MACROALGAL ECOPHYSIOLOGY IN RESPONSE TO HIGH AMMONIUM CONCENTRATIONS AND DIFFERENT IRRADIANCES

Dissertation submitted by

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SUMMARY

Reducing the net release of nutrients into the coastal environment has become an important issue of ecological and societal relevance. Efforts to remove excess nutrients have been made to utilize chemical or physical treatments, but they have not been successfully implemented, because they often negatively affect the environment. To utilize abundant macroalgae that have high nutrient uptake rates may become a sustainable alternative to removing excess nutrients, thus eliminating eutrophic conditions, and additionally producing a valuable by-product in the process. However, it is necessary to deepen the sustainable concept into focused studies in order to obtain incisive information for an application in such integrated aquaculture systems. Hence, investigations on macroalgal ecophysiological response to high ammonium concentrations and different irradiances are important in order to obtain a better understanding on macroalgal performance under environmental alterations.

Numerous studies have examined ammonium utilization by marine macroalgae, with the most current research focusing on their physiological responses to the polluted environment under low ammonium concentrations. There is comparatively few information on macroalgal resistance to high ammonium concentrations, a condition which is usually found in aquaculture systems, and is one of the predominant causes of eutrophication.

Thus, studies in three different experimental series under laboratory conditions of abundant pan-tropic macroalgal species have been conducted to investigate the maximum uptake and physiological tolerance to high ammonium concentration treatments, including ammonium toxicity symptoms and protein content, to test the feasibilities as nutrient bioabsorber. *Dictyota bartayresiana* J.V. Lamoroux and *Gracilaria verrucosa* (Hudson) Papenfuss were compared in relation to their ammonium uptake capacities in the first study. A series of perturbation experiments was conducted in a 360 minute experimental period. Ammonium concentrations used were 0, 50, 100, 200, and 400 µM; under 70 µmol photons $m^{-2}s^{-1}$ irradiation. In general, *G. verrucosa* showed higher nutrient uptake and photosynthetic performance. Final F_v/F_m of *G. verrucosa* remained high in all ammonium treatments, in contrast with *D. bartayresiana*. A surge in ammonium uptake rate was observed for both species in the first 15 minutes. *G. verrucosa* showed no saturation point over various time intervals. In contrast, *D. bartayresiana* showed a saturation point and discoloration after the first 15 minutes. A high ammonium supply and light availability might support photosynthesis and growth of *G. verrucosa*, but on the contrary, indicated ammonium toxicity in *D. bartayresiana*.

To investigate the maximum physiological tolerance of *G. verrucosa* under a very high ammonium concentrations and different irradiances was the focus of the second study. In the first experiment with 70 µmol photons $m^{-2}s^{-1}$ irradiation, uptake rate of *G. verrucosa* showed a surge phase in the first two days, and was saturated after day 4, while in the experiment with 300 µmol photons $m^{-2}s^{-1}$ irradiation, uptake saturation was shown from day 2 of the experimental period. The final F_v/F_m of both irradiances decreased significantly with increasing ammonium concentration treatments and irradiation. Tissue loss started from day 7 when exposed to high ammonium concentration treatment for both irradiances. Ammonium toxicity symptoms were found

in *G. verrucosa* with ammonium addition greater than 800 µM for both irradiations. High ammonium concentration treatments and different irradiance significantly affected accumulative biomass, total growth rate, pigments, and nutrient in tissue. However, further analysis of biochemical compounds might be necessary to explore ammonium toxicity symptoms of *G. verrucosa*.

The third study was conducted to obtain information on biochemical levels (e.g., protein content) to high irradiance and ammonium stress. A series of experiments under irradiation of 70 and 300 µmol photons $m^{-2}s^{-1}$ with 1200 µM ammonium concentration treatment had been conducted in a climate room for 7 days. In general, *G. verrucosa* showed low performance in most observed parameters, earlier than previously found. Massive discoloration as a symptom of infectious disease was found by day 4 in the macroalgae under 300 µmol photons $m^{-2}s^{-1}$ irradiation. Protein content in all treatments was lower than 0.5 µg/µL extract, which was not sufficient for the further protein analysis.

Overall, the most relevant findings of these studies were that 1) *G. verrucosa* exhibited a high performance in ammonium uptake and storage capacity, 2) unsaturated ammonium uptake shown by *G. verrucosa* was not a passive diffusion process, but actively transported due to charged ions of ammonium and huge storage capacity in macroalgal tissue, and 3) a very high ammonium concentration interacted with high irradiance to affect nutrient uptake, growth rate, and photosynthetic performance of *G. verrucosa*, and lead to toxicity symptoms.

The overall outcome of these studies advanced a different perspective on relevance of nutrient abundance and macroalgae, although in some points illustrate that high ammonium concentrations may contribute to substantial negative consequences to the macroalgae. To involve *G. verrucosa* in an integrated applied system to control the nutrients excess would be the next important step. However, how to achieve an appropriate balance of uptake and growth ratio is still a challenge. Thus, *G. verrucosa* cultivation in such integrated multitrophic aquaculture systems becomes the near future plan to be implemented.

ZUSAMMENFASSUNG

Eine Reduzierung der Nettoeinleitung von Nährstoffen in die Küstenumwelt ist zu einem wichtigen Thema von ökologischer und gesellschaftlicher Relevanz geworden. Bemühungen, überschüssige Nährstoffe durch chemische oder physikalische Behandlungen zu entfernen, konnten auf Grund ihrer negativen Einflüsse auf die Umwelt nicht erfolgreich angewandt werden. Der Einsatz von abundanten Makroalgen mit einer hohen Nährstoffaufnahmerate könnte eine nachhaltige Alternative sein, überschüssige Nährstoffe zu entfernen und damit eutrophen Bedingungen entgegenzuwirken. Zusätzlich wird in diesem Prozess ein wertvolles Nebenerzeugnis gewonnen. Es ist allerdings notwendig, das Konzept der Nachhaltigkeit dieser Methode durch fokussierte Studien zu bekräftigen, um prägnante Informationen über eine solche Anwendung in integrierten Aquakultursystemen zu gewinnen. Deshalb sind Studien über die ökophysiologische Reaktion von Makroalgen auf hohe Ammonium-Konzentrationen und unterschiedliche Lichtintensitäten notwendig, um somit ein besseres Verständnis der Leistung der Makroalgen unter Umweltveränderungen zu gewinnen.

Zahlreiche Studien haben die Ammonium-Nutzung von marinen Makroalgen untersucht, wobei die neueste Forschung sich auf ihre physiologische Reaktion auf Umweltverschmutzung unter niedrigen Ammonium-Konzentrationen konzentriert. Hingegen gibt es wenige Informationen über die Widerstandsfähigkeit von Makroalgen gegenüber hohen Ammonium-Konzentrationen, eine Bedingung, wie sie häufig in Aquakultursystemen auftritt und eine der vorherrschenden Ursachen für Eutrophierung ist.

Daher wurden in dieser Studie Untersuchungen an abundanten pantropischen Makroalgenarten in drei verschiedenen Experimentserien unter Laborbedingungen durchgeführt, um die maximale Aufnahme und physiologische Toleranz von hohen Ammonium-Konzentrationen, einschließlich der Symptome der Ammonium-Toxizität und des Proteingehalt zu erforschen und damit ihre Eignung als Bioabsorber zu prüfen.

In der ersten Studie wurden *Dictyota bartayresiana* J.V. Lamoroux und *Gracilaria verrucosa* (Hudson) Papenfuss hinsichtlich ihrer Ammonium-Aufnahmekapazität verglichen. Es wurden experimentelle Versuche über eine Inkubationsperiode von 360 Minuten durchgeführt, wobei Ammonium-Konzentrationen von 0, 50, 100, 200 und 400 µM unter einer Lichtintensität von 70 µmol Photonen m^{-2} s^{-1} verwendet wurden. *G. verrucossa* zeigte generell eine höhere Nährstoffaufnahme und photosynthetische Leistung. Finale F_v/F_m-Werte von *G. verrucosa* blieben, im Gegensatz zu *D. bartayresiana*, in allen Ammonium-Behandlungen hoch. In beiden Arten wurde ein plötzlicher Anstieg der Ammonium-Aufnahmerate in den ersten 15 Minuten beobachtet. *G. verrucosa* zeigte keinen Sättigungspunkt über verschiedene Zeitspannen, wohingegen bei *D. bartayresiana* eine Sättigung und Entfärbung nach den ersten 15 Minuten festgestellt wurde. Ein hohes Ammoniumangebot und eine hohe Lichtverfügbarkeit könnte Photosynthese und Wachstum von *G. verrucosa* gefördert haben, aber im Gegensatz dazu, in *D. bartayresiana* Ammoniumtoxizität hervorgerufen haben.

Das Ziel der zweiten Studie war es, die maximale physiologische Toleranz von *G. verrucosa* unter sehr hohen Ammonium-Konzentrationen und unterschiedlichen Lichtintensitäten zu untersuchen. Bei einer Lichtintensität von 70 µmol Photonen m^{-2} s^{-1}

zeigte die Aufnahmerate einen starken Anstieg in den ersten zwei Tagen, und eine Sättigung nach Tag 4, wohingegen unter 300 µmol Photonen m^{-2} s^{-1}, die Sättigung der Aufnahme bereits nach 2 Tagen Inkubation eintrat. Die finalen F_v/F_m-Werte bei beiden Lichtintensitäten nahmen signifikant mit zunehmender Ammonium-Konzentration und Lichtintensität zu. Ein Gewebeverlust der Algen setzte bei Inkubation mit hohen Ammonium-Konzentrationen bei beiden Lichtintensitäten an Tag 7 ein. Bei Ammonium-Konzentrationen höher als 800 µM wurde in *G. verrucosa* unter beiden Lichtintensitäten Symptome für Ammoniumtoxizität festgestellt. Hohe Ammonium-Konzentrationen und unterschiedliche Lichtintensitäten hatten einen signifikanten Einfluß auf die akkumulative Biomasse, die Gesamtwachstumsrate, die Pigmente und den Nährstoffgehalt des Gewebes. Es sind jedoch weitere Analysen von biochemischen Verbindungen notwendig, um die Symptome der Ammoniumtoxizität in *G. verrucosa* zu erforschen.

Die dritte Studie wurde durchgeführt, um Informationen über die Gehalte verschiedener biochemischer Substanzen (z.B. Proteingehalt) in *G. verrucosa* unter hohem Licht- und Ammoniumstress zu gewinnen. Dazu wurden in einem Klimaraum eine Reihe von Experimenten unter 70 und 300 µmol Photonen m^{-2} s^{-1} und einer Ammonium-Konzentration von 1200 µM über einen Zeitraum von 7 Tagen durchgeführt. *G. verrucosa* zeigte in den meisten Parametern generell eine geringe Leistung, und zwar bereits früher als in den vorherigen Studien gezeigt wurde. Massive Entfärbung der Makroalgen unter 300 µmol Photonen m^{-2} s^{-1} an Tag 4 weisen auf Symptome einer infektiösen Krankheit hin. Der Proteingehalt war in allen Ansätzen niedriger als 0.5 µg/µL Extrakt und somit nicht ausreichend für eine weitere Proteinanalyse.

Insgesamt, sind die wichtigsten Erkenntnisse dieser Studien, dass 1) *G. verrucosa* eine hohe Kapazität zur Ammoniumaufnahme und –speicherung zeigte, 2) die ungesättigte Ammoniumaufnahme von *G. verrucosa* nicht auf einen passiven Diffusionsprozess, sondern auf einen aktiven Transport von geladenen Ammonium-Ionen und eine enorme Speicherkapazität im Gewebe der Makroalge zurückzuführen ist und 3) sehr hohe Ammonium-Konzentrationen interagierend mit hohen Lichtintensitäten einen Einfluss auf die Nährstoffaufnahme, die Wachstumsrate und die photosynthetische Leistung von *G. verrucosa* haben und zu Toxizitätssymptomen führen.

Das Gesamtergebnis dieser Studien hat eine andere Perspektive auf die Relevanz von Nährstoffverfügbarkeit und Makroalgen vorangebracht, obgleich sich in einigen Ergebnissen gezeigt hat, dass hohe Ammonium-Konzentrationen einen erheblich negativen Einfluss auf die Makroalgen haben können. Um *G. verrucosa* in einem integrierten angewandten System zu verwenden, wäre es wichtig in einem nächsten Schritt die Belastung zu kontrollieren. Es ist allerdings noch eine Herrausforderung, ein entsprechendes Gleichgewicht zwischen Aufnahme und Wachstum zu erreichen. Daher ist es der zukünftige Plan, die Kultivierung von *G. verrucosa* in einem solchen integrierten, multitrophischen Aquakultur-System umzusetzen.

1. GENERAL INTRODUCTION

1.1 Overview

A wide range of problems plaguing near shore waters can be tied, directly or indirectly, to nutrient over enrichment (Nixon, 1995; Pew Oceans Commission, 2003; U.S. Commission on Ocean Policy, 2004). In addition to natural nutrient inputs, nutrients may be introduced by anthropogenic sources from agriculture, sewage, industry, and aquaculture (Morrand and Merceron, 2005). Nitrogen and phosphorous is now recognized as the most serious pollution problem facing coastal waters worldwide (GESAMP, 1990; Howarth et al., 2000).

Nutrient release from aquaculture and eutrophication

Expansion of aquaculture has often been accompanied by degradation of the natural environment. Aquaculture facilities release a significant quantity of nutrients as residual feed and excrements from fish, which thus form nutrients-rich sediments in confined areas. Generally, only about 30% of the nutrients added through feed will be removed through fish harvest (Holby and Hall, 1991; Hall et al., 1992), while the rest will be released as uneaten feed and metabolic wastes in a form of excretory products (Porter et al., 1987; Troell et al., 1997). Excretory products from fish and microbial degradation of leftover food generate ammonia and other nutrients in the crowded conditions of the intensive culture systems used for aquaculture (Krom et al., 1985; Neori et al., 2000). Dissolved nutrients in shrimp pond effluents are predominantly in the form of ammonium (Macintosh and Philips, 1992; Jones et al., 2001). Stead and Laird (2002) found that waste products from pen-based salmon aquaculture mainly consist of nitrogen, phosphorous, and carbon dioxide.

There is some criticism regarding the environmental impact of aquaculture due to its tendency to release particulate organic waste effluents containing elevated levels of nitrogen or phosphorous-rich compounds (Buschmann et al., 1994), which may lead to eutrophication of the environment (Neori et al., 1996; Rathakrishnan, 2001) and acute toxicity to animals (Troell et al., 1999; Neori et al., 2000). This occurs in countries with

intensive marine fish farms, such as Norway, Japan, or the Philippines (Morand and Merceron, 2005).

Eutrophication, resulting from excessive nutrient enrichment, is commonly recognized to affect alterations of nutrient availability and food web structure and result in a general pattern of community change (e.g., plant coverage, dominant species, species diversity, species interaction (Lin and Hung, 2001). While macroalgae are natural and vital components of estuaries, excessively dominant species can have negative ecosystem-wide effects, for example, algal bloom. These blooms are the natural response of algae to the environment, but when the environment changes and can no longer support such high algal populations, the algae that accumulate during this bloom later die and lead to oxygen decay (Göltenboth, 2006). For example, in Lee County, Florida, this problem reached a critical stage in 2003-2004 when massive rhodophyte blooms washed ashore, making beaches unsuitable for recreation and requiring an expensive removal program (Lapointe and Bedford, 2007). Continuously increasing nutrients in Upper Newport Bay, California, which is already a highly eutrophic estuary, were reported to be the trigger of macroalgal blooms (Kamer et al., 2001).

Unlike toxic phytoplankton blooms, such as red tides, macroalgal blooms can result in the displacement of indigenous species, habitat destruction, oxygen depletion, alteration of biogeochemical cycles, increased grazing, and die-off of seagrasses and coral reefs (Lapointe et al., 1994; McGlathery, 1995; ECOHAB, 1995; Valiela et al., 1997; NRC, 2000; Lapointe and Thacker, 2002). They can cause anoxic conditions by periodically covering sediments (Sfriso et al., 1987; Young et al., 1998), thereby causing shifts in sediment infaunal communities (Ahern et al., 1995). Cellular respiration by algal mats, either at night or during the day when light is below the compensation point in the bottom layers of the mats, can deplete oxygen in the water column (Valiela et al., 1992). This can result in fish and invertebrate mortality, which may ultimately affect birds and other fauna in the food web (Raffaeli et al., 1989). In other words, nutrient pollution is the common threat that links an array of problems including eutrophication, harmful algal blooms, bio-invasions, fish kills, shellfish poisonings, loss of seagrass and kelp beds, coral reef die-off, emerging marine diseases,

and marine mammal and seabird deaths (Howarth et al., 2000; Lapointe et al., 2004; 2005).

Nutrient bioabsorber

Reducing the net release of nutrients from euthropic estuaries, especially coming from aquaculture activities, into the environment has become an important issue of ecological and societal relevance. Chemical or physical treatments to remove excess ammonia from waste water and from culture ponds (Troell et al., 2003; Cripps, 1994) are expensive (Petrell, 1993) and not sustainable alternatives, as they may affect the environment (Troell et al., 1997) by causing erosion and the receding of certain beaches by approximately 10-12 m (Atkins et al., 1993).

Solutions for treating effluents from aquaculture must be sustainable, and not require large investments and energy consuming support. Abundant and commercially valuable macroalgae that have high nutrient uptake rates may be used to remove excess nutrients from aquaculture effluents, thus eliminating eutrophic conditions and producing a valuable by-product in the process.

During the past 10 years, claims for the use of ecological engineering tools in sustainable development have increased (Chopin et al., 2008). Monitoring of nutrient availability through the use of aquatic macrophytes is a potential method for evaluating the significance of nitrogen and phosphorous as potential growth-limiting factors (Lapointe, 1987; Lyngby, 1990). Studies have shown that waste water from fish cultivation is suitable as a nutrient source for macroalgae production (Krom et al., 1995; Neori et al., 1996). Integrating macroalgae in co-culture systems has been suggested as an efficient way to assimilate and utilize nutrients, to decrease nutrient excess, and to control red tides and biological diseases (Troell et al., 2006).

The use of macroalgae as nutrient bioabsorbers can provide benefits to the environment. Macroalgae can take up nutrients in high rates, which can reduce nutrient effluents to the environment. Excess nutrients in the water will be absorbed by the plants for use as fuel for physiological processes (Zhou et al., 2006), and at the same time can purify the medium (Morand and Merceron, 2005). Zhou et al., (2006) reported that laboratory seaweed and fish co-cultures were efficient nutrient pumps and could remove most nutrients from the system. Another advantage is that macroalgae can

provide more accurate information about water conditions and indicate community changes in the nutrient content. These advantages make macroalgae effective bioindicators. The use of macroalgae as bioindicators to infer changes in habitat characteristics is based on the ability of the plants to reflect water column concentrations of a preferred nutrient form (Fujita, 1985). This may give more relevant results than chemical water measurements, which probably do not assess actual availability of a particular nutrient for plant growth (Lyngby, 1990).

Some genera had been reported to have high nutrient uptake rates, such as *Ulva* (as *Enteromorpha* in Fujita, 1985; Duarte, 1995), *Ceramium* (Pedersen and Borum, 1997) and *Gracilaria* (Peckol et al., 1994). Fast growing macroalgae, including *Ulva lactuca* Linn., respond rapidly to changes in nutrient conditions, particularly to short-term N supply. This ability to rapidly take up and assimilate N contributes to the increasing occurrence of macroalgal blooms in heavily N loaded coastal ecosystems (Teichberg et al., 2006).

The effluent composition (Jones et al., 2001) and physiological characteristics of primarily cultivated species (Neori et al., 2004) play important roles in the choice of species candidates to be used as nutrient bioabsorbers (Jones et al., 2001; Neori et al., 2004). Obviously, the best macroalga to integrate into an animal aquaculture operation is the one characterized as a cosmopolite with rapid growth, high accumulation of nitrogen and phosphorous in the tissue (ecological value), and a high commercial price (economic value) (Troell et al., 2003; Neori et al., 2004; Carmona et al., 2006).

Dictyota bartayresiana

Species of *Dictyota* (Phaeophyceae) are very common in intertidal and shallow subtidal habitats along rocky coast worldwide (Tronholm et al., 2010), frequently found growing epiphytically on many reef species (Littler and Littler, 2000), or co-occurring other species in eulittoral pools (Tronholm et al., 2010). *Dictyota* has been frequently observed to occupied benthic community due to nutrient rich effluents (Beach et al., 2006; Walter and Beach, 2000), and overgrow corals and other macroalgae (Lirman and Biber, 2000). *Dictyota* spp. were found growing epiphytically on *Halimeda tuna*, *H. opuntia*, *Lobophora variegata*, *Galaxaura* sp., fire corals, hard corals, soft corals, bryozoans, and a variety of sponges on Conch Reef (Beach et al., 2003). The genus

reaches the highest diversity in tropical and warm-temperate environments where multiple species often coexist and reach high densities (Herren et al., 2006).

Variation in the responses by *Dictyota* spp. to irradiance and nutrient enrichment has been reported by Beach et al. (2006). Cronin and Hay (1996) found that light and nutrient availability contributed significant effects to secondary metabolite produced by *D. ciliolata* Kützing and *Sargassum filipendula* C. Agardh, and affected benthic herbivores community. Peckol and Ramus (1988) reported that thin flat species, such as *Dictyopteris hoytii* Tayl., *Dictyota cervicornis* Kütz, and *D. divacirata* Lamour., had higher photosynthetic capacity and greater pigment content than species with other thallus forms (calcified, coarsely branched, leathery).

Gracilaria verrucosa

The genus of *Gracilaria* is cosmopolitan in distribution, and has been reported from the arctic, temperate, and tropical regions. Greville set up the genus *Gracilaria* in 1830, which then comprised only four species. Agardh reexamined the genus in 1852, and increased it to 23 species. In 1876 and 1901 he re-identified them again and reported 61 species altogether. Since then, the numbers of *Gracilaria* species reported from many places over the world have reached about 150, with 24 species reported in China (FAO, 1990).

Gracilaria has rapidly gained in worldwide importance as a cheap source of dietary proteins and minerals, and as a source of commercial agar (Armisen and Galatas, 1987; Hurtado-Ponce, 1990), and it also has good hydrocolloid texture (Hurtado-Ponce and Umezaki, 1988). This genus has now practically replaced *Gelidium* as the most important source of agar in the world (Tseng and Xia, 1999).

Some species of *Gracilaria* have been reported to be useful as nutrient bioabsorbers in aquaculture systems, as reported for *G. lemaneiformis* (Yang et al., 2006) and *G. birdiae* (Marinho-Soriano, 2009). These local species reported to have rapid growth and high uptake of nitrogen and phosphor. It has been suggested that other species of *Gracilaria* may have a similar performance as the ideal macroalgae to remediate the ecological environment in eutrophic waters.

Gracilaria spp. has been used in a variety of studies investigating nutrient limitation and storage (Fujita, 1985; Lapointe, 1985; Hwang et al., 1987; Horrocks et

al., 1995). Most current research on *Gracilaria* focuses on their physiological response to the polluted environment under low nutrient concentrations (Amir et al., 2000). However, research on the physiological and biochemical characteristics of *Gracilaria* under high nutrient concentrations and its resistance to high nutrient concentrations are still very limited (Yu and Yang, 2008).

Physiological responses

Physiological responses of organisms are influenced by biotic and abiotic factors. For example, thallus morphology can strongly influence photosynthetic rates in addition to light and nutrient availability. On the other hand, change in abiotic factors can lead to different physiological responses. Generally, nutrient addition may increase photosynthetic activity and growth of macroalgae. In reverse, low availability of nutrients can limit photosynthesis and growth of macroalgae (McGlathery, 1992; Pedersen, 1995).

Nutrient uptake

Nutrient uptake may be affected by several factors. Light, temperature, water motion, and desiccation are mentioned as the physical factors that can influence the uptake rate. Light affects nutrient uptake indirectly through photosynthesis. Temperature affects ion-specific uptake rates. It is different among species (Lobban and Harrison, 1994). Water motion is another physical factor that is important in the movement of ions to the surface of the thallus (Wheeler and Neushul, 1981). Desiccation affects nutrient uptake by loss of water from the thallus during a frequent low tides (Lobban and Harrison, 1994). Chemical factors, such as concentration of nutrients being taken up and molecular form of the element, will also affect uptake rates. As an example, nitrogen in the form of ammonium is often taken up more rapidly than nitrate, urea, or amino acids in many macroalgae (Lobban and Harrison, 1994). Uptake rates can also be influenced by the concentrations of other ions in the medium. Ammonium may inhibit nitrate uptake by as much as 50% in many macroalgae (DeBoer, 1981). Biological factors that influence uptake rates include the surface area-volume ratio, hair formation, the type of tissue, the age of the plant, and interplant variability (Gerard, 1982). The morphological

characteristics of many macroalgae (such as surface area:volume ratio, simple sheet or filamentous thallus) growing in low-energy, high nutrient habitats, suggest that their nitrogen uptake efficiency is likely to be high. In contrast, other species may store nutrients and use several forms of nutrients for growth so that the rate of uptake is less important to their survival (Rosenberg and Ramus, 1982).

Nutrients transport a cell by moving across the boundary layer of water surrounding the cell in several ways. The kinetics of nutrient transport will depend on which uptake mechanism is being used. Active uptake is energy-dependent, while passive diffusion occurs without the expenditure of cellular metabolic energy. If transport occurs solely by passive diffusion, then the transport rate will be directly proportional to the external concentration. In contrast, facilitated diffusion and active transport will exhibit a saturation of the membrane carriers as the external concentration of the ion increases. The relationship between the uptake rate of the ion and its external concentration is generally described by a rectangular hyperbola, similar to the Michaelis-Menten equation for enzyme kinetics (Lobban and Harrison, 1994). The equation can generally describe the nutrient transport capabilities of various macroalgal species (Healey, 1980; Lobban and Harrison, 1994), by providing information of maximum uptake rate and half-saturation constant.

Photosynthesis

Photosynthesis is the biological conversion of light energy to chemical bond energy that is stored in the form of organic carbon compounds. Photosynthetic processes are affected by many abiotic factors, such as light, temperature, and nutrient availability. Light reactions of photosynthetic processes precede absorption of light and transfer of its energy to a reaction center, where the energy is used in electrical charge separation (Falkowski, 1994). There are also some biotic factors, intrinsic in the individuals that affect photosynthesis: morphology and ontogeny (Lobban and Harrison, 1994). The energy of absorbed photons is used to modify the electronic structure of pigment molecules to the extent that an electron can be physically transferred from a donor to an acceptor. Thus, the light reactions in photosynthesis are photochemically catalyzed oxidation-reduction reactions (Falkowski and Raven, 2007).

To maintain high activities of photosynthesis, many mechanisms involve enzymes are required. The synthesis of enzymes also pigments and proteins (e.g., amino acids) require nitrogen (Roy, 2000). Among the metabolic paths that are suspected to be most influenced by changing the nutritional conditions of the environment are the main nutrient assimilatory enzymes for CO_2, nitrate and phosphate, as well as the light-harvesting strategy. Carbonic anhydrase and nitrate reductase are well known as key regulatory enzymes for the carbon and nitrogen assimilation pathways, respectively (Gordillo et al., 2006). The degradation or inactivation of any protein involved in photosynthesis (e.g., the D1 protein or RuBisCo) is likely to lower the photosynthetic activity (Bischof et al., 2000; Aro et al., 1993).

Nitrogen limitation affects the rate of respiration. As nitrogen limitation leads to a reduction in growth and photosynthetic rates, so it leads to a reduction in respiratory rates (Geider, 1992). The rate of photosynthesis depends on the available irradiance, and ultimately on the irradiance absorbed. The relationship between photosynthesis and irradiance can be shown as a Photosynthesis-Irradiance (PI) curve. The curve is useful for comparing the physiology of light harvesting in different plants by showing saturating irradiance (I_k), photosynthetic capacity ($rETR_{max}$), and photosynthetic efficiency (α) as a useful indicator of quantum yield (Lobban and Harrison, 1994).

Growth rate

Growth and productivity of macroalgae are controlled in part by environmental factors, such as irradiance, temperature, nutrient availability, and water movement (Lobban and Harrison, 1994). Growth related to the limiting nutrient concentrations in the ambient water (Lobban and Harrison, 1994), and seasonal fluctuations in nutrient availabilities, especially nitrogen, will affect growth rates (Rosenberg and Ramus, 1982; Probyn and Chapman, 1982). In other systems, similar seasonal changes in the growth rates and production of macroalga have also been ascribed to nutrient limitation (Fujita et al., 1988; Borum and Sand-Jensen, 1996).

Recent experiments have shown that among all possible limiting elements, nitrogen most frequently limits the growth of macroalgae and microalgae in natural ecosystems (Hanisak, 1990; Lobban and Harrison, 1994). Nitrogen has been considered the limiting nutrient in temperate oceans both for microalgal (Twomey and Thompson,

2001) and macroalgal communities (Lobban and Harrison, 1994; Harris et al., 1996). It is well known that the addition of nitrogen can greatly enhance the growth rate and production of seaweeds under certain conditions (Smit, 2002), and will boost the growth rates Pedersen (1995). The growth rate and productivity of algae is controlled by the concentration of dissolved inorganic nitrogen in the aqueous medium surrounding the thallus (Rosenberg and Ramus, 1982; Lavery and McComb, 1991). The ability of an alga to utilize nitrogen for biomass production is determined by the rate at which dissolved inorganic nitrogen can traverse the boundary layer adjacent to the outer cell layer of the thallus, and the rate at which this nitrogen takes part in biochemical processes (Koch, 1994; Sanford and Crawford, 2000).

The level of irradiance needed for optimum growth of a species shows some correlation with its habitat (Lüning, 1981). Light modifies the nutrients requirement for maximal photosynthesis and growth by altering biochemical constituents that affect nutrient (e.g., nitrogen) levels (Lapointe and Duke, 1984).

Although the number of studies in nutrient uptake of macroalgae has been increasing so far, there is still a gap of information on the physiological responses of macroalgae to high concentrations of nutrients. Not much information of macroalgal response to high ammonium concentrations can be found. Therefore, this study aimed to investigate the physiological response of macroalgae to various ammonium concentrations under different irradiances.

1.2 Objectives

The primary objective of this study was to explore the physiological responses of tropical macroalgal species to different nutrient and light availabilities in order to obtain information of macroalgal suitability for nutrient bioabsorber. A series of experiments were conducted to observe the influence of various ammonium concentration treatments and different light irradiances on the nutrient uptake, photosynthesis performance, and growth rate of these macroalgae.

1.3 Research questions

Thus, this study aimed to answer the following research questions:

1. What is the maximum capacity of ammonium uptake in the tropical macroalgae *Dictyota bartayresiana* and *Gracilaria verrucosa*?
2. How do nutrient concentrations and light intensities affect the ammonium uptake capacity of these macroalgae?
3. How do nutrient concentrations and light intensities affect macroalgal photosynthesis and growth?
4. Are the results obtained in laboratory experiments transferable to applications in aquaculture systems?

2. GENERAL MATERIALS AND METHODS

2.1. Macroalgae

Macroalgae used in this study were *Dictyota bartayresiana* J.V. Lamoroux (Phaeophyceae) and *Gracilaria verrucosa* (Hudson) Papenfuss (Rhodophyceae). Both are tropical algal species and derived from Indonesia.

2.1.1 Collections

D. bartayresiana as collected in October 2008 from a reef flat in Spermonde Archipelago, Indonesia (4°52'33"S; 119°6'50"E), and was transferred and cultured in mesocosm tanks in the aquaculture facility at Leibniz-Zentrum für Marine Tropenökologie, Universität Bremen. *G. verrucosa* was collected from the Bay of Muaragembong, Indonesia (6^{o}0'19"S; 107^{o}1'28"E) in August 2009, and transported to the already mentioned aquaculture facility to be prepared for several experiments in the Marine Botany Laboratory.

2.1.2 Maintenance

Clean and healthy thalli of *D. batayresiana* were cultured in a mesocosm with recirculating artificial seawater and aeration, along with other reef organisms including fish, crustacean, corals, and macroalgae. Irradiation was applied in a 12:12 hr light:dark photoperiod at 300 µmol photons $m^{-2}s^{-1}$. *G. verrucosa* were acclimated in 60x45x30 cm^3 aquaria with gentle aeration and recirculation in filtered artificial seawater at room temperature (24^{o}C). Irradiation was applied in a 12:12 hr light:dark photoperiod at 90 µmol photons $m^{-2}s^{-1}$. Provasoli (PES) solution (McLachlan, 1973) as added with a concentration of 1.25 µL, once a week to meet the algal nutrient requirements.

2.1.3 Preconditioning

Preconditioning in this study refers to the starvation period before experiments. Approximately 50 g of *D. bartayresiana* or *G. verrucosa* were placed in 45x20x20 cm^3

aquarium with gentle aeration. Irradiation was applied in a 12:12 hr light:dark photoperiod at 90 μmol photons $m^{-2}s^{-1}$. Water temperature was maintained at 24°C. There was no nutrient addition during this period. The experiments were conducted after *D. bartayresiana* was starved for one week to obtain optimum uptake in the experiment. The same precondition procedures were done to prepare for the experiments of physiological response and protein content determination for *G. verrucosa*.

2.2 Medium

All the experiments being conducted in this study used artificial seawater as a medium. Artificial seawater was filtered with a sterile capsule (Sartobran P; 0.45 + 0.2 μm; Sartorius, Germany) to reduce the possibility of contamination of bacteria and epiphytes. Filtered seawater was frozen and stored in cold storage room at 0°C until analysis.

2.2.1 Enriched seawater

Enriched seawater was used for nutrient uptake and growth rate experiments. Ammonium as NH_4Cl was added to the filtered seawater in various concentrations as the nutrient enrichment. Desired ammonium levels were obtained by dilution of stock solution using the formula:

$$V_1.M_1 = V_2.M_2$$

Where V_1 is the initial volume from the stock solution (mL), M_1 is the initial concentration of stock solution (μM), V_2 is desired volume (mL), and M_2 is desired concentration (μM).

2.3 Tools preparations

All the tools derived from glass, plastics, and ceramics used in the experimental analyse were free of ammonium after being rinsed in HCl solution (10%) for 6 hours. Acid washing was followed by rinsing tools in distilled water and dried at room temperature.

Aluminum foil was used to wrap the tools to protect from ammonium contamination before sterilization procedure using the autoclave.

2.4 Lights and irradiation

Experiments in this study used two types of lamps. White fluorescence lamps with intensities of 100 µmol photons $m^{-2}s^{-1}$ were used for low exposure experiments. Aqua Medic white fluorescence lamps with light intensities of 300 µmol photons $m^{-2}s^{-1}$ were used for high exposure experiments. Desired light intensities were obtained by adjusting the position and the distance between the lamp and the object of the experiments (macroalgae in the beakers). Light intensities were measured by using a light meter (LI-1400 data logger).

2.5 Measurement of abiotic parameters

Abiotic parameters in this study refer to water dissolved oxygen concentrations, temperature, salinity, and pH. Measurement of abiotic parameters was carried out using a multiparameter checker WTW Multi 3430 SET F (Germany). The measurements were done initially and every other day through the experimental time period. The measurements were done before the medium was changed.

2.6 Photosynthetic performance

Photosynthetic activity was determined by measuring variable chlorophyll- fluorescence using a PAM 2100 device (Walz, Germany). Optimum quatum yield was calculated as the ratio of variable to maximum fluorescence (Fv/Fm) of the dark-acclimated plant (Bischof et al., 1998). The algal thalli were fixed to the end of the fiberoptics and placed in a beaker filled with seawater. The beaker was covered with aluminum foil to avoid light leakage from other light sources. After application of a 5-s far-red pulse (30 µmol.m-2s-1 at 735 nm), the samples were kept in darkness for 5 minutes. Afterwards, minimal fluorescence (F_o) was measured with a pulsed measuring beam (approximately 0.3 µmol $m^{-2}s^{-1}$, 650 nm). Then short pulses of saturating white light (0.4-0.8 s, 1500 up to 10,000 µmol $m^{-2}s^{-1}$) were provided to determine Fm. In ammonium uptake experiments, optimum quantum yield was measured initially and finally (after 360

minutes). In growth rates experiments, measurements of optimum quantum yield was taken after 0, 2, 4, 7, 11, and 14 days of experimental nutrient and light exposure.

To determine changes in the photosynthetic capacity, photosynthesis versus irradiance curves (PI-curve) were calculated following procedures described by Bischof et al. (1998). Algal samples were irradiated with increasing irradiance of actinic red light (10-700 μmol $m^{-2}s^{-1}$, 650 nm). After 30 s a saturating pulse was applied to measured effective quantum yield of photosynthesis and then actinic irradiation was increased. By multiplying quantum yield with photosynthetically active radiation (PAR) relative electron transport rates (rETR) were calculated as described by Schreiber et al. (1994):

$$rETR = \Delta F/Fm' \times PAR$$

with ΔF as difference of F_m (maximum fluorescence) and F_o (minimum fluorescence), F_m' as maximum fluorescence yield reached in a pulse of saturating light when the sample is preilluminated.

Relative ETR were plotted against irradiance of actinic light, and maximal relative electron transport rate (ETR_{max}, under saturating light) was determined by curve-fitting. PI-curves were monitored during growth experiments after 0, 2, 4, 7, 11, and 14 days of experimental nutrient and light exposure. Data were processed using the PAM Win programme and Kaleidagraph to obtain photosynthesis capacity ($rETR_{max}$), saturation point (I_k), and photosynthetic efficiency (α).

2.7 Growth rates

The growth of macroalgae was recorded as changes in the fresh weight of macroalgal fragments for a particular interval of time. Fragments were taken from beakers using pinset, and gently blotted on a paper to remove seawater before weighing. Biomass was measured by recording the fresh weight of macroalgal on an analytical digital balance Sartorius (Germany). The fresh weights were taken initially, and every 2^{nd} day until the end of the experiment.

Daily growth rates were calculated after measuring the accumulative biomass (in terms of fresh weight), following the formula described by Lignell and Pedersen (1989):

$$DGR\ (\%\ day^{-1}) = [(W_t/W_i)^{1/t}-1] \times 100$$

Where DGR stands for daily growth rates, W_i is initial fresh weight, W_t is fresh weight after particular time period (t days), and t is time (days) of cultivation. Changes in growth rates were measured between time intervals as well as accumulated biomass from initial measurements.

2.8 Nutrient analyses

Nutrients used in this study refer to N, particularly ammonium (NH_4^+). Analysis of NH_4^+ was measured in this study to examine the ammonium content in the water medium, ammonium uptake rates of macroalgae, and nitrogen content of the algal tissue.

2.8.1 Ammonium content in water samples

Water samples were analyzed for ammonium concentrations in a UV spectrophotometer Evolution 60 (USA) using modified indophenols blue methods of Koroleff (1983). An NH_4^+ standard solution was made fresh for each analysis. Ammonium concentrations were measured to determine nutrient uptakes rates. Solutions used were phenol reagent, alkaline (NaOH), chlorox, and mixed-reagent from hypochlorite and citrate. A stock solution of NH_4Cl was made to use for the standard curve. Concentrations used for the standard curve were 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 µM of ammonium. After being stored overnight in room temperature with added reagents, the absorbance was examined in the photometer at 620 nm wave-length. Water samples of 5 mL each were transferred to a sterile plastic tube. Dilutions were needed for all samples except the control in order to be in the sensitivity range of the analysis. The phenol reagent was added to the sample in the amount of 143 µL. After shaking for a few seconds, 143 µL of mixed reagent were added. As final shake as done and all samples were covered with aluminum foil to be stored overnight in room temperature and run on the photometer for

absorbance determination. Calculation of ammonium concentrations in the water samples were based on the calibration curve of the ammonium standard solutions.

2.8.2 Ammonium uptake

Ammonium uptake in this study was determined as the disappearance of ammonium in the water medium after being taken up by the macroalgae, and calculated for each time interval using a modified formula described by Rees et al. (1998):

$$A = (C_b - C_e)/B$$

Where A is the concentration of ammonium taken up by macroalgae (μM/g FW), C_b and C_e are ammonium concentration at the beginning and end of a time interval (μM) respectively, and B is the macroalgal biomass (g fresh weight).

Uptake rates were then calculated over the time at each interval according to equation described by Naldi and Wheeler (2002):

$$V = (\mu MN_i - \mu MN_f)/(t.B)$$

Where μMN_i and μMN_f are the initial and final concentration of ammonium in the medium (μM), t is time period (minute, hour, or day), and B is the macroalgal biomass (g fresh weight).

To determine the saturation kinetics of ammonium uptake of macroalgae from nutrient-enriched waters, Michaelis-Menten functions were fitted to uptake data by fitting a non-linear regression to the data using the programme ORIGIN 61. Uptake rates were plotted against the mean substrate concentration for each time interval, according to the formula described by Campbell (1999):

$$V = (V_{max} \times S)/(K_m + S)$$

Where V is the uptake rate, S is the ammonium concentration treatment, V_{max} is the maximum uptake rate, and Km is the half-saturation constant for uptake. Estimates of

uptake at the concentrations used were made by fitting the estimated V_{max} and K_{μ} constants to the Michaelis-Menten equation.

2.8.3 Ammonium content in macroalgal tissue

Filtrate for ammonium analyses were prepared following the method described by Lartigue and Sherman (2005) after being modified. Approximately 0.5 g crude extracts were prepared by grinding frozen algal material with ceramic mortar to a fine powder. The homogenized algal material boiled in 45 mL deionized water for 10 min and extracted for 24 hours at 4^{o}C. Extract was centrifuged in 3000 rpm for 10 min in 4^{o}C. The supernatant was taken by syringe and filtered through a 0.45 μm disposable filter (Sartorius) to get the clean filtrate and directly stored in a plastic sterile tube. The pellet was stored in -80^{o}C for back up. Filtrate samples were then analyzed in the UV spectrophotometer by the indophenols blue method of Koroleff (1983) using the same procedures for water sample analysis of ammonium.

2.8.4 C/N ratio of macroalgal tissue

The ratio of total nitrogen (%N) and carbon (%C) content, C:N, has been widely used to determine a potential limitation of microalgal (Donaghay et al., 1978) and macroalgal (Corzo and Niell, 1991; Campbell, 1999; Kang et al., 2011) growth by nitrogen availability. This study attempts to give explanation of C:N behavior of *D. bartayresiana* and *G. verrucosa* as a response to a variety of light intensities and high nitrogen availability. The observed behavior is explained in terms of the difference between the rates of increase in nitrogen and carbon content. The effect of light availability and various ammonium concentrations on total nitrogen content was measured. The results obtained were used to investigate physiological response of macroalagae involving nitrogen, in terms of photosynthesis and growth rates.

Samples for %N and %C were determined after being prepared as a homogenous-dried form. Samples were dried by exposure to constant heat at 60^{o}C for 48 hours. Dried samples were ground with mortar and pestal into a powder form. Powder samples were stored in a precombusted vial bottle until analysis in room

temperature. Approximately 1000 µg of sample (weighed using a Mettler Toledo balance) was transferred into a tin cup. The tin cup with sample inside was folded into a small ball shape. Every five samples were standardized using a control from leaf apple extract. %N and %C in algal tissue were then analyzed using a CHN auto analyzer EA 2100.

2.9 Pigment analyses

Pigment analysis was conducted in this study including chlorophyll-a and phycobiliproteins. Phycobiliproteins examined in this study were phycoerythrin (PE) and phycocyanin (PC).

2.9.1 Chlorophyll-a

Chlorophyll-a (chl-a) content was determined photometrically by following the method described by Inskeep and Bloom (1985). Approximately 0.1 g of algal sample was transferred into test-tubes containing 5 ml of dymethylformamide (DMF). Samples were stored in a refrigerator at 4^{o}C in darkness for 4 days. The extinction of the DMF solution was then measured at 664.5 nm in a UV spectrophotometer. The chlorophyll content was calculated using the formula:

$$\text{Chl-a (mg L}^{-1}) = 12.7 \times E_{664.5}$$

where E is the extinction of the DMF solution at wavelength 664.5 nm, with 12.7 as the constant.

2.9.2 Phycobiliproteins

Phycobiliproteins were extracted using the method described by Beer and Eshel (1985). Sample preparation was done using 0.03-0.1 g of algal tissue on average. Algal tissue was ground using a dismembrator for 3 min with 1500 RPM. All sample preparation was done under dark conditions and maintained frozen by emerging in liquid nitrogen. After being homogenized, samples were transferred into centrifuge test-tubes, and extracted in 5 mL of 1.0 M phosphate buffer (pH = 6.8). The extracts were centrifuged

at 6000 g in 4°C for 20 min. The supernatant was used for phycobiliprotein determination by measuring the absorbance in a UV spectrophotometer (Shimadzu, Japan). The phycoerythrin and phycocyanin concentrations were determined using the equations:

$$PE = ((A_{564} - A_{592}) - (A_{455} - A_{592})\ 0.20)\ 0.12$$
$$PC = ((A_{618} - A_{645}) - (A_{592} - A_{645})\ 0.51)\ 0.15$$

where PE and PC are phycoerythrin and phycocyanin, respectively, A is the absorbance in UV spectrophotometer under different wavelengths (λ = 455, 564, 592, 618, 645 nm).

2.10 Total protein content

Total protein content was measured following the method described by Bischof et al. (2000) as a modification of the Bradford method. Overall protein content in crude extracts was determined using a commercial Protein Assay (BioRad). Sigma Protein Standard was used as BSA stock solution. Protein content was determined by measuring extinction at wave length 595 nm using UVPC spectrophotometer (Shimadzu, Japan). Protein concentration was determined according to a calibration curve prepared with known concentrations of 1 mg/mL bovine serum albumin.

2.11 Statistical analyses

Mean values and their standard deviations were calculated from the different replicates per treatment. Uptake data were tested for assumptions of normality by examining heterogeneity of variance (Cochran's test). Non-normal data were subject to root transformation. Analyses of variance (ANOVA) was employed to examine for differences in total uptake, nutrients in macroalgal tissue content, and maximum quantum yield between ammonium treatments. The significance level used was $p < 0.05$. Tukey's test was used for post hoc analyses of multiple comparisons among treatment means from significant ANOVA test. The linear regression was used to analyze the correlation between two observed parameters. The computer software STATISTIKA 9.0 was used for all analyses.

2.12 Experiments

In this study, experiments were conducted in order to answer the research questions, on the physiological response of macroalgae under different irradiance and various ammonium concentrations. The following outline previously series of experiments were conducted:

1. Ammonium uptake experiments of *D. bartayresiana* and *G. verrucosa*.
2. Physiological responses of *G. verrucosa* under low irradiance and various ammonium concentrations.
3. Physiological responses of *G. verrucosa* under high irradiance with various ammonium concentrations.
4. Physiological response in terms of protein content determination of *G. verrucosa* under low and high irradiance with high ammonium concentrations.

2.12.1 Nutrient uptake experiments of *D. bartayresiana* and *G. verrucosa*

Short term experiments of 360 minutes were conducted to determine on nutrient uptake rates for *D. bartayresiana* and *G. verrucosa*. After starvation precondition treatments, nutrient uptake experiments were conducted in a climate chamber (24^{o}C) under irradiance of 70 µmol photons $m^{-2}s^{-1}$.

The experiments were conducted with approximately 1 g of algal tissue placed in glass beakers. Fresh weights of all algal material were measured after blotting with tissue and weighing using a balance (Sartorius), before placing in the beakers. The beakers were filled with 250 mL of filtered (0.2 µm) seawater enriched with ammonium, as NH_4Cl, in various concentrations. Concentrations of ammonium were 0, 50, 100, 200, and 400 µM. A series of beakers without algae with the same various ammonium concentrations were used as a control. Each treatment included 6 replicates. All glass beakers were placed on a magnetic plate with magnetic stir bars (2 cm length) inside the beakers, with a rotational speed of 120 rpm. This aimed to give periodic water movement in order to prevent localized depletion of ammonium in the medium.

Rates of ammonium uptake were determined from the depletion of ammonium content in the water samples. Water samples (10 mL) were taken using micropipette (Eppendorf) at 0, 15, 30, 60, 180, and 360 minutes of experimental period. Water samples were transferred in sterile plastic centrifuge tubes (15 mL) and stored at -80^{o}C

until the time of nutrient analysis. The photosynthesis performance (F_v/F_m) of algal tissue was measured initially and finally using the PAM 2100 fluorometer (Walz, Germany). Photos were taken during the experimental period to detect color change over time.

The experiments were followed by nutrient analysis in the water samples and algal tissue. This aimed to obtain the value of ammonium content in the water sample, ammonium uptake rates, ammonium content in macroalgal tissue, C:N of macroalgal tissue, and tissue nitrogen content (%N).

2.12.2 Physiological responses of *G. verrucosa* under low irradiance in various ammonium concentrations

An experiment of 14 days as conducted in climate room of 24^{o}C under irradiance of 70 µmol photons $m^{-2}s^{-1}$ in a 12:12 hr light:dark photoperiod. Approximately 1 g of algal material was placed in glass beakers with 1 L of enriched filtered (0.2 µm) seawater (NH_4Cl addition) in concentration of 0, 200, 400, 800, 1200, 2000 µM. Series of beakers without ammonium addition were used as controls. Four replicates were included for each concentration including the control. Stirrer bars were placed at the bottom of each beaker. All beakers were placed on magnetic plates and stirred with the lowest rotational speed (120 rpm). Culture media was changed every other day of the experiments to maintain ammonium concentrations at the initial level.

Water samples were taken initially, and every other day of the experiment up to day 14. Abiotic parameters of seawater and wet weight of macroalgae were measured on day 0, 2, 4, 7, 11, and 14. Photosynthetic performances were measured for day 0, 2, 4, 7, 11, and 14. Samples for ammonium in algal tissue, C:N, total nitrogen content, and pigment analysis were taken initially and finally. The samples were stored in -80^{o}C until analysis.

Laboratory analyses conducted were nutrient analyses (ammonium content in water sample, ammonium content in macroalgal tissue, C:N of macroalgal tissue, tissue nitrogen content), and pigment analyses (chl-a and phycobiliproteins). Data analyses for this experiment included abiotic parameters performance, nutrient analysis (uptake, uptake rates, C:N of macroalgal tissue, tissue nitrogen content (%N), photosynthetic

performance (F_v/F_m, PI curve, α, I_k, and $rETR_{max}$), accumulative biomass, and growth rates.

2.12.3 Physiological responses of *G. verrucosa* under high irradiance in various ammonium concentrations

An experiment was conducted for 14 days in a climate room (24°C) under irradiance 300 µmol photons $m^{-2}s^{-1}$ in a 12:12 hr light:dark photoperiod. Approximately 1 g of algal material was placed in glass beakers with 1 L of enriched filtered (0.2 µm) seawater.

Based on the former experiments of physiological response with low irradiance (70 µmol photons $m^{-2}s^{-1}$), ammonium concentration treatments of 400, 800, and 1200 µM were used for comparison of performance for the next experiments under high irradiance (300 µmol photons $m^{-2}s^{-1}$). As a control, no nutrient additions of NH_4Cl were also used.

Four replicates were used for each concentration treatment including the control. Stir bars were placed at the bottom of each beaker and all beakers were placed on magnetic plates with low rotational speed (120 rpm). Culture media were changed every other day to avoid nutrient depletion.

Water samples (10 mL) were taken initially, and every other day of the experiment. Abiotic parameters and wet weight were measured initially and at day 2, 4, 7, 11, and 14. Photosynthetic performance was measured initially and on day 2, 4, 7, 11, and 14. Macroalgal tissue was taken for analysis of ammonium content in the tissue, C/N ratio and nitrogen content (%N), and pigments (Chl-a and phycobiliprotein) were taken initially and finally. Those tissue samples were stored in -80°C until analysis.

Laboratory analyses were conducted for the water samples and macroalgal tissue. Laboratory analyses included nutrient analyses (ammonium content in water samples, C:N, and %N), and pigments analyses (chl-a and phycobiliprotein). Data analyses conducted for these experiments were abiotic parameters, nutrients (uptake rates, C:N, and %N), photosynthetic performance (F_v/F_m, PI curve, α, I_k, and $rETR_{max}$), accumulative biomass, and growth rate.

2.12.4 Ammonium and irradiance stress experiment of *G. verrucosa* under low and high irradiance in high ammonium concentrations

An experiment to examine the effects of light and ammonium concentration of protein content was conducted in a climate room (24^{o}C) under irradiance of 70 and 300 μmol photons $m^{-2}s^{-1}$ in a 12:12 hr light:dark photoperiod over seven days.

Approximately 1 g of macroalgal material was placed in glass beakers with 1 L of enriched filtered (0.2 μm) seawater (NH_4Cl addition) in a concentration of 1200 μM and 0 μM as a control. Three replicates were used in this experiment for each treatment. Stir bars (2 cm length) were placed in each beaker and all beakers were placed on magnetic plates. A low rotational speed of 120 rpm was used to avoid localized nutrient depletion. Electric fans were used around high irradiance chambers to ensure the air circulation. This was done in order to maintain the temperature at the desired value. Culture media was changed every other day to avoid depletion of nutrients. Fresh weight and photosynthetic performance were measured on day 0, 2, 4, and 7. Algal tissue were taken initially and finally and stored in -80^{o}C until total protein content analysis.

3. STUDY 1

Ammonium uptake of two tropical macroalgae: *Dictyota bartayresiana* (Phaeophyceae) and *Gracilaria verrucosa* (Rhodophyceae) under low irradiance

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Abstract

Macroalgae can use a wide variety of nitrogenous compounds to fulfill their nitrogen requirements and for growth, with NH_4^+ being the primary source in most circumstances. The ammonium uptake of *Dictyota bartayresiana* J.V. Lamoroux and *Gracilaria verrucosa* (Hudson) Papenfuss was determined in a series of perturbation experiments under various ammonium concentrations and low irradiance. Uptake experiments were conducted in 360 minutes. Ammonium concentrations used were 0, 50, 100, 200 and 400 µM and irradiation was 70 µmol photons $m^{-2}s^{-1}$. *G. verrucossa* had higher ammonium uptake rates than *D. bartayresiana*. Maximum quantum yield of *G. verrucosa* was higher in all ammonium treatments compared to initial values, while final maximum quantum yield of *D. bartayresiana* dropped. A surge ammonium uptake rate was observed in the first 15 minutes for both species. *G. verrucosa* showed no saturation point over the time intervals up to 360 minutes, whereas *D. bartayresiana* reached saturation and showed discoloration. These results indicated that *G. verrucosa* may be better suited for high ammonium environments.

Key words: ammonium, *D. bartayresiana*, *G. verrucosa*, irradiance, nutrient uptake, saturation point

Introduction

Eutrophication, resulting from excessive nutrient enrichment, is commonly recognized as in which substantially affect alterations of nutrient availability and food web structure result in general pattern of community change (e.g., plant coverage, dominant species, species diversity, species interaction) (Lin and Hung, 2004). Excessively dominant macroalgal species can have negative ecosystem-wide effects (Göltenboth, 2006). Opportunistic macroalgae which reported to have high nutrient uptake rates, such as *Ulva* and *Gracilaria*, frequently found as a blooming species in association with high nutrient load (Valiaela et al., 1992; Fujita 1985; Peckol et al., 1994; Rosenberg and Ramus, 1982).

The growth and impact of macroalgae on others communities (e.g., coral reefs, seagrass) had been shown to have strong correlation with nutrient effects (Lapointe, 1997; Stimson et al., 2001; Littler and Littler, 2000). Phase shift from coral to algal dominance have been increasingly common worldwide over the past several decades (Hughes et al., 1999; Lapointe 1997). Macroalgae commonly co-occuring on a variety of natural hard substrate communities (Stachowicz and Hay, 1999) or growing epiphytically on many reef species as *Dictyota* spp. frequently found (Littler and Littler, 2000; Lirman and Biber, 2000; Beach et al., 2006).

Nitrogen is an important factor controlling algal growth in marine environments (Lobban and Harrison, 1994). Numerous studies have examined nitrogen utilization by marine macroalgae, including studies on uptake physiology (e.g., determination of kinetics parameters, interactions between ammonium and nitrate uptake, influence of algal nitrogen content on uptake) (Campbell, 1999; Smit, 2002; Naldi and Wheeler, 2002). Other studies have examined the impact of macroalgal nitrogen requirements and uptake capacity on nitrogen cycling in the environment (Naldi and Wheeler, 2002).

In general, macroalgae can use a wide variety of nitrogenous compounds to fulfill their nitrogen requirements: ammonia, nitrate, urea, amino acid, and nucleosides may be taken up from the growth medium (Lobban and Harrison, 1994), with NH_4^+ being the primary sources in most circumstances. For most macroalgae, NH_4^+ is the preferred inorganic nitrogen form compared to NO_3^-. According to Rees (2003), ammonium uptake is more efficient than nitrate uptake in macroalgae. In addition, the

presence of ammonium can suppress the uptake and assimilation of less reduced forms of nitrogen, mainly nitrate, in macroalgae (DeBoer, 1981; Naldi and Wheeler, 2002).

The characteristics of ammonium uptake by macroalgae have been extensively studied (Fujita et al., 1988; Tyler and McGlathery, 2006; Naldi and Wheeler, 2002). Most current research of macroalgal nutrient uptake (e.g., *Gracilaria*) had focused on their physiological responses to the polluted environment under low nutrient concentrations (Amir et al., 2000). However, there is comparatively less information on the macroalgal resistance of high ammonium concentrations (Yu and Yang, 2008).

Gracilaria and *Dictyota* are commonly found species in eutrophic coastal area (Rosenberg and Ramus, 1982; Beach et al., 2006), occur as free-living, largely monospecific beds in a limited number of tropical coastal waters (Smit, 2002; Beach et al., 2006). The economic value of *Gracilaria* as hydrocolloids producers make *G. verrucosa* interesting to be observed in extended studies. As a species that frequently co-occurs with other species in high nutrient area, it will be interested to obtain information on the response of *D. bartayresiana* to various ammonium concentrations. Thus, it will extend the information to previous studies of *Dictyota* and *Gracilaria*.

The objectives of this study were to investigate the nutrient uptake capacity of two pan-tropical macroalgae: *D. bartayresiana* J.V. Lamoroux and *G. verrucosa* (Hudson) Papenfuss under laboratory conditions, and to determine preferential uptake concentrations. The results described ammonium uptake rates in various ammonium concentration treatments, tissue nutrient contents, and photosynthetic performance, as information for further examination of a physiological response.

Materials and methods

Macroalgae species. *D. bartayresiana* J.V. Lamoroux (Phaeophyceae) was collected in October 2008 from a reef flat in Spermonde Archipelago, Indonesia (4°52’33”S; 119°6’50”E), and was transferred and cultured in mesocosm tanks in the aquaculture facility at Leibniz-Zentrum für Marine Tropenökologie, Universität Bremen. *G. verrucosa* (Hudson) Papenfuss (Rhodophyceae) was collected from Bay of Muaragembong, Indonesia (6^{o}0’19”S; 107^{o}1’28”E), in August 2009, and transported to

the already mentioned aquaculture facility to be prepared for several experiments in the Marine Botany Laboratory.

Nutrient uptake experiments. Short term experiments of 360 minutes were conducted to determine ammonium uptake rates for *D. bartayresiana* and *G. verrucosa*. After starvation precondition treatments, nutrient uptake experiments were conducted in a climate chamber (24^{o}C) under irradiance of 70 µmol photons $m^{-2}s^{-1}$.

For each ammonium concentration, approximately 1 g of algal tissue was placed in glass beakers. Fresh weights of all algal material was measured after blotting with tissue and weighed (Sartorius balance), before placing into the beakers, filled with 250 mL of filtered (0.2 µm) seawater enriched with ammonium, as NH_4Cl. Concentrations of ammonium were 0, 50, 100, 200, and 400 µM (n = 6). A series of beakers without algae with the same ammonium concentrations were used as a control. All glass beakers were placed on a magnetic plate with magnetic stir bars (2 cm length) inside the beakers, with a rotational speed of 120 rpm. This aimed to give constant water movement in order to prevent localized depletion of ammonium in the medium.

Water samples (10 mL) were taken using micropipettes (Eppendorf) at 0, 15, 30, 60, 180, and 360 minutes of experimental period. Water samples were transferred in sterile plastic centrifuge tubes (15 mL) and stored at -80^{o}C until the time of nutrient analysis. The photosynthesis performance (F_v/F_m) of algal tissue was measured initially and finally using the PAM 2100 fluorometer (Walz, Germany). Photos were taken during the experimental period to detect color changes over time.

The experiments were followed by ammonium analysis of the seawater media and nutrient content of the algal tissue. This aimed to obtain the value of ammonium content in the water sample, ammonium uptake rates, ammonium content in macroalgal tissue, C/N ratio of macroalgal tissue, and tissue nitrogen content (%N).

Photosynthetic performance. Photosynthetic activity was determined by measuring variable chlorophyll-fluorescence using a PAM 2100 device (Walz, Germany). Maximum quatum yield was calculated as the ratio of variable to maximum fluorescence (F_v/F_m) of dark-acclimated algae (Bischof et al., 1998). The algal thalli were fixed to the end of the fiberoptics and placed into a beaker filled with seawater.

The beaker was covered with aluminum foil to avoid light leakage from other light sources.

Ammonium content in water samples. Ammonium concentrations of each time period were measured to determine nutrient uptake rates between intervals. Water samples were analyzed for ammonium concentrations on a UV spectrophotometer (Evolution 60, USA) using a modified indophenol blue method of Koroleff (1983). An NH_4^+ standard solution was made fresh for each analysis. Reagents used were phenol, alkaline (NaOH), chlorox, and mixed-reagent from hypochlorite and citrate. After being stored overnight in room temperature with added reagents, the absorbance was measured in the photometer at 620 nm wave-length. Dilutions were needed for all samples, except the controls, in order to be in the sensitivity range of the analysis.

Ammonium uptake. Ammonium uptake in this study was determined as the disappearance of ammonium in the water medium after being taken up by the macroalgae, and calculated for each time interval using a modified formula described by Rees et al. (1998):

$$A = (C_b - C_e)/B$$

Where A is the concentration of ammonium taken up by macroalgae (μM/g FW), C_b and C_e are ammonium concentration at the beginning and end of a time interval (μM), respectively, and B is the macroalgal biomass (g fresh weight).

Rates of ammonium uptake were determined from the depletion of ammonium content in the water samples, calculated over the time at each interval according to an equation described by Naldi and Wheeler (2002):

$$V = (\mu MN_i - \mu MN_f)/(t.B)$$

Where V is uptake rate; μMN_i and μMN_f are the initial and final concentration of ammonium in the medium (μM), t is time period (minute, hour, or day), and B is the macroalgal biomass (g fresh weight).

To determine the saturation kinetics of ammonium uptake of macroalgae from nutrient-enriched waters, the non-linear Michaelis-Menten function was fitted to the uptake data using the program ORIGIN 61. Uptake rates were plotted against the mean

substrate concentration for each time interval, according to the formula described by Campbell (1999):

$$V = (V_{max} \text{ x } S)/(K_m + S)$$

Where V is the uptake rate, S is the ammonium concentration treatment, V_{max} is the maximum uptake rate, and Km is the half-saturation constant for uptake. Estimates of uptake at the concentrations used were made by fitting the estimated V_{max} and K_μ constants to the Michaelis-Menten function.

Ammonium content in macroalgal tissue. Filtrate for ammonium analyses were prepared following a modified method described by Lartigue and Sherman (2005). Approximately 0.5 g frozen algal material was ground with ceramic mortar to get fine powder crude extracts. The homogenized algal material was boiled in 45 mL deionized water for 10 minutes and extracted for 24 hours at 4^{o}C. Extract was centrifuged in 3,000 rpm for 10 minutes at 4^{o}C. The clean filtrate of the filtered supernatant (0.45 µm disposable filter, Sartorius) was directly stored in a plastic sterile tube, and was then analyzed in the UV spectrophotometer by the indophenol blue method of Koroleff (1983) using the same procedures for water sample analysis of ammonium.

C/N ratio of macroalgal tissue. Samples of macroalgal tissue were prepared as a homogenous-dried form by exposure to constant heat at 60^{o}C for 48 hours. Dried samples were ground with mortar and pestal into a fine powder form, and were stored in a precombusted vial bottle at room temperature until analysis. The %N and %C in algal tissue were then analyzed using a CHN auto analyzer EA 2100.

Statistical analyses. Mean values and their standard deviations were calculated from the different replicates per treatment. Uptake data were tested for assumptions of normality by examining heterogeneity of variance (Cochran's test). Non-normal data were subject to root transformation. Analyses of variance (ANOVA) was employed to examine for differences in total uptake, nutrients in macroalgal tissue content, and maximum quantum yield between ammonium treatments. The significance level used was $p < 0.05$. Tukey's test was used for post hoc analyses of multiple comparisons

among treatment means from significant ANOVA test. The correlation between two observed parameters was analyzed using the linear regression. The computer software STATISTIKA 9.0 was used for all statistical analyses.

Results

D. bartayresiana

Initially, the F_v/F_m values in all ammonium treatments were similar, ranging between 0.743-0.751 (Fig.1). After 360 minutes of exposure in seawater enriched by various ammonium concentrations, *D. bartayresiana* decreased their performance in light utilization for photosynthetic processes, significantly (F = 1020.628; $p < 0.00$). The values dropped drastically to approximately 10% of initial values. Only macroalgae without ammonium addition maintained their high performance of quantum yield (0.749) at the end of the experimental period. The colors of *D. bartayresiana* in all ammonium treatments bleached after 15 minutes of exposure, except for the macroalgae with no ammonium addition.

In this study, ammonium concentrations of the seawater media refer to the amount of ammonium that was not absorbed by the macroalgae at each time interval. The results show high values of ammonium concentrations in water samples in all ammonium treatments at the end of the experiments (Fig.2a.). This indicates overall low nutrient uptake of macroalgae during the entire experimental period.

Curves of ammonium treatments of 50, 100, 200 and 400 µM showed a clear decline in the first hour. In the next two hours, uptake of ammonium in all treatments occured in very small amounts. This was shown by a slight decrease of ammonium concentration in the seawater media, as indicated by the flat curve in the last three hours of the experimental period. No change in ammonium concentration was observed in the absence of algae for all ammonium concentration treatments (Fig. 2b).

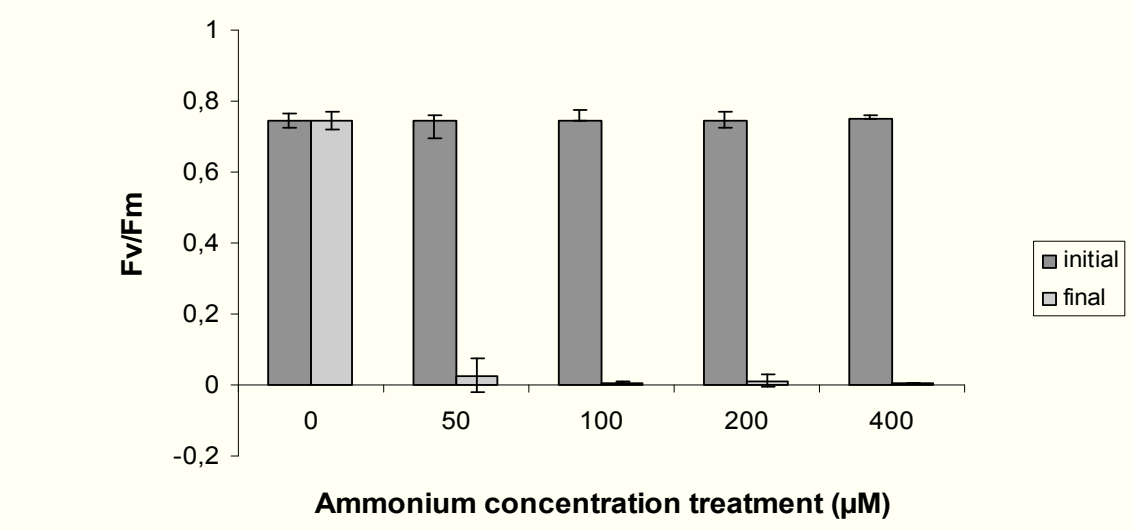


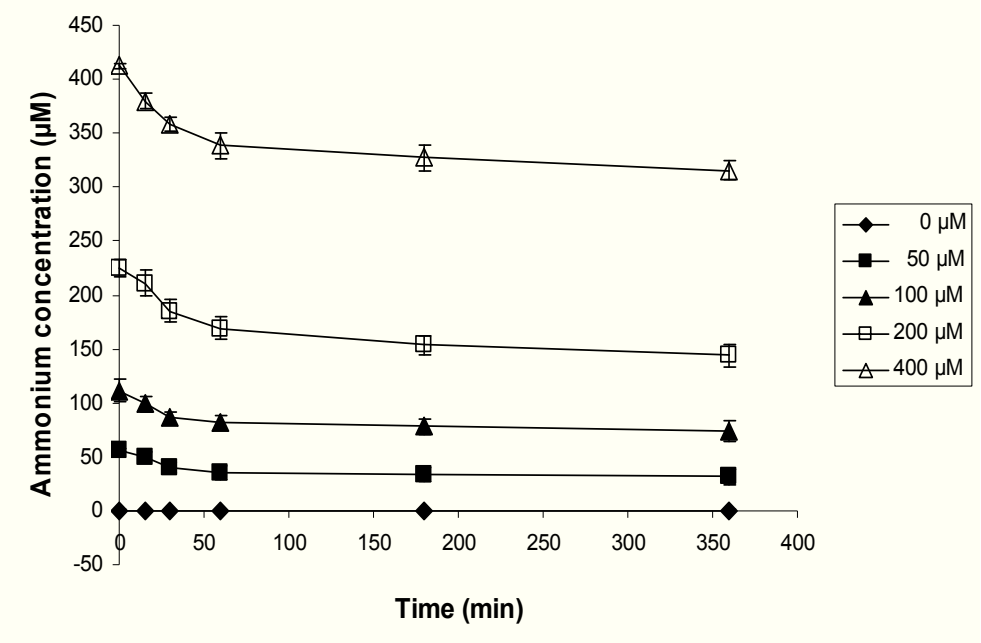
Fig.1. Maximum quantum yield (F_v/F_m) of *D. bartayresiana* before and after treatment with seawater enriched with ammonium concentration of 0, 50, 100, 200, and 400 µM under 70 µmol photons $m^{-2}s^{-1}$ irradiation and in a 360 minute experimental period.

Ammonium uptake of *D. bartayresiana* increased in the first 60 minutes in all ammonium treatments (Fig. 3). Surge uptake appeared in the first 30 minutes for macroalgae with ammonium addition. Maximum uptake of macroalgae for ammonium treatments of 50, 100, and 200 µM were reached at time interval 15-30 minutes. Macroalgae with ammonium addition of 400 µM reached maximum uptake in the first 15 minutes. Uptake generally decreased after one hour of exposure in all ammonium treatments.

The uptake rates were calculated as the uptake of ammonium per time in minutes of each interval period (Fig. 4). The surge phase was highest in the first 30 minutes for all macroalgae with ammonium addition (Fig. 4.) Macroalgae in 400 µM ammonium treatment reached maximum rates after 15 minutes, while macroalgae in ammonium treatments of 50, 100, and 200 µM reached maximum uptake rates in 30 minutes. After one hour, uptake rates remained constant and were near zero (Fig. 4.).

Uptake kinetics of *D. bartayresiana* indicated that ammonium was initially enhanced when macroalgae were exposed to ammonium (Fig. 5; Table 1). This is shown by the linear curve in the first 15 minutes (Fig. 5.), an indication of the highest maximal rate of uptake in this time interval. After 15 minutes, the uptake conformed to Michaelis-Menten saturation kinetics over the range of ammonium concentrations used (Fig. 5). Maximum uptake rates decreased over time intervals (Fig. 5; Table 1).

a)



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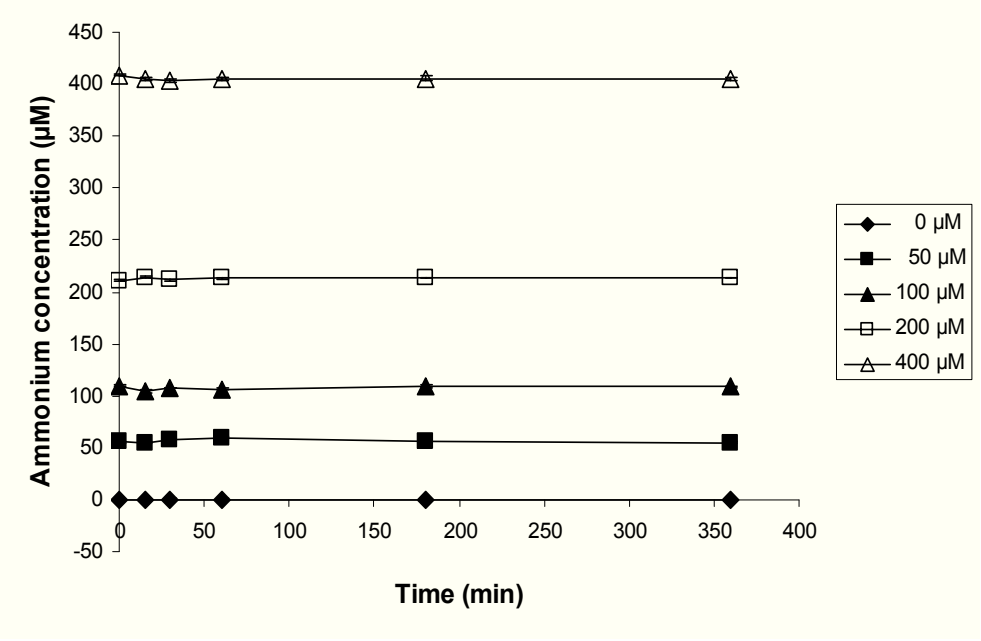


Fig. 2. Changes in ammonium concentration in a) seawater media of *D. bartayresiana* under various ammonium concentration treatments at 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 360 minute experimental period; and b) controls (beakers without macroalgae with ammonium concentration treatments included 0, 50, 100, 200, and 400 µM).

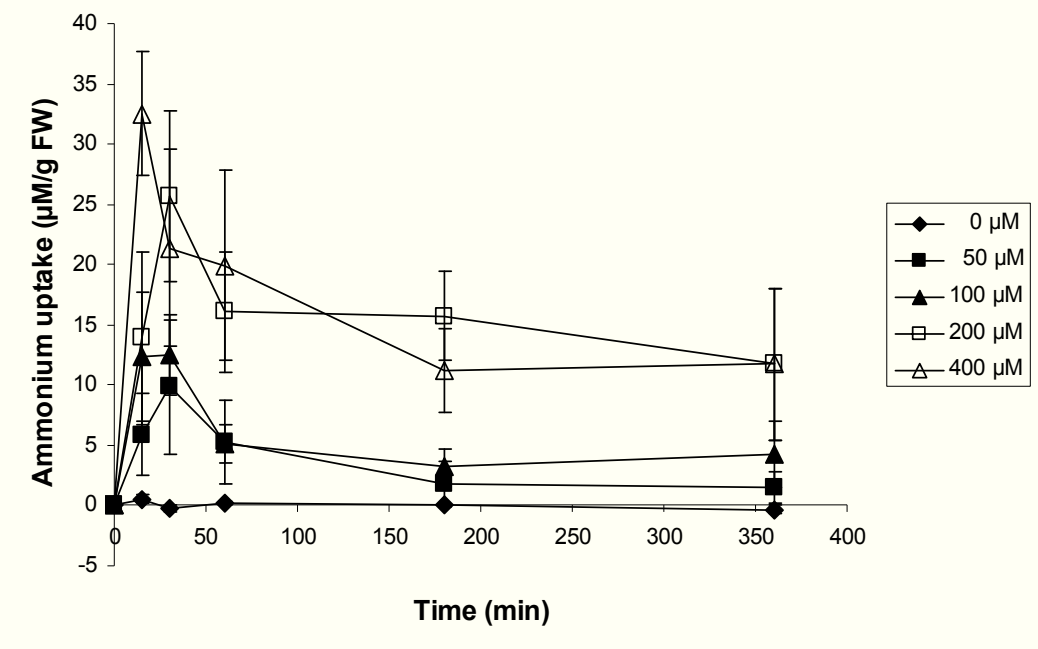


Fig. 3. Ammonium uptake of *D. bartayresiana* with ammonium concentration treatments of 0, 50, 100, 200, 400 µM under 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 360 minute experimental period.

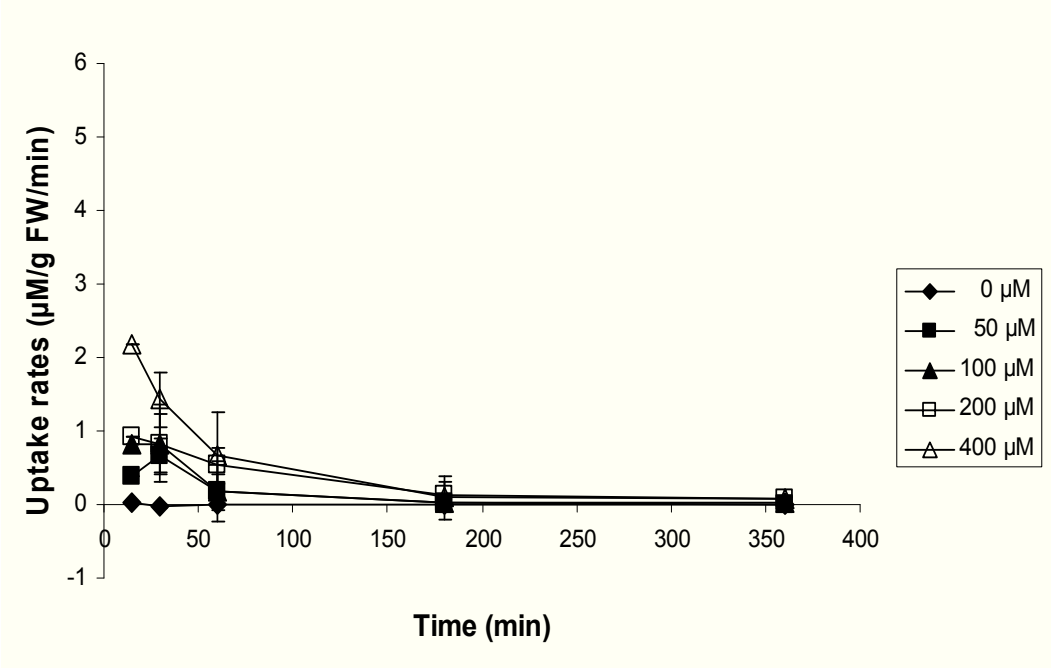


Fig. 4. Ammonium uptake rates of *D.bartayresiana* with ammonium concentration treatments of 0, 50, 100, 200, 400 µM under 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 360 minute experimental period.

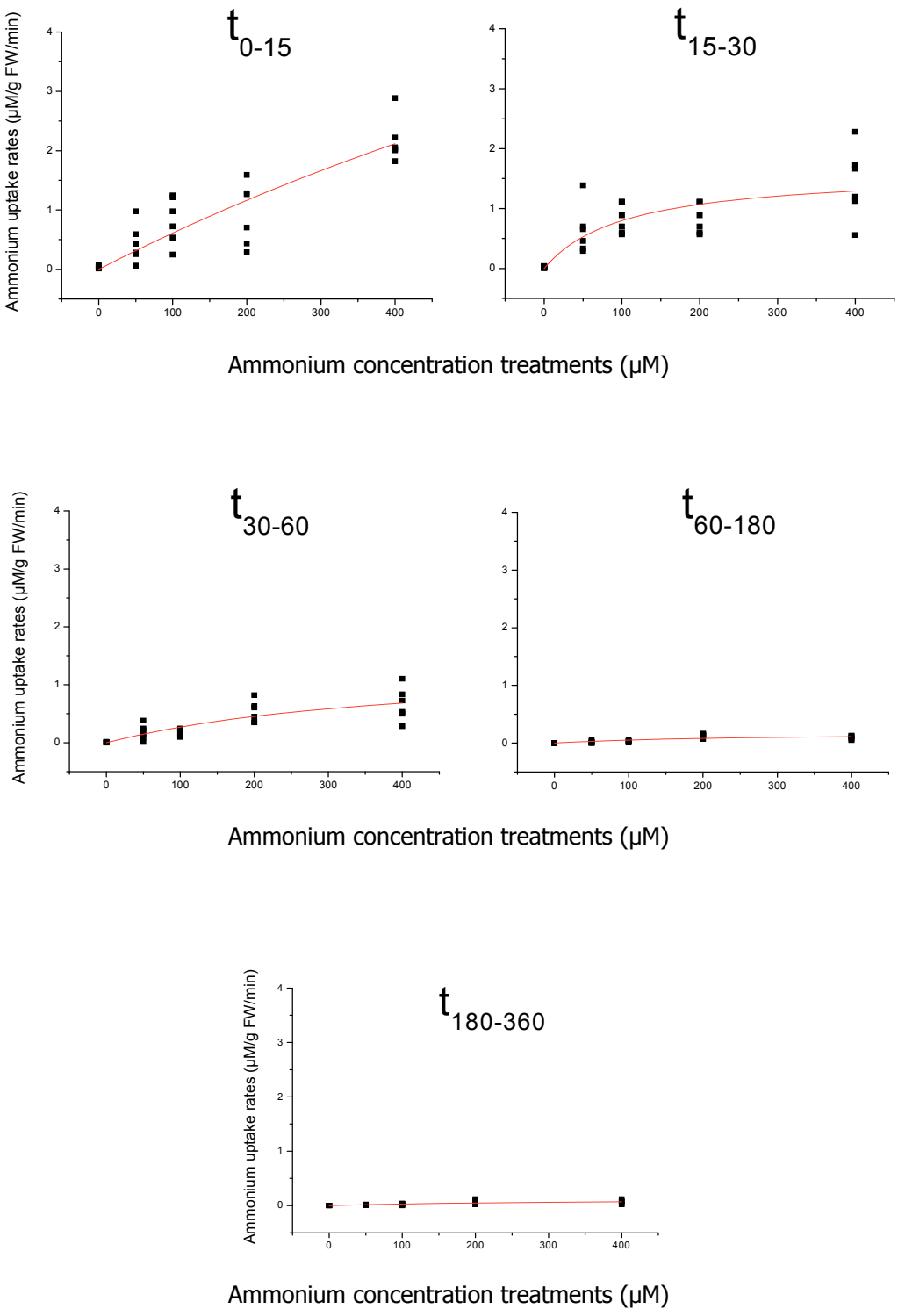


Fig.5. Rate of uptake of ammonium as a function of substrate concentration (Michaelis-Menten saturation curves) for *D. bartayresiana* under 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 360 minute experimental period.

Table 1. Parameters of the Michaelis-Menten saturation curves estimated by non-linear fitting of data from ammonium uptake experiments conducted with *D. bartayresiana* in a 360 minute experimental period; V_{max} is rate of saturation above a certain S (ammonium concentration treatment); K_m is the half saturation constant

Time interval (min)	V_{max} (µM/g FW/min)	K_m (µM)	R^2
0-15	11.69 ± 4.01	2565.33 ± 1809.72	0.77
15-30	1.63 ± 0.31	104.02 ± 55.15	0.61
30-60	1.40 ± 0.58	415.96 ± 287.52	0.68
60-180	0.18 ± 0.07	239.34 ± 169.57	0.60
180-360	0.12 ± 0.06	299.74 ± 267.13	0.51

The total ammonium uptake of *D. bartayresiana* in 360 minutes of experimental period increased significantly ($F = 110.83$; $p < 0.000$) with the increasing of ammonium treatments (Fig. 6). Post hoc test showed significant difference among treatments, except between 50 µM and 100 µM ($p = 0.13$), and between 200 µM and 400 µM ($p = 0.052$). The percentages of the total uptake in sequence were 24, 40, 37, and 48 for ammonium treatments of 50, 100, 200, and 400 µM, respectively.

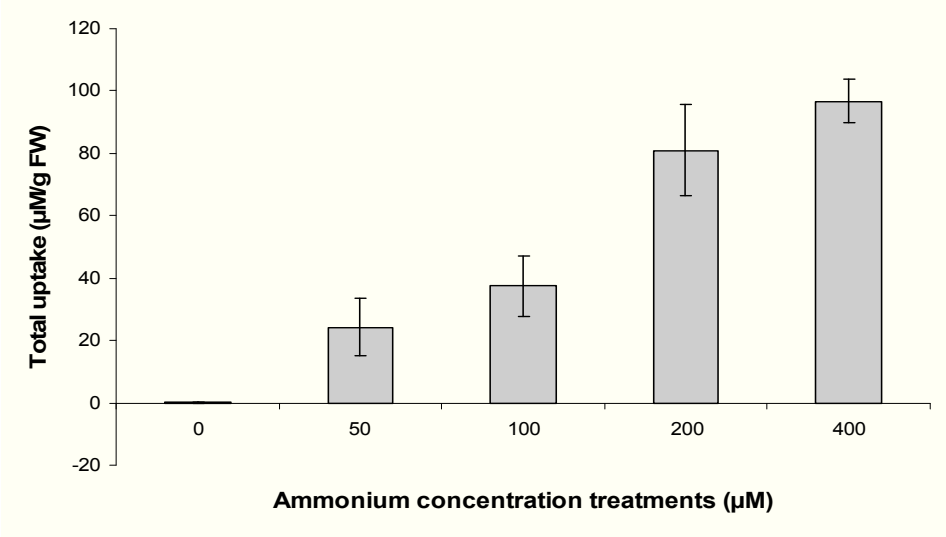


Fig. 6. Total ammonium uptake of *D. bartaresiana* with ammonium concentration treatments of 0, 50, 100, 200, 400 µM under 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 360 minute experimental period.

The ammonium content in the tissue of *D. bartayresiana* increased with the increasing total ammonium uptake during the experiment and showed a strong correlation ($r^2 = 0.95$) (Fig 7), as well as with the ammonium treatments ($F = 7.20$; $p < 0.000$) (Fig. 8a). However, from post hoc test of ammonium content in tissue, only macroalgae with 200 and 400 µM ammonium addition that showed significantly higher values than macroalgae with no ammonium addition ($p = 0.002$; $p < 0.000$, respectively).

The C:N values of *D. bartayresiana* were high, ranging at 14-20 (Fig. 8b). However, the ammonium concentration treatments showed no significant difference in C:N ($F = 1.83$; $p = 0.18$), and %N ($F = 2.02$; $p = 0.14$) of the tissue of *D. bartayresiana*.

Data of %N (Fig.8c) expressed low nitrogen content in tissue, ranging between 2.26-3.04% in all ammonium concentration treatments. Although the %N showed slightly decrease with the increasing of ammonium concentration treatments, the statistical analyses showed no significant difference among treatments ($F = 2.02$; $p = 0.14$).

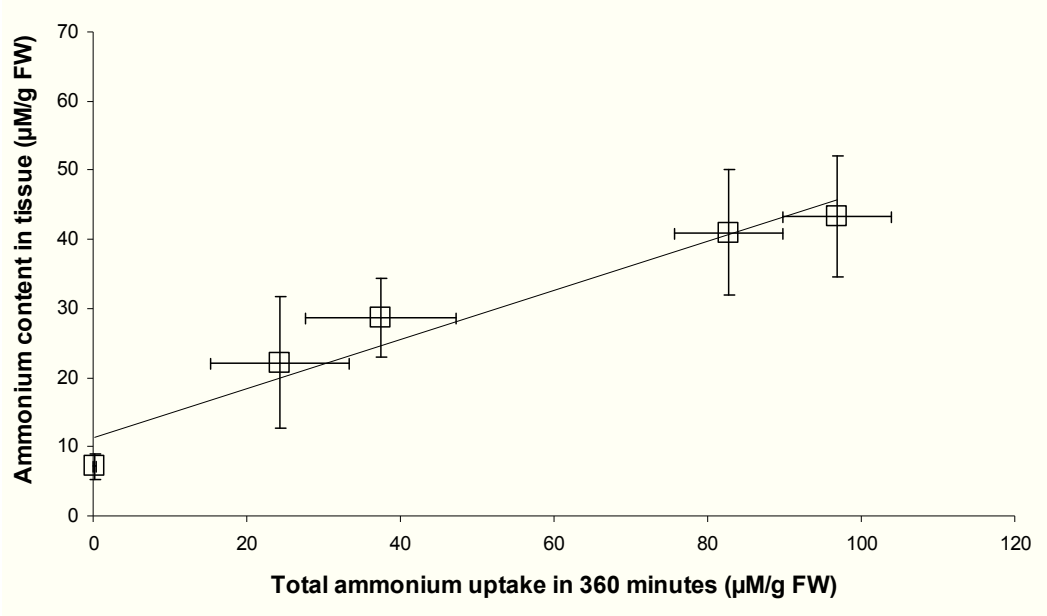
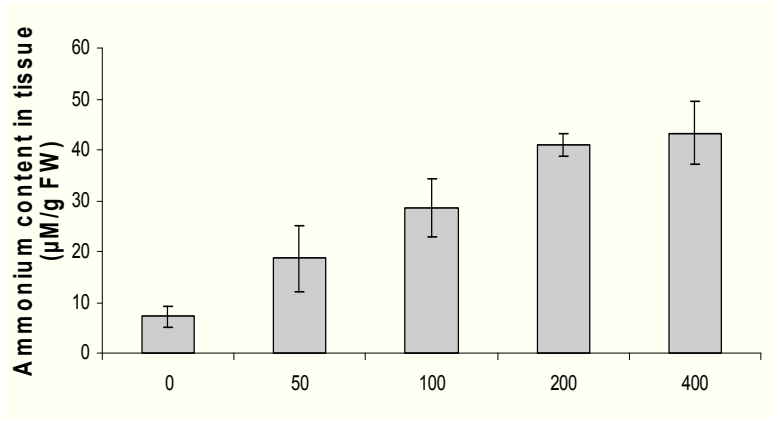
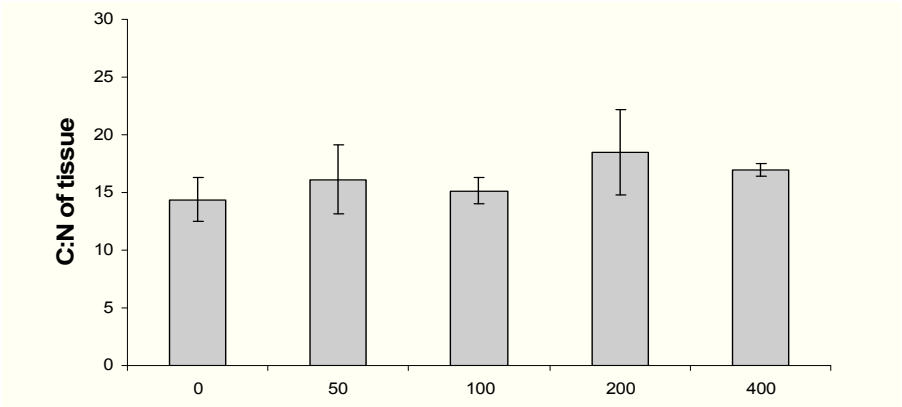


Fig. 7. The correlation between total uptake and internal content in tissue of *D. bartayresiana* with ammonium concentration treatments of 0, 50, 100, 200, and 400 µM under 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 360 minute experimental period.

a)



b)



c)

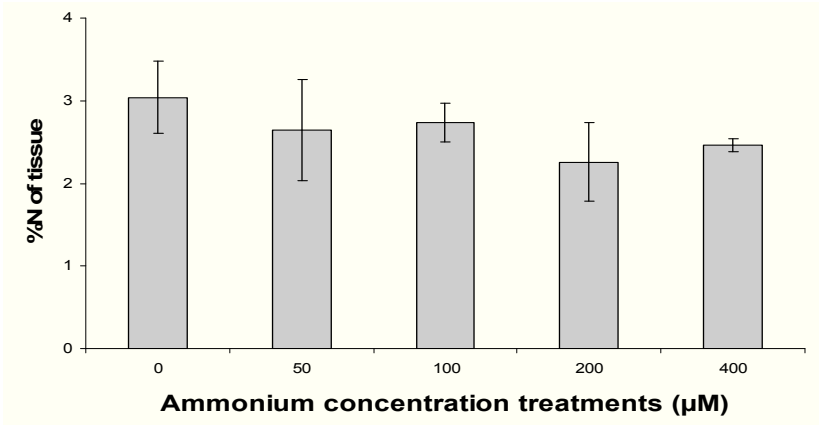


Fig. 8. Nutrient content in macroalgal tissue of *D. bartayresiana* with ammonium concentration treatments of 0, 50, 100, 200, and 400 µM under 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 360 minute experimental period; a) ammonium content in tissue; b) C:N of tissue; c) %N of tissue.

G. verrucosa

In contrast to *D. bartayresiana*, the initial and final maximum quantum yield (F_v/F_m) of *G. verrucosa* was high for all ammonium concentration treatments (Fig. 9). Although quantum yield was approximately 10% higher in macroalgae with ammonium addition at the end of the experiment, these differences were not statistically significant among treatments ($F = 0.58$; $p = 0.68$).

Ammonium concentrations in the media decreased throughout the experiment for all ammonium treatments, but were never completely exhausted (Fig. 10a). The greatest decrease was observed in the first hour of incubations in all ammonium concentration treatments. The ammonium concentration in the seawater media of macroalgae with ammonium addition of 400 µM had the greatest changes in the first hour, shown by the steep decrease of the curve. No change in ammonium concentrations were observed in the controls in the absence of macroalgae (Fig.10b).

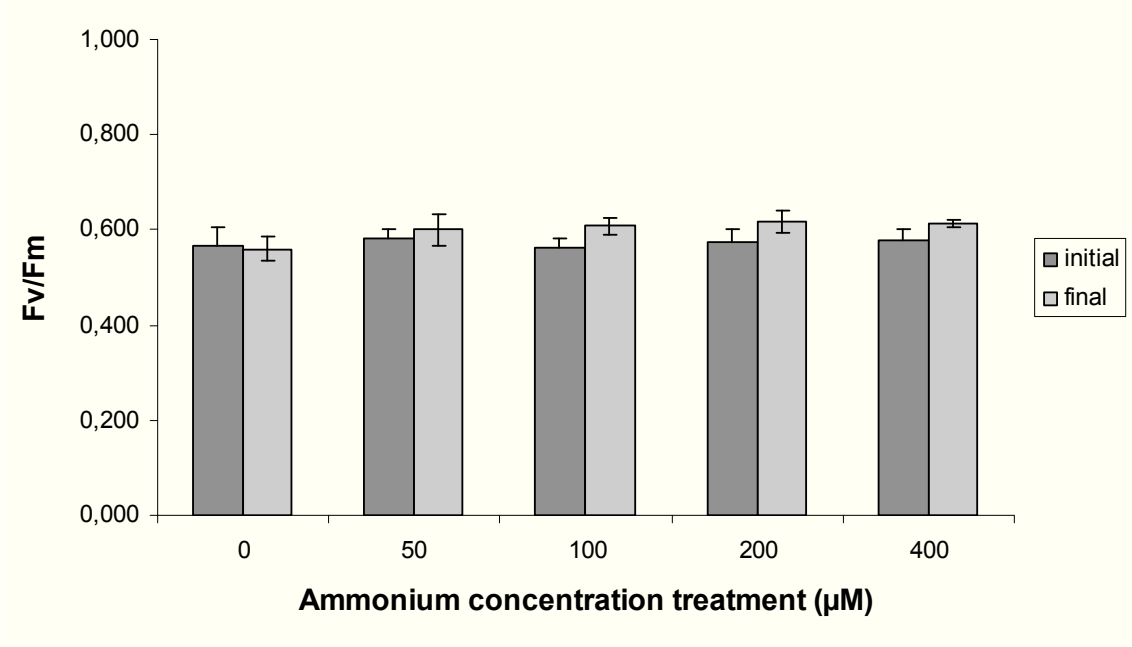
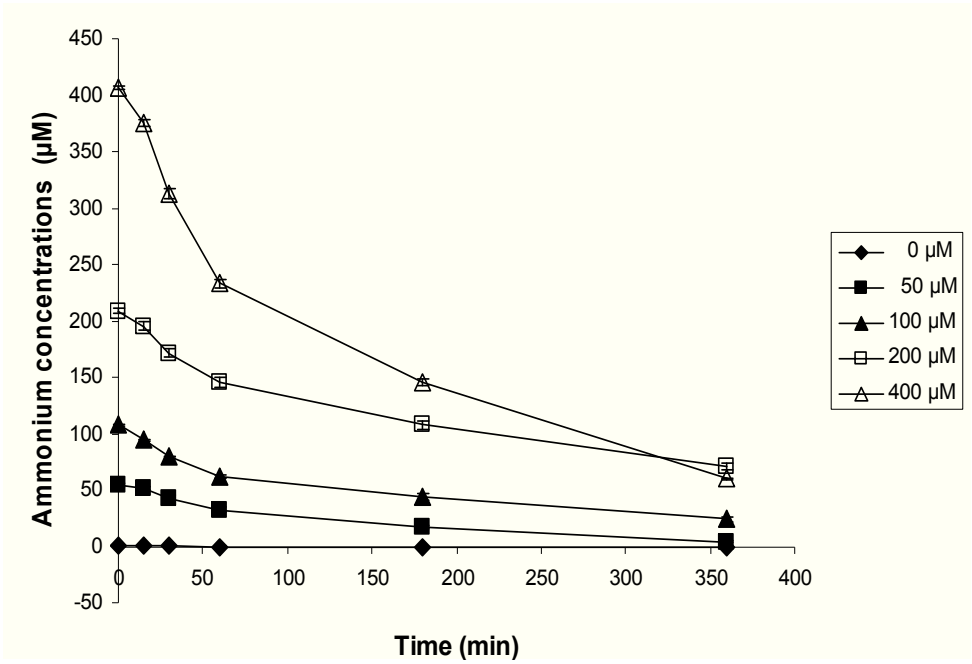


Fig. 9. Maximum quantum yield (F_v/F_m) of *G. verrucosa* before and after treatments using seawater enriched with ammonium concentration treatments of 0, 50, 100, 200, and 400 µM under 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 360 minute experimental period.

a)



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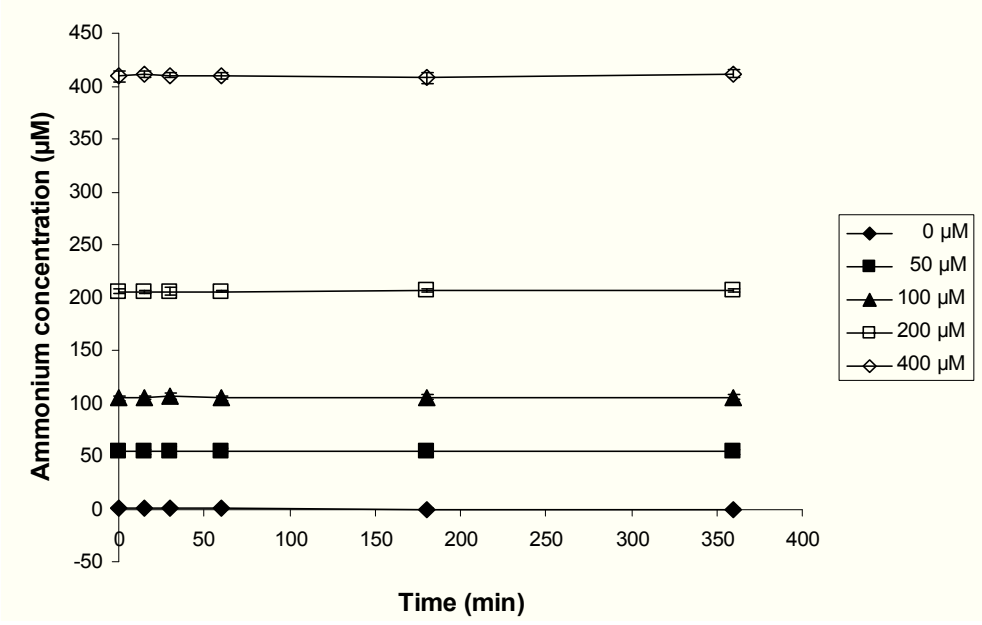


Fig. 10. Changes of ammonium concentrations in a) seawater media of *G. verrucosa* and b) beakers without macroalgae with ammonium concentration treatments were 0, 50, 100, 200, and 400 µM with exposure of 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 360 minute experimental period.

In general, ammonium uptake of *G. verrucosa* increased at the beginning of the experimental period (Fig. 11). *G. verrucosa* took up ammonium rapidly in the first hour for all treatments with ammonium addition. The highest uptake for most ammonium concentration treatments was reached at the interval of 60-180 minutes, with the exception of macroalgae with ammonium addition of 100 µM that reached the highest uptake at time interval 180-360 minutes. Uptake decreased after one hour of the experimental period.

The highest uptake rates of *G. verrucosa* in all ammonium treatments were reached at the time interval of 15-30 minutes, shown by a surge phase (Fig. 12). Over the duration of the experiments (360 minutes), the rate of ammonium depletion decreased. The curve of uptake rates declined after 30 minutes in all ammonium treatments, expressed by the decrease of uptake rates after 30 minutes of incubation. This was presumably due to nutrient depletion of the medium over time.

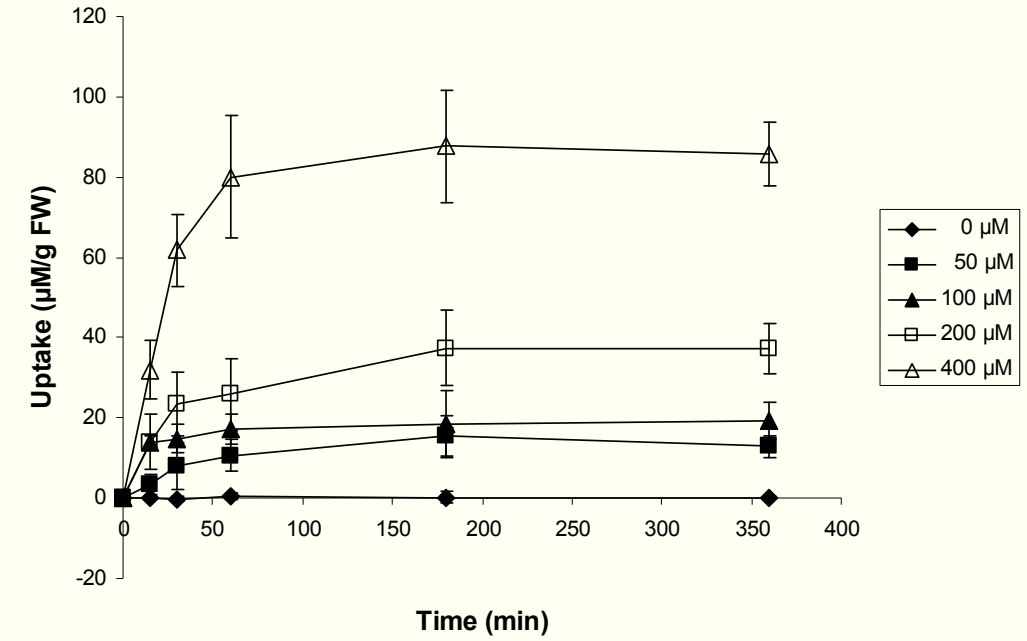


Fig. 11. Ammonium uptake of *G. verrucosa* with ammonium concentration treatments of 0, 50, 100, 200, and 400 µM under 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 360 minute experimental period.

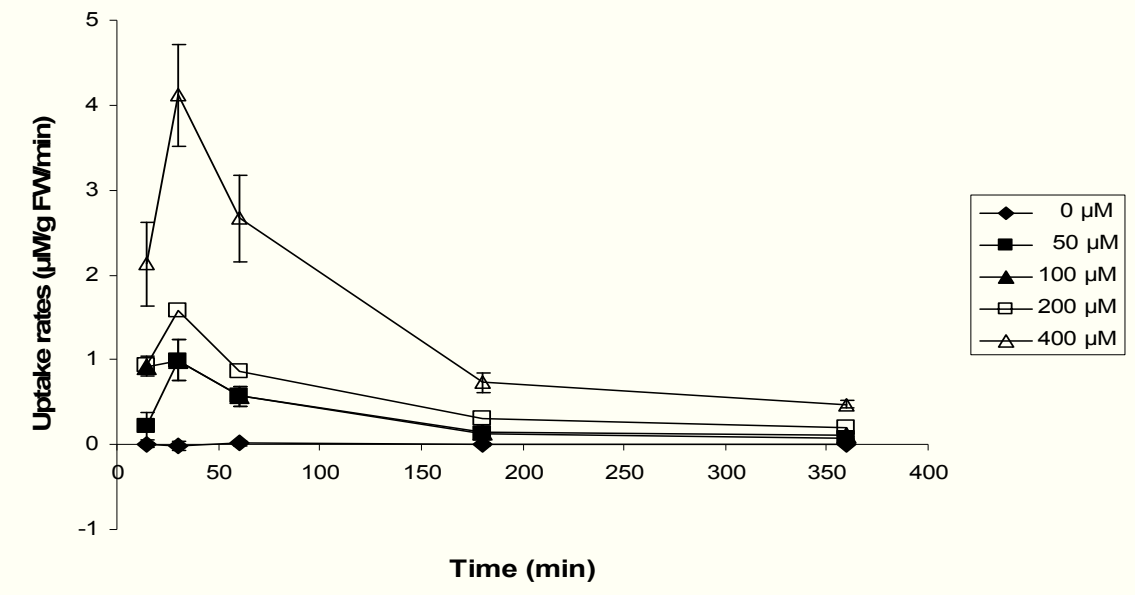


Fig. 12. Ammonium uptake rates of *G. verrucosa* with ammonium concentration treatments of 0, 50, 100, 200, and 400 µM under 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 360 minute experimental period.

The Michaelis-Menten saturation kinetics confirmed the results of decreasing uptake rates (Fig. 13). This was shown by decreasing steepness of the slope for each time interval. During the experiments, *G. verrucosa* reached no saturation, although the rates declined over time (Table 2). This was evident by the linear curves in all time intervals rather than following the Michaelis-Menten saturation kinetics. The greatest slope (α) showed during time interval 15-30 minutes, indicates the highest uptake rate.

Table 2. Parameters of the Michaelis-Menten function estimated by non-linear fitting of data from ammonium uptake experiments conducted with *G. verrucosa* under 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 360 minute experimental period; V_{max} is rate of saturation above a certain S (ammonium concentration treatment); K_m is the half saturation constant

Time interval (min)	V_{max} (µM/g FW/min)	K_m (µM)	R^2
0-15	12.66 ± 11.87	1851.58 ± 1831.24	0.81
15-30	*	*	0.89
30-60	*	*	0.87
60-180	*	*	0.89
180-360	*	*	0.94

* unsaturated

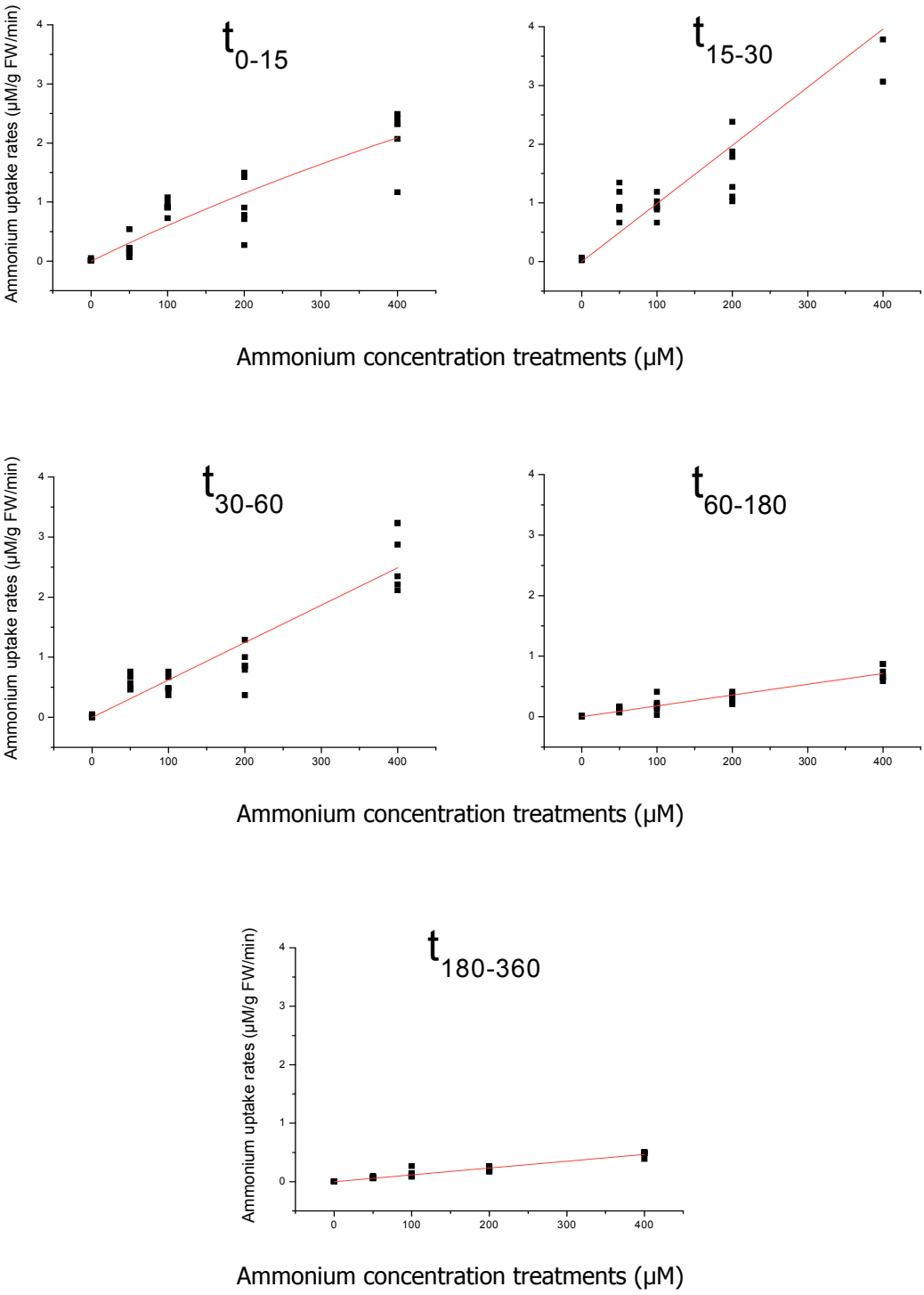


Fig.13. Rate of uptake of ammonium as a function of substrate concentration (Michaelis-Menten saturation curves) for *G. verrucosa* with ammonium concentration treatments versus ammonium uptake rates under 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 360 minute experimental period.

There were significant differences among treatments in total ammonium uptake of *G. verrucosa* ($F = 162.380$; $p < 0.000$). The total ammonium uptake of *G. verrucosa* in the 360 minute experimental period with various ammonium concentration treatments, indicated almost complete depletion of ammonium for all treatments (Fig.14). The percentages of total ammonium uptake in 360 minutes are 100, 83.6, 69.1, and 86.7, respectively for the treatments where ammonium was added.

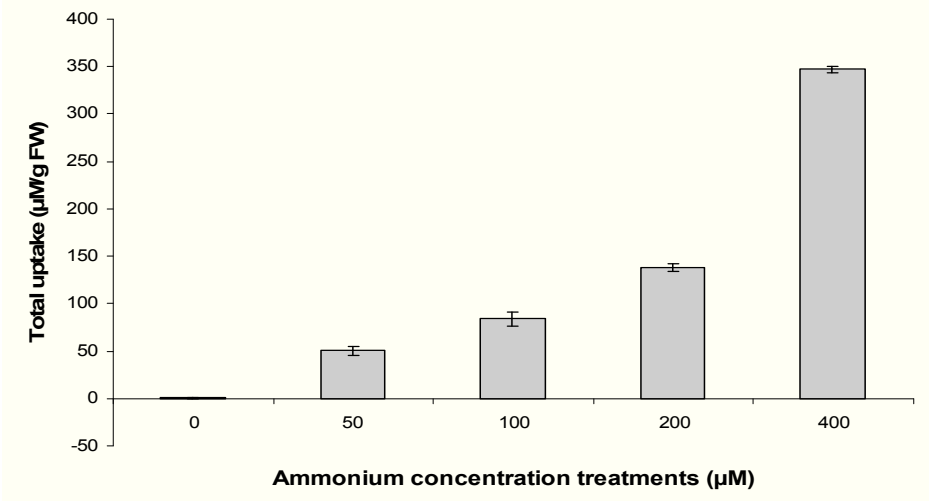


Fig. 14. Total ammonium uptake of *G. verrucosa* with ammonium concentration treatments of 0, 50, 100, 200, and 400 µM under 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 360 minute experimental period.

The increasing of ammonium content in tissue of *G. verrucosa* were strongly correlated to increasing total ammonium uptake during exposure ($r^2 = 0.99$) (Fig. 15). Content of ammonium in the algal tissue of *G. verrucosa* also increased significantly ($F = 82.67$; $p < 0.000$) with an increase of ammonium concentration treatment (Fig. 16a). However, a post hoc test showed only the ammonium content in tissue of *G. verrucosa* in the ammonium treatment of 400 µM had a significantly higher value. The values that exceeded the ammonium concentration in the treatment indicated that all specimens being tested already contained ammonium in their tissue before the experiments.

The %N of *G. verrucosa* tissue was showed no significant differences among treatments (F = 0.919; p = 0.47) (Fig. 16b). Unlike *D. bartayresiana, G. verrucosa* showed relatively lower values of %N in all ammonium treatments being tested. The values differ slightly at the range 1.59-1.78%. This is noteworthy that the results of %N in this experiment had contrary results with the total uptake (Fig. 14) that show high uptake (greater than 50%).

C:N of the macroalgae for all ammonium concentration treatments was greater than 15 (Fig. 16c). This indicated no nitrogen accumulation. However, there was no significant difference (F = 1.15; p = 0.36) among treatments in C:N of *G. verrucosa*. High values of total uptake and C:N of tissue, and low values of %N, indicated that *G. verrucosa* was nutrient (nitrogen) limited prior to the experiment. This could further explain why the macroalgae were not reaching ammonium saturation.

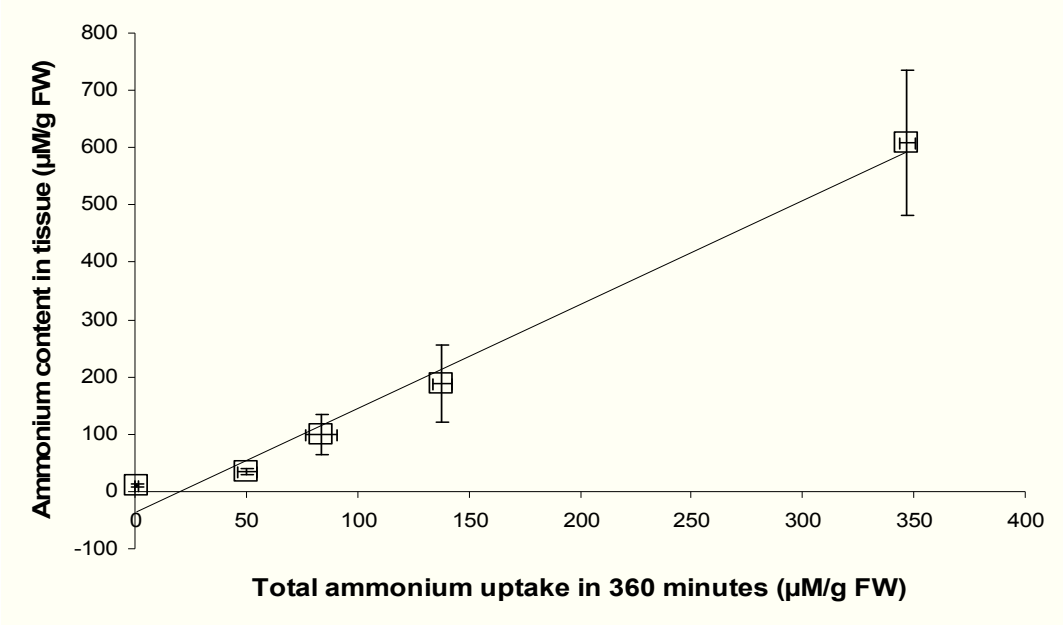
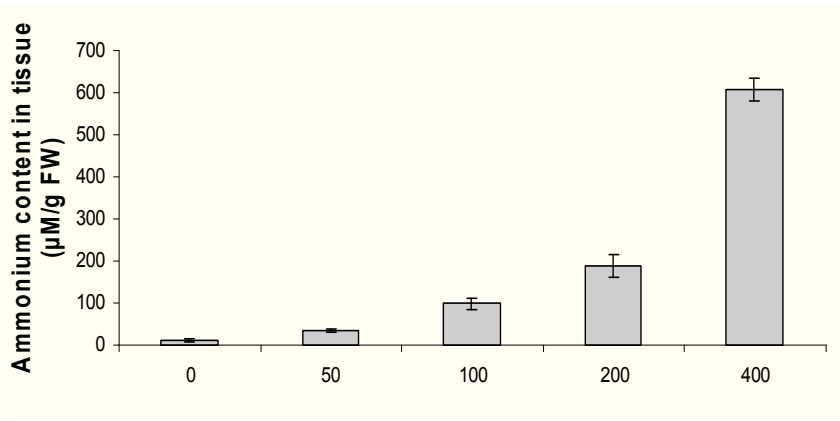


Fig. 15. The correlation between total uptake and internal content in tissue of *D. bartayresiana* with ammonium concentration treatments of 0, 50, 100, 200, and 400 µM under 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 360 minute experimental period.

a)



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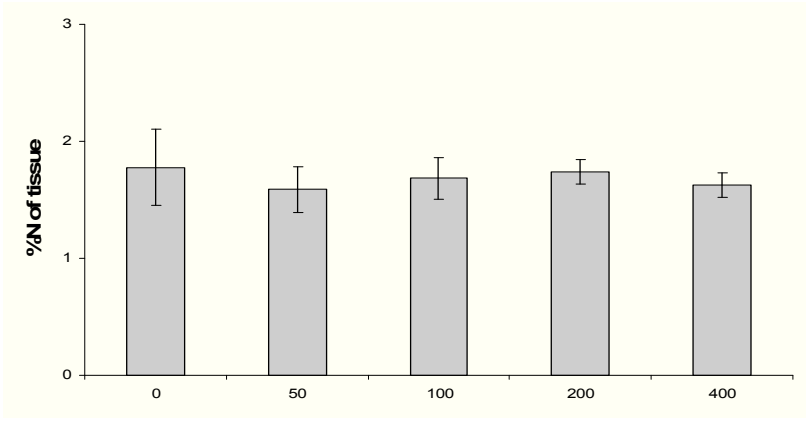

c)

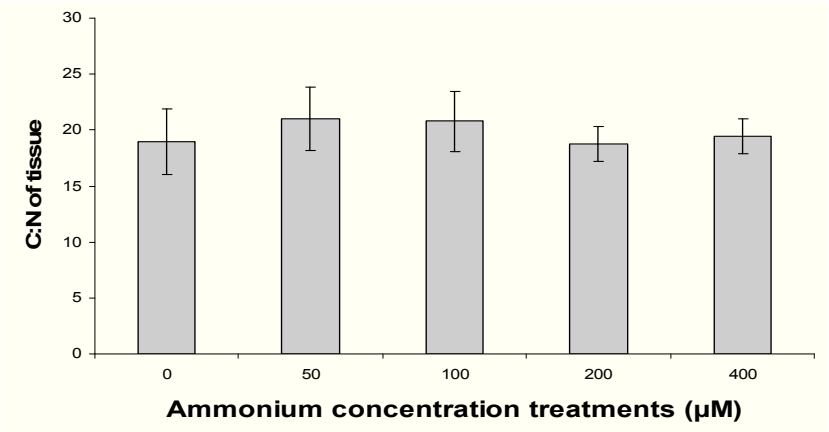

Fig. 16. Nutrient content in the macroalgal tissue of *G. verrucosa* with ammonium concentration treatments of 0, 50, 100, 200, and 400 µM under 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 360 minute experimental period; a) ammonium content in tissue; b) %N of tissue; c) C:N of tissue.

Discussion

In general, the various ammonium concentration treatments influenced the uptake rates of *G. verrucosa* and *D. bartayresiana*. The nutrient analysis results of both species showed mutual support of physiological performance in all parameters. In all ammonium concentration treatments, *D. bartayresiana* showed low absorption capabilities, which was in contrast to *G. verrucosa*. Total uptake of *G. verrucosa* in 360 minutes exposure was higher than 50% in all ammonium concentration treatments, indicating a fair tolerance of ammonium. This was contrary to total uptake of *D. bartayresiana*. The differences in ammonium tolerance of both species were comparable to differences of ammonium content in the macroalgal tissue, which were parallel to the increasing ammonium concentration treatment for *G. verrucosa*. The low values of ammonium content in tissue of *D. bartayresiana* indicated low ammonium absorption potential of this species. The value of C:N in *G. verrucosa* tissue showed no nitrogen accumulation, while *D. bartayresiana* had contrary results. The C:N tissue of *G. verrucosa* (18.97-21.01) gave clear indication of no nitrogen accumulation, even in the highest ammonium levels that had been given in this study. Irregularly high values ranging between 14-20 indicated no nitrogen saturation or nitrogen deficiency of both species, which is contrary with the results of ammonium content in water samples for *D. bartayresiana*. The rapid uptake at the beginning of the experimental period indicated that ammonium limitation during starvation period affects the uptake rates significantly for both species. However, short uptake experiments only showed difference in total uptake and ammonium content, but not in C:N or %N of the tissue. This might indicate that %N of the tissue was not affected after 360 minutes of exposure and that new ammonium content had not been assimilated. The result of uptake rates and the Michaelis-Menten saturation kinetics were consistent for *D. bartayresiana*. In all ammonium concentration treatments, *D. bartayresiana* showed saturation after the first 15 minutes of exposure. Conversely, *G. verrucosa* showed linear curves over time intervals which can be an indication of no saturation point being reached during the 360 minutes of the experimental period.

The same conclusion of curve linearity of relatively high nutrient concentrations was reported for *Kappaphycus alvarezii* (Hayashi et al., 2008), *Ulva* and *Chaetomorpha* (Lavery and McComb, 1991), *Chondrus crispus* (Amat and Braud, 1990), *G. pacifica*

(Thomas et al., 1987), *Laminaria groenlandica* (Harrison et al., 1986), *Fucus distichus* (Thomas et al., 1985). In some instances, the uptake of nutrients (e.g., ammonium) does not appear to be saturated even at high experimental concentrations (D'Elia and DeBoer, 1978), instead increases linearly (Lobban and Harrison, 1994) irrespective of nutritional history. In reality, such high Michaelis-Menten parameters would not be ecologically meaningful, and it is suggested that a linear, rate-unsaturated response would for all practical purposes adequately describe the uptake response (Smit, 2002). *Codium fragile* reported to have ammonium saturation kinetics, suggesting active transport (Hanisak and Harlin, 1978). For other species of macroalgae, the ammonium uptake rate does not saturate as the ammonium concentration is increased, but instead increases linearly. This linear increase in uptake rate at high ammonium concentrations may represent a second transport mechanism, perhaps diffusion via ion channels. However, definitive experiments have not been conducted to confirm this suggestion (Lobban and Harrison, 1994).

A steep slope of both species in the first 15 minutes showed that uptake rate was readily enhanced as soon as elevated ammonium concentration from the media is encountered. This is an indication of the affinity of uptake rate increase in response to elevated ammonium concentration in the media (Fujita, 1985). A high affinity implies that the macroalgae can effectively acquire nutrients at low concentrations (Smit, 2002), which presumably occurred in *D. bartayresiana*. The high affinity in the beginning of experimental period also reported for *Ulva lactuca* and *G. tikvahiae* (Fujita, 1985; Peckol et al., 1994). Nitrogen limitation increased the affinity for ammonium, though the uptake differs in mechanism (Fujita, 1985).

The results of the nutrient analyses in this study were strengthened by photosynthetic performance. The maximum quantum yield showed obviously low pattern in *D. bartayresiana* after 360 minute experimental period, which was in contrast with *G. verrucosa*. The light intensities used in this experiments was higher than the lower limit of irradiance requirement for macroalgae to grow (0.05-0.1% of the midday surface irradiance) as reported by Lüning and Dring (1979). However, the different uptake response of both species may correlate with the interaction between low irradiance and high ammonium concentration treatments. The ammonium uptake of *D. Bartayresiana* and *G. verrucosa* may contribute to previous nutrient-light interaction

studies, which is vary in results. Nishihara et al. (2005) found that nitrate and ammonium uptake of *Laurencia brongniartii* increased with increase in irradiance. Floc'h (1982) stated that nitrate uptake was affected by irradiance; while in the contrary, ammonium uptake has been found to be independent of irradiance (Wheeler, 1982; Smit, 2002). After all, further analysis is necessary to obtain more evidence of nutrient-light interaction on both species used in this study.

The low photosynthetic performance of *D. bartayresiana* was verified by the discoloration after 15 minutes of exposure. The discoloration of *D. bartayresiana* might indicate low tolerance as a response to relatively high ammonium concentrations. The visual symptoms of discoloration were similar to chlorosis as an indication of ammonium toxicity in higher plants (Britto and Kronzucker, 2002). The ammonium toxicity symptom in *D. bartayresiana* verified previous study of Waite and Mitchell (1972) who found that at concentration higher than 50 µM, ammonium may be toxic to some species of macroalgae. Gerendas, et al. (1997) found that ammonium toxicity could be the result of one or more of the following effects: (i) ammonium-induced mineral nutrient deficiency, arising from the impaired uptake of metal ions; (ii) growth inhibition arising from the acidification of the medium; (iii) alterations in intracellular pH; and (iv) uncoupling of photophosphorylation from electron transport, following the accumulation of ammonium in tissue. However, further investigation is necessary to evidence the ammonium toxicity.

These results suggested that the high supply of nitrogen may be sufficient to support growth in *G. verrucosa*, but on the contrary, indicated ammonium toxicity in *D. bartayresiana*. An exposure period of 360 minutes in ammonium concentration of 400 µM may have been insufficient to determine the maximum uptake capacity of *G. verrucosa*. Thus, it is necessary to conduct further examination in higher ammonium concentration treatments and light irradiance to observe the maximum tolerance and physiological behavior in an extended duration of observation.

Conclusion

The most important finding of this study showed that increasing ammonium concentrations influenced the uptake rates of *G. verrucosa* and *D. bartayresiana*. *G.*

verrucosa performed better than *D. bartayresiana* with a higher total uptake of *G. verrucosa* in 360 minutes of exposure. No ammonium saturation of *G. verrucosa* was observed during the experimental period. Higher supply of nitrogen may be sufficient to support growth in *G. verrucosa*, but became excessive in *D. bartayresiana*.

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4. STUDY 2

Physiological responses of the tropical macroalga *Gracilaria verrucosa* (Rhodophyceae) to various ammonium concentrations under different irradiances

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Abstract

The physiological responses of the tropical macroalga *Gracilaria verrucosa* to increasing ammonium concentrations under high and low irradiation were studied in two consecutive 14 day experiments. In the experiments macroalgae were incubated at 70 and 300 µmol photons $m^{-2}s^{-1}$ irradiation, and uptake rates, tissue nitrogen content, photosynthetic yield and performance, pigments, and growth were measured. Uptake rate of *G. verrucosa* under low irradiance showed surge phase in the first two days, and then become saturated after day 4, while under high irradiance, saturation was reached from day 2 of the experiment. The final F_v/F_m of the macroalgae under both irradiances decreased significantly with increasing ammonium concentration and irradiation. Tissue loss of *G. verrucosa* occurred from day 7, under high ammonium concentration treatments for both irradiances. Ammonium toxicity symptoms were evident with ammonium treatments greater than 800 µM. High ammonium concentrations and different irradiance significantly affect accumulative biomass, total growth rate, pigments, nutrient in tissue, external pH, and dissolved oxygen during the experiment.

Key words: ammonium, *Gracilaria verrucosa*, growth rate, physiological response, uptake rate, photosynthetic performance, saturation, toxicity.

Introduction

Expansion of aquaculture has often been accompanied by degradation of the natural environment. Aquaculture facilities release a significant quantity of nutrients as residual feed and excrements from fish, which thus form nutrient-rich sediments in confined areas. More than 70% of the excretory products from fish and microbial degradation of leftover food generate ammonia and other nutrients which are released into the natural environment (Porter et al., 1987; Krom et al., 1985; Neori et al., 1989). Dissolved nutrient effluents are predominantly in the form of ammonium (Macintosh and Philips, 1992; Jones et al., 2001).

There is some criticism of aquaculture regarding the environmental impact due to its tendency to release particulate organic waste effluents containing elevated levels of nitrogen or phosphorous-rich compounds (Buschmann et al., 1994), which may lead to eutrophication of the environment (Neori et al., 1991; Rathakrishnan, 2001) and acute toxicity to animals (Troell et al., 1999; Neori et al., 2000). This occurs in countries with intensive marine fish farms (Morand and Merceron, 2005).

Reducing the net release of nutrients from aquaculture activities to the environment has become an important issue of ecological and societal relevance. Chemical or physical treatments to remove excess ammonia from waste water and from culture ponds (Troell et al., 2003; Cripps, 1994) are expensive (Petrell, 1993) and not sustainable alternatives, as they may affect the environment (Troell et al., 1997) by causing erosion and the receding of certain beaches by approximately 10-12 m (Atkins et al., 1993). Solutions for treating effluents from aquaculture must be sustainable, and not require large investments and energy consuming support. Abundant and commercially valuable macroalgae that have high nutrient uptake rates may be used to remove excess nutrients from aquaculture effluents, thus eliminating eutrophic conditions and producing a valuable by-product in the process.

During the past 10 years, claims for the use of ecological engineering tools for more sustainable development have increased (Chopin et al., 2008). Studies have shown that waste water from fish cultivation is suitable as a nutrient source for macroalagae production (Krom et al., 1995; Neori et al., 1996). Integrating macroalgae in co-culture systems has been suggested as an efficient way to assimilate and utilize nutrients, to decrease nutrient excess, and to control red tides and biological diseases (Troell et al.,

2006). Excess nutrients in the water will be absorbed by the plants for use as fuel for physiological processes (Zhou et al., 2006), and at the same time can purify the medium (Morand and Merceron, 2005).

The ability of macroalgae to utilize nitrogen for biomass production is determined by the rate at which dissolved inorganic nitrogen can traverse the boundary layer adjacent to the outer cell layer of the thallus, and the rate at which this nitrogen takes part in biochemical processes (Koch, 1994; Sanford and Crawford, 2000). It is well known that the addition of nitrogen can greatly enhance the growth rate and production of seaweeds under certain conditions, and will boost the growth rates (Smit, 2002; Pedersen 1995).

The genus *Gracilaria* is cosmopolitan in distribution, and has been reported from the arctic, temperate, and tropical regions. A number of species of *Gracilaria* have been studied worldwide, because of its importance in diet (Cordero, 1984; Dawes, 1987; Dawes and Koch, 1990), as food source in co-culture with animals (Chiang, 1981), and as a water purifier (Zhou et al., 2006; Yang et al., 2006). Some species of *Gracilaria* have been reported to have advantageous features such as strong adaptability, rapid growth, and high uptake of nitrogen and phosphorous. It has been suggested that *Gracilaria* maybe ideal macroalgae to remediate the ecological environment in eutrophic waters (Yang et al., 2006; Marinho-Soriano et al., 2009). *Gracilaria* spp. has been used in a variety of studies investigating nutrient limitation and storage (Fujita, 1985; Lapointe, 1985; Hwang et al., 1987; Horrocks et al., 1995). Some species of *Gracilaria* had been reported to have relatively large nitrogen storage capacity (Fujita, 1985; Smit et al., 1997). This can be an advantage for using it for an integrated multitrophic aquaculture which is known to have high nutrient effluent.

Physiological responses of macroalgae had been used to explain the changes in tolerance of nutrient deprived macroalgae to environmental stress, such as shifts in temperature, salinity, or irradiance (Dawes and Koch, 1990; Kim et al., 2007; Ahn et al., 1998). Most current research on *Gracilaria* spp. focuses on their physiological response to the polluted environment under low nutrient concentrations (Amir et al., 2000). However, there are still few studies investigating the tolerance of macroalgae to high nutrient availability and the liability of ammonium toxicity, especially when it is intended to give in substantial quantities in applied aquaculture systems. Ammonium

toxicity of submerged aquatic plants has been given little attention (Van Katwijk et al., 1997). This study aimed to investigate the influence of high ammonium concentrations at different irradiances on physiological responses (uptake rates, photosynthetic performance, and growth rates) of *G. verrucosa* and to observe the maximum ammonium tolerance of *G. verrucosa*.

Materials and Methods

Plant materials. *Gracilaria verrucosa* (Hudson) Papenfuss (Rhodophyceae) was collected from the Bay of Muaragembong, Indonesia ($6^{o}0'19"S$; $107^{o}1'28"E$), in July 2010, and transported to the aquaculture facilities at Leibniz-Zentrum für Marine Tropenökologie, Universität Bremen to be prepared for several experiments in the Marine Botany Laboratory.

Physiological responses of *G. verrucosa* under various ammonium concentrations and low and high irradiance

Two experiments of 14 days were conducted in a climate room of $24^{o}C$ under irradiance of 70 and 300 µmol photons $m^{-2}s^{-1}$ in a 12:12 hour light:dark photoperiod. Approximately 1 g of algal material was placed in glass beakers with 1 L of enriched filtered (0.2 µm) seawater (NH_4Cl addition) in concentration treatments of 0, 200, 400, 800, 1200, and 2000 µM for the low irradiance treatment and 0, 400, 800, and 1200 µM in the high irradiance treatment. A series of beakers without ammonium addition were used as controls. Four replicates were included for each concentration treatment, including the control. Stirrer bars were placed at the bottom of each beaker. All beakers were placed on magnetic plates and stirred with the lowest rotational speed (120 rpm). Culture media were changed every other day of the experiments to maintain ammonium concentrations at the initial level.

Water samples (10 mL) for nutrient analysis were taken initially, and every other day of the experiment prior to replacement of water. Abiotic parameters and wet weight were measured initially and on day 2, 4, 7, 11, and 14. Photosynthetic performance was measured initially and on day 2, 4, 7, 11, and 14. Macroalgal tissue was taken for analysis of ammonium content in the tissue, C/N ratio and nitrogen content (%N), and

pigments (chl-a and phycobiliprotein) initially and end of the experiment. Tissue samples were stored in -80^{o}C until analysis.

Laboratory analyses were conducted for the water samples and macroalgal tissue, included nutrient analyses (ammonium content in water samples, ammonium content in macroalgal tissue, %N, and C/N ratio), and pigment analyses (chl-a and phycobiliprotein). Data analyses conducted for the experiments were abiotic parameters, ammonium concentrations (uptake rates, C/N ratio, and %N), photosynthetic performance (F_v/F_m, PI curve, α, I_k, and $rETR_{max}$), accumulative biomass, and growth rate.

Abiotic parameters. Abiotic parameters in this study refer to water dissolved oxygen concentrations, temperature, salinity, and pH. Measurement of abiotic parameters was carried out using a multiparameter checker WTW Multi 3430 SET F (Germany). The measurements were done initially and every other day through the experimental period. The measurements were done before the medium was changed.

Photosynthetic performance. Photosynthetic activity was determined by measuring variable chlorophyll-fluorescence using a PAM 2100 device (Walz, Germany). Maximum quantum yield was calculated as the ratio of variable to maximum fluorescence (F_v/F_m) of the dark-acclimated plant (Bischof et al., 1998). The algal thalli were fixed to the end of the fiberoptics and placed in a beaker filled with seawater. The beaker was covered with aluminum foil to avoid light leakage from other light sources.

To determine changes in the photosynthetic capacity, photosynthesis versus irradiance curves (PI-curve) were calculated following procedures described by Bischof et al. (1998). Algal samples were irradiated with increasing irradiance of actinic red light (10-700 µmol $m^{-2}s^{-1}$, 650 nm). After 30 seconds, a saturating pulse was applied to measure effective quantum yield of photosynthesis and then actinic irradiation was increased. By multiplying quantum yield with photosynthetically active radiation (PAR) relative electron transport rates (rETR) were calculated as described by Schreiber et al. (1994):

$$rETR = \Delta F/Fm' \times PAR$$

with ΔF as difference of F_m (maximum fluorescence) and F_o (minimum fluorescence), F_m' as maximum fluorescence yield reached in a pulse of saturating light when the sample is preilluminated.

Relative ETR were plotted against irradiance of actinic light, and the maximal relative electron transport rate (rETR, under saturating light) was determined by curve-fitting. Data were processed using the PAM Win program and Kaleidagraph to obtain photosynthetic capacity ($rETR_{max}$), saturation point (I_k), and photosynthetic efficiency (α).

Pigment analyses. Chlorophyll-a (chl-a) content was determined photometrically by following the method described by Inskeep and Bloom (1985). Approximately 0.1 g of algal sample was transferred into test tubes containing 5 ml of dymethylformamide (DMF). Samples were stored in a refrigerator at 4°C in darkness for 4 days. The extinction of the DMF solution was then measured at 664.5 nm in a UV spectrophotometer. The chlorophyll content was calculated using the formula:

$$\text{Chl-a (mg L}^{-1}) = 12.7 \text{ x } E_{664.5}$$

where E is the extinction of the DMF solution at wavelength 664.5 nm, with 12.7 as the constant.

Phycobiliproteins were extracted using the method described by Beer and Eshel (1985). Sample preparation was done using 0.03-0.1 g of algal tissue on average. Algal tissue was ground using a dismembrator for 3 min with 1500 RPM. All sample preparation was done under dark conditions and maintained frozen by emerging in liquid nitrogen. After being homogenized, samples were transferred into centrifuge test tubes, and extracted in 5 mL of 1.0 M phosphate buffer (pH = 6.8). The extracts were centrifuged at 6000 g in 4°C for 20 min. The supernatant was used for phycobiliprotein determination by measuring the absorbance in a UV spectrophotometer (Shimadzu, Japan). The phycoerythrin and phycocyanin concentrations were determined using the equations:

$$PE = ((A_{564} - A_{592}) - (A_{455} - A_{592})\ 0.20)\ 0.12$$

$$PC = ((A_{618} - A_{645}) - (A_{592} - A_{645})\ 0.51)\ 0.15$$

where PE and PC are phycoerythrin and phycocyanin, respectively, A is the absorbance in UV spectrophotometer under different wavelengths (λ = 455, 564, 592, 618, 645 nm).

Ammonium content in water samples. Water samples were analyzed for ammonium concentrations using modified indophenol blue method of Koroleff (1983). An NH_4^+ standard solution was made fresh for each analysis. Ammonium concentrations were measured on a UV spectrophotometer Evolution 60 (USA) to determine nutrient uptake rates. Solutions used were phenol reagent, alkaline (NaOH), chlorox, and mixed-reagent from hypochlorite and citrate. A stock solution of NH_4Cl was made to use for the standard curve. After being stored overnight in room temperature with added reagents, the absorbance was examined in the photometer at 620 nm wave-length. Water samples of 5 mL each were transferred to a sterile plastic tube. Dilutions were needed for all samples except the control in order to be in the sensitivity range of the analysis. The phenol reagent was added to the sample in the amount of 143 µL. After shaking for a few seconds, 143 µL of mixed reagent were added. A final shake was done and all samples were covered with aluminium foil to be stored overnight in room temperature and run on the photometer for absorbance determination. Calculations of ammonium concentrations in the water samples were based on the calibration curve of the ammonium standard solutions.

Ammonium uptake. Ammonium uptake in this study was determined as the disappearance of ammonium in the water medium after being taken up by the macroalgae. Rates of ammonium uptake were determined from the depletion of ammonium content in the water samples, calculated over the time at each interval according to the equation described by Naldi and Wheeler (2002):

$$V = (\mu MN_i - \mu MN_f)/(t.B)$$

Where μMN_i and μMN_f are the initial and final concentration of ammonium in the medium (µM), t is time period (minute, hour, or day), and B is the macroalgal biomass (g fresh weight).

To determine the saturation kinetics of ammonium uptake of macroalgae from nutrient-enriched waters, Michaelis-Menten saturation curve were fitted to uptake data by fitting a non-linear regression to the data using the program ORIGIN 61. Uptake rates were plotted against the mean substrate concentration for each time interval, according to the formula described by Campbell (1999):

$$V = (V_{max} \text{ x } S)/(K_m + S)$$

Where V is the uptake rate, S is the ammonium concentration treatment, V_{max} is the maximum uptake rate, and Km is the half-saturation constant for the uptake. Estimates of the uptake at the concentrations used were made by fitting the estimated V_{max} and K_μ constants to the Michaelis-Menten equation.

Ammonium content in macroalgal tissue. Filtrate for ammonium analyses were prepared following the method described by Lartigue and Sherman (2005) after being modified. Approximately 0.5 g frozen algal material were ground with ceramic mortar to get fine powder crude extracts. The homogenized algal material boiled in 45 mL deionized water for 10 min and extracted for 24 hours at 4^{o}C. Extract was centrifuged in 3000 rpm for 10 min in 4^{o}C. The clean filtrate of filtered supernatant (0.45 µm disposable filter, Sartorius) were directly stored in a plastic sterile tube, and were then analyzed in the UV spectrophotometer by the method of Koroleff (1983) using the same procedures for water sample analysis of ammonium.

C/N ratio of macroalgal tissue. Samples for %N and %C were determined after being prepared as a homogenous-dried form, by exposure to constant heat at 60^{o}C for 48 hours. Dried samples were ground with mortar and pestal into a fine powder form, and were stored in a precombusted vial bottle in room temperature until analysis; %N and %C in algal tissue were then analyzed using a CHN auto analyzer EA 2100.

Growth rates. The growth of macroalgae was recorded as changes in the fresh weight of macroalgal fragments for a particular interval of time. Fragments were taken from beakers using pinset, and gently blotted on a paper to remove seawater before weighing on an analytical digital balance Sartorius (Germany). Daily growth rates were calculated

after measuring the accumulative biomass following the formula described by Lignell and Pedersen (1989):

$$DGR\ (\%\ day^{-1}) = [(W_t/W_i)^{1/t}-1] \times 100$$

where DGR stands for daily growth rates, W_i is initial fresh weight, W_t is fresh weight after particular time period (t days), and t is time (days) of cultivation. Changes in growth rates were measured among time intervals as well as accumulated biomass from initial measurements.

Statistical analyses. Mean values and their standard deviations were calculated from the different replicates per treatment. Uptake data were tested for assumptions of normality by examining heterogeneity of variance (Cochran's test). Non-normal data were subject to root transformation. One-way Analyses of Variance (ANOVA) was employed to examine for differences in total uptake, nutrients in macroalgal tissue content, and maximum quantum yield among ammonium treatments. The significance level used was $p < 0.05$. Tukey's test was used for post hoc analyses of multiple comparisons among treatment means from a significant ANOVA test. Two-way ANOVA was employed to examine the interaction effects between ammonium concentration treatments and irradiances to physiological responses and abiotic parameters. Only the same concentration of ammonium treatments had been involved for this purpose. The computer software STATISTIKA 9.0 was used for all analyses.

Results

Physiological responses of *G. verrucosa* with increasing ammonium concentrations under low irradiance

Water analysis

Water temperatures increased in day 2, and fluctuated slightly at the range 26.8-29.4°C (Fig. 1a). The water temperatures remained at the same level to the end of experiments. The room temperature was maintained at 24°C during the experimental period. Salinities fluctuated slightly, ranging at 32.3-34.1 ppt (Fig. 1b). The pH of the seawater media was lower ($F = 12.23$; $p < 0.000$) in treatments with high ammonium

concentrations (Fig. 1c). In general, the pH ranged from 8.35 at the beginning of the experimental period, to 7.86 at the end. Concentration of dissolved oxygen was significantly lower (F = 197.09; p < 0.000) with higher ammonium concentration treatments (Fig. 1d).

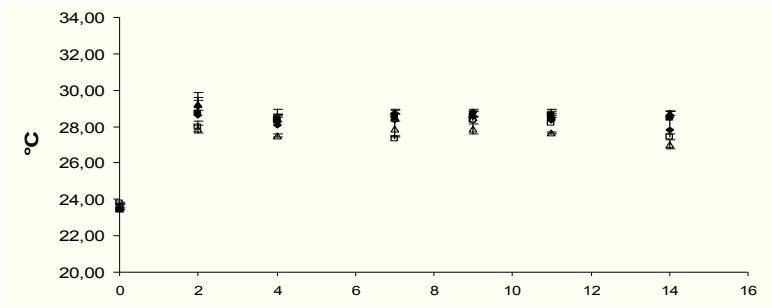
Ammonium analysis

In general, *G. verrucosa* took up ammonium in all concentration treatments, rapidly at time intervals d_{0-2} and d_{2-4} (Fig. 2), with the largest amount of ammonium being absorbed in the high ammonium concentration treatments (1200 and 2000 μM). The low concentration of ammonium in seawater media indicated the high uptake of *G. verrucosa*. The disappearance of ammonium in seawater media after day 4 declined in all concentration treatments.

The starvation period during precondition affected ammonium uptake rate of *G. verrucosa*. Surge phase of ammonium uptake of *G. verrucosa* appeared in the first two days of experimental period in all ammonium treatments being given (Fig. 3).

Ammonium uptake by *G. verrucosa* followed a linear relationship (Fig. 4; Table 1) instead of the Michaelis-Menten saturation curves at time intervals d_{0-2} and d_{2-4}. Starting at time interval d_{4-6}, ammonium uptake of *G. verrucosa* conformed to the Michaelis-Menten saturation kinetics. The steepness decreased with the duration of experimental period.

a)



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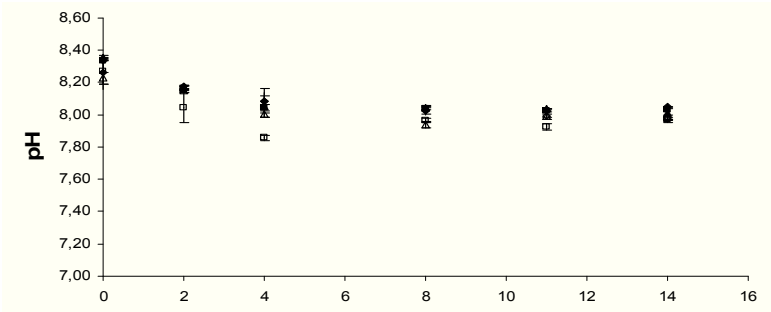
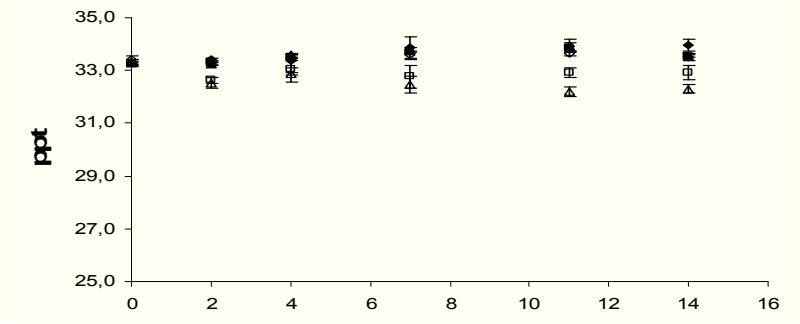


c)



d)

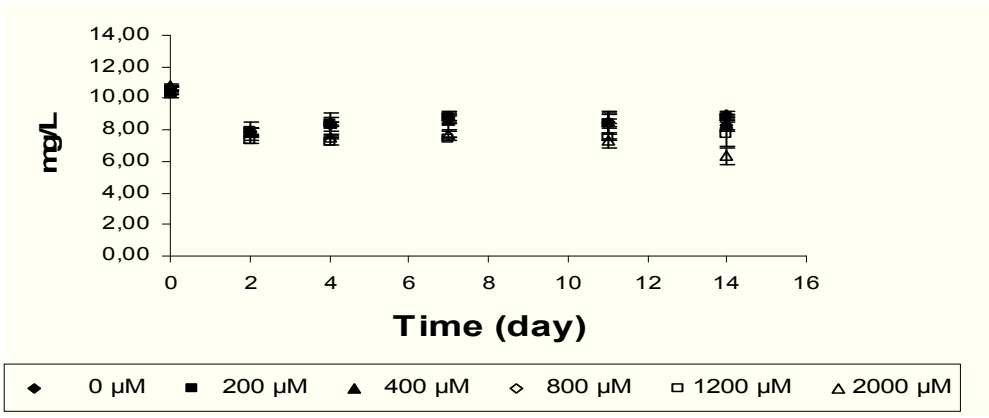


Fig. 1. Abiotic parameters of seawater media in the experiments of physiological response of *G. verrucosa* with ammonium concentration treatments of 0, 200, 400, 800, 1200 and 2000 µM under 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 14 day experimental period; a) water temperature; b) pH; c) salinity; d) Dissolved Oxygen (DO).

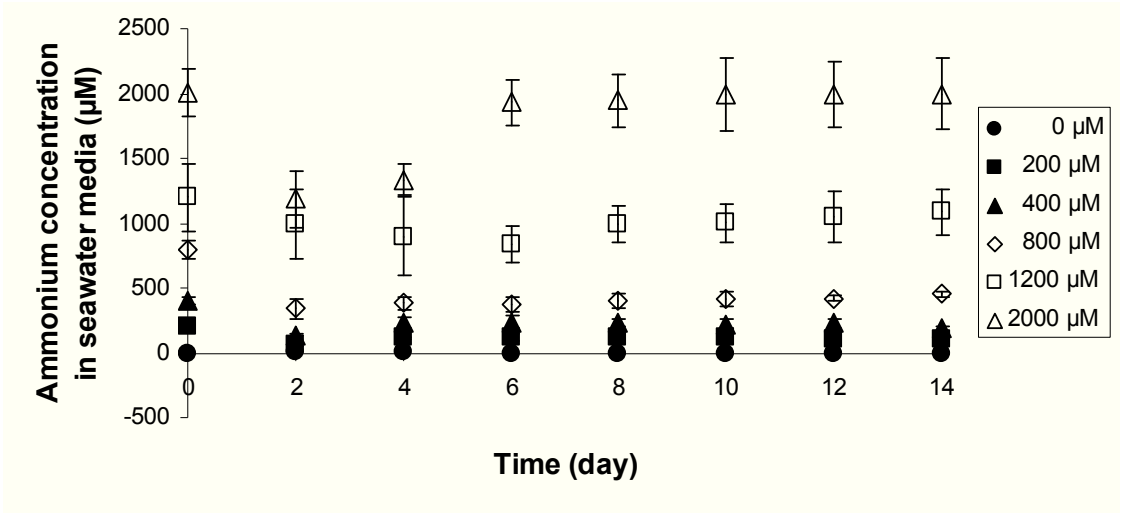


Fig. 2. Ammonium concentrations in the water for ammonium concentration treatments of 0, 200, 400, 800, 1200 and 2000 μM under 70 μmol photons $m^{-2}s^{-1}$ irradiation in a 14 day experimental period. Water was replaced every 2 days to starting ammonium concentrations after water samples were taken for analysis.

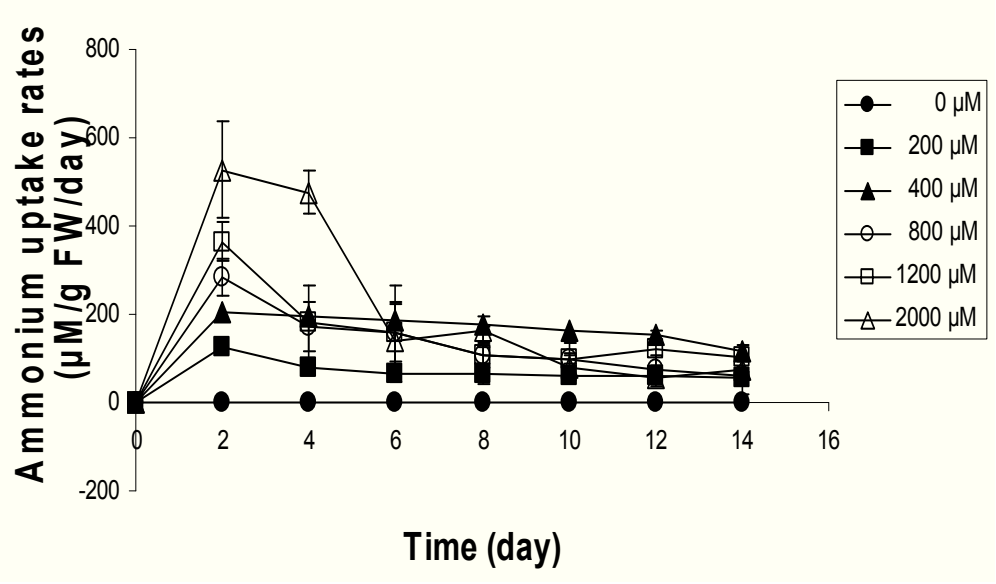


Fig. 3. Ammonium uptake rates of *G. verrucosa* in ammonium concentration treatments of 0, 200, 400, 800, 1200 and 2000 μM under 70 μmol photons $m^{-2}s^{-1}$ irradiation over a 14 day experimental period.

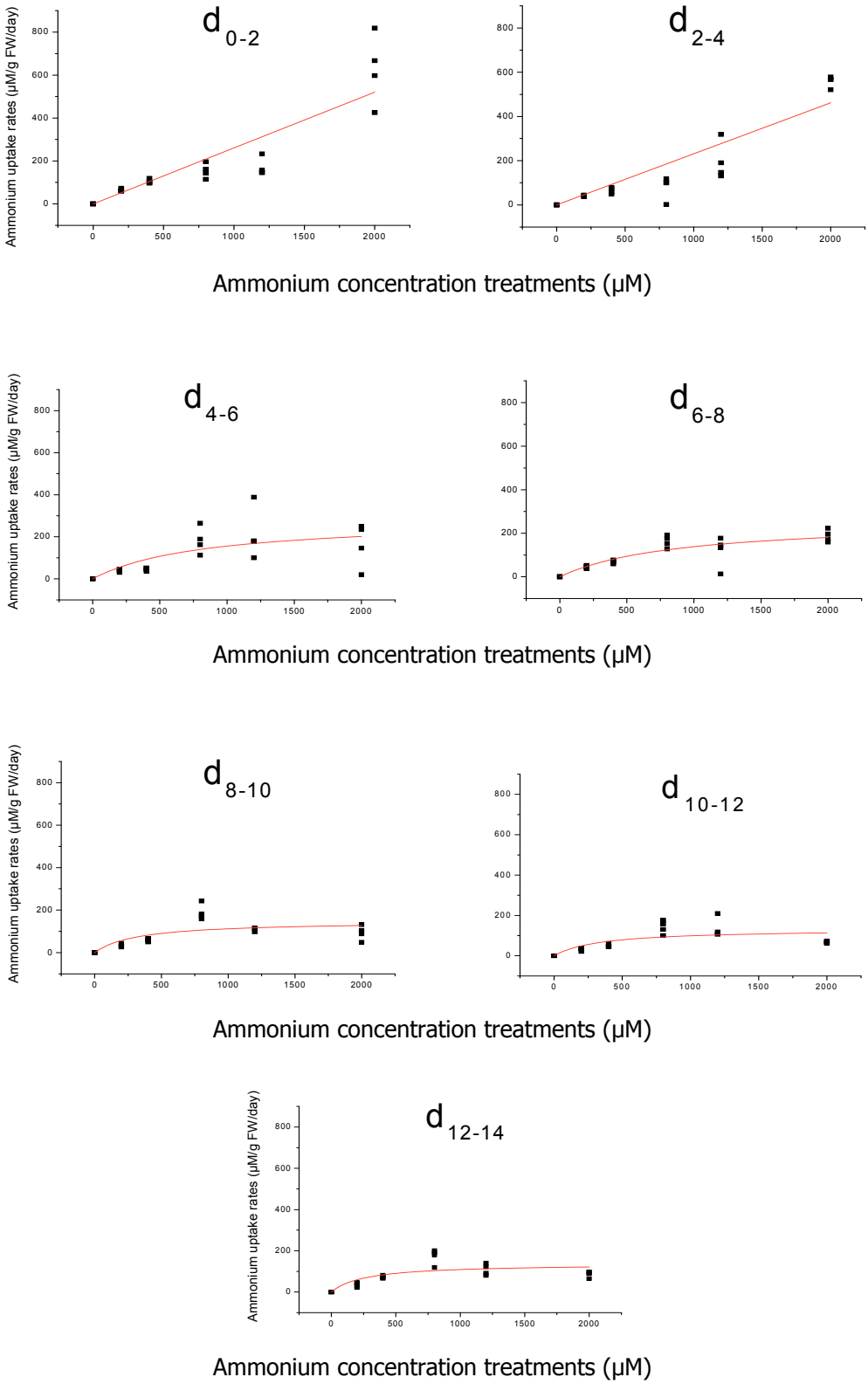


Fig. 4. Rate of uptake of ammonium as a function of substrate concentration (the Michaelis-Menten saturation curves) of *G. verrucosa* with ammonium concentration treatments of 0, 200, 400, 800, 1200, and 2000 µM under 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 14 day experimental period.

Table 1. Parameters of the Michaelis-Menten saturation kinetics estimated by non-linear fitting of data from ammonium uptake experiments conducted with *G. verrucosa* under 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 14 day experimental period; V_{max} is rate saturation above a certain S (ammonium concentration treatment); K_m is the half saturation constant.

Time interval (min)	V_{max} (µM/g FW/min)	K_m (µM)	R^2
0-2	*	*	0.79
2-4	*	*	0.84
4-6	285.05 ± 108.94	829.14 ± 725.81	0.51
6-8	259.99 ± 58.49	880.70 ± 443.08	0.74
8-10	148.09 ± 32.49	319.94 ± 240.03	0.53
10-12	132.54 ± 29.89	337.55 ± 254.19	0.53
12-14	137.26 ± 25.51	258.61 ± 179.04	0.56

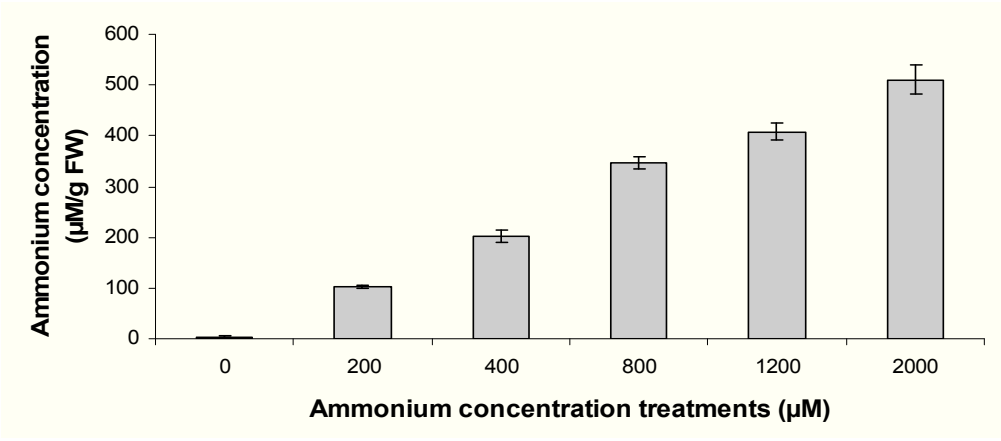
*unsaturated

The ammonium content in the macroalgal tissue increased significantly (F = 98.62; $p < 0.000$) with increasing ammonium concentration treatment (Fig. 5a) after 14 days of incubations.

All ammonium treatments showed a significant increase in %N in the tissue of macroalgae compare to the control treatment (F = 30.11; $p < 0.000$). The %N, which was greater than 3% (Fig. 5b) did not vary between the ammonium concentration treatments.

The C:N in tissue of *G. verrucosa* showed a significant difference (F = 110.46; $p < 0.000$) from the control and ammonium treatment (Fig. 5c). All CN ratios were lower than 10, ranging between 7.52 and 9.52.

a)



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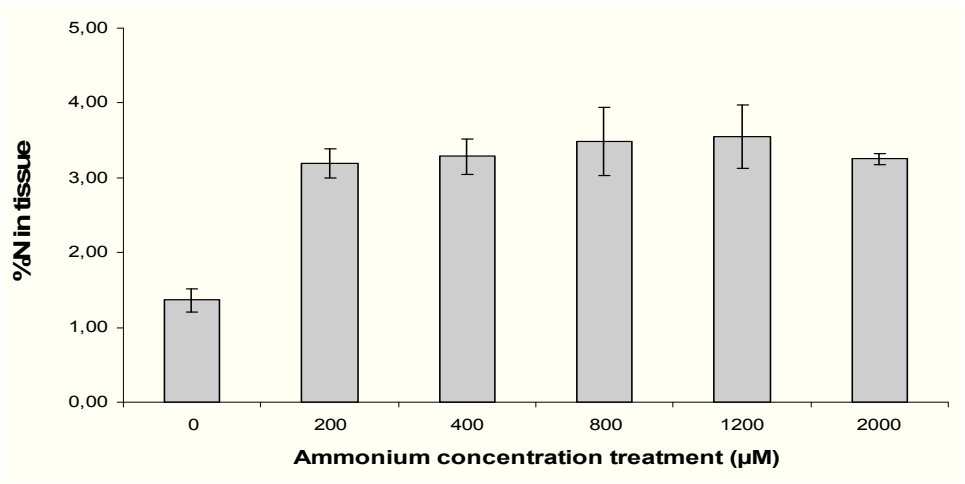

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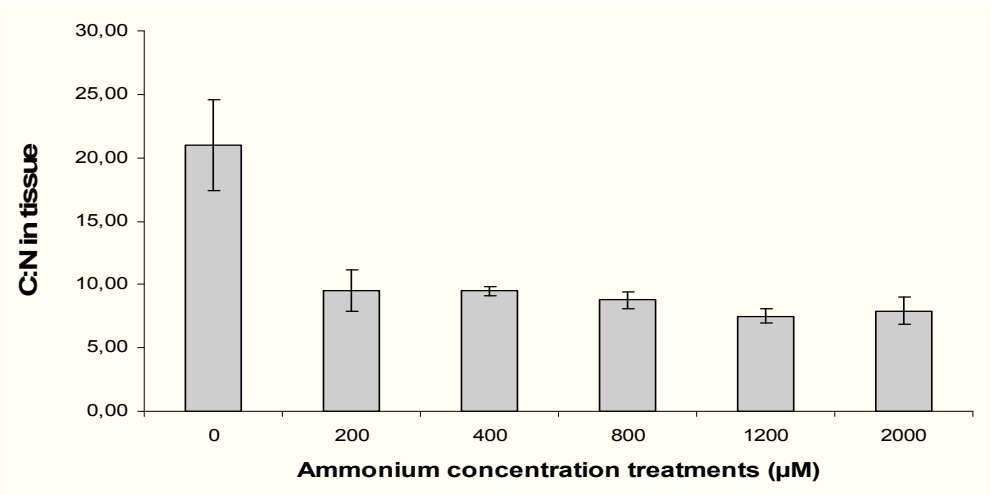


Fig. 5. Nutrient content in tissue of *G. verrucosa* in physiological response experiments with ammonium concentration treatments of 0, 200, 400, 800, 1200, and 2000 µM under 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 14 day experimental period; a) ammonium content in tissue; b) %N; c) C:N in tissue.

Photosynthetic performance

The F_v/F_m of *G. verrucosa* was initially high in all ammonium concentration treatments, including the control. In general, during 14 days of the incubation period in seawater media with varying ammonium concentrations, *G. verrucosa* showed decreasing performance in utilizing the light for photosynthetic processes. The ammonium treatments affected final F_v/F_m at the end of experimental period significantly ($F = 3.77$; $p = 0.02$). A noticeable decline was shown in macroalgae with no ammonium addition, which had the highest significant change of F_v/F_m ($p = 0.005$). The F_v/F_m for ammonium concentration treatments of 800, 1200, and 2000 µM were lower compare to those with ammonium treatments of 200 and 400 µM. This performance was expressed by the decrease in the time intervals d_{0-2} and d_{2-4}, and a gradual decline for the other time intervals. It is noteworthy that the F_v/F_m values of 200 µM ammonium concentration treatments were lower than those of 400 µM ammonium concentration treatments after a time interval d_{2-4}. Only F_v/F_m of 400 µM remained relatively high (0.609-0.425) compared to the other ammonium concentration treatments.

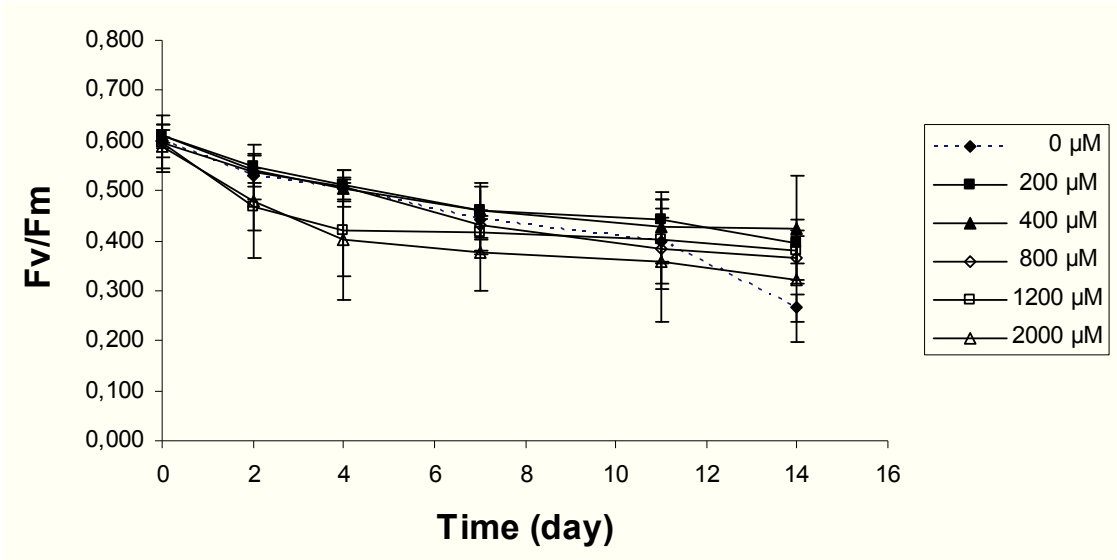


Fig. 6. Quantum yield (Fv/Fm) of *G. verrucosa* over time varying ammonium concentration treatments of 0, 200, 400, 800, 1200, and 2000 µM under 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 14 day experimental period.

The rETR of *G. verrucosa* (Fig. 7) over the duration of the experimental period showed less variation than F_v/F_m. With ammonium additions of 200, 400, 800 μM, the rETR increased considerably by day 2, while the rETR remained lower for macroalgae with 1200 and 2000 μM ammonium treatments. The rETR of those ammonium treatments reached the highest values at day 4, and decreased thereafter. In general, the rETR values of all ammonium concentration treatments decreased by day 7 and remained at the same levels during the further time of exposure to the end of experimental period. Ik and rETRmax were lower for the 1200 and 200 μM ammonium treatments, particularly between day 2 and 4.

Pigment analysis

In general, ammonium concentration treatments significantly affected pigments content (Fig. 9a) after the 14 days of incubation period. Chl-a was significantly ($F = 7.94$; $p < 0.000$) higher compared to phycocyanin ($F = 7.15$; $p < 0.000$) and phycoerythrin ($F = 6.05$; $p = 0.002$). In all ammonium concentration treatments, phycocyanin was found to be lower than phycoerythrin. Additionally, all pigments (content in tissue and percentage of initial values) were highest in the lower ammonium treatments (200 and 400 μM), and dropped considerably in the high ammonium treatments, particularly for chl-a. However, percentages of controls of PC were greater than PE and chl-a in all ammonium treatments (Fig. 9b).

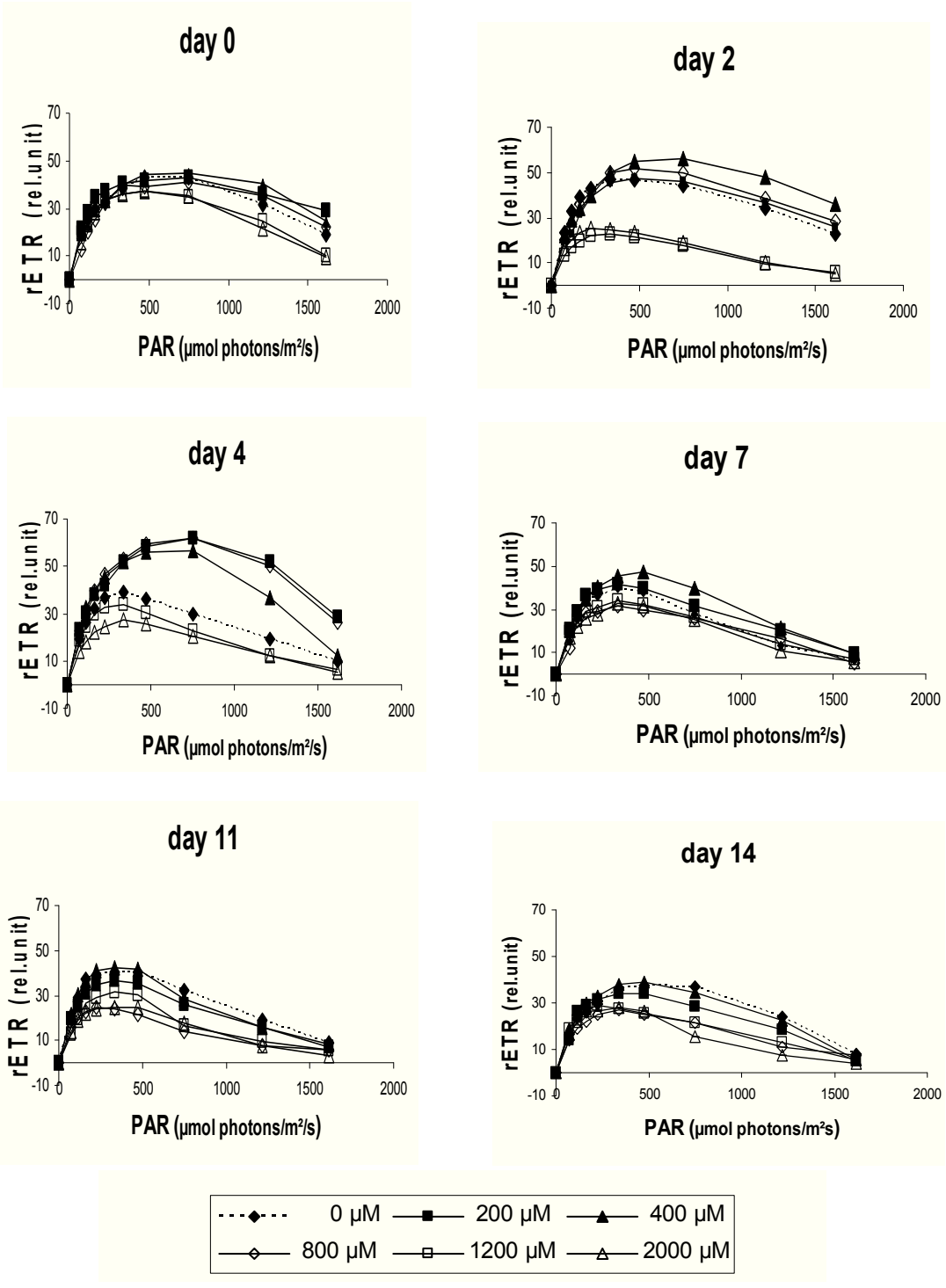
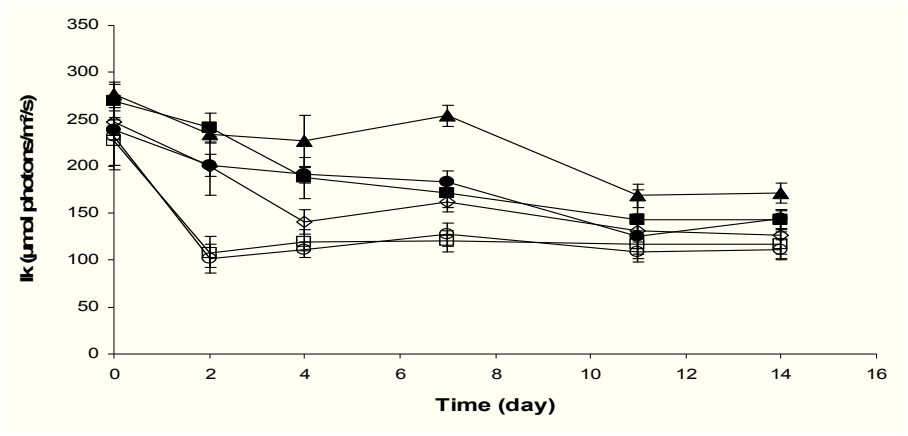


Fig. 7. ETR values of *G. verrucosa* in physiological response experiments with ammonium concentration treatments of 0, 200, 400, 800, 1200, and 2000 μM under 70 μmol photons $m^{-2}s^{-1}$ irradiation in a 14 day experimental period (mean ± SD).

a)



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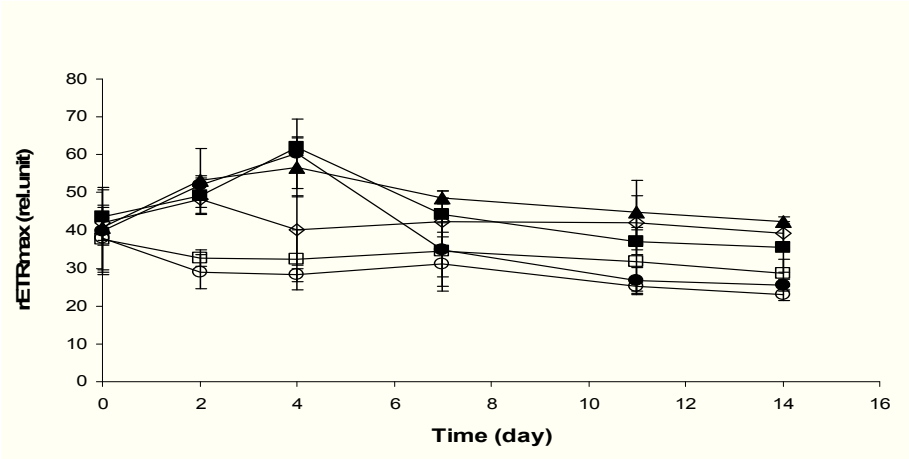

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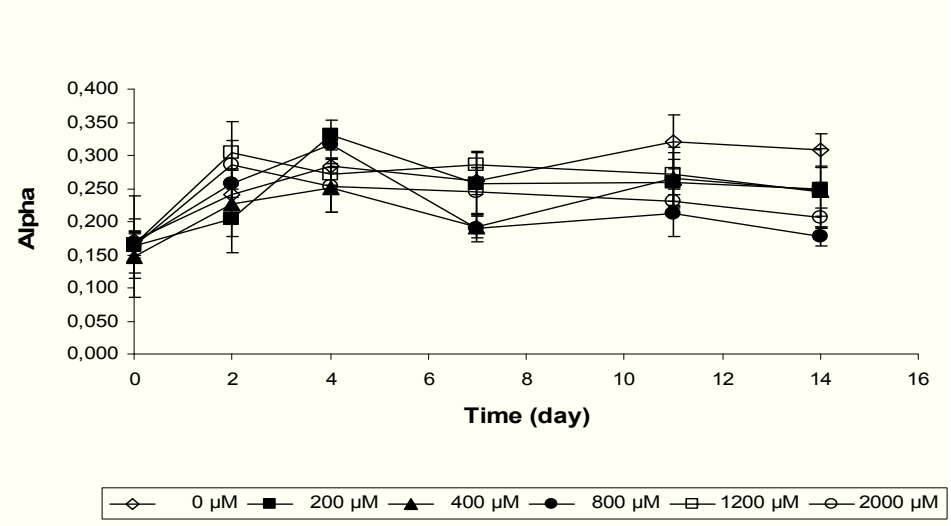
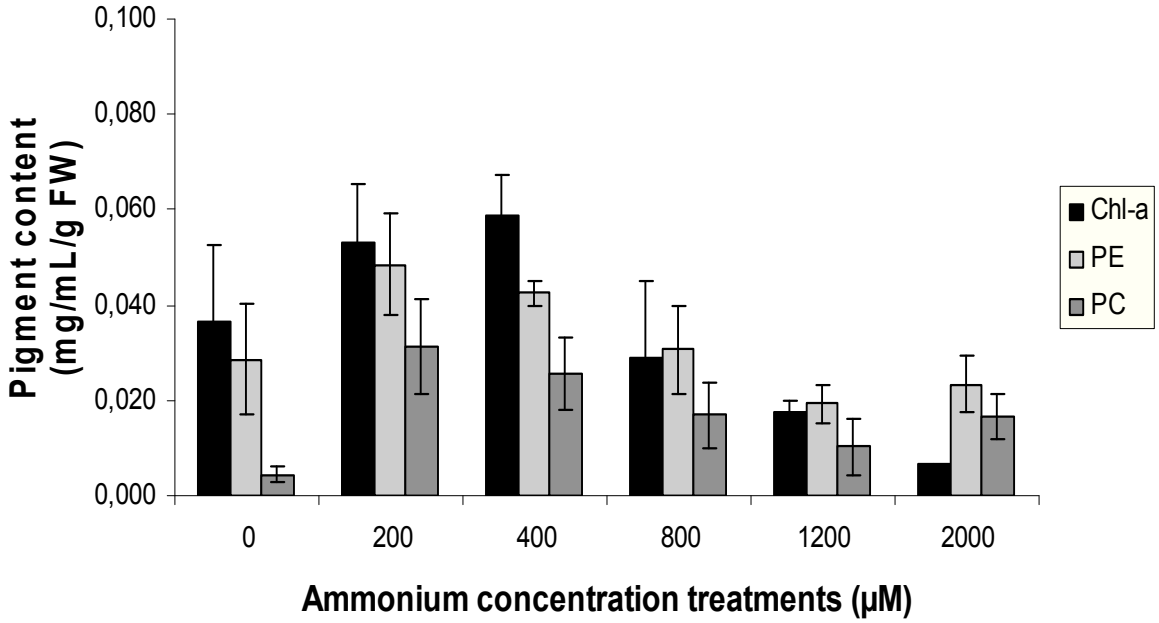


Fig. 8. Values of I_k, $rETR_{max}$, and α of *G. verrucosa* in physiological response experiments with ammonium concentration treatments of 0, 200, 400, 800, 1200, and 2000 µM under 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 14 day experimental period.

a)



b)

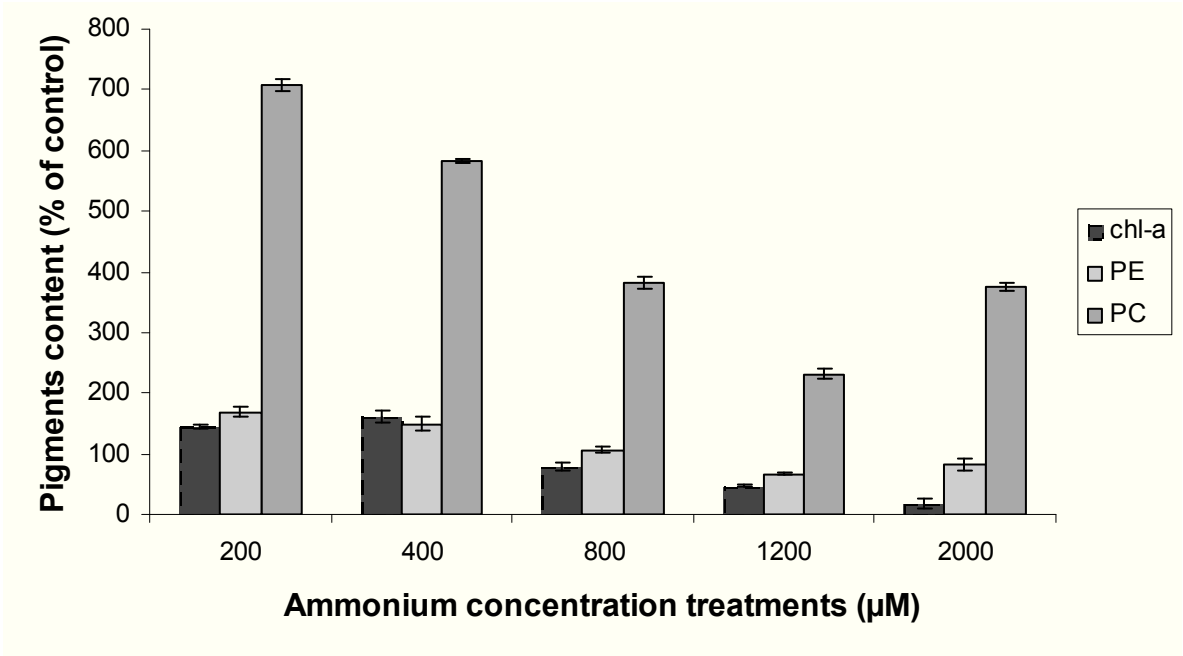


Fig. 9. Pigment content (a) and as percentage of control (b) of *G. verrucosa* with ammonium concentration treatments of 0, 200, 400, 800, 1200, and 2000 µM under 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 14 day experimental period.

Growth rate

The growth of *G. verrucosa* during the experimental period was expressed as accumulative biomass, daily growth rates, and total growth rates over experimental period (Fig. 10). Ammonium concentration treatments significantly effected macroalgal growth, in terms of accumulative biomass ($F = 13.69$; $p < 0.000$), and total growth rate ($F = 42.65$; $p < 0.000$). After day 4, macroalgae with ammonium additions of 1200 and 2000 μM decreased in accumulative biomass and became discolored.

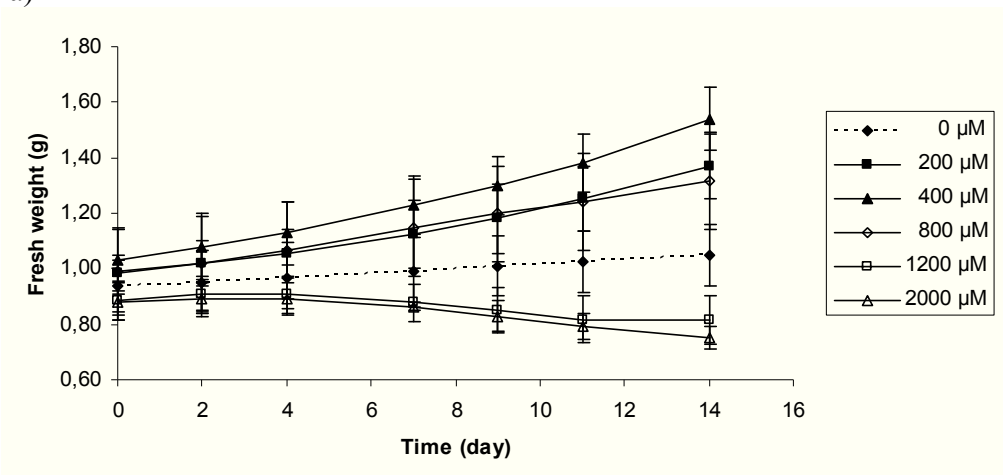
Daily growth rates (Fig. 10b) during the experimental period showed surge in time interval d_{0-2}, including control. Negative growth rate (Figs. 10b and 10c) were shown by macroalgae with ammonium additions of 1200 and 2000 μM. These negative values appeared when higher tissue fresh weight at initial measurement was compared with lower tissue fresh weight at final measurement in a certain time interval of the incubation period. This was due to the tissue loss, which started at time interval d_{4-7} and continued during further time of incubation. Algae with no ammonium addition (controls) showed relatively constant growth rates over time.

Physiological responses of *G. verrucosa* with ammonium concentration treatments of 0, 400, 800, and 1200 μM under high irradiance

Water analysis

The water temperature (Fig. 11a) was relatively constant during the study. The experiments started with the temperature 26.5^{o}C and increased to the highest value at 30.5^{o}C. The values were maintained at the same range to the end of the experiments. The salinity of seawater media (Fig. 11b) of *G. verrucosa* with various ammonium additions ranged between 34.2-35.3 ppt. In general, the salinity showed fluctuation, which was slightly higher from the former experiments with 70 μmol photons $m^{-2}s^{-1}$ irradiation. However, all the curves showed no particular patterns. In general, the final pH of seawater media (Fig. 11c) was significantly different ($F = 7.23$; $p = 0.005$) with ammonium treatments. The pH showed slight surge in day 4, and dropped on day 7. Ammonium additions also caused significant differences ($F = 39.95$; $p < 0.000$) in final dissolved oxygen in seawater media (Fig. 11d).

a)



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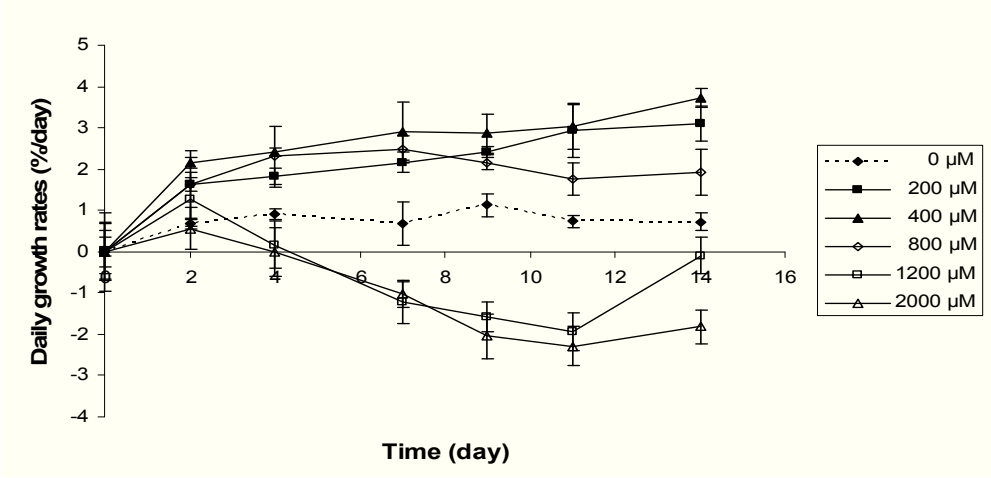

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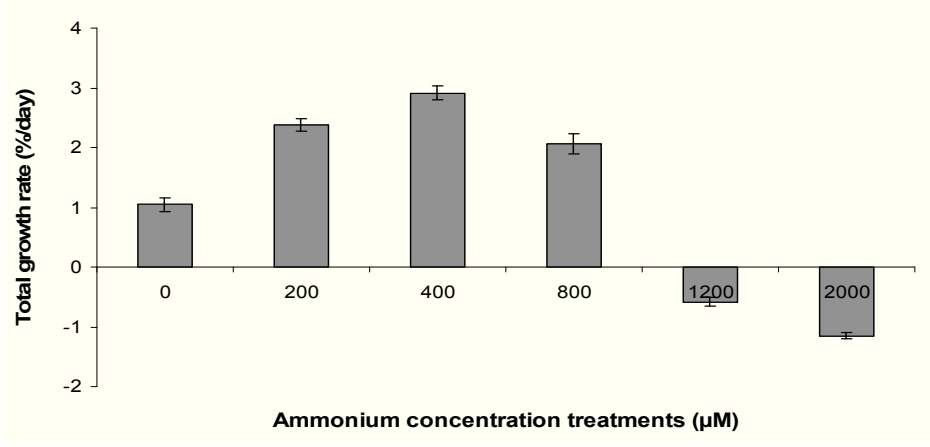


Fig. 10. Growth rates of *G. verrucosa* in physiological response experiments with ammonium concentration treatments of 0, 200, 400, 800, 1200, and 2000 µM under 70 µmol photons $m^{-2}s^{-1}$ irradiation in a 14 day experimental period; a) accumulative biomass; b) daily growth rates; c) total growth rates in 14 days.

Ammonium analysis

The ammonium concentration in the medium decreased with each time interval throughout the experiment (Fig. 12). During day 0 to day 6, ammonium concentration decreased in seawater media and had the lowest values in all treatments. This indicated that *G. verrucosa* took up significant amounts of ammonium within these time intervals. *G. verrucosa* took up ammonium in lower concentrations for the rest of the time intervals. *G. verrucosa* rapidly took up ammonium at all concentration treatments during the first two days of incubation (Fig. 13), shown by a surge phase at the beginning of the exposure time. Relatively constant uptake rates were observed for macroalgae with 400 µM ammonium treatment at time interval day 8 to day 14. Decreasing uptake rates after day 6 until the end of the experimental period were shown by macroalgae with higher ammonium treatments. Presumably, the decrease was due to slower ammonium uptake of *G. verrucosa* from the media.

Ammonium uptake by *G. verrucosa* conformed to the Michaelis-Menten saturation kinetics at time intervals d_{0-2}, d_{2-4}, $d_{4-6,}$ d_{6-8}, and d_{8-10} (Fig. 14 and Table 2). This showed that ammonium uptake of *G. verrucosa* in this experimental series reached saturation at these time intervals. This indicates that the higher irradiance of 300 µmol photons $m^{-2}s^{-1}$ affected the ammonium uptake of *G. verrucosa* compared to the low irradiance treatment. At time intervals d_{10-12} and $d_{12-14,}$ ammonium uptake kinetics did not follow the Michaelis-Menten saturation curve. Presumably, uptake saturation point had been already exceeded, and macroalgae showed toxicity symptoms.

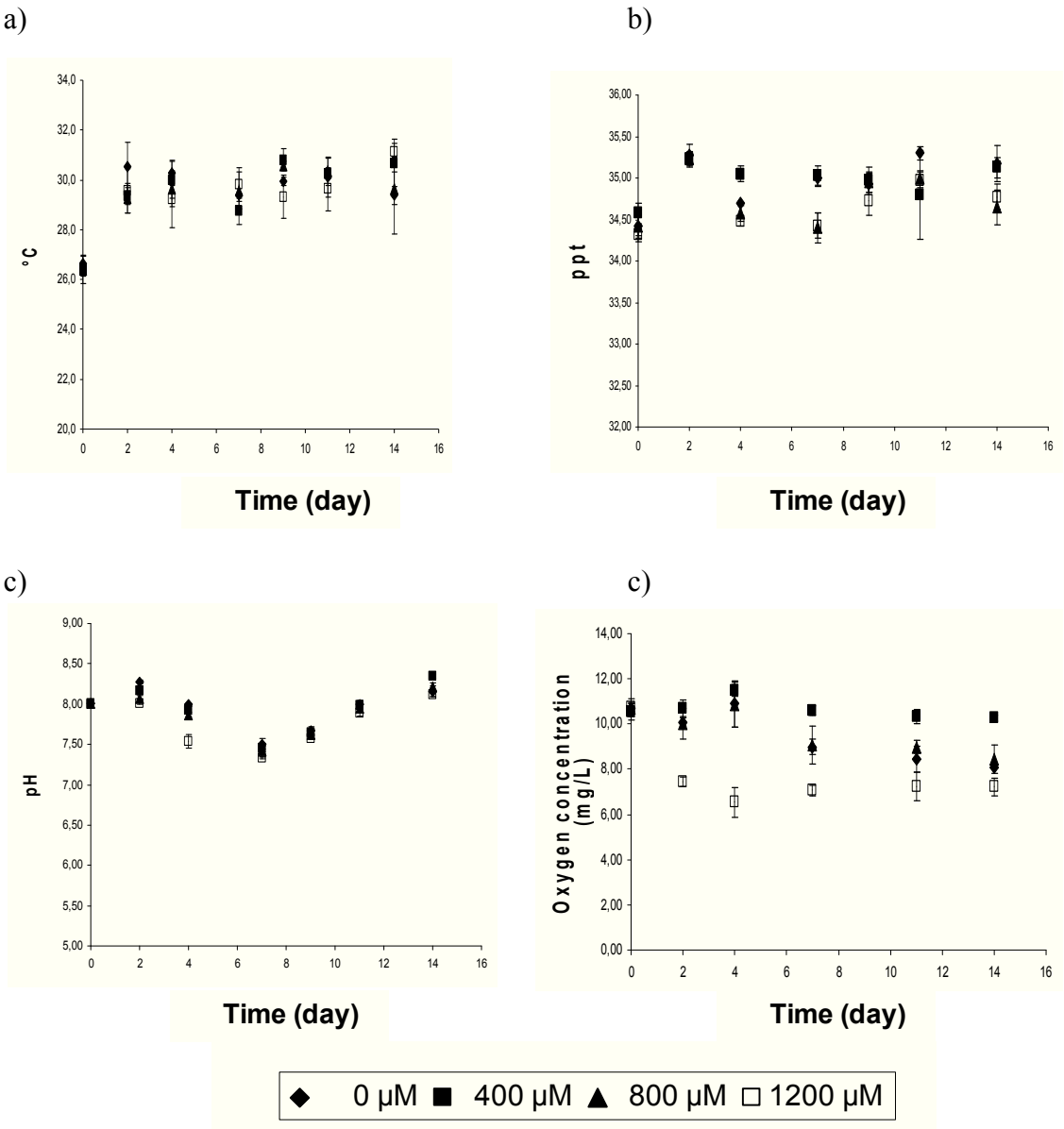


Fig. 11. Abiotic parameters of seawater media of *G.verrucosa* in physiological response experiments with ammonium concentration treatments of 0, 400, 800, and 1200 µM under 300 µmol photons $m^{-2}s^{-1}$ irradiation in a 14 day experimental period; a) water temperature; b) pH; c) salinity; d) dissolved oxygen

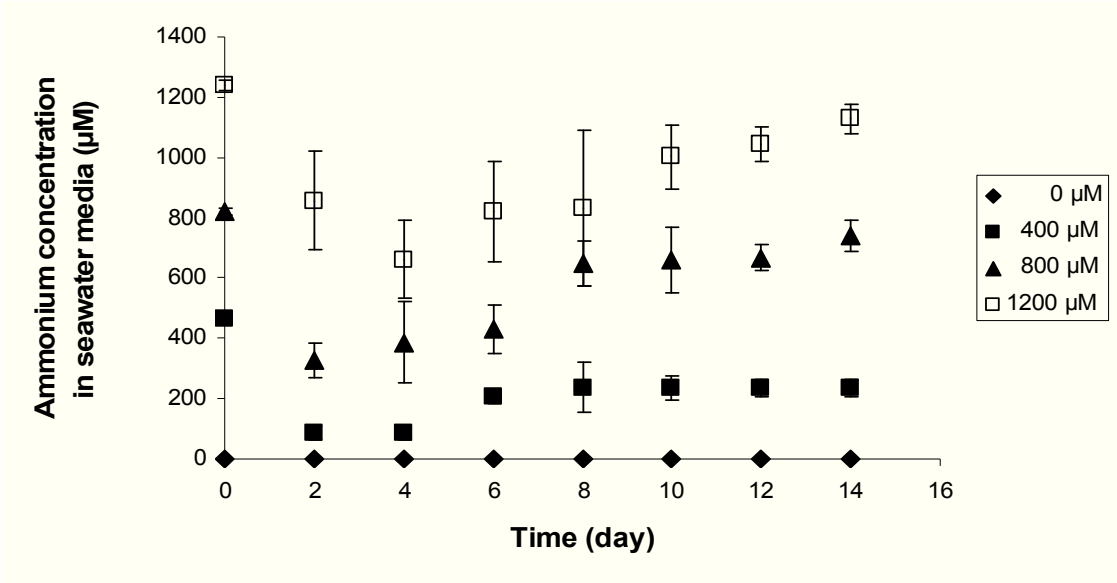


Fig. 12. Ammonium concentrations in the water for ammonium concentration treatments of 0, 400, 800, and 1200 µM under 300 µmol photons $m^{-2}s^{-1}$ irradiation in 14 a day experimental period. Water was replaced every 2 days to starting ammonium concentrations after water samples were taken for analysis.

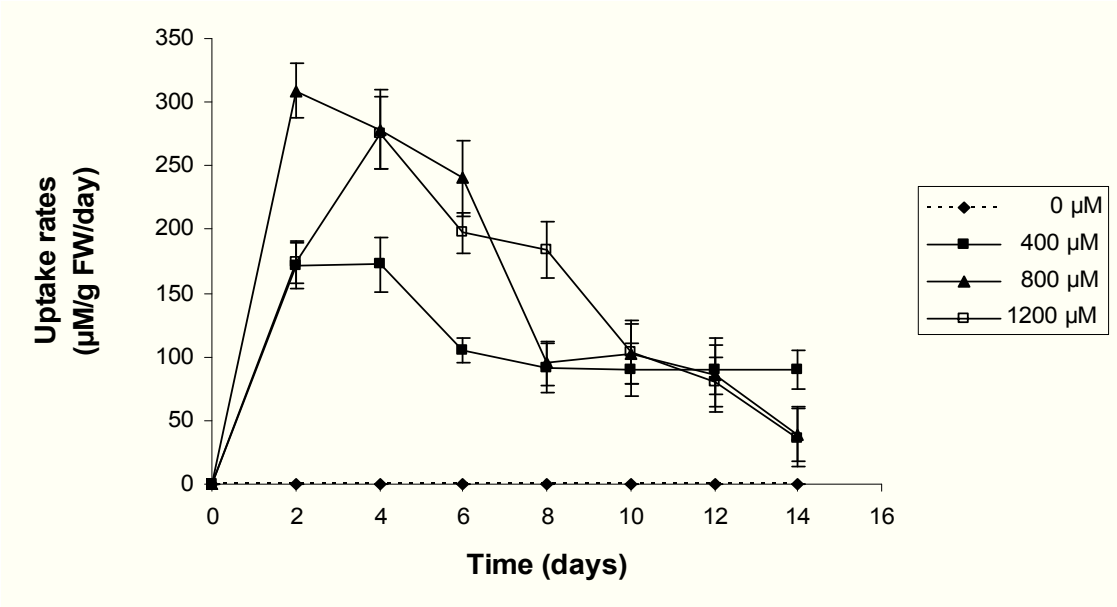


Fig. 13. Ammonium uptake rates of *G. verrucosa* with ammonium concentration treatments of 0, 400, 800, and 1200 µM under 300 µmol photons $m^{-2}s^{-1}$ irradiation in a 14 day experimental period.

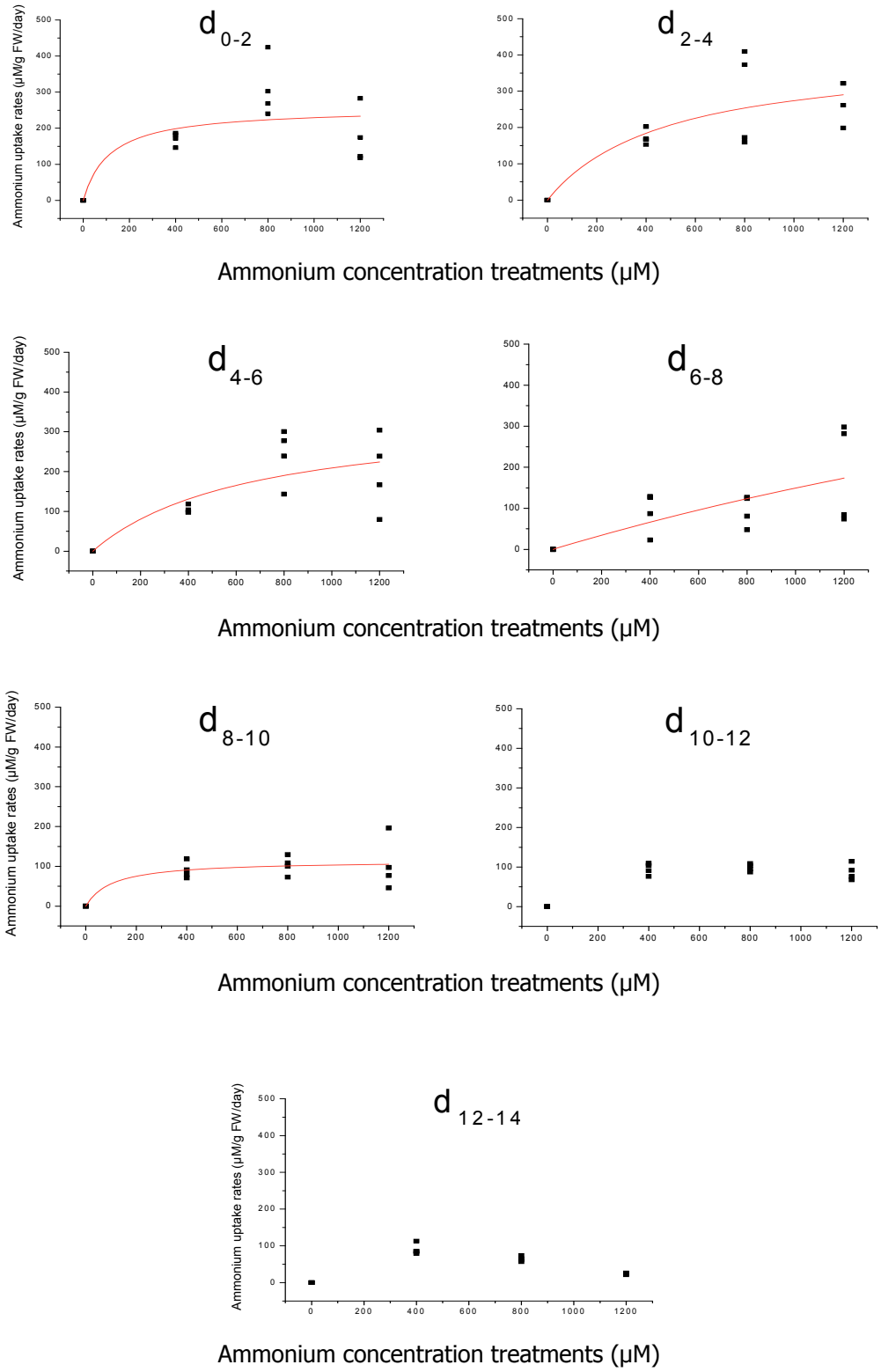


Fig.14. Rate of uptake of ammonium as a function of substrate concentration (the Michaelis-Menten saturation curves) for *G. verrucosa* with ammonium concentration treatments of 0, 400, 800, and 1200 µM under 300 µmol photons $m^{-2}s^{-1}$ irradiation in a 14 day experimental period.

Table 2. Parameters of Michaelis-Menten function estimated by non-linear fitting of data from ammonium uptake experiments conducted with *G. verrucosa* under 300 µmol photons $m^{-2}s^{-1}$ irradiation in a 14 day experimental period; V_{max} is rate saturation above a certain S (ammonium concentration treatment); K_m is the half saturation constant.

Time interval (min)	V_{max} (µM/g FW/min)	K_m (µM)	R^2
0-2	255.61 ± 70.80	115.01 ± 114.71	0.63
2-4	407.35 ± 132.16	484.85 ± 415.06	0.75
4-6	348.02 ± 163.45	663.22 ± 602.23	0.67
6-8	912.75 ± 374.63	5111.89 ± 2063.57	0.50
8-10	114.19 ± 29.72	104.19 ± 197.45	0.66
10-12	32.08 ± 4.92	*	0.85
12-14	26.54 ± 5.95	*	0.76

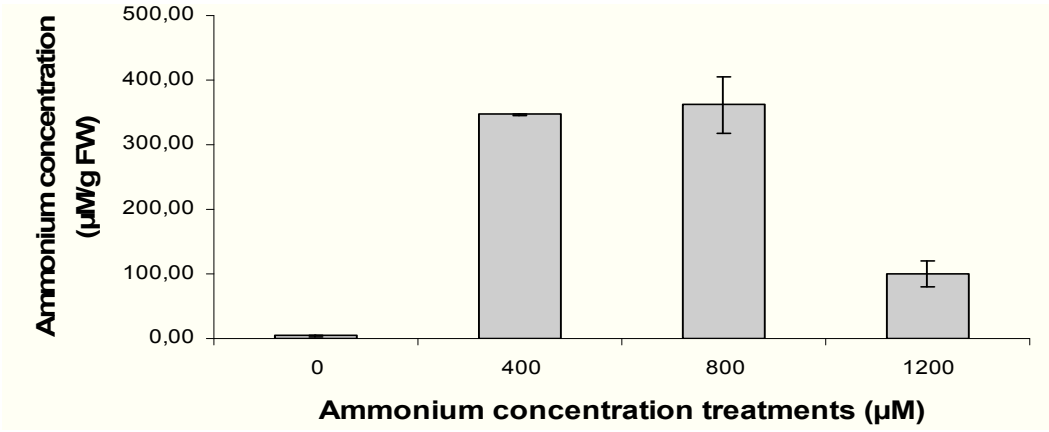
*not following the Michaelis-Menten saturation curve

After 14 days of incubation, ammonium content in tissue of *G. verrucosa* (Fig. 15a) had been affected significantly by the higher ammonium treatments ($F = 82.18$; $p < 0.000$). However, the ammonium in tissue content showed lower values than in the low irradiance treatment. The high ammonium treatments also affected %N significantly ($F = 64.01$; $p < 0.000$) and CN ratio ($F = 87.07$; $p < 0.000$) of *G. verrucosa* (Figs. 15b and 15c, respectively). It was noteworthy that the %N and CN ratio of macroalgae in 1200 µM ammonium addition showed the opposite results as found in the low irradiance treatment.

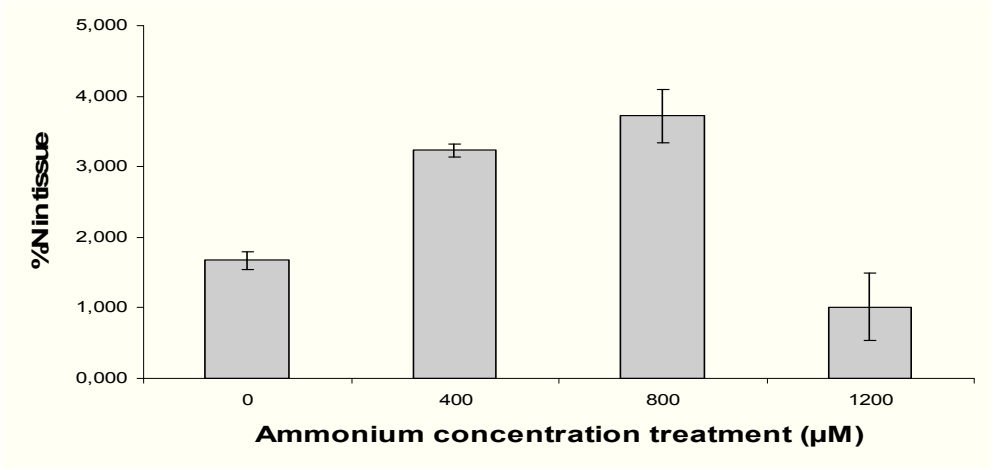
Photosynthetic performance

The maximum quantum yield (F_v/F_m) of *G. verrucosa* after 14 days of incubation were significantly affected ($F = 45.42$; $p < 0.000$) by variously high ammonium concentration treatments. In general, during 14 days of incubation, F_v/F_m of *G. verrucosa* decreased, with the best performance shown by macroalgae with 400 µM ammonium addition (Fig. 16). The F_v/F_m became relatively constant after day 7 until the end of the experimental period for all ammonium concentration treatments.

a)



b)



c)

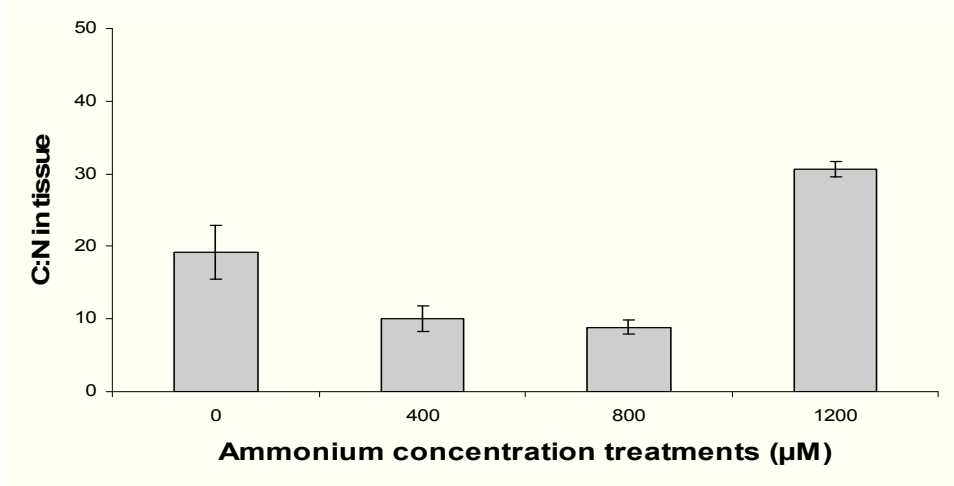


Fig. 15. Nutrient content in tissue of *G. verrucosa* with ammonium concentration treatments of 0, 400, 800, and 1200 µM under 300 µmol photons $m^{-2}s^{-1}$ irradiation in a 14 day experimental period; a) ammonium concentration in macroalgal tissue; b) %N; c) C:N in tissue.

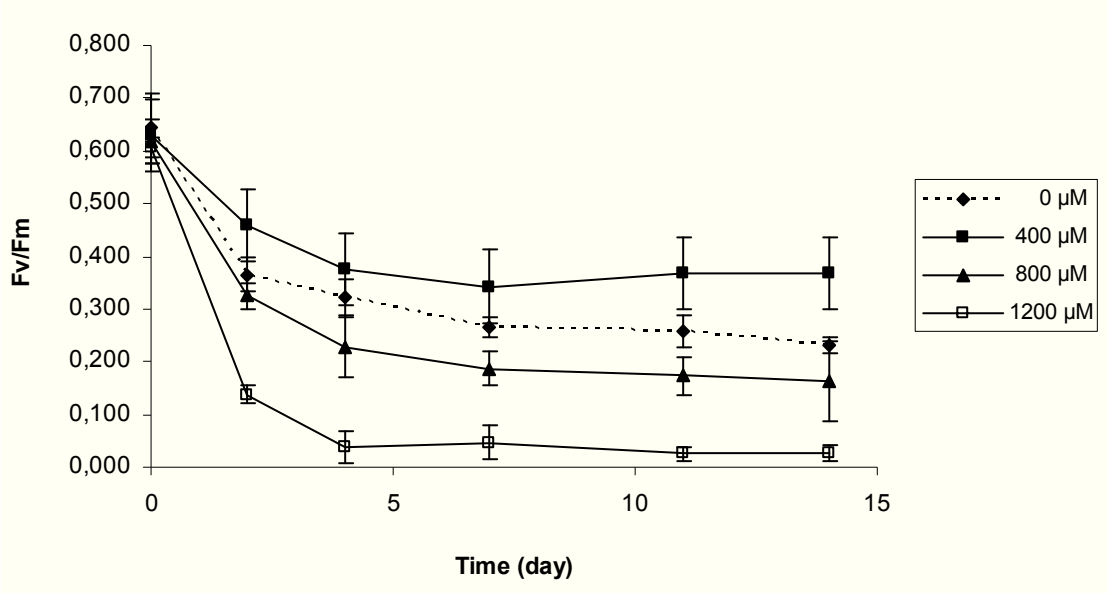


Fig. 16. Fv/Fm values of *G. verrucosa* with ammonium concentration treatments of 0, 400, 800, and 1200 µM under 300 µmol photons $m^{-2}s^{-1}$ irradiation in a 14 day experimental period.

On the initial day the rETR curve trends and pattern of *G. verrucosa* are similar to the control. After given ammonium addition, the rETR increased on day 2, in ammonium concentration treatments of 400 and 800 µM. The rETR of macroalgae with 1200 µM ammonium addition and controls failed to reach higher values after day 2. In general, rETR values for macroalgae with ammonium concentration treatments of 400 and 800 µM were higher compared to those with the treatments of 1200 µM during the experimental period.

Macroalgae with ammonium addition of 800 µM showed higher irradiance saturation point than the other ammonium concentration treatments. The highest saturation point for macroalgae with 800 µM ammonium addition was reached on day 4. At ammonium treatments of 400 and 800 µM, *G. verrucosa* reached the highest I_k on day 2. Relative ETR_{max} of macroalgae in the ammonium treatment of 1200 µM showed lower values compare to the other ammonium concentration treatments. Macroalgae with 800 and 1200 µM ammonium addition showed lower α than controls and macroalgae with 400 µM ammonium addition. This can be seen clearly starting on day 4.

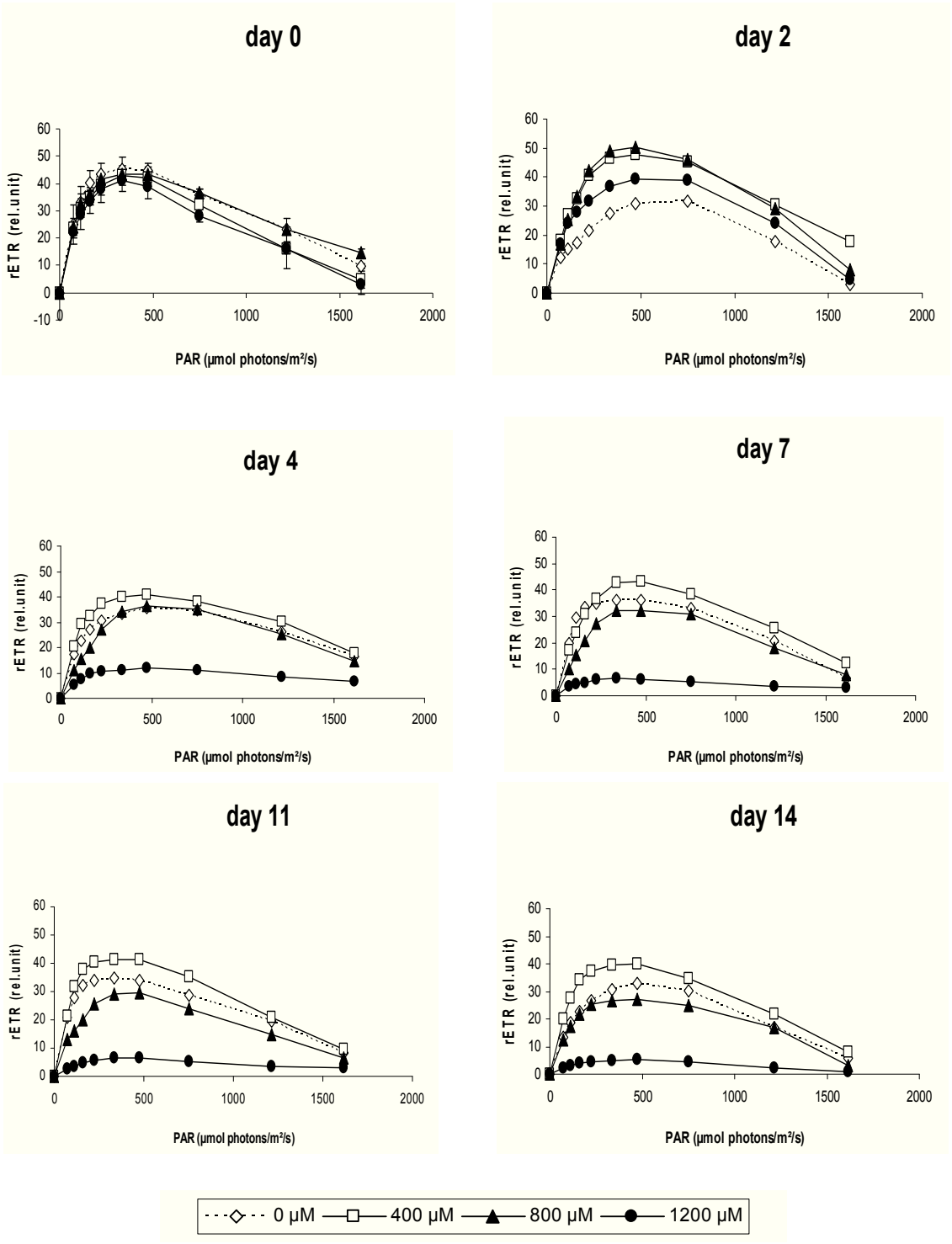
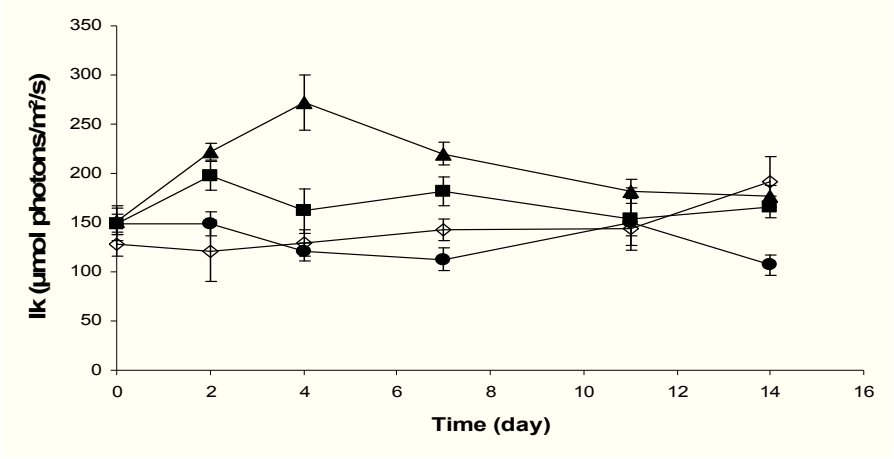
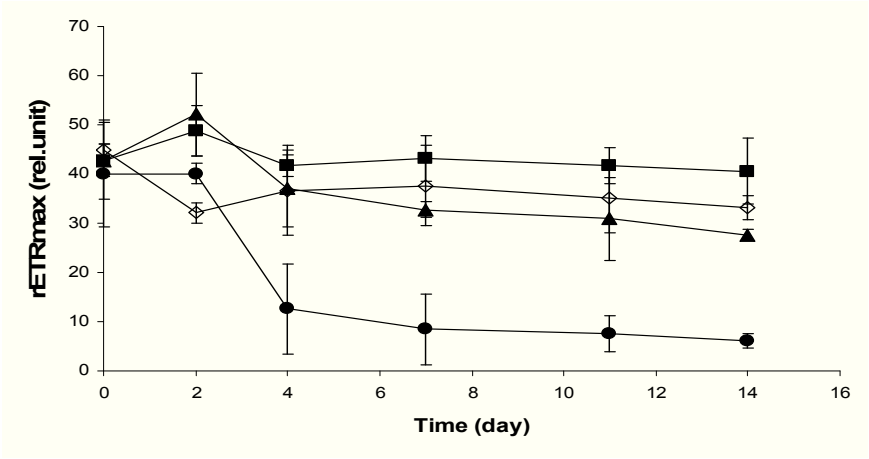


Fig.17. rETR values of *G. verrucosa* with ammonium concentration treatments of 0, 400, 800, and 1200 µM under 300 µmol photons $m^{-2}s^{-1}$ irradiation in a 14 day experimental period (mean ± SD).

a)



b)



c)

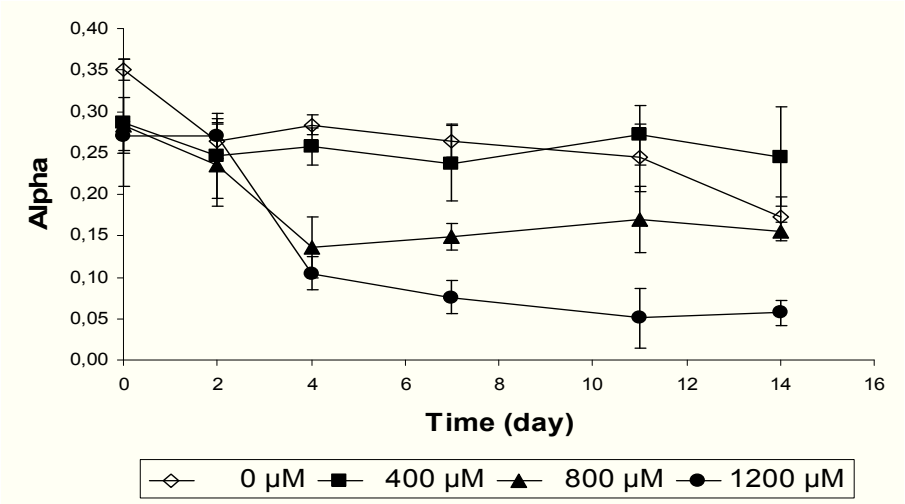


Fig. 18. Values of a) I_k; b) $rETR_{max}$, and c) α; of *G. verrucosa* with ammonium concentration treatments of 0, 400, 800, and 1200 µM under 300 µmol photons $m^{-2}s^{-1}$ irradiation in a 14 day experimental period.

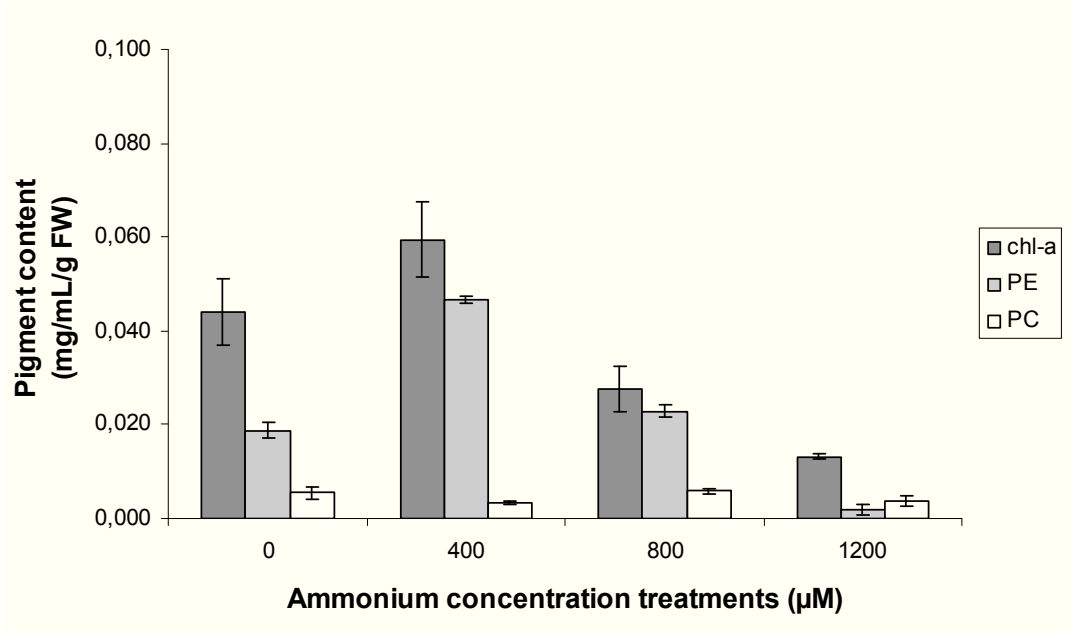
3.3.4 Pigment analysis

The pigment content (chl-a and PE) of *G. verrucosa* differed significantly among treatments ($F = 12.28$; $p < 0.000$; $F = 3.93$; $p = 0.003$, respectively), but not for PC ($F = 0.89$; $p = 0.51$). In general, chl-a contents had been found to be the highest compared to PE and PC, in all ammonium treatments, including control (Fig. 19a). With the exception of macroalgae with 1200 µM ammonium addition, PE contents show greater values compared to PC. However, the percentage of PE from controls were greater than PC and chl-a in lower ammonium treatments (400 and 800 µM) (Fig. 19b).

Growth rate

High ammonium concentration treatments significantly affected accumulative biomass ($F = 4.62$; $p = 0.02$) and total growth rates ($F = 16.81$; $p < 0.000$) of *G. verrucosa* (Fig. 20a and 20c, respectively). In all ammonium treatments, including controls, *G. verrucosa* showed rapid growth during the first time period (Fig. 20b). Tissue loss resulting in negative growth rates were observed in macroalgae with 1200 µM ammonium addition after one week of incubation. The highest growth rates of *G. verrucosa* over the 14 day experimental period was shown by macroalgae at 400 µM ammonium treatment, with an increasing biomass by about 2% day^{-1}.

a)



b)

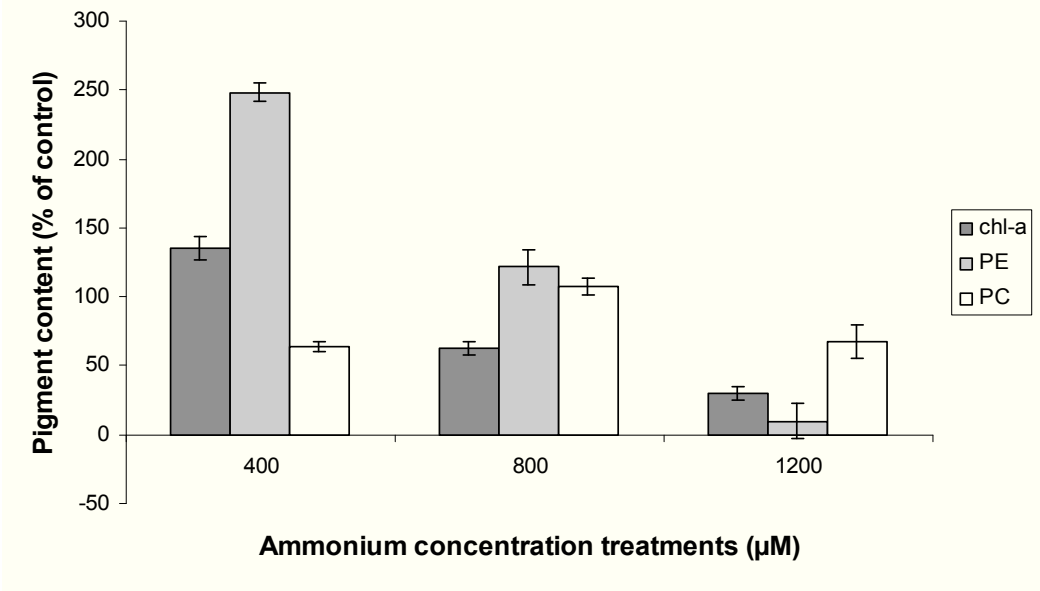
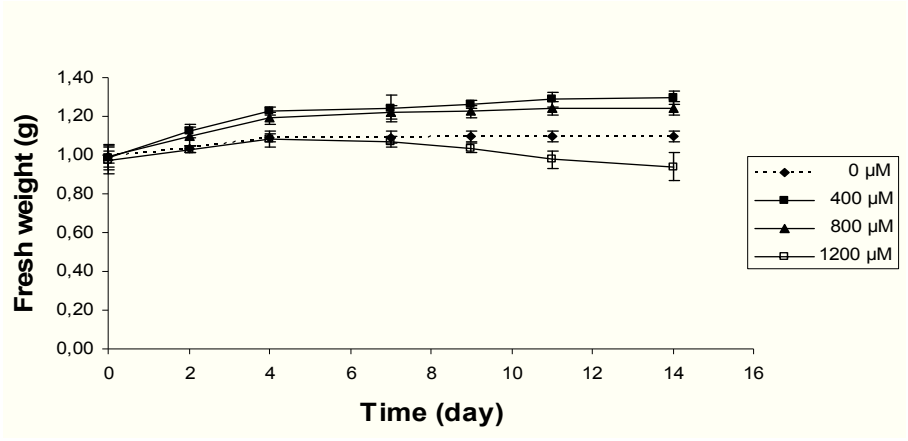
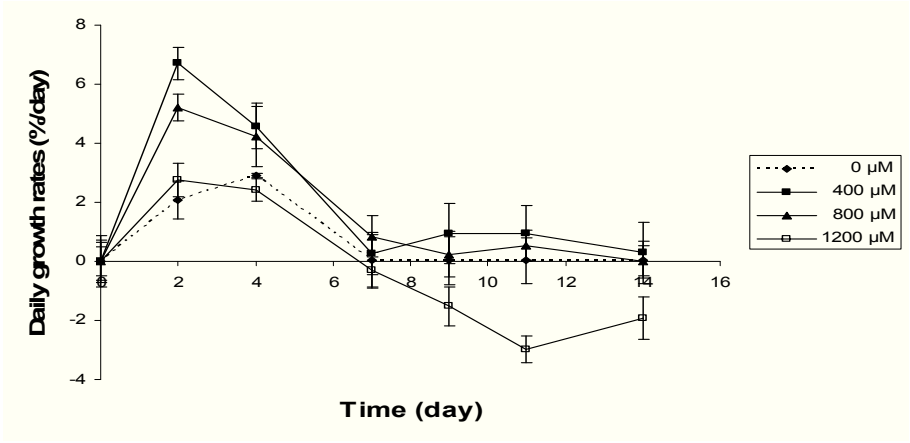


Fig. 19. Pigments content (a) and percentage of control (b) of *G. verrucosa* with ammonium concentration treatments of 0, 400, 800, and 1200 µM under 300 µmol photons $m^{-2}s^{-1}$ irradiation in a 14 day experimental period; chlorophyll (chl-a), phycoerythrin (PE), and phycocyanin (PC).

a)



b)



c)

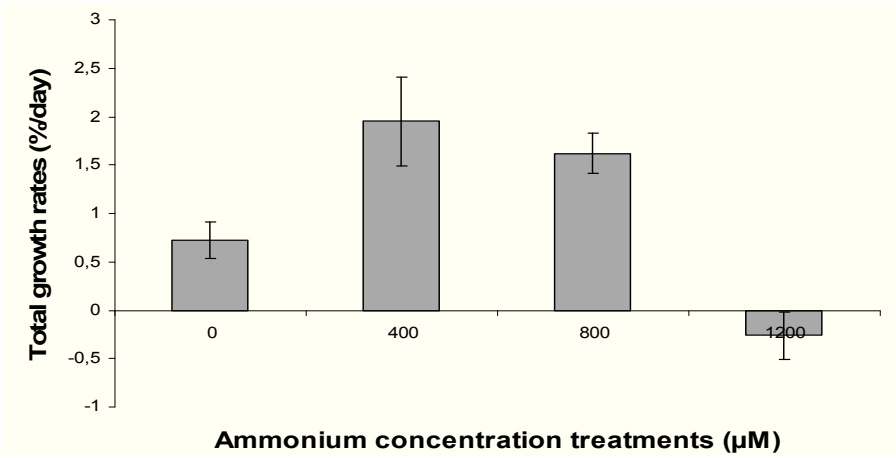


Fig. 20. Growth rates of *G. verrucosa* in physiological response experiments with ammonium concentration treatments of 0, 400, 800, and 1200 µM under 300 µmol photons $m^{-2}s^{-1}$ irradiation in a 14 day experimental period; a) accumulative biomass; b) daily growth rates; c) total growth rates in 14 days.

The results of the two-way ANOVA showed that irradiance and ammonium concentration treatments interacted differently in all parameters tested, except for phycoerythrin (Table 3). The results also showed that irradiances and ammonium treatments independently affected all parameters tested, except for irradiance to chl-a and ammonium concentration treatments to phycoerythrin (Table 3).

Table 3. Two-way ANOVA statistical analyses to test the interaction between ammonium concentration treatments and irradiances on the physiological response of *G. verrucosa* with ammonium concentration treatments of 0, 400, 800, and 1200 µM under 70 and 300 µmol photons $m^{-2}s^{-1}$ irradiation in a 14 day experimental period.

Parameters	groups	*F*	df	*p*
Ammonium content in tissue	a	10.11	1, 24	0.004
	b	322.96	3, 24	< 0.000
	c	54.34	3, 24	< 0.000
%N	a	20.60	1, 24	< 0.000
	b	66.33	3, 24	< 0.000
	c	35.86	3, 24	< 0.000
C:N	a	84.88	1, 24	< 0.000
	b	97.66	3, 24	< 0.000
	c	97.64	3, 24	< 0.000
Final F_v/F_m	a	62.27	1, 24	< 0.000
	b	11.54	3, 24	< 0.000
	c	34.61	3, 24	< 0.000
Chl-a	a	3.09	1, 24	0.09*
	b	11.74	3, 24	< 0.000
	c	3.18	3, 24	0.04
Phycoerythryn (PE)	a	66.12	1, 24	< 0.000
	b	2.59	3, 24	0.07*
	c	1.62	3, 24	0.21*
Phycocyanin (PC)	a	33.80	1, 24	< 0.000
	b	7.15	3, 24	0.001
	c	5.90	3, 24	0.004
Accumulative biomass	a	9.55	1, 24	0.005
	b	41.19	3, 24	< 0.000
	c	3.49	3, 24	0.031
Total growth rate	a	17.78	1, 24	< 0.000
	b	10.45	3, 24	< 0.000
	c	3.34	3, 24	0.036

a: between irradiances
b: among ammonium concentration treatments
c: interaction between irradiance and ammonium treatments
*: $p > 0.05$

Discussion

Nutrient uptake

Under 70 μmol photons $m^{-2}s^{-1}$ irradiation, *G. verrucosa* showed a surge phase of ammonium uptake in the early experimental period. The rapid uptake at the beginning of the incubation indicated that ammonium limitation during the starvation period affected the uptake rates. This also obviously described by the Michaelis-Menten saturation curve that showed linearity at time interval d_{0-2} and d_{2-4} in all ammonium concentration treatments. This result agreed with Peckol et al. (1994) and Fujita (1985), who found that ammonium uptake of *G. tikvahiae* is faster under nitrogen limiting conditions. A linear uptake response, particularly in nitrogen limitation (e.g. nitrogen starvation), point to a relatively large nitrogen storage capacity (Smit, 2002). Bird et al. (1982) reported that most studies of *Gracilaria* nutrient uptake had shown that *Gracilaria* spp. is able to take up ambient nitrogen rapidly and store it in organic form for later use during periods of nitrogen limitation. This could become an explanation of linear and surge uptake rates at the beginning of experimental period of *G. verrucosa*, under both irradiances.

The linearity of ammonium uptake kinetics in the early experimental period was also found in other studies of some macroalgal species, in which the macroalgae did not reach saturation with increasing ammonium concentration (Thomas et al., 1985; Friedlander and Dawes, 1985; Fujita, 1985; Lavery and McComb, 1991). This linearity at high ammonium concentration may represent passive diffusion via an ion channel (Lobban and Harisson, 1994). However, after day 4 the uptake kinetics curve of *G. verrucosa* showed saturation. At this time, the saturation indicated active transport, as also reported in *Codium fragile* by Hanisak and Harlin (1978).

Ryther et al. (1981) stated that *Gracilaria* can obtain and store enough nitrogen for non-limited growth. Thus, this storage is reflected in thallus nitrogen contents (3-5%), which can be substantially higher than those indicating nitrogen deficiencies (1.5-2%). The results of this study, both under 70 and 300 μmol photons $m^{-2}s^{-1}$ irradiation support Ryther et al. (1981) by showing high %N (3.2-3.5%) compare to control (1.4%). The high CN ratio in macroalgae is thought to be caused by their large amounts of structural and storage carbon, which vary taxonomically (Lobban and Harrison, 1994). Final ammonium content in tissue and %N of macroalgae with 1200 μM

ammonium addition under 300 μmol photons $m^{-2}s^{-1}$ irradiation showed lower values compared to other treatments, possibly due to stress in higher irradiance levels. *G. verrucosa* with ammonium concentration treatments no greater than 800 μM were able to assimilate nitrogen from ammonium in the tissue at both irradiances. This was ascertained by the high %N (> 3%) and low CN ratio (< 16). Continuously high ammonium concentrations in combination with high irradiance in a long duration of experimental period impaired ammonium uptake process of *G. verrucosa* in this study.

Photosynthetic performance

In general, photosynthetic performance was affected by the high ammonium concentration treatments and different irradiances. The results of different irradiance treatments indicated that 400 μM ammonium additions appeared to be the most convenient concentration for *G. verrucosa* to utilize the light for photosynthetic processes.

After one week of incubation, *G. verrucosa* under 300 μmol photons $m^{-2}s^{-1}$ irradiation showed lower photosynthetic performance, compared with macroalgae under 70 μmol photons $m^{-2}s^{-1}$ irradiation. Presumably, high ammonium concentration treatments cannot be tolerated by *G. verrucosa*, in higher irradiances. Reduction in photosynthethetic parameters of *G. verrucosa* under combined high ammonium and high irradiance were not entirely attributable to reduced concentrations of the components photosynthetic apparatus (e.g. pigments), because pigments, $rETR_{max}$ and F_v/F_m decreased relative to high ammonium concentration treatments.

In this study, increasing irradiance also caused an increase in water temperature, although room temperature was maintained at constant value. This condition contributed a slightly higher water temperature in experiments under 300 μmol photons $m^{-2}s^{-1}$ irradiation. Although other studies have shown that the temperature optima of macroalgae were higher than the highest mean monthly seawater temperature (Zupan and West, 1990; Sakanishi and Iizumi, 1998), the range of temperature optima for photosynthesis in some macroalgae became lower with a reduction or addition in irradiance (Kurashima et al., 1996; Sakanishi and Iizumi, 1998; Zou and Gao, 2005). Possibly, this condition might explain the decreasing photosynthetic performance of *G. verrucosa* under high irradiance, although the FAO (1990) reported that *Gracilaria* spp.

has a wide range of temperature tolerance to growth (15-32°C). The combination of high irradiance and high ammonium concentration treatment in duration of more than 7 days might depress photosynthetic process of *G. verrucosa*. It might also be the reason of the decreasing I_k, which suggested inefficient use of high irradiance rather than efficient use of low irradiance (Henley, 1993).

Irradiance modifies the nitrogen requirement for maximal photosynthesis and growth by altering biochemical constituents (i.e., pigments, RuBisCO, and nitrogen reserves) that affect the nitrogen level in the tissue (Lapointe and Duke, 1984). Macroalgae under 70 µmol photons $m^{-2}s^{-1}$ irradiation confirmed Lapointe and Duke (1984) by the demonstrated high %N in all ammonium concentration treatments. The similar results was also demonstrated by macroalgae under 300 µmol photons $m^{-2}s^{-1}$ irradiation, with ammonium additions of 400 and 800 µM, but not with ammonium addition of 1200 µM. This indicated that high irradiance in combination with high ammonium concentration treatments impaired nitrogen assimilation, which possibly impaired the synthesis of pigments.

The chl-a content demonstrated higher values than phycobiliprotein in lower ammonium treatments (400 and 800) under both irradiances, although percentage of controls showed contrary results. The values of phycoerythryn and phycocyanin in macroalgae under 300 µmol photons $m^{-2}s^{-1}$ irradiation were lower than in macroalgae under 70 µmol photons $m^{-2}s^{-1}$ irradiation). This was evident to the result reported by Jayasankar and Varghese (2000) that pigments in macroalgae decrease with increasing irradiance, and might indicate to some damages caused by high irradiance (Lobban and Harrison, 1994). A very high irradiance may cause photoinhibition, which involves damage to some components of the photosystems (especially PS II), such as the membranes or electron transport proteins, although the exact locations of damage and the mechanisms of resistance and repair in diverse plants remain uncertain (Lobban and Harrison, 1994). When macroalgae are shifted to high light, some of their pigment decreases through degradation (Falkowski and LaRoche, 1991).

Growth rates

The results of this study which were conducted under laboratory conditions, showed different growth rates of *G. verrucosa* at different ammonium concentration treatments

and irradiances. A surge of the growth rate was shown only at the beginning of the experimental period, and *G. verrucossa* grew slowly. However, ammonium availability affected macroalgal accumulative biomass. Daily growth rates reached the highest at 3.8% day^{-1} for macroalgae under 70 µmol photons $m^{-2}s^{-1}$ irradiation, and 6.9% day^{-1} for macroalgae with 300 µmol photons $m^{-2}s^{-1}$ irradiation. The results of this study contributed to the information of various growth rates that had been reported in other studies of *Gracilaria* spp. Hurtado-Ponce (1990) reported higher results (10.5% day^{-1}) for *Gracilaria* sp. from Philippines cultured in open water. Raikar et al. (2001) showed also higher daily growth rates for G. *lichenoides* from Malaysia (11.69% day^{-1}), *G. vermiculophylla* from Japan (22.32% day^{-1}), and *G. foliifera* from India (10.62% day^{-1}). Other studies reported slightly different growth rates from *G. verrucosa* than were obtained in this study. Marinho-Soriano et al. (2009) reported lower maximum daily growth rate of *G. birdie* (3.6% day^{-1}). *G. lichenoides* from Indonesia is also reported to reach maximum daily growth rate of 4.34% day^{-1} in open water cultivation (Atmadja and Sulistijo, 1980). In the peak season, *G. tenuistipitata* v. liui cultivated in open water in China reported to have reached maximum daily growth rate of 3.3% day^{-1} (FAO, 1990). Xu et al. (2008) reported mean daily growth rate of *G. lemaneiformis* (3.87% day^{-1}) in outdoor cultivation nearby several animals aquaculture in China. Troell et al. (1997) reported that *G. chilensis* co-cultivated with a salmon cage farm in southern Chile (10 m distance from the cages), had a mean growth rate of 7% day^{-1}. Although studies on the effects of various nutrient availabilities to macroalgae demonstrate relatively low values on growth rates, it is difficult to compare among studies and conclude that a particular general pattern exists. This situation might be due to the different experimental set ups and calculation formulas being used among studies.

Studies on the effects of nutrient addition to tropical species of the red algal *Gracilaria* have demonstrated that the highest growth rates occur under conditions of higher irradiance and water movement, although phycocolloid content usually decreases (Dawes, 1987). The increasing growth rate at the end of the experimental period at ammonium concentration treatments 200 and 400 µM might indicate better ammonium and light utilization of *G. verrucosa* compared to the other ammonium concentration treatments being given.

Ammonium tolerance and toxicity

There are very few macroalgal experiments conducted that use very high ammonium concentration treatments or macroalgal experiments intended to demonstrate ammonium toxicity. Thus, results of studies on higher plants are used to compare to this study concerning ammonium tolerance and ammonium toxicity symptoms. Higher plants, terrestrial and aquatic, had been reported to show ammonium toxicity (Ganmore-Neumann and Kafkafi, 1983; Van Katwijk et al., 1997; Gerendas et al., 1997; Wilcox et al., 1985). The symptoms obviously seen by chlorosis of leaves, and the overall suppression of growth (Breteler, 1973; Kirkby and Mengel, 1967; Britto and Kronzucker, 2002), or necrosis at the leaf tips (Van Katwijk et al., 1997).

Ammonium toxicity could be the result of one or more of the following effects: (i) ammonium-induced mineral nutrient deficiency, arising from the impaired uptake of metal ions; (ii) growth inhibition arising from the acidification of the medium; (iii) alterations in intracellular pH; and (iv) uncoupling of photophosphorylation from electron transport, following the accumulation of ammonium in tissue (Gerendas, et al., 1997).

Taking the accelerated export of ammonium uptake and assimilation to macroalgae under conditions of high irradiance into account, it might be expected that the ammonium tolerance increases when macroalgae were grown at a higher irradiance. However, a greater tolerance was not shown when *G. verrucosa* were exposed to 300 µmol photons $m^{-2}s^{-1}$ irradiation, especially when combined with high ammonium concentrations (1200 and 2000 µM). An uptake saturation curve already appeared in the early experimental period, although a surge phase was also observed at the same time.

At a concentration higher than 50 µM, ammonium may be toxic to some species of seaweed (Waite and Mitchell, 1972). In this study, a very high ammonium concentration treatment was used in combination with high irradiance. The treatments decreased the ammonium tolerance of *G. verrucosa* throughout the experiments. At the last two time intervals of the experimental period, *G. verrucosa* exceeded the saturation uptake rates and even showed toxicity symptoms, as discoloration and negative growth rates. This might be the reason of the low ammonium content in tissue for macroalgae with 1200 µM ammonium addition, although at the beginning of the exposure time they showed surge uptake. The visual symptoms of discoloration as chlorosis were similar to

ammonium toxicity as it happens in higher plants (Britto and Kronzucker, 2002; Van Katwijk et al., 1997).

Ganmore-Neumann and Kafkafi (1983), who conducted an experiment of ammonium toxicity in higher plants, reported that at high root temperatures, the ammonium tolerance of plants is often reduced. This was explained by the high carbon demand for ammonium detoxification, which competes with the increasing respiratory carbohydrate consumption at higher temperatures (Wakimoto and Yamada, 1985). This presumably happened in this study, conducted under high irradiance (300 μmol photons $m^{-2}s^{-1}$ irradiation). The treatment of higher irradiance resulted in a higher water temperature, although for *G. verrucosa* it was still at the range for optimal growth (FAO, 1990). Ammonium tolerance decreased with increasing irradiance.

Nitrogen supply exerts a strong influence on the charge balance and, therefore, ion homeostasis, which is reflected by an alteration of the tissue pH (Gerendas and Sattelmacher, 1990). The high supply of ammonium as cationic nitrogen source, induces significant acidification in media, and was reported to be responsible for ammonium induced growth depressions (Findenegg, 1987).

Conclusion

The most significant results of this study were that ammonium concentration treatments and different irradiances interacted significantly to influence physiological responses (nutrient uptake, photosynthetic performance, and growth rate) of *G. verrucosa*. The unsaturated ammonium uptake shown by *G. verrucosa* was not a passive diffusion process, but rapid active transport, possibly due to huge storage capacity. *G. verrucosa* showed the best physiological responses performance in the treatment of 400 μM ammonium addition under 70 μmol photons $m^{-2}s^{-1}$ irradiation. Ammonium tolerance of *G. verrucosa* decreased with increasing ammonium concentration treatments and irradiance. At a very high ammonium concentration treatment over one week of the incubation period, *G. verrucosa* showed toxicity symptoms. Rapid uptake and a significant growth rate in a relatively short time of *G. verrucosa* might contribute advantages to integrate this species with other organisms in such aquaculture systems in the future. However, further analysis of biochemical compound might be necessary to investigate ammonium toxicity of *G. verrucosa*.

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5. STUDY 3

Ammonium and irradiance stress of tropical macroalga *Gracilaria verrucosa* (Rhodophyceae)

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Abstract

High ammonium supply in combination with high irradiance to macroalgae may induce photoinhibition of photosynthesis and affect growth. Responses of *Gracilaria verrucosa* (Hudson) Papenfuss to high nutrient and light stress were studied under laboratory conditions. Changes in growth rates, protein content, and photosynthetic performance (F_v/F_m, PI curves, $rETR_{max}$, I_k, α, and NPQ) were studied in order to investigate the further response of *G. verrucosa* to ammonium and irradiation stress. A seven day experiment was conducted with *G. verrucosa* in a climate room with 1200 µM ammonium addition under 70 and 300 µmol photons $m^{-2}s^{-1}$ irradiation. In general, *G. verrucossa* showed low performance in most of the observed parameters. Protein content in all treatments showed values lower than 0.5 µg/µL extract. Discoloration as a symptom of an infectious disease occured on day 4 in macroalgae under 300 µmol photons $m^{-2}s^{-1}$ irradiation.

Key words: ammonium, discoloration, disease, *G. verrucosa*, irradiance, stress.

Introduction

Growth, survival, and reproduction of macroalgae are determined by different environmental factors, such as light (irradiance, light quality, and photoperiod) and temperature (Lobban and Harrison, 1997). Under high irradiance, the photosynthetic apparatus absorbs excessive light energy, and as a consequence, photosynthetic activity is depressed by photoinhibition (Osmond, 1994). Photoinhibition of photosynthesis by visible light occurs whenever light is absorbed at a greater rate than it can be processed (Krause, 1988). Excessive absorbed energy may cause damage to the photosynthetic apparatus. However, photodamage may vary with different wavelength ranges (Andersson et al., 1992).

During dynamic photoinhibition excessive energy is harmlessly dissipated as heat in the antenna complex causing reduced photosynthetic efficiency, which is fully reversible on a short time scale when the radiations stress decreases (Krause and Weis, 1991). In contrast, chronic photoinhibition leads to inactivation of the D1-protein in the reaction center of PSII, thus reducing the photosynthetic capacity for much longer time periods (Bischof et al., 1998).

Marine macrophytes perform different mechanisms against photoinhibition, such as movement of chloroplasts (Hanelt and Nultsch, 1991). Protein synthesis is necessary for recovery from photochemical damage (Ohad et al., 1984). These protective mechanisms are related to the ecological importance of photoinhibition. Hanelt et al. (1997) and Hanelt (1996) suggested that the ability to resist high light stress may be one of the factors determining the competitive ability of macroalgae at the upper limits of their zone.

Despite of a variety of adverse effects of solar ultraviolet radiation, the existence of repair and protective mechanisms indicate that macroalgae have developed a capacity of adaptation to high irradiance conditions. The efficiency of recovery was dependent on the concentration of ammonium (Huovinen et al., 2006). To predict the responses and resistance of *G. verrucosa* to high irradiance and ammonium concentration, information on physiological response and biochemical compounds (e.g., protein content) of *G. verrucosa* needs to be obtained. In this study inhibition and recovery of photosynthesis was observed as a protective mechanism to high irradiance and high ammonium stress.

Materials and methods

A 7 day experiment was conducted in a climate room (24^{o}C) under irradiances of 70 and 300 μmol photons $m^{-2}s^{-1}$ based on the lower irradiance limit by Lüning (1981) and irradiance saturation on the previous study, in a 12:12 hour light:dark photoperiod. Approximately 1 g of macroalgal material was placed in glass beakers with 1 L of enriched filtered (0.2 μm) seawater (NH_4Cl addition) in a concentration of 1200 μM and 0 μM as a control, with three replicates for each treatment. Stir bars were placed in each beaker and all beakers were placed on magnetic plates at low rotational speed of 120 rpm to avoid localized nutrient depletion. Electric fans were used around high irradiance chambers to ensure air circulation and maintain the temperature around the beakers as expected. Culture media were changed every other day to avoid depletion of nutrients. Fresh weight and photosynthetic performance were measured on day 0, 2, 4, and 7. Macroalgal tissue were taken initially and finally and stored in -80^{o}C until total protein content analysis.

Photosynthetic performance. Photosynthetic activity was determined by measuring variable chlorophyll fluorescence using a PAM 2100 device (Walz, Germany). Maximum quatum yield was calculated as the ratio of variable to maximum fluorescence (F_v/F_m) of the dark-acclimated plant (Bischof et al., 1998). The algal thalli were fixed to the end of the fiberoptics and placed in a beaker filled with seawater. The beaker was covered with aluminum foil to avoid light leakage from other light sources.

Photosynthesis versus irradiance curves (PI-curves) were calculated following procedures described by Bischof et al. (1998). Algal samples were irradiated with increasing irradiance of actinic red light (10-700 μmol $m^{-2}s^{-1}$, 650 nm). After 30 seconds, a saturating pulse was applied to measure effective quantum yield of photosynthesis and then actinic irradiation was increased. By multiplying quantum yield with photosynthetically active radiation (PAR) relative electron transport rates were calculated as described by Schreiber et al. (1994):

$$rETR = \Delta F/F_m' \times PAR$$

with ΔF as the difference of F_m (maximum fluorescence) and F_o (minimum fluorescence), F_m' as maximum fluorescence yield reached in a pulse of saturating light when the sample is preilluminated.

The relative ETR were plotted against irradiance of actinic light, and the maximal relative electron transport rate ($rETR_{max}$, under saturating light) was determined by curve-fitting. Data were processed using a PAM Win program and Kaleidagraph to obtain photosynthesis capacity ($rETR_{max}$), saturation point (I_k), and photosynthetic efficiency (α). Non-Photochemical Quenching (NPQ) was determined by measuring variable chlorophyll fluorescence and was calculated as described by Consalvey et al. (2005):

$$NPQ = (F_m - F_m')/F_m'$$

where F_m is maximal fluorescence yield of a dark adapted sample, and Fm' is a value of F_m substracted by minimum fluorescence yield of dark adapted sample.

Pigment analyses. Chlorophyll-a (chl-a) content was determined photometrically by following the method described by Inskeep and Bloom (1985). Approximately 0.1 g of algal sample was transferred into test tubes containing 5 ml of dymethylformamide (DMF). Samples were stored in a refrigerator at 4^oC in darkness for 4 days. The extinction of the DMF solution was then measured at 664.5 nm in a UV spectrophotometer. The chlorophyll content was calculated using the formula:

$$\text{Chl-a (mg L}^{-1}) = 12.7 \times E_{664.5}$$

where E is the extinction of the DMF solution at wavelength 664.5 nm, with 12.7 as the constant.

Phycobiliproteins were extracted using the method described by Beer and Eshel (1985). Sample preparation was done using 0.03-0.1 g of algal tissue on average. Algal tissue was ground using a dismembrator for 3 min with 1500 RPM. All sample preparation was done under dark conditions and maintained frozen by emerging in liquid nitrogen. After being homogenized, samples were transferred into centrifuge test

tubes, and extracted in 5 mL of 1.0 M phosphate buffer (pH = 6.8). The extracts were centrifuged at 6000 g in 4°C for 20 min. The supernatant was used for phycobiliprotein determination by measuring the absorbance in a UV spectrophotometer (Shimadzu, Japan). The phycoerythrin and phycocyanin concentrations were determined using the equations:

$$PE = ((A_{564} - A_{592}) - (A_{455} - A_{592})\ 0.20)\ 0.12$$
$$PC = ((A_{618} - A_{645}) - (A_{592} - A_{645})\ 0.51)\ 0.15$$

where PE and PC are phycoerythrin and phycocyanin, respectively, A is the absorbance in UV spectrophotometer under different wavelengths (λ = 455, 564, 592, 618, 645 nm).

Growth rates. The growth of macroalgae was recorded as changes in the fresh weight of macroalgal fragments for a particular interval of time. Fragments were taken from beakers, and gently blotted on a paper to remove seawater before weighing on an analytical digital balance Sartorius (Germany). Daily growth rates were calculated after measuring the accumulative biomass following the formula described by Lignell and Pedersen (1989):

$$DGR\ (\%\ day^{-1}) = [(W_t/W_i)^{1/t}-1] \times 100$$

where DGR stands for daily growth rates, W_i is initial fresh weight, W_t is fresh weight after particular time period (t days), and t is time (days) of cultivation.

Total protein content. Total protein content was measured following the method described by Bischof et al. (2000) as a modification of the Bradford method. Overall protein content in crude extracts was determined using a commercial Protein Assay (BioRad). Sigma Protein Standard was used as BSA stock solution. Protein content was determined by measuring extinction at wave length 595 nm using a UVPC spectrophotometer (Shimadzu, Japan). Protein concentration was determined according to a calibration curve prepared with known concentrations of 1 mg/mL bovine serum albumin.

Statistical analyses. Mean values and their standard deviations were calculated from the different replicates per treatment. The data were tested for assumptions of normality by examining heterogeneity of variance (Cochran's test). Non-normal data were subject to root transformation. Analyses of variance (ANOVA) were employed to examine for differences among ammonium treatments. The significance level used was $p < 0.05$. Tukey's test was used for post hoc analyses of multiple comparisons among treatment means from a significant ANOVA test. The computer software STATISTIKA 9.0 was used for all statistical analyses.

Results

Abiotic parameters

Water temperatures increased in day 2, and fluctuated slightly at the range 25.2-29.8°C (Fig. 1a). The water temperatures remained at the same level to the end of the experiments. The room temperature was maintained at 24°C during the experimental period. Salinities fluctuated slightly, ranging between 32.3-34.2 ppt (Fig. 1b). The final pH of the seawater media showed no significant differences ($F = 52.23$; $p = 0.08$) to initial values (Fig. 1c). In general, the pH ranged from 7.85 at the beginning of the experimental period to 8.21 at the end. Final concentration of dissolved oxygen decreased significantly ($F = 157.09$; $p < 0.000$) for both irradiances (Fig. 1d).

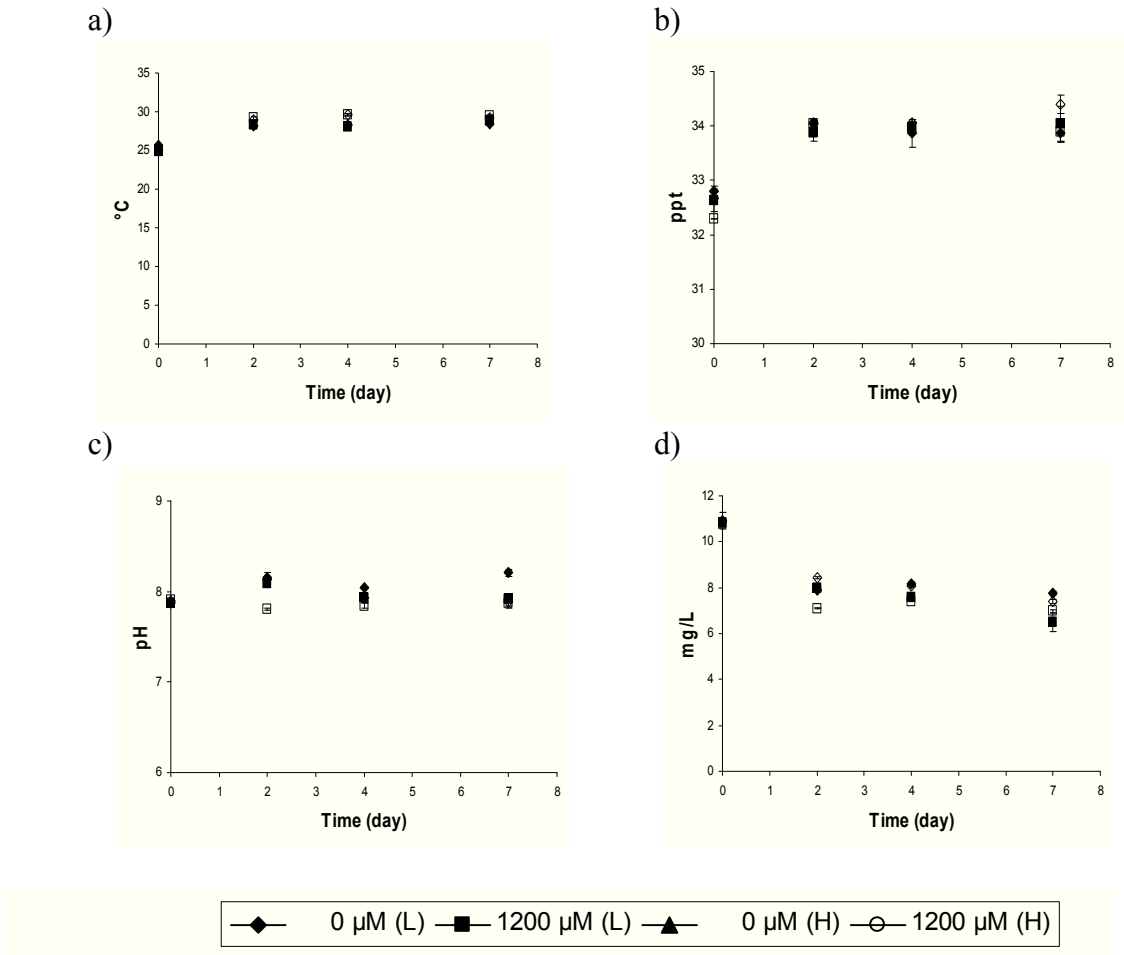


Fig. 1. Abiotic parameters of media seawater of *G. verrucosa* with ammonium concentration treatments of 0 and 1200 µM under 70 (L: low irradiance) and 300 (H: high irradiance) µmol photons $m^{-2}s^{-1}$ irradiation in a 7 day experimental period. Water was replaced every 2 days; a) water temperatures; b) salinity; c) pH; and b) dissolved oxygen.

Photosynthetic performance

The F_v/F_m significantly changed with ammonium addition and both irradiances of 70 and 300 µmol photons $m^{-2}s^{-1}$ (F = 5.87; p = 0.003). Under irradiation of 70 µmol photons $m^{-2}s^{-1}$, the F_v/F_m of *G. verrucosa* decreased slightly during the exposure time (Fig. 2). The F_v/F_m of macroalgae under 300 µmol photons $m^{-2}s^{-1}$ irradiation decreased drastically throughout the experiments, for both control and 1200 µM ammonium addition. The F_v/F_m of macroalgae under 70 and 300 µmol photons $m^{-2}s^{-1}$ irradiation were finally reduced to 70 and 30 % of the initial values, respectively.

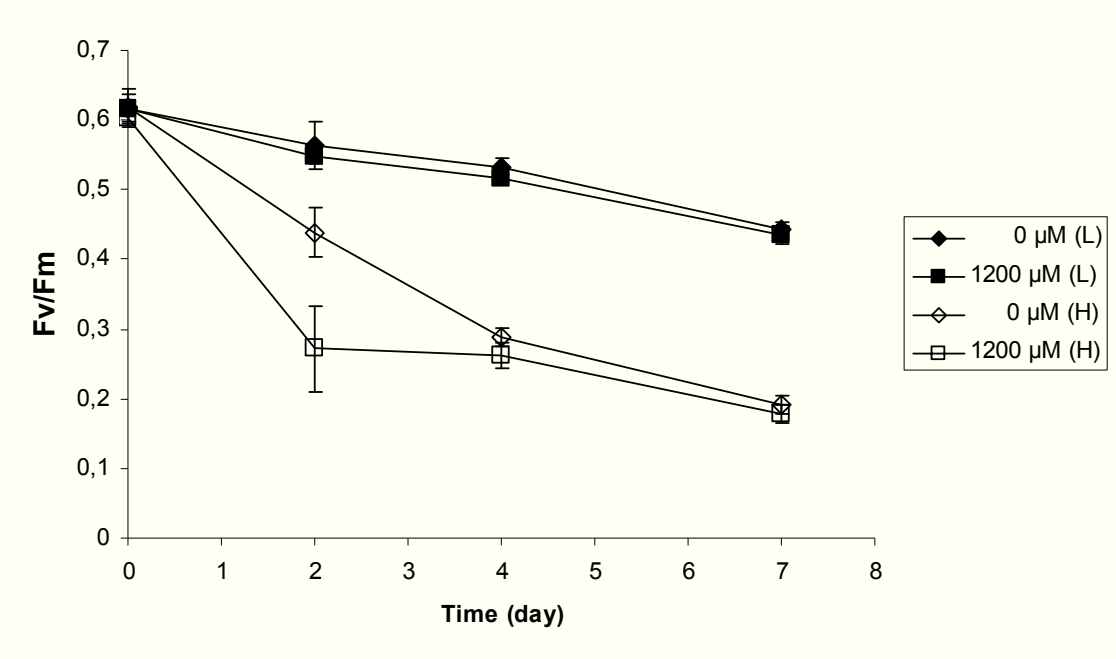


Fig. 2. Fv/Fm of *G. verrucosa* in physiological response experiments with ammonium concentration treatments of 1200 µM under 70 (L: low irradiance) and 300 (H: high irradiance) µmol photons $m^{-2}s^{-1}$ irradiation in a 7 day experimental period.

In general, rETR of all treatments reached the highest values at initial measurement, and decreased by the end of the experimental period. At initial measurements, the rETR curve of all treatments showed a similar curve pattern (Fig. 3). At day 2, rETR values of macroalgae with 1200 µM ammonium addition under 300 µmol photons $m^{-2}s^{-1}$ irradiation were higher than the other treatments and the control treatment at low light. However, the rETR of macroalgae with 1200 µM ammonium addition under 300 µmol photons $m^{-2}s^{-1}$ irradiation then decreased by day 4. By day 7, however, the lowest rETR were found in the high irradiance treatment.

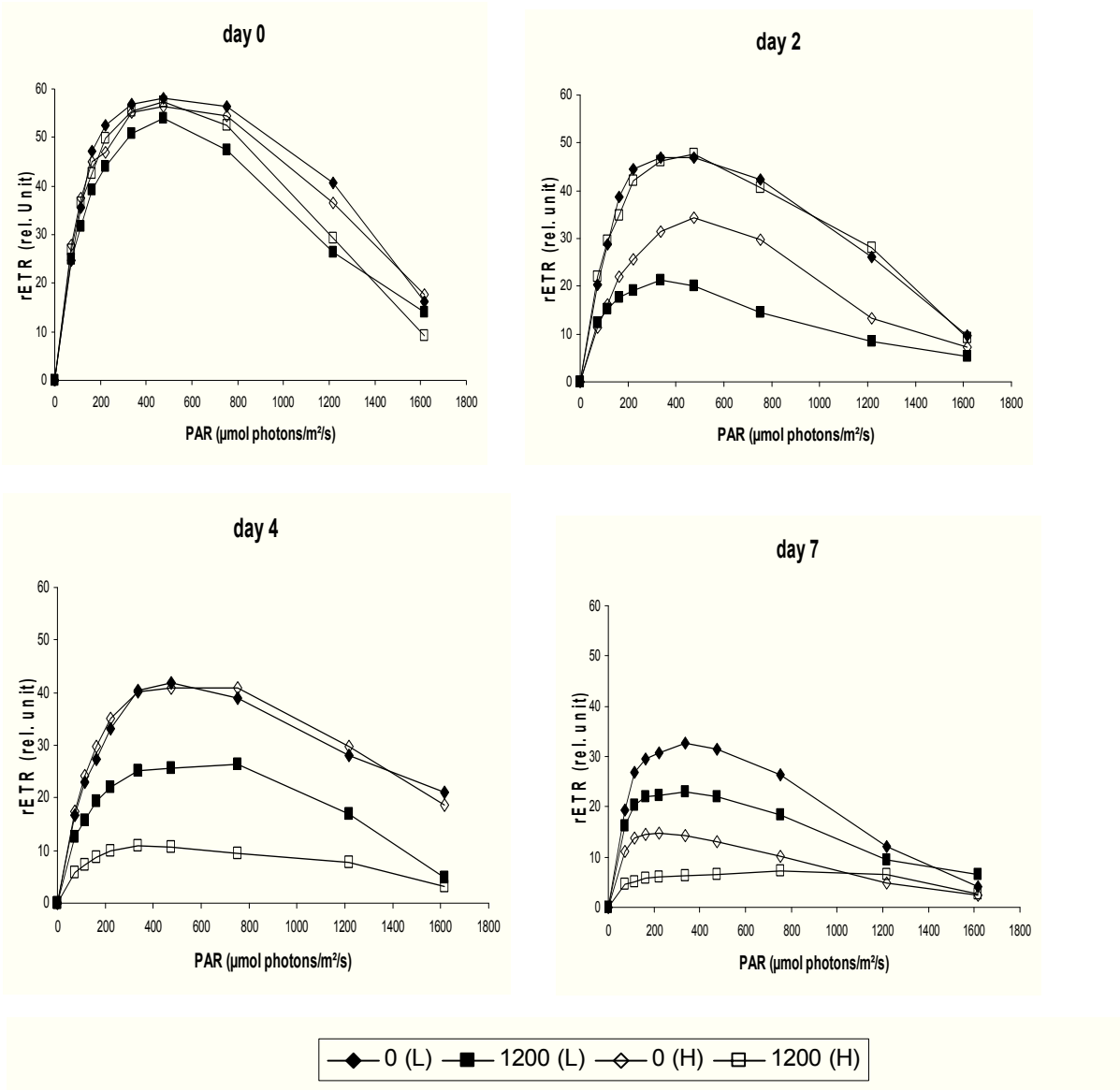
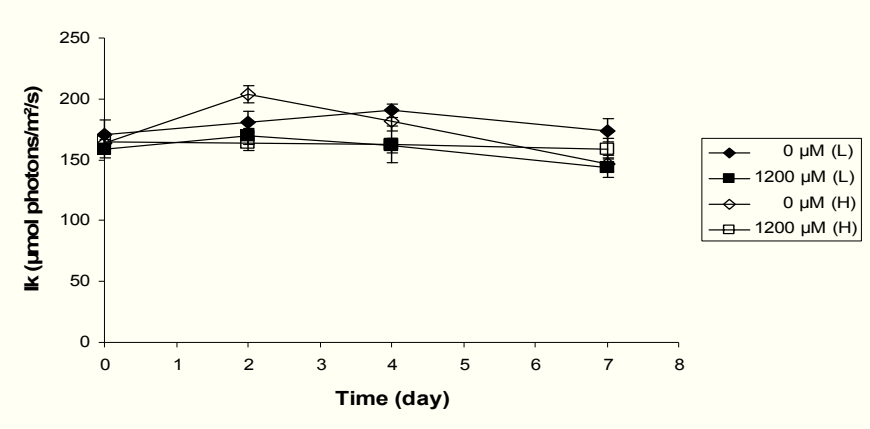


Fig. 3. PI curves of *G. verrucosa* in physiological response experiments with ammonium concentration treatments of 0 and 1200 µM under 70 (L: low irradiance) and 300 (H: high irradiance) µmol photons $m^{-2}s^{-1}$ irradiation in a 7 day experimental period (mean ± SD).

The ammonium addition of 1200 µM contributed to lower irradiance saturation point throughout the experiment relative to low ammonium controls for both 70 and 300 µmol photons $m^{-2}s^{-1}$ irradiation. Photosynthesis capacity ($rETR_{max}$) decreased in various patterns over time for all treatments including controls with high treatments being the most affected by the end of the experiment. Apparent photosynthetic efficiency (α) decreased over time with a similar pattern at $rETR_{max}$.

a)



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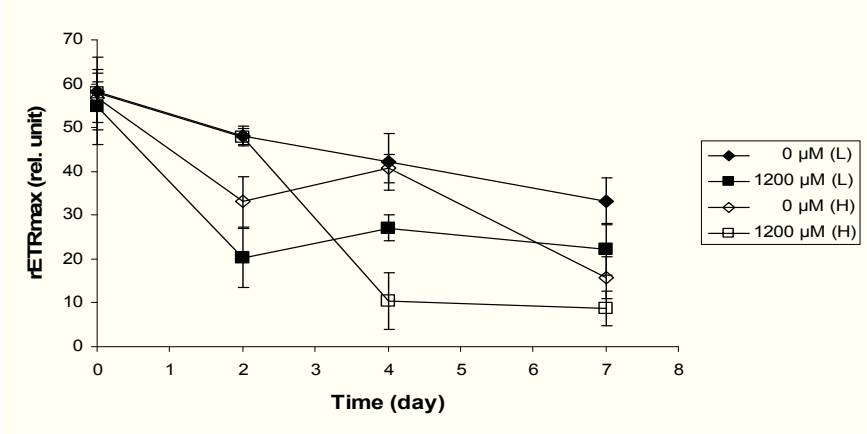

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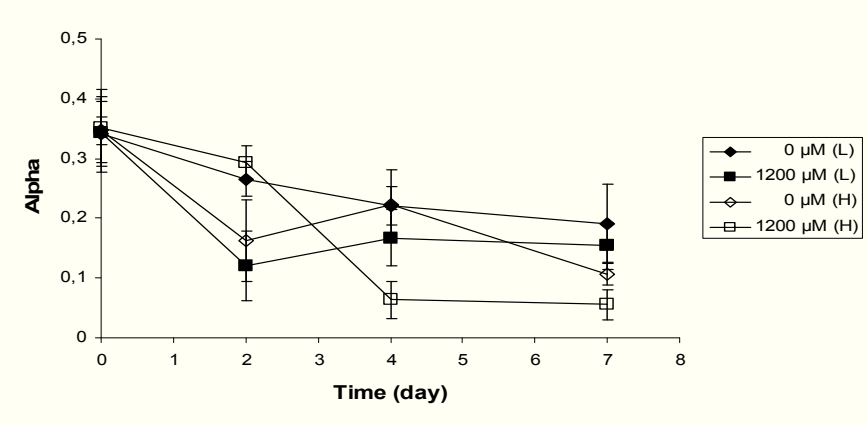


Fig. 4. Values of I_k, $rETR_{max}$, and α of *G. verrucosa* with ammonium concentration treatments of 0 and 1200 µM under 70 (L: low irradiance) and 300 (H: high irradiance) µmol photons $m^{-2}s^{-1}$ irradiation in a 7 day experimental period.

Non-photochemical quenching

The non-photochemical quenching (NPQ) hardly reacted differently to the treatments of 1200 µM ammonium addition under both irradiances ($F = 0.56$; $p = 0.47$) (Fig. 5). The response of NPQ macroalgae in the ammonium addition and irradiances treatments were plotted against the F_v/F_m values (Fig. 6). As the F_v/F_m decreased throughout the experiment, NPQ did not increase as expected.

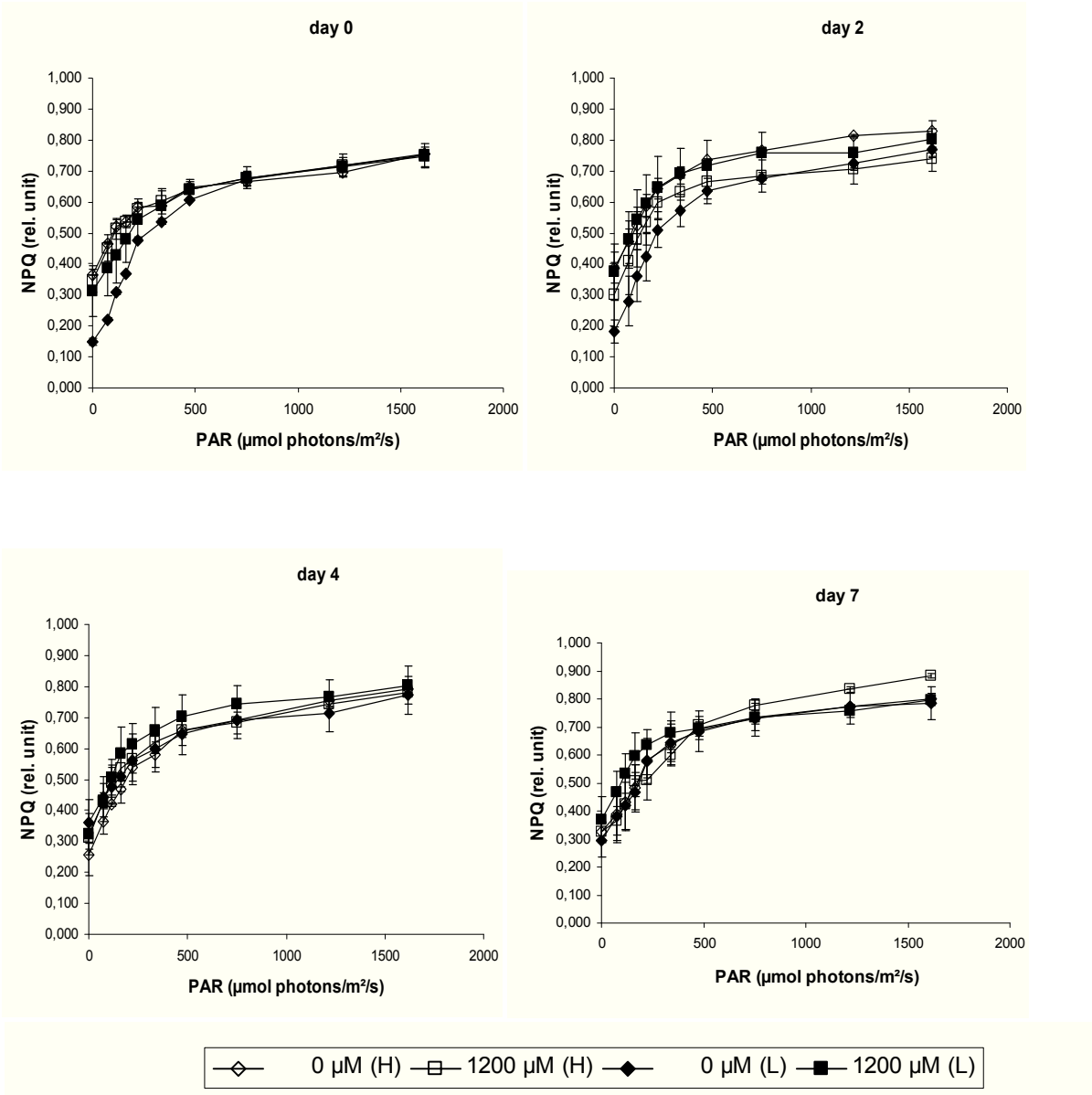


Fig. 5. Non-photochemical quenching of *G. verrucosa* in ammonium concentration treatments of 0 and 1200 µM under 70 (L: low irradiance) and 300 (H: high irradiance) µmol photons $m^{-2}s^{-1}$ irradiation in a 7 day experimental period.

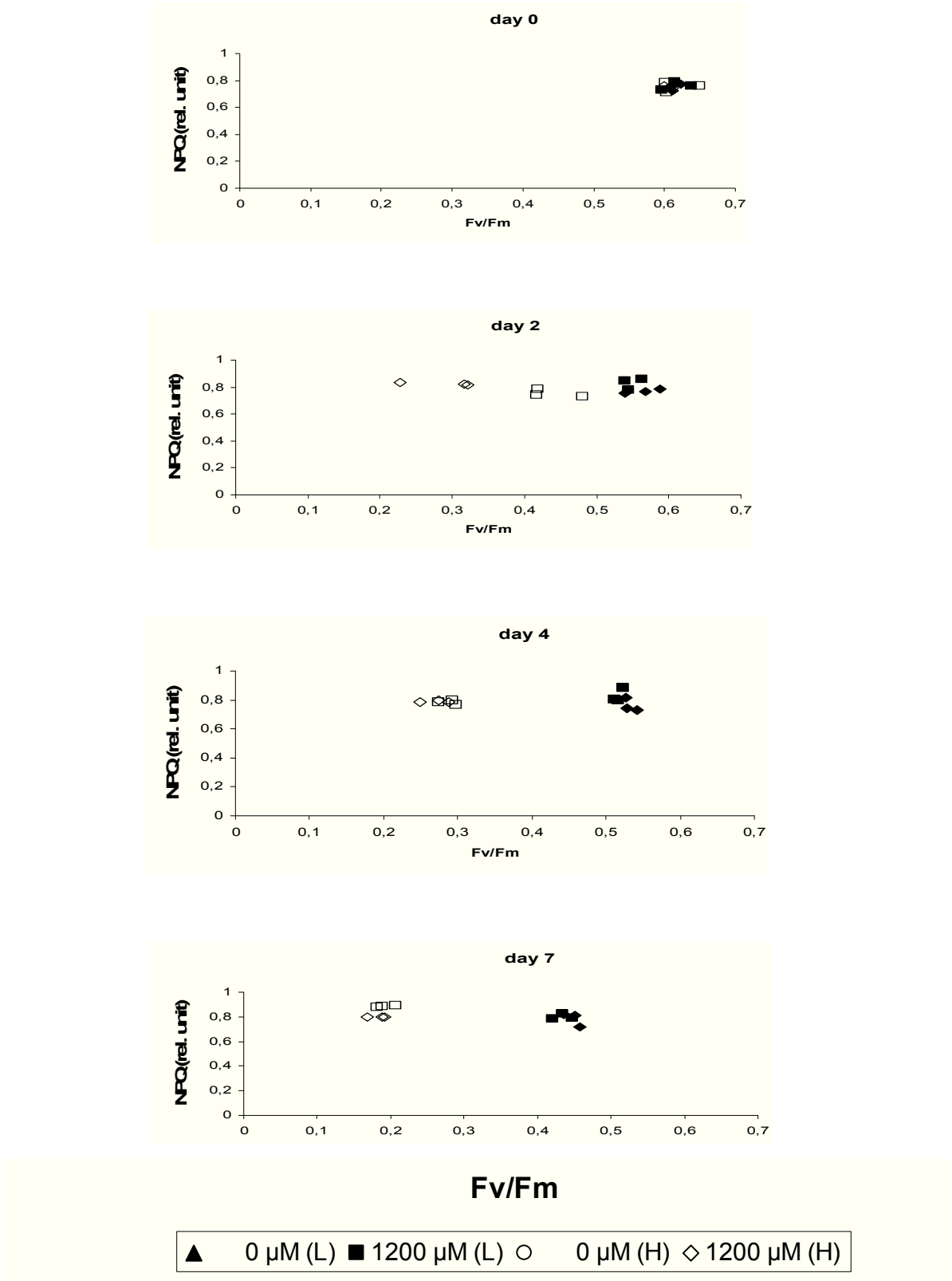
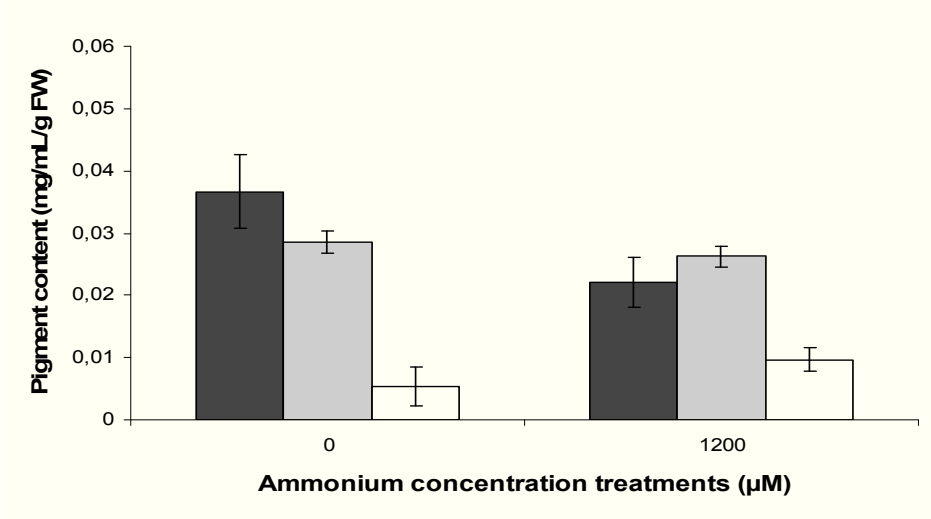


Fig. 6. Non-photochemical quenching versus Fv/Fm of *G. verrucosa* in ammonium concentration treatments of 0 and 1200 µM under 70 (L: low irradiance) and 300 (H: high irradiance) µmol photons $m^{-2}s^{-1}$ irradiation in a 7 day experimental period; NPQ values from the highest PAR (1600 µmol photons $m^{-2}s^{-1}$).

Pigments content

The ammonium concentration treatment of 1200 µM and different irradiances contributed significantly to chl-a content of *G. verrucosa* ($F = 103.36$; $p < 0.000$), but not to PE ($F = 0.09$; $p = 0.76$), and PC ($F = 1.11$; $p = 0.32$) content (Fig. 7).

a)



b)

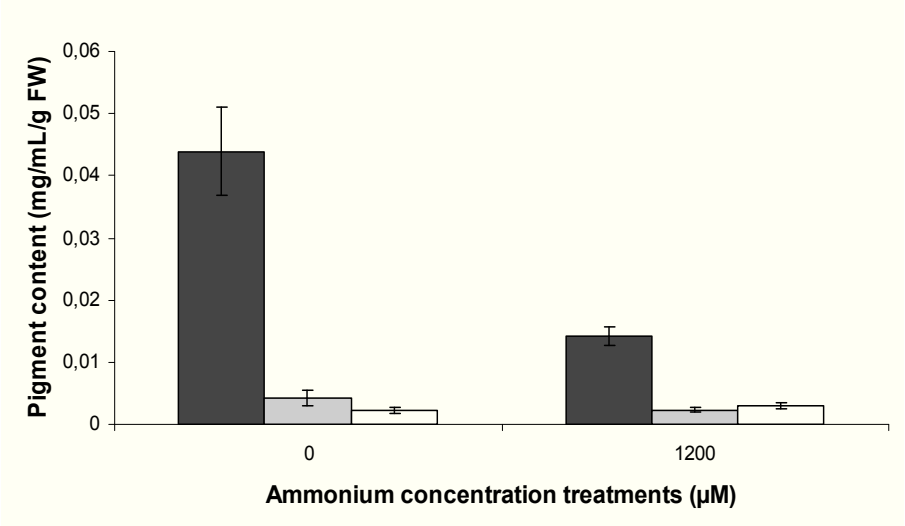


Fig. 7. Pigments content of *G. verrucosa* in physiological response experiments with ammonium concentration treatments of 0 and 1200 µM under 70 µmol photons $m^{-2}s^{-1}$ irradiation (a) and 300 µmol photons $m^{-2}s^{-1}$ irradiation (b) in a 7 day experimental period.

Growth rate

The treatments of ammonium addition and both irradiations contributed significantly to accumulative biomass and growth rates (F = 5.17, p = 0.04; F = 68.71, p < 0.000, respectively). Accumulative biomass from specimens with 1200 µM ammonium additions increased rapidly in the first two days of incubation, for both macroalgae under 70 and 300 µmol photons $m^{-2}s^{-1}$ irradiation (Fig. 8a). After day 4, macroalgae under 300 µmol photons $m^{-2}s^{-1}$ irradiation showed a decrease in biomass.

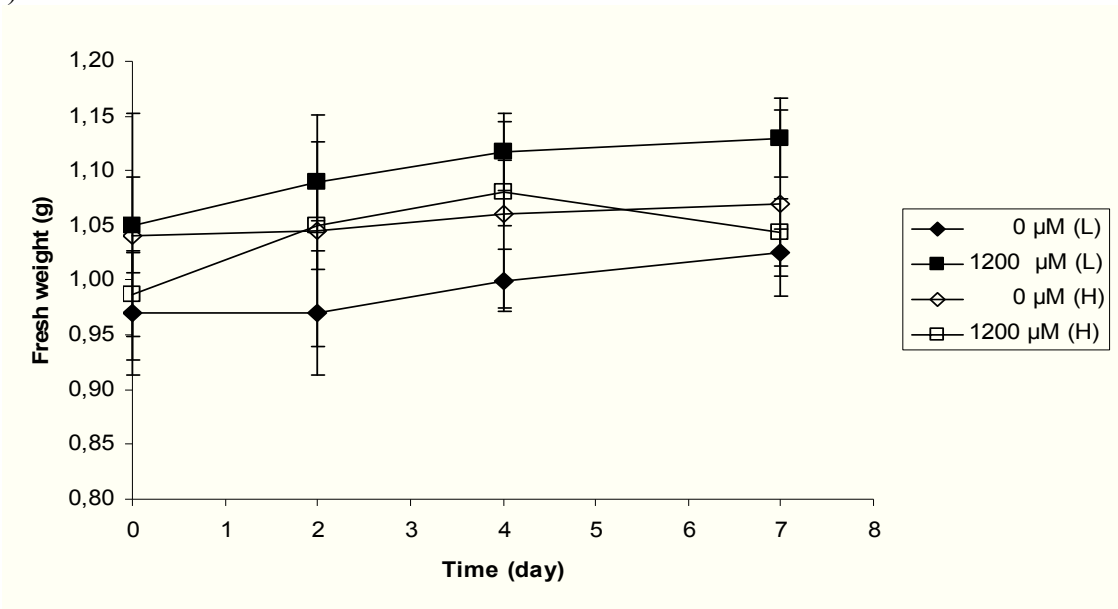
Macroalgal daily growth rates from both ammonium treatments and irradiances varied over the course of the experiment (Fig. 8b). Rapid growth of *G. verrucosa* on the first 2 days was shown by macroalgae with 1200 µM ammonium addition in both irradiances, and then dropped for the high irradiance treatment.

Protein content

At the end of experimental period, only approximately 9% of the initial protein content was measured under both irradiances. The treatments of 1200 µM ammonium addition under irradiances of 70 and 300 µmol photons $m^{-2}s^{-1}$ showed no significant interaction to macroalgal protein content (F = 0.23; p = 0.65). The protein contents of *G. verrucosa* (Fig.9) were slightly higher at macroalgae under 300 µmol photons $m^{-2}s^{-1}$ irradiation than macroalgae under 70 µmol photons $m^{-2}s^{-1}$ irradiation, for both controls and macroalgae with ammonium addition. However, the values were not significantly different (F = 0.51; p = 0.89).

Macroalgae exposed by high irradiance sustained discoloration. Due to the results of total protein content being lower than expected (< 1 µg/µL extract) and limitation of macroalgal material, it was not possible for further protein analyses (D1-turnover).

a)



b)

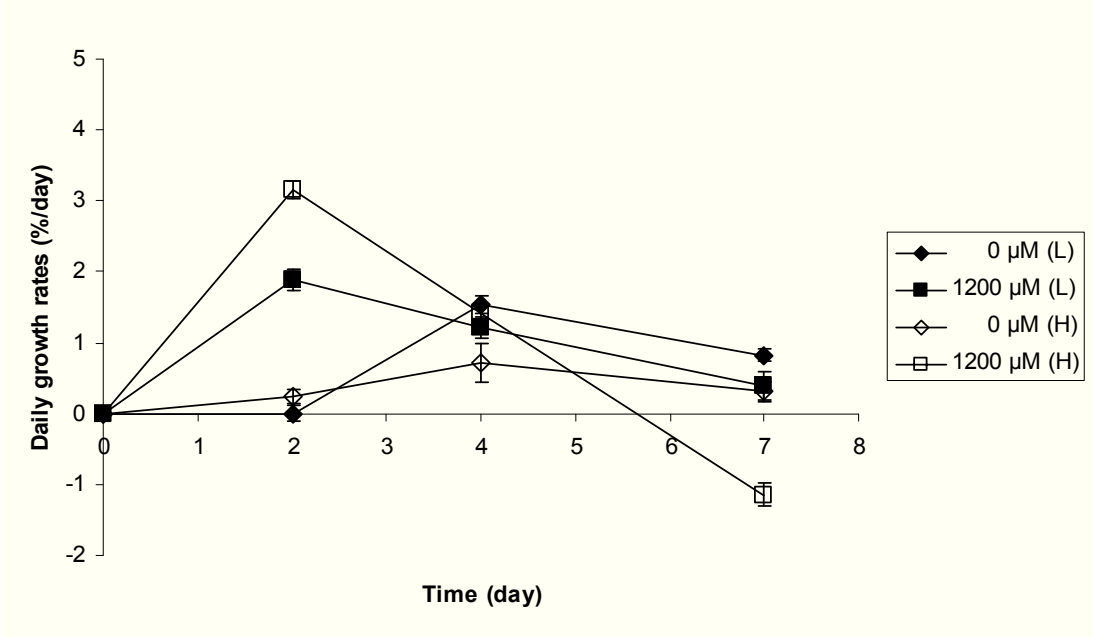


Fig. 8. Growth rates of *G. verrucosa* in protein content assessment with ammonium concentration treatments of 0 and 1200 µM under 70 (L: low irradiance) and 300 (H: high irradiance) µmol photons $m^{-2}s^{-1}$ irradiation in a 7 day experimental period; a) accumulative biomass; b) daily growth rates.

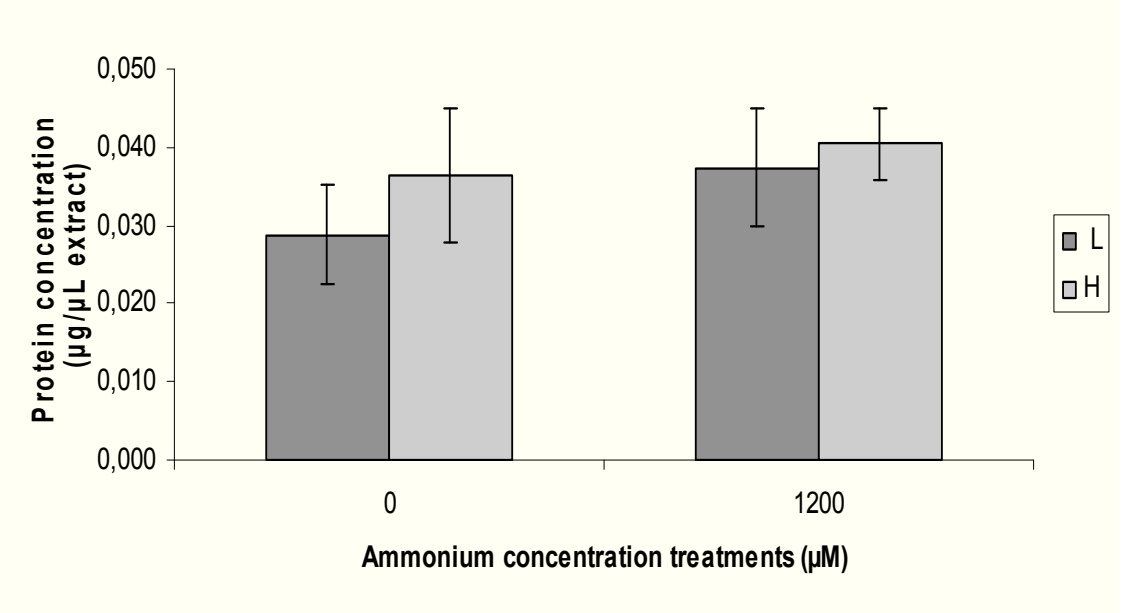


Fig. 9. Protein content of *G. verrucosa* in physiological response experiments with ammonium concentration treatments of 1200 µM under 70 (L: low irradiance) and 300 (H: high irradiance) µmol photons $m^{-2}s^{-1}$ irradiation in a 7 day experimental period.

Discussion

Higher irradiance in combination with high ammonium concentration influenced growth rate, maximum quantum yield, and chl-a of macroalgae throughout the experiment. Tissue losses appeared as an intolerant response of *G. verrucosa* to a combination of high irradiance and high ammonium addition. There was massive discoloration of macroalgae exposed to high irradiance after day 4, for both controls and macroalgae with ammonium addition. This might indicate that photosynthetic processes of *G. verrucosa* during incubation had suffered due to high irradiance and high ammonium concentration treatments.

Zheng et al. (1987) found that *G. tenuistipitata* and *G. asiatica* growth in a disc under laboratory conditions grew optimally under approximately 70 µmol photons $m^{-2}s^{-1}$ irradiation. Other studies reported by Lüning (1981) showed that sublittoral macroalgae (e.g. *Gracilaria*) require 100-250 µmol photons $m^{-2}s^{-1}$ irradiation to grow optimally. However, the early low performance of *G. verrucosa* during the exposure time of our experiment lead to a presumption that *G. verrucossa* had already low irradiance adapted during the maintenance period, which can lower the physiological

tolerance to higher irradiance and ammonium treatment. This might explain the rapid loss of total protein and phycobiliprotein in macroalgae under high irradiance. Bischof et al. (2000) found that the rapid loss of proteins might have been the result of cellular damage, probably resulting in excretion of protein into the surrounding medium. Structural disturbance to cellular membranes and finally membrane leakage, have previously been described as effects of excessive UV exposure (Tevini and Teramura, 1989).

A low irradiance adapted macroalgae will suffer from high irradiance. Under high irradiance, the photosynthetic apparatus absorbs excessive light energy, and as a consequence photosynthetic activity is depressed by photoinhibition (Osmond, 1994). Photoinhibition of photosynthesis by visible light occurs whenever light is absorbed at a greater rate than it can be processed, either by means of electron transport and carbon assimilation, or by fluorescence and thermal deexcitation (Osmond, 1981; Krause, 1988). Regulation of light and harvesting is necessary to balance the absorption and utilization of light energy, thereby minimizing the potential for photooxidative damage (Müller et al., 2001). Besides adjusting light absorption, macroalgae have ways of getting rid of excess light energy that has already been absorbed, called non-photochemical quenching. This mechanism is a protective process that quenches excited chlorophylls and harmlessly dissipates excess excitation energy as heat (Müller et al., 2001). Too much light can lead to increased production of damaging reactive oxygen species as by-products of photosynthesis. In extreme cases, photooxidative damage can cause pigment bleaching and death (Müller et al., 2001). However, NPQ results in this study void the presumption of the excessive light energy by showing hardly differences in reaction to the light treatments. This indicated there was no excess of energy to quench as heat as a response of chlorophyll excitement due to high irradiance.

The second presumption from our result is that *G. verrucosa* was struck by an infectious disease during the maintenance period, due to a massive discoloration in the early incubation time. The unexpected protein values indicated a rapid loss of protein in a short time. It might be due to cellular damage, resulting in an excretion of protein into the surrounding media. It is possible that unsatisfactory results in this study were due to an infectious disease, as reported by Jaffray and Coyne (1996), Friedlander and Gunkel (1994), Lavilla-Pitogo (1992). Since the cell wall of *Gracilaria* is composed primarily

of agar (McHugh, 1991), it is possible that agarases secreted by epiphytic bacteria may be responsible for disease symptoms, such as thallus discoloration and lesion formation (Jaffray and Coyne, 1996). Although cleaning was periodically done during maintenance, high ammonium supply during exposure time possibly evoked the growth of epiphytic bacteria. In addition, unfavorable factors, such as increased water temperatures, temporal nutrient deprivation during the starvation period, and suboptimal irradiation, may attenuate macroalgal resistance and induce the onset of bacterial disease in *G. verrucosa*, as also reported in *G. gracilis* (Anderson et al., 1996; Jaffray and Coyne, 1996), and *Kappaphycus alvarezii* Largo et al. (1995). However, it is difficult to state confidently concerning a disease due to the absence of a disease experiment in this study.

Conclusion

G. verrucosa showed low performance earlier than expected during the experimental period, and failed to utilize the availability of light and ammonium for photosynthetic processes. The infection of disease likely caused the unexpected results in this study. In particular, an assay of infectious disease would be an important topic to be conducted in order to obtain substantial information on macroalgal maintenance.

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6. GENERAL DISCUSSION

This study aimed to investigate the ecophysiological responses of macroalgae to various ammonium concentrations and irradiance was varied in results. From the ammonium uptake experiments, the various ammonium concentration treatments influenced the uptake rates of *G. verrucosa* and *D. bartayresiana.* Total uptake of *G. verrucosa* in 360 minutes exposure was higher than 50% in all ammonium concentration treatments, indicating a fair tolerance of ammonium. This is in contrast to *D. bartayresiana*. The uptake result in recent study of *G. verrucosa* was close to the results of *Ulva fenestrata* and *G. pacifica* reported by Naldi and Wheeler (2002). The differences in ammonium tolerance were comparable to differences in ammonium content in macroalgal tissue, which were in line with the increasing ammonium concentration treatments for *G. verrucosa*. CN ratios of *G. verrucosa* (18.97-21.01) also gave a clear indication of no nitrogen accumulation, even in the highest ammonium concentration treatments given in this study (400 μM). Uptake rates of *G. verrucosa* were linear in relation to concentration, an indication that saturation point was not reached during the experimental period. Conversely, *D. bartayresiana* show saturation after the first 15 minutes of exposure, conforming to Michaelis-Menten saturation curve. The nutrient results of both species were in line with the photosynthetic performance. The final maximum quantum yield of *D. bartayresiana* decreased, which was in contrast to *G. verrucosa*. This result was verified by the discoloration of *D. bartayresiana* after 15 minutes of exposure. The tissue discoloration resulting from high ammonium treatment was also reported by Yu and Yang (2008). The results in recent study suggest that the high supply of nitrogen may be able to support growth in *G. verrucosa*, but not in *D. bartayresiana*, which shows symptoms of ammonium toxicity at relatively low ammonium concentrations compared to *G. verrucosa*.

In the physiological response experiments under low irradiance, *G. verrucosa* showed a surge phase of ammonium uptake in the first two days. The rapid uptake at the beginning of the experimental period indicated that ammonium limitation during a pre-starvation period affected the uptake rates. This was also described by the Michaelis-Menten saturation curve that showed linearity in all ammonium concentration treatments at time intervals d_{0-2} and d_{2-4}. Ammonium saturation appeared by d_{4-6} and

after, as shown by the Michaelis-Menten saturation curves. The response of F_v/F_m to high ammonium addition was lower than initial values; although $rETR_{max}$ increased in day 4 for ammonium concentration treatments of 200, 400 and 800 μM. Tissue loss was found in *G. verrucosa* exposed to high ammonium concentration treatments (1200 and 2000 μM). Restricted growth in *G. lemaneiformis* with high ammonium treatment also reported by Yu and Yang (2008). However, in general, ammonium availability increased the accumulative biomass in the other ammonium concentration treatments. Taking into account the accelerated uptake and assimilation of ammonium to macroalgae under conditions of high irradiance, it might be expected that the ammonium tolerance would increase when macroalgae are grown at higher irradiances.

Unexpectedly, however, a greater tolerance was not shown in the experiments of high ammonium concentrations in combination with high irradiance. The highest uptake was observed only in time intervals d_{0-2} and d_{2-4} in all ammonium concentration treatments. A saturation curve already appeared by time interval d_{0-2}. Maximum quantum yield dropped in day 2 and remained low. A surge of growth rate was shown only in the beginning of the experimental period; however, in general, *G. verrucosa* grew slowly. Reduction in photosynthethetic performance of *G. verrucosa* under combined high ammonium and high irradiance were presumably attributable to reduced concentrations of photosynthetic apparatus components, because pigment concentration, $rETR_{max}$ and F_v/F_m decreased relatively to high ammonium concentration treatments.

Ammonium uptake and assimilation

The uptake rate of ammonium by seaweeds is influenced by a multitude of environmental conditions, such as light, temperature, water motions, and desiccation (Hanisak, 1983). However, uptake is not only simply a passive process relying on diffusion alone, but it is also controlled to some extent by factors intrinsic to the algae itself (e.g., morphology, life history) (Lobban and Harrison, 1994). Alternatively, it may be that some other upper limit is imposed on the rate at which nutrients can be incorporated into the thallus tissue (Pedersen, 1994).

The kinetics of nutrient uptake will depend on which uptake mechanism is being used. If the transport occurs solely by passive diffusion, then the transport rate will be directly proportional to the electrochemical potential gradient (external concentration).

In contrast, facilitated diffusion and active transport will exhibit a saturation of the membrane carriers as the external concentration of the ion increases (Lobban and Harrison, 1994). Active transport refers to the unidirectional transfer of ions or molecules across a membrane against an electrochemical potential gradient. The charge on the ion makes it difficult to penetrate a membrane that is electrically polarized and contains charged groups that either repel or immobilize the ions. In addition, ions usually are hydrophilic, and their particle sizes frequently are increased by substantial layer of the water of hydration. Both of these properties tend to decrease the rate of diffusion (Lobban and Harrison, 1994). Active transport typically is much slower than channel-mediated transport. Channels may allow 10^6 ions per second, which is much higher compare to 10^3-10^5 ions per second for active transport (Lobban and Harrison, 1994). Passive diffusion occurs without the expenditure of cellular metabolic energy; however, the electrical gradient that may drive passive cation movement is the result of cellular metabolism. In addition, no carriers are involved in diffusion, and therefore it is nonsaturable (Lobban and Harrison, 1994).

In some instances the uptake of nutrient (e.g., ammonium) does not appear to be saturated even at high experimental concentrations irrespective of nutritional history (D'Elia and DeBoer, 1978). In reality, such high Michaelis-Menten parameters would not be ecologically meaningful, and it is suggested that a linear, rate-unsaturated response would for all practical purposes adequately describe the uptake response (Smit, 2002).

In the uptake and physiological response experiments of this study, the surge uptake in the early period support the findings of other studies that report that *Gracilaria* is able to take up ambient nitrogen very rapidly and store it in organic form for later use during periods of nitrogen limitation (Bird et al., 1982). This ability to store ammonium was also reported by Harrison and Hurd (2001) and might also be the reason of unsaturated performance of *G. verrucosa* up to 400 μM ammonium concentration treatment in this study. The ammonium uptake rates obtained in these experiments show a large variation with ammonium concentrations and time intervals over which rates were calculated. The reason that the uptake rate varies is that under nitrogen limitation (e.g., starvation period), intracellular nitrogen pools may be low, and the initial enhancement in the uptake rate over the first 60 minutes may represent a pool-filling

phase (Fujita et al., 1988). As the pools fill, the decrease in the uptake rate maybe due to feedback inhibition (Harrison et al., 1989).

Pigments and associated proteins, such as phycoerythrin may serve as nitrogen storage compounds in the Rhodophyceae (Bird et al., 1982). Assimilation of nitrogen by nitrogen-deficient or nitrogen-limited cells is limited by the rate of protein synthesis (Syrett, 1956). This has been substantiated recently by evidence for the accumulation of internal pools of nitrate, ammonium, and free amino acids after the addition of nitrogen to nitrogen-limited culture of macroalgae (Thomas and Harrison, 1985). Such pools would not accumulate if the rates of protein synthesis were equal to or greater than the rates of membrane transport and subsequent metabolism to amino acids (Lobban and Harrison, 1994).

Tissue analysis determines the critical nutrient concentration of a species (Hanisak, 1990). In nutrient uptake experiments of *G. verrucosa* and *D. bartayresiana* at 400 μM ammonium addition showed 3.1% and 2.5% of nitrogen content, respectively. This result indicated a huge storage capacity for nitrogen in both *G. verrucosa* and *D. bartayresiana* tissue. However, *D. bartayresiana* appeared to have critical nitrogen storage at ammonium concentration treatment of 50 μM, after showing constant results of %N (no greater than 2.5%) in macroalgae with ammonium addition beyond 50 μM.

Ammonium toxicity

The discoloration of *D. bartayresiana* after 15 minutes of exposure might indicate a low response to relatively high ammonium concentration treatments. In physiological response experiments, this situation was also observed for *G. verrucosa* with a very high ammonium concentration treatment after the first week. The visual symptoms of discoloration, in addition with suppression of growth in *G. verrucosa* in a 14 day physiological response experiments, indicated ammonium toxicity as what happens in higher plants (Britto and Kronzucker, 2002). Ammonium toxicity is considered to be the result of one or more of the following effects: (i) ammonium-induced mineral nutrient deficiency, arising from the impaired uptake of metal ions; (ii) growth inhibition arising from the acidification of the medium; (iii) alterations in intracellular pH; and (iv) uncoupling of photophosphorylation from electron transport, following the accumulation of ammonium in the tissue (Gerendas, et al., 1997).

High ammonium concentration could inhibit the growth and reproduction (Lobban and Harrison, 1994), and stress resistance ability which might even lead the plant to die (Yu and Yang, 2008). Due to the necessity of charge balance, nitrogen affects not only the uptake of metal ions, but also alters the contents of organic acids (Gerendas et al., 1997) as an indispensable element incorporated into many organic macromolecules, such as proteins, nucleic acids, and pigments (Lobban and Harrison, 1994). These parameters are closely related to the electrolyte homeostasis, and Salsac et al. (1987) consequently mentioned the change of osmoregulation as one of the major causes of ammonium induced growth repressions.

Ammonium and growth

It is well known that the addition of nitrogen can greatly enhance the growth rate and production of macroalgae under certain conditions (Smit, 2002). Most studies have shown that *Gracilaria* is able to take up ambient nitrogen very rapidly and store it in organic form for later use during periods of nitrogen limitation (Bird et al., 1982). In line with other studies in relationship between nutrient availability and growth in some species of *Gracilaria* (Yu and Yang, 2008; Ryder et al., 2004), the results of this study showed that the growth rate of *G. verrucosa* decreased when ammonium concentration treatment exceeded 400 μM. The growth rate levelled off instead of continuing to increase when ammonium concentration treatments exceeded 800 μM, although uptake continued to occur at diminished rates over time. This was observed in experiments under high irradiance. This study gave similar results in uptake rates by showing a surge phase in all ammonium concentration treatments in the early exposure period. This was followed by a surge of growth rates in the early period of the experiments.

The results of this study, which was conducted under laboratory conditions, showed different growth rates of *G. verrucosa* at different ammonium concentration treatments and irradiances. Daily growth rates reached the highest at 3.8% day^{-1} for low irradiance, and 6.9% day^{-1} for high irradiance. These results were slightly different from other studies of growth rates of *Gracilaria* spp. (Marinho-Soriano et al., 2009; Atmadja and Sulistijo, 1980; Xu et al., 2008; Troell et al., 1997). Although studies on the effects of various nutrient availabilities to macroalgae demonstrate relatively low values on growth rates, it is difficult to compare among species and conclude a particular general

pattern, due to the differences in the species of *Gracilaria*, experimental set up, nutrient history, and calculation formula being used among studies.

Internal nutrient concentration of a species that just limits maximal growth can be determined by tissue analysis (Hanisak, 1990). Ryther et al. (1981) stated that *Gracilaria* can obtain and store enough nitrogen for non-limited growth. Thus, this storage is reflected in thallus nitrogen contents (3-5%), which can be substantially higher than those indicating nitrogen deficiency (1,5-2%). The result in this study in the physiological response experiments of *G. verrucosa* both under low and high irradiance, support Ryther et al. (1981) by showing high %N (3.2-3.5%) compared to control (1.4%). Better growth performance was also shown by macroalgae with high ammonium availability during the growth experimental period. However, macroalgae do not have just one critical tissue concentration of nitrogen when other factors, such as light, are also considered. This is because light modifies the nitrogen requirement for maximal photosynthesis and growth by altering biochemical constituents (i.e., pigments, nitrogen reserves) that affect the nitrogen concentration (Lapointe and Duke, 1984).

Hanisak (1990) found that there was a critical nutrient concentration for the growth of seaweed becoming saturated, based on an analysis of nutrient content within the seaweed. Ammonium concentration that was higher or lower than these critical points implied surplus or insufficient nutrient. In this study, ammonium concentration treatment at 400 µM combined with 300 µmol photons $m^{-2}s^{-1}$ irradiance appeared to be a critical point of growth. The growth rate of *G. verrucosa* decreased drastically when ammonium concentration was beyond the critical point, in combination with 300 µmol photons $m^{-2}s^{-1}$. As time progressed, the growth rate reduced gradually at each ammonium concentration. The reason for this might be that the growth rate of the macroalgae is not only related to the nutrient concentration in the external environment, but also to nutrient deposition within the cells.

Ammonium and photosynthetic performance

Interactive effects of light and nitrogen availability are part of particular interest. Nitrogen availability is often correlated with high irradiances in the previous studies. Ryther et al. (1981) reported that for *G. tikvahiae* cultivated under high-light (outdoor), the uptake rate was reduced after five hours in the dark, while under low-light

conditions (laboratory) no decreases in uptake rate occurred in the dark. This is similar to the results of the physiological response experiments in this study which showed decreasing uptake rates in macroalgae under low irradiance compare to the high ones. The ammonium uptake rates of macroalgae under low irradiance showed saturation after day 4; while under high irradiance, uptake rate saturation curve was shown from the beginning of exposure time.

In this study, the irradiances significantly affected the growth rate in the early incubation period. Under both irradiances, the early growth rates increased with increasing ammonium concentrations over time. This is consistent with the result of Yu and Yang (2008) who found that the growth rate of *G. lemaneiformis* increased under irradiance of 50 μmol photons $m^{-2}s^{-1}$ and high nitrogen concentrations up to 600 μM.

Differences in irradiance response were recorded for both optimum quantum yield and photosynthetic capacity during the experimental period. These results indicated that several mechanisms of inhibition may be involved (Bischof et al., 1998). Krause and Weis (1991) mention that decrease in photosynthetic efficiency is thought to be predominantly caused by reduced light harvesting efficiencies resulting in heat dissipation of excessively absorbed energy. This might explain why photosynthetic efficiency and quantum yield were inhibited to a similar concentration of ammonium under high irradiance, and thus resulted in slow growth rates.

Chlorophyll-a and PE are the key pigments that transform light energy into chemical energy during photosynthesis in red algae. Their cellular level is an important physiological index for photosynthesis of algae (Yan et al., 2007; Korbee et al., 2005). The results in this study indicated that when ammonium concentrations were lower than 400μM, the chlorophyll and phycobiliprotein increased with increasing ammonium concentration. This was implied to accelerate the synthesis of photosynthesis pigments and speed up photosynthesis, which improved the growth of *G. verrucosa*. These results confirmed the previous experiments reported by Lapointe (1981); Kursar and Alberte (1983) that phycobilisomes have been shown to increase in number in the presence of nitrogen. However, in physiological experiments with both low and high irradiances, chlorophyll and phycobiliprotein content went down instead of up when ammonium concentration exceeded 400 μM and incubation time was extended. This was due to the synthesis and normal metabolism of photosynthetic pigments and proteins impaired by

high ammonium (Wilson and Gritchley, 1997), which resulted in restricting the growth of *G. verrucosa*.

The ability of red algae to store significant amounts of pigments has been reported by Fujita (1985). Ryther et al. (1981) found that pigmentation and thallus color can be related to water nutrient concentrations. With extended period of low dissolved inorganic nitrogen (DIN), *Gracilaria* lose their dark red-brown coloration and become a pale straw yellow colour. The disappearance of the color could be the result of metabolism of PE as a nitrogen source used to synthesize other proteins (Ryder et al., 2004). The results of thallus color in this study were distinct from previous studies. The high ammonium concentration treatment caused discolorations in thalli instead of darkening of thalli when exposed to high ammonium concentrations. The color of *G. verrucosa* turned gradually from dark brown to light green or white after day 7 when the treatment exceeded ammonium concentration of 800 μM. A similar result was reported by Yu and Yang (2008) at nitrogen concentration of 600 μM, for *G. lemaineformis*. This indicates that high saturation of nitrogen combined with high irradiance in *G. verrucosa* could have negative impacts on both pigmentation and growth. When the nitrogen concentration exceeds the maximum allowable capacity of the nitrogen deposited, it will cause the histiocyte of macroalgae to undergo a series of physiological and biochemical reactions that will inhibit the growth and limit any increase in its pigment content, instead of accelerating them (Yu and Yang, 2008).

Without prejudice to the interest of other nutrient such as phosphorous, nitrogen (in the form of ammonium) found to contribute significant effect to physiological response of *G. verrucosa* in recent study. The presence of phosphorous combined with nitrogen as nutrient resources may contribute other possibilities in physiological response of *G. verrucosa*. Thus, study on nutrient combination of *G. verrucosa* is necessary in order to complete information of physiological response of this species to nutrients in wide perspective.

Ecological implication

Macroalgae play a major ecological role as primary producers and oxygen providers in aquatic ecosystems (Torres et al., 2007). The metabolic process in macroalgae can reduce eutrophication and at the same time contribute to water quality improvement,

such as increasing dissolved oxygen (Xu et al., 2008a) and decrease nutrient excess (Troell et al., 1997). They reduce significant amount of nutrient in the water by absorbing the excess nutrients (Buschmann et al., 1994; Neori et al., 1996) and use them as fuel for physiological processes (Zhou et al., 2006), and produce dissolved oxygen which can accelerate the decomposition of organics compound (Xu et al., 2008b). The increase of dissolved oxygen in correlation with ammonium and irradiance availability may give significant information for further application of macroalgae in such aquaculture systems.

In recent study, *G. verrucosa* has been shown to reduce efficiently the nutrient concentration. The relatively stable dissolved oxygen during the experimental period, noteworthy under high irradiance, confirmed other studies conducted by Xu et al. (2008a). Xu et al. (2008a) reported that dissolved oxygen was much more stable in the culture ponds of shrimp (*Litopenaeus vannamei*) and fish (*Epinephelus awoara*) with *G. lichenoides* compared with the ponds without macroalgae. However, it is necessary to contrive a sequence of applied research to get the best composition of macroalgae and nutrients in order to reach the optimum growth, availability of oxygen, and efficient nutrient excess. Thus, nutrient uptake studies on macroalgae may contribute significantly to the environment.

7. CONCLUSIONS AND FURTHER IMPLICATIONS

7.1 Conclusions

As discussed earlier, that various ammonium concentration and different irradiance affected differently to physiological response of *G. verrucosa* and *D. bartayresiana*. The difference in intrinsic factors, such as storage capacity, range of ammonium tolerance, and assimilation process were found to be the most substantial cause of different results. However, other extrinsic factor (e.g., temperatures, water movements, epiphytes, combination with other nutrient such as phosphorous) might also contribute as they need for further investigation.

Based on the rapid ammonium uptake and the significant growth rates of *G. verrucosa* in a relatively short time, it can be concluding that *G. verrucosa* has a high ability as nutrient bioabsorber. Thus, with reference to application, both environmental and economical advantages can be achieved by integrating *G. verrucosa* with other organisms in such multitrophic aquaculture systems. However, as the results are based on a laboratory scale experiments and conducted only for a relatively limited duration, and further research is necessary to investigate how suitable *G. verrucosa* is in a long-term, self-sustaining systems.

7.2 Implications to aquaculture

The efficiency of the different species for nutrient uptake, as well as their economic potential and growth ability, should be examined to decide which candidate would be best suited as a nutrient bioabsorber of aquaculture effluents. The recent study demonstrated that *G. verrucosa* has high nitrogen uptake and storage. Thus, makes it possible to benefit from high nutrient concentrations released in pulses from the aquaculture systems. This is a strong indication that this species could be cultured also as a potential by-product of fish or shrimp in an aquaculture system.

The growth of *G. verrucosa* might be affected by such contaminations as persistent organic compounds. Therefore, *G. verrucosa* may not be suitable for sewage treatment, but suited for an integrated land-based aquaculture system which can control most of the contaminations. This, however, remains to be tested in an up-scaled

approach in order to implement the laboratory results for further application in aquaculture systems. Along this line, the biggest challenge is still how to achieve an appropriate balance of animal and macroalgae ratio (biomass or density).

7.3 Outlook

When the results from the small and highly controlled laboratory scale are being extrapolated, there is a risk that productivity becomes overestimated due to the uncertain condition in upper scale may give different results, although this has not been proven to always be the case. Hence, further investigations on *G. verrucosa* cultivation along with other organisms in such aquaculture system are urgently needed. Furthermore, studies applying different nutrient combinations (nitrogen : phosphorous) are necessary in order to obtain extended information on physiological responses (e.g., storage capacity, growth rates) of *G. verrucosa* to reach high productivity. Subsequently, studies on the effects of environmental factors to hydrocolloid content in *G. verrucosa* are substantial to make a further productivity and economical analyses.

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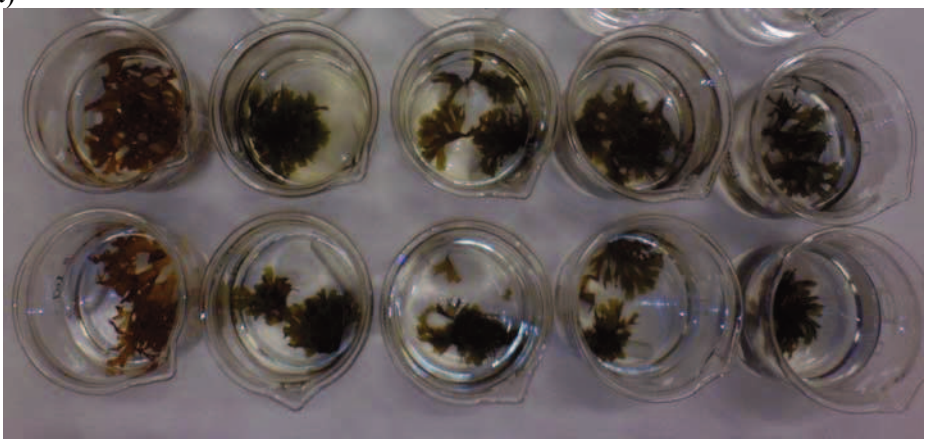
APPENDIX

a)



0 µM **50 µM** **100 µM** **200 µM** **400 µM**

b)

0 µM **1200 µM** **2000 µM**

c)

0 µM **1200 µM** **2000 µM**

Discoloration in *D. bartayresiana* (a); *G. verrucosa* by day 7 (b); and day 14 (c).

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Eidesstattliche Erklärung

Erklärung gemäß § 6 (5) Promo

(der Universität Bremen für die mathematischen, natur- und ingenieurwissenschaftlichen Fachbereiche vom 14. März 2007)

Ich erkläre hiermit, dass ich

die vorliegende Doktorarbeit mit dem Titel "MACROALGAL ECOPHYSIOLOGY IN RESPONSE TO HIGH AMMONIUM CONCENTRATIONS AND DIFFERENT IRRADIANCES " ohne unerlaubte fremde Hilfe angefertigt habe, keine anderen als die angegebenen Quellen und Hilfsmittel benutzt wurden, und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht wurden.

Bremen, August 2012

Tri Dewi K. Pribadi