Light, balanced and unbalanced growth and ammonium assimilation kinetics of *Ulva*

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Ulva pertusa pigmentation after a light gradient treatment

ABSTRACT

Macroalgae are important primary producers in coastal and estuarine waters and they depend on two essential resources: light and nutrients, but there are occasions when one or both can limit growth rate. Therefore, an improved understanding of how they acquire nutrients and how light and/or nutrient limitation impacts their growth and physiology is essential to understanding the ecology and aquaculture of macroalgae, particularly fast-growing, and potentially nuisance, species of *Ulva*. The kinetics of ammonium assimilation in *Ulva* was investigated using three techniques. The unifying component of all three methods is that the internally-controlled phase (V_i) of the perturbation method is numerically equal to the maximum rate of ammonium assimilation and therefore V_{max} of the Michaelis-Menten formula is effectively already defined. The first method used two phases (V_i and V_e) of the perturbation method to determine kinetics, the second used the multiple-perturbation method and the third the CCCP method, which, unlike the other two methods, has independent evidence to support its validity. The relationships between rate of ammonium uptake and ammonium concentration for the first two methods had little correspondence to the relationship between rate of ammonium assimilation and ammonium concentration using the CCCP method, suggesting that the first two methods were not measuring the kinetics of ammonium assimilation.

Following transfer of *U. pertusa* to decreased photon flux densities (10, 15, 30 and 50% ambient light) in outdoor cultures there was an initial phase (first 7 days) of unbalanced growth in which tissue at decreased photon flux density had similar, and not significantly different, growth rates measured as increases in total chlorophyll to that for the control (100% ambient light). Growth rates measured as increases in tissue fresh weight, dry weight, ash-free dry weight or surface area were significantly lower at decreased photon flux densities. After the period of unbalanced growth, a new steady-state was achieved and growth became balanced again, with no significant differences in the relationships between growth rate measured by any constituent and % ambient light. The q_{min} (photon flux density at which growth rate is zero) derived from the Droop relationship was 1.3 ± 0.1 mol photons m^{-2} d^{-1}. There was a strong positive relationship between total chlorophyll and nitrogen (as %N) content, suggesting that growth rates measured as increases in tissue nitrogen would be similar to those calculated as increases in total chlorophyll. *U. pertusa* maintained at

10% ambient light in spring had significantly greater rates of ammonium assimilation when measured at 30 or 300 µmole m^{-2} s^{-1} compared with those from 100% light, but there was little difference in rates of assimilation for *U. pertusa* maintained at 10% and 100% ambient light in autumn and winter, possibly because the growth rate at 10% was close to q_{min}.

Growth of *U. pertusa* in summer was N-limited and addition of nitrogen at low photon flux densities in indoor cultures led to high growth rates measured as increases in total chlorophyll. However, the relationship between total chlorophyll and nitrogen (as %N) content was poor, suggesting that increases in light harvesting pigment-protein complexes accounted for little of the increase in % N particularly in the 100% light treatment. Depriving *U. pertusa* of nutrients (nitrogen and phosphorus) resulted in even lower total chlorophyll content than with natural nitrogen deficiency. The increase in total chlorophyll content after addition of nitrate and phosphate at low photon flux density combined with the increase in biomass, yielded a growth rate measured as an increase in total chlorophyll of 0.67 d^{-1} that exceeds the highest growth rate ever measured in *Ulva*.

Issues relating to the use of the Michaelis-Menten and Droop equations in uptake kinetics and light-limited growth rate, respectively, the advantages and disadvantages of using indoor and outdoor cultures, the relationship between total chlorophyll and %N in N-deficient *U. pertusa* and future experiments are discussed.

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CHAPTER 1
GENERAL INTRODUCTION

Seaweeds, also referred to as macroalgae, are important primary producers particularly in coastal and estuarine waters (Hurd 2000). Although seaweed populations cover a small area of the world's oceans, their primary production makes up from 5 to 10% of total marine primary production (Wiencke & Bischof 2012). From an ecological perspective, one of their main roles is to convert energy in the form of light to fixed carbon that will eventually be used by the upper trophic layers. Depending on their location, macroalgae can account for almost 50% of the total fixed carbon in coastal environments (Ritschard 1992; Duarte & Cebrian 1996; Duarte et al., 2005; Gattuso et al., 2006).

1.1 Nutrient limitation of seaweed growth

Over 56 elements have been found in seaweeds, though most, such as metals, are present in very low quantities, with 21 required for normal metabolic and biosynthetic processes (DeBoer 1981). Seaweed rates of growth and photosynthesis can be limited by the availability of nutrients in their environment. The two forms of limitation are Liebig's (Liebig 1847) and Blackman's (Blackman 1905). Liebig's law of the minimum states that maximum yield will be limited by the nutrient presented in the minimum amount with respect to the organism's requirements (Falkowski 1994). Blackman limitation, which was originally applied to rates of photosynthesis, states that the maximum rate of a process is limited by the availability or rate of supply of a particular nutrient (Falkowski 1994).

Approximately 21 chemical elements are needed in the most important metabolic process in photosynthetic organisms, but not all of them are essential for growth (Hurd et al., 2014). The consensus is that nitrogen (N) is the main nutrient that limits growth in temperate coastal environments (Harrison & Hurd 2001; Mizuta et al., 2003; Tyler et al., 2003; Elser et al., 2007; Pedersen et al., 2010; Hurd et al., 2014), and phosphorus (P) in tropical climes or in areas rich in carbonate sediments (Littler et al., 1991). N is an important constituent of proteins and P in ribosomal RNA, transfer RNA and messenger RNA, all of which are involved in protein synthesis (Loladze & Elser 2011). Another nutrient that can possibly limit growth is iron (Fe) which is important in photosynthesis, respiration and nitrate assimilation (Viaroli et al., 2005).

In coastal marine environments, of the different forms of dissolved inorganic nitrogen (DIN), nitrate (NO_3^-) is the most abundant, followed by ammonium (NH_4^+) and nitrite (NO_2^-). Seaweeds have the ability to use different sources of N, but the affinity and uptake rate for these different sources are not the same (Rees 2003). In several species higher uptake rates for NH_4^+ have been observed, mainly because of the low energy cost required to assimilate this source of nitrogen (D'Elia & DeBoer 1978; Hanisak 1983; Naldi & Wheeler 1999; Dy & Yap 2001; Smit 2002; Rees 2003; Nishihara et al., 2005; Pereira et al., 2008). In comparison to nitrate the rate of ammonium uptake can increase steadily as the external concentration increases without becoming saturated, probably reflecting a low-affinity transport system, while nitrate uptake is largely due to a high-affinity transport system and demonstrates a saturation point when at higher concentrations the rate of uptake remains constant (McGlathery et al., 1996; Abreu et al., 2011).

Changes in environmental factors will influence seaweed metabolism and the rate at which nutrients are removed from the medium and assimilated (Figueroa et al., 2009). These factors depend on the season, and can be divided into external parameters, such as light, nutrients and temperature, or by internal factors, such as nutritional status, stage of development and the type of tissue. Alone or in combination with other factors, these parameters can generate physiological changes and differences in biochemical composition (Harrison & Hurd 2001; Luo et al., 2012; Hurd et al., 2014; Murphy et al., 2016; Xu et al., 2016).

1.2 Measuring uptake and assimilation kinetics

Uptake can be defined as the process by which a nutrient is removed from the surrounding medium, transported through the alga's membrane system (cell membrane and tonoplast) and, if necessary, stored in intracellular structures, usually vacuoles (Wheeler 1983). Assimilation depicts the following step where nutrients are, through enzymatic reactions, incorporated into small organic molecules such as amino acids and eventually larger compounds, such as proteins and nucleic acid (Dortch 1982), the latter being directly involved in growth.

Nutrient movement from the external medium across the membrane cell is done by three possible routes; passive (low energy cost), active (requires energy and can be low-affinity and/or high-affinity) and facilitated transport (combination of both) (D'Elia & DeBoer 1978; Dy & Yap 2001;

Harrison & Hurd 2001). Nutrient uptake kinetics will be influenced by the type of transport being used. Commonly, the relationship between nutrient uptake and these transport systems can be quantified and described as a rectangular hyperbola, similar to the Michaelis-Menten equation used for enzyme kinetics (Caperon & Meyer 1972; Pedersen 1994; Taylor et al., 1998; Taylor & Rees 1999; Abreu et al., 2011).

The standard ways to determine nutrient uptake and its kinetics are by the perturbation and multiple-flask method (Pedersen 1994). The perturbation method consists of incubations, usually for extended periods, with a high concentration of a limiting nutrient where the disappearance of this nutrient is monitored. For example, rates of uptake by *Ulva intestinalis* were followed for 12 hours in the presence of 400 µM ammonium (Rees et al., 1998).Three different uptake phases have been identified using this method, surge (V_s), internally controlled (V_i) and externally controlled (V_e) uptake. V_s is the first phase, characterized by an enhanced rate of uptake which leads to an abrupt decrease in the nutrient concentration. V_i is a phase where the rate of uptake is constant and is numerically the same as the rate of nutrient assimilation. In the last phase V_e, the rate of uptake decreases and depends on the concentration of the nutrient in the medium (Conway et al., 1976). One distinct advantage of the perturbation experiment is that it provides a measure of the maximum rate of assimilation (Harrison et al., 1989; Rees et al., 1998). With a nutrient-limited alga there will be a non-linear decrease in concentration over time, whereas the decrease is more likely to be linear if the alga is nutrient-replete (Harrison et al., 1989). One major disadvantage with this method is that as time passes the nutritional status of the alga will change, particularly if it is initially nutrient-limited.

The multiple-flask method involves a series of separate incubations with different concentrations of the nutrient in each flask and the same, relatively short incubation time. In principle, it could be used to determine the Michaelis-Menten kinetics of assimilation of the nutrient (Pedersen 1994), but there is no independent evidence that this is correct. In comparing the multiple-flask with the perturbation method, the duration of the multiple-flask experiment needs to exceed the duration of V_s. If the duration of the experiment is within the duration of V_s, then the kinetics will be the kinetic of V_s, which is a mixture of assimilation and storage. With reference to Pedersen (1994), the V_s phase of the perturbation experiment (his Figure 1B) lasts for at least 90 minutes. Consequently, most of the time intervals in his multiple-flask experiment (his Figure 4B) involve the V_s phase; only the 90-210 minute interval occurs during the V_i phase and has a V_{max} comparable to V_i. However, though V_{max} will be numerically equal to the maximum rate of assimilation, there is no

justification for assuming that at lower concentrations, what is being measured is the rate of assimilation. With lower initial concentrations, there is no V_i, only V_s and V_e (Pedersen 1994; his Figure 4A) and we have no idea what V_e represents.

Uptake kinetics of the main limiting nutrients, nitrogen (as ammonium or nitrate) and phosphorus (phosphate), has been thoroughly studied in a wide spectrum of macroalgal species (D'Elia & DeBoer 1978; Hanisak 1983; Hurd & Dring 1990; Ahn et al., 1998; Naldi & Wheeler 1999; Dy & Yap 2001; Smit 2002; Phillips & Hurd 2004; Nishihara et al., 2005). As might be expected, variations of the perturbation and multiple-flask method have been made in an attempt to improve or refine the method to measure uptake and assimilation kinetics (Dortch 1982; Fujita et al., 1988; Pedersen 1994; McGlathery et al., 1996; Rees et al., 1998; Taylor & Rees 1999; Barr et al., 2004).

One of these variations is the use of a multiple perturbation method, which combines a longer incubation period than is usual with multiple-flask experiments with a range of initial concentrations in different containers, to determine the kinetics of assimilation (Pedersen 1994), but is not clear if this is an appropriate method. In contrast, the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) method has been proven to equal the maximum ammonium assimilation rate and is directly comparable to the *in vivo* rate of glutamine synthesis (Rees et al., 1998; Taylor & Rees 1999; Barr et al., 2004).

1.3 Growth in macroalgae

Growth is defined as the increase in size and biomass of an organism over time that, with more "complex" organisms, involves development from a simpler system to a more complex level of organisation. Biomass yield or growth rate will often be restricted by the availability of nutrients, as described by Liebig's law of the minimum (Baar 1994) or Blackman rate limitation (Falkowski 1994), respectively. However, a more accurate representation of the effect of nutrient availability is the regulation of growth rate by the intracellular, rather than extracellular, nutrient concentration (Droop 1968) such that when cellular or tissue nutrient concentration is higher, growth will be enhanced (Flynn et al., 2010).

To understand and predict patterns of growth rate, models such as those of Monod (relationship of growth rate to external nutrient concentration) and Droop (relationship of growth rate to internal

nutrient concentration) have been developed (Shuter 1979; Schaechter 2006; Hernández et al., 2008; Lemesle & Mailleret 2008).

Conventionally, the Droop model (Droop 1968) is used to determine nutrient-limited growth in microorganisms, but it has been used to describe the nutrient relations of growth in macroalgae also (e.g. Hanisak 1979; Lavery & McComb 1991; Lamprianidou et al., 2015). One advantage of the Droop model is that it allows the derivation of two "constants", one, originally described by Droop, is q_{min} (the maintenance quota) or the minimum cell or tissue quota at which growth rate is zero, and the other, not described by Droop, is the critical q (usually described as critical N or critical P), which is the minimum cell or tissue quota at which growth rate is maximum (e.g. Hanisak 1979). However, microorganisms and macroalgae require other resources such as light that can limit growth rate and it could be argued that there is a q_{min} for photons (compensation point for growth).

From a physiological perspective macroalgae can be divided into two distinct forms: opportunistic and late-successional (Hurd et al., 2014). Perennial species have the ability to translocate stored nutrients from an area that is not involved in growth to specific zones that are metabolically active (e.g. meristematic tissue), hence decreasing nutrient uptake rates (Topinka 1978; Gerard 1982; Stephens & Hepburn 2016). Also, slow-growing perennial macroalgae have adapted to contain a larger nutrient pool, relative to the requirement for growth (Pedersen & Borum 1996), whereas opportunistic (fast-growing) algae have a higher nutrient uptake (Martínez et al., 2012) that is required to sustain a fast growth rate and higher nitrogen content at maximum growth rate (Pedersen & Borum 1997). Fast-growing algae are more susceptible to nutrient limitation than slow-growing species. *Ulva lactuca* and *Ceramium rubrum* (fast-growing species) cannot rely on stored P for long periods, thus requiring continuous supplies of dissolved inorganic phosphorus (DIP), whereas *Ascophyllum nodosum* and *Laminaria digitata* (slow-growth) have greater reliance on stored P during episodes of low DIP availability (Pedersen et al., 2010).

In opportunistic species growth rate will be limited by their capacity to store nutrients and they compensate for this limitation by increasing their uptake rates. This suggests that growth rate will be highly correlated with increased nutrient availability over short periods of time (Liu & Dong 2001), and by doing so they can compensate for this restricted temporal window (e.g. in the intertidal) to colonize and reproduce rapidly. For example, in spring *Ulva rigida* can exhibit a ten-fold increase in the total nitrogen content in their biomass (Naldi & Viaroli 2002). Consequently,

filamentous and monostromatic (consisting of cells arranged in a single layer of cells) macroalgae have higher uptake rates than slow-growing algae by increasing their surface area relative to their volume (SA:V) even with optimum photon flux density and temperature (Wallentinus 1984).

1.4 The effects of light on macroalgae growth

Genetic characteristics will define the basal capacities of an organism to assimilate nutrients, but differences in growth rate under natural conditions can largely be explained by the primary environmental factors (Lüning & tom Dieck 1989). Any physical or chemical factor that limits the cellular energy status can decrease rates of nutrient assimilation and, as a consequence, affect cell and tissue growth (Hurd et al., 2014).

In aquatic environments the primary limiting physical factors are light and temperature (Lapointe & Tenore 1981; Vergara et al., 1997; Altamirano et al., 2000). Light is one of the most important and complex abiotic factors that can disturb or enhance the metabolism and growth of autotrophs. In addition to its importance in photosynthesis, both the quantity and quality of light play key roles as environmental signals for the development and morphogenesis of algae (Korbee et al., 2005).

Light within the band of wavelength between 400 and 700 nm is known as photosynthetically active radiation (PAR) and all photosynthetic pigments absorb photons within this range of energy content. All eukaryotic photosynthetic organisms possess chlorophyll *a*, but differ in their accessory pigments, which play the dominant role in light absorption. In green algae the dominant accessory pigment is chlorophyll *b* (along with carotenoids), which has an absorption spectrum similar to that of chlorophyll *a*, but it absorbs at shorter wavelengths (higher energy content) in the red part of the spectrum than chlorophyll *a* and this is critical for transfer of energy to the reaction centres where ATP and reductant are produced. When a photon of a suitable energy content is absorbed by an accessory pigment, the energy is transferred to neighbouring pigment molecules as an electron, but some energy is lost in the transfer. Consequently, as energy is transferred from pigment molecule to pigment molecule, and ultimately to the trap chlorophylls in the reaction centres the wavelength of light with each successive transfer becomes longer in wavelength (Lüning & Dring 1985; Nobel 1991; Wiencke & Bischof 2012). Increasing the amount of chlorophyll (whether as increased size and/or number of photosynthetic units) increases the

chances of a photon being absorbed at low photon flux densities and for it to potentially perform useful work.

A green alga exposed to a decrease in photon flux density will acclimate by increased synthesis of both chlorophylls, but particularly chlorophyll *b* (Henley & Ramus 1989a; Gévaert & Rees 2015). However, it is worth noting that changes in total chlorophyll content are not exclusively due to light, as decreases in chlorophyll content occur with nutrient deficiency. Under low irradiance and adequate nutrient supply, whether seasonally or experimentally induced, photosynthetic pigments content will increase (Lapointe et al., 1984; Duke et al., 1986; Campbell et al., 1999) and prolonged exposure to high irradiance will provoke an increase in photoprotective pigments to mitigate associated stress and irreversible damage to the photosynthetic complex (Niyogi 1999).

In optimizing light absorption and increasing pigment synthesis, a significant reduction in growth rate will prevent the stored nutrient pools (primarily nitrogen) from being diluted in growth (McGlathery et al., 1996), and, as a result, growth rate may be uncoupled from other metabolic process (Fishov et al., 1995; Berman-Frank & Dubinsky 1999). This uncoupling mechanism becomes more pronounced during periods of low light where most of the metabolic budget is invested in optimizing photon capture (Dubinsky et al., 1986; Leonardos & Geider 2004). This metabolic shift is due to the chemical composition of chlorophylls, each molecule contains nitrogen atoms and this feature allows it to bind to proteins, specially chlorophyll *a*, to increase the absorption spectrum (Hurd et al., 2014).

Short-term changes in the photosynthetic complex have been reported for macroalgae (Lapointe & Duke 1984; Duke et al., 1986; Duke et al., 1989; López-Figueroa & Niell 1990; Altamirano et al., 2000; Makarov 2012). The main significant alterations were in photosynthetic pigment concentration and ratios, and in Rubisco activity (Calvin cycle). These changes could signify unbalanced growth (Fishov et al., 1995; Berman-Frank & Dubinsky 1999) and there are numerous potential examples of unbalanced growth in macroalgae. For example, when nutrients become limiting some kelp species will accumulate carbohydrate. Kelps store carbohydrates (primarily as laminarin) (Graiff et al., 2016) and consume it during periods when nutrients are not limiting growth, but light is (Suutari et al., 2015). During a light transition (high to low photon flux densities, and vice versa) carbohydrate pools serve as an energy reserve or a buffer to store available radiation or provide an energy source (Post et al., 1985).

Growth is defined as the increase in a measure of biomass (usually fresh weight with seaweeds) per unit of the same measure of biomass per unit time, which reduces to d^{-1} if days are the units of time. Any increase in any biological constituent can be expressed in the same way. For example, it is possible to express the increase in laminarin in kelps as a growth rate. If growth is balanced, the rate of increase in laminarin would be identical to the rate of increase in fresh weight. The major advantage of this approach is that all rates are in identical units: d^{-1} and are directly comparable.

1.5 *Ulva*

Species of *Ulva*, also referred to as sea lettuce, belongs to the phylum Chlorophyta, which includes the Ulvaceae. *Ulva* species can grow under a variety of conditions, from the high intertidal through the subtidal down to 40 m or more in depth, and they can be found attached to rocks or free-floating. It is considered to be a cosmopolitan species in marine and estuarine habitats (Runcie et al., 2003). *Ulva* species are difficult to identify as a species can be composed of a wide range of morphological variations. In general, this genus is mostly represented by a bladed or simple sheet-like or tubular shape that was previously known as *Enteromorpha* (Hurd et al., 2014). In New Zealand at least 19 species of this genus have been distinguished by molecular techniques, and the four most common are: *U. compressa*, *U. intestinalis*, *U. pertusa* and *U.* species 1; records of these species exist from the Kermadec Islands through to the Subantarctic islands (Park 2011; Nelson 2013).

U. pertusa morphology consists of tissue with a distromatic arrangement where both sides are exposed to the environment. This type of thallus morphology is adapted for quick growth since all cells are in direct contact with the medium and are photosynthetically active (Littler et al., 1980; Hanisak et al., 1990). Overall species of *Ulva* are considered to be opportunistic with a high nutrient uptake rate, large surface area/volume and a relatively low capacity to store nutrients. Its growth rate is highly correlated with increased nutrient availability over short periods of time (Liu & Dong 2001). Although the morphology of species of *Ulva* plays an important role in sustaining a high growth rate, it is their ability to readjust their internal allocations that makes them capable of inhabiting environments where light, temperature and nutrient can fluctuate regularly (Henley 1992; Pérez-LLoréns et al., 1996; Figueroa et al., 2009).

Because of these characteristic and its ability to efficiently remove nutrients from the environment *Ulva* species have been used as biofilters for aquacultural purposes (Neori et al., 1991). They are also known for generating massive accumulation of biomass in coastal areas, commonly referred to as "green tides" (Kim et al., 2004; Buapet et al., 2008; Park 2014; Xu et al., 2016; Alstyne 2018), and in New Zealand these have been registered in Tauranga and the Avon-Heathcote estuary (Nelson 2013; Port 2016).

Ulva is an appropriate seaweed to determine the effects of environmental changes, such as decreased photon flux density on metabolism and growth in both short-term and long-term experiments because of its simple morphology and high surface area : volume quotient.

1.6 Study sites and organisms

Biological material was collected from three different locations. *Ulva pertusa* (Chlorophyceae, Ulvales) fronds were collected from a depth of 1 to 5 m in the Mokohinau Islands, northeastern New Zealand (35°55′S, 175°07′E) and from Fergusson Park located in the Tauranga Harbour, Bay of Plenty (37°39′S, 176°07′E). *Ulva intestinalis* fronds were collected fresh from rock pools adjacent to the Leigh Marine Laboratory (36°16′S, 174°47′E) (Figure. 1.1).

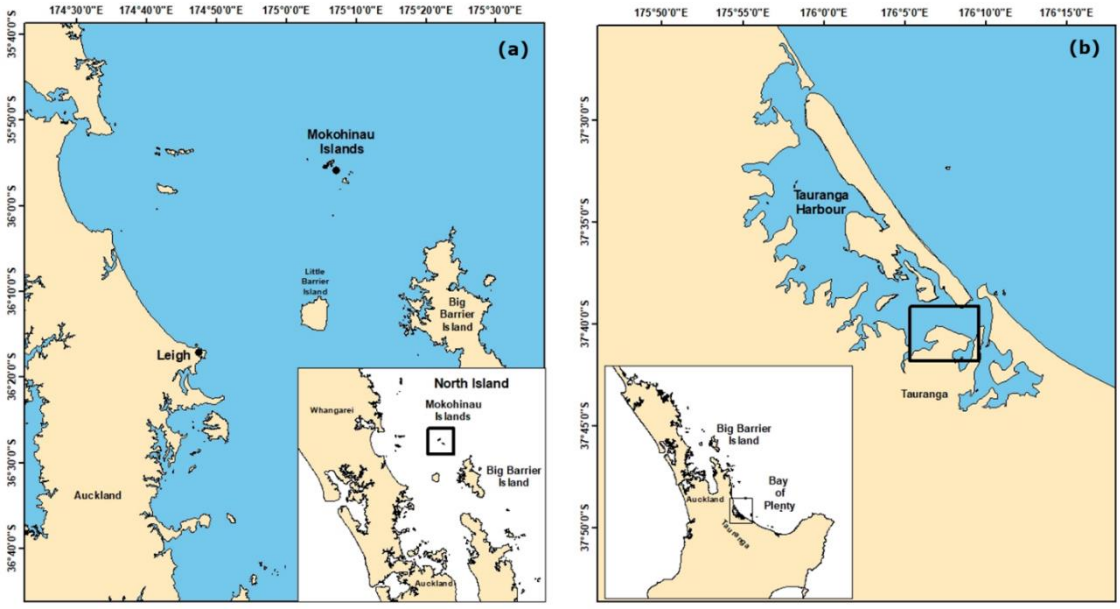

Figure 1. 1. Maps of the three locations where *Ulva* was collected. Figures correspond to: (a) *Ulva pertusa* (Mokohinau Islands) and *Ulva intestinalis* (Leigh), and (b) *Ulva pertusa* (Tauranga Harbour).

1.7 Thesis aim and outline

Growth is a fundamental characteristic and process in living organisms. The rate of growth is determined by the quality and quantity of diverse environmental parameters. It is generally thought that for seaweeds the two main factors that will affect their growth rate are light and nutrients. Due to seasonal variations, there are periods where one (or both) can become limiting factors, and a number of physiological and biochemical changes are crucial for them to acclimate to these conditions. The focus of this thesis was to improve our understanding of how macroalgae acquire nutrients and the short and long-term effects of light and/or nutrient limitation on their growth rate and physiology. By determining the effects of seasonal variation on a cosmopolitan genus *Ulva*, we can obtain valuable ecophysiological information that is potentially useful both ecologically and in the aquaculture of algae.

The thesis is divided into three experimental chapters. The aim of the first experimental chapter (Chapter 2), was to determine whether two methods involving the measurement of rates of ammonium uptake can be used to determine the kinetics of ammonium assimilation through a comparison with an established method for measuring assimilation (Rees et al., 1998). The two uptake methods involved (a) the identification of two distinct uptake phases: internally-controlled and externally-controlled uptake phases (V_i and V_e) with the perturbation method in *Ulva pertusa* (from Tauranga and Mokohinau Islands) and *U. intestinalis*, and (b) the multiple perturbation method in *U. intestinalis* (Pedersen 1994).

The aim of the second experimental chapter, Chapter 3, was (a) to investigate if light-limited growth was balanced or unbalanced by comparing growth rates of different constituents (fresh weight, dry weight, ash-free dry weight, surface area and total chlorophyll) and (b) whether a biosynthetic rate (of total chloropyll) can exceed the maximum growth rate. *U. pertusa* was grown at a range of percentage ambient light (10, 15, 30, 50 and 100%) for extended periods (up to 32 days) in outdoor cultures during autumn, winter and spring. Additionally, it was investigated whether the rates of ammonium uptake and assimilation "acclimated" to growth under decreased photon flux densities.

The third experimental chapter, Chapter 4, addressed whether (a) in summer growth of *U. pertusa* was nitrogen or phosphorus deficient and (b) with addition of nutrients (nitrogen and phosphorus) to nutrient-deficient *U. pertusa* at low photon flux densities (i.e. both conditions that promote

increased chlorophyll biosynthesis) did the rate of total chloropyll synthesis exceed the maximum growth rate.

Chapter 5 is the General Discussion in which key components of the thesis are discussed in a broader context, together with the implications of the results of the experimental chapters, their limitations and recommendations for future research.

CHAPTER 2
USE OF UPTAKE KINETICS FOR MEASURING ASSIMILATION KINETICS IN *ULVA*

2.1. INTRODUCTION

Uptake and assimilation are central components in the utilization of any nutrient by photosynthetic organisms, including nitrogen which is frequently the nutrient most likely to be limiting growth rate. For seaweeds, though nitrate is the main source of dissolved inorganic nitrogen in coastal marine environments, particularly in winter and early spring, ammonium is equally important despite being present at lower concentrations (Rees 2003). In several species higher uptake rates for NH_4^+ have been observed, mainly because of the low energy cost required to assimilate this source of nitrogen (D'Elia & DeBoer 1978; Hanisak 1983; Naldi & Wheeler 1999; Dy & Yap 2001; Smit 2002; Rees 2003; Nishihara et al., 2005; Pereira et al., 2008).

Uptake and assimilation rates have been measured using different methods with the most common being to follow the depletion of a nutrient such as ammonium in an enriched medium over a period of time (Fujita 1985; Pedersen 1994; Rees et al., 1998). Uptake is the process by which ammonium is taken up and assimilation is the conversion of ammonium into glutamine via glutamine synthetase (Bressler & Ahmed 1984; Rees et al., 1995; Taylor & Rees 1999). If the rate of uptake exceeds the rate of assimilation, ammonium can be stored in acidic intracellular organelles, usually vacuoles (Wheeler 1983). Uptake of ammonium in nitrogen-limited algae (with little or no ammonium present in storage pools), may be divided in three different phases for long time courses: surge (V_s), internally-controlled (V_i) and externally-controlled (V_e) uptake (Conway et al., 1976; Harrison et al., 1989; Pedersen 1994; Rees et al., 1998; Taylor & Rees 1999). V_s is the first stage which is characterized by an enhanced rate of uptake that includes assimilation and storage (Barr et al., 2004). This phase balances the nutrient deficit, detected by an abrupt decline in the concentration of ammonium in the medium and is in excess of the nitrogen required to sustain growth (Dy & Yap 2001). V_i is the next phase that consists of uptake that is numerically equal to the rate of assimilation only (the storage pool having been filled) (Rees et al., 1998). It is a period (the duration of which can be manipulated experimentally) with a constant uptake rate and is considered to be regulated by the nutritional status of the alga (Fujita et al., 1988). The last phase

is V_e, where the rate of uptake decreases as the concentration of the nutrient in the medium decreases (Conway et al., 1976).

The difficulty with the three phases of uptake is deciding what they represent, though intuitively it makes sense that V_s is a combination of uptake and assimilation and that V_i represents the maximum rate of assimilation. However, there was no independent evidence as to what each phase represented. That V_s is a combination of uptake and assimilation and V_i is numerically equal to the maximum rate of ammonium assimilation is now beyond doubt (Rees et al., 1998; Taylor & Rees 1999; Barr et al., 2004). However, what V_e represents is less clear.

The kinetics of ammonium uptake and assimilation is usually described using the Michaelis-Menten equation by comparing the relationship between ammonium concentration and the rate of ammonium uptake (Pedersen 1994; Taylor et al., 1998; Taylor & Rees 1999; Phillips & Hurd 2004; Abreu et al., 2011). Using this equation, uptake and assimilation are described as a hyperbola with a maximum saturation rate (V_{max}) and a half saturation constant (K_m) (Caperon & Meyer 1972). It should be noted that V_i is routinely used to determine the maximum rate of ammonium assimilation (e.g. Taylor et al., 1999) and that a combination of V_s and V_i are used to determine the apparent kinetics (K_m and V_{max}) of assimilation (Pedersen 1994).

The rate of ammonium assimilation is equal to the rate of *in-vivo* glutamine synthetase (GS) (Taylor & Rees 1999), which is the first enzyme involved in ammonium assimilation (Rees et al., 1995), followed by glutamate synthase (GOGAT) (Turpin & Harrison 1978). Occasionally, glutamate dehydrogenase (GHD) may be responsible for ammonium assimilation (Gayler & Morgan 1976; Sato et al., 1984; Taylor & Rees 1999; Inokuchi & Okada 2001), but assimilation and GS activity in *Ulva intestinalis* are completely inhibited by the GS inhibitor methionine sulphoximine, suggesting that there is no involvement of GDH in assimilation (Barr et al., 2004). After assimilation, amino acids are substrates for macromolecule (mainly protein) biosynthesis and this is termed *incorporation* (Fujita et al., 1988; Berges 1997).

The CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) method has been used to measure the rate of ammonium assimilation in marine algae (Rees et al., 1998), as it can distinguish between uptake and assimilation through its ability to release unassimilated ammonium during uptake experiments. The K_m and V_{max} of assimilation can be derived from this method, but it is important to know if methods that involve measuring uptake as the disappearance of ammonium (i.e.

multiple-perturbation incubations; Pedersen 1994) are equally valid. It should be emphasized that the evidence in favour of the CCCP method as a valid and reliable method for measuring the rate of assimilation are as follows:

i. The rate of ammonium assimilation matches the rate of *in-vivo* glutamine synthesis (Taylor & Rees 1999).
ii. Both glutamine synthetase activity and ammonium assimilation are completely inhibited by methionine sulphoximine (Barr et al., 2004).

No other apparent method for measuring the rate of ammonium assimilation as disappearance of ammonium has this level of support (in general, they have no independent verification of any description). An exception to this statement is that V_i is a valid measure of the maximum rate of ammonium assimilation. Consequently, the guiding principle of this chapter is that the CCCP method is the yardstick and that other possible methods must conform to the values obtained by the CCCP method to be valid.

The specific aim of this chapter was to investigate whether uptake can provide reliable information on the kinetics of ammonium assimilation by comparing two methods (perturbation and multiple-perturbation method) against the CCCP method (Taylor & Rees 1999). The CCCP method is supported by direct evidence that it can be used to determine ammonium assimilation kinetics, whereas the two uptake methods have no direct evidence to support their validity except that the uptake phase V_i during a perturbation experiment is numerically equal to the maximum rate of ammonium assimilation. However, V_i is only reached after an extended time interval during which the alga has been bathed in a high concentration of ammonium during which a number of physiological and biochemical changes may occurred. By comparing these methods the advantages and disadvantages of each technique will allow for better use of them in the future.

In addition, we currently have no understanding of what the third phase of uptake (V_e) in a perturbation experiment represents. Can this phase, together with V_i, be used to determine the kinetics of ammonium assimilation (as seen in Pedersen (1994) figure 2.2 but ignoring the V_s phase)? To answer this question, the ammonium concentrations at each time point in the V_i and V_e phase of the perturbation method was matched with the rate of uptake in the following time interval to construct a rate of uptake versus concentration plot and the data fitted to the Michaelis-Menten equation to estimate V_{max} and K_m.

2.2. MATERIALS AND METHODS

Ulva pertusa fronds were collected from the offshore Mokohinau Islands (26 June 2017) and from Tauranga Harbour (15 July 2017) located in the Bay of Plenty region. Different sites were chosen for logistic reasons (e.g. collection from the Mokohinau Islands involves an expensive boat trip and was combined with other research projects). *U. intestinalis* was collected fresh from rock pools adjacent to the Leigh Marine Laboratory (Figure. 1.1). *U. pertusa* was transferred to the Leigh Marine Laboratory and kept in outdoor circular tanks (1,600 L) with a flow rate of 11 L min^{-1}. Dump buckets were used to generate water circulation in the tanks (Barr et al., 2008).

All experiments were conducted in a constant temperature laboratory with a fixed temperature (18 °C) and photon flux density (300 ± 23 µmole photon m^{-2} s^{-1} photosynthetically active radiation). Incubations were performed using six, custom-made, Perspex chambers (250 mL) and water motion was provided by magnetic stirrers. In all experiments, each chamber was incubated with an individual algal frond and the total biomass used with each method was established from previous studies (Rees et al., 1998, Rees, unpublished data). Due to their duration, the perturbation and CCCP methods required an entire day to do the experiments and process the samples. The multiple perturbation experiment was completed in one day, but the CCCP method required three days to complete. On each day, all six concentrations were done and this was repeated on the subsequent days. In all experiments there were distinct and measurable decreases in ammonium concentration. All experimental treatments had three replicates and the order of the chambers was randomised in every experiment.

The dates of each experiment were as follows:
Perturbation method – *U. pertusa* (Tauranga) 24 July 2017, *U. pertusa* (Mokohinau Islands) 1 August 2017, and *U. intestinalis* 12 December 2017.
CCCP method – *U. pertusa* (Tauranga) 14 to 17 August 2017, *U. pertusa* (Mokohinau Islands) 5 to 7 September 2017, and *U. intestinalis* 22 to 24 November 2017.
Multiple-perturbation method - 7 May 2018 and compared to a CCCP experiment from 16 to 18 May 2018.

For the multiple-perturbation method only *U. intestinalis* was used (due the absence of the other two species in the field for a prolonged period).

2.2.1. CCCP method

The amount of alga used was 0.1 g (*Ulva intestinalis*) or 0.4 g (*U. pertusa*) fresh weight in 150 mL 1 µm filtered and UV-sterilized seawater enriched with ammonium chloride to give initial concentrations of 15, 30, 60, 90, 120 and 150 µM. The seawater used was supplied from the Laboratory's system (intake about 20 m offshore, about 1 m below mean low water spring) and always contained some ammonium, routinely less than 1 µM (Barr et al., 2008). This minor effect on the total concentration of ammonium after the addition of ammonium chloride was considered preferable to using methods to remove ammonium (e.g. preincubation with an alga such as *Ulva* to remove this ammonium) that may have led to contamination of the seawater with compounds released by the alga and/or uptake of other essential nutrients from the seawater. A 2 mL water sample was taken from the enriched medium at the beginning before the alga was added (a). All experiments were started at 08:00 and the alga was incubated for 20 (*U. intestinalis*) or 60 (*U. pertusa*) minutes, when a second 2 mL sample was taken (b). Immediately after this sample was taken, CCCP was added to the chamber to give a final concentration of 100 µM and left for another 140 (*U. intestinalis*; Rees et al., 1998) or 200 (*U. pertusa*; Rees, unpublished data) minutes to release all unassimilated ammonium after which, a further 2 mL sample was taken (c). To determine the initial amount of intracellular ammonium in the alga a separate incubation, with a different section of the same individual, was made of 0.1 or 0.4 g fresh weight with 100 µM CCCP in 1 µm filtered and UV-sterilized seawater (no additional nutrients) for 140 or 200 minutes and a 5mL sample (d) was taken. Samples a-c were diluted to 5 mL with 1 µm filtered and UV-sterilized seawater. Ammonium concentrations in seawater samples were determined as described by Koroleff (1983) with reference to standard curves with known amounts of ammonium in seawater. Ammonium reacts with phenol and hypochlorite at high pH to give a blue indophenol. Both Mg^{2+} and Ca^{2+} cause a precipitate to form with these reagents in seawater; this is prevented by complexing these ions with citrate. Uptake and assimilation were calculated as follows:

Uptake = a - b
Assimilation = (a+d) - c

2.2.2. Perturbation method (measuring V_i and V_e)

Each chamber contained 0.1 g fresh weight in 150 mL (*Ulva intestinalis*) or 0.4 g fresh weight in 250 mL (*U. pertusa*) ammonium (400µM)-enriched 1 µm filtered and UV-sterilized seawater,
A 2 mL sample was taken at the beginning before the addition of the alga. The time required for each species to reach the V_i and V_e phase was established with the results obtained from preliminary experiments. The start time of all these experiments was at 07:00, the total time course was 12 hours for *U. intestinalis* and 10 hours for *U. pertusa*, with 2 mL samples taken every 30 minutes throughout the time course. Samples were diluted to 5 mL with 1 µm filtered and UV-sterilized seawater and ammonium concentration measured as described above.

The amount of ammonium taken up by *Ulva* was calculated for each time interval as: $A= V_b (C_a-C_b)$; where A is the amount of ammonium taken up (µmole), V_b is the water volume (L) at the beginning of the time interval after the initial sample was taken and C_a and C_b the ammonium concentration (µM) at the start and end of the time interval, respectively. The rates of ammonium uptake for a given time interval during V_i and V_e were plotted against the ammonium concentration at the start of the given time interval.

2.2.3. Multiple-perturbation method

Due to the extended absence of *U. pertusa* in the field, this experiment was done only with *U. intestinalis*. The multiple-perturbation experiments were identical to that described for the perturbation method, with the exception of having six separate time courses each with a different initial ammonium concentration (15, 30, 60, 90, 120, 150 µM). Each chamber was inoculated with one of the concentrations and the incubation time lasted 120 minutes with 2 mL samples taken every 30 minutes. All the samples were diluted with 1 µm filtered and UV-sterilized seawater to 5 mL volume and the ammonium concentration determined as described above. One replicate consisted of six separate chambers each with a different algal frond in 15, 30, 60, 90, 120 and 150 µM. The second and third replicate consisted of the same six concentrations. Due to the short incubation period the three replicates were obtained on the same day by starting experiments at 07:00, 10:00 and 13:00.

At the end of all experiments dry weights were obtained by drying the tissue at 80°C for 48 hours to constant weight. Rates of uptake and assimilation are expressed as µmole ammonium g^{-1} dry weight h^{-1}.

2.2.4. Data analysis

The Michaelis-Menten equation was used to determine the kinetics of uptake and assimilation:

$$V = \frac{V_{max} * S}{K_m + S}$$

Where V is the rate of uptake or assimilation (µmole ammonium g^{-1} dry weight h^{-1}), V_{max} is the maximum uptake or assimilation rate (µmole ammonium g^{-1} dry weight h^{-1}), K_m the half saturation constant (µM) and S the substrate concentration (µM). In plots of rates of ammonium uptake or assimilation against ammonium concentration in SigmaPlot (version 14.0) the data was fitted to a two-function rectangular hyperbola (Michaelis-Menten equation). A Student's t-test was used to compare kinetic constants obtained by the different methods for measuring ammonium assimilation.

2.3. RESULTS

2.3.1. Comparing CCCP and perturbation methods

Two distinct uptake rate phases (V_i and V_e) were usually obtained with the perturbation method, but they showed poor correspondence to the ammonium assimilation kinetics from the CCCP method in *Ulva pertusa* from the Mokohinau Islands (Figure. 2.1), where there was a linear decrease in the uptake rate without any clear indication of the V_i phase, Tauranga Harbour (Figure. 2.2) and *U. intestinalis* from Leigh (Figure. 2.3). The correspondence for *Ulva pertusa* from the Mokohinau Islands (Figure. 2.1) and *U. intestinalis* from Leigh (Figure. 2.3) were particularly poor. The best correspondence between the two methods was with *Ulva pertusa* from Tauranga, because of the presence of a clear V_i phase (Figure 2.2), but there was no correspondence in the kinetic constants between the two methods for this alga (Table 2.1 and Table 2.3 in Appendix).

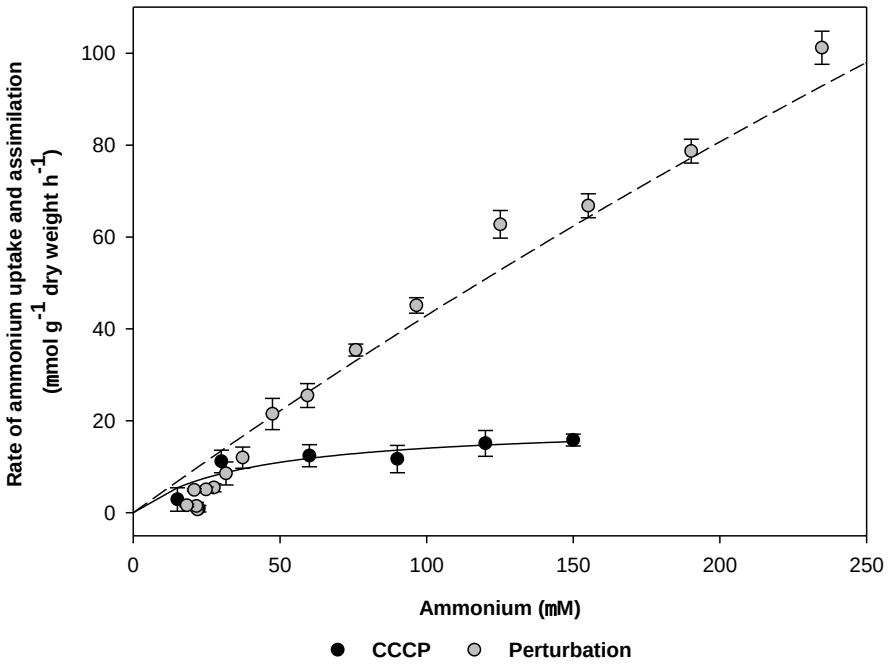

Figure 2. 1. Comparison between the kinetics of ammonium assimilation determined using the CCCP and perturbation methods in *Ulva pertusa* from the Mokohinau Islands. The rates of uptake during the nominal V_i (internally-controlled uptake) and V_e (externally-controlled uptake) phases from the perturbation experiment (grey symbols, dotted line) and the ammonium assimilation rates from the CCCP experiments (filled symbols, solid line) were plotted against the ammonium concentration. Values are means ± S.E. for three replicates.

There was a consistent hyperbolic relationship between the concentration of ammonium and the rate of assimilation using the CCCP method (Figures 2.1-2.3), but the V_{max} and K_m values were not comparable between methods (Table 2.1 and Table 2.3 in Appendix).

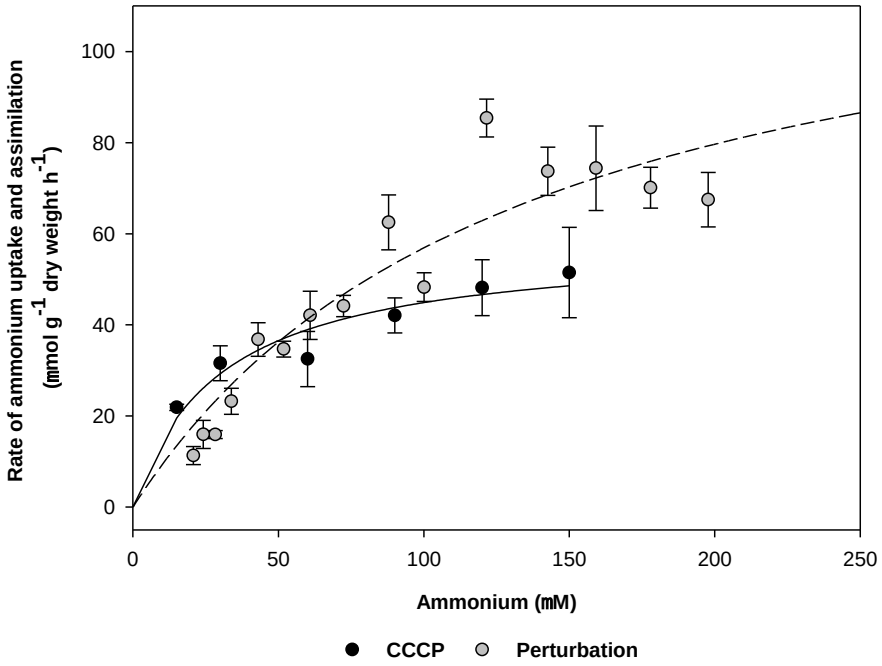

Figure 2. 2. Comparison between the kinetics of ammonium assimilation determined using the CCCP and perturbation methods in *Ulva pertusa* from Tauranga. The rates of uptake during V_i (internally-controlled uptake) and V_e (externally-controlled uptake) phases from the perturbation experiment (grey symbols, dotted line) and the ammonium assimilation rates from the CCCP experiments (filled symbols, solid line) were plotted against the ammonium concentration. Values are means ± S.E. for three replicates.

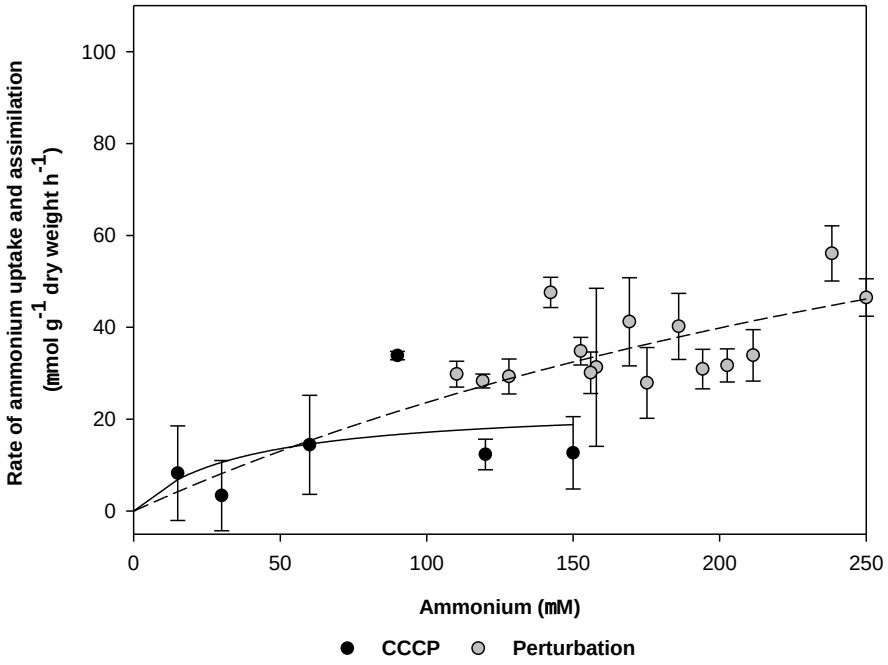

Figure 2. 3. Comparison between the kinetics of ammonium assimilation determined using the CCCP and perturbation methods in *Ulva intestinalis* from Leigh. The rates of uptake during V_i (internally-controlled uptake) and V_e (externally-controlled uptake) phases from the perturbation experiment (grey symbols, dotted line) and the ammonium assimilation rates from the CCCP experiments (filled symbols, solid line) were plotted against the ammonium concentration. Values are means ± S.E. for three replicates.

Table 2. 1. Summary of the kinetic constants (V_{max} and K_m) from the Michaelis-Menten equation for ammonium "assimilation" in *U. pertusa* (Tauranga), *U. pertusa* (Mokohinau Islands) and *U. intestinalis* using the perturbation and CCCP method. Asterisk indicates statistically significant differences between the kinetic constants for the perturbation method and CCCP method ($p < 0.05$). Values are means ± S.E. for three replicates.

	Perturbation method		**CCCP method**	
Species	V_{max} **(µmol·g^{-1} dry weight·h^{-1})**	K_m **(µM)**	V_{max} **(µmol·g^{-1} dry weight·h^{-1})**	K_m **(µM)**
***U. pertusa* (Mokohinau)**	-	-	19 ± 4	39 ± 22
***U. pertusa* (Tauranga)**	132 ± 26*	132 ± 49*	58 ± 6	30 ± 10
U. intestinalis	83 ± 44*	220 ± 211*	23 ± 17	35 ± 80

2.3.2 Comparing CCCP and multiple-perturbation uptake

Rates of ammonium uptake in multiple-perturbation ammonium uptake experiments were a saturable function of ammonium concentration and therefore yielded K_m and V_{max} values. However, though the V_{max} value for V^{0-30} and V^{30-60} were similar to the V_{max} for assimilation from the CCCP method (Table 2.2 and Table 2.4 in Appendix), neither the shape of the curves (Figure. 2.4) or the K_m values (Table 2.2 and Table 2.4 in Appendix) were similar to those for assimilation.

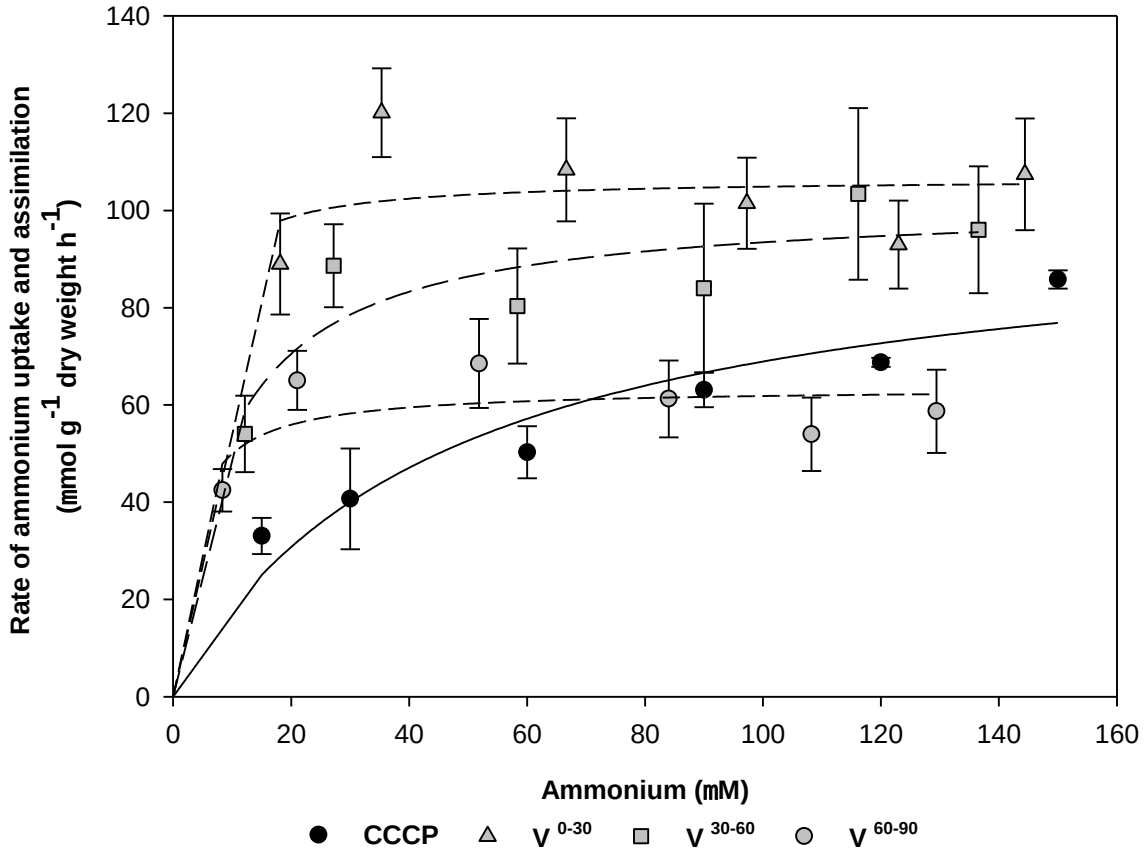

Figure 2. 4. Relationship between the rate of ammonium assimilation from the CCCP method (filled symbols, solid line) and the rate of ammonium uptake during different time intervals V^{0-30}, V^{30-60} and V^{60-90} measured using the multiple-perturbation method (grey symbols, dotted line) in *Ulva intestinalis* from Leigh. Values are means ± S.E. for three replicates.

Table 2. 2. Kinetic constants (V_{max} and K_m) for relationships between rates of ammonium uptake measured as V^{0-30}, V^{30-60}, V^{60-90} and V^{90-120} and rates of ammonium assimilation and ammonium concentration for *Ulva intestinalis*. Asterisk indicates statistically significant differences between the kinetic constants for the multi-perturbation and CCCP methods ($p < 0.05$). Values are means ± S.E. for three replicates.

Method	**Incubation**	**V_{max}** **(µmol·g^{-1} dry weight·h^{-1})**	**K_m** **(µM)**
Multiple-perturbation	V^{0-30}	107 ± 7	2 ± 3*
	V^{30-60}	102 ± 8	9 ± 4*
	V^{60-90}	64 ± 4*	3 ± 2*
	V^{90-120}	52 ± 5*	7 ± 4*
CCCP	Assimilation	100 ± 15	45 ± 18

2.4. DISCUSSION

2.4.1. Comparing CCCP and perturbation methods

With seaweeds, the perturbation method consists of long time courses (up to 10 hours) during which the decrease in ammonium uptake rate is followed (Taylor et al. 1999). As a rule, there are three distinct phases ($V_sV_iV_e$) (Conway et al., 1976; Harrison et al., 1989; Pedersen, 1994). As the V_e phase involves decreasing rates of uptake (to zero), it was hypothesized that by using plots of ammonium concentration against the rates of ammonium uptake during the V_i/V_e phases it would provide a reliable method of determining the kinetics of ammonium assimilation. This was not the case.

Pedersen (1994; his Figure 2) rejects the use of the perturbation method to obtain K_m and V_{max} values, but only because the inclusion of the V_s phase prevents fitting the Michaelis-Menten relationship. However, this does not prevent, in principle, the use of the V_i and V_e uptake phases to fit the Michaelis-Menten relationship as is clear from his Figure 2, which would have yielded a K_m value of about 5 µM. In other studies involving ammonium and nitrate uptake this method was used to estimate V_{max} and K_m values (D'Elia & DeBoer, 1978; Topinka, 1978; Smit, 2002), although there is no mention of its use as a method for measuring assimilation. In contrast, in Harrison et al., (1989), the approach to the use of the perturbation method is cautious and considered; with the advantage of estimating V_i and providing useful information about uptake kinetic parameters.

One reason for the poor correspondence between kinetics obtained using the V_i and V_e uptake phases and the kinetics of ammonium assimilation measured using the CCCP method was that the comparisons were not made at the same time. There is evidence for changes occurring in the physiology of control *U. pertusa* over a period of a month maintained under similar conditions (see Chapter 3) and this may have resulted in differences in the kinetic constants for ammonium assimilation using the two methods that were observed here.

Another reason for the poor correspondence was problems associated with the V_i phase during the experiments. The V_i phase should consist of a constant rate of uptake (Pedersen 1994; Rees et al., 1998), but it was either absent (*U. pertusa* from the Mokohinau Islands) or brief (*U. pertusa* from

Tauranga) or persistent (*U. intestinalis*). The absence of a constant V_i in *U. pertusa* from the Mokohinau Islands is easily explained by the diurnal cycle in the rates of ammonium assimilation that occurs in this alga from the same location (Gevaert et al., 2007) and this may apply equally to *U. pertusa* from Tauranga.

In addition, I consistently found a transient increase in the rate of uptake in the middle of the V_i phase. This peak (see Figures 2.5 and 2.6 in the Appendix) had a detrimental effect on the use of the V_i phase in the determination of assimilation kinetics. One explanation for this is again the diurnal cycle in the rate of ammonium assimilation (Gevaert et al., 2007). There is a peak in the rate of ammonium assimilation at 11:10 for *U. pertusa* from the Mokohinau Islands (Gevaert et al., 2007) and a peak occurred at 13:00 for the same alga from the same location (Figure 2.5). It should be noted that the only times that Gevaert et al., (2007) measured (directly) the rate of ammonium assimilation were at 6:45, 11:10, 15:15 and 18:45. Their peak at 11:10 may have increased further to a true peak at about 13:00 as occurs in Figure 2.5. It is less easy to explain the same characteristic of the rate of ammonium uptake during the V_i phase in *U. intestinalis* (Figure 2.6) as there is no other evidence for such a peak during V_i (Rees et al., 1998).

In terms of the three phases of uptake (V_s, V_i and V_e), we know what the first two represent. Surge uptake (V_s) consists of uptake to support assimilation and the filling of an internal pool (Rees et al., 1998; Barr et al., 2004). The internally-controlled phase (V_i) represents assimilation only, as the internal pool is now full (Rees et al., 1998; Barr et al., 2004). Unfortunately the precise nature of the externally-controlled phase (V_e) remains elusive. It is correctly referred to as the externally-controlled phase because as the external concentration of, in this instance, ammonium decreases towards zero, the rate of ammonium uptake also decreases. What is not clear is its relationship to assimilation and whether it can be used to determine the kinetics of ammonium assimilation. As the rate of uptake decreases, the rate of assimilation may, for an unknown period of time, remain constant, with ammonium in the storage pool being used to maintain a high rate of assimilation.

2.4.2. Comparing CCCP and multiple-perturbation uptake

Although the V_{max} values derived from uptake over the first 60 minutes ($V^{0\text{-}30}$ and $V^{30\text{-}60}$), were very similar to the V_{max} derived from the CCCP method the difference in the K_m values were sufficient to negate the use of the multiple-perturbation. However, this method has been widely

used to determine the kinetics of assimilation with (Pedersen 1994; Pedersen & Borum 1997; Liu & Dong 2001) or (apparently) without (Campbell 1999; Lotze & Schramm, 2000; Torres et al., 2004) any information from a full perturbation experiment. The full perturbation method is necessary to identify the V_i phase and establish what time series from the multiple-perturbation method corresponds to the maximum assimilation rate. Irrespective the multiple-perturbation experiment should not be used in determining the kinetics of ammonium assimilation.

In previous studies where the multiple-perturbation experiment was used to measure "assimilation", it was evident that V_{max} decreases with successive time intervals suggesting that the alga continued to be in V_s (Campbell 1999; Lotze & Schramm, 2000; Torres et al., 2004). V_s can last for over 600 minutes (Taylor et al., 1999), so assuming that V_i or "V_{assim}" has been reached after only 120 minutes may not be correct. When a full perturbation experiment is done before the multiple-perturbation experiment, V_i is known and this can provide a reliable guide to the V_{max} for the multiple-perturbation experiment. What is required is the correct time interval (e.g. 60-90 minutes) from the multiple-perturbation experiment that provides a relationship between the rate of ammonium uptake and ammonium concentration that gives a maximum rate of uptake close to V_i. However, the temptation to fit these data to the Michaelis-Menten equation and derive a K_m value for assimilation should be resisted. There is, of course, no independent evidence that this is a correct approach, but there is independent evidence to suggest that it is an incorrect approach.

With the exception of *Undaria pinnatifida* (Torres et al., 2004) that has an improbably high K_m, the K_m values for multiple-perturbation "V_{assim}" experiments involving ammonium uptake are usually low (< 15 µM) where the V_{max} matches or is close to the value for V_i (Pedersen 1994; Liu & Dong 2001) or the V_{max} for assimilation is simply stated with no supporting evidence (Campbell 1999; Lotze & Schramm 2000). In contrast, with the CCCP method, the K_m for assimilation is > 18 µM (Taylor & Rees 1999; Rees, unpublished data). The results from Table 2.2 are entirely consistent with this difference, with assimilation measured with the CCCP method giving a K_m of 45 µM and the multiple-perturbation method a K_m of 2-9 µM.

2.5. APPENDIX

2.5.1 Figures

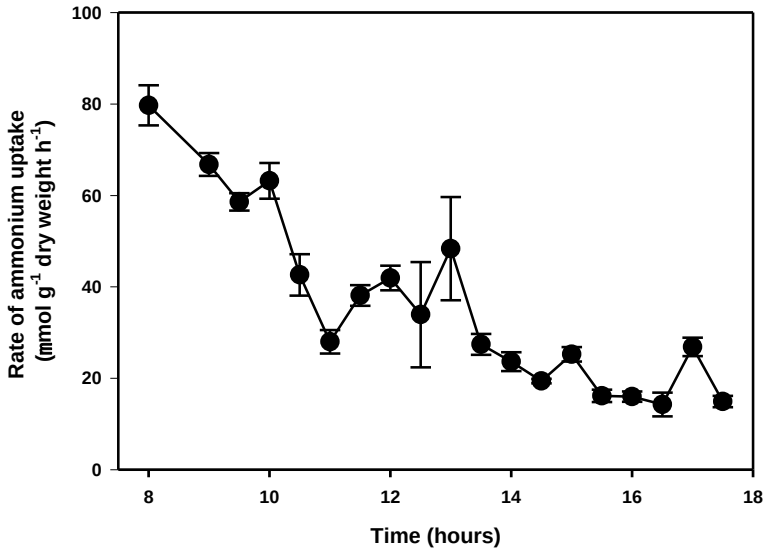

Figure 2. 5. Perturbation experiment with *Ulva pertusa* from the Mokohinau Islands on 29 August 2017. Note the increase in the rate of ammonium uptake, five hours after the start of the experiment at about 13:00. Values are means ± S.E. for three replicates.

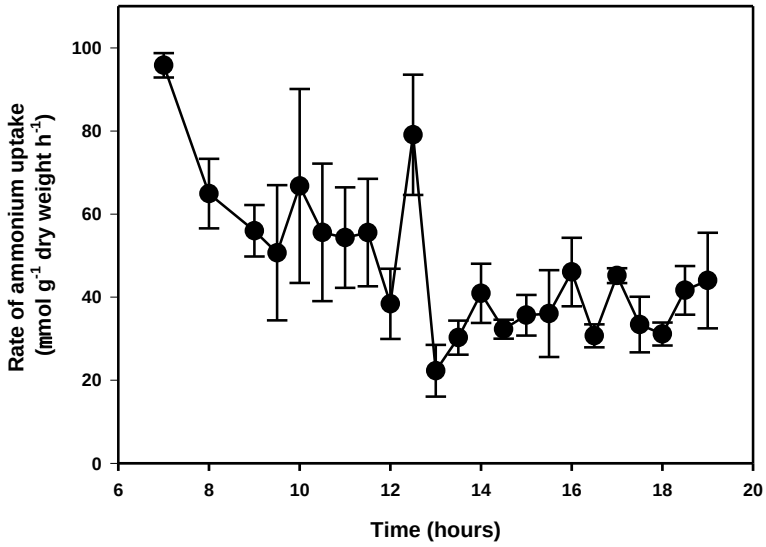

Figure 2. 6. Perturbation experiment with *Ulva intestinalis* on 29 November 2017. Note the marked increase in the rate of uptake, five hours after the start of the experiment at about 13:00. Values are means ± S.E. for three replicates.

2.5.2. Tables

Table 2. 3. Student's t-test comparison between the Michaelis-Menten equation variables from the CCCP and perturbation experiments with *U. intestinalis*, *U. pertusa* from the Mokohinau Islands and Tauranga. Values that are significantly different are in bold ($p<0.05$). Dates of the experiments are described in the methodology.

	Michaelis-Menten values					
Species	**V_{max} (µmol·g^{-1} dry weight·h^{-1})**			**K_m (µM)**		
	df	t	p	*df*	t	p
***U. pertusa* (Mokohinau)**	4	4.3	**0.012**	4	3.2	**0.030**
***U. pertusa* (Tauranga)**	4	4.3	**0.011**	4	5.3	**0.005**
U. intestinalis	4	4.8	**0.008**	4	6.3	**0.001**

Table 2. 4. Student's t-test comparison between the Michaelis-Menten equation variables from the CCCP and multiple perturbation experiments with *U. intestinalis*. Values that are significantly different are in bold ($p<0.05$). Dates of the experiments are described in the methodology.

	Michaelis-Menten values					
Time series	**V_{max} (µmol·g^{-1} dry weight·h^{-1})**			**K_m (µM)**		
	df	t	p	*df*	t	p
V^{0-30}	4	1.3	0.257	4	7.1	**0.002**
V^{30-60}	4	0.8	0.439	4	4.1	**0.014**
V^{60-90}	4	3.9	**0.016**	4	7.9	**0.001**
V^{90-120}	4	8.3	**0.001**	4	4.9	**0.007**

CHAPTER 3
LIGHT ACCLIMATION AND BALANCED AND UNBALANCED GROWTH IN *ULVA PERTUSA*

3.1 INTRODUCTION

Growth rate (μ) is the rate of increase of some measure of biomass. It assumes an exponential increase in biomass and is described by the following formula:

$$\mu = \frac{log_e\ (b_t - b_0)}{t}$$

where μ is growth rate (d^{-1}),
b_t is a measure of biomass (g) after time interval t (days),
b_0 is a measure of biomass (g) at the beginning of the time interval,
t is the time interval (days).

Growth rate (μ) is expressed as g biomass g^{-1} biomass d^{-1}, which reduces to d^{-1}.

If growth is balanced, all constituents (i.e. any measure of biomass) will increase exponentially at the same rate over time and under these steady-state conditions cellular composition and content stay fixed (Campbell 1957; Fishov et al., 1995; Neidhardt 1999; Schaechter 2006). However, environmental conditions (e.g. light, nutrients) change and under these circumstances the organism must acclimate to the new conditions. Organisms compensate for these changes by adjusting their composition and metabolism and as a result they uncouple their growth from other processes (e.g. light harvesting, nutrient storage) creating what may be called unbalanced growth (Fishov et al., 1995; Berman-Frank & Dubinsky 1999).

It is well documented that the rate of a variety of processes (e.g. photosynthesis, nutrient uptake) can change in response to changes in environmental conditions. For example, the rate of ammonium uptake in a number of phytoplankton species (Goldman & Glibert, 1982) and *Ulva lactuca* (Pedersen 1994) increases with increasing nitrogen-deficiency. However, what is not known is the biosynthetic rate associated with these changes (i.e. the rate at which the ammonium

transporters are synthesised). Growth rate is a biosynthetic rate and, with balanced growth, the biosynthetic rate of all constituents is the same. With changes to adverse environmental conditions, such as nutrient deficiency or light acclimation, is the maximum biosynthetic rate (i.e. for nutrient transporters or total chlorophyll) determined by the maximum growth rate, which cannot be exceeded? Or can the biosynthetic rate (e.g. of chlorophyll) exceed the maximum growth rate?

To grow an organism needs to synthesise all its constituents at the same rate (balanced growth). In unbalanced growth associated with acclimation to new environmental conditions, a few constituents must increase *markedly*, but this is against a background where the overall growth rate has decreased. With an alga acclimating to low light, growth rate decreases because there is insufficient energy available to maintain maximum growth rate and during the acclimation period the amount of chlorophyll per cell increases (Post et al., 1984). However, in marine microalgae the rate of acclimation can be surprisingly slow. Though acclimation measured as photosynthetic rate is complete within 10 hours in the diatom *Lauderia borealis* (Marra 1980), it takes at least 100 hours (4.2 days), measured as increases in chlorophyll *a*, to complete acclimation in the diatom *Thalassiosira weisflogii* (Post et al., 1984) and 3-7 days for increases in chlorophyll *a* and peridinin in the dinoflagellate *Prorocentrum mariae-lebouriae* (Harding 1988).

In the green alga *Ulva fasciata*, acclimation is complete after 3 days (Lapointe & Tenore 1981), but this is after a minor decrease (324 to 282 langleys d^{-1}) in ambient light. With a larger transition (100% to 9% ambient light), acclimation takes 4 to 5 days to complete in *U. rotundata* (Henley & Ramus 1989c). However, this is based on total chlorophyll values in the transition from 100% to 9% having similar values to those in the 9% control, but after 5 days there is no evidence for a plateau in total chlorophyll values, with values exceeding those in the 9% control (Henley & Ramus 1989c). Though growth rate is measured as increases in fresh weight (*U. fasciata*) and fresh weight and dry weight (*U. rotundata*), no measurements of growth rate as increases in total chlorophyll were made and it is impossible to calculate such a growth rate from the data provided.

Light is an essential resource for any photosynthetic organism including seaweeds (Hurd et al., 2014). Light quantity (Duke et al., 1986; Sand-Jensen 1988a, Sand-Jensen 1988b; Henley & Ramus 1989a; Henley & Ramus 1989b; Henley & Ramus 1989c; Henley 1990; Henley et al., 1991a; Henley, et al., 1991b; Henley 1992; Geertz-Hansen & Sand-Jensen 1992; Markager & Sand-Jensen 1994; Pérez-LLoréns et al., 1996; Vergara et al., 1997; Bischof 2002; Malta et al., 2003) can change growth rate, photosynthetic activity, development and morphogenesis of macroalgae. When

seaweeds are shifted from high to low light they undergo an "energy predicament" where most of their economy is diverted from lipid and carbohydrate synthesis to protein and photosynthetic pigment formation to enhance the photosynthetic apparatus (Duke et al., 1986; Floreto et al., 1994).

Earlier work (Henley & Ramus 1989c; Henley 1990) investigated balanced and unbalanced growth in *Ulva rotundata*. With relatively constant conditions in outdoor cultures of *U. rotundata* growth rates measured as changes in fresh weight, dry weight and surface area were very similar suggesting that growth was balanced (Henley & Ramus 1989c; Henley 1990). When the alga was transferred to decreased irradiance for one day, growth rate measured as dry weight decreases markedly (at least partly due to a decrease in starch content), but increased if measured as fresh weight or surface area (Henley & Ramus 1989c; Henley 1990), i.e. growth was unbalanced. Increase in surface area after the transfer to low photon flux density is due to cell division and cell expansion (in number), i.e. cell size is constant (Henley 1990).

Photosynthetic organisms increase the level of light-harvesting pigments when irradiance decreases, which for some macroalgae could also be a strategy to store nitrogen due to the proteins that bind these pigments (Kopczak 1994; Gévaert & Rees 2015). For example, *Ulva fasciata* grown under light-limitation for ten days doubled the amount of chlorophyll compared to individuals cultured under high light conditions (Lapointe & Tenore 1981). In contrast, high irradiance will result in an increase in photoprotective pigments to decrease photoinhibition (Niyogi 1999) and as a consequence reduce light harvest pigments synthesis, due to the associated high metabolic cost and negligible benefits (Raven & Hurd 2012).

Under natural conditions acclimation to low photon flux densities can be seen in algal mats, where a light gradient is generated with thalli at the surface exposed to high light and thalli at the base of the mat experiencing substantially reduced light (Vergara et al., 1997). In *Ulva* mats photon flux density decreases with depth, and, as a consequence of low light acclimation, the chlorophyll *a*:*b* ratio decreases (largely due to increases in the amount of chlorophyll *b*) (Bischof 2002; Malta et al., 2003). Tissue nitrogen levels are also affected (McGlathery et al., 1997; Malta et al., 2003), with surface thalli having lower values (~1%) and thalli at the base of the mats having higher values (2-3%).

Light availability also influences nitrogen uptake and assimilation because of the importance of nitrogen in chloroplasts and photosynthesis (Coleman et al., 1988; Evans 1989). In artificial mats

of *Chaetomorpha linum*, rates of ammonium uptake (with the ammonium added at the base of the mat) decreased in low light throughout the mat, but rates were sufficient to sustain demand at the base of the mat, but not at the surface (McGlathery et al., 1997). It is well known that uptake of nitrogen decreases significantly in low light or darkness during short periods of time (Fitzgerald 1968; Hanisak & Harlin 1978) but the long term effects of light-limited growth on ammonium uptake and assimilation has not been reported.

Ulva causes nuisance algal blooms in coastal waters throughout the world (Teichberg et al., 2010; Liu et al., 2013), including New Zealand (Park 2011; Nelson et al., 2015; Port 2016). Consequently, an increased understanding of the physiology and biochemistry of this important genus of algae is imperative. In this chapter, an outdoor multi-chamber culture system allowed me to naturally acclimate *Ulva pertusa* fronds to high and low photon flux densities for extended periods (more than 30 days). Over the duration of the experiment there were periods of balanced and unbalanced growth that allowed me to determine whether a biosynthetic rate (of total chloropyll) can exceed the maximum growth rate.

The main questions addressed in this chapter are as follows:

a. When *U. pertusa* is shifted from ambient light to a range of low photon flux densities (10, 15, 30 and 50% of ambient light), is growth initially unbalanced and how quickly does growth achieve a new, balanced growth rate?

b. During the unbalanced growth phase, does the rate of increase in chlorophyll (measured as a growth rate) that accompanies acclimation to decreased photon flux density equal to or exceed the balanced (maximum) growth rate in the control?

c. What is the effect of light-limited growth on rates of ammonium uptake and assimilation?

Three methods were used in measuring rates of ammonium uptake and assimilation that I refer to as: optimal conditions, *in-situ* conditions and acclimatory response. Using the optimal conditions method, rates for tissue acclimated to 10, 15, 30, 50 and 100% ambient light were all measured under "optimal" laboratory conditions. This experiment addressed whether the maximum rate of assimilation had increased in response to light acclimation. With the *in-situ* conditions, this addressed what the relative rates of assimilation were under the five light treatments. The acclimatory response addressed whether, in acclimating to 10% ambient light, the tissue assimilated ammonium more rapidly at 10% ambient light than if they had not acclimated (100% acclimated tissue incubated at 10% ambient light).

3.2. MATERIALS AND METHODS

Ulva pertusa fronds were collected from Otūmoetai, Tauranga Harbour (Figure 1.1) located in the Bay of Plenty region of New Zealand. A single collection of fresh material was made before each experiment. The 30 to 40 thalli selected from the field were in good condition, i.e. fronds were not eroded, were a rich green colour and a length above 30 cm and sufficient in number for each experiment. The thalli were transferred in a plastic container with seawater to the Leigh Marine Laboratory and kept in outdoor circular tanks (1,600 L) with a flow rate of 11 L min^{-1} delivered via dump buckets (Barr et al., 2008). Collections were made approximately a week before each experiment in autumn (April 29), winter (July 17), spring (October 1) 2017 and winter (August 11) 2018 and left to acclimatise in the tanks prior to the experiments.

3.2.1. Growth under different photon flux densities

Experiments were done using outdoor cultures located in a clear area (i.e. away from any building that would shade the cultures from direct sunlight) at the University of Auckland's Leigh Marine Laboratory, based on the description in Barr et al. (2008) (except that no nutrients were added to the seawater that fed the tubs). Each module consisted of a large shallow tank that held 16 polypropylene tubs with dimensions of 47 cm length x 18 cm width x 13 cm height and 4.2 L capacity. Constant water flow was provided through a 100 µm filtered seawater flow-through system that connected to all the tubs. The outflow from the tubs drained into the large shallow tank that helped (together with the relatively rapid flow of seawater through the tubs) to maintain a relatively constant temperature within the tubs.

A PVC dump bucket that provided wave surge movements that promoted aeration and mixing of the seawater surrounding the alga was installed above each tub. To obtain a similar water flow rate the dump bucket tipping point was adjusted using a set screw (attached to each dump bucket) to increase or decrease the inclination and water exited through a slot on the opposite end of the tub. Flow rate was set to 1 L min^{-1} and it took approximately 4 minutes to replace all the water in each tub (water residence time). To prevent the alga from clumping at the far end of the tub and to ensure even exposure to light, the thallus was pressed to the bottom with a clear plastic structure that consisted of 6 x 3mm thin rows of plastic rods. The main water line was checked regularly and, to prevent any blockage from fine sediment, the pipes were cleaned and flushed periodically.

The effects on growth under different light intensities were achieved by clipping on top of the tubs layers of neutral density cloth to reduce natural light and give a range of photon flux densities: 10%, 15%, 30%, 50% and 100% (no neutral density screens) ambient light. Each treatment had three replicates. Fifteen tubs (5 treatments x 3 replicates) were arranged within the large tray in a random block design, and each container held a thallus with a known initial fresh weight. The initial biomass was ~4g fresh weight and twice per week they were weighed and trimmed back to their original fresh weight. Experiments lasted up to 32 days. A photographic comparison of the tissue after 0 and 30 days of the culture can be seen in the Appendix (Figure 3.18).

Experiments were started in autumn (May), winter (July) and spring (October) 2017 and winter (August) 2018. An experiment in summer 2018 was planned, but *Ulva pertusa* disappeared from Tauranga Harbour from December 2017 until mid-July 2018. Though the alga was present during summer (December – February) 2018/19, the amount of material was limited and experiments were done indoors (Chapter 4) because less material was needed.

3.2.2. Measurements of tissue

Two times a week, each thallus was taken indoors, spun in a salad spinner for 15 seconds and gently blotted dry between paper towels. The thalli were weighed on an electronic balance (Shimadzu) and trimmed back to their initial weight (~ 4g). Samples for chlorophyll content (chlorophyll *a* and *b*), fresh weight, dry weight, ash-free dry weight and surface area were also collected at the same times as the fresh weights.

The growth rate (μ) between every time interval over the course of the experiment was determined as:

$$\mu = \frac{log_e(b_t - b_0)}{t}$$

Where μ is the growth rate (d^{-1}), b_t is the measure of biomass after t days, b_0 is the initial measure of biomass and t is the time interval between samples (days). The basic unit of measurement was fresh weight, but values for dry weight, etc were substituted for fresh weight to calculate μ for each constituent as described below.

Dry weights were obtained using the excess frond material after the alga had been trimmed. Tissue of known fresh weight was dried in an oven at 80°C for more than 48 hours to reach constant weight. After this period the material was weighed to obtain the final dry weight. Fresh weights obtained during the experiment were converted to dry weight by dividing each value with the fresh weight to dry weight ratios that corresponded to that measurement.

Ash-free dry weight was determined by burning the dry weight samples in a muffle furnace (Nabertherm) at 500 °C for eight hours. The remaining ash was weighed and subtracted from the dry weight to give the ash-free dry weight. The dry weight to ash free dry weight ratio for each sample was used to convert each dry weight to its ash-free dry weight as described above.

Surface area was calculated using a disc cutter to obtain circular discs of 1.9 cm diameter (3.6 cm^2) and at every sample interval discs were cut from each excess thallus material to obtain their fresh weight. The total area of the thallus (one side only) (cm^2) was calculated as fresh weight divided by the fresh weight : surface area ratio.

It should be noted that ash-free dry weight and surface area values were not obtained for the first 7 days of the experiment in October 2017, but were for the steady-state phase later in the experiment.

Chlorophylls were extracted according to Mackinney (1941), using 1 g tissue in 10 mL methanol for a minimum of 24 hours at 4°C in darkness. After this period the solution was decanted and centrifuged at 4000 rpm at 4°C for 5 minutes. Spectrophotometer readings were made at 650, 665 and 750 nm. Concentration of chlorophyll was derived from the following equations:

Chlorophyll *a* (mg/L) = 16.5 (A_{665}-A_{750})-8.3 (A_{650}-A_{750})
Chlorophyll *b* (mg/L) = 33.8 (A_{665}-A_{750})-12.5 (A_{650}-A_{750})

The extracted tissue was dried in an oven at 80°C for 48 hours then weighed. Total chlorophyll is the sum of chlorophyll *a* and *b* and is expressed in mg g^{-1} dry weight. The reason for using total chlorophyll is the strong relationship between total chlorophyll and nitrogen content of tissue (Gévaert & Rees 2015). The total amount of chlorophyll in the tissue at each time interval was calculated by multiplying the value for chlorophyll (mg g^{-1} dry weight) by the dry weight : fresh

weight ratio, which in turn was multiplied by the total fresh weight of the tissue for that time interval.

The relationship between growth rate during the steady-state phase (standardised as growth measured over the last 10 days of the experiment) and % ambient light was fitted to a saturation curve using the Droop equation:

$$\mu = \bar{\mu}\,(1 - \frac{q_{min}}{q})$$

Where μ and $\bar{\mu}$ are growth rate (d^{-1}) and theoretical maximum growth rate at $q \rightarrow +\infty$ (d^{-1}), respectively, q is the percentage ambient light and q_{min} the percentage ambient light at zero growth rate. To convert the value for q_{min} into daily photon flux density (mole photons m^{-2} d^{-1}) the average daily irradiance from each experiment was used as the 100% light value. The daily irradiance was the sum of all photon flux measurements throughout the day (see below).

3.2.3. Ammonium uptake and assimilation

At the end of the autumn (2017) and winter (2017 and 2018) experiments, rates of ammonium uptake and assimilation were determined in the laboratory as described above (Chapter 2.2.1). It should be noted that making the same measurements *in situ* is logistically extremely difficult because of the need for an electricity supply for the magnetic stirrers. Due to low biomass at the end of the experiment in the 10 and 15% ambient light treatments (because of slow growth) only one experiment was done in September 2018. In experiments (b) and (c) outlined below, caps made of layers of neutral-density shade cloth were used to give 10, 15, 30 and 50% of ambient light in the constant temperature room. Each measurement of rates of ammonium uptake and assimilation consisted of a complete set of the five light treatments, repeated three times on successive days. Three different approaches were used to determine the effects of extended light periods on the rates of ammonium uptake and assimilation:

a) **Optimal conditions**.- Rates were measured under "optimal" laboratory conditions. Acclimated tissue from the 10, 15, 30, 50 and 100% ambient light treatments were incubated separately under constant conditions: 18 °C and 300 ± 23 μmole photon m^{-2} s^{-1} , without any neutral density caps. The experiment was done on 7, 8 and 9 June 2017.

b) ***In situ* conditions**.- Rates were measured under identical conditions to a) above, except that the acclimated tissue from the 10, 15, 30, 50 and 100% ambient light treatments were incubated at the equivalent photon flux densities (10, 15, 30, 50 and 100%) of 300 ± 23 µmole photon m^{-2} s^{-1}, by covering the Perspex chambers with caps made of layers of neutral-density shade cloth. The three replicate determinations were made on 17, 18 and 19 August 2017.

c) **Acclimatory response**.- Rates were determined for 100% and 10% ambient light acclimated thalli that were incubated at either 10% or 100% of 300 ± 23 µmole photon m^{-2} s^{-1}. Tissue that had been grown at 10% ambient light was incubated in six chambers. Three chambers were randomly assigned and covered with a 10% photon flux density cap (n=3) and the other stayed uncapped (n=3) at 300 ± 23 µmole photon m^{-2} s^{-1}. The experiment with tissue that had been grown at 100% treatments was done in exactly the same way on the following day. Rates for 10% acclimated thalli were determined on 19 September 2018 and the 100% acclimated thalli on 20 September. Photographs of the chambers from the (b) and (c) experimental setup can be seen in the Appendix (Figure 3.17).

3.2.4. Nitrogen content analysis

Tissue was dried in an oven at 80°C for more than 48 hours and then ground in a mortar. The dry fine powder was placed in Eppendorf tubes and stored in a plastic bag (compressed to minimise oxygen content) and stored at -80 °C. The samples (5 mg) were analysed using a CHNOS Elemental Analyzer (vario EL cube) using the protocol for vegetative tissue with 90 seconds of O_2 with acetanilide as the standard. The nitrogen content (%N) of *Ulva pertusa* was determined for tissue from the start and conclusion of each culture.

3.2.5. Environmental conditions

Photon flux densities (photosynthetically active radiation) were measured using Light-Odyssey sensors in the single empty tub from the fifteen selected to contain an alga. Using the software Odyssey data logging (version 2.0) the loggers were set to take irradiance levels at intervals of 5 minutes for the duration of the experiment. At the end of every experiment the light sensor raw data (lumens m^{-2}) was extracted and transformed into photon flux density as µmol m^{-2} s^{-1} and all the information for one day was summed to obtain total daily photon flux density (mol m^{-2} d^{-1}). To convert between units a LI-COR data logger (LI-1400) was used in conjunction with Light-

Odyssey sensors for an hour in an open area (car park) during a clear day, and the resulting data was graphed with a fixed intercept value (i.e. a = zero) to calculate the linear regression equation (y= a + bx) and the slope (b) used to convert all the values.

Water temperature was recorded by a temperature logger about 1 m below the water surface and about 200 m offshore from the Leigh Marine Laboratory. Temperature was logged at 09:00 daily.

3.2.6. Data analysis

Two distinct phases were apparent: an initial period lasting 7 days when there was a marked increase in total chlorophyll in the 10% treatment (hereafter "Unbalanced phase") and a period of 10 days at the end of the experiment when there was little or no change in total chlorophyll (hereafter "Balanced phase") (see Results & Figures 3.13 to 3.16 in the Appendix). It should be noted that the use of the last 10 days was conservative as acclimation was largely complete after 7 days (see Figures 3.13 to 3.16 in the Appendix). In one instance, winter 2017, the experiment lasted 22 days rather than 32 days, so that the last 10 days occurred before the last 10 days of the 32-day experiments. In other words, using the last 20 days of the experiment would have been as valid as using the last 10 days.

The effect of photon flux density on growth rate of *U. pertusa* was compared with a one-way analysis of variance (ANOVA). Before any statistical analysis, all growth rates were log-transformed to reduce skewness and asymmetry between the units of measurement. I asked, for each of the unbalanced [first 7 days] and balanced [final 10 days] phases, whether growth rates varied with respect to light level when expressed as fresh weight, dry weight, ash-free dry weight, chlorophyll and surface area.

Data groups were checked with a normality test (Shapiro-Wilk) and equal variance test (Brown-Forsythe) prior to performing the ANOVA. If the equal variance test passed (p=>0.05), a single-factor ANOVA was used to determine statistical differences between groups, and to isolate the groups that differed from the others a pairwise multiple comparison procedure (Bonferroni test) was used. If the equal variance test failed (p=<0.05), a nonparametric Kruskal-Wallis ANOVA on ranks test was used to determine statistical differences, and to isolate the groups that differed from

the others an all pairwise multiple comparison procedure (Tukey Test) was used. All analysis were performed in SigmaPlot (version 14.0).

The effects of acclimation at different light intensities on ammonium uptake and assimilation rates were also analysed using a single-factor ANOVA and the same principles were applied as the description above. Ordinary least squares regression (Sokal & Rohlf 1995) was used to describe relationships between %N and total chlorophyll and temporal changes in mean daily photon flux density and total chlorophyll. For the latter analyses the line-fitting package SMATR Ver. 2.0 (Warton et al., 2006; http://www.bio.mq.edu.au/ecology/SMATR/) was used.

3.3. RESULTS

3.3.1. Mean daily temperatures and total photon flux densities

The lowest temperatures were recorded in winter. The mean water temperature in autumn 2017 was greater than in spring 2018 (Table 3.1). As the mean temperature increased, so did the range: 0.6 °C in winter, 1.4 °C in spring and 1.9 °C in autumn.

Table 3.1. Mean, maximum and minimum daily seawater temperatures (°C) for each experiment.

		2017		2018
	Autumn	**Winter**	**Spring**	**Winter**
		Temperature (°C)		
Mean	17.6	14.5	16.1	14.6
Minimum	16.8	14.2	15.4	14.2
Maximum	18.7	14.8	16.8	14.8

In winter and spring 2017 photon flux density varied on a daily basis, but overall there was no trend. In contrast, in winter 2018 there was an increase in photon flux density with time. The mean daily photon flux density in winter 2017 (13 mol photon m^{-2} d^{-1}) was lower than winter 2018 (23 mol photon m^{-2} d^{-1}) and spring 2017 (35 mol photon m^{-2} d^{-1}) (Figure 3.1).

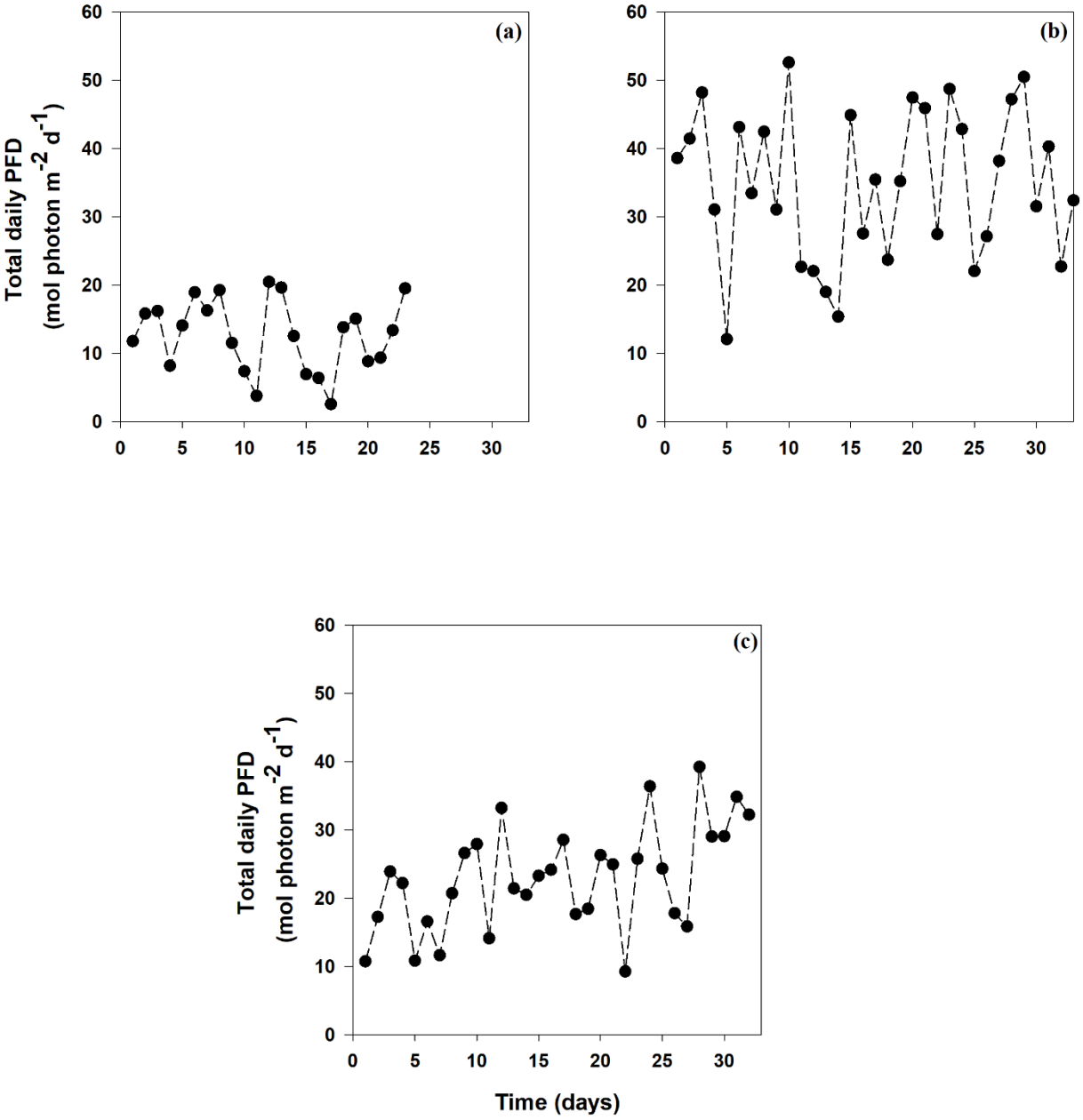

Figure 3. 1. Total daily photon flux density (PFD; mol photon m^{-2} d^{-1}) during the outdoor experiments in (a) winter 2017, (b) spring 2017 and (c) winter 2018.

3.3.2. Total photon flux densities and chlorophyll content

In most experiments though photon flux density fluctuated, sometimes markedly, there was no overall change during the experiment and there was no change in total chlorophyll content, e.g. spring 2017 (Figure. 3.2.a). An exception was the experiment in winter 2018 when there was an overall increase in photon flux density during the experiment; this was accompanied by a decrease

in total chlorophyll content in *U. pertusa*, albeit not significant (Figure. 3.2.b). The summary of the total chlorophyll content (mg g^{-1} dry weight) under different % ambient light in outdoor cultures can be seen in the appendix section Figure 3.19.

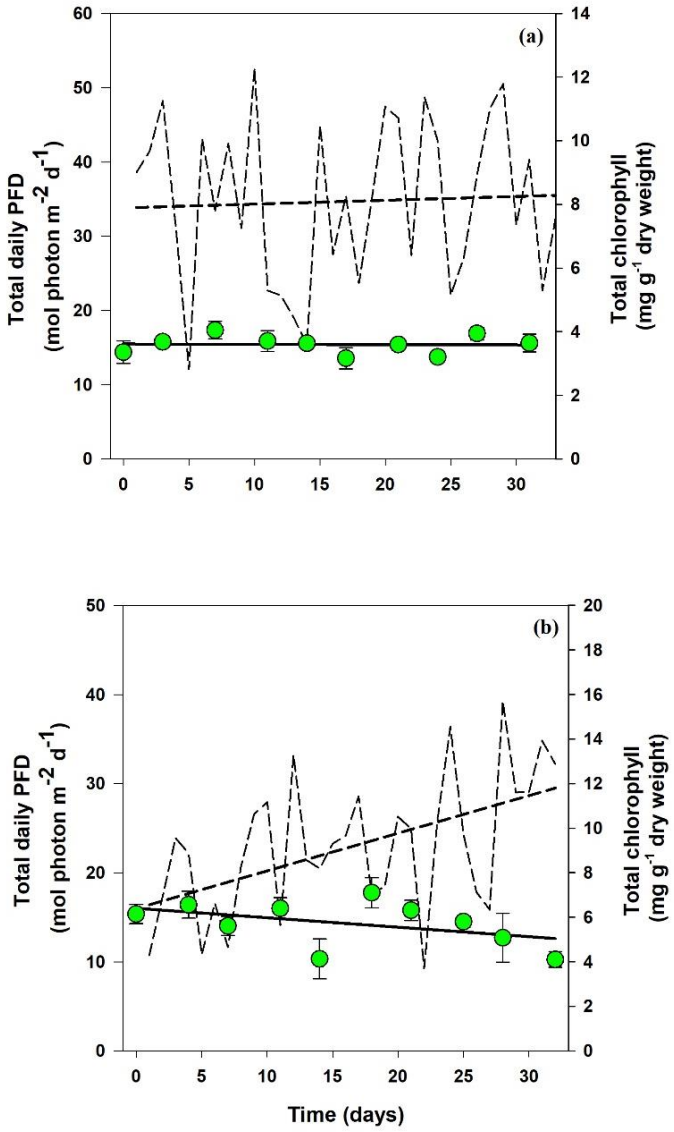

Figure 3. 2. Temporal values for total chlorophyll content (mg g^{-1} dry weight) in *Ulva pertusa* in the 100% ambient light treatment and total daily photon flux density (PFD; mol photon m^{-2} d^{-1}) in outdoor cultures during (a) spring 2017 and (b) winter 2018. Ordinary least squares equation and coefficient of determination are (a) total chlorophyll content: y = 3.6 + -0.0008x, r^2 = 0.001, p (slope = 0) 0.933 and total daily photon flux density: y = 33.8 + 0.05x, r^2 = 0.002, p (slope = 0) 0.802 and (b) total chlorophyll content: y = 6.4 + -0.04x, r^2 = 0.199, p (slope = 0) 0.196 and total daily photon flux density: y = 16.0 + 0.42x, r^2 = 0.263, p (slope = 0) 0.003. Total chlorophyll values are means ± S.E. for three separate samples.

3.3.3. Unbalanced growth (acclimatory) and balanced growth (steady-state) periods during exposure to different photon flux densities

There were two distinct phases: an initial period lasting 7 days when there was a marked increase in total chlorophyll in the 10% treatment and a period of 10 days at the end of the experiment when there was little or no change in total chlorophyll. These two phases are described in more detail below.

3.3.4. Balanced growth under different photon flux densities ($\bar{\mu}$ and q_{min})

The values for the mean growth rate for each constituent (fresh weight, dry weight, total chlorophyll, ash-free dry weight and surface area) over the last 10 days of each experiment (when a new steady-state had been reached in all the treatments) was plotted against % ambient light and the data fitted to the Droop equation. In general there was good agreement between the growth rates for each constituent (Figure. 3.3) and there was no significant difference between the growth rates except for the values in 50% ambient light in winter 2018 (see Table 3.3 in Appendix).

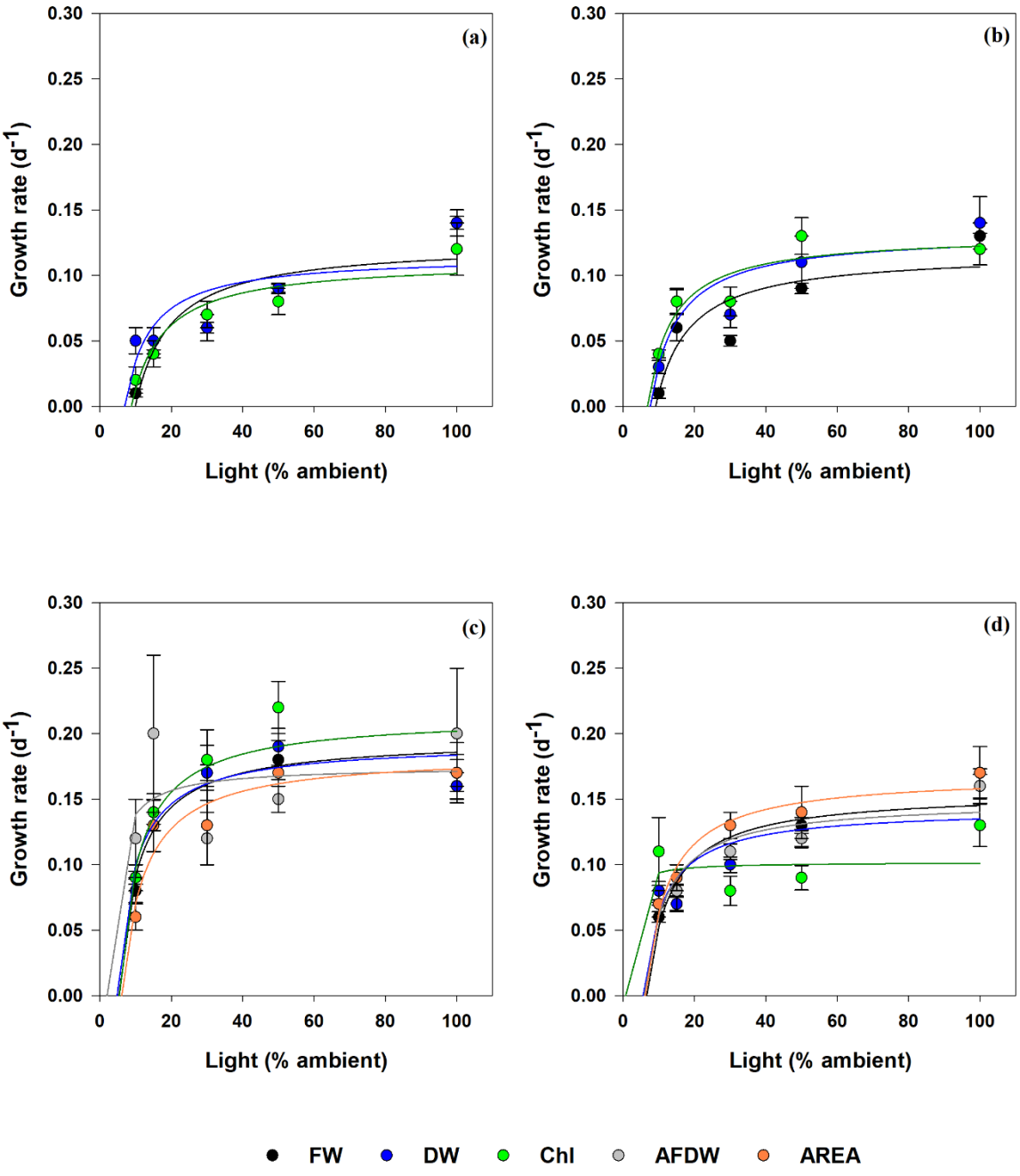

Figure 3. 3. Relationship between mean growth rate (d^{-1}) measured as increases in fresh weight (FW), dry weight (DW), total chlorophyll (Chl), ash-free dry weight (AFDW) and surface area (AREA) over the last 10 days of the experiment and light (% ambient light) with data fitted using the Droop equation for: (a) autumn 2017, (b) winter 2017, (c) spring 2017 and (d) winter 2018. Values are means ± S.E. for three separate determinations.

Table 3. 2. Values for q_{min} (ambient light at zero growth rate) expressed as % ambient light or as daily photon flux density (mol photon m^{-2} d^{-1}) for autumn, winter and spring 2017 and winter 2018. Autumn 2017 is only presented as a % as no photon flux density data was available. Values are means ± S.E. for three separate determinations.

	2017			2018	2017		2018
Group	**Autumn**	**Winter**	**Spring**	**Winter**	**Winter**	**Spring**	**Winter**
	q_{min} (% ambient light)				**q_{min} (mol photon m^{-2} d^{-1})**		
FW	9.9 ±1.5	9.1 ±1.8	5.3±0.7	6.6±0.7	1.2 ±0.2	1.8 ±0.2	1.5 ±0.2
DW	6.9± 2.3	7.6 ±1.5	4.8±1.0	5.6±1.6	1.0 ±0.2	1.7 ±0.3	1.3 ±0.4
Chl	8.8 ±1.1	6.8 ±1.2	5.4±1.2	0.7±3.1	0.9 ±0.2	1.9 ±0.4	0.2 ±0.7
AFDW	-	-	2.0±3.0	5.9±1.1	-	0.7 ±1.0	1.4 ±0.3
AREA	-	-	6.1±0.9	6.2±0.6	-	2.1 ±0.3	1.4 ±0.1

There were differences in values for q_{min} expressed as % ambient light (Table 3.2), but, to a degree, these were due to seasonal differences in photon flux density (Figure 3.2) which became less marked when % ambient light was converted to daily photon flux density (Table 3.2). The very low q_{min} for chlorophyll growth rate in winter 2018 was largely due to a poor fit between growth rate and % ambient light (Figure 3.3.d; Table 3.3 in Appendix).

If growth rate measured as fresh weight is plotted against the mean daily photon flux density (mol photon m^{-2} d^{-1}), growth rates in spring were greater than in winter at the same mean daily photon flux density (Figure. 3.4), suggesting that the availability of photons was not the only factor influencing growth rate.

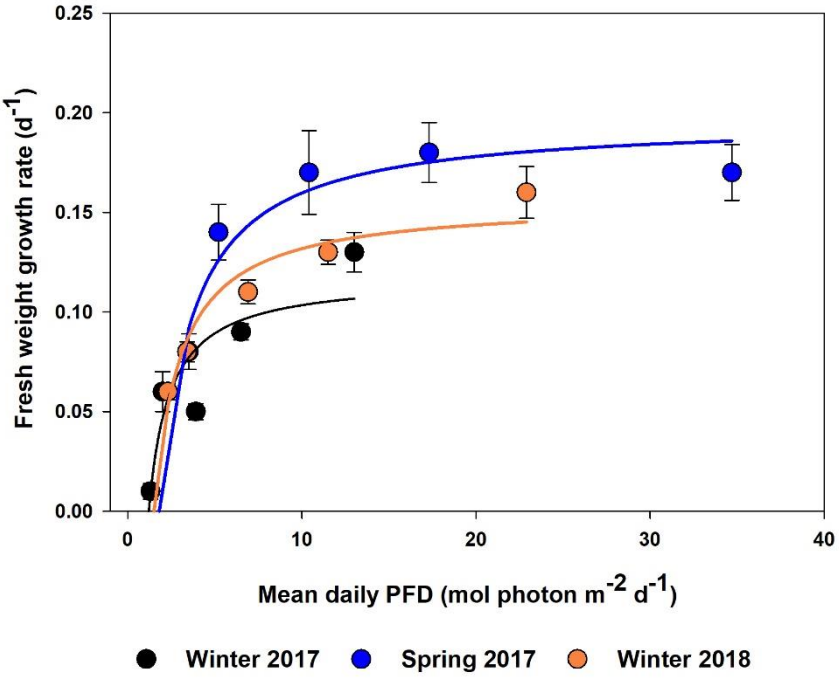

Figure 3. 4. Relationships between mean growth rates (d^{-1}) measured as increases in fresh weight and mean daily photon flux densities (PFD; mol photon m^{-2} d^{-1}) during the steady-state phase after acclimation (last 10 days of the experiments) in winter and spring 2017 and winter 2018. Values were fitted to the Droop equation. Values are means ± S.E. for three separate determinations.

3.3.5. Acclimation to low light and unbalanced growth

In 10% and 15% (Figures 3.5 - 3.8) ambient light, growth rate measured as increases in total chlorophyll were consistently and significantly greater than those measured as increases in other constituents during the initial (first 7 days) acclimatory phase. Although there were occasional significant increases in growth rate measured as increase in total chlorophyll at 100% and 30% ambient light compared to other measures (Figure 3.5 and 3.7), in general the rates of increase of total chlorophyll were the same as the rates of increase of any other measured constituent at 30%, 50% and 100% ambient light.

Only the total chlorophyll growth rates showed no statistically significant differences between the light treatments, indicating the ability of light-limited *Ulva* to increase their chlorophyll content (Figure 3.5– 3.8). In spring 2017 and winter 2018 the total chlorophyll growth rates at 10% and

15% ambient light exceeded those for tissue grown at 100% ambient light. Detailed information about the ANOVA analysis can be found in the Appendix in Tables 3.4 and 3.5.

Autumn 2017

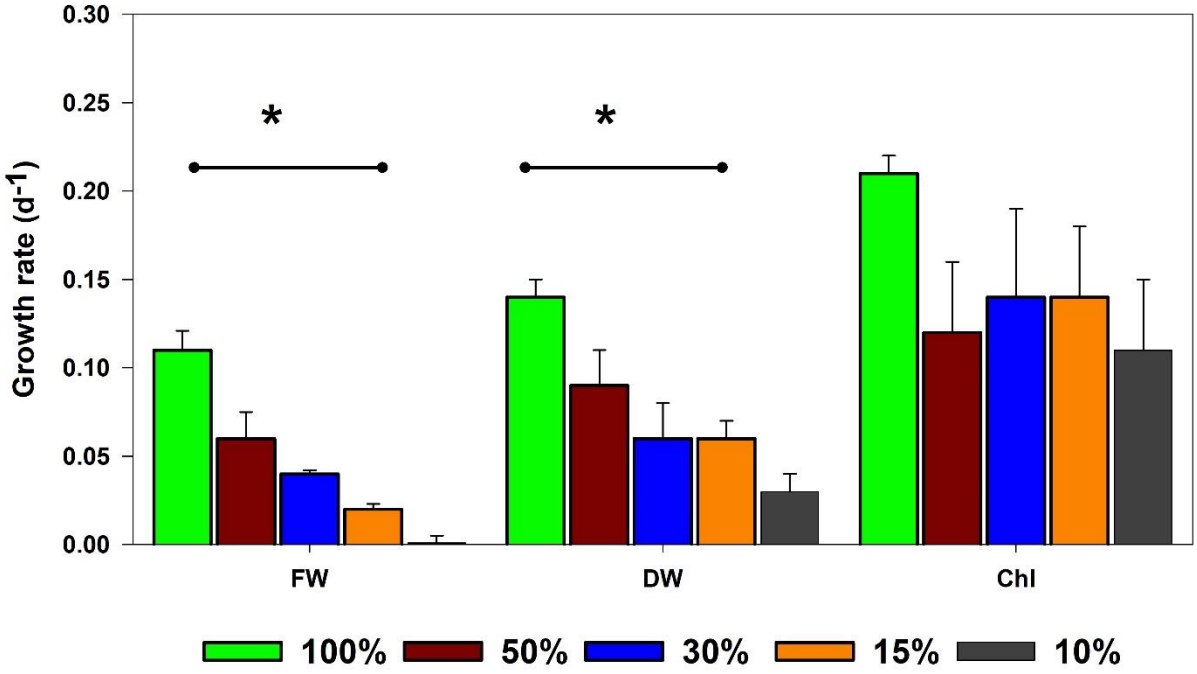

Figure 3. 5. Growth rates (d^{-1}) measured as increases in fresh weight (FW), dry weight (DW) and total chlorophyll (Chl) at a range of ambient light from 10 to 100% in autumn 2017. An asterisk denotes significant differences between growth rates ($p < 0.05$). Values are means ± S.E. for three separate determinations.

Winter 2017

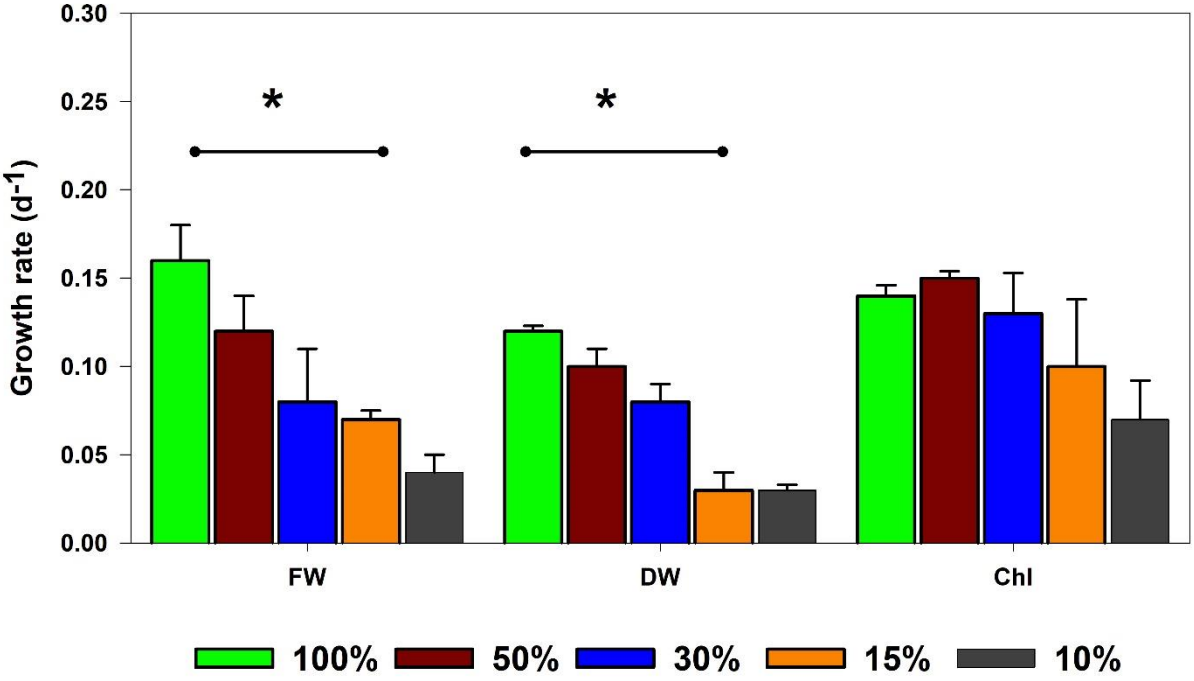

Figure 3. 6. Growth rates (d^{-1}) measured as increases in fresh weight (FW), dry weight (DW) and total chlorophyll (Chl) at a range of ambient light from 10 to 100% in winter 2017. An asterisk denotes significant differences between growth rates ($p < 0.05$). Values are means ± S.E. for three separate determinations.

Spring 2017

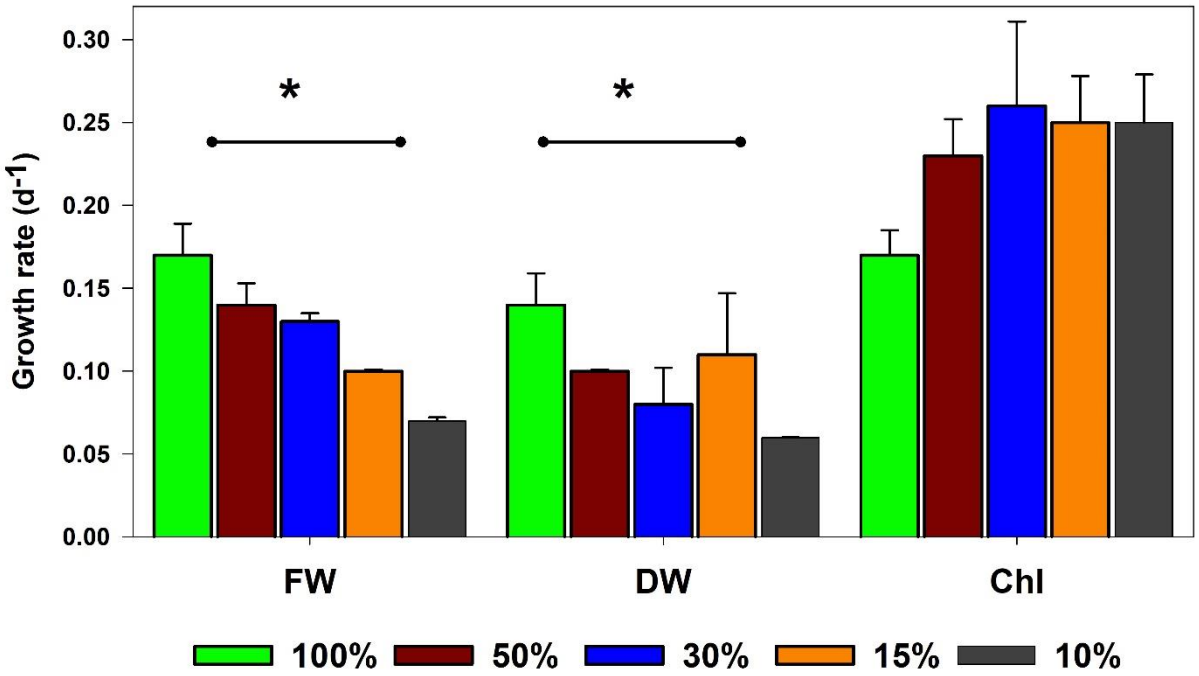

Figure 3. 7. Growth rates (d^{-1}) measured as increases in fresh weight (FW), dry weight (DW) and total chlorophyll (Chl) at a range of ambient light from 10 to 100% in spring 2017. An asterisk denotes significant differences between growth rates ($p < 0.05$). Values are means ± S.E. for three separate determinations.

Winter 2018

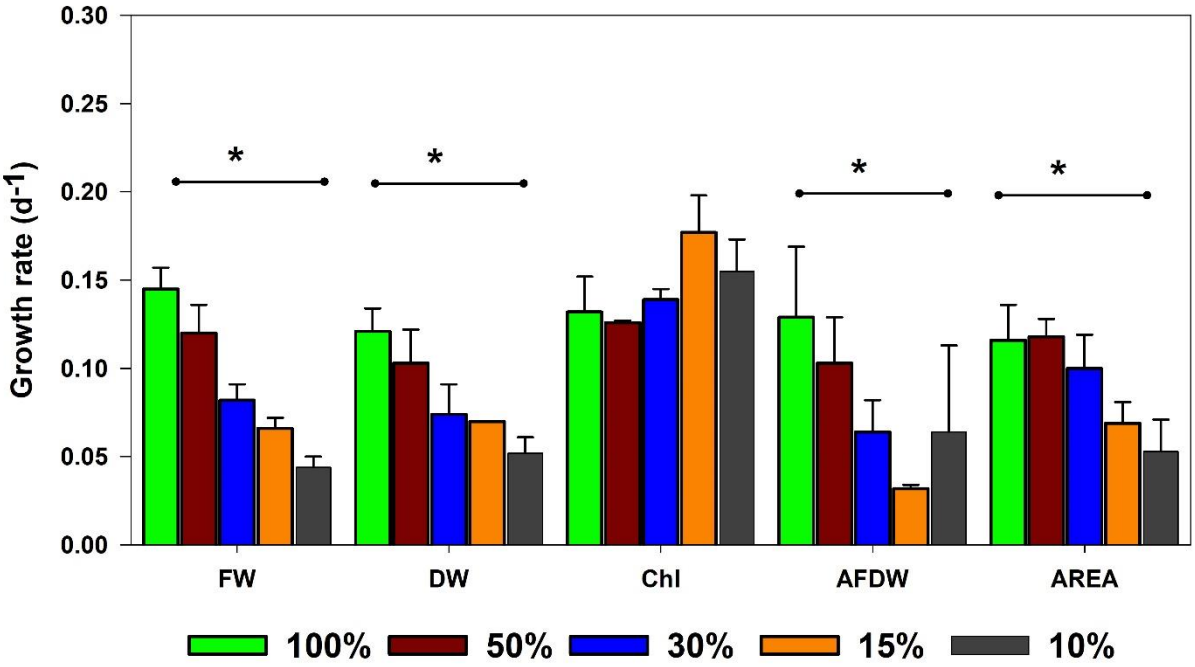

Figure 3. 8. Growth rates (d^{-1}) measured as increases in fresh weight (FW), dry weight (DW), total chlorophyll (Chl), ash-free dry weight (AFDW) and surface area (AREA) at a range of ambient light from 10 to 100% in winter 2018. An asterisk denotes significant differences between growth rates ($p < 0.05$). Values are means ± S.E. for three separate determinations.

3.3.6. Relationship between %N and total chlorophyll in *Ulva pertusa*

There was a strong positive relationship between % N and total chlorophyll levels (Figure. 3.9). All %N values are given in the Appendix (Table 3.6).

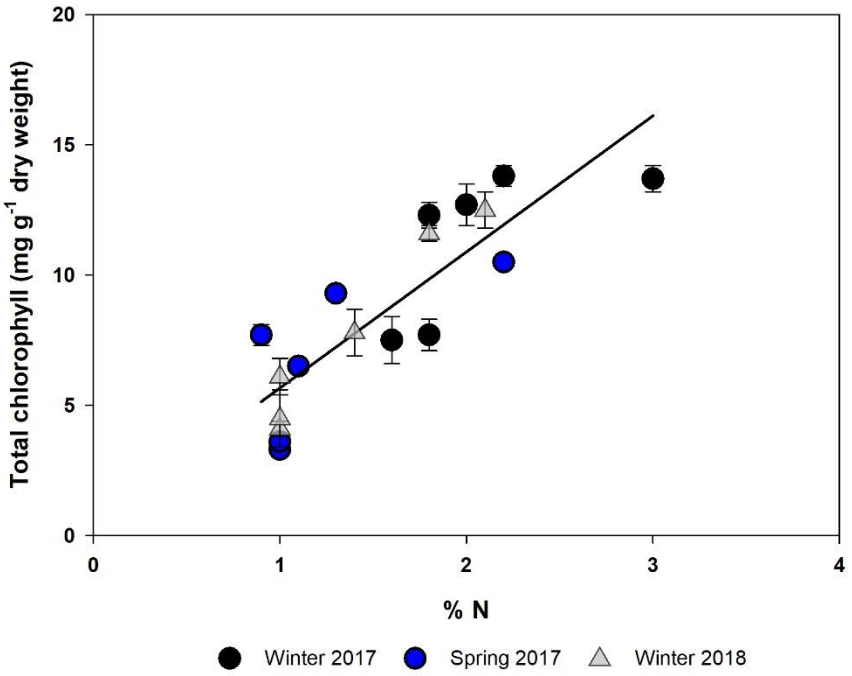

Figure 3. 9. Relationship between tissue nitrogen (% N) and total chlorophyll (mg g^{-1} dry weight) content in *Ulva pertusa* in outdoor experiments in 2017 and 2018. Ordinary least squares equation and coefficient of determination are: total chlorophyll (mg g^{-1} DW) = 5.22 × (%N) – 0.43, r^2 = 0.74. Values are means ± S.E. for three separate samples.

3.3.7. Light acclimation and rates of ammonium uptake and assimilation

Rates of uptake and assimilation measured under optimal conditions

The rate of ammonium uptake was significantly less (by 24%) in *Ulva pertusa* that had acclimated to 10% ambient light compared to the control that was exposed to ambient light (p = 0.034). In contrast, there were no statistical differences in the rates of ammonium assimilation (p = 0.110). between tissue grown at the five photon flux densities treatments (Figure. 3.10).

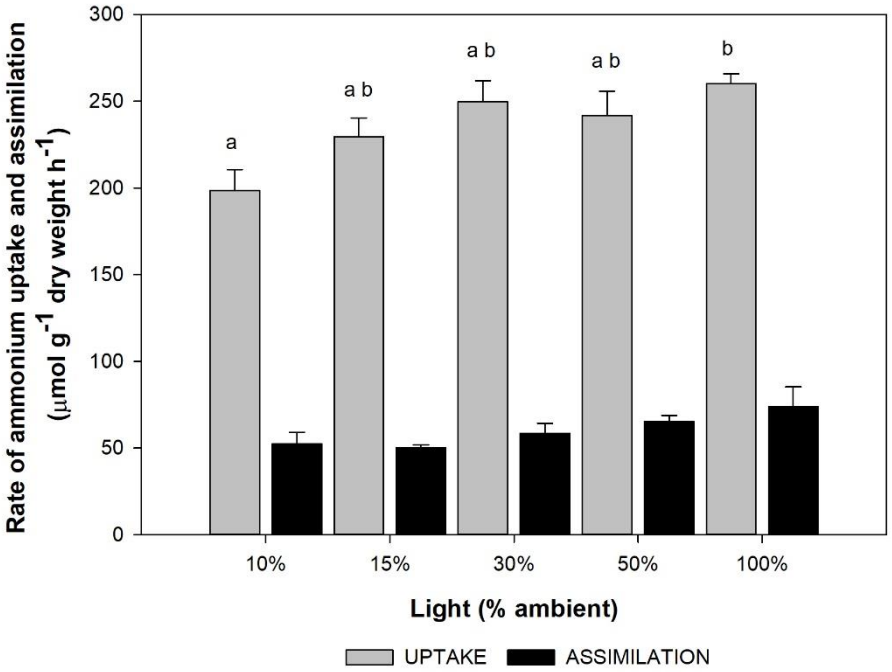

Figure 3. 10. Rates of ammonium uptake and assimilation by *Ulva pertusa* that had acclimated to different ambient light conditions (10, 15, 30, 50 and 100%) for 32 days in June 2017. Measurements were made under identical laboratory conditions. Uptake rates that are labelled with the letters "*a*" and "*b*" only are significantly different from each other. Values are means ± S.E. for three separate replicates.

Rates of uptake and assimilation measured under "in situ" conditions

In this experiment rates of ammonium uptake and assimilation were determined in *Ulva pertusa* that had been acclimated to different ambient light conditions (10-100% ambient) for 21 days and were incubated at the same % ambient light in the laboratory. There were no significant differences between rates of ammonium uptake ($p = 0.111$) or assimilation ($p = 0.910$) (Figure. 3.11).

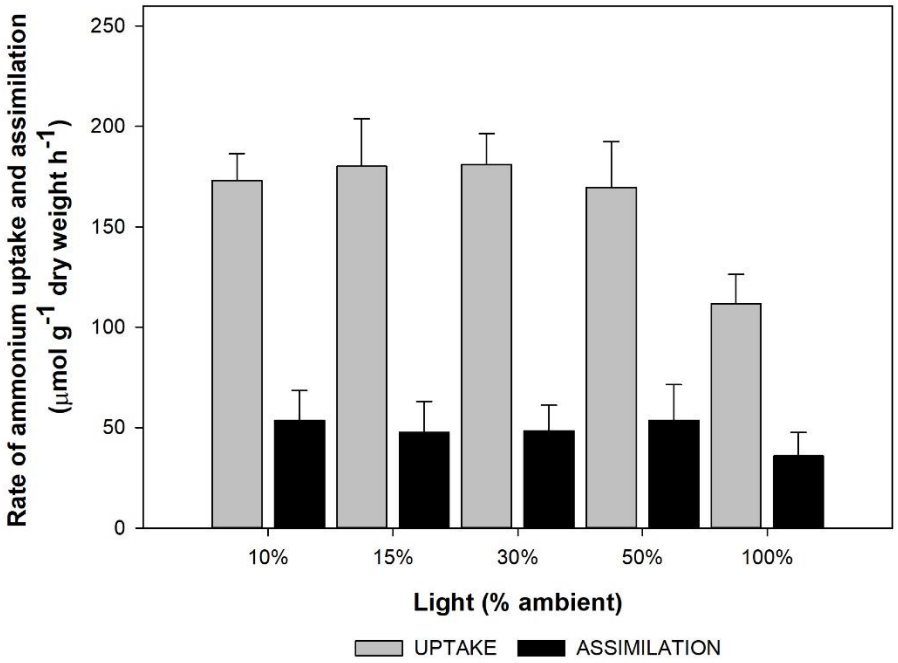

Figure 3. 11. Rates of ammonium uptake and assimilation by *Ulva pertusa* acclimated to different ambient light conditions for 21 days in August 2017. Measurements were made under "in-situ" conditions, e.g. the tissue that had acclimated to 10% ambient light was incubated under 10% of full photon flux density (300 µmole m^{-2} s^{-1}) in the laboratory. Values are means ± S.E. for three separate replicates.

Rates of uptake and assimilation - acclimatory response

Only *Ulva pertusa* acclimated at 10% and 100% of ambient light for 32 days in September 2017 were used in this experiment. For tissue from both treatments, rates of ammonium uptake and assimilation were measured in the laboratory at a photon flux density of either 30 or 300 µmole m^{-2} s^{-1}. *U. pertusa* acclimated at 10% ambient light had significantly greater rates of ammonium assimilation when measured at both 30 or 300 µmole m^{-2} s^{-1} compared with those from 100% light (Figure. 3.12). Full statistical analysis of rates of ammonium uptake and assimilation are given in Table 3.7 in the Appendix.

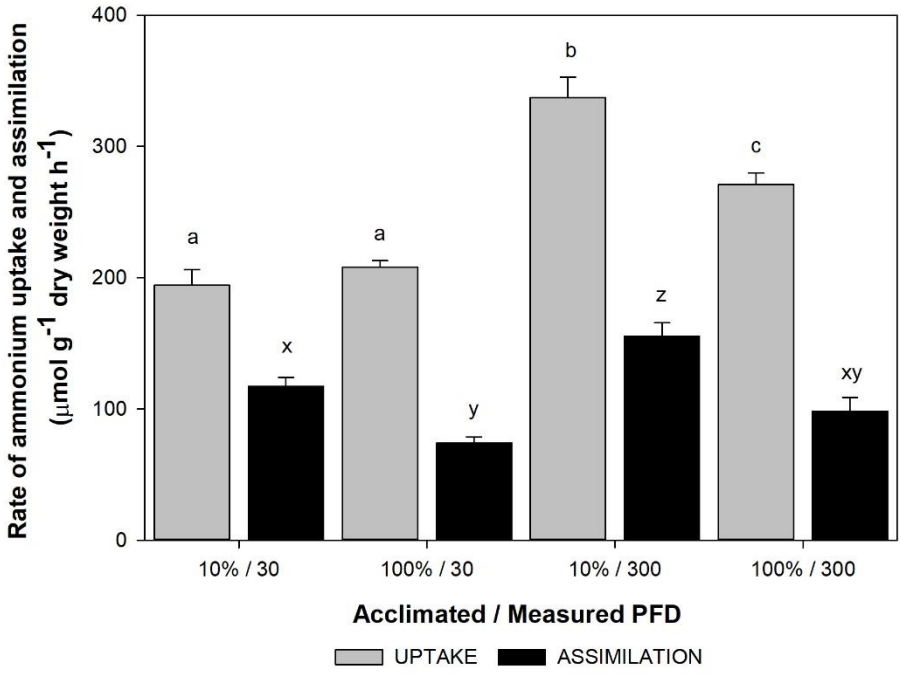

Figure 3. 12. Rates of ammonium uptake and assimilation by *Ulva pertusa* acclimated to 10% and 100% ambient light conditions for 32 days in spring 2017. Measurements were made for both treatments at 30 or 300 µmole m^{-2} s^{-1} (10% or 100% full photon flux density PFD) in the laboratory. Letters indicate statistically significant differences between treatments and measurements ($p < 0.05$). Values are means ± S.E. for three separate replicates.

3.4. DISCUSSION

3.4.1. Use of Droop equation and balanced growth

The Droop equation (Droop 1968) was used originally to define the relationship between growth rate (of a single-celled alga) and the amount of an internal nutrient (cell quota of vitamin B_{12}). Clearly ambient light is not an internal nutrient. Though most uses of the Droop equation with macroalgae have concentrated on the relationship between nitrogen or phosphorus content and growth rate (Campbell, 2001; Malta et al., 2005; Hernández et al., 2008), it has also been used to describe the relationship between N:P ratio and growth rate in marine microalgae (Terry et al., 1985; Klausmeier et al., 2004). The latter suggests that the use of the Droop equation has a wider use than Droop may have envisaged originally. Therefore, the compensation point for growth (my q_{min}) is at least analogous to Droop's minimum cell quota. Using an equation that is used for describing the relationship between rates of photosynthesis and irradiance (Webb et al., 1974) gave a poorer fit to the data, but the Webb et al. (1974) equation gave a better fit to photosynthetic data for *Ecklonia radiata* (Caitlin Blain, pers. comm.) than the Droop (1968) equation (data not shown).

The similarities between the use of the Droop equation here and its original use to describe the relationship between internal nutrient content and growth rate is that they both involve (a) growth rate and (b) zero growth rate occurring at a greater than zero value for internal nutrient concentration and ambient light (see section 5.1 for a full discussion of the use of the Droop equation and how it compares with another possible model). To my knowledge, this is the first use of the Droop equation to describe the relationship between % incident photon flux density and growth rate and its first use with growth rates measured as increases in a constituent that changes markedly in response to changes in photon flux density (total chlorophyll) (Figure 3.3). Other curve-fitting equations (derived from descriptions of the relationship between rates of photosynthesis and irradiance) have been used (e.g. Markager & Sand-Jensen 1990) to describe the relationship between growth rate and incident light, but growth rates were measured as increases in carbon or surface area (Sand-Jensen 1988a; Markager & Sand-Jensen 1992).

Under autotrophic conditions *Ulva* clearly has a q_{min} or compensation point (both refer to the photon flux density at which the net growth rate is zero) for growth (Figure 3.3). For *U. lactuca* this can vary from 0.33 to 2.5 µmole photons m^{-2} s^{-1} or 0.03 to 0.22 mole photons m^{-2} d^{-1} for growth

in continuous light for growth measured as increases in carbon after 7 to 70 days of acclimation in *U. lactuca* (Sand-Jensen 1988a; Markager & Sand-Jensen 1992) as much as there is for photosynthesis (Sand-Jensen 1988b). The compensation point for growth in *U. lactuca* is generally lower than the values that I obtained (Table 3.2). However, the data obtained for *U. lactuca* was for laboratory cultures grown at 7 °C with ample nutrients, as opposed to a range of mean temperatures of 14.5-16.1 °C. As the compensation point is lower in winter than in spring, growth at a low temperature may decrease the compensation point; it would be interesting to investigate this further.

3.4.2. Rate of light acclimation – unbalanced growth

During acclimation to low photon flux density most of the metabolic budget is invested in optimizing energy capture (increasing light-harvesting pigment-protein complexes) whereas a reduction in the cell quota of the light harvesting system will reduce photo-oxidative stress under high light (Dubinsky et al., 1986; Leonardos & Geider 2004). Changes associated with light acclimation in macroalgae include increased photosynthetic pigment levels (particularly for the accessory pigments) (Lapointe & Duke 1984; Duke et al., 1986; Duke et al., 1989; López-Figueroa & Niell 1990; Altamirano et al., 2000; Makarov 2012; Wang et al., 2016). In this analysis, the time taken for *Ulva pertusa* to readjust its total chlorophyll content in response to decreased photon flux density was about 7 days (Figure 3.5 - 3.8), similar to that for *U. rotundata* (at least 5 days) (Henley & Ramus 1989c). During the 5 days after *U. rotundata* was transferred from 100% to 9% ambient light, total chlorophyll increased 2.1-fold (from 4.1 to 8.6 µmole chlorophyll *a*+*b* g^{-1} dry weight). Increases in total chlorophyll in *U. pertusa* were slightly less at 1.8-fold (3.7 to 6.7 mg chlorophyll *a*+*b* g^{-1} dry weight) (Appendix Figure 3.19.c).

The literature relating to unbalanced growth is limited for algae, with most relating to bacteria (Campbell 1957; Fishov et al., 1995; Neidhardt 1999; Henley 1990; Berman-Frank & Dubinsky 1999; Schaechter 2006). One day after transfer of *Ulva rotundata* to decreased irradiance, growth rate measured as dry weight decreases markedly, but not if measured as fresh weight or surface area (Henley & Ramus 1989c; Henley 1990). There was no difference in growth rate expressed as either dry weight or fresh weight after three days in 10% ambient light in *U. pertusa* (Appendix Figures 3.13 - 3.16) This does not negate the observations with *U. rotundata*, but suggests that they are likely to be transient, as indeed are other changes during light acclimation.

One of the benefits of measuring changes in tissue constituents as growth rates is that it provides a way of comparing rates with the same units (d^{-1}). Of particular interest is growth rate measured as tissue total chlorophyll at 10% and 100% ambient light. The growth rate measured at 100% ambient light is the maximum growth rate that can be achieved under those conditions. This is also a biosynthetic rate that describes the rate of total chlorophyll synthesis and the rate at which the proteins that bind chlorophyll molecules are made. There must be an obligatory relationship between the two as chlorophyll is always found bound to protein. The rate of total chlorophyll biosynthesis was essentially identical in both the 10% and 100% ambient light treatments over the first seven days, except possibly in spring 2017 (Figures 3.5 – 3.8) . In spring 2017, rates of total chlorophyll increase were greater in 10 to 30% treatments with a maximum growth rate of 0.25 - 0.26 d^{-1}, compared to a growth rate of 0.17 d^{-1} for the 100% treatment (Figure 3.7).

One of the questions that this chapter intended to address was: can the biosynthetic rate of total chlorophyll exceed the maximum growth rate? Based on the data presented here, the answer is equivocal and depends on the season. Why does the biosynthetic rate of total chlorophyll exceed the growth rate measured as increases in fresh weight in spring, but not autumn or winter? One possibility is that 10% of ambient light in autumn and winter is so low that *U. pertusa* had insufficient available energy to acclimate rapidly. In winter 2017 the mean photon flux density was 13 mol photon m^{-2} d^{-1}, in winter 2018 it was 23 mol photon m^{-2} d^{-1} and in spring 2017 35 mol photon m^{-2} d^{-1} (Figure 3.1). Consequently, 10% of these values are 1.3, 2.3 and 3.5 mol photon m^{-2} d^{-1}. Q_{min} values for winter 2017, winter 2018 and spring 2018 were (for growth rate measured as increases in fresh weight) 1.2, 1.5 and 1.8 mol photon m^{-2} d^{-1} respectively (Figure 3.3 and Table 3.2). This suggests that in winter 2017 *U. pertusa* at 10% ambient light was at the compensation point for growth and only slightly above the compensation point in winter 2018. In contrast, in spring 2018, the available photon flux density at 10% ambient light was comfortably in excess of the compensation point. However, even in winter 2017, when the growth rate measured as average increases in fresh weight was 0.02 d^{-1}, the growth rate measured as increases in total chlorophyll was 0.05 d^{-1} – an indication of the importance of light acclimation at any cost (Appendix Figure 3.14). Virtually all resources under these conditions were devoted to light acclimation.

A doubling time can be calculated from a known growth rate (μ) as follows:

$$doubling\ time = \frac{\log_e 2}{\mu}$$

where μ and doubling time are expressed in days.

The growth rate measured as increases in fresh weight in 100% ambient light was consistently 0.17 d^{-1} or less over the initial 7-day period (Figure 3.6), which corresponds to a doubling time of about 4 days or less. In contrast, the growth rate measured as increases in total chlorophyll in 10% ambient light varied between 0.07 d^{-1} in winter 2017 and 0.25 d^{-1} in spring 2017 (Appendix Figures 3.14 and 3.15). The latter corresponds to a doubling time of 2.8 days. Therefore, it can take less than a generation time for acclimation to be completed in *U. pertusa* and some (Prézelin & Matlick 1983 and references therein), but not all microalgae (Post et al., 1984; Harding 1988). Why there should be these marked differences in the time it takes for an alga to fully acclimate is unknown.

3.4.3. Seasonal effects on total chlorophyll and nitrogen content in *Ulva pertusa*

Ulva consists of a thin, two-cells thick thallus. It is adapted for rapid growth, with all cells being photosynthetically active and in direct contact with the medium (Littler & Littler 1980; Hanisak et al., 1990). Maximum chlorophyll levels are higher during autumn-winter and tend to decrease in spring and summer (Altamirano et al., 2000; Kim et al., 2004). There are similar patterns in the tissue nitrogen and phosphorus content, reflecting an equilibrium between elemental composition and dilution due to growth. In winter *Ulva* spp. tissue N and P were higher reaching 4.0 to 4.5 %N and 0.14 to 0.18 %P and in summer they decrease to 1.5 %N and 0.8 %P (Malta & Verschuure 1997; Rivers & Peckol 1995; Villares et al. 1999). *Ulva pertusa* displayed similar patterns with greater chlorophyll and nitrogen levels in winter (Figure 3.9 and Table 3.6).

Most of the % N values range between 1 and 2% N, which could be interpreted as N-deficiency (Figure 3.9). However, though critical N values for *Ulva* can be 2% or greater (Björnsäter & Wheeler 1990; Lavery & McComb 1991), they can be as low as 1.2% N (Angell et al., 2014). Without constructing a Droop relationship between tissue N content and growth rate for *U. pertusa*, it is impossible to know whether any of the values in Figure. 3.12 represent N-deficiency. Chlorophyll can be thought of as an indirect form of nitrogen storage, as all the chlorophyll is bound to proteins located in the thylakoid membranes. The light harvesting system constitutes 20% to 43% of the total nitrogen in *U. pseudolinza* (Gévaert & Rees 2015), with higher values occurring

under light limitation. This high allocation of nitrogen to the light harvesting system during light limitation is another example of the importance acclimation has to the organism.

3.4.4. Seasonal effects on maximum growth rate and the compensation point for growth

Despite being the season when dissolved inorganic nitrogen and phosphorus concentrations in seawater are at a maximum, winter is also characterized by short days, low photon flux densities and low temperatures (Lotze et al., 1999; Lotze & Schramm 2000; Rees 2003), and it would be expected that growth rates would be lower than at any other time of the year. However, the lowest growth rates were recorded in autumn (Figure 3.3), despite higher temperatures than either winter or spring (Table 3.1). As the effect of temperature (over a much wider range) on the growth rate of caged *U. pertusa* in Japan is relatively minor (Yoshida et al., 2015), this may be equally true in New Zealand. The temperature in winter 2017 and 2018 were essentially identical, but growth rates were higher in 2018 possibly because the average daily photon flux density was greater (Figures 3.3 and 3. 4). This suggests that both temperature and photon flux density affect growth rate, but that photon flux density is more important.

The compensation point for growth varies between 0.7 and 9.9% ambient light (Figure 3.3). However, ambient light changes seasonally and when the compensation point is given as the mean daily photon flux density the range is smaller at 0.2 to 2.1 mol photons m^{-2} d^{-1} (Table 3.2). The very low value for total chlorophyll growth rate in winter 2018 is due to a poor fit to the Droop equation and if this value is ignored the ranges are 2.0 to 9.9% ambient light and 0.7 to 1.9 mol photons m^{-2} d^{-1}. Spring is considered an optimal season for growth as photon flux density and temperature increase and there are sufficient nutrients in seawater (Martínez et al., 2012). The % values for compensation point were lowest in spring 2017, but were the highest (except for ash-free dry weight) for mol photons m^{-2} d^{-1} (Table 3.2). The former is due to there being more light in spring and the latter probably to a greater requirement for photons to fuel an increased growth rate (Figure 3.1).

3.4.5. Light acclimation of rates of ammonium uptake and assimilation

Perfect light acclimation would allow an alga to maintain the same maximum growth rate under low photon flux density as it exhibits under optimum photon flux densities. In general, this does

not occur. However, even some acclimation is preferable to none and *Ulva pertusa* appears to fall into this category with total chlorophyll growth rate. Does a similar acclimation occur with ammonium uptake and assimilation?

Macroalgae increase the level of photosynthetic pigments when irradiance decreases and nutrients are available (e.g. Vermaat & Sand-Jensen 1987). As there is a strong positive relationship between nitrogen content and total chlorophyll content, acclimation to low light should be accompanied by an increase in rates of nitrogen assimilation. In *Ulva fasciata* there is an inverse relationship between growth photon flux density and rate of nitrate uptake: with limited light, the uptake rate is higher (Lapointe & Tenore 1981).

The first two experiments (optimal and *in situ* conditions) were done in autumn and winter and there were no significant differences in rates of ammonium assimilation (Figures 3.10 and 3.11). This is probably not surprising as the growth rates were extremely low. The compensation point for growth was 6.9 to 9.9 % ambient light in autumn, and 6.8 to 9.1% in winter, which are only slightly less than 10% (Table 3.2). Nitrogen assimilation would continue to be important (tissue grown at 10% had a high total chlorophyll content and, consequently, a high nitrogen content), though, as with growth rate, the nitrogen specific rate of ammonium assimilation would be low.

In spring, rates of assimilation were greater in *U. pertusa* maintained at 10% irrespective of whether rates were measured at 30 or 300 µmole photons m^{-2} s^{-1} (Figure 3.12). The investment in a greater rate of ammonium assimilation at low growth irradiance may reflect the importance of nitrogen acquisition in maintaining the alga's ability to maintain high levels of light-harvesting pigment-protein complexes combined with a much greater growth rate at 10% ambient light than in autumn and winter. However, this was only possible because at 10% ambient light in spring, *U. pertusa* was sufficiently above the compensation point for growth to allow it to invest absorbed light energy in the manufacture of the proteins (e.g. glutamine synthetase) required to increase the rate of ammonium uptake and assimilation (Table 3. 2).

To conclude, the results provided from this chapter give us a better understanding of the effects on growth during long-term light limitation periods. Under natural circumstances it would be difficult to evaluate this effect as the acclimation process requires an extended period. During the 32-day acclimation to low light (10% ambient light), *U. pertusa* showed periods of unbalanced and balanced growth, that eventually led to measurable physiological changes (increased rates of

ammonium assimilation). The most relevant acclimation response was the total chlorophyll growth rates at low photon flux density, which exceeded that of high-light grown *U. pertusa* (Figures 3.5 & 3.6) and how this was linked to an increase in rates of ammonium assimilation to maintain these high chlorophyll growth rates (Figure 3.12).

3.5. APPENDIX

3.5.1. Figures

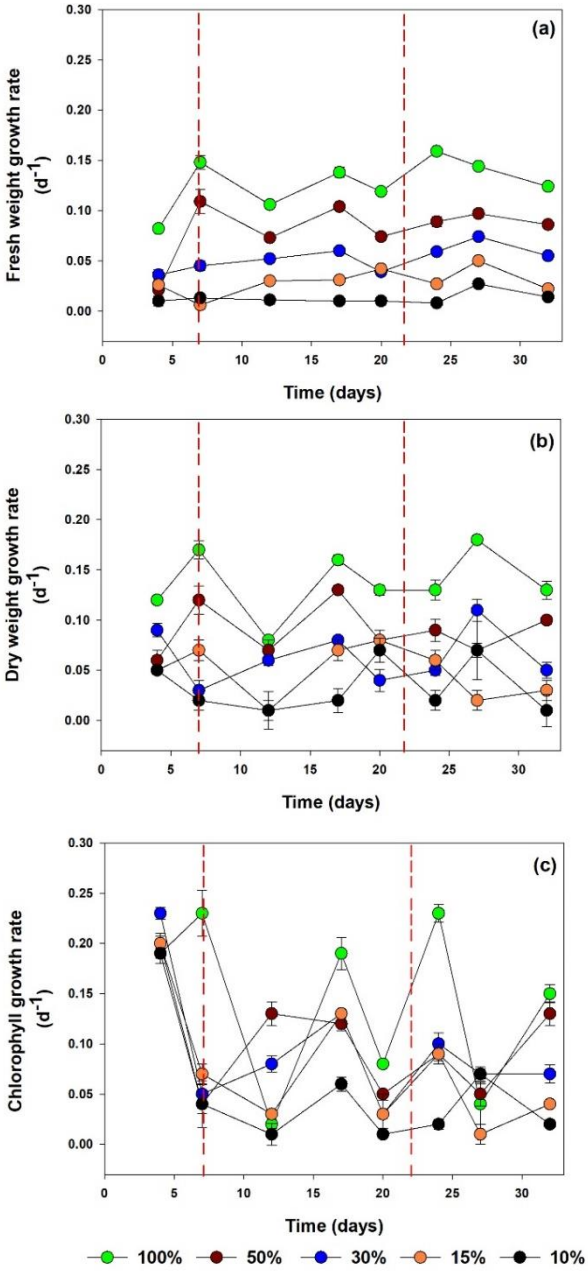

Figure 3. 13. Growth rates measured as increases in fresh weight (a), dry weight (b) and total chlorophyll (c) at a range of ambient light from 10 to 100% in autumn 2017 for 32 days in outdoor cultures. Values are means ± S.E. for three separate determinations. The red dashed lines correspond to the first seven days (acclimation phase) and the last ten days (steady-state growth phase) of the experiment.

Winter 2017

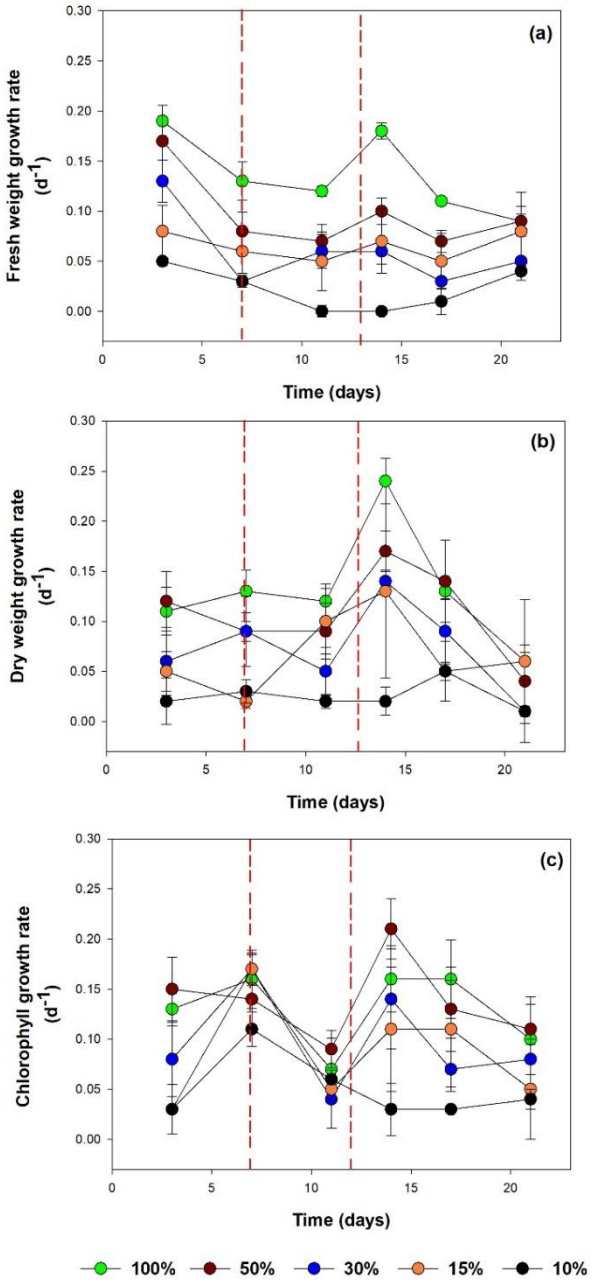

Figure 3. 14. Growth rates measured as increases in fresh weight (a), dry weight (b) and total chlorophyll (c) at a range of ambient light from 10 to 100% in winter 2017 for 21 days in outdoor cultures. Values are means ± S.E. for three separate determinations. The red dashed lines correspond to the first seven days (acclimation phase) and the last ten days (steady-state growth phase) of the experiment.

Spring 2017

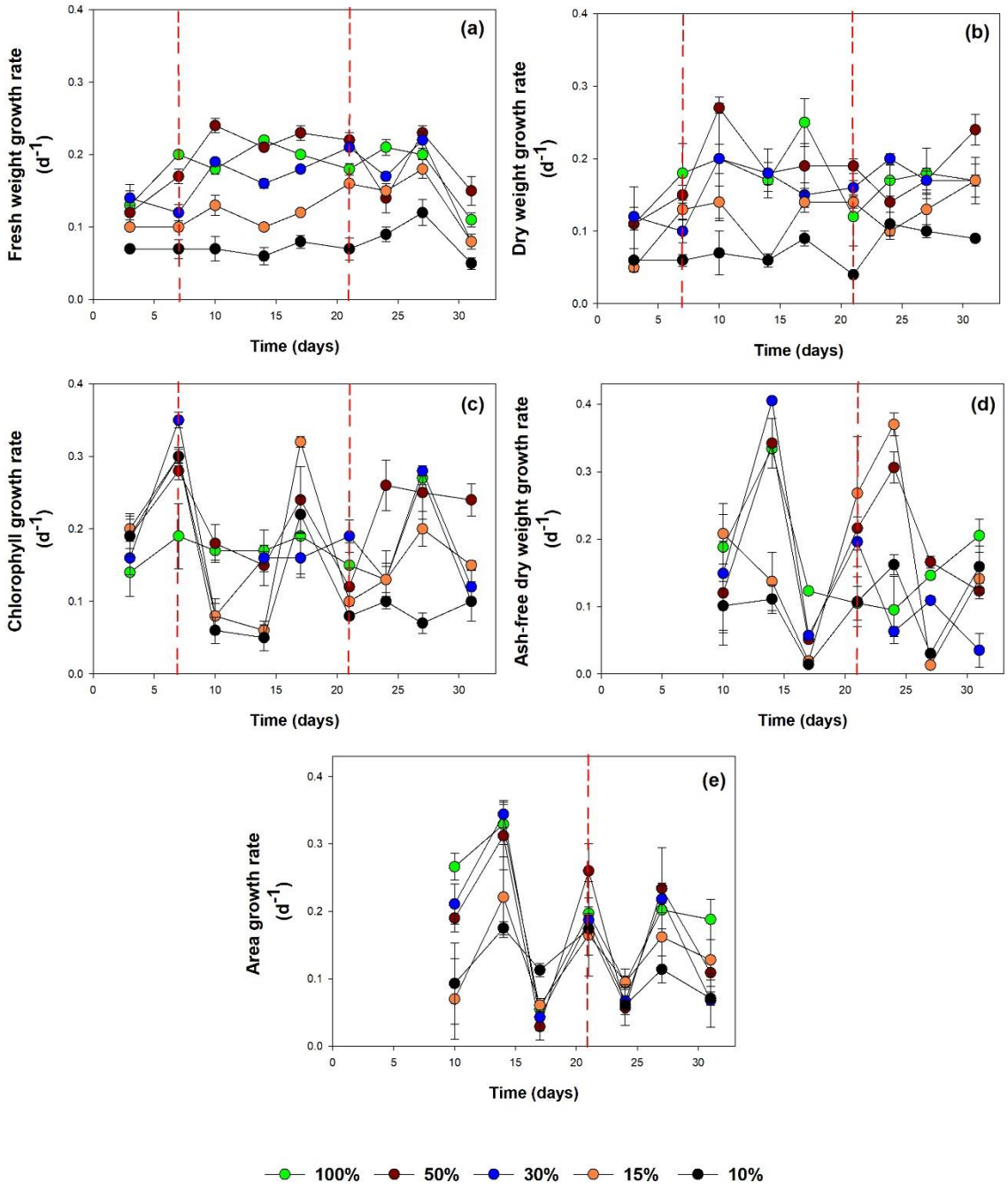

Figure 3. 15. Growth rates measured as increases in fresh weight (a), dry weight (b), total chlorophyll (c), ash-free dry weight (d) and surface area (e) at a range of ambient light from 10 to 100% in spring 2017 for 32 days in outdoor cultures. Values are means ± S.E. for three separate determinations. The red dash lines correspond to the first seven days (acclimation phase) and the last ten days (steady-state growth phase) of the experiment.

Winter 2018

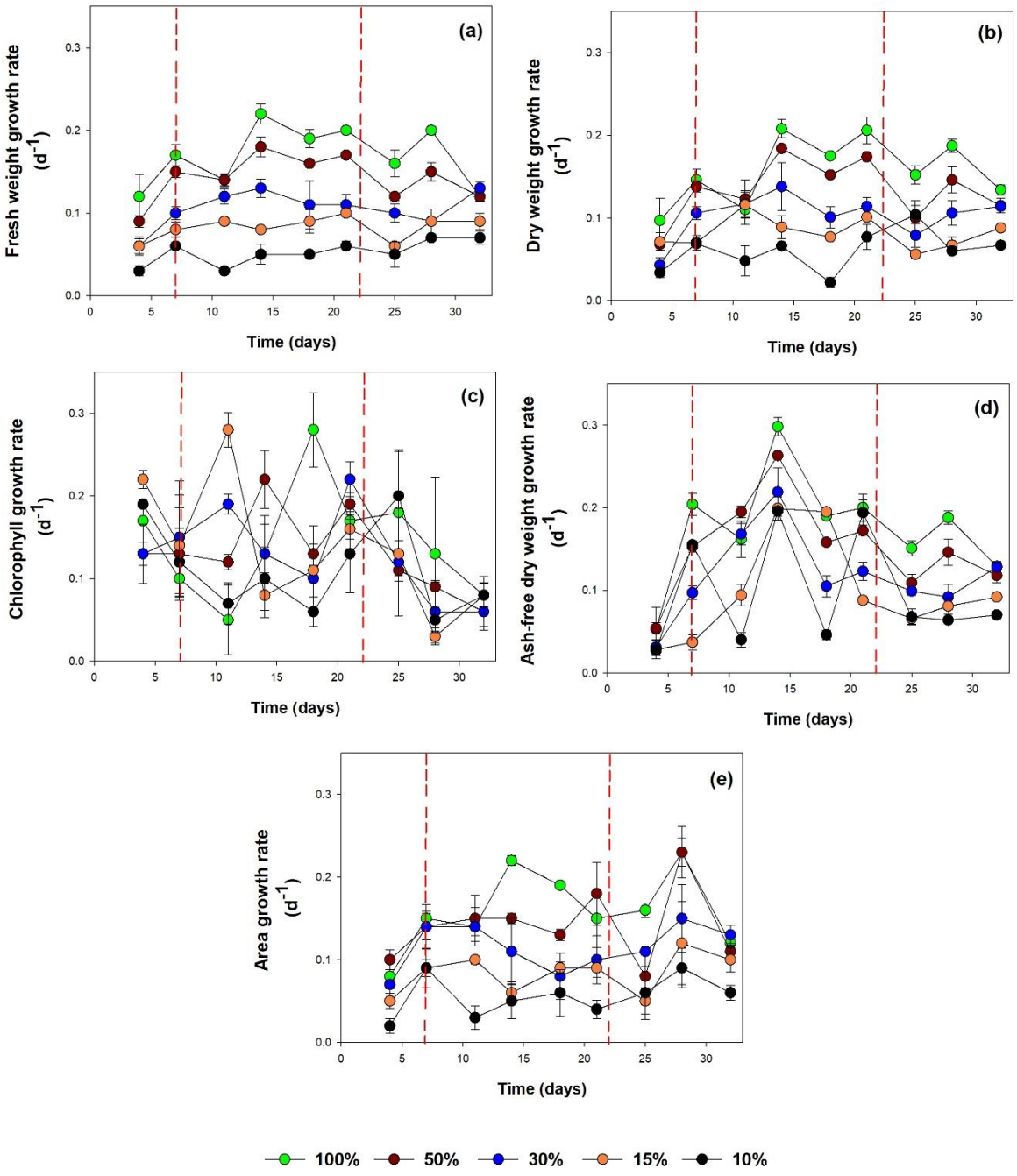

Figure 3. 16. Growth rates measured as increases in fresh weight (a), dry weight (b), total chlorophyll (c), ash-free dry weight (d) and surface area (e) at a range of ambient light from 10 to 100% in winter 2018 for 32 days in outdoor cultures. Values are means ± S.E. for three separate determinations. The red dash lines correspond to the first seven days (acclimation phase) and the last ten days (steady-state growth phase) of the experiment.

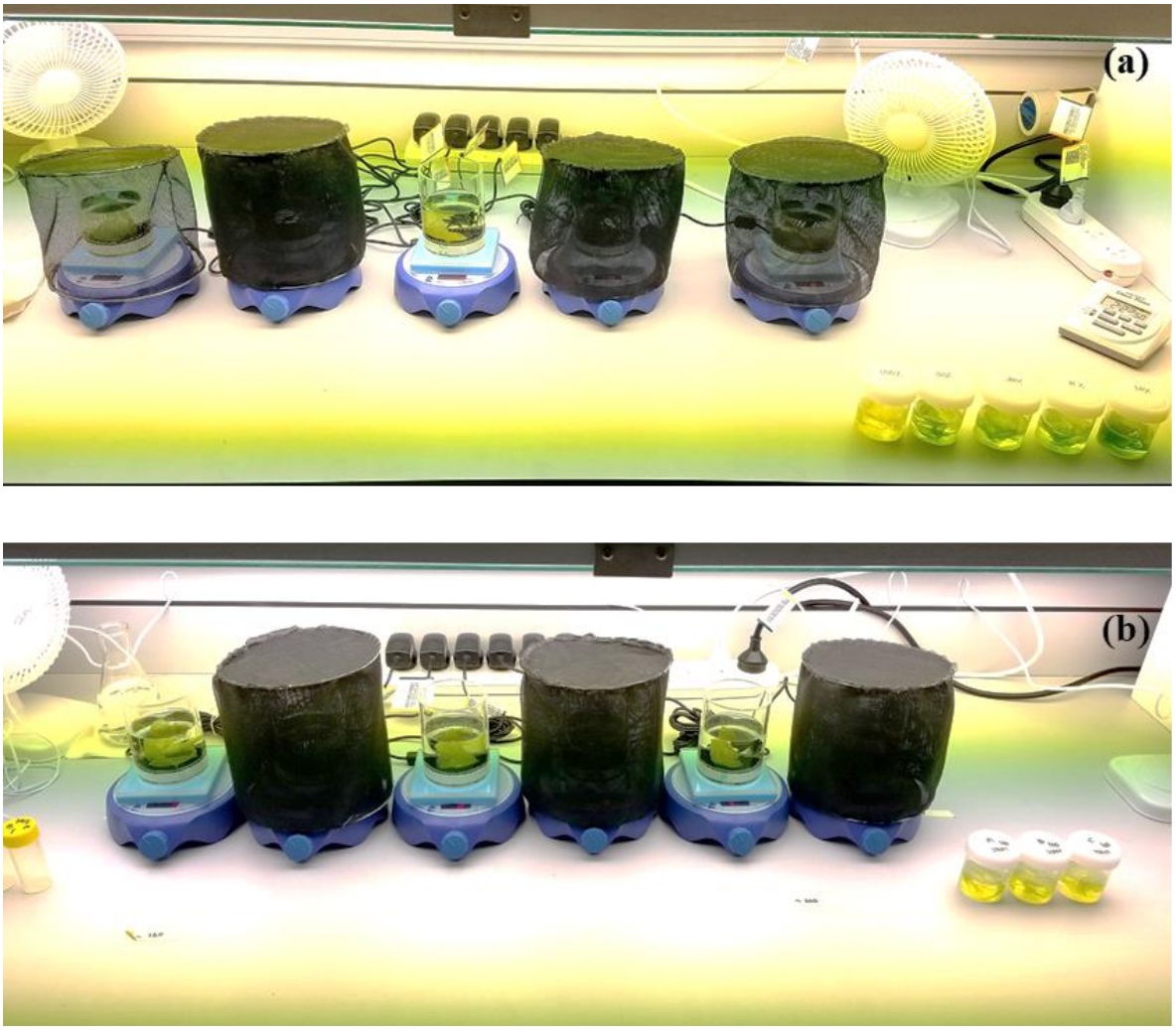

Figure 3. 17. Experimental design for the ammonium uptake and assimilation experiments in *U. pertusa* acclimated to 10%, 15%, 30%, 50% and 100% ambient light for extended periods. Experiments correspond to (a) *in situ* field condition and (b) acclimatory response experiments. Each experiment consisted of three replicates each with different tissue.

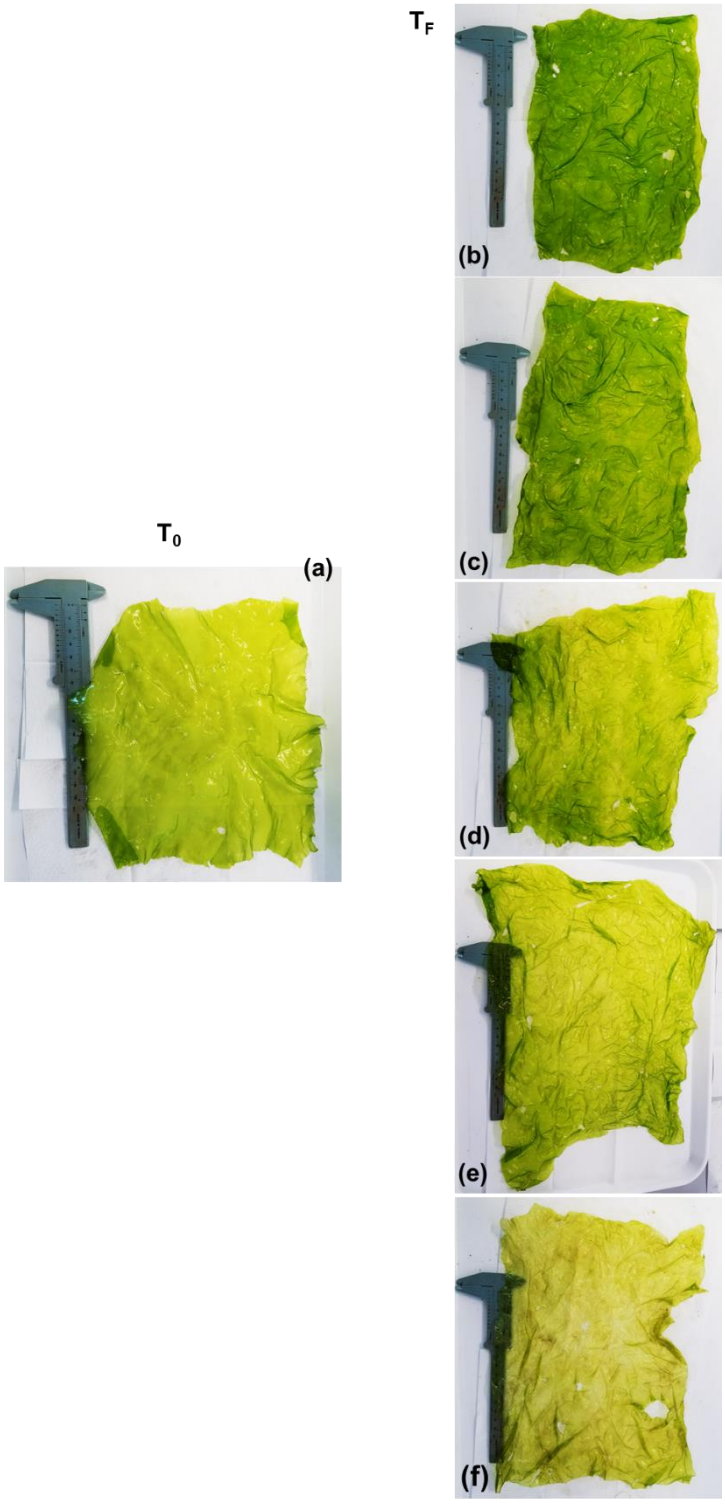

Figure 3. 18. Effects of ambient light on the colour of *U. pertusa* after 30 days growth in spring (October) 2017. Images are (a) initial and after 30 days in; (b) 10%, (c) 15%, (d) 30%, (e) 50% and (f) 100% ambient light.

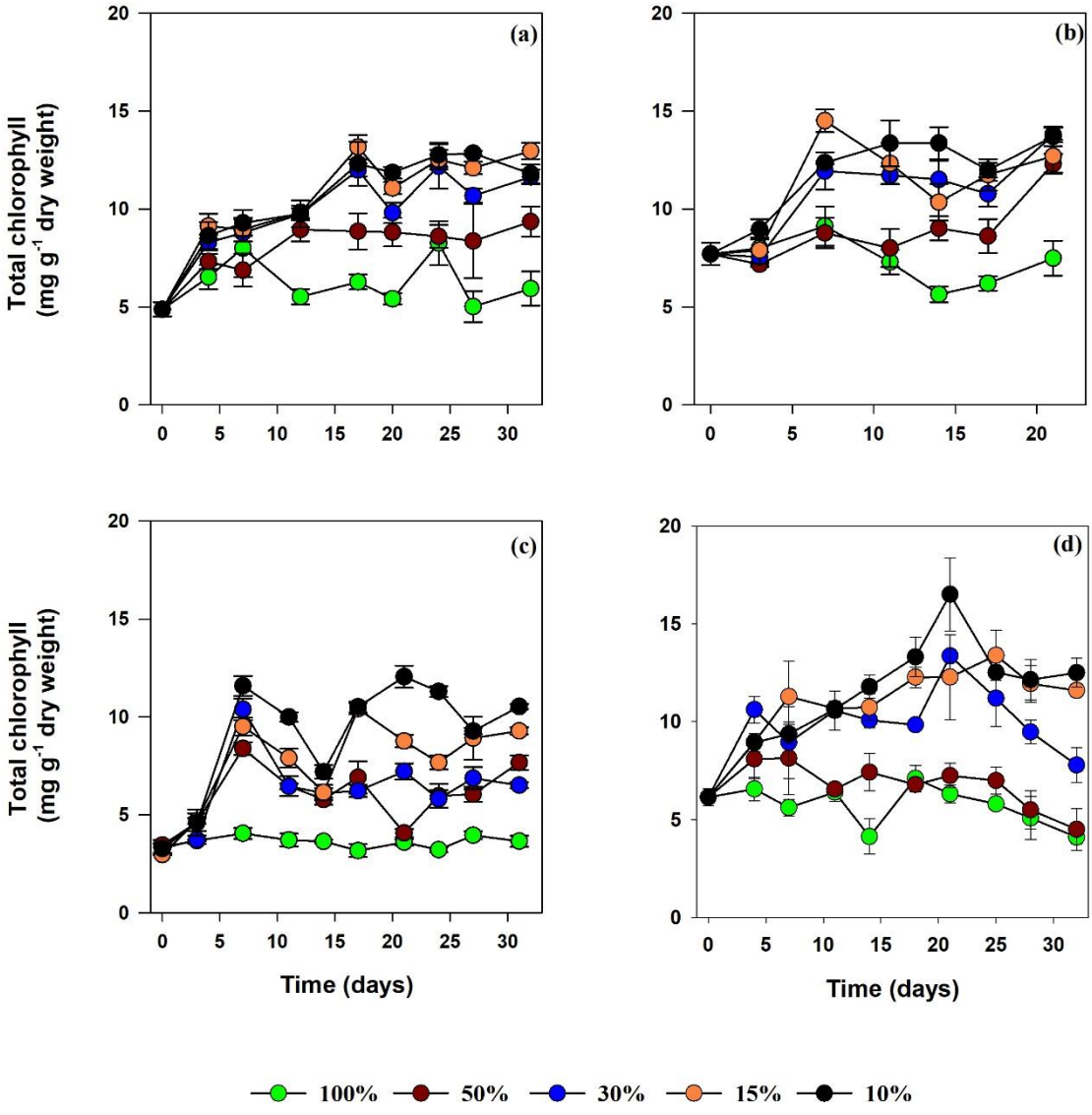

Figure 3. 19. Total chlorophyll content (mg g dry weight) under different % ambient light in outdoor cultures. The figures correspond to: (a) autumn 2017, (b) winter 2017, (c) spring 2017 and (d) winter 2018. Values are means ± S.E. from three replicates.

3.5.2. Tables

Table 3. 3. Summary of one-way ANOVAs (α=0.05) for the effects of photon flux density (treatment) on growth rates (d^{-1}) measured as increases in each of the five constituents (fresh weight, dry weight, total chlorophyll, ash-free dry weight and surface area) in *U. pertusa* during the steady-state growth phase (last 10 days of the experiments). Significant differences between constituents are displayed in bold ($p<0.05$).

Season	Treatment	Steady-state growth phase
Autumn 2017	100%	*df* = 2,11, F = 0.26, p = 0.773
	50%	*df* = 2,11, F = 0.23, p = 0.796
	30%	*df* = 2,11, F = 0.20, p = 0.819
	15%	*df* = 2,11, F = 0.48, p = 0.621
	10%	*df* = 2,11, F = 0.87, p = 0.917
Winter 2017	100%	*df* = 2,11, F = 0.89, p = 0.829
	50%	*df* = 2,11, F = 2.08, p = 0.353
	30%	*df* = 2,11, F = 0.43, p = 0.650
	15%	*df* = 2,11, F = 0.11, p = 0.895
	10%	*df* = 2,11, F = 0.06, p = 0.935
Spring 2017	100%	*df* = 4,11, F = 0.06, p = 0.991
	50%	*df* = 4,11, F = 1.62, p = 0.804
	30%	*df* = 4,11, F = 2.32, p = 0.676
	15%	*df* = 4,11, F= 0.08, p = 0.986
	10%	*df* = 4,11, F = 2.37, p = 0.668
Winter 2018	100%	*df* = 4,11, F = 7.69, p = 0.103
	50%	*df* = 4,11, F = 11.75, **p = 0.019**
	30%	*df* = 4,11, F = 7.36, p = 0.118
	15%	*df* = 4,11, F = 2.68, p = 0.612
	10%	*df* = 4,11, F = 2.70, p = 0.608

Table 3. 4. Summary of ANOVAs for growth rates (d^{-1}) measured as increases in fresh weight, dry weight, total chlorophyll and, where appropriate, ash-free dry weight and surface area grouped as % ambient light treatment during the acclimation phase (first 7 days) for the experiments in 2017 and 2018. Growth rates that displayed a significant difference are in bold ($p<0.05$).

Season	Treatment (ambient light)	Acclimation phase
Autumn 2017	100%	*df* = 2,5, F = 7.11, **p = 0.012**
	50%	*df* = 2,5, F = 0.68, p = 0.528
	30%	*df*= 2,5, F = 2.33, **p = 0.043**
	15%	*df* = 2,5, F = 9.16, **p = 0.005**
	10%	*df* = 2,5, F = 5.79, **p = 0.021**
Winter 2017	100%	*df* = 2,5, F = 1.50, p = 0.255
	50%	*df* = 2,5, F = 0.10, p = 0.904
	30%	*df* = 2,5, F = 0.89, p = 0.431
	15%	*df* = 2,5, F = 6.03, **p = 0.049**
	10%	*df* = 2,5, F = 4.45, **p= 0.030**
Spring 2017	100%	*df* = 2,5, F = 0.12, p = 0.882
	50%	*df* = 2,5, F = 7.87, **p = 0.005**
	30%	*df* = 2,5, F = 7.45, **p = 0.024**
	15%	*df* = 2,5, F = 10.15, **p = 0.006**
	10%	*df* = 2,5, F= 9.08, **p = 0.011**
Winter 2018	100%	*df* = 4,5, F = 2.40, p = 0.663
	50%	*df* = 4,5, F = 2.13, p = 0.711
	30%	*df* = 4,5, F = 6.53, p = 0.163
	15%	*df* = 4,5, F = 12.67, **p = 0.013**
	10%	*df* = 4,5, F = 3.27, **p = 0.035**

Table 3. 5. Summary of ANOVAs for growth rates (d^{-1}) during the acclimation phase (first 7 days) at 10, 15, 30, 50 and 100% ambient light. Growth rates were measured as increases in fresh weight: FW, dry weight: DW, or total chlorophyll: Chl and, where appropriate, ash-free dry weight: AFDW, or surface area: SA for the experiments in 2017 and 2018. Growth rates that displayed a significant difference are in bold ($p<0.05$).

Season	Constituent	Acclimation phase
Autumn 2017	FW	*df* = 4,5, F = 11.29, **p <0.001**
	DW	*df* = 4,5, F = 5.15, **p = 0.005**
	Chl	*df* = 4,5, F = 1.24, p = 0.322
	AFDW	-
	AREA	-
Winter 2017	FW	*df* = 4,5, F = 5.69, **p = 0.002**
	DW	*df* = 4,5, F = 6.64, **p <0.001**
	Chl	*df* = 4,5, F = 0.70, p = 0.594
	AFDW	-
	AREA	-
Spring 2017	FW	*df* = 4,5, F = 5.16, **p = 0.004**
	DW	*df* = 4,5, F = 2.25, p = 0.688
	Chl	*df* = 4,5, F = 0.86, p = 0.500
	AFDW	-
	AREA	-
Winter 2018	FW	*df* = 4,5, F = 16.92, **p <0.001**
	DW	*df* = 4,5, F = 6.52, **p = 0.002**
	Chl	*df* = 4,5, F = 1.98, p = 0.135
	AFDW	*df* = 4,5, F = 11.11, **p = 0.025**
	AREA	*df* = 4,5, F = 7.08, **p = 0.001**

Table 3. 6. Tissue nitrogen content (as % dry weight) during at the beginning (T^0) and end (T^f) for each ambient light treatment in each experiment with *Ulva pertusa*. Values are means ± S.E. for three separate samples.

	Seasons [N %]							
Treatment (%)	Winter 2017		Spring 2017		Winter 2018		Spring 2018	
T^0	1.8 ±	0.08	1.0 ±	0.02	1.0 ±	0.03	1.1 ±	0.26
T^f-100%	1.6 ±	0.16	1.0 ±	0.04	1.0 ±	0.02	-	-
T^f-50%	1.8 ±	0.15	0.9 ±	0.08	1.0 ±	0.12	1.0 ±	0.31
T^f-30%	2.2 ±	0.22	1.1 ±	0.04	1.4 ±	0.08	0.8 ±	0.06
T^f-15%	2.0 ±	0.74	1.3 ±	0.04	1.8 ±	0.10	1.1 ±	0.08
T^f-10%	3.0 ±	0.07	2.2 ±	0.03	2.1 ±	0.02	1.1 ±	0.28

Table 3. 7. Pairwise multiple comparison (Holm-Sidak method) from ANOVA for growth at 10% & 100% ambient light with rates of ammonium uptake and assimilation measured at a photon flux density of 30 or 300 μmole photons m^{-2} s^{-1} in winter 2018. Values that are significantly different are in bold ($p<0.05$).

Comparison (alga/PFD)	**Uptake**		**Assimilation**	
	t	p	t	p
10%/300 vs 10%/30	9.2	**<0.001**	3.2	**0.040**
10%/300 vs 100%/30	8.2	**<0.001**	6.7	**<0.001**
10%/300 vs 100%/300	4.2	**0.008**	4.7	**0.007**
100%/300 vs 10%/30	4.9	**0.005**	1.6	0.152
100%/300 vs 100%/30	4.0	**0.008**	2.0	0.155
100%/30 vs 10%/30	0.9	0.399	3.6	0.029

CHAPTER 4
NUTRIENT DEFICIENCY AND LIGHT ACCLIMATION IN *ULVA PERTUSA*

4.1. INTRODUCTION

The consensus is that nitrogen (N) is the main nutrient that limits growth in temperate coastal waters (Lotze & Schramm 2000; Harrison & Hurd 2001; Mizuta et al., 2003; Elser et al., 2007; Hurd et al., 2014), and phosphorus (P) in tropical waters or in areas rich in carbonate sediments (Littler et al., 1991). Generally the availability of these nutrients at the end of spring and during summer is the main limiting factor in growth and primary production in marine macroalgae (Hanisak 1983; Kim et al., 2004), whereas photon flux densities remain sufficient to maintain optimal growth. Both N and P are potentially limiting nutrients (Patey et al., 2008; Pérez-Mayorga et al., 2011), but N deficiency is more common in temperate coastal waters (Hurd et al., 2014).

The absence of one or both of N and P will decrease growth rate of a macroalga and cause alterations in its cellular composition, the nature of which will depend on which nutrient becomes limiting. One response that most nutrient deficiencies have in common is a decrease in the rate of photosynthesis and the levels of photosynthetic pigments (Lapointe et al., 1984; Duke et al., 1986; Campbell et al., 1999) mainly because of a reorganization of thylakoid membranes inside the chloroplast (Post et al., 1985) so as to minimise energy capture during decreased growth rate (Healey 1973).

The most obvious changes associated with N-deficiency in macroalgae are a decrease in growth rate and in nitrogen-containing compounds, particularly photosynthetic pigments (Henley et al., 1991; Kopczak 1994; Chopin et al., 1995; Rivers & Peckol 1995; Hurd et al., 2014; Gévaert & Rees 2015). The recovery process following addition of N to N-deprived algae is known as “regreening” (Grimme & Porra 1974) and in *Chlamydomonas reinhardtii* is more rapid than the loss of photosynthetic activity during N-deprivation (Preininger et al., 2015).

Individually, addition of nutrients to a nutrient-deficient green alga (Grimme & Porra 1974; Preininger et al., 2015) and the imposition of light-limitation on a nutrient-replete green alga (Henley & Ramus 1989c) each result in an increase over time in the amount of total chlorophyll,

and, consequently, an increase in the chlorophyll growth rate. However, the increase in chlorophyll growth rate following the addition of nutrients to a nutrient-deficient green alga that also occurs under light-limitation has not been investigated. How does this chlorophyll growth rate compare with the maximum growth rate measured as increases in fresh weight under optimum conditions of nutrients and photon flux density?

There is considerable evidence that macroalgae in temperate coastal waters are N-deficient in summer, when concentrations of inorganic sources of N (nitrate, nitrite and ammonium) are depleted (e.g. Pedersen & Borum 1996). In New Zealand, there is evidence for N-deficiency during summer in marine phytoplankton (Gibbs & Vant 1997; Carter et al., 2005) and macroalgae (Brown et al., 1997; Phillips & Hurd 2003; Phillips & Hurd 2004; Roth 2014; Stephens & Hepburn 2016). However, the information in New Zealand is restricted to the South Island, with the exception of growth of the green alga *Microdictyon umbilicatum* in Tryphena Harbour, Great Barrier Island in March 2013 being limited by N (Roth, 2014). However, only two of these experiments (Gibbs & Vant 1997; Roth 2014) investigate the effect of P addition alone and only one (Roth 2014) that of N and P together at the same time as the effect of N addition alone. Given that there is evidence that the photosynthesis of the red alga *Acanthophora spicifera* from Twin Cays, a mangrove ecosystem in Belize, is limited by both N and P (Lapointe et al., 1987), it is important (if not vital) that the effects of both N and P on growth are investigated.

The main questions addressed in this chapter were:

a. Is growth of *U. pertusa* from Tauranga limited by N and/or P in summer?
b. When *U. pertusa* is deprived of nutrients (N and P) and the total chlorophyll decreases markedly, does the total chlorophyll growth rate exceed the maximum growth rate measured as an increase in fresh weight when nutrients are added to this tissue under light-limitation?

4.2. MATERIALS AND METHODS

Ulva pertusa fronds were collected from Tauranga Bay in the Bay of Plenty region (Figure. 1.1). The biological material was transferred to the Leigh Marine Laboratory and kept in outdoor circular tanks (1,600 L) with a flow rate of 11 L min^{-1}. The algae were left to acclimatise in the tanks for at least one week prior to the experiments.

4.2.1. Indoor cultures

All cultures were grown at 18 °C and under six Phillips 40W cool-white fluorescent tubes providing a continuous photon flux density of 300 ± 23 µmole photon m^{-2} s^{-1}. Twelve to 18 individual, cylindrical (9.5 cm width x 15.5 cm height) polyethylene chambers (the specific number for each experiment is described below) containing 1L medium were used, each fitted in a rectangular plastic structure with 18 slots to ensure stability of the chambers and aeration was provided to each chamber via two water pumps (Aqua One 9500) delivering air to the outlets from 18 gang valves. Aeration was adjusted to ensure adequate mixing, but minimising any loss due to evaporation which was about 1-2% of the total volume over the maximum time period.

As a standard procedure before inoculation each thallus was spun in a salad spinner for 15 seconds and gently blotted dry between paper towels before being weighed on an electronic balance (Shimadzu) and put in the appropriate chamber. The biomass used in each experiment ranged from 0.1 to 2 g, depending on the experiment. Three times each week, the tissue in each treatment was weighed, trimmed to its original weight and returned to the chamber that contained fresh medium.

Chlorophyll *a* and *b* were measured as described in Mackinney (1941). Because growth rate in some treatments were slow, the amount of tissue for the analysis was reduced to 0.1 g in 5 mL methanol and incubated for more than 24 hours at 4°C in darkness. After this period the solution was decanted and centrifuged at 4,000 rpm at 4°C for 5 minutes. Spectrophotometer readings were made at 650, 665 and 750 nm wavelengths. Concentrations of chlorophyll *a* and *b* were calculated from these equations:

Chlorophyll *a* (mg/L) = 16.5 (A_{665}-A_{750})-8.3 (A_{650}-A_{750})
Chlorophyll *b* (mg/L) = 33.8 (A_{665}-A_{750})-12.5 (A_{650}-A_{750})

Tissue was left to dry in an oven at 80°C for 48 hours then weighed. Total chlorophyll is the sum of chlorophyll *a* and *b* and is expressed as mg g^{-1} dry weight.

Growth rate (μ) was measured as the increase in fresh weight or total chlorophyll and calculated as:

$$\mu = \frac{log_e\,(b_t - b_0)}{t}$$

Where μ is the growth rate (d^{-1}), b_t is the final biomass as fresh weight or total chlorophyll (g), b_0 is the initial biomass as fresh weight or total chlorophyll (g) and t the duration in days (d).

4.2.2. Seawater or artificial seawater as growth media

Initial experiments were devised to determine whether 1 μm filtered and UV-sterilized seawater or Red Sea salts (35g kg^{-1}) was the better medium for growth of *Ulva pertusa*. Growth of *Codium fragile* may be improved by the addition of trace metals, vitamins etc (Hanisak 1979). The effect of this addition on growth was also tested. The alga was grown with one of the following two combinations:

a) 500 μM $NaNO_3$ plus 50 μM Na_2HPO_4 (N+P),

b) 500 μM $NaNO_3$ plus 50 μM Na_2HPO_4 plus Hanisak's enriched seawater medium (Hanisak, 1979; his Table 1) (N+P+H).

Biomass per chamber was kept at 0.2g. The experiment lasted 9 days and each treatment had three replicates in random order. These experiments were performed during August 2018.

4.2.3 Is *Ulva pertusa* N and/or P limited in summer?

To determine if *Ulva pertusa* was N or P deficient an experiment was done with the following treatments as additions to the artificial seawater (Red Sea salts) medium:

a. No addition (RS)
b. P (50 µM Na_2HPO_4) only (RS+P)
c. N (500 µM $NaNO_3$) only (RS+N)
d. N+P (500 µM $NaNO_3$ and 50 µM Na_2HPO_4) (RS+N+P)

The tissue was collected from Tauranga Harbour on 16 February 2019 and kept in outdoor circular tanks (1,600 L) with a flow rate of 11 L min^{-1} for 4 days before the experiment. The experiment lasted for 12 days.

4.2.4. Time course of nutrient deficiency in *Ulva pertusa*

As not all the experiments described below were done in summer, a time course experiment was done to determine the amount of time needed to make nutrient-replete *Ulva pertusa* fully nutrient-deficient. The control consisted of Red Sea salts plus 500 µM $NaNO_3$ and 50 µM Na_2HPO_4 and the treatment was Red Sea salts only. The control consisted of 3 replicates and the treatment had 15 replicates that were grouped into sets of three in random order. The reason for this design is that there was sufficient growth between each time interval in the control for total chlorophyll determinations. In contrast, due to slow growth in the treatments, three of the 15 replicates were sacrificed (after the biomass had been measured) at each time interval to ensure sufficient biomass for total chlorophyll analyses. Biomass in every remaining chamber was measured at each time interval and biomass per chamber was kept close to 0.35 g. The duration of the experiment was 14 days. The experiment was done in October (spring) 2018.

4.2.5. Effect of addition of nutrients to N- or nutrient-deficient *Ulva pertusa* at high and low photon flux densities

Two experiments were done. The first in mid-March 2019 involved no pre-incubation of *Ulva pertusa* as it was assumed that, based on total chlorophyll content, the tissue was N-deficient. This experiment used a biomass between 1.0 and 1.5 g. and concentration of $NaNO_3$ and Na_2HPO_4 were 500 µM and 50 µM, respectively.

The second experiment was done in June 2019 and involved a pre-incubation (based on the results of the time-course experiment described above) of 9 days nutrient deficiency (i.e. no additions to Red Sea salt medium). The intention with this pre-incubation was to (a) maximise the decrease in total chlorophyll and (b) to maximise the response when nutrients were added. The tissue consisted of discs that were cut from intact thalli, using a 6 cm diameter PVC cylinder disc cutter. Discs weighed close to 0.2 g and concentration of $NaNO_3$ and Na_2HPO_4 were 100 µM and 10 µM, respectively.

The treatments for both experiments consisted of the following:

(a) 100% light (100% L - nutrients),
(b) 100% light plus NO_3 + PO_4 (100% L + nutrients),
(c) 10% light (10% L - nutrients),
(d) 10% light plus NO_3 + PO_4 (10% L + nutrients).

For both experiments the 100% light treatment was 300 µmole photon m^{-2} s^{-1}. The 10% light treatments was obtained by covering the chambers with four layers of shade cloth. The visual differences between the fronds can be seen in the Appendix section under Figure 4. 6.

4.2.6. N content analysis

Tissue was dried in an oven at 80°C for more than 48 hours and then ground in a mortar. The dry fine powder was placed in Eppendorf tubes and stored in a plastic bag (compressed to minimise oxygen content) and stored at -80 °C. The samples (5 mg) were analysed using a CHNOS

Elemental Analyzer (vario EL cube) using the protocol for vegetative tissue with 90 seconds of O_2 with acetanilide as the standard. The nitrogen content (%N) of *Ulva pertusa* was determined for tissue from the start and conclusion of each culture.

4.2.7. Data analysis

The effects of treatments on growth rate and total chlorophyll content were determined with a one-way analysis of variance (ANOVA) and a Student's t-test when comparing two groups. To reduce skewness and asymmetry, all growth rates were log-transformed. Data sets were checked with a normality test (Shapiro-Wilk) and equal variance test (Brown-Forsythe) prior to performing the ANOVA in SigmaPlot (version 14.0).

4.3. RESULTS

4.3.1. Seawater and artificial seawater as media for growth

Growth rates, frond survival and absence of fragmentation were better in artificial seawater medium than in seawater. Though the differences were clear there were no significant differences between treatments (Table 4.1). The addition of Hanisak's (1979) enriched seawater medium had a minor and inconsistent effect and was not used in the other experiments described below.

Table 4. 1. Growth rate (d^{-1}) and survival (%) of *Ulva pertusa* during growth in seawater (SW) and artificial seawater medium (RS) with added nitrate and phosphate in the presence or absence of Hanisak's enriched seawater medium (H). Both experiments were done in August 2018. There were no significant differences between treatments ($p > 0.05$). Values are means ± S.E. for three replicates.

Experiment	Treatment	μ (d^{-1})	Survival (%)	ANOVA
1	SW+N	0.09 ± 0.05	33.3	*df*= 11,3, F= 0.85, p= 0.47
	SW+N+H	0.05 ± 0.06	33.3	
	RS+N	0.13 ± 0.02	100	
	RS+N+H	0.19 ± 0.04	100	
2	SW+N	0.15 ± 0.04	33.3	*df*= 11,3, F= 0.12, p= 0.94
	SW+N+H	-0.03 ± 0.08	33.3	
	RS+N	0.24 ± 0.03	100	
	RS+N+H	0.24 ± 0.04	100	

4.3.2. Does N and/or P limit growth in *Ulva pertusa*?

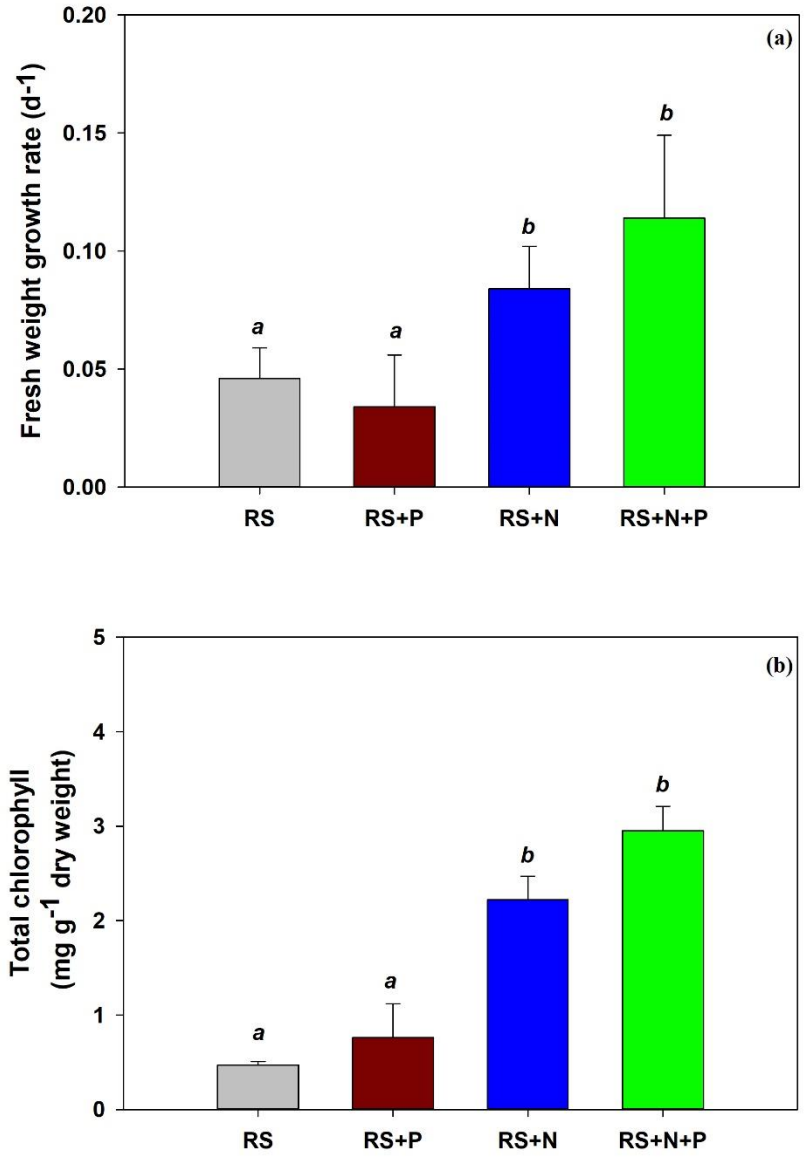

Figure 4. 1. Determination of whether *Ulva pertusa* in February/March 2019 was N, P or N+P deficient, measured as (a) mean growth rate (d^{-1}) measured as changes in fresh weight and (b) mean total chlorophyll (mg g^{-1} dry weight) over the 12 days of the experiment. Different letters indicate statistical differences ($p < 0.05$). Values are means ± S.E. for three replicates.

In the +P treatment, growth rate was low and total chlorophyll levels decreased to very low values. There was no significant difference between the +P treatment and the control (no additions). Only in the N and N+P treatments growth rates and total chlorophyll levels were significantly greater than in control cultures (Figure 4.1). Both the growth rate and total chlorophyll were greater in the N+P treatment than the N treatment, but not significantly different. The summary of the ANOVA analysis for Figure 4.1 is in the Appendix, Table 4.2.

4.3.3. Effect of photon flux density and nitrogen on growth rate and total chlorophyll content in *Ulva pertusa*

The experiment described in this section was done soon after that shown in Figure. 4.1 and the total chlorophyll levels were identical. Consequently, it was reasonable to assume that the tissue at the start of the experiment was N-deficient. However, there was no effect of N addition on growth rate as measured by increases in fresh weight in the 100% light treatment. The growth rate was lower in 10% light, but there were no significant differences ($df = 3,19$ $F = 2.28$, $p = 0.09$) between treatments (Figure. 4.2. a).

In contrast, there were marked differences in growth rates measured as increases (or decreases) in total chlorophyll ($df = 3,19$ $F = 3.14$, $p = 0.033$) (Figure 4.2.b) and in total chlorophyll content ($df = 3,23$ $F = 46.05$, $p < 0.001$) (Figure 4.2.c). Despite a positive fresh weight growth rate, the chlorophyll growth rate in 100% light -N was negative, mainly because of a marked decrease in total chlorophyll content (Figure 4.2.c). The decrease in total chlorophyll content was less in the 10% light +N, and the chlorophyll growth rate was positive (Figure 4.2.b). Chlorophyll growth rates were 0.33 d^{-1} in 100% light +N and 0.44 d^{-1} in 10% light +N; these values exceeded that for fresh weight growth rate by 386% in 100% light +N and by 642% 10% light +N (Figure 4.2.b) because of marked increases in total chlorophyll content at least over the first two days (Figure 4.2.c). Total chlorophyll content increased little after two days in 10% light +N and decreased in 100% light +N (Figure 4.2.c). The summary of the ANOVA analysis for Figure 4.2 is in the Appendix, Table 4.3.

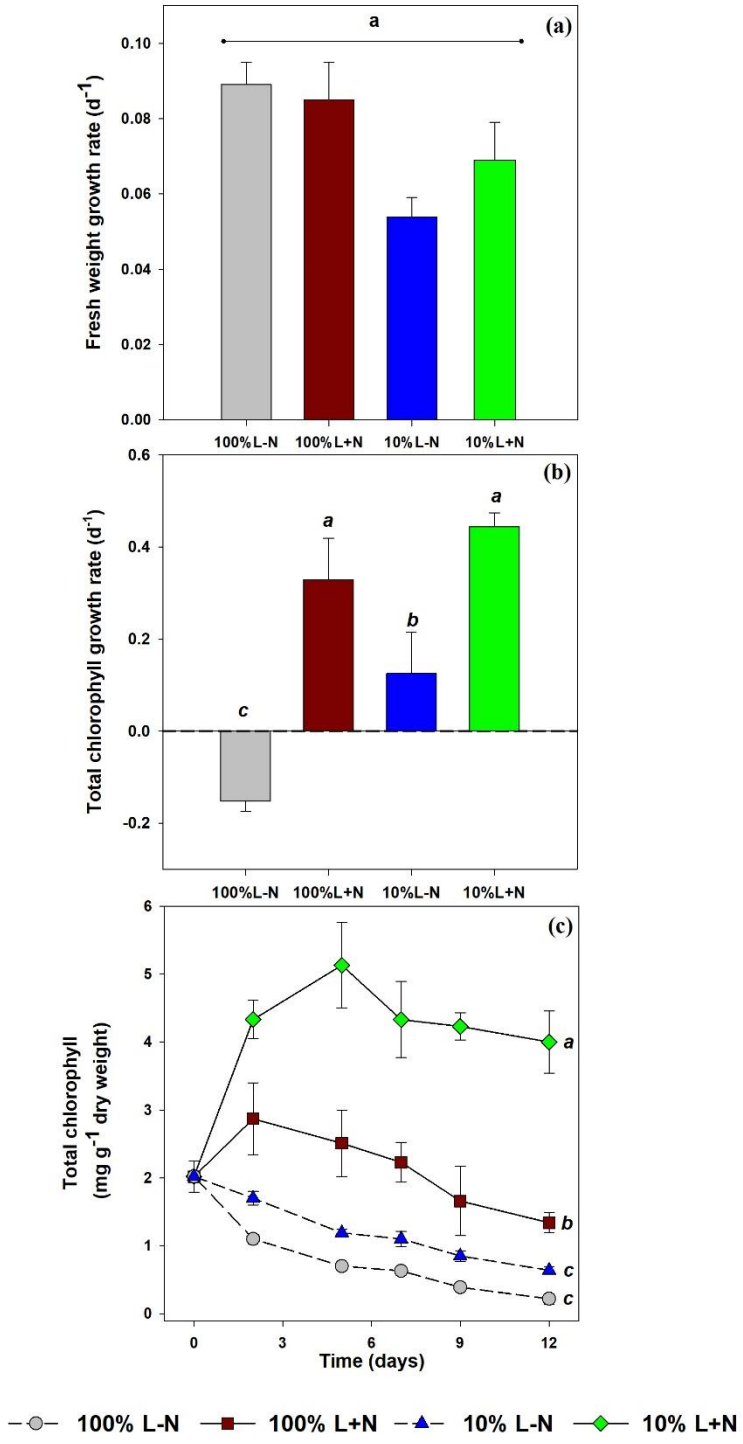

Figure 4. 2. Effect of nitrogen additions in 100% or 10% light on (a) growth rate (d^{-1}) measured as increases in fresh weight over the first two days, (b) growth rate (d^{-1}) measured as increases in total chlorophyll over the first two days, and (c) total chlorophyll content (mg g^{-1} dry weight) of nitrogen-deficient *Ulva pertusa* over 12 days. The experiment was done in mid-March 2019. Treatments with different letters are statistically different ($p<0.05$). Values are means ± S.E. for three replicates.

At the start of the experiment the tissue had a low nitrogen content that supports, but does not confirm, the assumption that the tissue was N-deficient and at the end of the experiment had decreased in both 100% and 10% light treatments to which no nitrogen was added (Fig. 4.3). The decrease in 100% light was greater than in 10% light. In the 10% light treatment %N increased 4-fold from the initial value and in the 100% light +N treatment the increase was 8-fold after the addition of N (Figure 4.3).

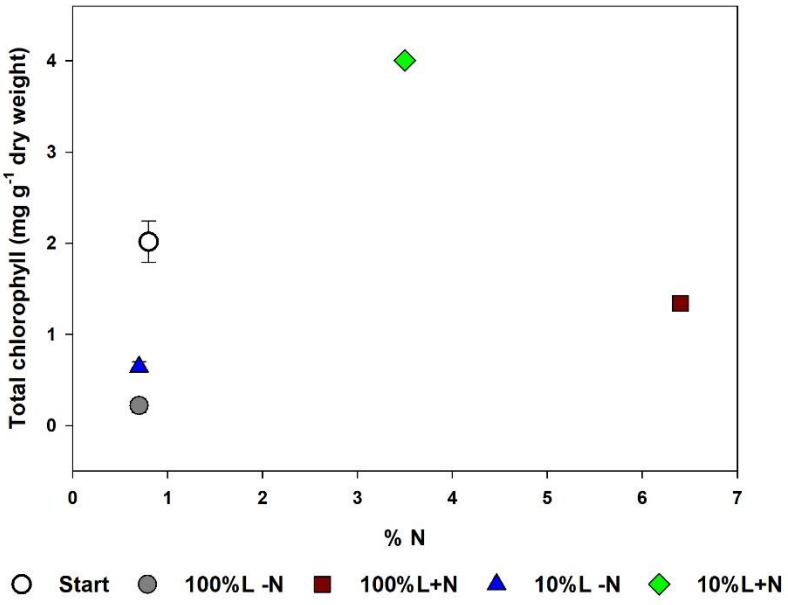

Figure 4. 3. Relationship between tissue nitrogen (%N) and total chlorophyll (mg g^{-1} DW) in *Ulva pertusa* at end of experiment described in Fig. 4.2. Values are means ± S.E. (n=3), except for 10% +N and 100% +N, where n=2.

4.3.4. Nutrient deficiency time course

For indoor experiments that were not done in summer, it was necessary to determine the time taken for nutrient-replete tissue to become nutrient deficient. Both growth rate and total chlorophyll content remained constant throughout the experiment in the control, suggesting that both the concentration of N and P used and the frequency with which the medium was changed were sufficient to ensure "steady-state" conditions. In contrast, in the nutrient-deficient treatment both growth rate and total chlorophyll content decreased markedly, reaching minima after 9 days (Figure

4.4). The percentage decrease was 86% for growth (t-test, *df* = 8, t = 11.80, p <0.001) and 85% for total chlorophyll (t-test, *df* = 8, t = 9.43, p <0.001).

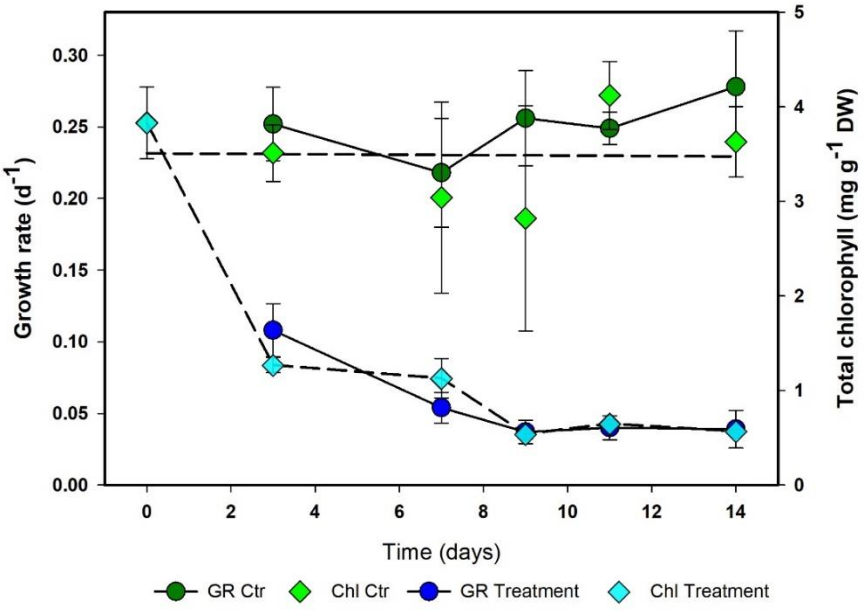

Figure 4. 4. Growth rate, GR (d^{-1}) measured as changes in tissue fresh weight and total chlorophyll content, Chl (mg g^{-1} dry weight) of *Ulva pertusa* during deprivation of nutrients in artificial seawater medium. The experiment was done in October 2018. Values are means ± S.E. for three replicates.

4.3.5. Effect of photon flux density and nutrients on growth rate and total chlorophyll content in *Ulva pertusa* discs

Following a pre-treatment of 9 days in the absence of nutrients, growth rate measured as increases in fresh weight had decreased from 0.15 to 0.10 d^{-1} and total chlorophyll content had decreased from 8.0 mg g^{-1} dry weight 0.7 mg g^{-1} dry weight (Figure 4.5). Irrespective of subsequent conditions (with or without added nutrients; 10% or 100% light), there was no significant difference in growth rate measured as increases in fresh weight (Figure 4.5.a). The only significant increase in total chlorophyll content occurred in the 10% light with nutrients treatment (Figure 4.5.c). Consequently, there was a small positive growth rate measured as increases in total chlorophyll in the 100% light plus nutrients, but a very large value (0.67 d^{-1}) in the 10% light plus nutrients treatment (Figure. 4.5.b). The summary of the ANOVA analysis for Figure 4.5 is in the Appendix, Table 4.4.

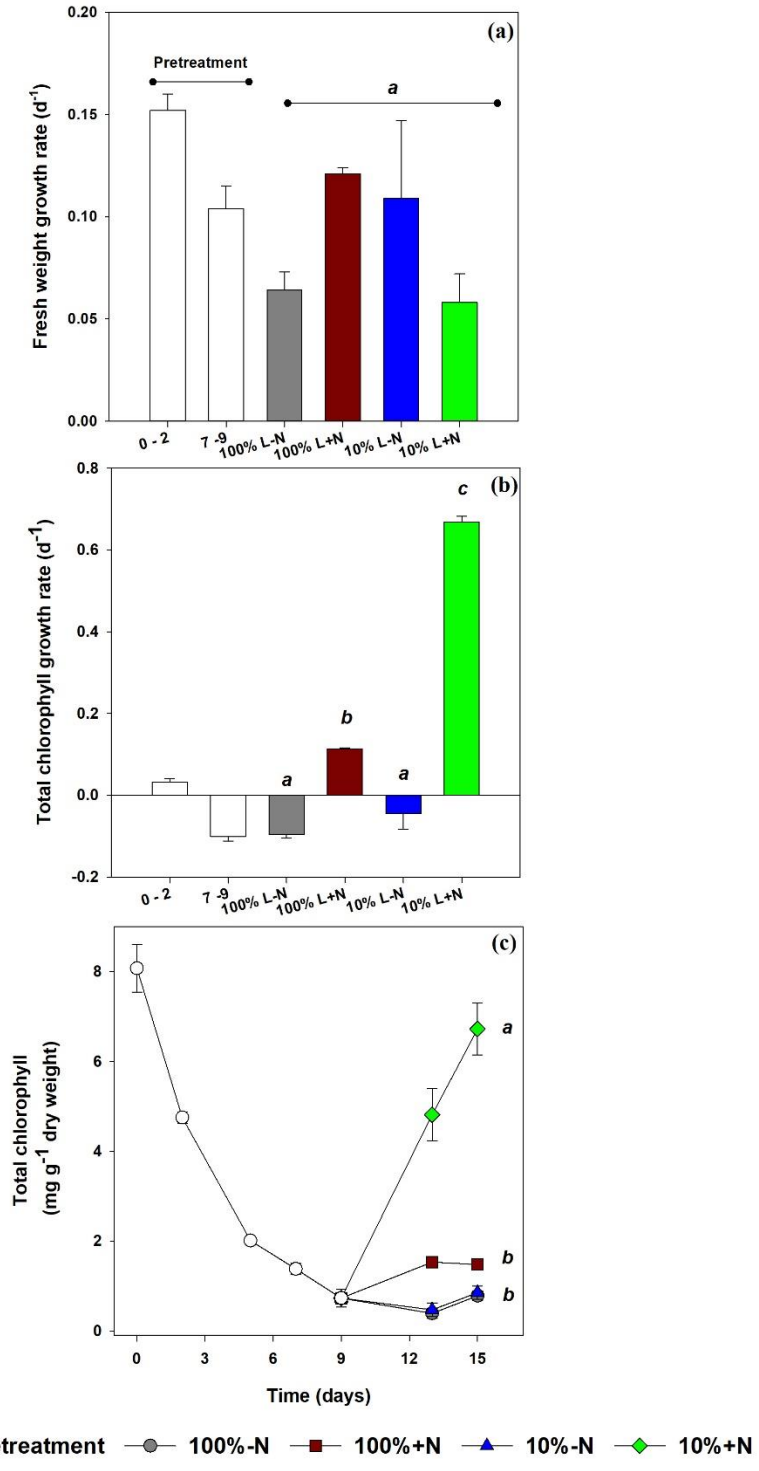

Figure 4. 5. Effect of nutrient (N+P) additions in 100% or 10% photon flux density on (a) growth rate (d^{-1}) measured as increases in fresh weight over the first 3 days, (b) growth rate (d^{-1}) measured as increases in total chlorophyll over the first 3 days, and (c) total chlorophyll content (mg g^{-1} dry weight) of nutrient (N+P)-deficient *Ulva pertusa*. The experiment was done in June 2019. Treatments with different letters are statistically different ($p<0.05$). Values are means ± S.E. for three replicates.

4.4. DISCUSSION

4.4.1. Artificial medium versus seawater

There were advantages and disadvantages to the use of seawater for these experiments. The obvious advantage was that it is the medium that *Ulva* normally grows in and presumably contains all the elements that it requires. The disadvantage was that for experiments where the aim was to deprive the alga of nitrogen and/or phosphorus, seawater contains dissolved inorganic nitrogen and phosphorus even in summer. These nutrients can be removed by pre-incubating seawater with an alga (such as *Ulva*) for a few days, but it would be extremely difficult to know whether these were the only difference in the composition of seawater after the pre-incubation. In contrast, the manufacturer of artificial seawater (Red Sea salts) states that it does not contain any inorganic nitrogen or phosphorus, and any nitrate, nitrite, ammonium and reactive phosphorus present was below the detection limit of the respective methods (data not shown).

4.4.2. N or P limitation of growth of *Ulva pertusa* in summer

It is tempting, based on the available evidence, to simply assume that all macroalgae in temperate coastal waters, including New Zealand, are N deficient in summer (Brown et al., 1997; Phillips & Hurd 2003; Phillips & Hurd 2004; Stephens & Hepburn 2016). In the most extensively studied system, the Pelorus Sound, phytoplankton are N-deficient in summer (Gibbs & Vant 1997; Carter et al., 2005) and, not P deficient (Gibbs & Vant 1997). However, growth rates of the red alga *Adamsiella chauvinii* in Otago Harbour were greatest in summer and there was no evidence for N-limitation (Kregting et al., 2008).

The problem with making the assumption that all macroalgae in temperate coastal waters (including New Zealand) are N deficient in summer is that there is evidence for both N and P limiting growth in macroalgae (Lapointe et al., 1987; Littler et al., 1991; Fong et al., 2003) and it is therefore important to test the effect of P too. Of even greater concern is that within the same small area (Curlew Bay, Belize), one species (*Halimeda opuntia*) is N deficient and another (*Dictyota divaricata*) is P deficient (Lapointe et al. 1987). These examples are for tropical algae, but this does not negate the requirement for those working on temperate algae to be equally thorough and careful.

In general, temperate coastal macroalgae are N-deficient in summer (Hurd et al., 2014), but *U. laetevirens* from the temperate Venice Lagoon is P-deficient (Teichberg et al., 2010) (see paragraph above). In June, growth of *U. intestinalis* from Upper Newport Bay estuary (Kamer et al., 2004) and the Western Baltic Sea (Lotze & Schramm 2000) increased with N enrichment, but not with P. Also in June, the total chlorophyll content of *Chaetomorpha linum* from a Spanish coastal lagoon increased after 4 days with nitrate, but not with phosphate (Menéndez et al., 2002). Growth rate of *U. lactuca* from Waquoit Bay in July increased with the addition of N, but not P (Teichberg et al., 2008). Only with *U. intestinalis* from Upper Newport Bay estuary (Kamer et al., 2004) was the increase in growth rate greater with N+P than with N-enrichment alone. The data presented here for *U. pertusa* provides evidence for N-limitation of growth in summer, and, possibly, for secondary P-limitation (Figure 4.1). With increasing inputs of nitrogen, algal populations are likely to be driven towards P limitation (Turner et al., 2003).

4.4.3. Responses of N-limited *Ulva pertusa* to the addition of N at high and low photon flux densities

"Regreening" following addition of N to N-deficient *Chlorella fusca* occurs before cell division in synchronous cultures (Grimme & Porra 1974), but takes up to 5 days in asynchronous cultures of *C. vulgaris* (Chu et al., 2015) or 4 days in *Chlamydomonas reinhardtii* (Preininger et al., 2015). The effects of different photon flux densities during regreening were not investigated. The only directly comparable experiment to those described here is the effect of nutrient deprivation on light acclimation in the dinoflagellate *Gonyaulax polyedra* (Prézelin & Matlick 1983). The onset of pigment synthesis on transfer to low photon flux density *Gonyaulax polyedra* is delayed in nutrient-limited cultures, and the final amount of pigment is much less than in nutrient-replete cultures (Prézelin & Matlick 1983). Addition of nutrients to nutrient-limited cultures reverses these effects. There are similar experiments with *Ulva lactuca* (Duke et al., 1986), but these involved investigating the effects of different nitrogen loadings on growth and biochemical characteristics of the macroalga when grown at different photon flux densities, rather than the effects of regreening of N-deficient tissue at different photon flux densities.

The regreening of N-deficient *U. pertusa* was far more pronounced at low photon flux densities, but there was no significant difference in the growth rates measured as increases in total chlorophyll in 10 or 100% light because growth rate measured as increases in fresh weight was

greater in 100% light (Figure 4.2). In this experiment the relationship between total chlorophyll content and %N broke down, with higher %N, but lower total chlorophyll content at 100% light +N than in 10% light +N. To explain this difference, the high %N in the 100% light +N treatment must be due to some N-containing compounds other than light-harvesting pigment complexes (Figure 4.3).

Nitrate is one possible form of N storage with up to 7% of total tissue N in *Ulva fenestrata* (Naldi & Wheeler 1999), 15% in *Chaetomorpha linum* (McGlathery et al., 1996) and *U. rigida* (Naldi & Viaroli 2002). Nitrate expressed as %N in the tissue reaches a maximum of about 0.7% N in *C. linum* and *U. rigida*; these values are due to an accumulation of about 500 μmol nitrate g^{-1} dry weight (McGlathery et al., 1996; Naldi & Viaroli 2002). The nitrate content of *U. lactuca* can reach 583 μmol nitrate g^{-1} dry weight (Duke et al., 1986), or 0.8% N. Free amino acids in *U. pertusa* can be as high as 350 μmole N g^{-1} dry weight (mainly as asparagine) (Barr 2007), or 0.5% N and up to 307 μmole N g^{-1} dry weight in *U. fenestrata* (Naldi & Wheeler 1999). Therefore, it is likely that nitrate and free amino acids make a relatively minor contribution to the total N content. A more likely possibility is protein storage, possibly as increased size of the proteinaceous pyrenoid that is dominated by Rubisco (Badger et al., 1998) or as protein bodies (Pueschel & Korb 2001), though there is no evidence for the latter in *Ulva*. *U. pertusa* contains a single chloroplast usually containing two pyrenoids (López et al., 2007) and pyrenoid size in the microalga *Isochrysis galbana* decreases markedly during N deprivation (Roopnarain et al., 2014). During N-limited growth of *U. curvata* and *U. lactuca*, Rubisco activity is greater at low than high photon flux densities, but Rubisco activity increases and total chlorophyll content decreases with increasing photon flux densities during N-replete growth (Duke et al., 1986). Further work would be required to elucidate whether one or more of these possibilities applies to *U. pertusa*.

4.4.4. Responses of nutrient-limited *Ulva pertusa* to the addition of nutrients at high and low photon flux densities

A preliminary experiment showed that a 9-day period of nutrient deficiency was required to decrease growth rate and total chlorophyll content to a minimum (Figure 4.4). Following this pre-treatment, total chlorophyll levels were lower than with N-deficiency. Following the addition of nutrients (N+P), the increase in total chlorophyll was far more marked in the 10% light treatment and the total chlorophyll growth rate was a mean value of 0.67 d^{-1} and, for an individual within the

replicates, 0.70 d^{-1} (Figure 4.5). The maximum measured growth rate for *U. pertusa* is 0.34 d^{-1} (Rees, unpublished data) and for *Ulva* is a mean value of 0.51 d^{-1} and, for an individual within the replicates, 0.55 d^{-1} (Pedersen & Borum 1997). The proteins associated with the light-harvesting complexes are synthesized on cytosolic ribosomes (Nechushtai et al., 1995; Leister 2003). Presumably, before tissue is transferred to low photon flux density, cytosolic ribosomes are synthesizing proteins required for a high, balanced growth rate. On transfer to low photon flux density, synthesis of many proteins are now not required and ribosomes can be diverted to the synthesis of proteins associated with the light-harvesting complexes.

Irrespective of the mechanisms involved, the total chlorophyll growth rate can exceed the highest growth rate ever measured in *Ulva* (Pedersen & Borum 1997; measured as increases in dry weight). There are two possible reasons for this. The first is that there is a "rate-limiting" process in growth rate (obviously not the rate at which total chlorophyll can be synthesized), which determines the overall maximum growth rate. All that is known about metabolic control analysis would argue against this suggestion (Oliver 2006). The other possibility is that the total chlorophyll growth rate is a measure of what is possible with growth in *U. pertusa*. In other words, if the ideal growth conditions were known, it would be possible to grow a balanced *U. pertusa* at 0.67 d^{-1}. It may be that it is easier to engineer the conditions (nutrient deficiency coupled with low photon flux density) that lead to a high total chlorophyll growth rate than the "perfect" conditions that allow all the constituents of *U. pertusa* to grow at 0.67 d^{-1}.

4.5. APPENDIX

4.5.1. Figures

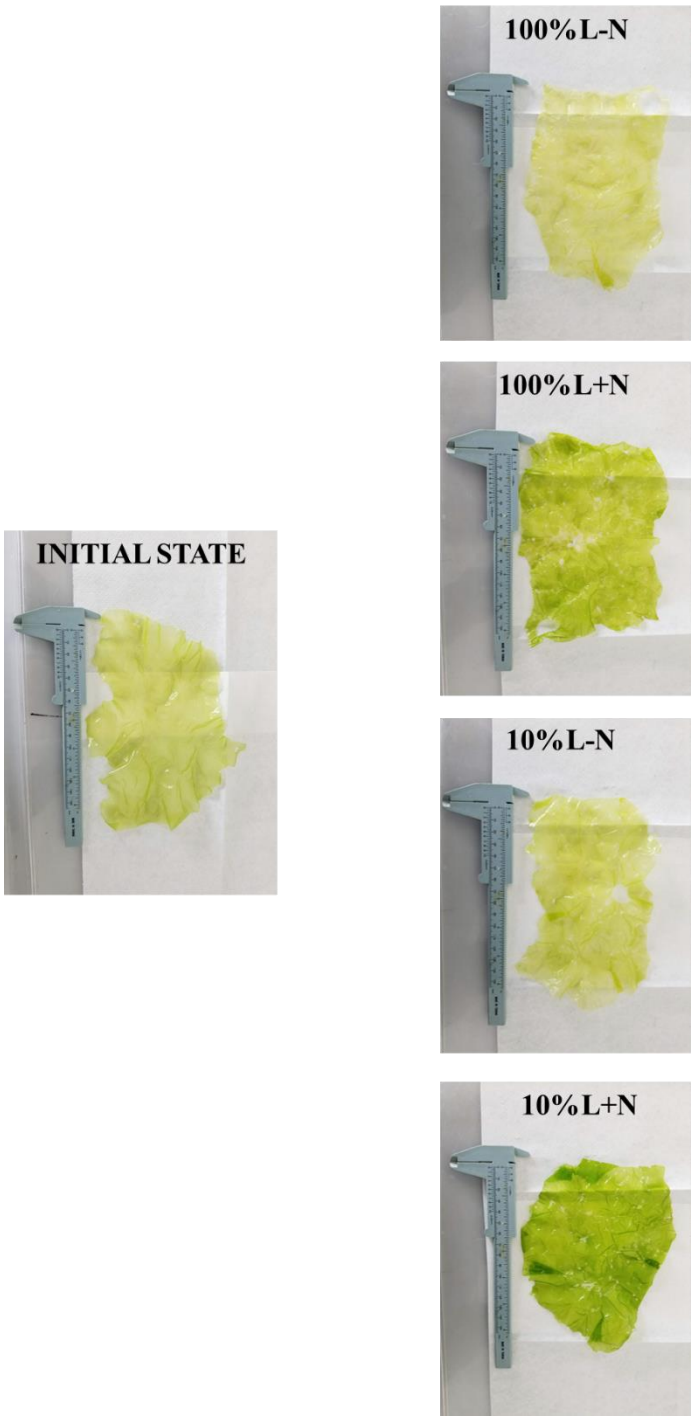

Figure 4. 6. Re-greening effect after five days of the addition of nutrients (N+P) at 100% or 10% photon flux density with *Ulva pertusa* fronds. The treatments consisted of; 100% L – nutrients (100%L-N), 100% light plus NO_3 + PO_4 (100% L + N), 10% light- nutrients (10% L-N) and 10% light plus NO_3 + PO_4 (100% L + N). The experiment was done in mid-March 2019.

4.5.2. Tables

Table 4. 2. Summary of ANOVAs for the February/March 2019 experiments to determine if *Ulva pertusa* was N, P or N+P deficient over a 12 day period.

Units	Comparison	
Fresh weight growth rate (d^{-1})	All treatments	*df*= 3,19 F= 4.4, **p= 0.008**
	RS & RS+P	*df*= 3,19 F= 1.6, p= 0.607
	RS+N & RS+N+P	*df*= 3,19 F= 0.3, p= 1.000
Total chlorophyll (mg g^{-1} dry weight)	All treatments	*df*= 3,23, F= 15.8, **p= <0.001**
	RS & RS+P	*df*= 3,23, F= 0.2, p= 1.000
	RS+N & RS+N+P	*df*= 3,23, F= 2.2, p= 0.181

Table 4. 3. Summary of ANOVAs for the effect of nitrogen addition at 100% or 10% photon flux density on *Ulva pertusa* in March 2019. For the total chlorophyll growth (d^{-1}) rate the number of subjects was estimated from the first two days.

Units	Comparison	
Total chlorophyll growth rate (d^{-1})	All treatments	*df*= 3,3 F= 20.8, **p= <0.001**
	100%L+N & 10%L+N	*df*= 3,3 F= 1.4, p= 0.100
	100%L-N & 10%L-N	*df*= 3,3 F= 3.4, **p= 0.046**
Total chlorophyll (mg g^{-1} dry weight)	All treatments	*df*= 3,23, F= 46.0, **p= <0.001**
	100%L+N & 10%L+N	*df*= 3,23, F= 6.0, **p= <0.001**
	100%L-N & 10%L-N	*df*= 3,23, F= 1.4, p= 0.837

Table 4. 4. Summary of ANOVAs for the effect of nitrogen addition at 100% or 10% photon flux density on *Ulva pertusa* in June 2019. For the total chlorophyll growth rate (d^{-1}) and content (mg g^{-1} dry weight) the number of subjects was estimated from the first two days.

Units	**Comparison**	
Fresh weight growth rate (d^{-1})	All treatments	*df*= 3,11 F= 3.2, p= 0.066
Total chlorophyll growth rate (d^{-1})	All treatments	*df*= 3,3 F= 440.4, **p= <0.001**
	100%L+N & 10%L+N	*df*= 3,3 F= 21.8, **p= <0.001**
	100%L-N & 10%L-N	*df*= 3,3 F= 2.1, p= 0.055
Total chlorophyll (mg g^{-1} dry weight)	All treatments	*df*= 3,3, F= 44.2, **p= <0.001**
	100%L+N & 10%L+N	*df*= 3,3, F= 7.7, **p= <0.001**
	100%L+N & 10%L-N	*df*= 3,3, F= 1.5, p= 0.584
	100%L-N & 10%L-N	*df*= 3,3, F= 0.5, p= 1.000

CHAPTER 5
GENERAL DISCUSSION

5.1. Michaelis-Menten and Droop equations

In Chapter 2, the use of Michaelis-Menten kinetics to derive a K_m for rates of ammonium assimilation from rates of ammonium uptake is criticized. This is not a criticism of the Michaelis-Menten equation itself, but the use to which it has been put. However, in Chapter 3, the Droop equation is used to (a) describe the relationship between % ambient light and growth rate, and (b) derive a value for the compensation point for growth. This is not what the Droop equation was originally intended for.

The Michaelis-Menten formula was originally used to describe enzyme kinetics. Whether it is appropriate to use the same formula to describe uptake kinetics, given that uptake is a vectorial process, in contrast to enzyme activity which is scalar in nature, is open to question. However, the use of the Michaelis-Menten formula to describe the relationship between substrate concentration and rate of uptake is now so common as to resist any temptation to criticize. However, this was not the point of Chapter 2, which was a criticism, in effect, of using one measure (uptake) as a reliable measure of a completely different process (assimilation) without any independent evidence for its validity. The validity of the criticism only increases when the kinetics using the multiple-perturbation technique bears very little resemblance to kinetics derived from the CCCP technique that does have independent supporting evidence.

The problem with the Michaelis-Menten formula is that it can be used with all manner of biological data. For example, it could have been applied to the growth rate data in Chapter 3. Indeed, species accumulation curves (cumulative number of species plotted against number of sampled areas of defined size) could probably be fitted to a Michaelis-Menten curve and the relationship between mean fish diversity and miles from Baytown, Texas (Magurran 1988) certainly can be with a K_m of 2.2 km. However, this does not make it an appropriate model for the data. Do the same considerations apply to the Droop equation and the compensation point for growth?

Intuitively, the use of the Droop equation to describe the relationship between incident light and growth rate makes sense. The similarities between this use of the Droop equation and the original use to describe the relationship between internal nutrient content and growth rate is that they both

involve (a) growth rate and (b) zero growth rate occurring at a greater than zero value for internal nutrient concentration and ambient light.

The Droop equation (Droop 1968) was used to define the relationship between growth rate (of a single-celled alga) and the amount of an internal nutrient (cell quota of vitamin B_{12}). Ambient light is not an internal nutrient and it may be more appropriate to use "internal photons" or absorptance (defined as the fraction of incident light absorbed by the tissue). With increasing chlorophyll content in *Ulva lactuca* the absorptance also increases as does the proportion of total absorptance that is attributable to chlorophyll (Markager & Sand-Jensen 1994). Absorptance multiplied by the incident light is a measure of the amount of photons available for growth. Absorptance includes the contribution of structural material, but following acclimation to low photon flux density, nearly 80% of absorptance can be accounted for by chlorophyll *a* and *b* (Markager & Sand-Jensen 1994). If absorptance was used, then as the % ambient light decreased, the absorptance would increase and the range of the amount of photons available for growth (absorptance x incident light) would narrow relative to the range (10-100% ambient light) reported here.

5.2. Use of outdoor and indoor cultures

The use of outdoor cultures in studies of macroalgal growth and physiology are common, particularly in aquaculture studies (e.g. Lapointe & Tenore 1981; Henley & Ramus 1989a; Msuya & Neori 2008). There are a number of advantages in their use. One is the generation of water movement and it is also much easier to ensure a nutrient supply, and, to a large extent, the temperature they are likely to encounter in nature. These are far more difficult to achieve with indoor cultures, but there are also disadvantages. The main disadvantage is that it is very difficult, if not impossible, to control even the variable of interest (in this instance photon flux density). Other obvious factors that are difficult to control are temperature and nutrients, though the latter can be controlled (Barr et al., 2008). The advantages and disadvantages of laboratory cultures are almost the opposite of those for outdoor cultures.

The use of outdoor cultures are to be preferred for studies with an ecological focus. For example, it is difficult to see how the data for Fig. 3.13 could be acquired with an indoor culture and it provides useful ecological (and physiological information). Many of the relevant environmental conditions (light, temperature, water motion, nutrient concentrations) are closer to natural with

most outdoor culture designs. These environmental conditions also change naturally and this can be a disadvantage. With aquaculture, the main reason for outdoor cultures is that it is far easier and cheaper to achieve an increase in scale. It is the absence of change that makes indoor cultures attractive. Light and temperature can be controlled easily, though the spectral composition of the light from fluorescent tubes does not bear much resemblance to solar radiation. However, water motion is much more difficult to engineer, though aeration goes some way towards solving this problem. Initial nutrient concentrations can be controlled, but with most indoor designs it is much more difficult to maintain a constant nutrient concentration.

5.3. Unbalanced growth (total chlorophyll and nitrogen)

During unbalanced growth, the total chlorophyll growth rate can equal or exceed the maximum growth rate measured as increases in fresh weight in the control. For the outdoor experiments, there was a strong positive relationship between tissue %N content and total chlorophyll content. Therefore, the nitrogen growth rate was probably close to that for total chlorophyll. In contrast, the relationship between tissue %N content and total chlorophyll content was poor in the indoor experiment. There appear to be two reasons for this. Firstly, the total chlorophyll content started at very low values, lower than any of the values obtained for the outdoor experiment. This was because they were severely nitrogen deficient. Secondly, and more importantly, the increase in total chlorophyll content after the addition of nitrogen, though proportionately dramatic in the 10% light treatment, yielded total chlorophyll contents that were at the lower end of the values obtained in the outdoor cultures. Clearly, relatively little of the increase in %N was caused by increases in the light-harvesting pigment-protein complexes.

5.4. Future experiments

In perturbation experiments, V_e represents remains an enigma. It is probable that during this phase, the rate of assimilation continues at the maximum value, with stored ammonium (from V_s) being used to add to that being taken up during V_e. It would be possible to test this using CCCP to release unassimilated ammonium during V_e, but this would not be an easy experiment to do, because tissue removed to determine the amount of unassimilated ammonium is not going continue to take up ammonium. There would need to be a number of separate groups of tissue, with one being used to follow V_e and the others used to determine stored ammonium at each of the time intervals.

A useful subject for future experimental study would be the relationship between absorptance, amount of photons available for growth and growth rate. This would enable Droop plots of the relationship between growth rate and amount of photons available for growth to be constructed, with the latter representing an internal resource or quota. The amount of photons available for growth would obviously be less at 10% of ambient light compared to 100%, but, once balanced growth had resumed, there would be occasions when the range of photons available for growth would be only about 3-fold (because of the increase in total chlorophyll increasing absorptance) compared to the 10-fold difference in ambient light.

The nature of "regreening" after extreme nitrogen deficiency would be useful to reinvestigate. The quantitative (rather than proportional) increases in total chlorophyll content after the addition of nitrogen to such tissue causes a marked increase in tissue nitrogen content, but this is not reflected in a similar increase in total chlorophyll. What are the effects on photosynthetic activity? What is the increased nitrogen used for and in what compounds is it present?

5.5. Balanced and unbalanced growth and aquaculture

Data presented here provides unequivocal evidence that the total chlorophyll growth rate (unbalanced) in *Ulva pertusa* during recovery from nutrient-deficiency at low photon flux density can comfortably exceed the highest growth rate (balanced) ever achieved for *Ulva* in culture. One aim of macroalgal aquaculture is to grow macroalgae of interest as, for example, food sources as rapidly as possible. My data raises the possibility that growth rate in macroalgae could be engineered to exceed those currently achievable. Much more work would need to be done and we need a far greater understanding of what controls and determines maximum growth rate in any organism (Oliver 2006), let alone a macroalga. However, the rewards for the ability to enhance the maximum growth rate in macroalgae are potentially great and offer exciting possibilities for future research.

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