol·L^{-1}, 0.4 μmol·L^{-1}, 0.8 μmol·L^{-1}, 1.5 μmol·L^{-1}, 3.0 μmol·L^{-1} and 6.0 μmol·L^{-1}) and a non-limiting concentration of dissolved inorganic nitrogen (DIN: 50 μmol·L^{-1}). The installed DIP concentrations, as well as DIN concentration, covered the range of observed natural concentrations in coastal areas of the NE Atlantic, respectively neighboring seas like the

North Sea, which seasonal extremes (winter concentrations) show an overall average of 2-4 μmol·L^{-1} for DIP and 60-90 μmol·L^{-1} for DIN for the years 2006-2014 (OSPAR assessment report 2017; https://www.oap.ospar.org/en/ospar-assessment/intermediate-assessment-2017/pressures-human-activities/eutrophication/nutrient-concentration; retrieved in August 2018). The investigation on higher DIP concentrations, as in nominal concentration of 6.0 μmol·L^{-1} could be of interest for nursery operations of the seaweeds, as well as integrated multi-trophic aquaculture (IMTA) activities with young sporophytes or bioremediation purposes.

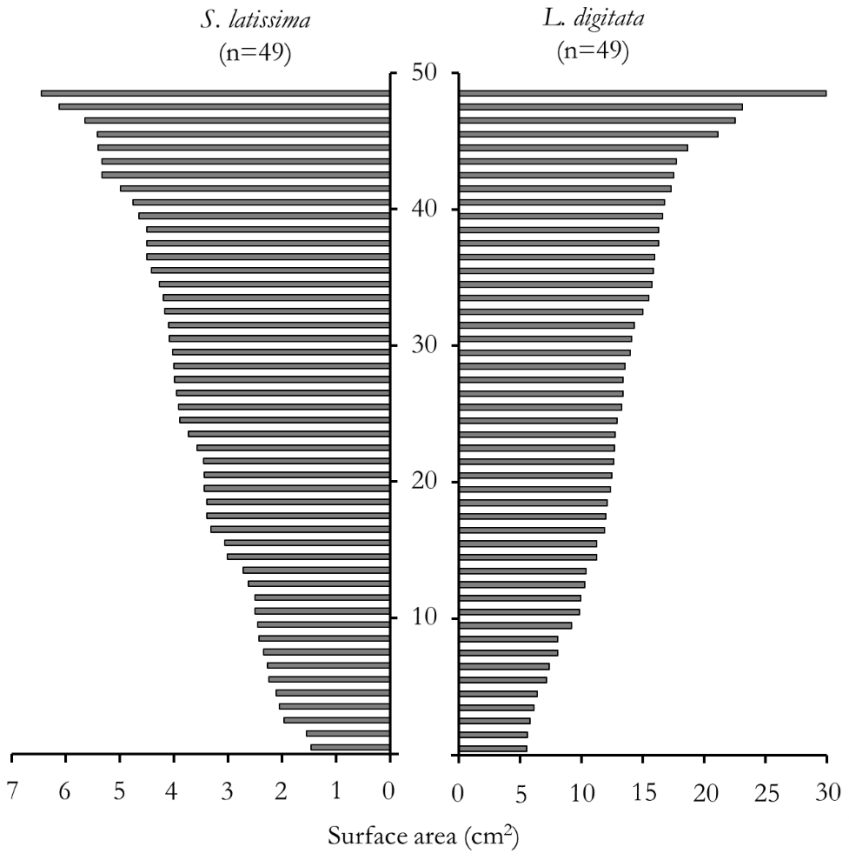

Figure 4-1. Size range of initial surface area (cm^2) of *Saccharina latissima* and *Laminaria digitata* sporophytes applied in experiments on nutrient uptake kinetics (experimental approach 1).

The seawater medium was refreshed ("pulsed") and samples of the day-old medium were taken ("chased") for dissolved nutrient analysis on a daily basis for 3 weeks, as the removal of DIP and DIN from the seawater medium were referred to uptake rates by the seaweed. After daily refreshment of the seawater medium, all flasks were randomly distributed to minimize differences in light availability on a rotating table, which provided moderate water movement at a speed of 100 rpm. This constant water movement was maintained for optimal mixing and, hence, availability of nutrients by decreasing the diffusion boundary layers between tissues and the growing medium (e.g. Gonen et al. 1995, Hurd 2000).

Seawater medium

The base for the seawater medium was nutrient-poor seawater from the North Atlantic Ocean (salinity 34.5) with low phosphate (PO_4^{3-}; 0.008 $\mu mol \cdot L^{-1}$), ammonium (NH_4^+; 0.022 $\mu mol \cdot L^{-1}$) and nitrate (NO_3^-; 0.003 $\mu mol \cdot L^{-1}$) concentrations. The seawater was pasteurized (80 °C for 2 h) and salinity was adjusted to 29.5 to reflect the values measured at the NIOZ Seaweed Research Centre and around the island of Texel by mixing with ultrapure water (Milli-Q, Merck KGaA, Massachusetts, USA). Afterwards, potassium-dihydrogen-phosphate (KH_2PO_4) and potassium nitrate (KNO_3) were added as sources for DIP and DIN to create the desired DIP concentrations of 0.0, 0.2, 0.4, 0.8, 1.5, 3.0 and 6.0 $\mu mol \cdot L^{-1}$ and DIN concentration of 50 $\mu mol \cdot L^{-1}$. The pH of the medium, after pasteurization and DIN and DIP addition, was 8.1±0.1 (n=14) as measured with a pH-Meter (GHM-3511, Greisinger, Germany).

Nutrient analysis

Dissolved inorganic nutrients (DIP and DIN) were measured with colorimetric analysis using a Technicon TRAACS 800 auto-analyzer (Seal Analytical, Germany) in the NIOZ Texel nutrient laboratory. DIP was measured as ortho-phosphate (PO_4^{3-}) at 880 nm after the formation of molybdophosphate complexes (Murphy & Riley 1962). For DIN measurements (nitrate and nitrite), nitrate was first reduced to nitrite through a copperized cadmium coil and color intensity

was measured at 550 nm after complexation with sulphanylamide and naphtylethylenediamine (Grasshoff et al. 1983). Ammonium (NH_4^+) was measured at 630 nm after the formation of an indophenol blue complex with phenol and sodium hypochlorite at a pH of 10.5. Citrate was used as a buffer and complexant for calcium and magnesium at this pH (Koroleff 1969 and optimized by Helder & de Vries 1979). The low NH_4^+-concentration (0.022 $\mu mol \cdot L^{-1}$) was not further considered, as no NH_4^+ was added in the experiments. The precision for all the measured channels within the automated nutrient analyzer was higher than 0.25 % (personal communication K. Bakker, NIOZ).

DIP and DIN uptake dynamics

DIP and DIN uptake refers to the removal of these nutrients from the medium by *S. latissima* and *L. digitata*. Daily uptake rates (V) were derived from changes in the nutrient concentrations of the seawater medium each day, which were normalized for SA (cm^2) and time (d) using the following calculation:

$$V = (T_1 - T_2) \times SA^{-1} \cdot t^{-1},$$

with T_1 as the initial nutrient concentration, T_2 as the nutrient concentration before water exchange after 24 h, SA as surface area (cm^2) and t as the incubation time (hours).

Two different uptake rates were classified over time: surge uptake (V_S) after starvation and maintenance uptake with filled nutrient pools (V_M). V_S was calculated from uptake rates under conditions of non-limiting nutrient concentration using the following equation:

$$V_S = (V_2 - V_1) \times (d_2 - d_1)^{-1} = \Delta V \times \Delta d^{-1},$$

where V_1 and V_2 are daily uptake rates on days before a significant decline in uptake rate occurs and no significant variations in nutrient uptake follow. The difference operator between the two days is represented by d_1 and d_2. V_M is calculated as the average uptake rate under non-limiting

nutrient concentration after a significant decrease has occurred and subsequent uptake rates show no significant variations.

Surface area analysis

Sporophytes of both species were individually spread flat on a white background, placed next to a ruler for scale, and covered with a transparent Plexiglas sheet to avoid folding of the frond. Photographs (using a Panasonic Lumix DMC-FT5) were taken on a weekly basis, enabling analysis of surface area (SA) by using the open source software ImageJ (ImageJ, U. S. National Institutes of Health, Maryland, USA). Photographs were converted into grayscale (type 8-bit) and transformed into a binary image before SA analysis. The obtained SA represents one side of the frond. Differences in SA over time were used as indices of growth, with relative growth rates (μ) calculated according to Kain (1987) as follows:

$$\mu = (\ln SA_1 - \ln SA_2) \times t^{-1},$$

where SA_1 represents the initial surface area, and SA_2 represents the final surface area after incubation time t.

Experimental approach 2

In experimental approach 2, young sporophytes of *S. latissima* and *L. digitata* were placed inside 20 L bottles filled with 15 L DIP and DIN enriched seawater medium (DIP: 3 μmol·L^{-1}, DIN: 50 μmol·L^{-1}) for a 21-day adaptation phase under laboratory conditions. The nutrient-enriched seawater was renewed every other day to ensure saturated storage pigments after the adaptation phase. In experimental approach 2, individual sporophytes of *S. latissima* (n=20) and *L. digitata* (n=20) were transferred from the 20 L bottles into 500 ml glass jars filled with 200 ml seawater medium, which were either DIP and DIN enriched (DIP: 3 μmol·L^{-1}, DIN: 50 μmol·L^{-1}, n=5), DIP depleted and DIN enriched (DIP: 0 μmol·L^{-1}, DIN: 50 μmol·L^{-1}, n=5), DIP enriched and DIN depleted (DIP: 3 μmol·L^{-1}, DIN: 0 μmol·L^{-1}, n=5), or DIP and DIN depleted (n=5).

The seawater media were refreshed on a daily basis throughout the experiment. Before refreshment of the seawater medium, fluorescence measurements (F_v/F_m) were conducted every other day and after daily refreshment of the seawater medium, all jars were placed on a rotating table (100 rpm) to provide a moderate water movement, while a random distribution of the jars minimized differences in light availability.

Fluorescence measurements

Fluorescence measurements to determine photosynthetic efficiency (F_v/F_m, F_v refers to variable fluorescence and F_m refers to maximum fluorescence) were conducted every other day over a period of 66 days for *S. latissima* (n=5) and 54 days for *L. digitata* (n=5) in all four treatments of experiment II. Sporophytes were dark-adapted for 20 minutes before photosynthetic efficiency was measured using a pulse-amplitude modulated fluorimeter (JUNIOR-PAM, Walz, Effeltrich, Germany; settings: measuring light intensity=10, pulse width=0.8s, gain=2) attached to a laptop. These measurements were carried out under minimum light conditions (laptop screen as the only light source) in a temperature-controlled room set to 12 °C around the same daytime. Each sporophyte was measured twice at different locations on the frond in an interval of 40 seconds.

Internal storage capacity

The internal storage capacities (ISC) for DIP and DIN were derived from the response of seaweed F_v/F_m when cultured in DIP- and DIN-depleted seawater. All, under the premise that internal storages for DIP and DIN had been filled during adaptation phase and the seaweed was not nutrient starved at the start of the experiment. Either DIN or DIP concentrations that were retrieved as non-limiting in previous experimental set-up I were pulsed for potentially optimal conditions. A control was installed, adding both nutrients, DIP and DIN concentrations. A significant decrease in F_v/F_m under limitation/depletion conditions was postulated to reflect a stress reaction by seaweeds to internal DIP and/or DIN depletion, as parameters, like temperature,

light and hydrodynamics were fully controlled. As V_M is considered equal to the rate of assimilation (Taylor & Rees 1999, Barr et al. 2004), the ISC was calculated as follows:

$$ISC = \Delta t \cdot V_M,$$

where Δt represents the duration (days) with initially filled internal nutrient storages under depletion conditions, before a significant decrease in F_v/F_m occurred, and V_M represents the daily maintenance or metabolic uptake rate.

Statistics

Data of both experimental approaches were tested for normality with the Kolmogorov-Smirnoff test (KS test) for cumulative probability distribution. A two-sided repeated measures ANOVA was applied to test for significant differences in growth, nutrient uptake rates, and Fv/Fm within and between treatments with different nutrient concentrations.

4.4 Results

Experimental approach 1

Surface area analysis

The increase in SA, as a measure of growth of *S. latissima* and *L. digitata* sporophytes displayed significant differences between DIP treatments over time (respectively ANOVA df=6, F=2.24, p=0.042; df=6, F=9.47, p<0.001). The highest growth rates for *S. latissima* were found in low to intermediate DIP treatments receiving nominal concentrations of 3.0 $\mu mol \cdot L^{-1}$ or less, which were not significantly different in growth from each other (ANOVA df=5, F=2.28, p=0.545). Mean SA increased by the factor 1.84±0.14 in 23 days (n=42, Figure 4-2), representing a growth rate of 4 % d^{-1}. *S. latissima* cultured in high nominal DIP concentrations of 6.0 $\mu mol \cdot L^{-1}$ exhibited significantly lower growth compared with sporophytes in other treatments (ANOVA

df=1, F=4.04, p=0.004). Mean SA increased by the factor 1.19±0.21 in 9 days, before growth stagnated after 15 days and a negative growth with signs of texture loss and disintegration of the sporophytes was observed on day 23, the final measurement of SA (n=7, Figure 4-2).

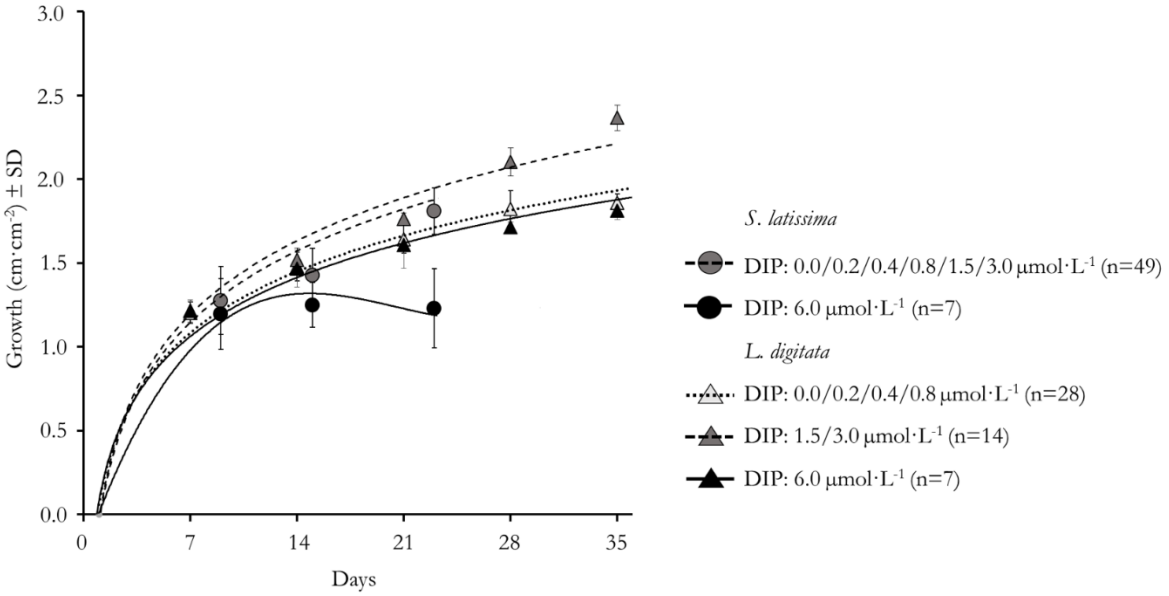

Figure 4-2. Mean growth ± SD (cm·cm^{-2}) of young *Saccharina latissima* and *Laminaria digitata* cultivated in different DIP concentration (0 - 6 µmol·L^{-1}, n=7) and saturating DIN concentration (50 µmol·L^{-1}) in a 'pulse-and-chase' assay over 5 weeks. Data is depicted according to significant differences in increase of surface area (growth) of the sporophytes in different DIP concentrations.

Laminaria digitata showed the highest growth rates when exposed to intermediate nominal DIP concentrations of 1.5 µmol·L^{-1} and 3.0 µmol·L^{-1}, and there were no significant differences in relative increase of SA among these treatments (ANOVA df=1, F=0.46, p=0.502). Mean SA increased by the factor 2.37±0.08 in 35 days (n=14, Figure 4-2), exhibiting a growth rate similar to *S. latissima* in low to intermediate DIP treatments. Sporophytes cultivated under low nominal DIP conditions of 0.8 µmol·L^{-1} or less, showed a significantly smaller increase in SA (ANOVA df=3, F=3.39, p<0.001), which was comparable to *L. digitata* exposed to high nominal DIP concentration of 6.0 µmol·L^{-1} (ANOVA df=1, F=11.1, p=0.001). The relative increase in SA of sporophytes in

these treatments increased by the factor 1.86±0.05, respectively 1.81±0.05, in 35 days (n=28, respectively n=7, Figure 4-2), which translates to a growth rate of 2 % d^{-1}.

DIP-uptake dynamics

Sporophytes of *S. latissima* exposed to very low nominal DIP concentration of 0.2 μmol·L^{-1}, 0.4 μmol·L^{-1} and 0.8 μmol·L^{-1} depleted all supplied DIP within the daily sampling period of 24 hours throughout the experiment, which indicates non-saturating DIP concentrations to the nutrient starved sporophytes (not depicted). When exposed to nominal DIP concentrations of 1.5 μmol·L^{-1}, all supplied DIP was depleted until day 9, after which uptake significantly decreased (ANOVA df=1, F=6.37, p=0.021) and mean uptake rates levelled off from 0.30±0.03 μmol·cm^{-2}·d^{-1} to 0.22±0.01 μmol·cm^{-2}·d^{-1} until day 22 (n=7, Figure 4-3 A) with no significant variations (ANOVA df=12, F=1.38, p= 0.220), indicating saturating DIP conditions. Similarly but with uptake declining earlier, sporophytes grown in a nominal DIP concentration of 3.0 μmol·L^{-1} depleted all daily supplied DIP until day 4, followed by a significant decline of mean uptake rates (ANOVA df=1, F=5.91, p=0.007) from 0.80±0.03 μmol·cm^{-2}·d^{-1} to 0.40±0.04 μmol·cm^{-2}·d^{-1} on day 7 (n=7, Figure 4-3 B), after which no significant variations in DIP uptakes rates were found (ANOVA df=14, F=1.29, p=0.226). *S. latissima* exposed to a high nominal DIP concentration of 6.0 μmol·L^{-1} showed highly significant variations in DIP uptake both within treatment (ANOVA df=6, F=7.31, p<0.001) and over time (ANOVA df=21, F=5.79, p<0.001). Sporophytes depleted all supplied DIP on days 1 and 2 with a mean uptake rate of 1.66±0.10 μmol·cm^{-2}·d^{-1} (n=7), which was followed by a daily decline and final collapse of mean DIP uptake rates to 0.05±1.28 μmol·cm^{-2}·d^{-1} (n=7) on days 21 and 22 (Figure 4-3 C). At this point, 5 of 7 young sporophytes had lost their texture and started to disintegrate.

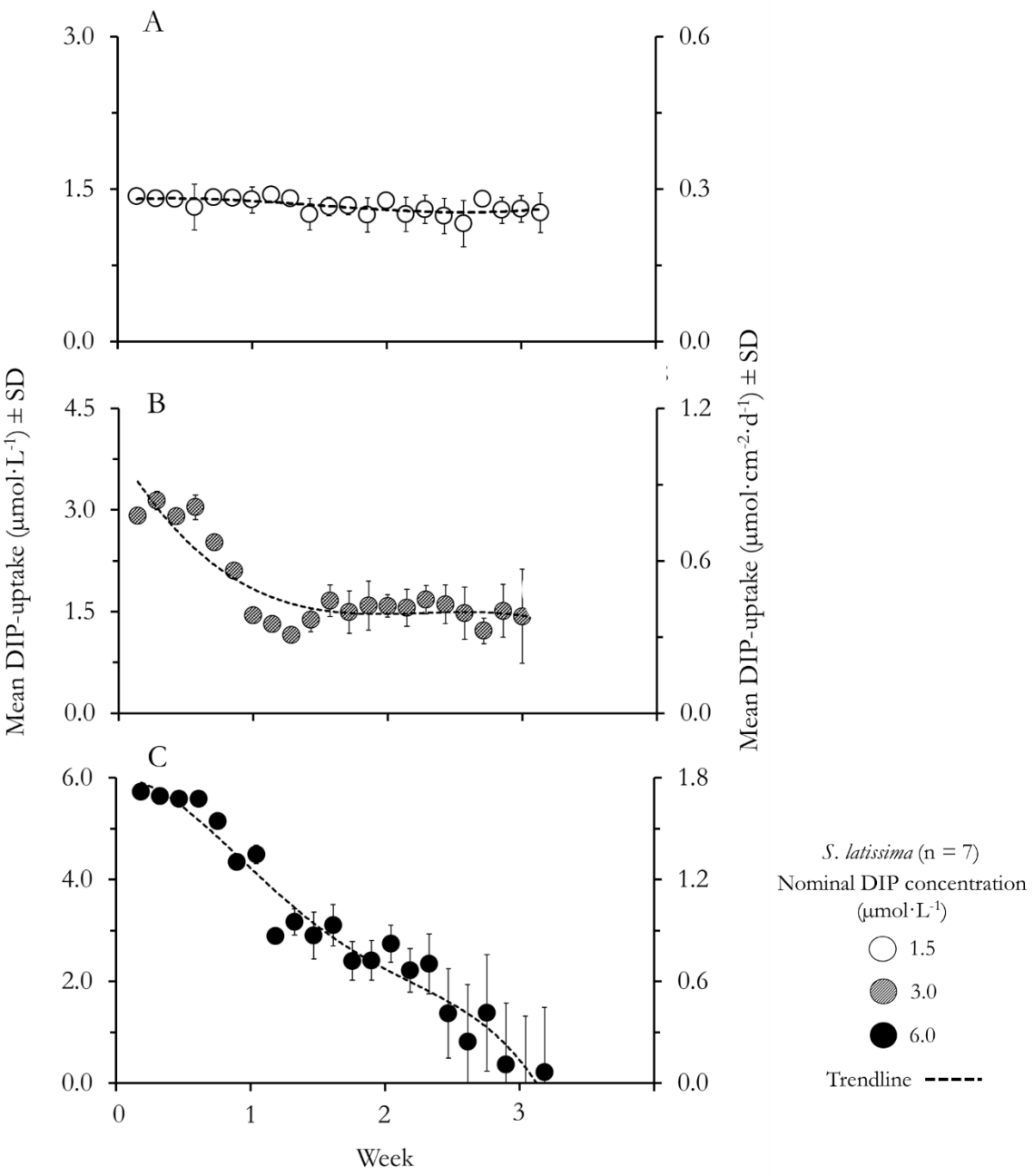

Figure 4-3. Mean DIP uptake (µmol·L^{-1}) ± SD of young *Saccharina latissima* (n=7) in saturating nominal DIP concentrations of (A) 1.5 µmol·L^{-1}, (B) 3.0 µmol·L^{-1} and (C) 6.0 µmol·L^{-1} and corresponding standardized daily DIP uptake (µmol·cm^{-2}·d^{-1}) in a 'pulse-and-chase' assay over 3 weeks.

Based on DIP uptake rates of *S. latissima* under saturated states in nominal DIP concentrations of 1.5 μmol·L^{-1} and 3.0 μmol·L^{-1}, the calculated V_M-DIP was 0.30±0.09 μmol·cm^{-2}·d^{-1} (n=14). V_S for DIP was calculated to be 0.80±0.03 μmol·cm^{-2}·d^{-1} (average ± SD, n=7), which was based on DIP uptake rates of sporophytes exposed to a nominal concentration of 3.0 μmol·L^{-1} on days 1 to 4. A maximum surge uptake rate was calculated to be 1.66±0.10 μmol·cm^{-2}·d^{-1} (average ± SD, n=7), based on DIP uptake rates of the young sporophytes exposed to a nominal DIP concentration of 6.0 μmol·L^{-1} on day 1. Sporophytes in this treatment disintegrated within 3 weeks and high uptake rates were referred to as a stress reaction to the unusually high DIP concentrations. Sporophytes of *L. digitata* cultured in nominal DIP concentrations of 0.2 μmol·L^{-1}, 0.4 μmol·L^{-1}, 0.8 μmol·L^{-1}, 1.5 μmol·L^{-1}, and 3.0 μmol·L^{-1} depleted all of the supplied DIP within the 24-hour sampling period throughout the experiment, which indicates non-saturating DIP concentrations (depicted for 3.0 μmol·L^{-1}, Figure 4-4 A). In contrast, mean DIP uptake of *L. digitata* cultured in a high nominal concentration of 6.0 μmol·L^{-1} did not lead to depletion throughout the experiment, thus indicating a saturating concentration. Mean DIP uptake rates varied around 0.37±0.03 μmol·cm^{-2}·d^{-1} between day 1 and day 10 before a significant decrease occurred (ANOVA df=1, F=8.50, p=0.013). Within day 11 and 21, mean uptake rates stabilized at 0.24±0.04 μmol·cm^{-2}·d^{-1} (n=7, Figure 4-4 B). The V_M-DIP of *L. digitata* was calculated to be 0.22±0.01 μmol·cm^{-2}·d^{-1} (n=14), while V_S-DIP was determined to be approximately twice as high as V_M-DIP at 0.37±0.03 μmol·cm^{-2}·d^{-1} (average ± SD, n=7).

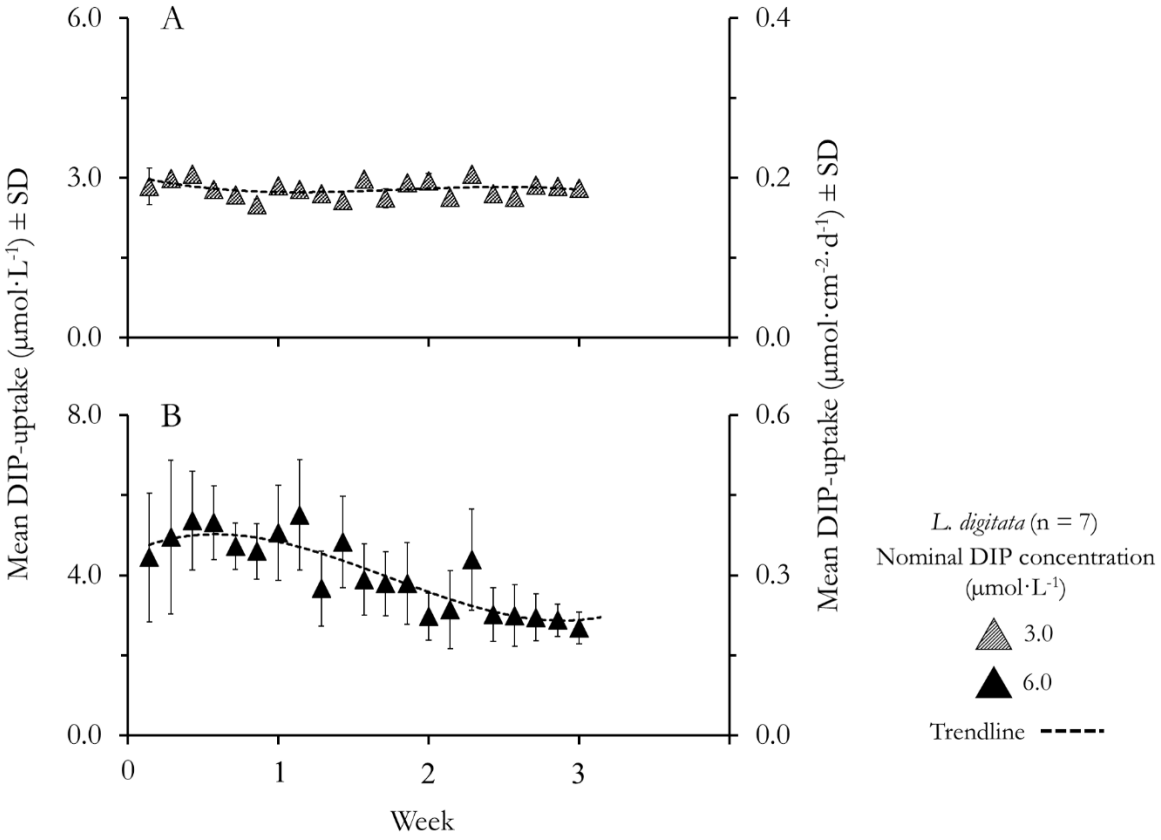

Figure 4-4. Mean DIP uptake (µmol·L^{-1}) ± SD of young *Laminaria digitata* (n=7) in (A) un-saturating nominal DIP concentration of 3.0 µmol·L^{-1} and (B) saturating nominal DIP concentration of 6.0 µmol·L^{-1} and corresponding standardized daily DIP uptake (µmol·cm^{-2}·d^{-1}) in a 'pulse-and-chase' assay over 3 weeks.

DIN-uptake dynamics

Saccharina latissima showed no significant differences in daily DIN uptake rates among different DIP treatments (ANOVA df=6, F=1.71, p=0.116) but did display a highly significant difference in uptake over time (ANOVA df=21, F=5.35, p<0.000). No correlation between DIN and DIP uptake (R=0.415) was found. The mean DIN uptake oscillated downwards from a high of 11.26±0.56 µmol·cm^{-2}·d^{-1} (n=49) on day 1, which represents the V_S-DIN of *S. latissima*, to 5.46±0.77 µmol·cm^{-2}·d^{-1} (n=49) by day 14. After these two weeks, the DIN uptake stayed around 4.07±0.82 µmol·cm^{-2}·d^{-1} without any significant variation (ANOVA df=6, F=1.94, p=0.097) until

the end of the experiment on day 22 (Figure 4-5). A V_M-DIN of 3.94±0.67 µmol·cm^{-2}·d^{-1} was conclusively calculated, which is approximately three times lower than V_S-DIN.

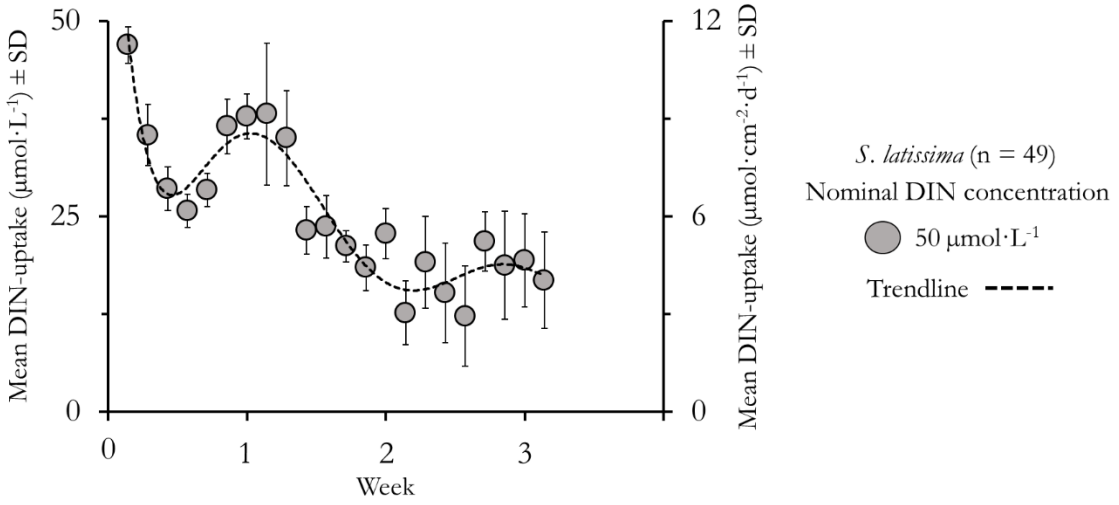

Figure 4-5. Mean DIN uptake (µmol·L^{-1}) ± SD of young *Saccharina latissima* (n=49) cultivated in nominal DIN concentration of 50 µmol·L^{-1} and corresponding standardized daily DIN uptake (µmol·cm^{-2}·d^{-1}) in a 'pulse-and-chase' assay over 3 weeks.

Laminaria digitata also showed no significant differences in DIN uptake rates among different DIP treatments (ANOVA df=6, F=1.21, p=0.306), but exhibited a highly significant difference in DIN uptake over time (ANOVA df=20, F=28.46, p<0.001). Similar to *S. latissima*, no correlation between DIN and DIP uptake (R=0.229) was found. DIN uptake rates showed no significant variations within day 1 and 8 (ANOVA df=6, F=0.27, p=0.897) with a mean uptake of 3.72±0.56 µmol·cm^{-2}·d^{-1} (n=49, Figure 4-6). In correspondence, a V_S-DIN of 3.92±0.08 µmol·cm^{-2}·d^{-1} (n=49) was calculated. A significant decrease in uptake rates was observed within day 9 and 14 (ANOVA df=5, F=5.44, p=0.001). After this, DIN uptake stabilized without significant variations (ANOVA df=4, F=0.70, p=0.590) at 1.81±0.38 µmol·cm^{-2}·d^{-1} between day 16 and 21 (Figure 4-6), which also represents V_M-DIN for *L. digitata*.

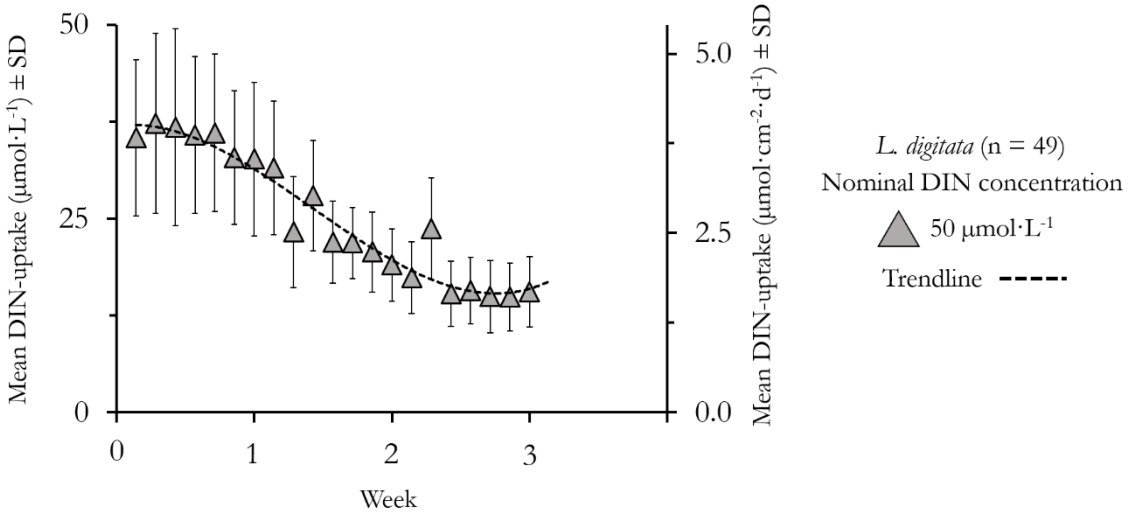

Figure 4-6. Mean DIN uptake (µmol·L^{-1}) ± SD of young *Laminaria digitata* (n=49) cultivated in nominal DIN concentration of 50 µmol·L^{-1} and corresponding standardized daily DIN uptake (µmol·cm^{-2}·d^{-1}) in a 'pulse-and-chase' assay over 3 weeks.

Experimental approach 2

Based on the results on DIP and DIN uptake kinetics for *S. latissima* and *L. digitata* and in regard to saturating concentrations in experimental approach 1, nominal concentrations of 3.0 µmol·L^{-1} DIP and 50 µmol·L^{-1} DIN were chosen in experimental approach 2 for the control group (n=5) to ensure non-limiting nutrient availability throughout the experiment without inducing nutritional stress.

Fluorescence measurements

The mean photosynthetic efficiency (F_v/F_m) of *S. latissima* showed significant variations between treatments (ANOVA df=3, F=17.78, $p<0.001$) and over time (ANOVA df=34, F=5.09, $p<0.001$). The control group exposed to treatments with DIP and DIN additions of 3 µmol·L^{-1} and 50 µmol·L^{-1}, respectively, *S. latissima* expressed no significant differences in mean F_v/F_m (ANOVA df=1, F=0.18, p=0.686), but displayed moderate fluctuations around a F_v/F_m of

0.78±0.04 (n=5) over 10 weeks (Figure 4-7 A). A comparable performance in F_v/F_m was observed in treatments under DIP-depleted conditions, where no significant difference in photosynthetic efficiency was found (ANOVA df=1, F=3.58, p=0.095) and mean F_v/F_m stayed around 0.77±0.04 throughout the experiment (n=5, Figure 4-7 B). When exposed to DIN-depleted conditions, however, mean F_v/F_m significantly decreased (ANOVA df=23, F=2.04, p=0.007) from 0.78±0.04 to 0.70±0.08 after 6 weeks (n=5, Figure 4-7 C) with no significant variations thereafter (ANOVA df=10, F=0.17, p=0.998). Similarly, mean F_v/F_m of *S. latissima* sporophytes exposed to total DIP and DIN depletion displayed no significant variations until week 7 (ANOVA df=22, F=1.35, p=0.177), followed by a significant decrease (ANOVA df=23, F=2.37, p=0.002) from 0.77±0.03 to 0.65±0.16 during week 10 (n=5, Figure 4-7 D). The photosynthetic efficiency of *L. digitata* also exhibited significant differences between different treatments (ANOVA df=3, F=11.79, p<0.001) and over time (ANOVA df=29, F=5.26, p>0.001). Sporophytes of the control group exposed to a DIP and DIN concentration of 3 $\mu mol \cdot L^{-1}$ and 50 $\mu mol \cdot L^{-1}$, respectively, showed no significant variations in F_v/F_m over time (ANOVA df=6, F=0.77, p=0.406) and the mean photosynthetic efficiency was 0.74±0.06 throughout the experiment (n=5, Figure 4-7 E). However, photosynthetic efficiency was more sensitive to DIP and DIN depletion than in *S. latissima*. When exposed to DIP-depleted, DIN-depleted, and both DIP and DIN-depleted seawater medium, the mean F_v/F_m significantly decreased after 6 weeks (ANOVA DIP-depleted, df=22, F=2.41, p=0.025; DIN-depleted, df=6, F=8.51, p=0.043; DIP/DIN-depleted, df=23, F=2.40, p<0.001). Under DIP depletion, the mean photosynthetic efficiency dropped from 0.73±0.05 to 0.50±0.20 during week 8 (n=5, Figure 4-7 F), while mean F_v/F_m under DIN depletion decreased comparably from 0.67±0.10 to 0.53±0.25 during week 8 (n=5, Figure 4-7 G). *L. digitata* sporophytes under DIP and DIN depletion displayed a moderate decrease in mean F_v/F_m from 0.67±0.07 to 0.55±0.15 between week 3 and 6, after which the mean photosynthetic efficiency significantly dropped to 0.34±0.24 during week 8 (n=5, Figure 4-7 H).

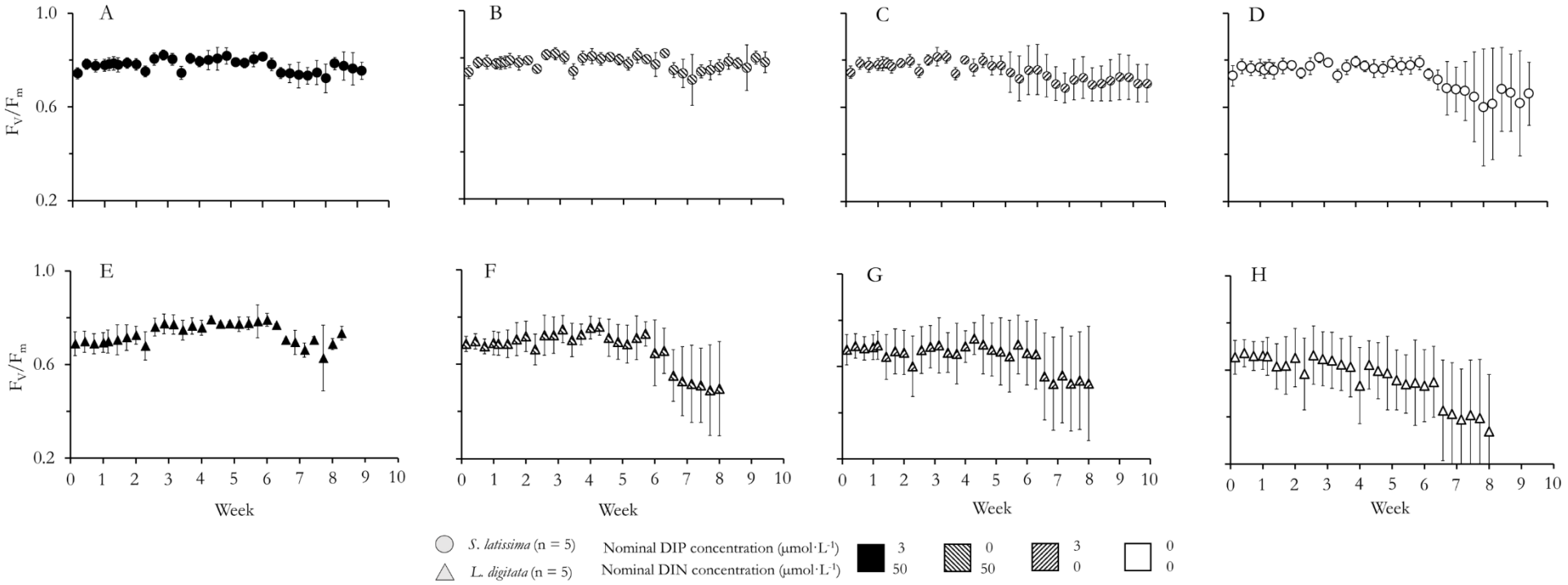

Figure 4-7. Photosynthetic efficiency F_v/F_m of *Saccharina latissima* (A – D, n=5) and *Laminaria digitata* (E – H, n=5) cultivated in DIP/DIN-enriched (A and E), DIP-depleted (B and F), DIN-depleted (C and G), and DIP/DIN-depleted (D and H) seawater medium in a 'pulse-and-chase' assay over 10 weeks, respectively 8 weeks.

Internal storage capacity

Using the fluorescence measurements and duration time, before a significant decrease in F_v/F_m in different treatments occurred and the daily DIP and DIN uptake rates under V_M, we calculated an internal storage capacity (ISC) of 10 μmol·cm^{-2} (n=7) for DIP and 80 μmol·cm^{-2} (n=49) for DIN in *L. digitata*. An ISC-DIN of 160 μmol·cm^{-2} (n=49) was calculated for *S. latissima*, while ISC-DIP could not be calculated from the experimental data collected on this species as no indication of phosphorus nutritional stress was exhibited and no significant decrease in mean F_v/F_m was observed over 66 days (Figure 4-7 B). However, based on the DIP requirements according to V_M over 66 days and consistent with a DIP:DIN uptake ratio of 1:6 under steady state conditions, we estimated an ISC-DIP of 27 μmol·cm^{-2} (n=14) for *S. latissima*.

4.5 Discussion

The growth, productivity and geographical distribution of seaweeds are controlled by environmental factors, such as temperature, irradiance, water movement and nutrient availability. Seasonal fluctuations in nutrient availabilities can also reflect differences in the seasonal growth patterns of seaweeds (Gagne et al. 1982, Zimmerman & Kremer 1986), as for *S. latissima* and *L. digitata* (Conolly & Drew 1985). This study adds to the physiological understanding of dissolved inorganic P and N uptake in *S. latissima* and *L. digitata*, which in turn enables estimation of ecological effects on nutrient availability.

Uptake kinetics are usually expressed as functions of either fresh weight (FW), dry weight (DW) or surface area to volume (SA:Vol), which makes it difficult to compare data. Furthermore, uptake kinetics expressed as a function of dry weight (DW) necessitates destructive sampling through harvesting living biomass. Standardized determination by fresh weight (FW) is even more problematic as small variations in the amount of water attached to the living (and growing) seaweed

can lead to large differences in its measured weight, not only between different samples and over time, but also amongst different experimenters. As seaweeds take up nutrients throughout their whole frond, the SA represents a reasonable function to determine uptake kinetics. Standardization of uptake kinetics by SA would allow for intra- and interspecific comparisons over time, for example with observations on the green seaweed *Ulva lactuca* Linneaus (Chapter 2). Moreover, phenotypic plasticity of seaweed strongly depends on predominant hydrodynamics of the site (Gerard 1987, Demes et al. 2011), but can also be affected by biotic stress (Molis et al. 2015), which can make comparison of functions of SA:Vol troublesome. Comparisons between uptake kinetics, such as V_S, V_M, and ISC, of different seaweed species would allow for general insights into seaweed survival and competition in natural environments. It is also an important aspect in scaling up operations to levels of commercial viability, as it enables to estimate the carrying capacity of a cultivation site in regard to nutrient availability and nutrient demand of cultivated species, as well as it allows to adjust duration and quantity of potential nutrient additions according to size (seaweed SA·m^{-2}) and growth of the operation.

Our results on growth rates for both, *S. latissima* and *L. digitata*, with only a slightly sub-optimal increase of SA observed under non-saturating external DIP conditions within the first 28 days of the first experimental approach, suggests that previously filled internal phosphate storages were utilized during the experiments and were able to compensate for external DIP deficiency (after Probyn & Chapman 1982, Pederson & Borum 1996). This is supported by the reduced, but continuing growth of both species when exposed to DIP-depleted seawater, which clearly indicates that internal phosphate storages had not been depleted after two weeks of starvation during the adaptation phase. The mean growth rates of 4 % d^{-1} under optimal DIP conditions for *S. latissima* and *L. digitata* are within the reported growth rates for both species from the North Sea area (*S. latissima*, Nielsen et al. 2014, Boderskov et al. 2015; *L. digitata*, Gomez & Lüning 2001). The reduced growth of *L. digitata* exposed to DIP concentrations of 6.0 μmol·L^{-1}, which are above the optimal levels, has also been observed for *Ulva lactuca* by Waite & Mitchell (1972) and Steffensen (1976).

These studies showed that phosphate concentrations above 4.76 μmol·L^{-1} had negative effects on growth rates for the green seaweed. Similarly, the daily pulsing of high DIP concentrations had a fatal effect on *S. latissima*.

The texture loss and disintegration of juvenile *S. latissima* sporophytes exposed to nominal DIP concentration of 6.0 μmol·L^{-1} within 3 weeks of exposure could have been caused by epiphytic bacteria. In many cases the level of bacterial populations found on seaweed surfaces depend on the species, thallus section and season (Armstrong et al. 2000, Bengtsson et al. 2010). One cause of a seasonal shift in marine and epiphytic bacterial communities may be a change in external conditions or physical and chemical parameters, such as nutrient stoichiometry and availability. However, studies have demonstrated the ability of certain marine bacteria to degrade various seaweed polymers (Goecke et al. 2010), thus leading to fouling and disintegration of the seaweed. However, *S. latissima* sporophytes only started to disintegrate after 3 weeks of exposure to nominal DIP concentration of 6.0 μmol·l^{-1}, and the daily uptake rates within the first week showed the ability of juvenile sporophytes to manage pulses of high DIP concentration for a short time. This ability could also be altered when stress reactions to high external nutrient concentration are initiated (e.g. Fourcroy 1999, Jiang & Yu-Feng 2008), allowing for mobilization and uptake of sufficient DIP to provide temporary relief.

Two different phases of transient responses to nutrient pulses, an initial surge uptake rate (V_S) after starvation and a maintenance or steady state uptake rate (V_M), which is considered equal to the rate of assimilation (Taylor & Rees 1999, Barr et al. 2004) were clearly seen for DIP and DIN-uptake in *S. latissima* and *L. digitata* in our first experimental approach. Depending on total DIP availability, V_S-DIP in *S. latissima* was maintained until the internal storages had been filled and uptake rates gradually decreased to V_M-DIP levels. This is supported by a significant decrease of DIP uptake found in the treatments with saturating nominal DIP concentrations of 1.5 μmol·L^{-1} and 3.0 μmol·L^{-1} on day 9 and day 4, respectively. Similar uptake characteristics were found for

L. digitata exposed to a nominal DIP concentration of 6.0 μmol·L^{-1}; thus, similarly starved *L. digitata* exposed to a nominal DIP concentration twice as high may lead to a shift in uptake rates from V_S to V_M in approximately half the time. This time-shifted phenomenon has also been described for DIP uptake in the green seaweed *Ulva lactuca* (Chapter 2) and corroborates evidence that the filling of internal nutrient pools is uncoupled from growth (Conway et al. 1976, Chapman et al. 1978). The high DIP uptake rates of *S. latissima* exposed to nominal concentrations of 6.0 μmol·L^{-1} within the first 2 days, although referred to as a stress reaction allowing for temporary relief, shows the ability of *S. latissima* to "handle" high DIP concentrations for a short time indicated by a stagnation of growth after 9 days, and in turn the calculated V_S-DIP observed from sporophytes in 3.0 μmol·L^{-1} within 4 days might have been underestimated. It should be mentioned that the daily offered nominal concentration of 6.0 μmol·L^{-1} would correspond to an initial daily DIP availability of approximately 1.6 μmol·cm^{-2}·d^{-1} for *S. latissima* and 0.4 μmol·cm^{-2}·d^{-1} for *L. digitata*, depending on the SA of the sporophytes. Therefore, the nominal concentration does not represent a fully comparable measure between these species.

DIN uptake rates of *S. latissima* and *L. digitata* under fully saturating DIN conditions followed the same response as DIP uptake rates under saturating DIP conditions. V_M-DIN was attained when internal DIN pools had been filled. In regard to the filling of internal DIN pools, *S. latissima* showed a V_S three times higher than its V_M for DIN, as well as V_S for DIN in *L. digitata*. These values are comparable to the V_S and V_M for DIN, respectively 12.5±5.2 μmol·cm^{-2}·d^{-1} and 2.3±0.9 μmol·cm^{-2}·d^{-1}, found in the green seaweed *U. lactuca* (Table 4-1; Chapter 2). Nutrient uptake rates can also vary according to the seaweeds age, as uptake of nitrate in first-year plants of *Laminaria groenlandica* Rosenvinge was three times higher than in second- and in third year plants (Harrison et al. 1986).

Table 4-1. Calculated dissolved inorganic phosphate (DIP) and dissolved inorganic nitrate (DIN) surge uptake rates (V_S), metabolic uptake rates (V_M), and internal storage capacity (ISC) of *Saccharina latissima*, *Laminaria digitata* and *Ulva lactuca*.

	DIP			DIN		
	V_S	V_M	ISC	V_S	V_M	ISC
	μmol·cm^{-2}·d^{-1}	μmol·cm^{-2}·d^{-1}	μmol·cm^{-2}	μmol·cm^{-2}·d^{-1}	μmol·cm^{-2}·d^{-1}	μmol·cm^{-2}
Saccharina latissima	0.80±0.03	0.30±0.09	27*	11.26±0.56	3.94±0.67	160*
n	7	14	14	49	49	49
Laminaria digitata	0.38±0.03	0.22±0.01	10*	3.92±0.08	1.81±0.38	80*
n	7	14	7	49	49	49
Ulva lactuca**	0.66±0.12	0.07±0.04	0.7±0.1	12.5±5.2	2.3±0.9	23±7
n	3	6	9	24	24	24

*approximation based on V_M, DIP:DIN uptake ratio, and photosynthetic efficiency Fv/Fm over time

**data derived from Chapter 2

The oscillatory decrease of DIN uptake in *S. latissima* during V_S suggests that DIN uptake was limited by internal aspects, such as the physical transfer of nutrients to inner tissue and/or enzymatic activity by feedback-controlled processes at the molecular level. Evidence shows that cellular processes are intrinsically rhythmic and follow a circadian metabolic timekeeping. Although the molecular basis of circadian rhythms in seaweed is poorly understood, many circadian rhythms have been described for microalgae (Wijnen & Young 2006). For example, the green single-cell alga *Chlamydomonas reinhardtii* shows maximal daily uptake of DIN at dawn and maximal nitrate reductase activity around midday (Pajuelo et al. 1995). This rhythmic expression aids in the synchronization of mutually coupled dynamical systems (Progromsky & Nijmeijer 1998). Furthermore, it has been demonstrated that species diversity could be enhanced by different temporal nutrient uptake pattern in micro-algae and even under limitation conditions a coexistence was possible (Ahn et al. 2002).

Survival and growth of perennials also depends on the duration of internal nutrient storages to overcome seasonal minima in nutrient availability. In experimental approach 2, the photosynthetic efficiency (F_v/F_m) was measured as an indication to nutrient stress. F_v/F_m of various seaweeds has often been applied to indicate stress resulting from desiccation (Varela et al. 2006, Schagerl & Möstl 2011, Flores-Molina et al. 2014), photo-period (Magnusson 1997) or light-intensity (Hanelt et al. 1997, Gevaert et al. 2002). The use of F_v/F_m as an indication of nutrient-related stress in the marine sector has been more common in microalgae (Kromkamp & Peene 1999) and corals (Wiedenmann et al. 2012). F_v/F_m values in the range of 0.79 to 0.84 are considered optimal for many plants, while values significantly below are considered to indicate stress (Kitajima & Butler 1975, Maxwell & Johnson 2000). Accordingly, *S. latissima* and *L. digitata* first indicated nutritional stress by a significant decrease in F_v/F_m after 9, respectively 7 weeks of exposure to DIP- and DIN-depleted seawater. This decrease in F_v/F_m can be inferred to indicate depletion of internal storage pools of DIP and/or DIN, as abiotic parameters like light, temperature, and hydrodynamics were kept constant during the pulse-and-chase approach and in relation to the control.

The inferred internal storage capacity (ISC) for DIP and DIN in *S. latissima* and *L. digitata*, derived from uptake kinetics (experimental approach 1) and the observed photosynthetic efficiency F_v/F_m (experimental approach 2) are realistic for perennial seaweeds like *S. latissima* and *L. digitata*, which are considered K-strategists that seasonally store reserves. The reserve ratio of the ISC for DIN and DIP of 6:1 in *S. latissima* and 8:1 in *L. digitata*, compared to V_M, the rate of assimilation with DIN and DIP uptake ratios of approximately 13:1 for *S. latissima* and 8:1 for *L. digitata*, suggests that *S. latissima* is twice as likely to fall under N limitation than P limitation, while in *L. digitata* the storage ratio is equivalent to $V_{M..}$ A pattern that was reflected by our results on F_v/F_m, which showed a decrease in photosynthetic efficiency to DIN and/or DIP depletion conditions at the same time *in L. digitata*, while *S. latissima* exhibited a decrease in F_v/F_m to DIN depletion, but none to DIP depletion, after 6 weeks of exposure. The high demand for DIN (and

DIP) in *S. latissima* was also reflected by its high uptake rates under V_S (Table 4-1), which is comparable to the V_S for DIN and DIP in the opportunistic green seaweed *U. lactuca* (Chapter 2), which is considered a promising seaweed for biofiltration purposes (Neori et al. 2003). Unlike *U. lactuca*, which flourishes at relatively high temperatures and light intensities, *S. latissima* can be regarded as a winter species, and this could allow for crop rotation in mariculture. Our data provides evidence that *S. latissima* is an effective candidate for bioremediation, for example in close proximity to marine fish farms, potentially able to balance nutrient loads from fish cages, while a relatively fast growth provides valuable biomass at the same time (Reid et al. 2013, Handå et al. 2013, Freitas et al. 2015).

Based on our results on DIP and DIN uptake kinetics and calculated ISC, *S. latissima* is predicted to outcompete *L. digitata* in the struggle for nutrients, despite similar spatial and temporal distribution. As mentioned before, multiple environmental factors regulate geographical distribution, and there is no available information about sporophyte recruitment strategies and the intra- and interspecific competitiveness of gametophytes of *S. latissima* and *L. digitata*. Reed (1990) showed that intra-and interspecific competition was more intense when settlement of gametophytes of *Macrocystis pyrifera* and *Pterygophora californica* were at high densities, but not at low densities. On the other hand, no evidence of competition was found among gametophytes of *Nereocystis luetkeana* (Vadas 1972). Delaying development can also ameliorate the negative effects of intra- and interspecific competition among seaweed gametophytes (Carney & Edwards 2010).

Our standardized data add to the physiological understanding of *S. latissima* and *L. digitata* and can contribute to the development and modification of applications in a bio-based economy, such as in integrated multi-trophic aquacultures (IMTA). Likewise, the obtained physiological data can help to identify potential locations for commercial cultivation and facilitates predicting yields of seaweed biomass in different locations under different environmental conditions using various models (Broch & Slagstadt 2012, Van der Molen et al. 2018). These are important applications, as

the interest in industrialisation of seaweed culture has increased in Europe throughout the last decades (Holdt & Kraan 2011, Wijesinghe & Jeon 2012).

4.6 Acknowledgements

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Chapter 5

Texture analysis of *Laminaria digitata* (Phaeophyceae) thallus reveals toughness gradient along lamina

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5.1 Abstract

Texture analysis is a method to test physical properties of a material by compression and tension. Growing interest in commercialisation of seaweeds for human food products vindicates research into physical properties of seaweed tissue. These are important parameters for selection and survival of stationary organisms, exposed to steady turbulent flow and its varying drag-forces, and not least tactile properties affect the perception and acceptance of consumers. Here we present the first standardised data on physical properties in the brown seaweed *Laminaria digitata*, known to be prevalent on exposed coastlines around the northern Atlantic Ocean. Morphological features of a healthy *L. digitata* thallus (lamina) seem effectively pronounced to its physical distress from

hydrodynamic forces. Reciprocal responses to compression and tension along the lamina and an age gradient indicate a twined structural alignment to optimise constituent tissue toughness and flexibility. A positive toughness gradient of 75 % from young to old tissue by means of tensile strength was found. Based on our results, a short morphological, ecological and physiological interpretation of the heterogeneity of a *L. digitata* lamina is given.

5.2 Introduction

Texture analysis is a method to test physical properties of a material by compression and tension. These parameters allow to calculate multiple properties such as resilience, hardness, breaking-point, firmness, spread-ability, and others, depending on the physical state of the materials, ranging from fluid to solid. Texture analysis has been commonly used in the food industry since the early 1960's to evaluate and standardize tactile properties of food products (Szczesniak 1963), as its haptic information affects the perception and acceptance of consumers enormously, in addition to visual evaluation (e.g. Szczesniak & Kleyn 1963, Peck & Childers 2003). Tests on typical conventional food ranges from processed pastry like biscuits to raw fruits like watermelons. Edible seaweeds are on the verve to enter the market for human food in the western hemisphere, and the general demand of seaweed products have been increasing globally during the last decade hence stimulating the efforts towards (mass) cultivation next to wild harvests (e.g. Neori 2008, Bixler & Porse 2011, Holdt & Kraan 2011, Kraan 2013).

Only limited standardised data about physical properties as strength and resilience (elasticity) of tissue in seaweeds has been attained, which is an important parameter for the selection and survival of these stationary organisms, exposed to steady turbulent flow and its varying drag forces during their development. Water velocities can be as high as 14 $m \cdot s^{-1}$ and can cause massive hydrodynamic forces on intertidal and shallow subtidal marine plants like seaweed (e.g. Koehl 1984, Denny 1994). This is an aspect of great ecological, morphological and

physiological interest, but also very relevant in marine plant cultivation. The hydrodynamic environment influences the morphology and eco-biomechanics in seaweed and hydrodynamic drag can be related to the occurrence of diffusion boundary layers (DBL), hence drag has an impact on the acquisition of essential resources (Hurd 2000).

A small number of studies investigated the tensile strength of different seaweed *in situ* with pull-tests applied horizontally to the substratum using a spring scale until dislodgment of the algae (Carrington 1990, Hawes & Smith 1995, Bell 1999). Dislodge of seaweed can be associated to its survival and distribution (Norton 1991, Denny 1995) and knowledge about physical properties can help to understand how population dynamics and community organization could develop with increasing weather extremes (Berg & Ellers 2010, Young et al. 2011, Coumou & Rahmsdorf 2012). Stipe-extension and stipe-strength examinations were done to determine break forces (Holbrook et al. 1991, Utter & Denny 1996, Smith & Bayliss-Smith 1998, Duggins et al. 2001). Breakage does not necessarily occur at the stipe during storms (Carrington 1990, Shaughnessy et al. 1996, Milligan & DeWreede 2000) and thallus morphology has been suggested to be the central element to mitigate break forces (Denny 1995, Boller & Carrington 2006). Thallus damage, e.g. caused by herbivores, can lead to (extended) rupture and loss of distal tissue to the damage (Koehl & Wainwright 1977, Santelices et al. 1980, Munoz & Santelices 1989). Herbivore-like damage is commonly measured as punctuation (compression) of the tissue, either with a gravitational penetrometer or an industrial texture analyser. The physiological resistance to compression is referred to (tissue-)toughness and is examined in only few ecological studies on changes in phenotypic plasticity of seaweed as a response to biotic and abiotic stress (e.g. Lowell et al. 1991, Pratt & Johnson 2002, Toth & Pavia 2007, Molis et al. 2015). Likewise, knowledge on morphological and physical properties can be important to select and adjust adequate pre-treatment to reduce size of raw material prior bio-refining processes (Zhu & Pan 2010). Yet information on morphology and physical properties as strength and toughness of seaweed appear fragmented and dispersed (Thomsen & Wernberg 2005).

In this study we present a standardised texture analysis of tissue strength and toughness along the central lamina of the thallus of cultivated *Laminaria digitata* for the first time. Breaking points by means of tensile and compression forces, as well as total elongation and thickness of the tissue are evaluated and discussed in an ecological, physiological and morphological context.

5.3 Material and methods

Experimental set-up

A cohort of one year old *Laminaria digitata* were obtained from cultivation tanks at the NIOZ seaweed centre (www.nioz.nl/seaweedcentre) in April 2015 and were kept in a cool box (15 L), filled with ambient seawater (14 °C, salinity 29.3), during analysis in the laboratory. Individuals ranged from 36 to 68 cm in length, based on measurements of the central lamina of the thallus from stipe to tip, and showed no physical damage, nor epiphytes. Tissue samples were punched out, using a polyethylene vial (Ø = 11 mm) for round stamps to test the load needed to pierce through the tissue, the ultimate piercing load (UPL). A custom built (120 x 20 mm), double-winged press block with a narrow centre of 3 mm (Figure 5-1) was used for stamps to test pulling forces necessary for tissue rupture, hence the ultimate tensile strength (UTS) of the tissue. All stamps were taken from the central lamina in repetitive pattern along an age gradient from stipe to tip (Figure 5-2). Young tissue develops from the meristem located at the basis near the stipe in *L. digitata*, consequently leaving the oldest tissue at the tip (apex). Three or four sets of samples for UPL and UTS measurements were punched out, limited by the lamina's length (Figure 5-2), and their relative distance from the stipe was determined for each individual (n=11). Tissue thickness around the (narrow) centre of the stamp was measured with a digital vernier calliper (accuracy ± 0.1 mm) in duplicates and averaged for data treatment.

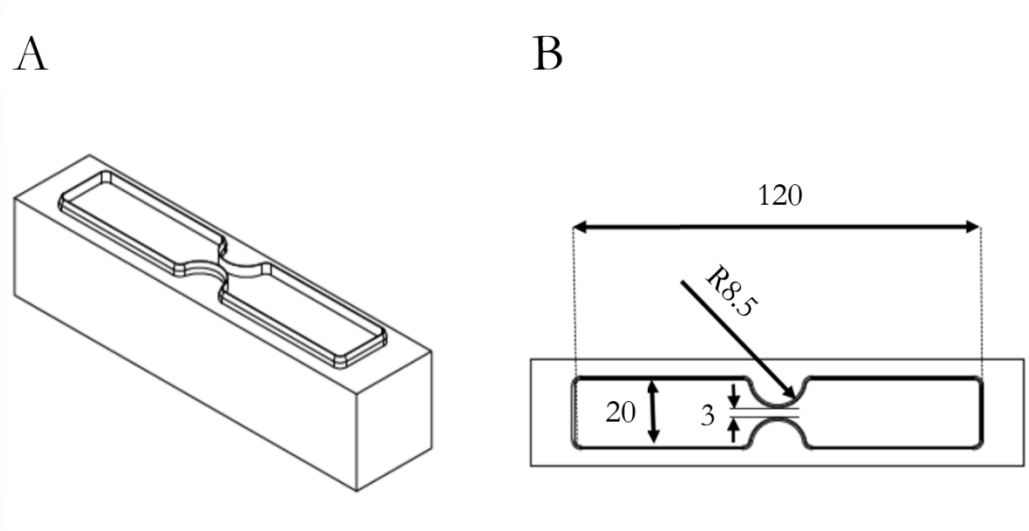

Figure 5-1. (A) Sketch of press block to stamp tissue samples for UTS analysis in diagonal view. (B) Schematics in top view including metric dimensions (mm) of the stamp.

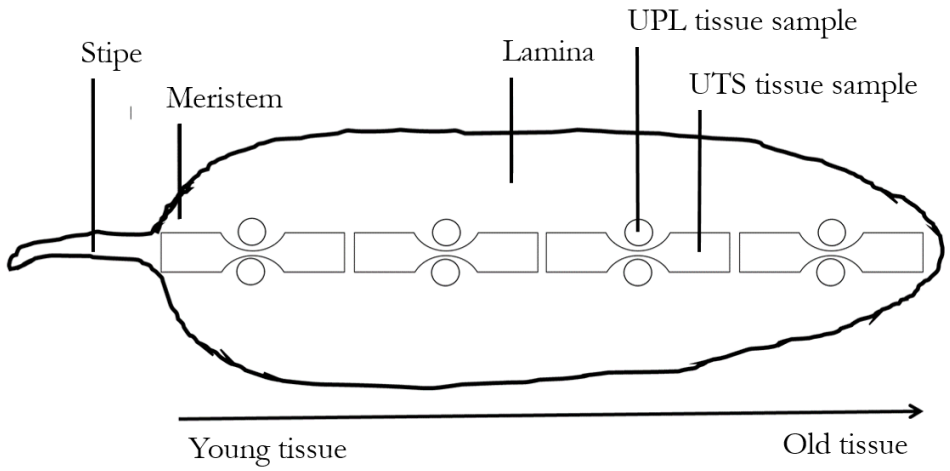

Figure 5-2. Sketch of a seaweed including stamp pattern of lamina tissue for UPL and UTS analysis along the central axis and age gradient. Young tissue develops from the meristem, consequently the oldest tissue is found at the apical tip.

Analysis of UTS and UPL by means of tension and compression were conducted with a texture analyser (CT3, Brookfield Engineering, USA; kindly provided by the Department of Aquatic Biotechnology and Bioproduct Engineering, Faculty Mathematics and Natural Sciences, University of Groningen), equipped with a 1000 g load cell. Custom built clamps (Figure 5-3) and mounts (Figure 5-4) to hold the sample in place during measurements were built at the NIOZ workshop. All customized items attached to the analyser were weighed and a corresponding pre-programmed 'trigger load' (2 g) was chosen (Brookfield TexturePro CT software package, firmware version 2.1). The test speed in both approaches, tension and compression, was set to a constant velocity of 0.2 mm s^{-1}.

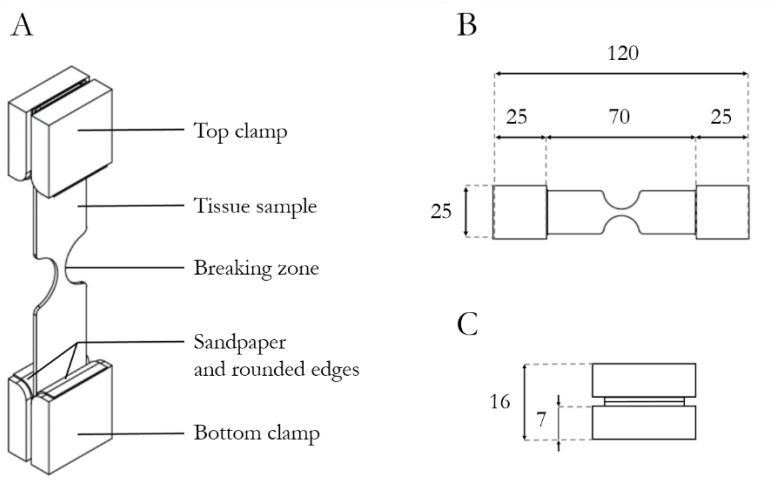

Figure 5-3. (A) Sketch of set up to analyse UTS. The tissue sample is fixated between top and bottom clamp (attached to the texture analyser). The clamps have rounded edges to avoid damage and a layer of sandpaper prevents the sample from slipping during UTS analysis. (B) Front view and metric dimensions (mm) of the set up with clamps. (C) Top view and metric dimensions (mm) of a clamp (round edges not illustrated).

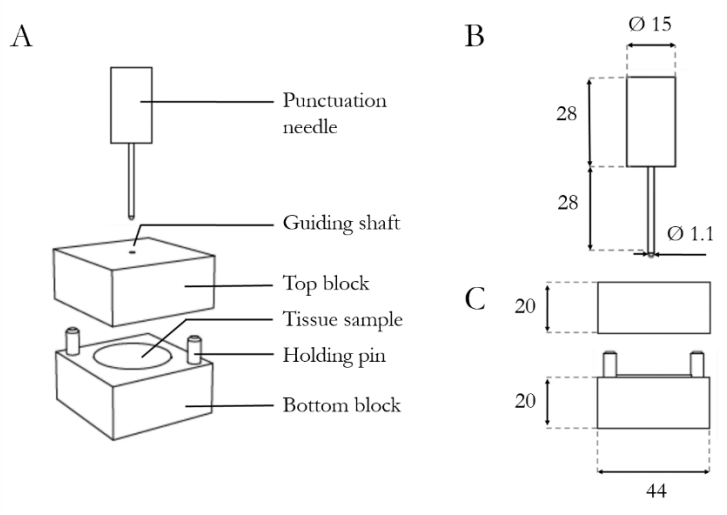

Figure 5-4. (A) Sketch of set up to analyse UPL. The tissue sample is fixated between top and bottom block. A shaft (Ø 1.2 mm) guides a punctuation needle through both blocks, which are held in alignment by pins on the bottom and their negatives in the top block. The punctuation needle is attached to the texture analyzer. (B) Side view and metric dimensions (mm) of the punctuation needle. (C) Side view and metric dimensions (mm) of top and bottom block (guiding shaft is not illustrated).

UTS examination required the double-winged tissue sample to be evenly fixated with each end to a top and a bottom clamp (Figure 5-3 A-C), which were rigidly attached to the beams of the texture analyser. One clamp basically consisted of two solid aluminium plates (25 x 25 x 7 mm) facing each other (Figure 5-3 B, C). Rounded edges (90°) and a layer of sandpaper (P100) on the inner sides of the plates prevented the fixated wet sample from unwanted influence by the plates' outskirts and slipping when subjected to tension forces, and ensured an accurate reconstruction of UTS with the narrow centre of the sample as the pre-determined breaking zone (Figure 5-3 A).

For UPL analysis a round tissue sample was placed between two solid PVC blocks (44 x 44 x 20 mm) with a guiding shaft (Ø = 1.2 mm) for a punctuation needle (Ø = 1.1 mm) in the centre (Fig. 5-4 A-C). Two holding pins on the bottom block (and their counter form sunk in the top block) guaranteed an exact alignment of the superimposed guiding shafts of each block and hence a 'barrier-free' slide for the stainless steel punctuation needle with a plain tip. To minimise friction in the shaft, the needle was firmly coated with a lubricant (Vaseline).

Data treatment

Typical graphs of UTS and UPL measurements of *L. digitata* are illustrated in Figure 5-5 (A, B). During UTS examination the sample is exposed to a linearly increasing pulling force (t_1) until it snaps (t_2). The UTS represents the maximum applied force (load) before the tissue snaps (Figure 5-5 A) and obtained data was normalized to tissue thickness, respectively the cross section of the narrow breaking point:

$$UTS = F_m \times w^{-1} \times d^{-1},$$

with F_m = recorded load (g), w = width of the sample at breaking point (3 mm, compare Figure 5-3 B) and d = thickness of the sample (mm). Before the UTS was reached the tissue sample stretched linearly to pulling forces (Figure 5-5 A) and a relative tissue elongation (ε_t) was calculated:

$$\varepsilon_t = L_t \times (t_2 - t_1)^{-1} \times v_{tx}^{-1},$$

with L_t = Length of the tested area (70 mm, compare Figure 5-3 B), t_1 = starting time of applied force, t_2 = time of tissue rupture, and v_{tx} = test velocity of texture analyser (0.2 $mm \cdot s^{-1}$).

UPL analysis showed two breaking points (Figure 5-5 B). The breaking point at t_2 represents the maximum applied load before the external side of the cell wall collapses (UPL), and t_3 depicts the internal disruption of the cell wall on the opposite side. Measured UPL was normalized to surface area (mm^2), while internally initiated breaking of the cell wall was neglected:

$$UPL = F_m \times (\pi \times r^2)^{-1},$$

with F_m = recorded load (g) and r = radius of the plain tip of the punctuation needle (0.55 mm, compare Figure 5-4 B).

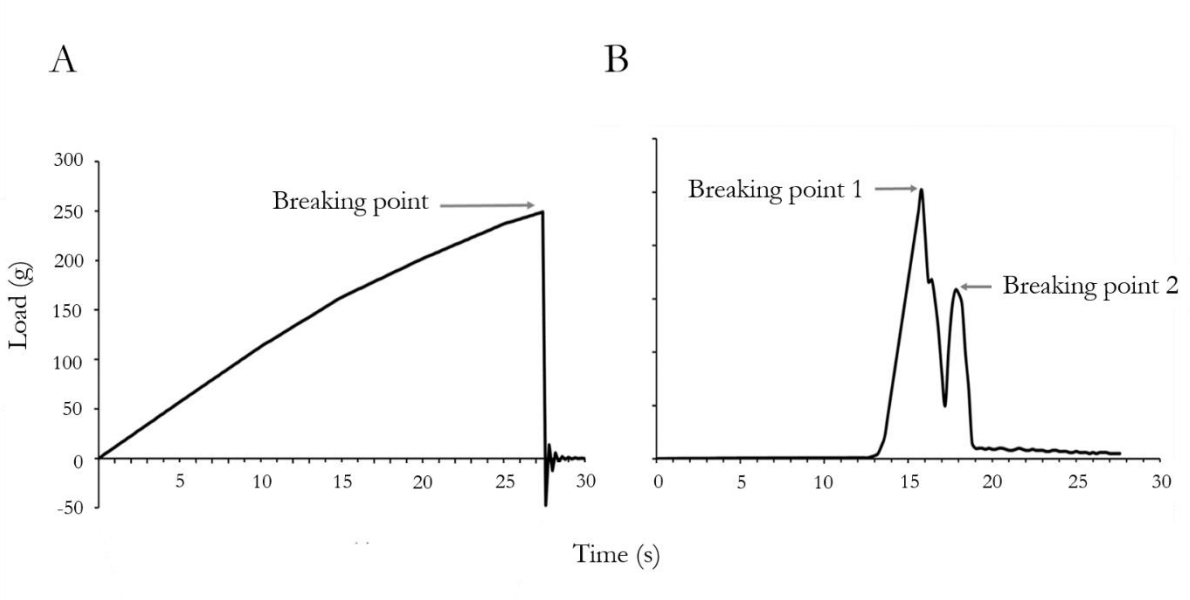

Figure 5-5. Typical graph of tension (A) and compression (B) measurements during texture analysis of *L. digitata* samples. (A) The breaking point indicates the rupture of the sample, referred to the ultimate tensile strength (UTS) of the tissue. (B) Breaking point 1 indicates the rupture of the external cell wall by punctuation needle, the ultimate piercing load (UPL). Breaking point 2 represents the internally initiated rupture of the cell wall on the opposite side.

Statistics

All data was tested for normality applying the Kolmogorov-Smirnoff test (KS test) for cumulative probability distribution. Single factor analysis of variances was performed to test significant variances of tissue thickness, ε_t and UTS, as well as UPL between all individuals and within each lamina in relative distances from the stipe. Statistical comparison of two interdependent datasets within individuals was conducted with a paired T-test. Significance level in all analysis was ≤ 0.05.

5.4 Results

The lamina showed no significant variations in thickness between individuals (group), but a highly significant difference in thickness within the lamina (Table 5-1).

Table 5-1. Analysis of variance of thickness, elongation (ε_t), ultimate tensile strength (UTS) and ultimate piercing load (UPL) between individuals (group) and within the lamina.

Parameter		df	*F*	*p*
Thickness				
	Group	10	1.10	0.396
	Lamina	3	3.92	0.006
Elongation				
	Group	10	0.76	0.529
	Lamina	3	0.69	0.656
UTS				
	Group	10	0.45	0.907
	Lamina	3	8.83	<0.001
UPL				
	Group	10	3.11	0.078
	Lamina	3	12.49	<0.001

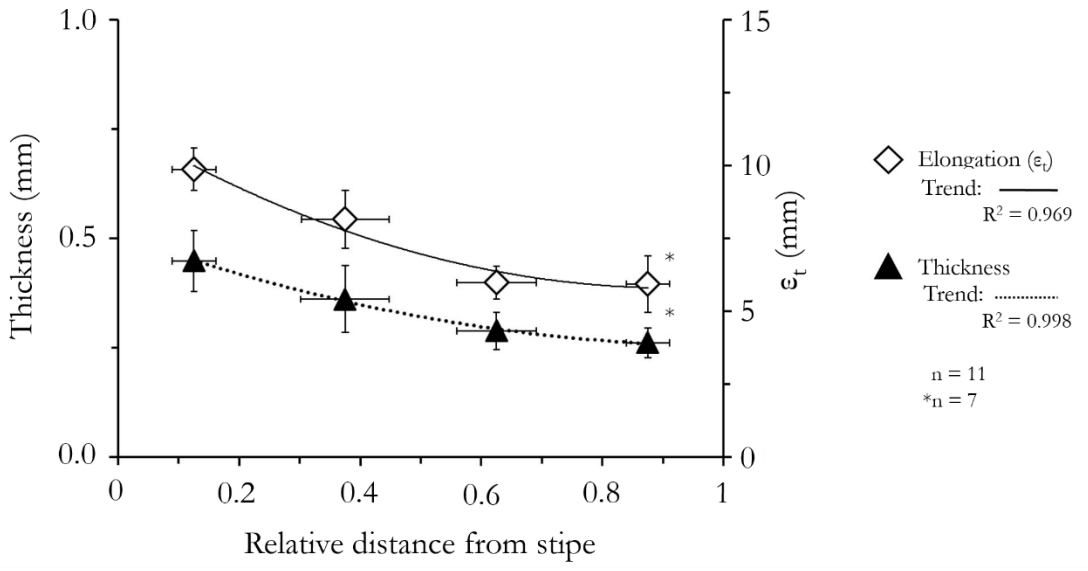

Figure 5-6. Tissue thickness (mm) and total elongation (mm) of tested *L. digitata* samples in relative distance from the stipe ± SD (n=11; *n=7).

Tissue thickness correlated negatively to its distance from the stipe (*R*=-0.968), respectively to age, and decreased from young tissue with a thickness of 0.45±0.07 mm towards the tip with a thickness of 0.26±0.03 mm by 40 % (Figure 5-6). Tissue thickness showed a positive correlation (*R*=0.698) to tissue elongation (ε), observed during texture analysis and exposure to pulling forces. Mean ε of all tested tissue samples before rupture was 7.54±1.45 mm, resulting in a relative elongation (ε_t) of 11±2 %. No significant difference in ε_t between individuals and within the lamina was found (Table 5-1, 5-2). No significant difference in ultimate tensile strength (UTS) along the lamina in relative distance from the stipe between individuals, but highly significant variances within the lamina (Table 5-1) and a positive correlation (*R*=0.701) to the relative distance from the stipe, respectively age of tissue, was observed. Young tissue, 12.5±3.6 % of the lamina length from the stipe, showed a mean UTS of 246±36 g·mm^{-2} and was significantly weaker than tissue located 37.5±7.3 % from the stipe with a mean UTS of 389±52 g·mm^{-2} (Table 5-2). Maximum pulling forces necessary for tissue rupture were measured approximately at two thirds (62.5±6.6 %) of the lamina length from the stipe with a mean load of 429±76 g·mm^{-2}, 74.5 % stronger than mean UTS of young tissue (Figure 5-7).

Like UTS, UPL showed no significant variance between individuals and highly significant differences within lamina (Table 5-1). In contrast to UTS, no correlation (R=-0.188) between UPL and a relative distance from the stipe were found. Young tissue at 12.5 ± 3.6% of the lamina length from the stipe had a mean UPL of 327±71 g·mm^{-2} and was significantly weaker than tissue found after one third, 37.5±7.3 %, of the lamina's length (Table 5-2) with a mean UPL of 432±80 g·mm^{-2}, the measured maximum UPL of the lamina. A continuous and significant decrease of UPL after the first third of the lamina's length towards the tip (Table 5-2) with 292±39 g·mm^{-2} resulted in an UPL gradient of 32.3 % throughout the lamina's length (Figure 5-7).

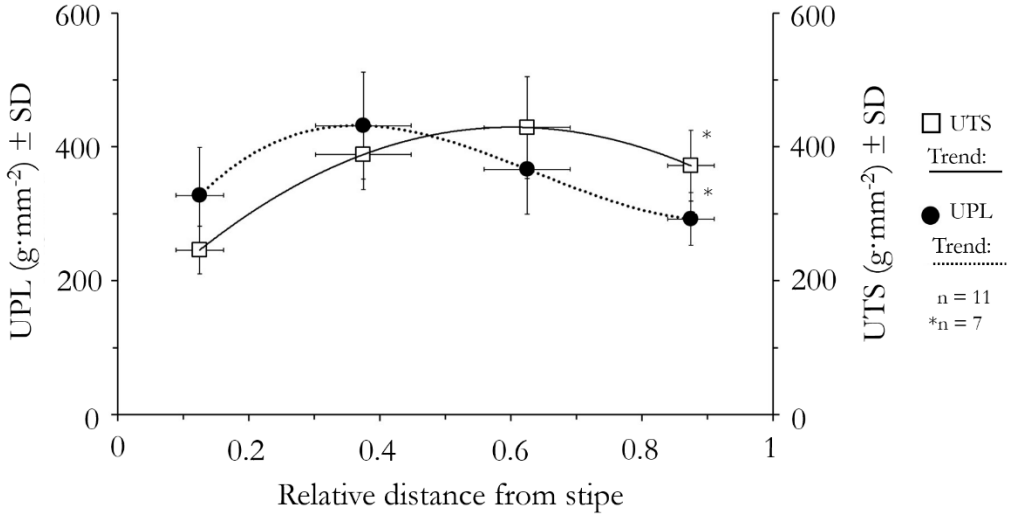

Figure 5-7. UTS and UPL (g·mm^{-2} ± SD) of the central lamina of *L. digitata* in relative distance from the stipe ± SD (n=11; *n=7).

Table 5-2. *P*-values (paired T-test) of differences (significance p = 0.05) in thickness, elongation (ε_t), ultimate tensile strength (UTS) and ultimate piercing load (UPL) within the central lamina in relative distance from the stipe (in %).

	Relative distance from stipe (%)			
Parameter	**12.5±3.6**	**37.5±7.3**	**62.5±6.6**	**87.5±3.6***
Thickness		0.080	0.021	0.050
Elongation		0.819	0.286	0.383
UTS		<0.001	0.061	0.014
UPL		0.021	0.043	0.005

n = 11, * n = 7

Trades between UTS and UPL alternate between the basal and apical third of the lamina, while the central part shows maximum UTS and UPL. UPL was significantly higher than UTS in young tissue (p<0.001), UTS was significantly higher than UPL in old tissue (p<0.001), while maximum UPL did not show a significant difference (p=0.467) to maximum UTS. In the young third of the lamina UPL was 23.2 % stronger than corresponding UTS and within the apical third UTS was 23.3 % stronger than analogous UPL (Figure 5-7).

5.5 Discussion

This study offers a standardised method to examine for example effects of hydrodynamic forces on seaweed individuals and provides an example of the heterogeneity of a *L. digitata* thallus (lamina). Based on our measurements we give a short morphological, ecological and physiological interpretation of tissue toughness to its fundamental physical modulation as adaptation to survive in a wave exposed habitat. In addition, design options for seaweed support structures for cultivation purposes are discussed.

Morphology

In the intertidal and subtidal zone, the seaweed habitat, water currents change their direction frequently and seaweed toughness and flexibility are key factors for them to endure mechanical stress caused by hydrodynamic forces. Drag forces are a function of the area of a seaweed (Carrington 1990), consequently drag will change as the seaweed grows over time (Denny et al. 1985, Denny 1988).

Different strategies are used by different seaweeds to effectively reduce impact of kinetic energy (E_K) on the load-bearing stipe and holdfast to prevent breakage or detachment from the substrate. A hydrodynamic streamlining, with narrower and flatter blades, as well as higher rates of cell elongation have been reported for *Laminaria saccharina*, when subjected to constant longitudinal drag compared to individuals grown without stress (Gerard 1987). A similar morphological characteristic is represented by a decrease of tissue thickness of a *L. digitata* lamina along an age gradient from newly formed, young tissue near the meristem to old tissue at the apex (Figure 5-6). The relative tissue elongation (ε) of 11±2 % showed no significant variations throughout the lamina (Table 5-1) and the decrease of thickness can be asserted to cell elongation, as they age, rather than an abrasion of outer cell layers climaxing towards the apex. An elongation results in a reduction of relative (residual) biomass towards the apex of the lamina. A reduction of biomass reduces the movement acceleration of the lamina in opposite direction by transferring kinetic energy (E_K) along the lamina to an increasing residual mass, which helps to mitigate impact of E_K on critical attachment areas of stipe and holdfast.

Adjustments to morphology and mechanical properties in seaweed include modification of the microstructural composition of the cells, including incorporation of cellulose, the structure of alginate blocks, or the packing density of cells (Koehl 1986, LaBarbera 1985, Mackie & Preston 1974). As alignment, structure, composition and density of cells influence the mechanical properties and their feedback to tensile and compressive forces, these aspects can be asserted to

the alternating dominance of UTS (highest in apex) and UPL (highest in meristem) throughout the lamina along an age gradient (Figure 5-7). UTS and UPL alternated within the same range, and minimum and maximum values were not significantly different from another, with UPL 23 % tougher than UTS in young tissue and 23 % stronger UTS than UPL in old tissue.

Generally, most cells undergo a rapid cell expansion after they grow out of the meristem and before they differentiate into mature cells. During cell expansion and elongation, support tissue becomes obligate to counter hydrodynamic forces, while support for vertical growth is provided by buoyancy of the tissue in the surrounding water. As cells mature, UTS and UPL increase (Figure 5-7). This could be attributed to the incorporation of support tissue like cellulose, which forms into microfibrils with high tensile strength. The reciprocal dominances of UPL and UTS within the lamina could be based on the rearrangement of the microstructural composition and alignment to maximise traits between toughness and flexibility, leading to a high UTS. A microstructural alignment to increase UTS becomes distinct in the apical third of the lamina, where UTS nearly doubled and reached maximum values, while UPL continued to decrease until it attained initial values of young tissue (Figure 5-7). A significant decline of UTS around the apex (Table 5-2) can be explained by the lag of necessity to support distal portions of tissue. Similar principles of a twined alignment can be found in e.g. historical manufacturing processes of ropes and in modern nanotechnology. For example, Liu et al. (2009) pointed out that tensile strength of nanotube yarns depended not only on the diameter, but also on the twisting angle of the yarn. It must be mentioned that a growing organism has to provide this alignment during growth and likely by cell to cell communication, while manufactured products, like a rope, are completed with a 'twist and lock' after the basic construct has been finished and length is determined. A rearrangement of microstructural composition and alignment follows over time, as no significant variations in UPL and UTS in relative distance from the stipe between individuals with different lamina length were observed (Table 5-1).

Ecology

Seaweed morphology also serves as a mechanical defence against herbivores (Mauricio 1998) and tissue toughness is the first physical barrier to overcome. UPL is referred to the force mandible-bearing mesograzer (amphipods, isopods) have to apply in order to "bite" and feed on pieces of seaweed tissue. According to the optimal defense theory (ODT; after Rhoades 1979) seaweed parts with a great ecological value to the plant are protected more intensively than other parts, and many seaweeds may combine several types of defenses without paying considerable trade-offs (Koricheva et al. 2004). The meristem represents tissue of great ecological value and the texture of young tissue, near the meristem, in *L. digitata* appears to be morphologically protected according to the ODT. A 40 % greater thickness of young tissue, near the meristem, within the basal third of the lamina, compared to the apical third (Figure 5-6) impedes initial access for mesograzer to young tissue. The decreasing thickness within the basal third of lamina comes with a 32 % increase of tissue toughness, as measured in UPL, and would increase grazing efforts. Morphological modifications and chemical defences in seaweeds in respond to biotic and abiotic mediated stress and the ecological effects have been well documented for some seaweeds (e.g. Agrawal 2001, Toth & Pavia 2007, Utsumi 2011, Molis et al. 2015). For example, tissue toughness in *Fucus vesiculosus* adjusted plastically to the prevailing level of wave exposure, which in turn affected the phenotypic plasticity of the radula of the grazing flat periwinkle, *Littorina obtusata* (Molis et al. 2015). The accessibility for small grazers to seaweed in wave-swept, rocky coastlines with high water velocities is restricted to periods of weak hydrodynamic impact and it can be assumed that morphological features of a healthy *L. digitata* lamina are pronounced to its physical distress from hydrodynamic forces. On the other hand, seaweeds become more and more popular as a food source for humans in Europe, the "new grazers". Our standardized method offers opportunities to quantify tissue toughness in different parts of seaweeds enabling selection of the most favourable parts to be consumed.

Physiology

As seaweed morphology can be affected by hydrodynamics, the morphology affects the hydrodynamics around the seaweed, which influences physiological processes like nutrient uptake. Seaweed can actively engineer its own microhabitat through morphological features like hydrodynamic streamlining, hyaline hairs, small corrugations and edge undulations, that affect the water velocities above the surface of the seaweed, create turbulences and have an influence on the thickness of the diffusive boundary layer (DBL). Thereby it can help to facilitate resource (nutrient) supply (Hurd et al. 1993, Hurd 2000, Hurd & Pilditch 2011). Flexible, smooth seaweeds transfer E_K into harmonic motions of the lamina, which are perpetuated by water turbulences created by a current flow over the lamina, generating an opposed drag and slowing down the relative water movement above the seaweed surface (Koehl 1986, Hurd & Stevens 1997), hence affecting the DBL. An optimal nutrient availability and general resource exchange can be achieved by decreasing diffusion distances through the DBL (Wheeler 1980), or increasing concentration gradients within the DBL (Hurd & Pilditch 2011). These aspects are not only important for the physiology of seaweeds, it is also very relevant for the design of seaweed supporting structures. In a seaweed cultivation set-up, optimisation should be achieved in ensuring optimal nutrient availability, also in large cultivation farms in combination with structural support elements. When, for example, seaweed is cultivated for carbohydrates, a flexible cultivation set-up should allow for multiple hydrodynamic forcing on a flexible seaweed, in order for the seaweed to invest in structural elements of the cell wall.

More knowledge on the phenotypic plasticity and physical trade interaction, also on cellular level, is inevitable not only to understand and develop tools to modify mechanical properties for cultivation purposes, but also to understand morphological, ecological and physiological responses of seaweed and seaweed communities to changing environmental terms. The presented approach

also allows for standardised methods of inferring the effects on nutrient availability and varying hydrodynamic forces on seaweed individuals.

5.6 Acknowledgements

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Chapter 6

Dissolved inorganic phosphate uptake and corresponding dissolved inorganic nitrate uptake in the seaweed *Palmaria palmata* (Rhodophyceae): ecological and physiological aspects of nutrient availability

In revision

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6.1 Abstract

Uptake dynamics of dissolved inorganic phosphate (DIP) and dissolved inorganic nitrate (DIN) in young *Palmaria palmata* (n=49), cultivated in a range of DIP concentrations (0.0-6.0 μmol·L^{-1}) and non-limiting DIN concentration (50 μmol·L^{-1}) under fully controlled laboratory conditions, were quantified in a 'pulse-and-chase' approach over 5 weeks. Two different uptake

rates were specified: (1) surge uptake (V_S) after starvation and (2) maintenance uptake with filled nutrient pools (V_M). V_S for DIP of 1.57±0.29 μmol·cm^{-2}·d^{-1} and DIN of 15.6±4.3 μmol·cm^{-2}·d^{-1}, as well as V_M for DIP of 0.57±0.22 μmol·cm^{-2}·d^{-1} and DIN of 5.6±2.1 μmol·cm^{-2}·d^{-1} were calculated. In addition, an absolute size of the internal storage capacity (ISC) for DIP of 22 μmol·cm^2 and DIN of 222 μmol·cm^2 was determined. A DIP to DIN uptake ratio of 1:10 under V_M showed a weekly rhythmic uptake pattern, highlighted by a high correlation between DIP and DIN uptake (R=0.943). V_S for DIN did not occur under DIP depletion, but uptake rates increased with increasing DIP availability. Hence, DIP availability limited access to DIN, which was also reflected by total dissolvable protein concentrations in sporophytes, which ranged from 10.2±2.5 % to 24.6±8.0 % dry weight depending on DIP availability. Similarly, total dissolvable carbohydrate concentration ranged from 22.1±3.6 % to 54.3±12.3 % dry weight. The data presented in this study opens further insight into ecological and physiological aspects of nutrient availability in *P. palmata* and allows for an optimization in cultivation.

6.2 Introduction

Dissolved inorganic phosphorus (DIP) and dissolved inorganic nitrogen (DIN) are two of the most important macro-nutrients in the metabolism and growth of seaweeds. A nutrient limitation can significantly affect growth, physiology, reproduction, and internal composition of seaweeds and thus can affect the nutritional value, as well as render their spatial and temporal distribution (Lobban and Harrison 1994, Pederson et al. 1996). Thus resource availability is a key element for survival of species in any given environment and hence drives the outcome of biological interactions, shaping community composition and structure (Chapin III et al. 2000). It has been demonstrated that species diversity in microalgae was enhanced by temporal stratification of nutrient uptake, resulting in oscillating or rhythmic pattern, and even under limitation conditions a coexistence was possible with this strategy (Ahn et al. 2002). The general nutrient uptake

mechanisms in seaweeds are basically known (e.g. Lüning 1992, Lobban and Harrison 1994, Harrison and Hurd 2001). However, there is a paucity of information on nutrient uptake pattern in seaweeds, particularly on phosphate uptake and its relationship to nitrogen utilization. New approaches are needed to fully understand nutrient uptake dynamics in seaweeds in order to gain knowledge on effects of nutrient limitation and shifts in limitation from one element to another. This can also contribute to economical endeavours, as it allows to identify potential locations for mariculture and enables optimization in cultivation. The demand of seaweed products, for example alginates or carrageenan, have been increasing globally during the last decades (Bixler & Porse 2011, Porse & Rudolph 2017). Edible seaweeds are on the verve to enter the market for human food in the western hemisphere, as they are marketed as super-food with high values of various minerals, vitamins, carbohydrates, proteins and a low fat content.

The red alga *Palmaria palmata* (Linnaeus) F. Weber & D. Mohr is a temperate seawater species, which can be found in the intertidal zone along the North Atlantic Ocean. Due to its nutritional value with protein levels higher than soybeans (Morgan et al. 1980, Arasaki and Arasaki 1983, Galland-Irmoulli et al. 1999) and with its distinctive umami flavour (when dried, roasted, or fried), *P. palmata* is considered a novel and tasteful marine vegetable. With an increasing interest in novel and functional foods and the successful commercialisation of *P. palmata* for feed in aquaculture, for example in abalone farms (Evans and Langdon 2000, Rosen et al. 2000), the natural resources of *P. palmata* have become short in supply. As a result, studies on the development of cultivation methods have been performed thoroughly to understand the life cycle and hence control reproduction (Van der Meer and Todd 1980, Wikfors and Ohno 2001, Grote 2019). A few studies have aimed at the yield and effectiveness of bioremediation by *P. palmata* in pilot scale offshore cultivation, for example in the vicinity of fish farms (Sanderson et al. 2012), as well as in land-based tank production (Gall et al. 2004, Pang and Lüning 2004, Corey et al. 2014, Grote 2016). A majority of these studies have focussed on yield and the efficiency to remove nitrogenous compounds like ammonium (NH_4^+) and nitrate (NO_3^-) from the water column. Less

attention has been paid to phosphate uptake and the potential of co-limitation between nutrients with one limiting nutrient hindering uptake of a second nutrient (Harpole et al. 2011), which has been observed in many microalgae (e.g. Rhee 1974, Haines and Wheeler 1978, D'Elia and DeBoer 1978). Martinez and Rico (2004) demonstrated a biphasic nutrient uptake for DIP and DIN in *P. palmata* by incubating sporophytes in various DIN and DIP concentrations and following the uptake rates over approximately 6 hours. A biphasic nutrient uptake has often been described for nutrient-starved seaweeds (e.g. Fujita 1985, Dy and Yap 2001), including a surge uptake (V_S), which refers to the filling of internal nutrient pools, uncoupled from growth, and an internally or metabolic uptake (V_M), which is considered equal to the rate of assimilation (Taylor and Reed 1999, Barr et al. 2004). Little focus has been rewarded given to the absolute size and thus (in combination with daily requirements) the time internal nutrient pools or internal storage capacity (ISC) would be sufficient to overcome seasonal minima in nutrient availability without significant forfeit to growth (Fujita 1985, Pedersen and Borum 1996, 1997, Pedersen et al. 2010). Perennial seaweeds, like *P. palmata*, rely on stored N and P, which are gained during autumn and winter, when nutrient availability is high, and benefit from this internal storage during spring and summer with increased day length, temperatures and typically low nutrient availability (e.g. Martínez and Rico 2002). In *P. palmata*, protein has been described as the major N storage pools, which constitutes a large fraction of the seaweed's dry weight (DW) (Morgan et al. 1980).

This study adds to eco-physiological research of *P. palmata* under fully controlled laboratory conditions and contributes to ecological aspects of nutrient uptake dynamics and nutrient management strategy for DIP and DIN. Uptake dynamics and ISC were quantified and standardized for surface area (SA), comparable to experiments on the green seaweed *Ulva lactuca* Linnaeus and the brown seaweeds *Saccharina latissima* (Linnaeus) C.E.Lane, C.Mayes, Druehl & G.W.Saunders and *Laminaria digitata* (Hudson) J.V. Lamouroux by Lubsch and Timmermans (2018, 2019). In addition, total dissolvable protein concentration and total dissolvable carbohydrate concentration in the fronds were determined after 5 weeks exposure to limiting and

non-limiting nutrient concentrations. More information on the eco-physiology of seaweeds, presented in a comparable and comprehensive fashion, would strengthen the ecological understanding of eco-system dynamics and could initiate and expand bio-based activities in a responsible manner.

6.3 Material and methods

Experimental set-up

All experiments and analysis were conducted at the Royal Netherlands Institute for Sea Research (NIOZ), Texel, The Netherlands. Young sporophytes of *P. palmata*, which parental plants had originated from the Irish coastline, were cultivated at the NIOZ Seaweed Research Centre (https://www.nioz.nl/en/expertise/seaweed-research-centre) and brought to a temperature-controlled room (set 12 °C) for a 10-day adaptation phase under laboratory conditions. During this adaptation phase the sporophytes received DIP- and DIN-depleted seawater medium to ensure nutrient starvation. After adaptation, 49 randomly collected sporophytes with a mean surface area (SA) of 1.9±0.7 cm^2 were individually transferred into 200 ml glass jars filled with 100 ml seawater medium, enriched with a range of DIP-concentrations (0.0 – 0.2 – 0.4 – 0.8 – 1.5 – 3.0 – 6.0 $\mu mol \cdot L^{-1}$) and a DIN concentration of 50 $\mu mol \cdot L^{-1}$. The seawater medium was exchanged/refreshed ("pulsed") on a daily basis throughout the experimental time, which not only provided a constant daily pulse of nutrients, but also would mitigate effects of elevated or low CO_2 levels on growth rates (Kübler and Raven 1995). Samples of the seawater medium for dissolved nutrient analysis were taken ("chased") for the initial 20 days of the experiment. After water exchange, all flasks were randomly distributed on a custom platform (100 x 60 x 1 cm) on a rotating table, slowly brought to a speed of 100 rpm to provide a moderate water movement. This constant water movement was maintained for optimal mixing and, hence, availability of nutrients by decreasing the diffusion boundary layers between tissue and growing medium (e.g. Gonen et al.

1995, Hurd 2000). Two tubular fluorescence lamps (OSRAM L18 Watt 965, Deluxe cool daylight) attached 50 cm above the flasks provided a light intensity of 60±8 µmol photons·m^{-2}·s^{-1} to reach light saturation for the sporophytes (Kübler and Raven 1995) and a light/dark period of 16/8 h was arranged (Pang and Lüning 2004). Growth rates as a measure of surface area (SA) and the photosynthetic efficiency F_v/F_m were followed on a weekly basis for an additional 2 weeks and total dissolvable protein concentration, as well as the total dissolvable carbohydrate concentration of all sporophytes (n=49) were determined after a total experimental time of 5 weeks.

Seawater medium

Natural, filtered (0.2 µm) North Atlantic seawater with low phosphate (PO_4^{3-}: 0.011 µmol·L^{-1}), ammonium (NH_4^+: 0.032 µmol·L^{-1}) and nitrate (NO_3^-: 0.004 µmol·L^{-1}) concentrations and a salinity of 34.5 was used as a base for the seawater medium. After pasteurization of the seawater (80 °C for 2 h), the salinity was adjusted to 29.5 by mixing ultrapure water (Milli-Q, Merck KGaA, Massachusetts, USA) to bring the salinity to levels of the cultivation tanks. Afterwards potassium-dihydrogen-phosphate (KH_2PO_4) and potassium nitrate (KNO_3) were added as sources for DIP and DIN to create DIP concentrations of 0.2 - 0.4 - 0.8 - 1.5 - 3.0 and 6.0 µmol·L^{-1} and a DIN concentration of 50 µmol·L^{-1}. The mean pH of all seven seawater medium stocks with varying DIP concentration was 8.1±0.1 (n=14), measured with a pH-Meter (GHM-3511, Greisinger, Germany).

Seawater analysis

Dissolved inorganic nutrients (DIP and DIN) were measured with a colorimetric analysis using a Technicon TRAACS 800 auto-analyzer (Seal Analytical, Germany) in the NIOZ Texel nutrient laboratory. DIP was measured as ortho-phosphate (PO_4^{3-}) at 880 nm after the formation of molybdophosphate complexes (Murphy and Riley 1962). DIN (nitrate and nitrite) was calculated, after nitrate reduction to nitrite through a copperized cadmium coil and measured at 550 nm, posterior to a complexation with sulphanylamide and naphtylethylenediamine (Grasshoff

and Hansen 1983). Ammonium (NH_4^+) was measured at 630 nm, after the formation of an indophenol blue complex with phenol and sodium hypochlorite at a pH of 10.5. Citrate was used as a buffer and complexant for calcium and magnesium at this pH (Koroleff 1969 and optimized by Helder and de Vries 1979). The low NH_4^+-concentration (0.022 $\mu mol \cdot L^{-1}$) were not further considered, as no NH_4^+ was added for the experiments. Nominal nitrite concentration were measured in all cases <0.05 $\mu mol \cdot L^{-1}$ and hence played only a subordinate role in the (nitrate dominated) DIN concentration. Precision for all measured channels within the automated nutrient analyzer was better than 0.25 % (personal communication K. Bakker, NIOZ).

Surface area (SA) analysis

The sporophytes were individually spread flat on a white plastic board next to a ruler, used for scale comparison, and covered with a transparent Plexiglas sheet to avoid corrugations. Photographs (Panasonic Lumix DMC-FT5) of the samples were taken from a 90° angle, enabling an analysis of surface area (SA) by using the open source software ImageJ (ImageJ, U. S. National Institutes of Health, Maryland, USA). The images of *P. palmata* were converted into grayscale (type 8-bit) and transformed into a binary image before the SA was analyzed. The obtained SA represents one side of the frond.

Growth

Differences in SA over time were interpreted as growth with relative growth rates (μ) calculated according to Kain (1987), as follows:

$$\mu = (\ln SA_1 - \ln SA_2) \times t^{-1},$$

where SA_1 represents the initial surface area, and SA_2 represents the final surface area after incubation time t. The results were used to calculate DIP and DIN uptake dynamics on days with no measurements of SA.

DIP and DIN uptake dynamics

Uptake is referred to the removal of dissolved inorganic phosphate (DIP), dissolved inorganic nitrate (DIN) by *P. palmata* sporophytes. Determination of daily uptake rates was comparable to the analysis by Lubsch and Timmermans (2018) on *Ulva lactuca*. Daily uptake rates (V_D) were derived from changes in the nutrient concentrations of the seawater medium during each day, normalized for SA (cm^2) and time (d), and calculated using the following equation:

$$V_D = (T_1 - T_2)\, SA^{-1} \times t^{-1},$$

with T_1 as the initial nutrient concentration, T_2 as the nutrient concentration before water exchange after 24 h, SA as surface area (cm^2) and t as the incubation time.

Two different uptake rates over time were categorized: surge uptake (V_S, S for surge) after starvation, and maintenance uptake with filled nutrient pools (V_M, M for maintenance). V_S was calculated from uptake rates in non-limiting nutrient concentrations (indicated by remaining nutrients after sampling interval), using the following equation:

$$V_S = (V_2 - V_1) \times (d_2 - d_1)^{-1} = \Delta V \times \Delta d^{-1},$$

with V_1 and V_2 as daily uptake rates on days before a significant decline in uptake rates occurs and no significant variations in nutrient uptake follow. The difference operator between the two days is represented by d_1 and d_2.

The internal storage capacity (ISC) for DIP was calculated, based on V_M and the response of the photosynthetic efficiency F_v/F_m under DIP-limitation, respectively depletion. The ISC was calculated, also accounting for the 10-day adaptation phase under depletion conditions, as follows:

$$ISC_{DIP} = n_M \times V_M,$$

where n_M represents the number of days under DIP-depletion before F_v/F_m significantly decreased and V_M accounts for the daily DIP uptake under saturating conditions.

Photosynthetic efficiency F_v/F_m

Photosynthetic efficiency F_v/F_m was determined on a weekly basis for 5 weeks. All sporophytes were dark-adapted for 20 minutes in glass jars, before F_v/F_m was determined with a pulse-amplitude modulated fluorimeter (JUNIOR-PAM, Walz, Effeltrich, Germany; settings: measuring light intensity=10, pulse width=0.8s, gain=2) by measuring each sporophyte twice on different locations of the frond with an interval of 40 seconds between the two measurements. The measurements were done under minimum light conditions (laptop screen as the only light source) in a temperature controlled room (set to 12 °C) at approximately the same daytime.

Total dissolvable protein and carbohydrate analysis

All sporophytes were individually rinsed in fresh (MilliQ™) water to remove saltwater residue, immediately frozen (-40 °C), freeze-dried (24 h) and homogenized for the determination of total dissolvable protein concentration (after Lowry et al. 1951), as well as total dissolvable carbohydrate concentration (Anthrone method after Trevelyan et al. 1952). A suspension of a homogenized *P. palmata* sample (10 mg) and MilliQ™ (10 ml) was made and the Lowry reagents, respectively the Anthrone reagents, were added. The solution with Lowry reagents was incubated at room temperature for 10 minutes, before the Folin/Cioccalteus reagent was added and the solution incubated for 30 minutes at room temperature. The solution with Anthrone reagents was placed in a heating chamber for 6 minutes at 95 °C. Both solutions developed a blue colour, which was analysed with a photometer (SpectraMax M2, Molecular Devices, LLC, CA, USA) at a wavelength of 660 nm, respectively 620 nm. The total dissolvable protein concentration was calculated using a calibration curve based on a bovine serum albumin (BSA) stock solution with known protein concentration. Comparably, the total dissolvable carbohydrate concentration was determined by a glucose stock solution with known concentration.

Statistics

All data were tested for normality with the Kolmogorov-Smirnoff test (KS test) for cumulative probability distribution. A two-sided ANOVA with repetition was performed to test whether growth rates, nutrient uptake rates, total dissolvable protein, carbohydrate content, and F_v/F_m varied significantly within and between different nutrient concentrations over time.

6.4 Results

Growth

The increase of surface area (SA), referred to as growth, of *P. palmata* showed no significant variations among treatments with different DIP concentrations (ANOVA $F(6, 210)=1.06$, $p=0.391$), but a significant difference in growth rates was detected over time (ANOVA $F(4, 210)=11.30$, $p<0.001$). The interaction between growth rates and different DIP concentrations showed no significant variations within 5 weeks (ANOVA $F(24, 210)=0.72$, $p=0.832$), thus the averaged growth of all sporophytes in different DIP concentrations was depicted (Figure 6-1). The SA of all young sporophytes (n=49) showed a mean increase of 0.12 ± 0.04 cm^2 in week 1, which decreased to mean growth of 0.01 ± 0.04 cm^2 in week 3 and displayed a subsequent increase in week 4 to week 5 with a mean growth of 0.05 ± 0.02 cm^2 per week (Figure 6-1). The total increase in SA over 5 weeks resulted in a daily growth rate of 1 %.

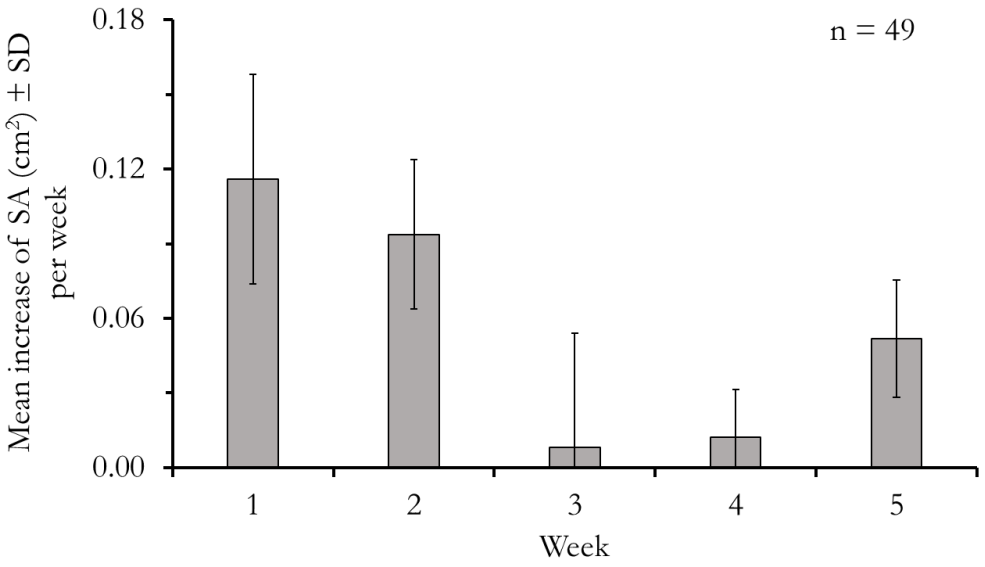

Figure 6-1. Mean increase in surface area (cm^2) ± SD of young *Palmaria palmata* sporophytes (n=49) cultivated in a range of DIP concentration (0-6 $\mu mol \cdot L^{-1}$) and saturating DIN concentration (50 $\mu mol \cdot L^{-1}$) in a 'pulse-and-chase' assay over 5 weeks.

DIP and DIN uptake dynamics

Palmaria palmata sporophytes exposed to nominal DIP concentrations of 0.2, 0.4, and 0.8 $\mu mol \cdot L^{-1}$ depleted all offered DIP within the daily sampling of 24 hours and throughout the experimental time (here referred to as limiting concentrations of PO_4^{3-}). The daily supplied DIN concentration of 50 $\mu mol \cdot L^{-1}$ was non-limiting in all treatments and over experimental time. In treatments with DIP additions, DIN uptake was significantly higher than DIN uptake under DIP depletion conditions (ANOVA $F(1, 40)=10.70$, $p=0.002$) and mean uptake rates increased in accordance with DIP availability. A strong positive correlation between DIP and DIN uptake was found ($R=0.943$). When exposed to DIP depleted seawater medium, sporophytes showed a mean DIN uptake rate of 5.8 ± 1.0 $\mu mol \cdot cm^{-2} \cdot d^{-1}$ (Figure 6-2) without significant variations over 20 days (ANOVA $F(19, 6)=1.31$, $p=0.182$). Similarly, no significant variation in DIN uptake rates of sporophytes in nominal DIP concentration of 0.2 $\mu mol \cdot L^{-1}$ were found (ANOVA $F(19, 6)=0.04$, $p=0.838$), but a mean DIN uptake rate of 7.6 ± 2.0 $\mu mol \cdot cm^{-2} \cdot d^{-1}$ over 20 days was moderately

higher than uptake rates under DIP depletion. An analogous increase of DIN uptake rates in accordance to DIP availability was observed for sporophytes in treatments of DIP concentrations of 0.4 and 0.8 μmol·L^{-1} with mean uptake rates (n=7) of 11.0±2.1 and 16.9±6.1 μmol·cm^{-2}·d^{-1} on day 1 (Figure 6-2). In nominal DIP concentrations of 1.5, 3.0, and 6.0 μmol·L^{-1}, initial DIN uptake rates showed no significant variations to initial uptake rates in nominal DIP concentration of 0.8 μmol·L^{-1} (ANOVA $F(3, 20)=2.33$, $p=0.099$). Mean DIN uptake rates (n=7) in these treatments were 16.0±4.3, 11.8±2.7, and 11.3±1.0 μmol·cm^{-2}·d^{-1} on day 1, respectively 15.6±4.3, 17.9±3.8, and 15.6±2.3 μmol·cm^{-2}·d^{-1} on day 2 (Figure 6-2). At the same time, maxima in mean DIP uptake rates (n=7) of 1.42±0.37 and 1.64±0.13 μmol·cm^{-2}·d^{-1} were observed in nominal DIP concentration of 3.0 and 6.0 μmol·L^{-1}. Available DIP was not limiting in these treatments, unlike DIP in nominal concentration of 1.5 μmol·L^{-1}. Sporophytes exposed to 1.5 μmol·L^{-1} had depleted all daily supplied DIP until day 3, before a significant decrease in DIP removal from the seawater medium occurred on day 4 (ANOVA $F(3, 6)=13.05$, $p<0.001$) with a mean uptake rate of 0.29±0.05 μmol·cm^{-2}·d^{-1} (Figure 6-2). DIP depletion was also recorded on days 9 and 14 in this treatment. Based on uptake rates of *P. palmata* in nominal DIP concentration of 3.0 and 6.0 μmol·L^{-1}, a mean V_S of 1.57±0.29 μmol·cm^{-2}·d^{-1} for DIP (n=14) was calculated for sporophytes in both treatments.

After elevated uptake rates on day 1 and day 2, sporophytes in treatments with DIP availability >0.8 μmol·L^{-1} showed a rhythmic DIP and DIN uptake pattern with recurring maxima in uptake rates within the magnitude of initially elevated uptake on days 9 and 14, and minima with very low or hardly any detectable DIP and DIN uptake on days 12 and 18 (Figure 6-2). For example, mean DIP uptake rates as low as 0.08±0.10 and 0.10±0.10 μmol·cm^{-2}·d^{-1} were measured in treatments with nominal DIP concentration of 1.5 μmol·L^{-1} during minima on day 12, respectively day 18. At the same time, low DIN uptake rates of 4.0±1.0 and 2.2±0.6 μmol·cm^{-2}·d^{-1} were observed in this treatment (Figure 6-2). The rhythmic recurrence of minima and maxima in DIP and DIN uptake rates resulted in mean uptake rates of 0.31±0.15 μmol·cm^{-2}·d^{-1} for DIP and

5.4±2.2 μmol·cm^{-2}·d^{-1} for DIN during the last rhythmic interval recorded in week 3. Similarly, the rhythmic uptake pattern of sporophytes in nominal DIP concentration of 3.0 and 6.0 μmol·L^{-1} showed mean uptake rates of 0.59±0.23 μmol·cm^{-2}·d^{-1} for DIP and 5.6±3.1 μmol·cm^{-2}·d^{-1} for DIN, respectively 0.56±0.23 μmol·cm^{-2}·d^{-1} for DIP and 7.8±4.3 μmol·cm^{-2}·d^{-1} for DIN, during in the rhythmic interval in week 3. Based on the data, V_M of 0.57±0.22 μmol·cm^{-2}·d^{-1} (n=14) for DIP and V_M of 5.6±2.1 μmol·cm^{-2}·d^{-1} (n=28) for DIN were calculated. Uptake rates for DIP and DIN under V_M were a threefold smaller than uptake rates under V_S and DIP:DIN uptake ratio under V_M was 1:10.

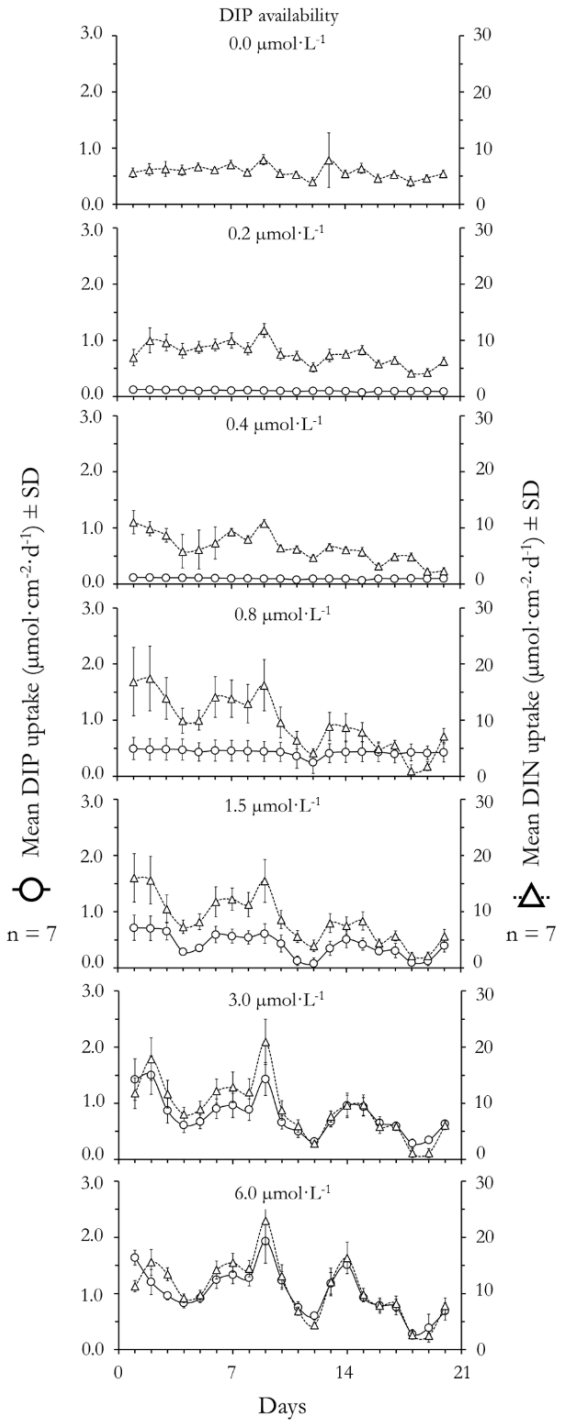

Figure 6-2. Mean uptake rates (µmol·cm^{-2}·d^{-1}) ± SD of dissolved inorganic phosphorus (DIP) and dissolved inorganic nitrate (DIN) of young *Palmaria palmata* sporophytes (n=7) exposed to a range of available DIP concentration (0.0 – 0.2 – 0.4 – 0.8 – 1.5 – 3.0 – 6.0 µmol·L^{-1}) and saturating DIN concentration (50 µmol·L^{-1}) in a 'pulse-and-chase' assay over 20 days.

Internal storage capacity

An internal storage capacity (ISC) for DIP of 22.2 μmol·cm^{-2} was calculated based on V_M and the response in F_v/F_m under depletion conditions, including adaptation phase. In correspondence to a DIP:DIN uptake ratio of 1:10 under V_M, an ISC for DIN of 222 μmol·cm^{-2} was deduced. The internal storages for both, DIP and DIN, are equivalent to 40 days to maintain growth under depletion conditions.

Photosynthetic efficiency F_v/F_m

Sporophytes of *P. palmata* showed no significant variation in photosynthetic efficiency (F_v/F_m) when growing under different DIP concentrations until week 3 (ANOVA $F(6, 42)=0.75$, $p=0.612$). Mean F_v/F_m of all sporophytes was 0.61±0.04 (n=49) (Figure 6-3). After week 3, F_v/F_m of sporophytes exposed to limiting DIP concentrations of 0.0, 0.2, 0.4, and 0.8 μmol·L^{-1} significantly decreased (ANOVA $F(2, 27)=3.87$, $p=0.027$) to a mean value of 0.49±0.06 (n=28) with no significant variations among sporophytes (ANOVA $F(27, 54)=1.17$, $p=0.305$). Sporophytes exposed to non-limiting DIP concentrations of 1.5, 3.0, and 6.0 μmol·L^{-1} showed neither significant variations among sporophytes (ANOVA, $F(20, 40)=1.18$, $p=0.315$), nor a significant decrease within the first 3 weeks of the experiment (ANOVA $F(2, 20)=0.52$, $p=0.595$). Photosynthetic efficiency of sporophytes exposed to limiting DIP concentration of 0.0, 0.2 and 0.4 μmol·L^{-1} continued to significantly decrease between week 4 and 5 (ANOVA $F(1, 20)=18.97$, $p<0.001$) to a mean value of 0.44±0.06 (n=21). Although no significant variation in F_v/F_m among sporophytes in limiting DIP treatments were found until week 4 (ANOVA $F(20, 80)=1.17$, $p=0.299$), photosynthetic efficiency levelled off in accordance with available DIP concentrations. Sporophytes exposed to a concentration of 0.0 μmol·L^{-1} DIP showed the steepest and deepest drop in F_v/F_m with a mean of 0.46±0.09 (n=7) in week 4 and 0.40±0.08 in week 5, compared to values exhibited by sporophytes in DIP concentration of 0.2 and 0.4 μmol·L^{-1}, which decreased to 0.51±0.04, respectively 0.49±0.07 in week 4 and reached values of 0.41±0.03, respectively

0.43±0.03 in week 5 (Figure 6-3). In contrast, F_v/F_m of sporophytes in DIP concentration of 0.8 µmol·L^{-1} showed no significant variation between week 4 and 5 (ANOVA F(1, 6)=4.21, p=0.086) and a mean value of 0.51±0.04 persisted (Figure 6-3).

Similar to *P. palmata* exposed to limiting DIP concentrations, F_v/F_m of sporophytes in non-limiting DIP concentrations of 1.5, 3.0, and 6.0 µmol·L^{-1} showed no significant variation among treatments (ANOVA, F(20, 84)=0.93, p=0.557), but a significant variation over time (ANOVA F(4, 20)=6.71, p=0.007). Unlike a steep decrease of F_v/F_m in sporophytes exposed to limiting DIP concentrations, mean values moderately decreased from 0.60±0.04 in week 3 to 0.54±0.04 in week 5 (Figure 6-3).

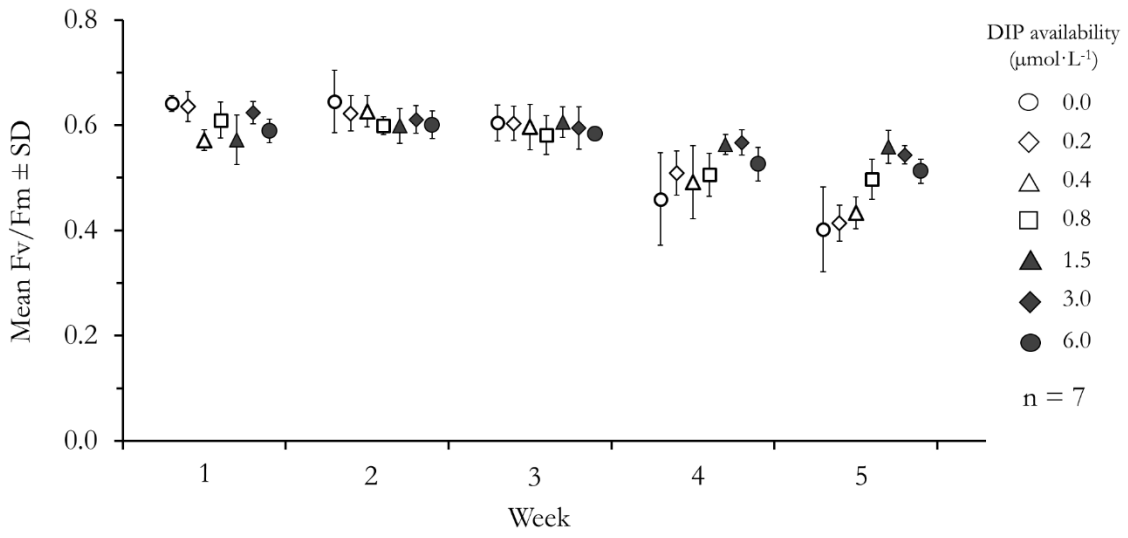

Figure 6-3. Mean photosynthetic efficiency F_v/F_m ± SD of young *Palmaria palmata* sporophytes (n=7) cultivated in a range of available dissolved inorganic phosphorus (DIP) concentrations (0.0 – 0.2 – 0.4 – 0.8 – 1.5 – 3.0 – and 6.0 µmol·L^{-1}) and a dissolved inorganic nitrate concentration of 50 µmol·L^{-1} in a 'pulse-and-chase' assay over 5 weeks.

Total dissolvable protein and carbohydrate concentrations

The total dissolvable protein concentration in *P. palmata* showed significant variations among treatments with different nominal DIP concentrations (ANOVA $F(6, 40)=9.01$, $p<0.001$), as did the total dissolvable carbohydrate concentration (ANOVA $F(6, 40)=6.41$, $p<0.001$). Mean dissolvable protein concentration in sporophytes was 102±25 $\mu g \cdot mg^{-1}$ DW (n=7) after DIP depletion conditions for 6.5 weeks (adaptation and experimental time) (Figure 6-4) and showed significant variations to mean protein concentration of sporophytes with DIP availability (ANOVA $F(6, 30)=7.37$, $p<0.001$). Mean dissolvable protein concentration of sporophytes increased to 202±47 $\mu g \cdot mg^{-1}$ DW (n=7), as DIP availability increased to a nominal DIP concentration of 0.8 $\mu mol \cdot L^{-1}$ (Figure 6-4). No significant variation of dissolvable protein concentration in sporophytes exposed to nominal DIP concentration of 0.8 $\mu mol \cdot L^{-1}$ and higher were found (ANOVA $F(3, 22)=1.62$, $p=0.214$) and mean dissolvable protein concentrations in sporophytes exposed to nominal DIP concentrations of 1.5, 3.0 and 6.0 $\mu mol \cdot L^{-1}$ (n=7) was 186±33, 246±80 and 206±28 $\mu g \cdot mg^{-1}$ DW, respectively (Figure 6-4).

The total dissolvable carbohydrate concentration in *P. palmata* showed no significant variation, when exposed to limiting DIP concentrations of 0.0, 0.2, 0.4 and 0.8 $\mu mol \cdot L^{-1}$ (ANOVA $F(3, 24)=1.81$, $p=0.171$) and mean dissolvable concentrations were 383±230 μg, 535±140, 543±125 and 432±156 $\mu g \cdot mg^{-1}$ DW, respectively (Figure 6-4). Similarly, no significant variation in dissolvable carbohydrate concentrations among treatments with non-limiting DIP concentrations were found (ANOVA $F(2, 16)=1.53$, $p=0.247$), but concentrations of dissolvable carbohydrates were significantly lower than concentrations in sporophytes exposed to limiting DIP concentrations (ANOVA $F(4, 30)=6.11$, $p<0.001$). This threshold in mean dissolvable carbohydrate content resulted in concentrations of 221±36, 275±119 and 294±60 $\mu g \cdot mg^{-1}$ DW were measured after 5 weeks exposure to non-limiting DIP concentrations of 1.5, 3.0 and 6.0 $\mu mol \cdot L^{-1}$, respectively (Figure 6-4).

In DIP depletion and limitation conditions (0.0 - 0.4 μmol·L^{-1}), the protein: carbohydrate ratio in the sporophytes ranged from 0.27 to 0.31, the critical protein: carbohydrate ratio. A ratio of 0.47 was exhibited, when daily DIP pulses of 0.8 μmol·L^{-1} were supplied. Sporophytes exposed to DIP concentrations >0.8 μmol·L^{-1} showed a protein: carbohydrate ratio as high as 0.89.

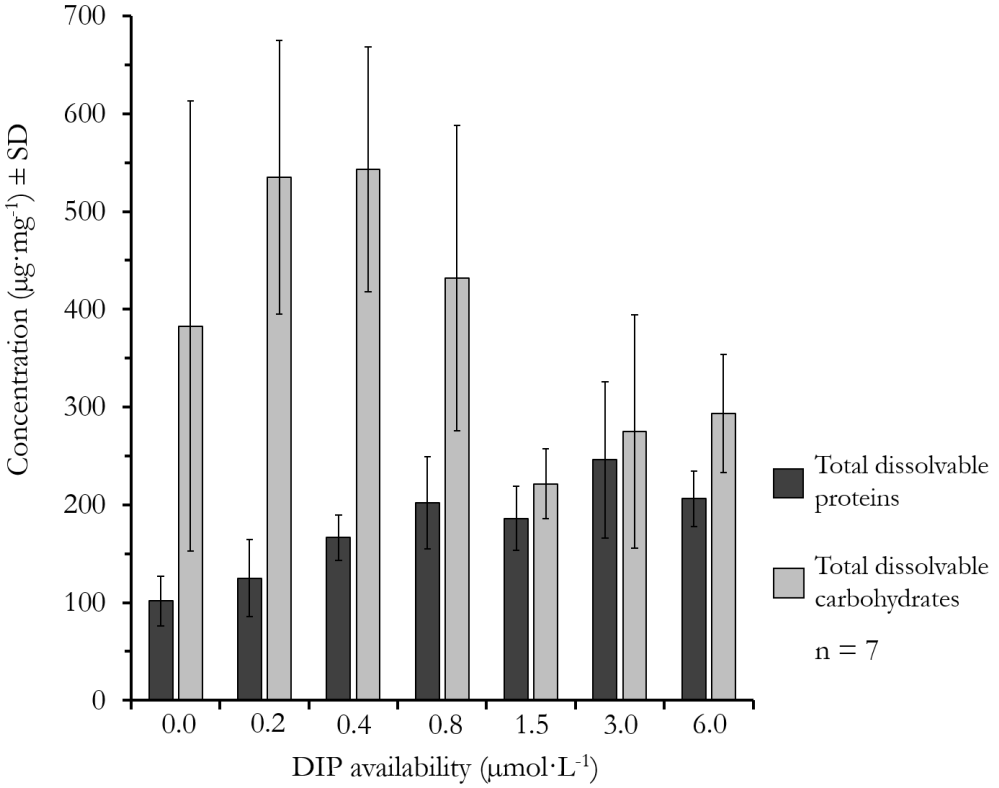

Figure 6-4. Mean total dissolvable protein and carbohydrate concentration (μg·mg^{-1} of dry weight) ± SD of young *Palmaria palmata* sporophytes (n=7), after cultivation in a range of dissolved inorganic phosphate (DIP) concentrations (0.0 – 0.2 – 0.4 – 0.8 – 1.5 – 3.0 – and 6.0 μmol·L^{-1}) and a dissolved inorganic nitrogen (DIN) concentration of 50 μmol·L^{-1} in a 'pulse-and-chase' assay for 5 weeks.

6.5 Discussion

Seaweed, as well as microalgae, acquire their resources from the surrounding seawater by uptake across their entire SA, and growth rates of both seaweed and microalgae in nature are often constrained by rates of uptake and assimilation of nutrients per cm^2 surface area (Rees 2007). The determination of the SA as a non-destructive method to infer to growth showed no significant variations in treatments with different nominal DIP concentrations, thus the nutrient supply was not decisive for growth. This was supported by the on-going growth and high photosynthetic efficiency of *P. palmata* sporophytes under DIP depletion, which clearly demonstrated that internal storage pools were not completely depleted, during the 10-day adaptation phase. The latter was during the experiments confirmed, after calculation of maintenance uptake and ISC for *P. palmata*. Moreover, the determination of SA revealed a rhythmic growth pattern in *P. palmata*, conceivably in a monthly growth cycle. Circadian rhythms in growth have been often documented for plants, including seaweeds, and are attributed to survival and competitive advantages, although the contribution to plant fitness remain unknown (e.g. Michael et al. 2003, Dodd et al. 2005). Reproduction and growth cycles in monthly, respectively moon-related periods, have been reported for several brown seaweed genera, including *Fucus*, *Dictyota*, and *Sargassum* (Schad 2001). Similar lunar or semilunar periodicities were also found in the green seaweeds *Ulva*, *Enteromorpha*, *Halimeda*, and *Halicystis* (Schad 2001). Obviously most seaweeds live in (inter-)tidal zones of coastal habitats and it is not surprising that physiological pattern have adapted to their environment in a more or less strict, genetically fixed amount (Schad 2001).

Biphasic responses to nutrient pulses are well known for seaweeds (Hurd and Dring 1990, Lotze and Schramm 2000, Lubsch and Timmermans 2018, 2019) and also have been reported for *P. palmata* in short time experiments (Martínez and Rico 2004). The responses to DIP and DIN pulses in *P. palmata* reported by Martínez and Rico (2004) showed a biphasic uptake with a V_{max} for DIP of 6.29 to 10.21 $\mu mol \cdot h^{-1} \cdot g^{-1}$ DW and V_{max} for DIN of 16.96 to 24.67 $\mu mol \cdot h^{-1} \cdot g^{-1}$ DW

(V_{max} refers to the maximal uptake rate from the Michalis-Menten model, which is equivalent to V_S in this study), followed by a regression in uptake rates. This regression was described by the half saturation concentration K_s from the Michaelis-Menten model and values for DIP were 11.64 to 25.40 μmol·L^{-1}, and K_s for nitrate was reported to be between 15.28 and 30.53 μmol·L^{-1} (Martínez and Rico 2004). A comparison of uptake kinetics to results of this study is troublesome, as no conversion factor for DW to SA for *P. palmata* was available. Uptake kinetics expressed as a function of dry weight (DW) necessitates destructive sampling through harvesting living biomass and as seaweeds take up nutrients throughout their whole frond, the SA represents a more appropriate function to determine uptake dynamics in this study, comparable to publications on uptake kinetics and management strategies in *U. lactuca* (Lubsch and Timmermans 2018), *S. latissima* and *L. digitata* (Lubsch and Timmermans 2019) (Table 6-1). Rees (2007) reviewed available data, i.a. maximum uptake rates of nitrate and growth rates in several marine microalgae and macroalgae, which provided values or enabled to calculate for these parameters in relationship to their SA. The reported maximum uptake rates per SA and hour in macroalgae by Rees (2007) ranged from 40.3 nmol·cm^{-2}·h^{-1} for *Ulva intestinales* (after Taylor et al. 1998) to 342.0 nmol·cm^{-2}·h^{-1} for *Fucus spiralis* (after Topinka 1978 and Nielsen and Sand-Jensen 1990), which meet the result on V_S in *P. palmata* (325 nmol·cm^{-2}·h^{-1}) in this study. Nevertheless, it has to be clarified that results on uptake dynamics in this study refer to one side of the front (see material and methods), which is an important factor, particularly for the implementation of data in 3-D models.

Table 6-1. Results on uptake dynamics (surge uptake V_S, maintenance uptake V_M, uptake ratios, internal storage capacity ISC, and growth) for dissolved inorganic nitrate (DIN) and dissolved inorganic phosphate (DIP) in *Ulva lactuca* (Chlorophyceae), *Saccharina latissima*, *Laminaria digitata* (Phaeophyceae), and *Palmaria palmata* (Rhodophyceae), conducted in 'pulse-and-chase' experiments under controlled conditions for light (light/dark: 16/8; *U. lactuca*[1]: 80 µmol photons $m^{-2}\cdot s^{-1}$, *S. latissima*[2], *L. digitata*[2]: 18 µmol photons $m^{-2}\cdot s^{-1}$, *P. palmata*: 60 µmol photons $m^{-2}\cdot s^{-1}$), temperature (12±1 °C) and hydrodynamics over several weeks.

Class		Chlorophyceae	Phaeophyceae		Rhodophyceae
Species		***U. lactuca***	***S. latissima***	***L. digitata***	***P. palmata***
V_S ($\mu mol\cdot cm^{-2}\cdot d^{-1}$)	DIN	12.5±5.2	11.3±0.6	3.9±0.1	15.6±4.3*
	DIP	0.66±0.12	0.80±0.03	0.38±0.03	1.57±0.29
V_M ($\mu mol\cdot cm^{-2}\cdot d^{-1}$)	DIN	2.3±0.9	3.9±0.7	1.8±0.4	5.6±2.1
	DIP	0.07±0.04	0.30±0.09	0.22±0.01	0.57±0.22
	N:P-ratio	10:1	13:1	8:1	10:1
ISC ($\mu mol\cdot cm^{-2}$)	DIN	23±7	49	80	222
	Duration (days)	10	42	45	40
	DIP	0.7±0.1	14	>10	22
	Duration (days)	10	90	>45	40
Daily growth (%)		4	3	2	1

* Strong dependency (R=0.943) on DIP availability for nitrate uptake.

Clearly, elevated DIP and DIN uptake rates in saturating DIP-treatments at the beginning of the assay can be attributed to V_S and the filling of internal nutrient pools, before V_M is attained, which is considered equal to the rate of assimilation (Taylor and Rees 1999, Barr et al. 2004). The

DIP and DIN uptake rates quantified during V_S and V_M showed a ratio of 3:1, which is consistent with observations on uptake kinetics in *P. palmaria* by Martínez and Rico (2004). Uptake rates under V_M for both nutrients, DIP and DIN, were notably rhythmic in approximately weekly intervals, in contrast to an almost linear DIP and DIN uptake pattern during V_M in the brown seaweeds *S. latissima* and *L. digitata* (Lubsch and Timmermans 2019) and the green seaweed *U. lactuca* (Lubsch and Timmermans 2018). The synchronized rhythmic uptake pattern of *P. palmata* in different treatments provides evidence for a physiological cell synchronization. Synchronisation of nutrient uptake, growth, and reproduction has often been applied to harmonize the response of seaweed cultures and can be achieved, for example, by the regulation of abiotic factors, for example photoperiod, temperature and nutrient supply over an extended period of time (e.g. Lüning 1993, Gomez and Lüning 2001, Bogaert et al. 2016) A weekly oscillating or rhythmic uptake pattern as observed during our experiments with *P. palmata*, can be referred/linked to a physiological response to intra- and interspecific competition. Arino et al. (2003) demonstrated that a competitor-mediated coexistence, respectively a competitive exclusion strategy, can result in oscillatory coexistence of more than one species in regards to nutrient uptake and growth (yield) of microorganisms in mathematical simulations. The rhythmic uptake pattern under V_M and high nutrient uptake rates under both V_S and V_M suggest a competitive exclusion strategy by *P. palmata*, which can often be found as large epiphytic populations, for example on *Laminaria* stipes (Whittick 1983). It is conceivable that *Laminaria* stipes resemble a beneficial substratum to settle on, as big *Laminaria* fronds can provide shade and reduce effects of harmful light intensities to *P. palmata*, such as ultraviolet-induced genotoxicity (Atienzar et al. 2000). *Laminaria* stipes can mitigate the impact by hydrodynamic forces on *P. palmata*, and by this avoid damage or dislodgement by drag, the primary wave-induced force to intertidal seaweeds (Denny and Gaylord 2002). High uptake rates, especially during V_S, in *P. palmata* suggest a competitive advantage for nutrients, compared to nutrient uptake rates in *L. digitata*, which showed a V_S and V_M for DIP of 0.38 ± 0.03 and 0.22 ± 0.01 $\mu mol \cdot cm^{-2} \cdot d^{-1}$, respectively for DIN 3.9 ± 0.1 and 1.8 ± 0.4 $\mu mol \cdot cm^{-2} \cdot d^{-1}$ in comparable

conditions of light, temperature and hydrodynamics (Table 6-1; Lubsch and Timmermans 2019). The rhythmic uptake strategy during V_M by *P. palmata* bypasses the strategy of linear uptake under V_M in *L. digitata* (Lubsch and Timmermans 2019), and by that ensures a coexistence in regard to nutrient resources.

The strong correlation of DIP to DIN uptake, as well as the uptake ratio of 1:10 under V_M show the importance of DIP for metabolism and growth in *P. palmata*, especially compared to the opportunistic seaweed *U. lactuca*, which showed an uncorrelated DIP and DIN uptake with a ratio of 1:32 under V_M (Chapter 2). Moreover, an elevated DIN uptake, as in V_S, did not occur in *P. palmata* under DIP depletion. Comparably, Martinez & Rico (2004) reported that an enrichment with DIN did not increase growth, if DIP was not added to the cultivation medium and a proper enrichment with both, N and P, was the only way to enhance growth rate in *P. palmata*.

The ISC calculated for DIP is an approximation, as internal nutrient pools had not been depleted during the adaptation/starvation phase, indicated by an on-going growth, as well as high values of F_v/F_m under DIP depletion into 3 weeks, respectively 4 weeks of the assay. Nevertheless, a rational estimation of ISC for DIP was possible based on the decrease in F_v/F_m of sporophytes that levelled off in accordance with the dosage of limiting DIP availability under completely controlled conditions for light, temperature, and hydrodynamics over the 5 weeks experimental period. It can be estimated that it will take some 40 days for *P. palmata* to maintain its assimilation rate (equivalent to V_M) with filled internal DIP and DIN storages under external depletion conditions. This is comparable to the ISC in the perennial seaweeds *S. latissima* and *L digitata* with an approximate 45 days capacity to storage of DIN. With completely filled internal pools for DIP, it can be estimated that *S. latissima* can maintain its assimilation rate under DIP-depleted conditions for approximately 90 days (Chapter 4). In contrast, the opportunistic seaweed *U. lactuca* exhibited an ISC that would last 10 days for DIP and DIN (Fujita 1985, Chapter 2). It should be realised that the estimation of ISC is largely based on the response of photosynthetic efficiency F_v/F_m to

nutritional stress. Seaweeds can exhibit a broad range of physiological responses to stress-related conditions, notably an immediate change in the photosynthetic efficiency F_v/F_m (Parkhill et al. 2001). An F_v/F_m value between 0.79 and 0.84 is considered the optimal value for many plants, while values significantly below that range are considered to stress (Maxwell & Johnson 2000). *P. palmata* showed values significantly below the considered optimum of 0.79 to 0.84, when exposed to non-limiting DIP and DIN treatments, but were in agreement within the optimum range of F_v/F_m generally measured in red algae (Bose et al. 1988, Hanelt et al. 1993, Dring et al. 1996), including *P. palmata* (Liu & Pang 2010). Accordingly, *P. palmata* first indicated nutritional stress by a significant decrease in F_v/F_m, in limiting DIP concentrations.

The total dissolvable protein- and carbohydrate concentrations in *P. palmata* were in the range of reported values for this species (Morgan et al. 1980, Galland-Irmoulli et al. 1999, Harnedy and FitzGerald 2013). The dissolvable protein concentrations, ranging from 10-25 % DW in sporophytes exposed to different limiting and non-limiting DIP treatments in our experiments, perfectly aligned with observed seasonal variations of protein concentrations in natural populations of *P. palmata*: the lowest protein concentrations of approximately 8 % DW were measured during summer months, when nutrient concentration of the seawater were lowest, and protein concentrations of approximately 30 % DW were found during winter and early spring, when nutrient concentrations of the seawater showed an annual high (Martinez and Rico 2002, Rødde et al. 2004). Galland-Irmoulli et al. (1999) reported a protein content as high as 21.9±3.5 % DW in natural populations of *P. palmata*. The significant differences in dissolvable protein concentration in our experiments with *P. palmata* under different treatments of limiting DIP concentrations can not only reflect seasonal variations, but also show the strong dependency on available DIP in order to take up nitrate, as nitrogen represents a key element in the protein production, respectively amino-acid synthesis. For *P. palmata*, it has been shown that some forms of N increase growth rate, whereas other forms increase tissue N, and therefore protein content (Morgan and Simpson 1981, Grote 2016). Nitrate was found superior to ammonium as a source

of nitrogen for growth and *P. palmata* supplied with ammonium accumulated more tissue nitrogen than plants supplied with nitrate within the same time span (Morgan and Simpson 1981b). Similar to N and P, carbon can be stored as reserves in the form of carbohydrates and can be utilized to profit during times of high external DIP and DIN availability. In addition to nutrient availability, light (irradiance) has been identified as a main factor affecting nutrient reserves in *P. palmata.* Sun-acclimated *P. palmata* in northern Spain showed lower N and P and higher C content than shade-acclimated individuals, irrespective of transient high nutrient concentrations due to upwelling (Martínez and Rico 2008). The storage of C from high light exposure was shown to be the driving factor for metabolic adjustments at the end of summer. Environmental parameters vary according to season and the ecological conditions can stimulate or inhibit the biosynthesis of chemical composition in seaweed (Lobban and Harrison 1994).

In this study, the storage of C during times of low external DIN and DIP availability was clearly shown by high concentrations of dissolvable carbohydrates and low to moderate concentrations of dissolvable proteins in sporophytes exposed to limiting DIP concentrations, and vice versa in non-limiting DIP conditions. Total dissolvable carbohydrate concentrations ranged from 20-55 % DW, and concentrations were consistent with reported values for this species. For example, Mutripah et al. (2014) reported a carbohydrate concentration of 469.8 $mg \cdot g^{-1}$ DW in *P. palmata*, the highest carbohydrate content of 20 seaweed species evaluated. Our results showed that increased DIP availability led to increased nitrate uptake in *P. palmata*, which in turn increased the protein: carbohydrate ratio. Similar protein: carbohydrate ratios were found, for example, in seasonal patterns in natural populations of the red alga *Gracilaria verrucosa* (Hudson) Papenfuss, with the highest ratio in the winter months associated with high inorganic nitrogen concentration of the seawater, low water turbidity and low temperatures (Bird 1984). A critical protein: carbohydrate ratio for the subtropical *G. verrucosa* was documented at 0.38. It was suggested that the thallus constituents of protein and carbohydrates could be used to evaluate the nutrient deficiency status of *G. verrucosa* (Bird 1984).

The results on DIP and DIN uptake dynamics, as well as on dissolvable protein and carbohydrate concentrations under limiting and non-limiting DIP conditions, that are presented in this study match existing information on *P. palmata* and add to the ecophysiological understanding, help to interpret nutrient management strategies and open further insight into ecological aspects of nutrient availability. Moreover, our data allows to contribute to a viable seaweed mariculture, as well as a modern land-based cultivation in an economical and environmentally responsible manner. For example, our data on uptake dynamics and growth rates supports *P. palmata* to be a potent species for bioremediation purposes in layered multi-species cultures, while a considerable amount of valuable proteins and carbohydrates is produced at the same time. For example, to improve efficiency in bioremediation and enhance yield, the slow growth rates and oscillating uptake strategy by *P. palmata* can be complemented by *S. latissima*, which showed a mean growth of 4 % d^{-1} and similarly high, but uncorrelated and linear uptake rates of DIP and DIN in a ratio of 1:13 under V_M in comparable conditions (Table 6-1). Such a multi-species culture can be useful especially in close proximity to fish farms, which commonly generate large amounts of effluents in fluctuating quantities, including nitrogenous compounds and phosphates. Limitations or shifts in limitation from one element to another can be accounted for by nutrient additions in appropriate frequency and ratio, as well as by crop rotation of different species in accordance to DIP and DIN uptake ratios.

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Chapter 7

'Manual for nutrient uptake kinetics in seaweed cultivation'

The studies in this thesis were conducted with clear fundamental and applied objectives to aim for a better understanding of the ecophysiology of the 4 seaweeds and at the same time contribute to applications and implications in the development of a new bio-based economy emerging in the Western hemisphere, and in particular around the North Sea area. In the following chapter specific examples on the implementation of our results into seaweed operations, such as offshore cultivation, tank cultivation, IMTA applications and bio-filtration activities are given. Generally, our data on uptake kinetics and nutrient management (Chapter 2, 4 & 6) can perfectly well be integrated into dynamical models, such as ERSEM (European Regional Seas Ecosystem Model) to project the effects of environmental variables on growth and composition in seaweed species. Dynamical models allow not only an estimation on growth of seaweed and its composition, for example, the seasonal growth and composition of *S. latissima* (Broch & Slagstad 2012) and its potential production in seaweed farms (for the North Sea and UK coastal waters: van der Molen et al. 2018), but also support to project the economic feasibility of sustainable seaweed production (for the North Sea area: van den Burg et al. 2016).

7.1 Offshore cultivation

Offshore cultivation of seaweed opens opportunities for large scale operations to produce food, feed and chemical compounds for further utilization (McHugh 2003, Bartsch et al. 2008, Holdt & Kraan 2011, Fernand et al. 2016). Although offshore cultivation leaves relatively little control to environmental variables, some factors can be manipulated. Some structural systems for seaweed cultivation are designed to be moved vertically, thus allowing to submerge the system to depth where light conditions are favourable for growth or higher nutrient concentrations are

present. In addition, a submergible system can be moved to reduce hydrodynamic impact on the seaweed and the structure itself during severe oceanic weather conditions (Buck & Buchholz 2004). During periods of low nutrient concentration in the surrounding ocean water, fertilizer can be added, for example by deploying porous containers with slow-release fertilizer (Neushul et al. 1992). Information on nutrient ecophysiology, as studied for *U. lactuca* (Chapter 2 & 3), *S. latissima*, *L. digitata* (Chapter 4), and *P. palmata* (Chapter 6) in this thesis, offer good opportunities to locate potential cultivation sites in relation to nutrient availability and manipulate nutrient additions appropriately on site, as well as adjust the frequency of additions and ratios.

The following solely serves as a hypothetical example on nutrient uptake management in *S.latissima*, as observed in our studies (Chapter 4) and demonstrates how the fundamental data on nutrient uptake kinetics and management can be applied: a suitable location, based on nutrient availabilities, to culture *S. latissima* should offer (more or less constant) nutrient concentrations in the seawater of approximately 19.5 $\mu mol \cdot L^{-1}$ DIN and 1.5 $\mu mol \cdot L^{-1}$ DIP for optimal growth at any time (see V_M in relation to nominal offered concentration; Table 8-1). A seaweed farm with a total line length of 2 km (whether long line arrangement or ring system) and monocultures of young *S. latissima*, have, for example, an averaged SA of 0.04 m^2 (400 cm^2) per meter of line. This amounts to a total SA of 800.000 cm^2 for 2 km of line. Initial daily requirement of DIN and DIP would account for 43.7 g N and 7.4 g P, according to our data on V_M in *S. latissima* (Table 8-1; Chapter 4). A daily growth of 4 %, as observed for *S. latissima* in our study and by others (Nielsen et al. 2014, Boderskov et al. 2015), increases the total SA to its tenfold in 59 days, calculated as follows:

$1.04^t = 10 \rightarrow t = \ln(10) \times \ln(1.04)^{-1}$,

with t as time (days) needed to 10-fold the SA at a growth rate of 4%. After 118 days, the initial SA has increased by the factor 100 to 4m^2 per meter of line and the total daily DIN and DIP requirements in this scenario have expanded likewise, and account for 4.4 kg N and 0.74 kg P. Fertilizers (N and P in the appropriate concentration and ratio) can be added in periods of nutrient

limitations. Also, by tracking seasonal changes of DIN and DIP availability in the seawater, an estimate on the eco-physiological performance of the seaweed can be given based on the information on the ISC, and thus can reduce the necessity for tissue content analysis of N and P. The high ISC and an uncoupled nutrient uptake in *S. latissima* allows for up to 7 weeks of DIN and DIP depletion or limitations without major forfeit in growth. For example, when nutrient concentrations in the seawater show a DIN concentration of approximately 10 $\mu mol \cdot L^{-1}$ over 4 weeks, the rate of assimilation (V_M) in *S. latissima* is only half-saturated. This external limitation can be compensated by utilization of internal DIN pools. Approximately one third of the ISC for DIN in *S. latissima* would compensate for the limitation, accounting to 54.6 $\mu mol \cdot cm^2$, calculated as follows:

$$\Delta ISC = d_M \times (V_M - T_c),$$

with d_M as days of nutrient limitation, V_M as metabolic uptake rate, and T_c as limiting nutrient concentration. This estimate allows to adjust appropriate nutrient additions and duration: V_s for DIN is approximately 3 (2.9) times more efficient than V_M in *S. latissima* and a nominal DIN concentrations in the seawater of approximately 19.5 $\mu mol \cdot L^{-1}$ optimally saturates V_M. Therefore the addition of DIN to create a seawater concentrations of approximately 60 $\mu mol \cdot L^{-1}$ (56.5 $\mu mol \cdot L^{-1}$) over the next 8 (7.4) days is advisable to allow *S. latissima* to refill internal N pools in this scenario, calculated as follows:

$$d_{ISC} = \Delta ISC \times (V_S - V_M)^{-1},$$

with d_{ISC} as days needed to refill ISC, ΔISC as available capacity, V_S as the surge uptake rate, and V_M as the metabolic uptake rate. The addition of costly nutrients to establish higher concentrations is superfluous, as V_S is limiting, and hence excessive nutrients are 'washed' away in an open system and are lost for the operation.

Naturally, this over-simplified example is not able to adequately represent the complex and combined mechanisms of many environmental factors impacting physiology, growth and composition of a seaweed. However, suitable nutrient uptake and nutrient management data for the 4 species used during this thesis is very scarce, and our data can be integrated into dynamic ecological models, such as ERSEM for a more detailed projection and can support economic (feasibility) studies on seaweed farming and can be used for studies of resulting impacts on marine ecosystem services. An approach in seaweed farming is a multi-layered poly-culture of seaweed (Reith et al. 2005), that is, different groups of seaweed have differing light requirements, so that green, brown, and red seaweeds can be grown at various depths, allowing for integration of crops through a layered growth of different seaweed groups. The nutrient demand of poly-cultures, for example *S. latissima* integrated with *P. palmata*, can be evaluated accordingly using the data presented in this thesis. Poly-cultivation is also a promising approach for IMTA activities, also related to bioremediation purposes and/or ecosystem services. In ecosystem services, coastal seaweed farms can act as the ultimate barrier to (re-)capture/recycle dissolved phosphate, before it is diluted to the deep sea. Phosphorus is often a limiting nutrient in (terrestrial) agriculture, due to its low availability and mobility in soils. Soil fertility has been identified as one of the most significant challenges in achieving food security in the developing world (Elser 2012).

7.2 Integrated multi-trophic aquaculture (IMTA)

In most cases the stimulus for the integration of seaweeds in a multi-trophic aquaculture is based on cleaning the seawater from increased concentrations of dissolved nutrients, originated from fish farm activities, and by this prevent eutrophe conditions (Troell et al. 1997). Most fish farms are located close to the coastline, typically in sheltered bays and estuaries, which are vulnerable ecosystems with regard to eutrophication. Several IMTA systems, including multi-layered cultures of different seaweed species, have been proposed to combine seaweed cultivation and fish farms in order to mitigate effects of eutrophication and at the same time produce valuable

biomass for food, feed and fertilizer (Reith et al. 2005). It is important to note that fish (by definition) produce organic waste, and seaweeds (by definition) prefer the inorganic form of nutrients. Organic nutrient sources can be differentiated from inorganic nutrients in that they must be decomposed/re-mineralised before they are available to the seaweed. This is done by microorganisms such as bacteria. Moreover, large fractions of the waste produced by fish are in particulate material. This is a mismatch from the beginning. Filter-feeders may be much more efficient in removal of waste products in the immediate vicinity of fish farms (e.g. Soto & Mena 1999, Mazzola & Sarà 2001). The extreme enrichment of N and P in the water column deriving from fish farms (Aure & Stigebrandt 1990, Gowen 1994), paired with their continuous operation throughout the year, and often surrounding (natural) restrictions of available space, require an efficient and yearlong functioning approach.

In our studies, *S. latissima* and *P. palmata* showed the highest uptake rates for both, DIP and DIN with a ratio of 1:13, respectively 1:10, and growth rates of 3 % d^{-1}, respectively 1 % d^{-1} (Table 8-1), which makes them promising specimen for multi-layered poly-cultures in close vicinity to fish aquaculture. The constant nutrient uptake by *S. latissima* (Chapter 4) and the oscillating nutrient uptake, as well as the strong dependency on available DIP for an increased DIN uptake in *P. palmata* (Chapter 6) complement each other in DIP and DIN removal. In terms of bioremediation, this poly-culture could represent the 'main body' of the seaweed cultures, with supplementary configuration on size and integration of other species in correspondence to efficiency, nutrient removal ratios of DIN and DIP, as well as season. Downstream the IMTA operation, nutrient concentrations (and ratios) should be on the natural level, to minimize effects on the ecosystem. A DIN limitation in the seawater can create a downstream gradient with a high DIP to DIN ratio. According to our results, the integration of *L. digitata* with a DIP:DIN-uptake ratio of 1:8 under V_M (Table 8-1; Chapter 4) could help to diminish such a gradient. A DIP limitation, creating a high DIN concentration in the downstream could be mitigated by the integration of *U. lactuca*, which showed the lowest DIP uptake rates and considerably high DIN

uptake rates (Table 8-1). Another possibility would be the harvest of *P. palmata* and replacement with *S. latissima*. As our study showed, *P. palmata* strongly depends on the availability of DIP to increase DIN uptake, in order to increase the total dissolvable protein concentration, hence the nutritional value (Chapter 6). Its replacement by *S. latissima* with a DIP to DIN uptake ratio of 1:13 under V_M (Table 8-1; Chapter 4) could scale down the forming of a high DIN concentration under DIP limitation. During the summer months, *S. latissima* and *P. palmata* can/will reduce their growth due to an increase in water temperature (discontinued growth in *S. latissima*: >20 °C (Pedersen 2015); in *P. palmata*: >18 °C (Morgan & Simpson 1981)), and an integration of *U. lactuca* (>25 °C Fortes & Lüning 1980) could complement the poly-culture on the surface layer to maintain an efficient bioremediation, produce biomass, as well as shade and protect *S. latissima* and *P. palmata* in deeper layers from harmful light intensities. It is also conceivable that an integration of *U. lactuca* could divert certain (meso-) grazers to minimize feeding impact on *P. palmata* and *S. latissima* from spring to autumn. Generally it is assumed that fast growing seaweeds with filamentous texture can reduce the impacts on herbivory by escaping consumers in space, time or high growth rates (Lubchenco & Gaines 1981). However, seaweed-herbivore interactions and feeding preferences on different seaweed species represent a largely unexplored facet of seaweed ecology (e.g. Paul et al. 2006, Toth & Pavia 2007, Molis et al. 2015).

In spite of the many opportunities as described above, major fundamental challenges remain for IMTA: in addition to a possible mismatch of organic versus inorganic nutrients and the large fraction of particulates in waste from fish, the balance of waste stream and size of seaweed site, as well as position, is difficult. Large seaweed populations are required to absorb the dissolved waste of large quantities of fish, and the other way around. In practice this will be difficult to match.

7.3 Tank cultivation

In tank cultivation of seaweeds, it is possible to control (almost) all environmental factors and thus knowledge of the ecophysiology is very important to maximise growth rates, yields, and/or acquire desired products. Information on the optimum nutrient requirements in order to calculate the nutrient supply rate to a tank are essential. This includes not only knowledge on V_S and V_M for DIN and DIP of the applied species, but also information on the optimum nutrient uptake ratio to determine the most economical additions of for example macro- or micro-nutrients (Harrison & Hurd 2001). In addition, information on the ISC can help to mitigate and control the potential entry of epiphytes and/or microalgae. By starving the seaweed for a suitable time period, before the ISC for DIN and DIP have decreased to critical levels, the growth of epiphytes could be hampered, respectively controlled in cultivation practices (Pickering et al. 1993). The longer the period of time before the ISC of the cultivated species is depleted, the better the control of contamination by epiphyte and/or microalgae. Typically, V_S of nutrient starved seaweed is multiple times greater than V_M (Conway et al. 1976) and hence the seaweed is able to quickly overcome its nutrient deficiency, after the addition of saturating nutrient concentration. For example, a tank monoculture of *P. palmata* with a total SA of 10.0 m^2 (100,000 cm^2) incubated in a 4000 L aerated cultivation tank, constant water temperature of 12 °C and a 16/8 h light-dark rhythm with a light supply of 70 μmol photons $m^{-2} \cdot s^{-1}$ would require a daily addition of 7.8 g N and 1.8 g P, calculated as follows:

$n = m/M \rightarrow m = n \times M$,

with n as the daily amount of substance (or chemical amount, or daily requirements by the seaweed), M as the molar mass (N=14 $g \cdot mol^{-1}$; P=31 $g \cdot mol^{-1}$), and m as the required mass of N, respectively P.

$m_N = 0.56 \text{ mol} \times 14 \text{ g} \cdot \text{mol}^{-1} = 7.84 \text{ g}$

$m_P = 0.057 \text{ mol} \times 31 \text{ g·mol}^{-1} = 1.76 \text{ g}$

Naturally, N and P are only available as chemical compound and the required concentrations of N and P can be added, for example, in the forms of potassium nitrate (KNO_3; 101.1 $g·mol^{-1}$) and potassium-dihydrogen-phosphate (KH_2PO_4; 136.1 $g·mol^{-1}$). In this case, the daily additions would amount to 56.6 g KNO_3 and 7.8 g KH_2PO_4. After addition, the initial DIN and DIP concentration in the seawater inside a 4000 L tank, should have a DIN concentration of 140 $\mu mol·L^{-1}$, respectively 14.25 $\mu mol·L^{-1}$ (N:P-ratio of 1:10, neglecting potential background concentrations already present in the seawater), calculated as follows:

$c_x = n_x \times V^{-1}$,

with c_x as the concentration, n_x as the total daily molarity required, and V as the volume of the tank filled with seawater. Due to an oscillating nutrient uptake by *P. palmata* in a weekly rhythm (Chapter 6), a weekly pulse of approximately 400 g KNO_3 and 100 g KH_2PO_4, rather than a constant daily supply, would be ideal in this scenario. Simultaneously, the weekly nutrient pulses will result in starvation of epiphytes- and/or microalgae between the nutrient additions. A weekly addition of 400 g KNO_3 and 100 g KH_2PO_4 would create an initially very high DIN and DIP concentration of 980 $\mu mol·L^{-1}$, respectively 100 $\mu mol·L^{-1}$ in the cultivation tank. The high uptake rates of *P. palmata* under V_S and V_M conditions (Table 8-1) are capable of refilling the ISC in a short time under these saturating nutrient conditions. The weekly dosage can be adjusted to growth rates. An averaged growth rate of 1 % d^{-1} has often been reported for *P. palmata* (Sanderson et al. 2012, Corey et al. 2014), similar numbers are reported in this thesis (Chapter 6). A growth rate of 1 % d^{-1} is calculated as follows:

$f(t) = a \times (1 + p·100^{-1})^t$,

with *a* as the total initial SA, p as growth rate, and t as the number of cultivation days. Accordingly, the total SA of the *P. palmata* culture will increase from 10 m^2 to 11.5 m^2, after 2 weeks (f(t) =

100,000 $cm^2 \times (1 + 1/100)^{14}$ = 100,000 $cm^2 \times 1.01^{14}$ = 115,000 cm^2). In tank cultivation of *P. palmata*, it is advisable to regularly control DIP concentration in the water and ensure DIP availability, due to the strong necessity for elevated DIN uptake and thus growth (Chapter 6). When young sporophytes of *S. latissima* are cultivated in tanks, it is not advisable to pulse extremely high nutrient concentrations, as shown in the study in Chapter 4: all sporophytes (n=7) exposed to daily high DIP and DIN pulses perished within 3 weeks.

7.4 Biofiltration

In order to predict the efficiency of a particular seaweed in water treatment facilities (biofilters), for example in land-based tank systems or in *situ* applied biofilters at inlets of cooling water for power plants, information about uptake kinetics are indispensable and can help to control effluent and productivity in environmentally responsible practices (e.g. Robertson-Andersson et al. 2008, Copertino et al. 2009). The opportunistic seaweed *U. lactuca* has been identified as a promising species in biofilter systems and in IMTA systems (e.g. Cohen and Neori 1991, Neori et al. 2003). The majority of studies related to the efficiency of N and P removal from seawater have been conducted under field conditions (Cohen & Neori 1991, Naldi & Viaroli 2002, Neori et al. 2003) and verify the feasibility of *U. lactuca* for biofiltration, but a quantification on total filtration (or nutrient assimilation) capacity was unknown. Our results on the uptake kinetics support that *U. lactuca* can efficiently be applied in biofiltration systems for an excess nutrient uptake, leading to less eutrophic waters (Chapter 2). Despite the quickly filled ISC and the corresponding declines in nutrient uptake rates of approximately 90 % for DIP and 80 % for DIN in saturating concentrations, V_M in *U. lactuca* can still significantly contribute to the reduction of nutrient loads. Although our results on nutrient uptake kinetics showed higher uptake rates in the perennials *S. latissima* and *P. palmata,* higher growth rates (Table 8-1), as well as a greater SA (to take up nutrients) in relation to biomass favor the opportunistic *U. lactuca* to be employed in filtration systems. The correlation factors presented in this thesis for SA with FW and DW in *U. lactuca* (Chapter 2) enables

conversions between these standardization units. This allows for an accurate estimation of the efficiency and sustainability of a (large scale) bio-filtration system, as industrial enterprises typically determine the FW rather than SA for practical reasons. Moreover, efficiency and sustainability of these biofilter systems can be maintained by controlling the effluent and/or adapting the biomass, also in accordance to growth rates. In the case of *U. lactuca*, for example, a bio-filtration system containing a biomass of 100 kg FW would be an equivalent SA of approximately 7.7 million cm^2, given our conversion factor for FW (y=0.013x). This SA could take up a total of around 538,000 µMol DIP and 17.7 million µMol DIN in a day, according to the uptake rates under V_M for DIP and DIN in *U. lactuca* (Table 8-1). The daily reduction of DIP and DIN from the seawater by *U. lactuca* corresponds to 16.7 g of P and 250 g of N, calculated as follows,

$n = m/M \rightarrow m = n \times M$,

with n as the amount of substance, *M* as the molar mass (N= 14 $g \cdot mol^{-1}$; P= 31 $g \cdot mol^{-1}$) and m as the mass. A moderate growth rate for *U. lactuca* of averaged 4 % d^{-1}, as observed in this thesis (Chapter 2), would double the initial biomass of 100 kg FW in approximately 18 days, according to an exponential increase, determined by the following function:

$t_2 = \ln(2) \times \ln(1.04)$,

with t_2 as doubling time, ln(2) as the logarithm for doubling the biomass and ln(1.04), resembling the daily growth rate of 4 %. However, this example on the quantification of the bio-filtration capacity by *U. lactuca* requires optimal mixing of the water column for nutrient distribution and constant biomass circulation to avoid self-shading and provision of light for each individual. Related to growth rates, the filtration tanks should offer sufficient room for the increase in biomass (Figure 7-1).

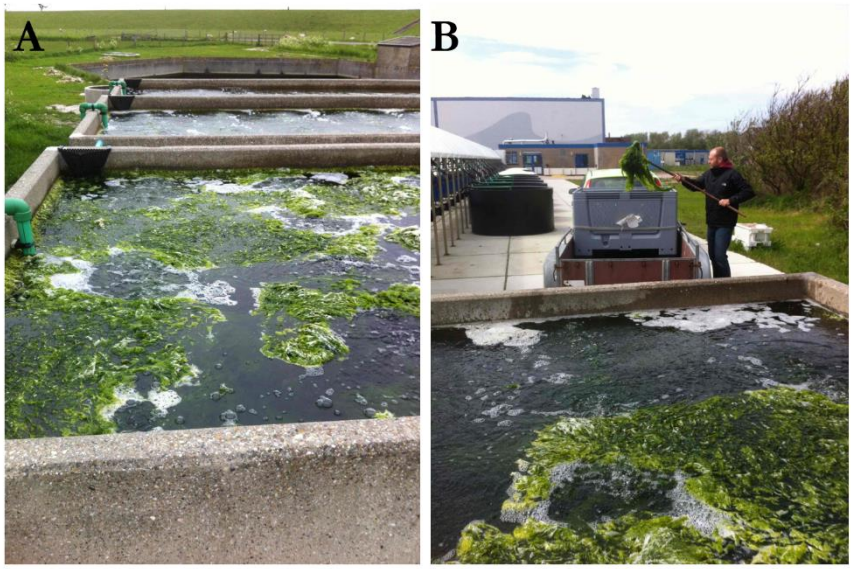

Figure 7-1. Carrying capacity. (A) *Ulva lactuca* piling up to '*Ulva*-bergs' in a bio-filtration tank and (B) harvest of piled up biomass with a pitchfork at the NIOZ Seaweed Research Centre on Texel in summer 2015.

Chapter 8

Synthesis

Fundamental scientific curiosity to understand the processes that determine, for example, the success and competitiveness of native North Sea seaweed species in relation to nutrient availability is essential to explore possibilities for finding the balance between preserving marine ecosystem services and unlocking its potential for sustainable food, feed and fuel production. There is a growing interest in seaweeds in Western Europe and efforts are being made to establish a viable mariculture, as the commercial exploitation of seaweeds for food, feed, energy, and chemical compounds is outlined (Dhargalkar & Pereira 2005, Thangaraju 2008, Holdt & Kraan 2011, Milledge et al. 2014, Fernand et al. 2016, Porse & Rudolph 2017). Efforts to investigate the economic feasibility of seaweed production in Europe have been implemented (Reith et al. 2005, Taelman et al. 2015, van der Molen et al. 2018). Although a bio-based economy is promoted and under development, often aspects of sustainability and resource availability are addressed only to a limited extent (Staffas et al. 2013). In order to understand the physiology, and most notably the nutrient kinetics of seaweeds and their successful implementation into a sustainable bio-based economy, it is necessary to understand basic concepts, such as the response to nutrient additions, nutrient uptake, nutrient uptake ratios, and storage capacity (i.e. nutrient management) in relation to resource availability. This all is interesting from a pure fundamental scientific point of view, urging scientific inquiries into seaweed physiology and ecology. What conditions are favourable, and which are disadvantageous to seaweed species? What are the similarities/differences among species? How long can species grow/survive under limitation conditions, etc.? Although fundamental scientific questions were always governing my research, the results clearly have implications for mariculture (Chapter 7). The results can contribute to a sustainable seaweed production, evaluate the bio-filtration/bioremediation potential, and at the same time gain a better

understanding of the ecophysiology in relation to resource availability. An innovative concept of colorimetric analysis of seaweed fronds related to the nutritional value (and indirectly to nutrient availability/history) was successfully tested on the example of *U. lactuca* (Chapter 3) and results were implemented into an easy-to-use application (www.eyeonwater.org/ulva), freely accessible for everyone with a smartphone. Another novelty is the introduction of a standardized method to infer to physical properties by compression and tension of seaweed, using an industrial texture analyser, demonstrated on the example of *L. digitata* thalli (Chapter 5). These are important parameters for selection and survival of stationary organisms, exposed to steady turbulent flow and its varying drag-forces, and also to determine tactile properties of seaweeds, which for example affect the perception and acceptance of consumers. The general findings and innovative aspects of this thesis are given in the following sections. In addition, conclusions from this thesis are drawn and a future outlook on research on seaweed is proposed.

8.1 General findings

In this thesis, research covered a wide range of eco-physiological experiments on seaweeds and many fundamental aspects related to ecological conditions and economic interests were addressed. Hatchery and cultivation of the 4 seaweed species gave a great insight into seaweed physiology in general, and in particular into the DIP and DIN uptake kinetics, as well as nutrient management strategies of *U. lactuca*, *S. latissima*, *L. digitata*, and *P. palmata*. All this led to a unique set of data and greatly increased my experience on seaweed research. In particular: Chapter 2 - planning, preparing and managing experiments, designing and building custom applications, for example light cabinets and custom-made extensions for rotating tables. Chapter 3 - applying optical measurements on seaweeds, including fluorescence, spectral and colorimetric measurements techniques. Chapter 5 - designing and developing fixation clamps for the texture analysis of seaweed samples and working with an industrial texture analyser. Chapter 6 - establishing standardized protocols for post-experimental treatment of seaweeds, for example the

homogenisation of different seaweed samples for further analysis of total dissolvable protein- and total dissolvable carbohydrate concentration.

Common to all my experimental work during my PhD project was the "pulse-and-chase" set-up, which was used for all 4 species, rendering equal conditions and thus results for the species can be compared. In essence, seaweed individuals were starved in nutrient depleted seawater to bring all individuals in the same physiological status, after which they were exposed to a constant (range of) concentration of one limiting nutrient, while the other essential resources were supplied in non-limiting concentrations. In practice this was done by replacing/refreshing the seawater medium in each experimental treatment on a daily basis. Low nutrient concentrations in the seawater medium were depleted per day, while supplement of high concentrations had nutrients remaining after 24 hours. The quantity of nutrients remaining after 24 hours changed and showed clear, coherent patterns with basically increasing concentrations being left over in the seawater medium over time (compare Figure 1-5). The difference between daily supplied and nutrients remaining in the seawater after 24 hours was referred to uptake by the seaweed. The combination of non-limiting (high) nutrient concentration and a range of limiting (low) nutrient concentrations gave insights into physiological responses related to nutrient availability and more importantly, enabled an accurate calculation of uptake kinetics. These are labour intense experiments, requiring large quantities of well-defined seawater medium, and represent an optimal and essential method to perform these scientific inquiries into seaweed physiology (in the laboratory).

In Chapter 2, 4, and 6 the nutrient uptake dynamics of *U. lactuca*, *S. latissima*, *L. digitata*, and *P. palmata* were quantified in 'pulse-and-chase' experiments over extended time periods. All seaweed species showed a biphasic response in nutrient uptake rates; surge uptake (V_S), as a response to nutrient-starvation in the initial phase of the experiment, followed by a maintenance uptake (V_M), after internal nutrient pools had been filled. Most striking by comparison was the linear regression in uptake rates from V_S to V_M, when internal nutrient pools had been filled. This

was, also in relation to available nutrient concentrations in the seawater medium, followed by a relatively constant V_M in the green seaweed *U. lactuca* (Chapter 2) and the brown seaweeds *S. latissima* and *L. digitata* (Chapter 4). In contrast, the red seaweed *P. palmata* showed an oscillating V_M in a weekly pattern (Chapter 6). *Palmaria palmata* also showed the highest uptake rates of all 4 seaweeds for DIP and DIN under V_S and V_M, although paired with the lowest growth rates. At first sight, the highly competitive nutrient uptake rates seem dominating over uptake rates of the other 3 seaweeds, but a more detailed view into the uptake strategy revealed a rhythmic uptake in a weekly pattern, which allows seaweeds with lower uptake rates (i.e. *L. digitata*) to compete for nutrients. Moreover, a strong dependency on DIP availability for elevated DIN uptake in *P. palmata* limits the competitiveness of high uptake rates to times of the abundance of both nutrients.

In contrast to the K-strategy of *P. palmata* in relation to nutrients, *U. lactuca* showed a typical r-strategy with a comparable V_S for DIN, higher growth rate, and smaller ISC. The 'strategic intent' of r-strategy is to flood/invade/dominate in numbers a habitat with progeny so that, regardless of predation or mortality, at least some of the progeny will survive to reproduce. *Ulva lactuca* has the ability to reproduce rapidly with little energy investments and thrives during summer months when light conditions are favourable. Under high nutrient concentration conditions or eutrophe conditions this can lead to the forming of 'green tides', as increasingly is observed in coastal zones of areas with dense industry and/or intense agriculture (Teichberg et al. 2008, 2010). The data on uptake kinetics in *U. lactuca* with relatively low V_S and very low V_M for DIP and high uptake rates for DIN, support the conclusion that N is the main element driving *Ulva* blooms. The opportunistic strategy by *U. lactuca* is also reflected by the (limited) ISC, by the low concentration of total dissolvable protein and high concentration of total dissolvable carbohydrate concentration, and by their fast turn-over rates in frond colour, which can change significantly within a day (Chapter 3). In contrast, the high ISC in the perennial seaweeds *S. latissima*, *L. digitata* and *P. palmata* allows these *K*-strategists to endure long periods under DIN and DIP limiting conditions, typically in the summer months, and live close to the carrying capacity of their habitat.

Similar to *U. lactuca* and *P. palmata*, a high V_S for DIN was also found in *S. latissima*. V_S is not necessarily high (a multi-fold of V_M) in each species, as nutrient uptake rates in *L. digitata* displayed. The perennials *S. latissima* and *L. digitata*, are both considered winter species and live in the same underwater zonation. In Britain and Ireland, bedrock that was subjected to moderate to strong tidal water movements was characterized by dense *L. digitata* populations, while similar shores that were not tide-swept were generally characterized by mixed *S. latissima* and *L. digitata* or just *S. latissima* (Connor et al. 2003). This fits well to the eco-physiological data presented in this thesis, and suggests a niche separation of *L. digitata* from *S. latissima* in relation to hydrodynamics. The texture analysis of *L. digitata* (Chapter 5) showed that fronds were perfectly adapted to mechanical stress in terms of tensile and compression forces, similar to hydrodynamic forces experienced in a wave-swept habitat.

Another result noteworthy during the experimental work was that young sporophytes of *S. latissima* did not survive high DIP concentrations for an extended period of time (3 weeks), unlike the perennials *L. digitata* (Chapter 4) and *P. palmata* (Chapter 6). This intolerance to high DIP concentrations would inhibit *S. latissima* to colonize areas during eutrophe conditions. Although nutrient concentrations, as high as concentrations supplied in the experiments are very unlikely to occur in nature, this information can play a role in the settlement of young *S. latissima* sporophytes. An overview on the results on V_S, V_M, uptake- ratios and strategy, ISC for DIN and DIP, and growth rates for *U. lactuca*, *S. latissima*, *L. digitata* and *P. palmata* can be found in the following table:

Table 8-1. Overview of results on uptake management (surge uptake V_S, maintenance uptake V_M, uptake ratios, internal storage capacity ISC, favourable nominal nutrient concentration for cultivation, and growth) for dissolved inorganic nitrate (DIN) and dissolved inorganic phosphate (DIP) in *Ulva lactuca* (Chlorophyta), *Saccharina latissima*, *Laminaria digitata* (Phaeophyta), and *Palmaria palmata* (Rhodophyta), conducted in 'pulse-and-chase' experiments under controlled conditions for light (light/dark: 16/8 h; *U. lactuca*: 80 µmol photons $m^{-2}\cdot s^{-1}$, *S. latissima*, *L. digitata*: 18 µmol photons $m^{-2}\cdot s^{-1}$, *P. palmata*: 60 µmol photons $m^{-2}\cdot s^{-1}$), temperature (12±1 °C) and hydrodynamics over several weeks. The ISC was evaluated after the response in fluorescence signal (F_v/F_m) in relation to duration of DIN and DIP depletion/limitation conditions.

Devision		Chlorophyta	Phaeophyta		Rhodophyta
Species		***U. lactuca***	***S. latissima***	***L. digitata***	***P. palmata***
V_S (µmol·cm^{-2}·d^{-1})	DIN	12.5±5.2	11.3±0.6	3.9±0.1	15.6±4.3*
	DIP	0.66±0.12	0.80±0.03	0.38±0.03	1.57±0.29
V_M (µmol·cm^{-2}·d^{-1})	DIN	2.3±0.9	3.9±0.7	1.8±0.4	5.6±2.1
	DIP	0.07±0.04	0.30±0.09	0.22±0.01	0.57±0.22
	N:P-ratio	10:1	13:1	8:1	10:1
	strategy	linear	linear	linear	rhythmic
ISC (µmol·cm^{-2})	DIN	23±7	49	80	222
	DIP	0.7±0.1	14	>10	22
Nominal seawater concentration (µmol·L^{-1})	DIN	60	20	24	15
	DIP	6	1.5	3	1.5
Daily growth (%)		4	3	2	1

* Strong dependency (R=0.943) on DIP availability for nitrate uptake under V_S.

Resource availability is key for the survival, growth and reproduction of species in any given environment and hence drives the outcome of biological interactions, shaping final community composition (Chapin III et al. 2000). A high V_S shows the ability of a seaweed to rapidly take up nutrients when the concentration in the seawater is high, but is not indicating the affinity for nutrients at low concentrations, which can make an accurate evaluation of interspecific competition for nutrients difficult. Often K_M (Michaelis-Menten constant) is used to compare an organisms (here: seaweed) ability to take up nutrients at low concentrations, determined by plotting the nutrient uptake rate (V) versus the nutrient concentration (M). The resulting curve is described by the Michaelis-Menten equation:

$$V = V_{max} (M \times K_M^{-1} + M),$$

with V_{max} as the maximal uptake rate, M as the nutrient concentration, and K_M as the nutrient concentration where $V = V_{max}/2$. It was stated that K_M is dependent on V_{max} and it is more accurate to use α (the initial slope of the V versus M hyperbole) to compare uptake affinities of two or more species at low nutrient concentration (Harrison et al. 1989, Ritchie & Prvan 1996). In my experiments on uptake kinetics, the sampling interval of 24 h did not allow a detailed insight into uptake affinities, but on longer term allowed reliable quantification of V_S, V_M, and ISC, hence nutrient management strategies.

8.2 Innovative aspects / highlights of the thesis

I report in my thesis on several new scientific findings regarding the ecophysiology of N and P dynamics in 4 species of native North Sea seaweed species. Albeit not revolutionary new, my 'pulse-and-chase' approach applied under fully controlled laboratory conditions over several weeks (Chapter 2, 4 & 6) turned out to be a very successful and unique tool in my experimental work. This method is labour, seawater and time consuming, but resulted in detailed and reliable insights into the eco-physiology and nutrient management strategies of *U. lactuca*, *S. latissma*, *L. digitata* and *P. palmata*. Certainly in combination with constant measurements of the fluorescence

signal F_v/F_m. At the same time it contributed to economically important and practical aspects for a bio-based economy. Next to the contributions to the ecophysiology of North Sea seaweed species, I describe two innovations. The first innovation is the spectral and colorimetric analysis of *U. lactuca* and the implementation of the results into a freely available smartphone application 'EyeOnUlva' to evaluate the nutritional value of this green seaweed, found globally (Chapter 3). The second innovation is the texture analysis on seaweed individuals, which allows for standardised methods of inferring the effects on nutrient availability, varying hydrodynamic forces, and seaweed-herbivore interactions on phenotypic plasticity and particular traits (Chapter 5). Both innovations developed out of experience and curiosity on the subjects.

8.3 Conclusions

Seaweeds offer interesting options for fundamental research and applications. It is crucial to conduct eco-physiological laboratory studies, as it gives insight in the functioning of seaweeds. This insight is needed before a scale-up can be done to larger (outdoor) facilities, such as tank cultivation and mariculture operations in an ecological and economic responsible manner. In this thesis, uptake dynamics, uptake strategy, growth, and insights into cellular biochemistry and elemental stochiometry in 4 ecological important and economic interesting seaweed species native to the North Sea, were assessed in relation to DIN and DIP availability under fully controlled laboratory conditions. . Nutrient uptake dynamics were investigated through analyses of removal of dissolved nutrients. Growth was followed by combining non-destructive measurements during the experiment, with destructive harvests (for determination of the cellular composition) at the end of the experiments. Photosynthetic efficiency (F_v/F_m) was measured using PAM fluorometry. Total dissolvable protein and carbohydrate composition was measured using standard assays. In a separate chapter, results were presented in a comprehensive way, akin to a "manual for nutrient uptake kinetics in seaweed cultivation" (Chapter 7). Each of the 4 seaweed species has different adaptations to (changing) environmental conditions, also shown by their nutrient uptake

management and strategies (Table 8-1), which in turn can be used in mariculture. Although interactions of environmental factors as nutrients, light, temperature and hydrodynamics (addressed in Chapter 5) were not included, the high nutrient requirements not only show the ecological importance of seaweeds in terms of the ecosystem services they provide, but also in nutrient cycling, especially for N and P, which results in seaweed biomass being produced. Biomass that can be used by other organisms for nursery, shelter or food, and biomass that can be used as fertilizer or refined for other utilization by humans. Furthermore, the data presented in this thesis allows to manipulate and project production in seaweed cultivation and for bioremediation purposes, as well as it enables a comprehensive insight into ecological effects of nutrient limitations and shifts in limitations.

Supporting tools to assess and interpret the eco-physiological status were proposed and developed. A new avenue to conduct standardized method to determine tactile properties in seaweed, using a texture analyzer, was proposed. This enables comparable measurements on responses to abiotic and biotic stress on seaweeds, and allows species selection related to hydrodynamics in mariculture and adjust adequate pre-treatment in biorefinery. Another aspect is the development of the smartphone application 'EyeOnUlva', which is a good example on combining fundamental research of different disciplines, as biology and physics, leading to a new and timely application tool for environmental monitoring, also suitable for the general public.

8.4 Research outlook

Seaweed research in Europe is at its infancy and many aspects about the ecophysiology and biochemistry in seaweed in remain unknown. Aside from the 4 seaweed species that were the main players during the research for this thesis, there are plenty of other ecological important and economical promising North Atlantic seaweed species, for example *Saccorhiza polyschides* (Lightfoot) Batters, *Alaria esculenta* (Linnaeus) Greville, *Undaria pinnatifida* (Harvey) Suringar, and *Laminaria hyperbora* (Gunnerus) Foslie (Werner & Kraan 2004). Another interesting species to study in an

ecological and economical context is *Sargassum muticum* (Yendo) Fensholt, which firmly established itself (as an invasive species) in the North Sea (Kraan 2008), but it remains unclear exactly how this translates to changes in community composition and finally ecosystem functioning.

Follow-up studies could address the combined effects of nutrient availability (DIN, DIP), relevant physicochemical (e.g. light, temperature) and hydrodynamic conditions (e.g. turbulence) on growth and composition in seaweeds, leading to greater insight in seaweed organic matter dynamics and optimal production. As seaweeds have different morphologies, differences in nutrient uptake are suggested under similar hydrodynamics forcing (Gerard 1987, Kawamata 2001), with the highest uptake rates occurring under conditions of the highest currents and turbulence (Morris et al. 2008, for seagrasses). Apart from affecting nutrient uptake by reducing boundary effects, hydrodynamic forcing may also be expected to impose a direct effect on morphology and/or cellular composition (La Nafie et al. 2012 for seagrasses, Molis et al. 2015). In this context, the texture analysis as introduced in Chapter 5 can be applied, for example in a study aiming to shed light on niche separation of different seaweeds. In this thesis, it was shown that *S. latissima* was the superior competitor for N and P (Chapter 4), but the ultimate outcome of competition will not only be determined by availability of nutrients. In the same chapter it was clear that young sporophytes of *S. latissima* did not survive high DIP concentrations. Similarly, (differences in) toughness, indicative of resistance to hydrodynamic stress, can contribute to the ultimate outcome of competition.

Environmental conditions will not only determine growth, but through the effects on composition, for example induction of anti-herbivory substances, or anti-viral compounds (Holdt & Kraan 2011) it may also affect loss factors. Loss factors, as grazing, viral lysis and erosion are hardly taken into account (certainly not in relation to varying environmental conditions) although essential for a proper ecological understanding and sustainable production. Herbivory is known to take place in seaweeds and can be a substantial loss factor: fish, sea urchins and mesograzers

(amphipods, copepods, polychaetes) have been described to feed on seaweeds (Carpenter 1986). The exact nature of herbivory, and the relation with environmental variables however is hardly ever quantified. Mesocosm studies would allow to conduct (choice-) feeding experiments with different seaweed species and/or seaweed parts (stipe, frond, etc.) to test feeding preferences of (meso-)grazers related to food availability/composition. At the same time, effects on texture and composition of seaweeds to biotic stress can be investigated to get further insights into community interactions, defence strategies and biochemistry. For example, it was shown that tissue toughness in *Fucus vesiculosus* adjusted plastically to the prevailing level of wave exposure, which in turn affected the phenotypic plasticity of the radula of the grazing flat periwinkle, *Littorina obtusata* (Molis et al. 2015). It can be envisioned that this ecological "race of arms" can also be adopted to microscopic interactions, such as viral infections, which could be investigated by techniques of flow-cytometry and virus detection methods, for example enzyme-linked immunosorbant assay (ELISA) and real-time polymerase chain reaction (PCR).

Viral infection has been observed for seaweeds, even with remarkable cross infection between genera (Kapp 1998). The severity of infection symptoms show a broad range; while some infected plants undergo severe morphological changes or become completely unable to produce spores, others have only mild expression of symptoms. The epidemic dieback of the kelp *Ecklonia radiata* has been associated with the presence of viruses (Easton et al. 1997), and viral infected *Saccharina* sp. showed spiral growth and dwarfism.

The research on seaweeds is mostly unchartered territory and there many remaining research opportunities to "dive" into.

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Summary

Seaweeds are found throughout the world's oceans and seas. Seaweeds serve many important functions in ecosystems. As so-called ecosystem engineers, they can influence the availability of resources by influencing sedimentation and erosion. Seaweeds also provide a food source for primary consumers and at the same time offer protection from predators and can serve as a nursery for many animal species, thus increase diversity and significantly contribute to the structural complexity of an ecosystem. Furthermore, seaweeds play a considerable role in the world's carbon cycle, approximately 6 % of the global net primary production is realised by seaweeds, although they only inhabit 0.1 % of the seafloor. Unlike terrestrial crops, seaweeds do not require agricultural land for cultivation and many species grow in saltwater or brackish water, avoiding competition for land and precious freshwater. In addition seaweeds take up dissolved nutrients from the sea and do not need pesticides to protect their biomass. All these characteristics make that seaweeds play an important role in marine ecosystem services, for example in bioremediation and as the ultimate barrier to capture precious nutrients, like phosphate, before it is dilute into the deep sea.

Not surprising, there is a growing interest in seaweed cultivation in Western Europe (including in The Netherlands), as seaweeds are an attractive marine source of biomass for cultivation. The North Sea, located on the European continental shelf, belongs to one of the world's most productive marine areas. The overall nutrient budget of the North Sea ecosystem is influenced both by oceanic inflow from the north-east Atlantic Ocean, riverine and atmospheric input. Spatial differences in nutrient concentrations exist in coastal areas, largely affected by the run-off waters of several rivers, including Rhine, Meuse, and Thames. These run-off waters often contain considerable amounts of inorganic phosphorus (P) and nitrogen (N) from anthropogenic land-based activities. Besides natural fluctuations, the anthropogenic discharge of nutrients can

generate concentration gradients and limitations, which are often observed along coastal zones of the North Sea, often causing eutrophication.

Nutrient limitation and shifts in limitation from one element/compound to another can significantly affect the internal composition, physiology and growth of seaweeds. Each seaweed species has its own growth characteristics, internal composition and bottlenecks related to nutrient availability. Some species thrive on large amounts of nitrogen and can handle low concentration of phosphorus, while others require larger quantities of phosphorus, and can cope with relatively low nitrogen concentration. Therefore, fundamental knowledge on nutrient uptake kinetics and nutrient management strategies of different seaweed species is essential, for both ecological, as well as economic aspects. This knowledge can shed light on questions, like what conditions are favourable and which are disadvantageous to seaweed species? What are the similarities/differences among species? How long can species grow under limitation conditions, etc.?

In this thesis, 4 ecologically and economically important seaweed species native to the North Sea were investigated: the opportunistic green seaweed *Ulva lactuca* (Chlorophyceae), the perennial brown seaweeds *Saccharina latissima* and *Laminaria digitata* (Phaeophycea), and the perennial red seaweed *Palmaria palmata* (Rhodophyceae). A full factorial design was used to determine uptake kinetics (surge uptake V_S, maintenance uptake V_M, and uptake ratios) of dissolved inorganic phosphate (DIP) and dissolved inorganic nitrate (DIN), as well as internal storage capacity (ISC) of DIP and DIN under laboratory conditions, and all were standardized to surface area as seaweeds take up nutrients throughout their surface. All seaweeds tested showed a biphasic response in nutrient uptake rates: V_S as a response to nutrient-starvation and V_M after internal nutrient pools had been filled. A high V_S for both nutrients was exhibited by *U. lactuca* (DIN: 12.5±5.2 μmol·cm^{-2}·d^{-1}, DIP: 0.66±0.12 μmol·cm^{-2}·d^{-1}), *S. latissima* (DIN: 11.3±0.6 μmol·cm^{-2}·d^{-1}, DIP: 0.80±0.03 μmol·cm^{-2}·d^{-1}), and *P. palmata* (DIN: 15.6±4.3 μmol·cm^{-2}·d^{-1}, DIP:

1.57±0.29 μmol·cm^{-2}·d^{-1}), while *L. digitata* showed significantly lower surge uptake rates (DIN: 3.9±0.1 μmol·cm^{-2}·d^{-1}, DIP: 0.38±0.03 μmol·cm^{-2}·d^{-1}). V_M for DIP and DIN was significantly lower than V_S for both nutrients in all species tested: *U. lactuca* (DIN: 2.3±0.9 μmol·cm^{-2}·d^{-1}, DIP: 0.07±0.04 μmol·cm^{-2}·d^{-1}), *S. latissima* (DIN: 3.9±0.7 μmol·cm^{-2}·d^{-1}, DIP: 0.30±0.09 μmol·cm^{-2}·d^{-1}), *L. digitata* (DIN: 1.8±0.4 μmol·cm^{-2}·d^{-1}, DIP: 0.22±0.01 μmol·cm^{-2}·d^{-1}), and *P. palmata* (DIN: 5.6±2.1 μmol·cm^{-2}·d^{-1}, DIP: 0.57±0.22 μmol·cm^{-2}·d^{-1}). The quantification of the ISC for DIN and DIP enables to estimate the time, during which N and P reserves can be used, before nutrient limitations cause significant forfeit to growth and losses of yield. A large ISC was quantified for perennial seaweeds *P. palmata* (DIN: 222 μmol·cm^{-2}, DIP: 22 μmol·cm^{-2}), *S. latissima* (DIN: 49 μmol·cm^{-2}, DIP: 14 μmol·cm^{-2}), and *L. digitata* (DIN: 80 μmol·cm^{-2}, DIP: 10 μmol·cm^{-2}). The ISC for both nutrients in the opportunistic *U. lactuca* (DIN: 23±7 μmol·cm^{-2}, DIP: 0.7±0.1 μmol·cm^{-2}) was significantly smaller than ISC in the perennial seaweeds. In addition to the quantification of uptake kinetics and ISC of DIN and DIP, the uptake strategies or long term performance to environment and/or competition for (limited) nutrient resources of the 4 seaweeds were described. For example, a rhythmic DIN and DIP uptake in weekly intervals in *P. palmata*, in contrast to a linear uptake under V_M in *L. digitata* provides evidence for a niche separation in relation to nutrient availability. Moreover, DIP availability limited access to DIN in *P. palmata*, which consequently was mirrored by the total dissolvable protein- and carbohydrate concentration, thus the nutritional value of the seaweed. Total dissolvable protein content ranged from 10.2±2.5 % to 24.6±8.0 % dry weight, depending on DIP availability. Similarly, total dissolvable carbohydrate content ranged from 22.1±3.6 % to 54.3±12.3 % dry weight.

In *U. lactuca*, colour differences of fronds appeared to be related to the nutritional value, respectively total dissolvable protein concentration. This led to a novel study, which examined the possibility to deploy spectro-radiometry and colorimetric techniques to evaluate the total dissolvable protein concentration from frond colour of this green seaweed. Based on the concept of colorimetric techniques, we developed the smartphone application 'EyeOnUlva' for IOS and

Android systems, which records the frond colour and provides an inexpensive, reliable, safe and easy-to-use method to give a fast evaluation on the total dissolvable protein concentration in *U. lactuca*. The 'EyeOnUlva' application not only represents a useful and timely tool to the aquaculture industry to assess the nutritional value of their seaweed crop and determine its feeding quality in a cost-effective way, but is also applicable in environmental surveys, including citizen science programs.

Another novel approach, which allows for standardised methods of texture analysis to infer to effects of nutrient availability and varying hydrodynamic forces on seaweed was introduced on the example of *L. digitata*. Texture analysis is a method to test the physical properties of a material by tension and compression. The trade-off in tissue responses to tensile and compression forces in *L. digitata* along the lamina, linked to an age gradient, indicates a twined structure aligned to optimise the toughness and flexibility of constituent tissue. Tensile strength increased from young to old tissue along a positive toughness gradient of 75 %. Morphological features of a healthy *L. digitata* frond seem modified to withstand physical forces from hydrodynamics in its wave-swept habitat. These tactile properties also affect consumer perception and acceptance. This accounts not only for marine herbivores that graze on seaweeds, but also for humans in Europe, the "new grazer" as seaweeds become more popular as an alternative food source. The ecophysiological data on *U. lactuca*, *S. latissima*, *L. digitata*, and *P. palmata*, as well as the innovative aspects related to seaweed research that are introduced in this thesis, showed that each of the 4 seaweed species has developed adaptations to (changing) environmental conditions, also shown by their different nutrient uptake management and diverse strategies. Although the interactions of environmental factors as nutrients, light, temperature and hydrodynamics were not included, the data presented enables a comprehensive insight into ecological effects of nutrient limitations and shifts in limitation, as well as shows the ecological importance of seaweeds in terms of ecosystem services they provide in matter cycling, especially for N and P. Furthermore, the data also allows to manipulate and project production in seaweed cultivation and for bioremediation purposes.

Samenvatting

Zeewier komt in alle oceanen en zeeën over de hele wereld voor. Het heeft veel belangrijke functies in ecosystemen. Zeewier fungeert als een soort "ecosystem engineer" of "bio-bouwer" binnen het ecosysteem het kan de beschikbaarheid van resources beïnvloeden door sedimentatie en erosie. Zeewier vormt daarnaast een voedingsbron voor primaire consumenten (herbivoren), het biedt bescherming tegen predatoren, en het kan dienen als kraamkamer voor allerlei diersoorten. Zo vergroot zeewier de biodiversiteit en draagt het aanzienlijk bij aan de structurele complexiteit van een ecosysteem. Zeewier speelt ook nog eens een belangrijke rol in de mondiale koolstofcyclus: zo'n 6% van de netto primaire productie wordt geproduceerd door zeewier. Dit ondanks dat zeewier maar 0,1% van de zeebodem bedekt. In tegenstelling tot landplanten is voor zeewier geen landbouwgrond nodig en veel soorten groeien in zout of brak water, zodat er competitie om grond of kostbaar zoet water vermeden wordt. Ten slotte neemt zeewier opgeloste voedingsstoffen op uit de zee en zijn er geen pesticiden nodig om hun biomassa te beschermen. Al deze eigenschappen zorgen ervoor dat zeewier een belangrijke rol speelt bij processen binnen het mariene ecosysteem diensten, bijvoorbeeld bij bioremediatie. Zo vormt zeewier de laatste barrière om kostbare voedingsstoffen zoals fosfaten op te vangen in de kustzone voordat ze in de diepzee verdwijnen.

Er is in West-Europa (en ook in Nederland) steeds meer belangstelling voor het kweken van zeewier, omdat het een aantrekkelijke mariene biomassa is om te verbouwen. De Noordzee, is één van de productiefste kustzeeën ter wereld. De balans van opgeloste voedingsstoffen in het ecosysteem van de Noordzee wordt beïnvloed door de instroom van water uit de noordoostelijke Atlantische Oceaan, rivierwater en atmosferische input. Ruimtelijke verschillen in voedingsstoffenconcentraties treden op in kustgebieden, met name onder invloed van water uit verschillende rivieren, waaronder de Rijn, de Maas en de Theems. Dit rivierwater bevat vaak aanzienlijke hoeveelheden anorganische fosfor (P) en stikstof (N) afkomstig van anthropogene

activiteiten op het land, en kunnen locaal eutrofiering veroorzaken. De anthropogene afvoer van voedingsstoffen kan, naast natuurlijke fluctuaties, ook concentratiegradiënten en -limitaties veroorzaken. Deze worden vaak gezien langs de kusten van de Noordzee.

Limitaties van voedingsstoffen en/of fluctuerende limitaties van verschillende elementen/verbindingen kunnen van aanzienlijke invloed zijn op de interne samenstelling, de fysiologie en de groei van zeewier. Elke zeewiersoort heeft zijn eigen groei-eigenschappen, interne samenstelling en kritische grenzen met betrekking tot de beschikbaarheid van voedingsstoffen. Sommige soorten doen het goed op grote hoeveelheden stikstof en hebben voldoende aan een lage concentratie fosfor, terwijl andere soorten grotere hoeveelheden fosfor en relatief weinig stikstof nodig hebben. Fundamentele wetenschappelijke kennis over de opnamekinetiek en de verwerking van voedingsstoffen door verschillende zeewiersoorten is daarom belangrijk, zowel om ecologische als economische redenen. Deze kennis kan helpen om vragen te beantwoorden als: wat zijn goede en wat zijn minder goede omstandigheden voor bepaalde zeewiersoorten? Wat zijn de overeenkomsten en verschillen tussen zeewiersoorten? Hoe lang kan een bepaalde soort groeien onder limiterende omstandigheden, etcetera?

In dit proefschrift zijn vier ecologisch en economisch belangrijke inheemse zeewiersoorten uit de Noordzee onderzocht: het opportunistische groene zeewier *Ulva lactuca* (zeesla, Chlorophyceae), de (meerjarige) bruine zeewieren *Saccharina latissima* (suikerwier) en *Laminaria digitata* (vingerwier) (Phaeophycea), en het (meerjarige) rode zeewier *Palmaria palmata* (Rhodophyceae). Er is gebruikgemaakt van een volledig factoriele opzet ("full factioral design") om de opnamekinetiek te bepalen van opgelost anorganisch fosfaat (*dissolved inorganic phosphate*; DIP) en opgeloste anorganische stikstof (*dissolved inorganic nitrate*; DIN), evenals de interne opslagcapaciteit (*internal storage capacity*; ISC) van DIP en DIN onder laboratoriumomstandigheden. Al deze parameters zijn gestandaardiseerd naar bladoppervlakte, omdat zeewieren voedingsstoffen opnemen via hun gehele bladoppervlakte.

Alle geteste zeewieren vertoonden een bifasische reactie bij de opnamesnelheid van voedingsstoffen: V_S ("surge" of versnelde opname) als reactie op een tekort aan voedingsstoffen en V_M ("maintenance" of "onderhoud" opname) nadat de interne voedingsstoffenvoorraad was aangevuld. Een hoge V_S voor beide voedingsstoffen was te zien bij *U. lactuca* (DIN: 12,5±5,2 μmol·cm^{-2}·d^{-1}, DIP: 0,66±0,12 μmol·cm^{-2}·d^{-1}), *S. latissima* (DIN: 11,3±0,6 μmol·cm^{-2}·d^{-1}, DIP: 0,80±0,03 μmol·cm^{-2}·d^{-1}), en *P. palmata* (DIN: 15,6±4,3 μmol·cm^{-2}·d^{-1}, DIP: 1,57±0,29 μmol·cm^{-2}·d^{-1}), terwijl *L. digitata* een significant lagere V_S vertoonde (DIN: 3,9±0,1 μmol·cm^{-2}·d^{-1}, DIP: 0,38±0,03 μmol·cm^{-2}·d^{-1}). V_M voor DIP en DIN was significant lager dan V_S voor beide voedingsstoffen in alle geteste soorten: *U. lactuca* (DIN: 2,3±0,9 μmol·cm^{-2}·d^{-1}, DIP: 0,07±0,04 μmol·cm^{-2}·d^{-1}), *S. latissima* (DIN: 3,9±0,7 μmol·cm^{-2}·d^{-1}, DIP: 0,30±0,09 μmol·cm^{-2}·d^{-1}), *L. digitata* (DIN: 1,8±0,4 μmol·cm^{-2}·d^{-1}, DIP: 0,22±0,01 μmol·cm^{-2}·d^{-1}), en *P. palmata* (DIN: 5,6±2,1 μmol·cm^{-2}·d^{-1}, DIP: 0,57±0,22 μmol·cm^{-2}·d^{-1}). Door de interne opslagcapaciteit ISC voor DIN en DIP te kwantificeren kan de tijd worden geschat waarbinnen N- en P-reserves kunnen worden gebruikt voordat voedingsstoftekorten leiden tot significante groeiproblemen en verminderde opbrengst. Er werd een grote ISC gemeten voor de meerjarige zeewier soorten *P. palmata* (DIN: 222 μmol·cm^{-2}, DIP: 22 μmol·cm^{-2}), *S. latissima* (DIN: 49 μmol·cm^{-2}, DIP: 14 μmol·cm^{-2}) en *L. digitata* (DIN: 80 μmol·cm^{-2}, DIP: 10 μmol·cm^{-2}). De ISC voor beide voedingsstoffen in het opportunistische *U. lactuca* (DIN: 23±7 μmol·cm^{-2}, DIP: 0,7±0,1 μmol·cm^{-2}) was significant lager dan de ISC in de meerjarige zeewieren.

Naast de kwantificering van de opnamekinetiek en de ISC van DIN en DIP worden de opnamestrategieën of prestaties op langere termijn in relatie tot de omgeving en/of de competitie om (limiterende) bronnen van voedingsstoffen van de vier zeewieren beschreven. Zo bleek er een ritmische DIN- en DIP-opname met wekelijkse intervallen voor *P. palmata* te bestaan. Dit in tegenstelling tot een lineaire opname van V_M in *L. digitata*. Deze verschillen kunnen een indicatie zijn dat er tussen beide soorten een scheiding van niches is, die gestuurd wordt door beschikbaarheid van voedingsstoffen. De beschikbaarheid van DIP leidde bovendien tot

verminderde toegang tot DIN in *P. palmata* wat vervolgens terug te zien was in de totale oplosbare eiwit- en koolhydratenconcentratie, en daarmee de voedingswaarde van het zeewier. De totale oplosbare eiwitconcentratie varieerde van 10,2±2,5 % tot 24,6±8,0 % op basis van droog gewicht, afhankelijk van de beschikbaarheid van DIP. Ook de totale oplosbare koolhydratenconcentratie varieerde van 22,1±3,6 % tot 54,3±12,3 % (droog gewicht).

In *U. lactuca* bleken kleurverschillen in de bladen het gevolg te zijn van de voedingswaarde, of te wel de totale concentratie oplosbare eiwitten. Dit leidde tot nieuw onderzoek, waarin de mogelijkheid werd onderzocht om spectrofotometrische en colorimetrische technieken te gebruiken om de totale concentratie oplosbare eiwitten te bepalen op basis van de kleur van het blad van dit groene zeewier. Op basis van het concept van colorimetrische technieken ontwikkelden we EyeOnUlva, een app voor iOS- en Android-smartphones die de bladkleur vastlegt en een goedkope, betrouwbare, veilige, snelle en eenvoudig te gebruiken manier biedt om de totale concentratie oplosbare eiwitten in *U. lactuca* te bepalen. 'EyeOnUlva' is een handige app waarmee bedrijven binnen de aquacultuur sector op een goedkope manier de voedingswaarde en voedingskwaliteit van zeewier kunnen bepalen. De app kan bovendien worden gebruikt voor milieuonderzoek, waarbij burgers gevraagd kan worden mee te werken (zgn. citizen-scienceprogramma's).

Een andere nieuwe benadering in dit proefschrift, is textuur analyse, een methode om de fysieke eigenschappen van een materiaal (hier: zeewier) te testen door middel van spanning en compressie. Er werd een gestandaardiseerde mogelijkheden ontwikkeld voor de analyse van de textuur van het bruine zeewier *L. digitata*. Textuur analyse is een methode om de fysieke eigenschappen van een materiaal te testen door middel van spanning en compressie. Bij deze soort werden de effecten van de beschikbaarheid van voedingsstoffen en variabele hydrodynamische krachten op textuur van het blad bepaald. Resultaten wijzen op een ineengestrengelde structuur in de bladen die de sterkte en flexibiliteit van samengesteld weefsel optimaliseert. De spanningskracht

nam van jong tot oud weefsel toe met circa 75 %. De morfologische kenmerken van een gezond blad van *L. digitata* leken te zijn aangepast om de hydrodynamische krachten te weerstaan in de door golven gedomineerde leefomgeving van deze soort. De textuur zal ook van invloed zijn op de perceptie en acceptatie door de consument. Dit geldt niet alleen voor marine herbivoren die leven van zeewier, maar ook voor mensen in Europa, de 'nieuwe herbivoren', nu zeewier steeds populairder wordt als alternatief voedingsmiddel.

De ecofysiologische gegevens van *U. lactuca*, *S. latissima*, *L. digitata* en *P. palmata*, en de innovatieve aspecten van zeewieronderzoek die in dit proefschrift worden geïntroduceerd, laten zien dat elk van de vier zeewiersoorten zich heeft aangepast aan (veranderende) omgevingsomstandigheden. Dit blijkt ook uit de verschillende manieren waarop deze soorten voedingsstoffen opnemen en beheren. Hoewel niet alle interactie van omgevingsfactoren zo als voedingsstoffen, licht, temperatuur en hydrodynamica is beschreven, bieden de gepresenteerde gegevens een breed inzicht in de ecologische effecten van limiterende hoeveelheden voedingsstoffen en veranderingen van de limitaties. Bovendien laten ze het ecologisch belang van zeewier voor ecosysteem diensten zien, bij voorbeeld voor cycli van voedingsstoffen, met name voor N en P. De gegevens kunnen ten slotte ook worden gebruikt om de productie van zeewier te manipuleren en te voorspellen, en voor doeleinden op het gebied van bioremediatie.

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much time on nitrates to measure uptake rates. "Why do bacteria like nitrates so much? They are cheaper than day rates."

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Appendix

List of abbreviations and acronyms

ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
°C	Degree Celsius
Chl *a*	Chlorophyll *a*
c	concentration
cm	Centimeter
DBL	Diffusive boundary layer
DIN	Dissolved inorganic nitrate
DIP	Dissolved inorganic phosphate
DIY	Do It Yourself
DNA	Deoxyribonucleic acid
DW	Dry weight
d	Day
E_K	Kinetic energy
ELISA	Enzyme-linked immunosorbant assay
ERSEM	European Regional Seas Ecosystem Model
e.g.	Exempli gratia ('for example')
ε	Total strain deformation/elongation
FAO	Food and Agriculture Organization of the United Nations
F_v/F_m	F_v refers to variable fluorescence; F_m refers to maximum fluorescence
FW	Fresh weight
GPS	Global Positioning System
g	Gram
Hz	Hertz
h	hour
IMTA	Integrated multi-trophic aquaculture
IOS	Internet Operating System
ISC	Internal storage capacity
i.a.	Inter alia ('amongst other things')
i.e.	Id est ('in other words')

K_m	Michaelis-Menten constant
K_s	Half saturation concentration
kg	Kilogram
km	Kilometer
L	Liter
ln	Natural logarithm
M	Molar mass
m	Mass
μ	Relative growth rate
N	Nitrogen
n	Amount of substance (chemical amount)
NE	North East
NIFT	Nutrient-Induced Fluorescence Transient
NIOZ	Royal Netherlands Institute for Sea Research
NPP	Net primary production
ODT	Optimal defense theory
P	Phosphorus
PAM (fluorometry)	Pulse-amplitude-modulation (fluorometry)
PCR	Polymerase chain reaction
RGB	Red Green Blue (colour scale)
Rrs	Remote sensing reflectance
SA	Surface area
T_c	Nutrient concentration
t	Time
UK	United Kingdom
UPL	Ultimate piercing load
UTS	Ultimate tensile strength
Vol	Volume
V	Uptake rate
V_e	Externally controlled uptake
V_M	Maintenance uptake rate
V_S	Surge uptake rate

About the author

Alexander, man of the family Lubsch, was born on the 14th day in October of the year 1980 in Lengerich, Westphalia in Germany. Alexander has always been interested in a diversity of science, but especially natural science has interested him most. After his Abitur he started to study Geophysics and Geology. Participating in many excursions, including the German coastline, his interest in marine life was aroused and he finally studied Bioscience at the Westfälische Wilhelms Universität (WWU) in Münster, Germany, before he could intensify his studies on marine science at the University of Rostock, Germany, and at Alfred-Wegener Institute for Marine and Polar Research (AWI) on the island of Helgoland, Germany, where he worked during his Master's degree in Marine Biology. He successfully completed his Master's research project investigating the palatability of different seaweed parts on various meso-grazers, such as isopods, amphipods, and gastropods. After graduating in 2011, Alexander worked on many sea-going surveys on the North Sea, including shrimp-, herring-, mackerel-, cod-, and flatfish migration and stocktaking, as well as testing fishing techniques and working on hydro-acoustics surveys, all executed by the Johann Heinrich von Thünen-Institute (VTI) in Hamburg and Rostock, Germany. He could enhance his international working experience in Rio de Janeiro, Brazil in 2012, where he investigated the impact of industrial ashes on soil and carbon availability to bacterial communities at the Pontifícia Universidade Católica do Rio de Janeiro (PUC-Rio). Being back in Europe, Alexander started a PhD project at the Royal Netherlands Institute for Sea Research (NIOZ), the Netherlands, focussed on nutrient uptake dynamics and management strategies of 4 native North Sea seaweeds, as well as their cultivation. During his project he worked on biotechnological applications, such as texture analysis of seaweed fronds and the colorimetric analysis of a seaweed frond to evaluate its protein concentration, as well as experienced many facettes of work and development of the NIOZ Seaweed Research Centre. Professor dr. Klaas R. Timmermans and prof. dr. Tjeerd J. Bouma supervised Alexander's PhD project at the NIOZ, which finally led to this thesis.