ANA LÍVIA NEGRÃO LEITE RIBEIRO

Efeitos do aumento da temperatura e dos níveis de CO_2 e do nitrogênio na água do mar no metabolismo de rodofíceas marinhas bentônicas

Tese apresentada ao Instituto de Botânica da Secretaria do Meio Ambiente, como parte dos requisitos exigidos para a obtenção do título de DOUTOR em BIODIVERSIDADE VEGETAL E MEIO AMBIENTE, na Área de Concentração de Plantas Avasculares e Fungos em Análises Ambientais.

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Celso, irmãos Ana Carolina e
André Luis, e companheiro Alan
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“A natureza pode suprir todas as necessidades do homem, menos a sua ganância.”

Mahatma Gandhi

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Resumo

O crescimento desordenado das cidades, mudanças no uso da terra, o aumento da queima de combustíveis fósseis, desmatamento e descarga de efluentes em ambientes aquáticos são algumas das causas do aumento das emissões de CO_2 atmosférico, que causa a elevação da temperatura global, e do enriquecimento das águas marinhas com nutrientes causadores de eutrofização como o nitrogênio e o fósforo. No primeiro capítulo deste trabalho, verificamos os efeitos do aumento da disponibilidade de nitrogênio (na forma de nitrato ou amônio) e fósforo no crescimento da rodofícea *Hypnea aspera* Kützing (Rhodophyta, Gigartinales) e seu potencial de captar, remover e assimilar esses nutrientes na forma de pigmentos fotossintetizantes, proteínas solúveis totais e conteúdos de C, H, N e P no talo. A espécie teve um aumento do crescimento com o aumento da disponibilidade de nitrato, entretanto altas concentrações de amônio e o aumento de fósforo no meio de cultura (relação N:P de 10:1) inibiram o seu crescimento. *H. aspera* pode ser usada como um potencial biofiltro, uma vez que o excesso de nutrientes disponíveis na água do mar foram acumulados na forma de pigmentos, proteínas e elementos no talo das algas. *H. aspera* também removeu quase 100 % do nitrato, amônio e fosfato disponíveis nos diferentes tratamentos. No segundo, terceiro e quarto capítulos, foram avaliados os efeitos do aumento da temperatura (21, 25 e 30°C) e dos níveis de CO_2 (0, 380 e 1000 ppm) segundo projeções climáticas do Intergovernmental Panel on Climate Change (IPCC) para os dias atuais e para o ano de 2100, bem como o aumento das concentrações de nitrato (0, 125 e 500 µM) ou amônio (0, 50 e 100 µM) na água do mar no crescimento, conteúdo de pigmentos, proteínas, C, H e N no talo e fotossíntese das rodofíceas *H. aspera* (capítulo 2), *Dichotomaria marginata* (J. Ellis & Solander) Lamarck (Rhodophyta, Nemaliales) ((capítulo 3), uma espécie com deposição de carbonato de cálcio na forma de aragonita) e *Amphiroa fragilissima* (Linnaeus) J.V. Lamouroux (Rhodophyta, Corallinales ((capítulo 4), uma espécie com deposição de carbonato de cálcio na forma de calcita). Para essas duas últimas espécies, também foi avaliado a porcentagem de calcificação. *H. aspera* foi sensível aos aumentos das concentrações de CO_2 para 1000 ppm e elevação da temperatura para 30°C e essas condições foram letais para a espécie. Altos valores para todas as váriaveis analisadas foram observados em 125 e 500 µm de nitrato ou 50 e 100 µM de amônio, à 21 e 25°C e

sem adição de CO_2 e com 380 ppm. *D. marginata* cresceu em todos os tratamentos testados com CO_2, temperatura e disponibilidade de nitrogênio, e os valores ótimos foram observados em 125 e 500 µM de nitrato ou 50 µM de amônio, com 0 ou 380 ppm de CO_2 em todas as temperaturas testadas. O aumento do CO_2 e da temperatura causaram efeitos negativos no crescimento, calcificação e fotossíntese de *D. marginata*. Nessas condições, as algas acumularam pigmentos, C e H no talo, que podem estar relacionados ao aumento da síntese de polissacárideos em situações de estresse. O aumento do CO_2 afetou negativamente a fisiologia e metabolismo da alga coralinácea *Amphiroa fragilissima*, entretanto em alto CO_2 (1000 ppm) e com adição de 500 µM de nitrato à 25°C e disponibilidade de 50 µM de amônio à 30°C, a espécie apresentou alta porcentagem de calcificação, assimilação de compostos nitrogenados e fotossíntese. Além disso, nessas condições, o pH da água do mar, a disponibilidade de carbonato e o estado de saturação da calcita não diminuiram, como seria esperado. Esses resultados sugerem que *A. fragilissima* pode se aclimatar as mudanças climáticas globais. Os resultados obtidos fornecem subsídios para que estudos futuros possam ser realizados e contribui para ampliar o conhecimento científico sobre os efeitos das mudanças climáticas globais e o enriquecimento de nutrientes na água do mar em rodofíceas marinhas bentônicas.

Palavras-chave: *Amphiroa fragilissima*, aragonita, calcita, CO_2, *Dichotomaria marginata*, fósforo, *Hypnea aspera*, nitrogênio, temperatura.

Abstract

The disorderly growth of cities, changes in land use, increased burning of fossil fuels, deforestation and effluent discharge in aquatic environments are some of the causes of the increase in atmospheric CO_2, global warming, and high nutrient availabilities, as nitrogen and phosphorus in aquatic ecosystems, as a result of eutrophication. In the first chapter of this study, the effects of increased nitrogen levels (as nitrate or ammonium) and phosphorus in growth of *Hypnea aspera* Kützing (Rhodophyta, Gigartinales) were evaluated as well as its potential to uptake, remove and assimilate these nutrients as photosynthetic pigments, soluble protein content and C, H, N and P in the thallus. This species had an increase in growth with increasing of nitrate availability, however high ammonium concentrations and high availabilities of phosphorus in the culture medium (N/P of 10: 1) inhibited its growth. *H. aspera* can be used as a potential biofilter, since this species could incoporate the excess of nutrients available in seawater as pigments, proteins and elements in the thallus. *H. aspera* also removed nearly 100% of nitrate, ammonium and phosphate available in different treatments. In the second, third and fourth chapters, we evaluated the effects of increased temperature (21, 25 and 30°C) and CO_2 levels (0, 380 and 1000 ppm) under climate projections of the Intergovernmental Panel on Climate Change (IPCC) to the present day and the year 2100, as well as the increase of nitrate concentrations (0, 125 and 500 µM) or ammonium (0, 50 and 100 µM) in the seawater on growth, pigment, protein, C, H and N content, in photosynthesis of the red algae *H. aspera* (chapter 2), *Dichotomaria marginata* (J. Ellis & Solander) Lamarck (Rhodophyta, Nemaliales) ((chapter 3), a species with calcium carbonate deposition as aragonite), and *Amphiroa fragilissima* (Linnaeus) J.V. Lamouroux (Rhodophyta, Corallinales ((Chapter 4), a species with calcium carbonate deposition as calcite). For these two coralline species, the percentage of calcification was also evaluated. *H. aspera* was sensitive to increases in CO_2 concentrations to 1000 ppm and temperature up to 30°C, and these conditions were lethal to the species. High values for all variables were observed in 125 and 500 µM of nitrate or 50 and 100 µM of ammonium at 21 and 25°C and without addition of CO_2 and 380 ppm. *D. marginata* grew in all treatments tested with CO_2, temperature and nitrogen

availability, and the optimal values were observed in treatments with 125 and 500 µM of nitrate or 50 µM of ammonium, with 0 or 380 ppm CO_2 at all temperatures tested. The increase of CO_2 and temperature caused negative effects on growth, calcification and photosynthesis of *D. marginata*. Under these conditions, the species accumulated pigments, C and H on the thallus, which may be related to the increase of polysaccharides synthesis due to stress conditions. The increase of CO_2 affected negatively the physiology and metabolism of coralline alga *Amphiroa fragilissima*, however, in high CO_2 (1000 ppm) and with 500 µM of nitrate at 25°C and 50 µM of ammonium at 30°C, the species showed high percentage of calcification, assimilation of nitrogenous compounds and photosynthesis. Moreover, under these conditions, the pH of seawater, the availability of carbonate and the calcite saturation state did not decrease, as expected. These results suggest that *A. fragilissima* acclimated to global climate change. The results provide information for future studies to be performed and contribute to the knowledge about the effects of global climate change and high availabilities of nutrients in seawater on Rhodophyta species.

Key-words: *Amphiroa fragilissima*, aragonite, calcite, CO_2, *Dichotomaria marginata*, *Hypnea aspera*, nitrogen, phosphorus, temperature.

Introdução geral

Desde a Revolução Industrial, nos séculos XVIII e XIX, houve um aumento da produção de gases causadores do efeito estufa, como o dióxido de carbono (CO_2), metano (CH_4), óxido nitroso (N_2O), clorofluorocarbonos (CFCs) e compostos orgânicos voláteis (COVs) devido ao aumento das atividades antrópicas. Essas atividades modificam o fluxo de carbono no ar-oceano, aumentando as concentrações de carbono inorgânico dissolvido nos oceanos, além de elevar a entrada de nutrientes inorgânicos, como nitrogênio e fósforo. Os efeitos das mudanças climáticas combinados com os efeitos de impactos locais antrópicos, como o aumento das concentrações de nutrientes devido aos efluentes domésticos, industriais e agrícolas, promovem mudanças ecológicas e fisiológicas nas populações de macroalgas marinhas bentônicas e dos ecossistemas marinhos (Roessing *et al.* 2004, Andria *et al*. 2009, Hood & Broadgate 2009, Russell *et al.* 2009).

1.1 Metabolismo do nitrogênio (N) e fósforo (P) e seus efeitos nas macroalgas marinhas bentônicas

O crescimento desordenado das cidades nos últimos anos tem gerado impactos antrópicos no meio marinho, sendo a descarga de efluentes nas águas costeiras um dos maiores problemas que tem causado o enriquecimento das águas marinhas com nutrientes causadores da eutrofização, como o nitrogênio e o fósforo (Andersen *et al.* 2006, Cloern 2001). O nitrogênio está disponível na água do mar na forma de nitrato (NO_3^-), nas concentrações de 0 a 40 μmol N l^{-1}, e essas concentrações podem variar ao longo da superfície do mar e nas diferentes latitudes, como mostra a Figura 1 (NOAA 2016). Além do nitrato, o nitrogênio pode ser encontrado na forma de nitrito (NO_2^-, de 0 a 1 μmol N l^{-1}), amônio (NH_4^+, entre 0 a 1μmol N l^{-1}) e nitrogênio orgânico dissolvido (NOD) (Tyrrel

1999). O nitrogênio é o principal elemento que limita o crescimento das algas marinhas bentônicas e controla a produtividade primária dos oceanos, sendo o nitrato e o amônio importantes íons que influenciam o crescimento desses organismos e responsáveis pela formação de amino ácidos, purinas, pirimidinas, açúcares e aminas (Lobban & Harrison 2004).

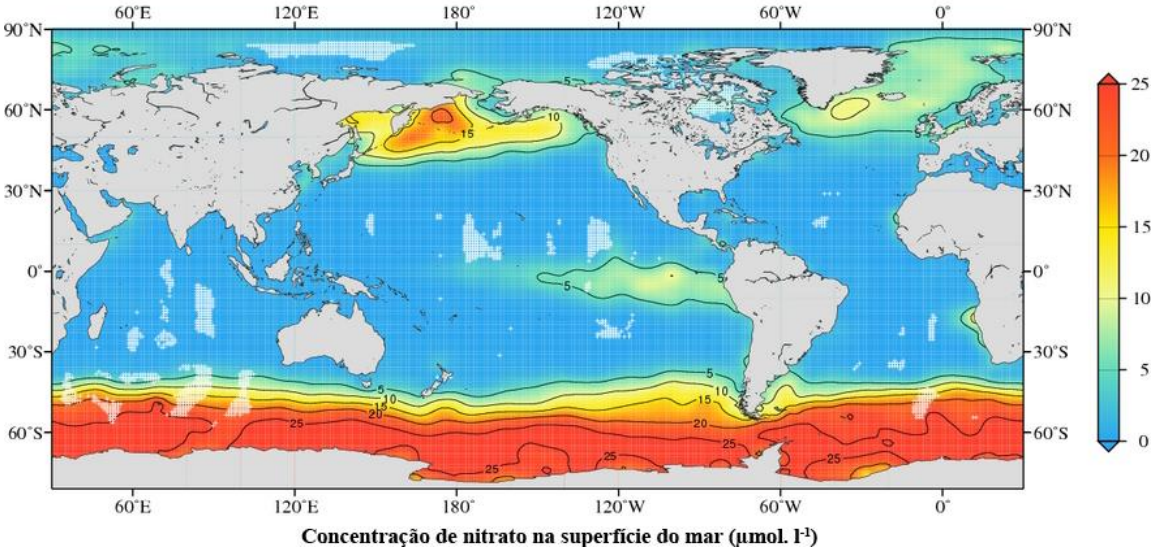

Figura 1: Concentração de nitrato (µmol.l^{-1}) na superfície do mar para o ano de 2013, segundo o World Ocean Atlas Climatology (NOAA, 2016). Disponível em: https://www.nodc.noaa.gov/cgi-bin/OC5/woa13f/woa13oxnuf.pl.

A utilização das diferentes fontes de N pelas macrolgas marinhas variam de acordo com o seu metabolismo, uma vez que, para a captação de NO_3^- ocorre um maior gasto energético do que para a captação de NO_2^- e, consequentemente, para a captação de NH_4^+. O metabolismo do N nas algas consiste na absorção do NO_3^- através da membrana plasmática por transporte ativo, tendo gasto de energia na forma de ATP (Figura 2). O NO_3^- presente no citoplasma pode ser armazenado no vacúolo para posterior utilização ou pode ser reduzido à NO_2^- pela ação enzimática da nitrato redutase, utilizando-se o NADPH como fonte de elétrons. O NO_2^-, resultante da redução do NO_3^- ou captado

diretamente da água do mar, é reduzido no cloroplato à NH_4^+ pela ação enzimática da nitrito redutase que utiliza a ferrodoxina como fonte de elétrons. O NH_4^+ produzido pela redução do NO_2^- ou captado diretamente do meio marinho, se incorpora ao glutamato pela ação da glutamina sintetase, que é uma enzima catalisadora. Essa glutamina transfere o grupo amino a um alfacetoglutarato que forma duas moléculas de glutamato pela ação enzimática da glutamato sintetase, que utiliza o NADH como fonte de elétrons. Os grupos aminas das moléculas de glutamato são transferidas a alfacetoácidos pela ação da transaminase, formando aminoácidos e alfacetoglutarato. Por fim, os aminoácidos serão incorporados na forma de pigmentos e proteínas e o alfacetoglutarato será utilizado em processos metabólicos como o Ciclo de Krebs na respiração (Lobban & Harrison 2004).

Assim como o nitrogênio, o fósforo é um nutriente limitante no desenvolvimento das algas marinhas, responsável por funções estruturais e transferência de energia (como formação de ATP, por exemplo) e está disponível principalmente na forma de ortofosfato (HPO_4^{-2}), que corresponde a 97% do fosfato inorgânico presente na água do mar (DeBoer 1981, Lüning 1990, Tyrrel 1999). O fósforo também está disponível na forma de outros íons fosfato, como 1% de íon fosfato (PO_4^{-3}) e 2% como fosfato dihidrogênio ($H_2PO_4^-$) (Lobban & Harrison 2004). No Oceano Pacífico, a variação das concentrações de fosfato é de 0 à 0,5 µmol. l^{-1}, aumentando próximo à região Antártica, onde as concentrações variam de 1,5 até 2,5 µmol. l^{-1} (Fig. 3).

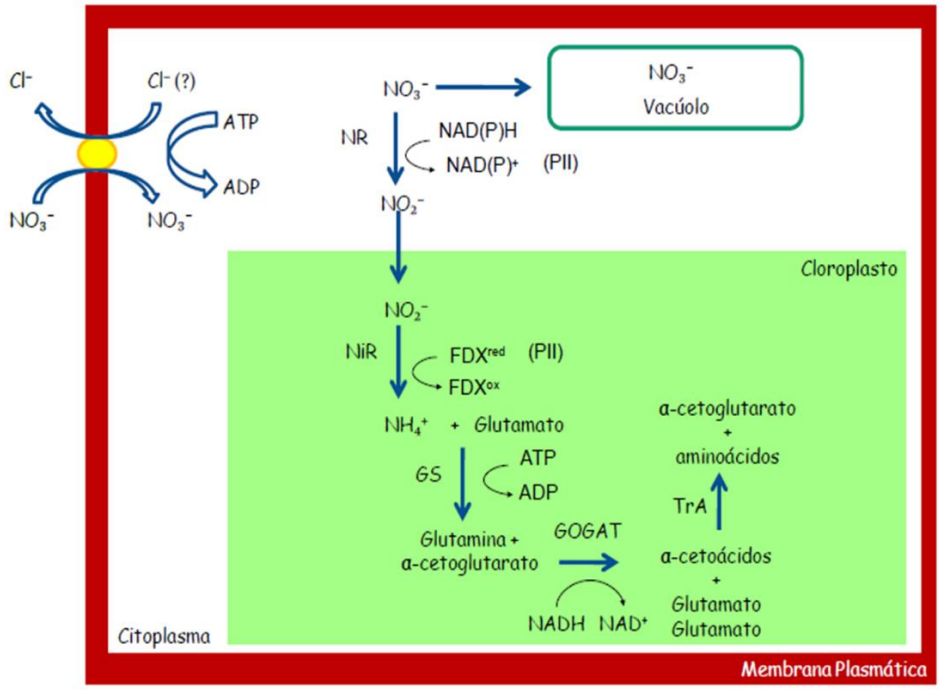

Figura 2: Esquema do metabolismo do nitrogênio em macroalgas marinhas bentônicas segundo DeBoer (1981), com modificações. NR = nitrato redutase, NiR = nitrito redutase, FDX = ferrodoxina, GS = glutamina sintetase, GOGAT = glutamato sintetase, NAD = nicotinamida adenina dinucleotídeo, NADH = nicotinamida adenina dinucleotídeo reduzido, NADP = nicotinamida adenina dinucleotídeo fosfato, NADPH = nicotinamida adenina dinucleotídeo fosfato reduzido, TrA = transaminase, PII = fotossistema II, ATP = adenosina trifosfato, ADP = adenosida difosfato.

O metabolismo do fósforo nas macroalgas marinhas bentônicas está esquematizado na Figura 4. O fosfato inorgânico presente na água do mar é captado pela alga por transporte ativo, havendo gasto de energia na forma de ATP. Esse fosfato inorgânico pode ser armazenado no citoplasma em quatro formas de polifosfatos (Poly-A, B, C e D) que podem formar moléculas de DNA, RNA, ou podem ser utilizados na formação de fosfolipídeos e como fonte de energia na forma de ATP nos processos de respiração e

fotossíntese. O fosfato inorgânico pode ser armazenado no vacúolo e, em excesso, pode retornar ao meio marinho na forma de fosfato orgânico dissolvido.

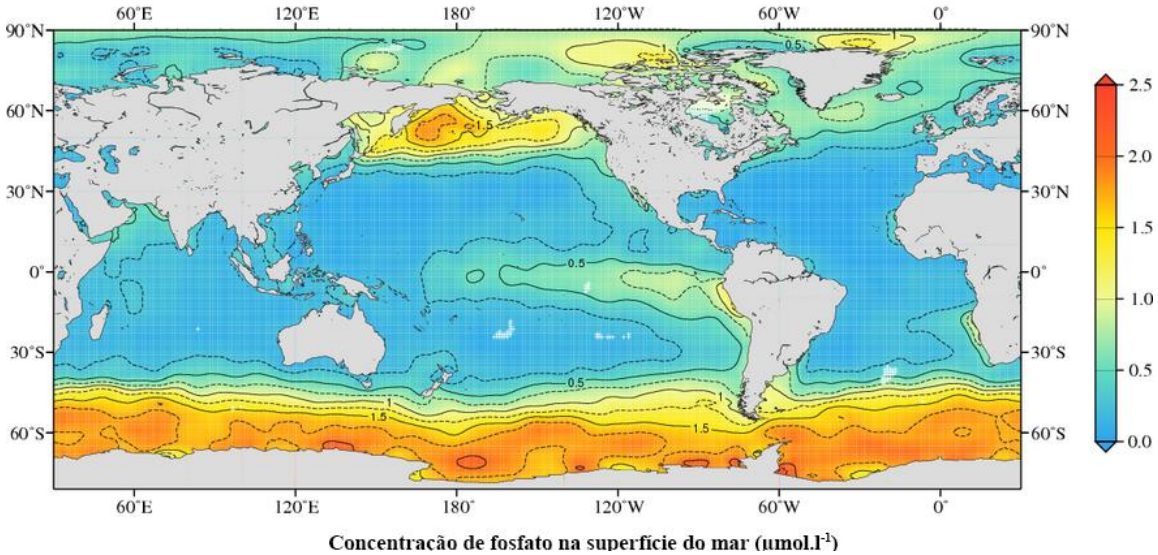

Figura 3: Concentração de fosfato (µmol.l^{-1}) na superfície do mar para o ano de 2013, segundo o World Ocean Atlas Climatology (NOAA, 2016). Disponível em: https://www.nodc.noaa.gov/cgi-bin/OC5/woa13f/woa13oxnuf.pl.

As macroalgas são eficientes na remoção desses nutrientes em excesso na água, atuando como biofiltradoras, ou seja, removendo íons como o nitrato e amônio do ambiente aquático e que serão incorporados (bioacumulados) na forma de pigmentos ou aminoácidos (Lobban & Harrison 2004, Nunes 2006, Carneiro 2007). Diversos estudos demonstraram a capacidade das macroalgas marinhas bentônicas de absorver/remover nitrogênio e fósforo do meio marinho, como mostra a tabela 2. Muitos desses estudos visam o cultivo de macroalgas junto ao cultivo de animais marinhos, como ostras, peixes e camarão, que possuem grande importância econômica. Nos sistemas de cultivos multi-tróficos (IMTA), as algas além de possuirem grande importância comercial, passam a ter um papel fundamental na biorremediação da água do mar. Os resíduos oriundos de

monocultivos de peixes, ostras e camarões aumentam as concentrações de compostos nitrogenados e fosfatados na água, provenientes dos excrementos e alimentação, causando a eutrofização do ambiente. Dessa forma, por possuirem alta capacidade de biofiltragem de N e P do meio marinho e usarem no seu metabolismo e desenvolvimento, as algas se tornam fundamentais para manter a saúde do sistema, diminuindo assim os impactos da eutrofização e de descartes provenientes de monocultivos (Neori *et al.* 2000, Chopin *et al.* 2001, Neori *et al.* 2004).

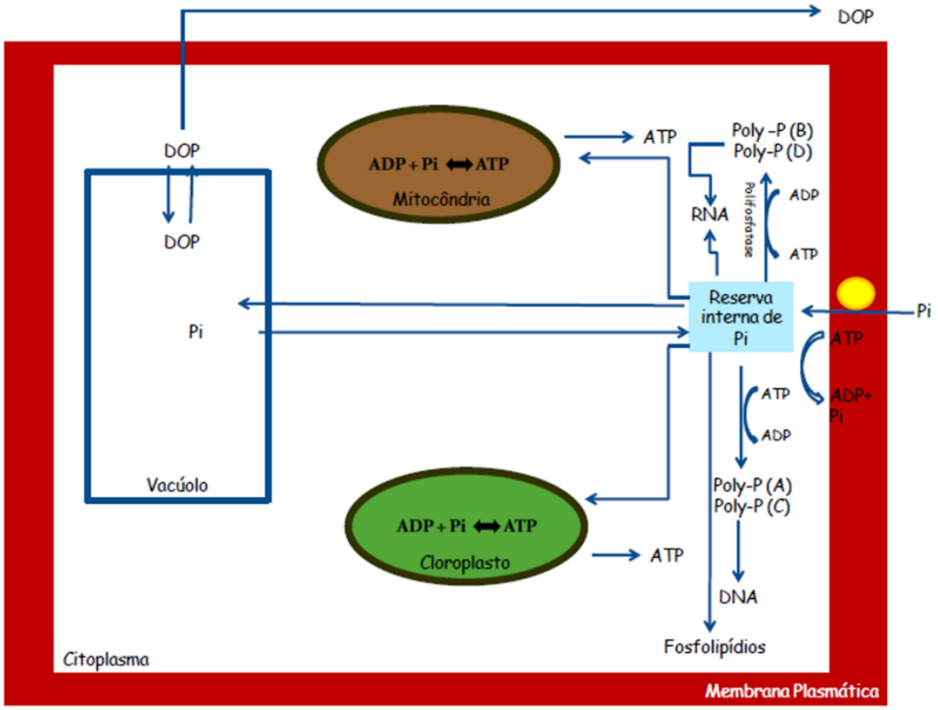

Figura 4: Esquema do metabolismo do P em macroalgas marinhas bentônicas, modificado segundo Lobban & Harrison (2004). Pi = fosfato inorgânico, DOP = fosfato orgânico dissolvido, Poly – P = polifosfato.

Tabela 1: Remoção máxima de nutrientes efetuada por algas marinhas atuando como biofiltradoras em experimentos de laboratório ou em cultivos integrados com animais marinhos.

Espécie	Tipo de cultivo	% de remoção de nutrientes	Autores
Ulva rotundata Bliding	Cultivo de peixe	96,2% de fosfato	Martínez-Aragón *et al.* (2002)
Ulva intestinalis Linnaues		99,2% de fosfato	
Gracilaria gracilis (Stack.) M. Steentoft. L.M.Irv. & W.F. Farnham		98% de fosfato	
Ulva lactuca Linnaeus	Cultivo de camarão	83% de nitrato 83% de amônio 53% de fosfato	Costa (2006)
Gracilaria birdiae Plastino & E.C. Oliveira	Cultivo de camarão	100% de nitrato 34% de amônio 93,5% de fosfato	Nunes (2006)
Gracilaria cervicornis (Turner) J. Agardh	Cultivo em laboratório	97,5% de nitrato 85,3% de amônio 81,2% de fosfato	Carneiro (2007)
Hypnea pseudomusciformis Nauer, Cassano & M.C. Oliveira Citada como *H. musciformis* (Wulfen) J.V. Lamouroux Linhagem marrom	Cultivo em laboratório	99,98% de nitrato 94,89 % de nitrito 44,16% de amônio 98,33 % de fosfato	Martins (2007)
Linhagem verde clara		99,98% de nitrato 93,92 % de nitrito 38,88% de amônio 99,31 % de fosfato	
Kappaphycus alvarezii (Doty) Doty ex P.C. Silva	Cultivo de peixe (*Trachinotus carolinus* L.)	18,20% de nitrato 50,84% de nitrito 70,54% de amônio 26,76% de fosfato	Hayashi *et al.* (2008)
Ulva lactuca	Cultivo de abalone (gastrópode *Haliotis midae* L.)	80% de amônio 77% de nitrato 75% de nitrito	Robertson-Anderson *et al.* (2008)
Porphyra dioica J. Brodie & L.M. Irvine	Cultivo em laboratório	98% de nitrato 88% de fosfato	Pereira *et al.* (2008)
Gracilariopsis tenuifrons (C.J. Bird & E.C. Oliveira) Fredericq & Hommersand	Cultivo em laboratório	99,96% de nitrato 72,28% de fosfato	Wanderley (2009)

Continuação Tabela 2

Espécie	Tipo de cultivo	% de remoção de nutrientes	Autores
Gracilaria lemaneiformis (Bory de Saint-Vincent) E.Y.Dawson, Acleto & Foldvik	Cultivo de vieira (molusco *Chlamys farreri* Jones et Preston**)**	80 % amônio 70,4 % de fosfato	Mao *et al.* (2009)
Ulva chlathrata (Roth) C. Agardh	Cultivo de camarão (*Litopenaeus vannamei* Boonei)	85 % de DIN em 10 dias 44% de fosfato em 10 dias	Copertino *et al.* (2009)
Ulva lactuca	Cultivo em laboratório	80% de fosfato	Tsagkamilis *et al.* (2010)
Palmaria palmata (Linnaeus) F.Weber & D.Mohr *Chondrus crispus* Stackhouse	Cultivo em laboratório	37% de nitrato 100% de amônio 87% de nitrato 100% de amônio	Corey *et al.* (2013)
Ulva fasciata S.F. Gray *Gracilaria birdiae*	Sistema outdoor	100 % de nitrato 100% de amônio 62,1 % de fosfato 99,2% de nitrato 100% de amônio 62,1% de fosfato	Castelar *et al.* (2015)

Espécies do gênero *Hypnea* J.V. Lamouroux apresentam potencial como biofiltro em ambientes com níveis elevados de N e P, tendo uma rápida absorção desses nutrientes e estocando-os na forma de pigmentos e proteínas. Haines & Wheeler (1978) verificaram uma alta porcentagem de remoção de NH_4^+ e NO_3^- com o aumento das concentrações de até 20 µM do que com 25 µM, para ambos os nutrientes. Martins (2007) observou que as linhagens de cor verde clara (VC) e marrom (MA) de *H. pseudomusciformis* (citada no estudo como *H. musciformis*) removeram 99,96% de NO_3^- da água do mar quando cultivada em 100 µM de nitrato, assimilando o nitrogênio na forma de pigmentos, como por exemplo ficobiliproteínas. A linhagem VC ainda removeu 93,92% de NO_2^- e 38,88 % de NH_4^+ em 60 µM de NO_3^-, e cerca de 60% de PO_4^{3-} em tratamentos com adição de 40, 60 e 80 µM de NO_3^-. O mesmo foi observado para a linhagem MA que removeu

94,89% de NO_2^- e 44,16 % de NH_4^+ em tratamento com adição de 60 µM de NO_3^-, e cerca de 58% de PO_4^{3-} quando cultivada com 40, 60, 80 e 100 µM de NO_3^- (Martins 2007). Segundo Suárez-Álvarez *et al.* (2011), *H. spinella* (C.Agardh) Kützing removeu 100% de NH_4^+ quando cultivada com 140 µM desse nutriente e em concentrações de 360, 750 e 1600 ppm de CO_2. Além disso, a capacidade de espécies de *Hypnea* em assimilar N e P como proteínas solúveis totais e pigmentos fotossintetizantes foi observada por Ribeiro (2012) em *H. aspera* (citada como *H. musciformis*) e em *Hypnea* sp., e por Ribeiro *et al.* (2013) em *H. cervicornis*, e ambos estudos utilizaram concentrações de até 500 µM de NO_3^- e até 50 µM de NH_4^+ em relações de N:P de 10:1 e 100:1.

As espécies do gênero *Hypnea* apresentam uma grande importância econômica por ser a principal fonte de κ-carragenana e ι-carragenana que são utilizadas na indústria alimentícia e farmacêutica (Schenkman 1986, Knutsen *et al.* 1995, Oliveira 1998, Rodrigues *et al.* 2011). Além disso, produz lectinas, que são glicoproteínas utilizadas como agentes pró e anti-inflamatórios, empregadas no diagnóstico e terapia do câncer, e como agentes antibióticos e antifúngicos (Cordeiro *et al.* 2006, Nagano *et al.* 2005, Nascimento *et al*. 2006). Devido a grande importância econômica do gênero, é necessário conhecer as melhores condições para o cultivo dessas algas. Além disso, espécies de *Hypnea* apresentam uma grande importância ecológica, podendo ser utilizadas no processo de biorremediação de áreas impactadas.

1.2 Mudanças climáticas globais e seus efeitos nos oceanos

As concentrações atmosféricas de dióxido de carbono (CO_2) aumentaram consideravelmente desde 1750, com o início da Revolução Industrial. Seu acúmulo acarreta uma maior retenção de calor pelo sistema terrestre, o que causa uma elevação da temperatura atmosférica e oceânica e diminui o pH dos oceanos. A Figura 5 mostra a

diminuição do pH da superfície dos oceanos em função do aumento do CO_2, desde o ano de 1990 até 2010.

O IPCC (Intergovernmental Panel on Climate Change) é o orgão internacional que avalia as mudanças climáticas globais, por meio de modelos climáticos. No último relatório em 2013, esse orgão apresentou o Modelo Climático CMIP5 (Coulped Model Intercomparison Project Phase 5) que mostrou projeções climáticas para o ano de 2100, através de quatro novos cenários climáticos (RCP – Representative Concentrations Pathways) que incluem as emissões e concentrações de gases do efeito estufa, aerossóis e uso da terra:

1. Cenário RCP 2,6: estima o aumento de 1,7°C na temperatura global e aumento do CO_2 atmosférico para 400 ppm;
2. Cenário RCP 4,5: estima o aumetno de 2,6°C na temperatura global e aumento do CO_2 atmosférico para 500 ppm;
3. Cenário RCP 6,0: estima o aumento de 3,1°C na temperatura global e aumento do CO_2 atmosférico para 650 ppm;
4. Cenário RCP 8,5: estima o aumento de 4,8°C na temperatua global e aumento do CO_2 atmosférico para 1000 ppm.

Segundo o IPCC, estima-se também uma diminuição de pH de aproximadamente 0,3 até o ano de 2100 (Figura 6), o que poderá agravar a acidificação dos oceanos e seus efeitos nos organismos marinhos (Fabry *et al.* 2008, Roleda *et al.* 2011).

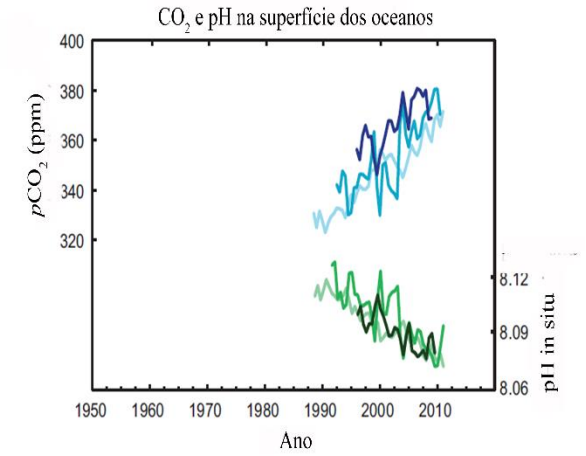

Figura 5: Pressão parcial do CO_2 na superfície dos oceanos (curvas azuis) e pH *in situ* (curvas verdes), como medida da acidez da água do mar para os anos de 1990 até 2010. As medidas foram feitas em três estações do Oceano Atlântico (curvas azul escuro, azul, verde e verde escuro) e do Oceano Pacífico (curvas azul claro e verde claro) (IPCC 2013).

O carbono inorgânico dissolvido (DIC) é encontrado em três formas no meio marinho quando em pH 8,0, sendo 88 % na forma de íons bicarbonato (HCO_3^-), 11 % de íons carbonato (CO_3^{2-}) e 0,5 % de dióxido de carbono aquoso (H_2CO_3). Na água do mar, o CO_2 dissolvido ($CO_{2(aq)}$) forma dióxido de carbono (H_2CO_3) e dissocia em bicarbonato (HCO_3^-) e carbonato (CO_3^{2-}) e a variação do pH pode mudar a disponibilidade do sistema carbonato na água do mar, como mostrado na figura 7 (Follows *et al.* 2006). Aproximadamente metade do CO_2 antropogênico produzido nos últimos 200 anos tem sido absorvido pelos oceanos, acarretando em um aumento na liberação de íons H^+, diminuindo o pH e levando a um processo de acidificação (Sabine *et al.* 2013).

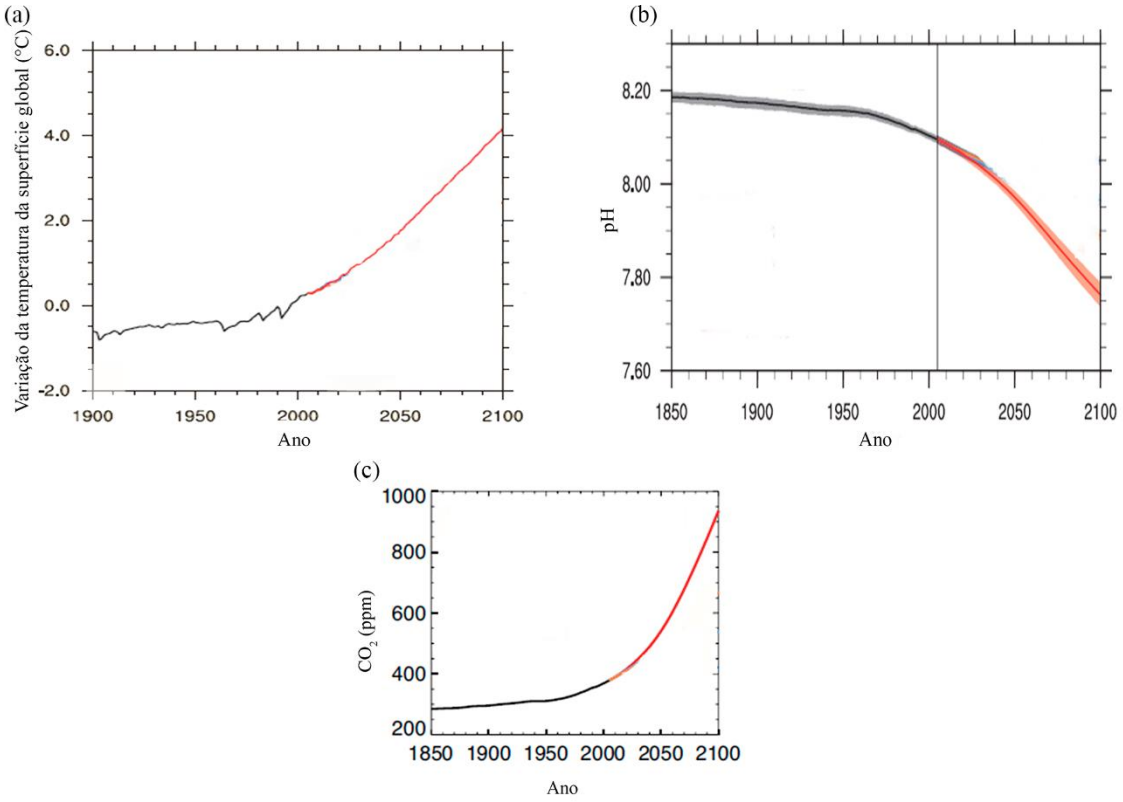

Figura 6: Variação dos valores de temperatura da superfície global (a), pH da superfície dos oceanos (b) e CO_2 atmosférico (c) do período pré-industrial até 2100, segundo projeções do modelo climático CMIP5, RCP 8,5, modificado segundo IPCC (2013).

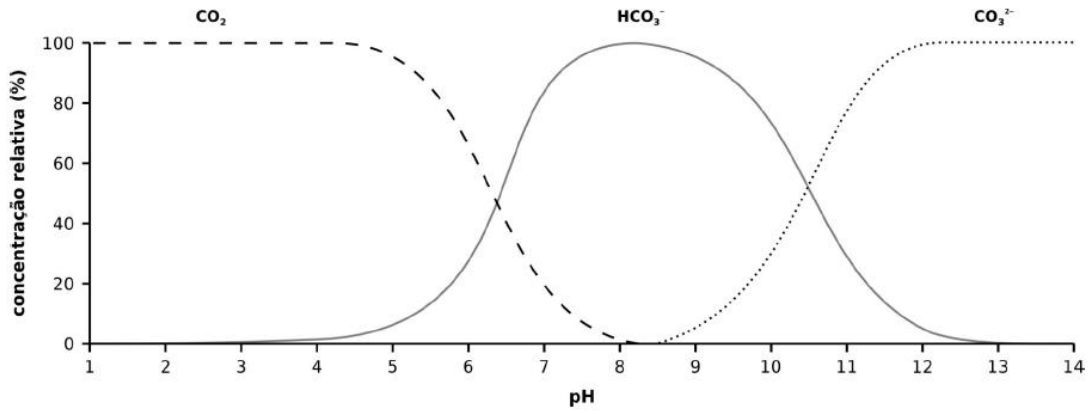

Figura 7: Variação do sistema carbonato da água do mar em função do pH segundo Raven (2005), adaptado por Amancio (2007).

1.3 Efeitos do CO_2 nas macroalgas marinhas

Estudos sobre os impactos ecológicos e fisiológicos das mudanças das concentrações de CO_2 nas macroalgas marinhas bentônicas tiveram início na década de 1970 (Borowitzka & Larkum 1976, Axelsson 1988), mas foi a partir da década de 1990 que houve um aumento expressivo no número de estudos, o que pode estar relacionado com a crescente preocupação com o aumento das concentrações de CO_2 atmosférico e acidificação dos oceanos. Alguns autores observaram efeitos positivos do aumento de CO_2 na água do mar no metabolismo das algas em condições de cultivo controladas, como na taxa de crescimento (Gao *et al.* 1991, Israel & Hophy 2002, Swanson & Fox 2007, Suárez- Álvarez *et al.* 2011, Gordillo *et al.* 2016), na fotossíntese (Gao *et al.* 1993, Mercado *et al.* 1999, Russell *et al.* 2009, Egilsdottir *et al.* 2016), no conteúdo de clorofila *a* (Johnson *et al.* 2012), no conteúdo de C e N do talo (Kübler *et al.* 1999) e na atividade da Rubisco, nitrato redutase, anidrase carbônica e conteúdo de carboidratos (Martins *et al.*2016). Esses efeitos positivos podem ser atribuidos à presença de mecanismos de concentração de carbono (CCM) apresentado por várias espécies de macroalgas marinhas. Algas que apresentam esse mecanismo são capazes de captar tanto o CO_2 difuso na água, quanto o HCO_3^- disponível, e fazem a conversão do HCO_3^- à CO_2 pela ação enzimática da anidrase carbônica, utilizando o CO_2 como substrato para a Rubisco no processo de fotossíntese (Israel & Hophy 2002, Gordillo *et al.* 2016).

Em contrapartida, diversos estudos mostraram os efeitos negativos do aumento do CO_2 no metabolismo das algas, como no crescimento (Zou 2014), respiração (Hoffmann *et al.* 2015), fotossíntese (Beer & Koch 1996), conteúdo de pigmentos fotossintetizantes (Gao & Zheng 2010, Sinutok *et al.* 2011) e, principalmente, na ocorrrência de espécies de algas calcáreas (Kuffner *et al.* 2007, Jokiel *et al.* 2008, Porzio *et al.* 2011) e no processo de calcificação desses organismos (Gao *et al*. 1993, Langdon *et al*. 2000, Büdenbender *et*

al. 2011, Diaz-Pulido *et al*. 2012, Noisette *et al.* 2013). A diminuição do pH decorrente do aumento do CO_2 na água do mar reduz a ocorrência de espécies de algas com calcificação na forma de aragonita ou calcita (Porzio *et al.* 2011).

1.4 Processo de calcificação em macrolgas marinhas

Ao reagir com a água, o CO_2 atmosférico reduz a disponibilidade de CO_3^{2-} que é utilizado por macroalgas calcáreas para a formação de carbonato de cálcio ($CaCO_3$). Além disso, o $CaCO_3$ também pode se formar a partir do HCO_3^- (Frankignoulle *et al.* 1994, Fabry *et al*. 2008).

A deposição de carbonato de cálcio ocorre nas espécies de algas calcáreas na forma de calcita (cristal hexagonal-rombohédrico) ou na forma de aragonita (cristal ortorrômbico), e estas duas formas não ocorrem juntas na mesma alga. A aragonita possui cristais mais frágeis e é a principal forma de deposição de carbonato de cálcio encontrada nas algas calcáreas verdes, pardas e vermelhas (como observado em espécies de *Dichotomaria* Lamarck) e a calcita é encontrada nas algas vermelhas pertencentes à família Corallinaceae (Lobban & Harrison 2004). Algumas formas de calcificação são apresentados na tabela 3.

Tabela 2: Diferentes formas de calcificação em algas, segundo Simkiss & Wilbur (1989) modificado por Lobban & Harrison (2004).

Local	Forma	Exemplos de espécies
Extracelular		
Superfície da parede celular	Bandas concêntricas de agulhas de aragonita	*Padina* Adanson (Dictyotaceae)
	Encrustação de cristais de calcita	*Chaetomorpha* Kützing (Cladophoraceae)
intercelular	Agulhas finas de aragonita	*Halimeda* J.V. Lamouroux , (Halimedaceae) *Udotea* J.V. Lamouroux (Udoteaceae) *Neomeris* J.V.Lamouroux (Dasycladaceae)
	Cristais de calcita ou aragonita	*Liagora* J.V. Lamouroux (Liagoraceae) *Galaxaura* J.V. Lamouroux (Galaxauraceae)
Membrana	Agulhas de aragonita na membrana externa	*Penicillus* Lamarck (Udoteaceae), *Udotea*
	Grupos irregulares de cristais de calcita	*Plectonema* Turet ex Gomont (Oscillatoriaceae)
Entre paredes celulares	Cristais de calcita	*Lithophyllum* Phillipi (Corallinaceae), *Lithothamnion* Heydrich (Hapalidiaceae)
Intracelular		
Vesículas de Golgi	Placas de várias formas, geralmente de calcita	*Emiliania* W.W. Hay (Noelaerhacdaceae), *Cricosphaera* Braarud (Hymenomonadaceae)

O processo de calcificação das macroalgas marinhas ainda é desconhecido, mas sabe-se que há uma relação da calcificação com a fotossíntese, uma vez que o CO_2 proveniente da precipitação do HCO_3^- e da respiração são utilizados no processo de fixação de C. O CO_2 reage com a água do mar e entra na célula por transporte ativo onde será utilizado no processo de fotossíntese no cloroplasto, assim como o HCO_3^- proveniente da reação de CO_2 com a água do mar, que pode ser reduzido a ácido carbônico

(CO_2^-) pela ação da anidrase carbônica. Além disso, o CO_2 proveniente do processo de respiração pode ser utilizado diretamente no processo de fotossíntese (Borowitzka & Larkum 1977, Lobban & Harrison 2004). O CO_3^{2-} resultante da reação do CO_2 com a água se liga ao cálcio (Ca^{2+}) formando o carbonato de cálcio no espaço intercelular de *Halimeda* spp. (Figura 8). Toda reação de dissociação do CO_2 até CO_3^{2-} libera íons H^+ que acificam a água do mar e o espaço intercelular (Digby 1977, Lobban & Harrison 2004). Dessa forma, o processo de calcificação das algas contribui para o equilíbrio no fluxo de gases da água do mar e depósitos de algas calcárias podem ser considerados reservas de carbono ou, como recentemente foi chamado, de estoque de "carbono azul" , tendo importante papel na mitigação dos impactos das mudanças climáticas (Tokoro *et al.* 2014, Trevathan-Tackett *et al.* 2015).

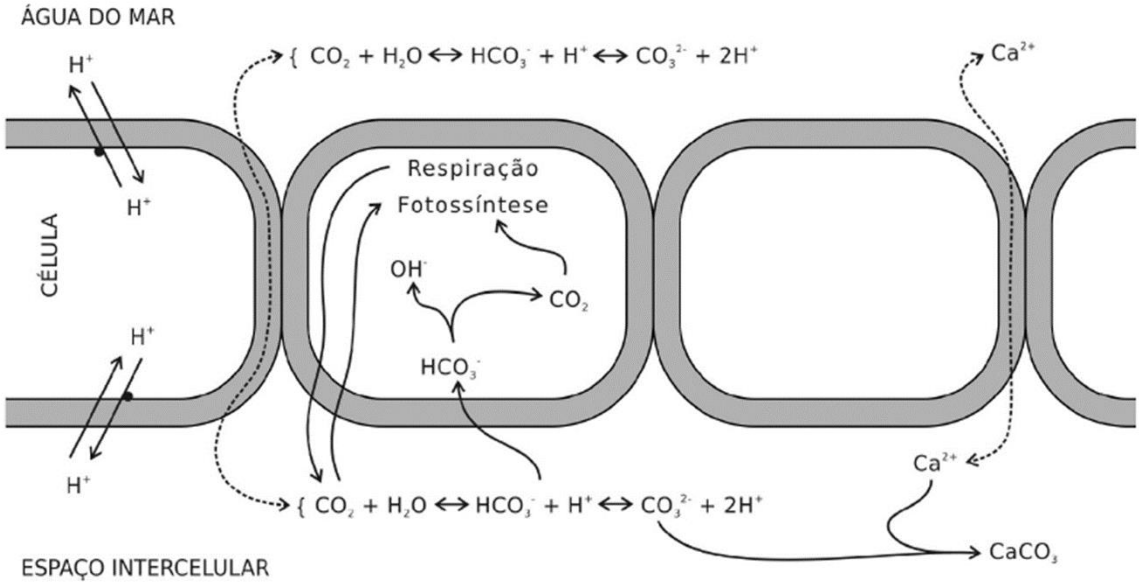

Figura 8: Modelo de calcificação da alga verde *Halimeda* J.V. Lamouroux. Os pontos em preto na plasmalema indicam possível transporte ativo. Segundo Amancio (2007) e adaptado de Borowitzka & Larkum (1977).

1.5 Temperatura e seus efeitos em algas marinhas bentônicas

O aumento das concentrações de CO_2 atmosférico afeta a dissipação do calor, o que acarreta num aumento de temperatura, que pode influenciar na fisiologia das algas marinhas, afetando todos os processos metabólicos, como a fotossíntese e a afinidade da enzima Rubisco pelo CO_2. Em altas temperaturas, pode ocorrer um aumento da respiração, aumentando a dissipação térmica devido ao excesso de energia absorvida (Lobban & Harrison 2004, Necchi 2004).

A maioria dos estudos mostram os efeitos isolados da temperatura no metabolismo e fisiologia das algas marinhas. Alguns autores observaram que temperaturas acima de 35°C diminuiram a fotossíntese e aumentaram a respiração em espécies como *Ulva compressa* Linnaeus, *Ishige okamurae* Yendo, *Sargassum fusiforme* (Harvey) Setchell, *Padina arborescens* Holmes, *Gloiopeltis complanata* (Harvey) Yamada, *Ahnfeltiopsis flabelliformis* (Harvey) Masuda e *Gelidium amansii* (J.V. Lamouroux) J.V. Lamouroux (Yokohama 1973) e *Cladophora sericea* (Hudson) Kützing (Katayama *et al.* 1985). Temperatura de 30°C diminuiu o crescimento de *Pterocladiella capillacea* (S.G. Gmelin) Santelices & Hommersand (citada como *Pterocladia capillacea* (S.G.Gmelin) Bornet), *Gracilaria cornea* J. Agardh, *G. caudata* J. Agardh (citada como *G. verrucosa* (Hudson) Papenfuss, *G. chilensis* C. J. Bird, McLachlan & E.C. Oliveira e *Meristotheca gelidium* (J.Agardh) E.J. Faye & M. Masuda, enquanto que estimularam o crescimento de *Hypnea cornuta* (Kützing) J.Agardh e *Hypnea pseudomusciformis* (citada como *H. musciformis*, Yokoya & Oliveira 1992) e de linhagens marrom e verde de *H. pseudomusciformis* (citada como *H. muscirformis*) cultivadas em fotoperíodo de 10 h (Yokoya *et al*. 2006) . A fotossíntese e respiração da alga *Laurencia brongniartii* J. Agardh diminuiram em temperaturas a partir de 28°C (Nishihara *et al.* 2004). Em *Gracilariopsis lemaneiformis* (Bory de Sanit-Vicent) E.Y. Dawson, Acleto & Foldvik a diminuição da fotossíntese e

do crescimento ocorreu em temperaturas de 26°C (Zou & Gao 2014), assim como observado em *Asparagopsis armata* Harvey (Zanolla *et al.* 2015).

Além da dimuição do crescimento, linhagens de cor de *Kappaphycus alvarezii* (Doty) Doty ex P. Silva apresentaram menor concentração de ficobiliproteínas, clorofila *a* e proteinas principalmente quando coletadas durante estação chuvosa e cultivadas à 32°C (Araújo *et al*. 2014). Em contrapartida, carposporófitos de *Gracilaria domingensis* (Kützing) Sonder ex Dickie apresentaram um aumento do seu crescimento em temperaturas de 30°C (Ramlov *et al.* 2012). Temperaturas acima de 22°C aumentaram o branqueamento dos talos de *Delisea pulchra* (Greville) Montagne e estimularam a produção de furanona em talos saudáveis aumentando a defesa da alga contra herbivoria (Campbell *et al*. 2011).

Poucos estudos focam os efeitos da temperatura juntamente com a elevação de CO_2 em macroalgas. O crescimento e a fotossíntese de espécies que formam os "turfs", compostos principalmente por *Feldmania* sp., aumentaram com a elevação da temperatura de 17 para 20°C e do CO_2 de 380 para 550 ppm. O crescimento desses "turfs" inibiu o recrutamento de *Ecklonia radiata* (C. Agardh) C. Agardh (Connell & Russell 2010). O crescimento de *Chondrus crispus* Stackhouse aumentou com adição de 700 ppm de CO_2 à 24°C, enquanto que o conteúdo de ficobiliproteínas, clorofila *a* e a fotossíntese diminuíram nessas condições (Sarker *et al.* 2013). *Gracilariopsis lemaneiformis* apresentou um aumento da fotossíntese quando cultivada em baixa temperatura (12°C) e concentração de 4,4 mM de carbono inorgânico, enquanto o aumento da temperatura (26°C) diminuiu a fotossíntese da espécie (Zou & Gao 2014). A calcificação e o crescimento das algas calcárias *Halimeda macroloba* Decaisne, *H. cylindracea* Decaisne e *Porolithon onkodes* (Heydrich) Foslie diminuíram com o aumento da temperatura e queda do pH devido ao aumento da concentração de CO_2 na água do mar (Sinutok *et al.*

2011, Diaz-Pulido *et al.* 2012). A calcificação, o conteúdo de caroteno e clorofila *a* de *Lithothamnion corallioides* (P. Crouan & H. Crouan) P. Crouan & H. Crouan diminuiram em 19°C e 1000 ppm de CO_2, enquanto que o conteúdo de zeaxantina aumentou (Noisette *et al.* 2013). Temperatura de 15°C e alto CO_2 (700 ppm) contribuiram para o aumento da herbivoria em *Fucus vesiculosus* Linnaeus (Gutow *et al.* 2014) e *Pyropia haitanensis* (T.J.Chang & B.F.Zheng) N.Kikuchi & M.Miyata apresentou baixo crescimento e alta fotossíntese quando cultivada em 22°C e 390 ppm de CO_2 (Liu & Zou 2015).

1.6 Impactos antrópicos locais e mudanças climáticas: Efeitos do aumento do nitrogênio, CO_2 e temperatura em algas marinhas

A urbanização resultante da Revolução Industrial nos séculos XVIII e XIX mudarou o uso da terra, aumentando o desmatamento de florestas e o uso de combustíveis fósseis, o que resultou em um aumento na concentração e produção de nitrogênio e o CO_2 (Horta *et al.* 2012). Como já mencionado anteriormente, o aumento do CO_2 atmosférico contribui para o aumento das temperaturas globais, causando o aquecimento, que junto ao aumento da poluição local aumentam os impactos negativos nos ecossistemas costeiros e nos organismos marinhos, alterando a composição química da água do mar. Em algas marinhas, os processos metabólicos do carbono e do nitrogênio são acoplados, desde a fixação do CO_2 que forma esqueletos de carbono necessários para a assimilação do N (Turpin 1991).

Existe pouca informação sobre os efeitos sinérgicos do carbono e nitrogênio na composição química e nos processos fotossintetizantes das macroalgas marinhas bentônicas (Andria *et al.* 2009). O aumento das concentrações de NO_3^- e CO_2 diminuiram o crescimento, o conteúdo de carboidratos e a relação C:N de *Gracilaria* sp., enquanto que aumentou as concentrações de N no talo, o conteúdo de ficocianina, ficoeritrina e

clorofila *a* (Andria *et al.* 2009). Em *Feldmanina* sp. foi observado maior massa seca e rendimento quântico efetivo quando submetida a maiores concentrações de CO_2 e nitrogênio. Entretanto, na alga calcária *Lithophyllum* sp., esses parâmetros foram menores nas condições elevadas de CO_2 e nitrogênio, devido a redução da estrutura de carbonato de cálcio causada pela diminuição do pH da água (Russell *et al*. 2009). O recrutamento do kelp *Ecklonia radiata* aumentou em altas concentrações de CO_2 e nitrogênio (Falkenberg *et al*. 2012). Alta concentração de NO_3^- e PO_4^{3-} e baixo CO_2 estimularam a fotossíntese de *Dictyota* sp. e a calcificação de *Halimeda opuntia* (Linnaeus) J.V. Lamouroux (Hofmann *et al.* 2015). Em contrapartida, altas concentrações de nutrientes diminuiram a respiração das algas e o crescimento de *H. opuntia* (Hofmann *et al.* 2015).

Alguns estudos mostram os efeitos conjuntos das alterações de temperatura e nitrogênio em macroalgas marinhas bentônicas, como observado para *Pyropia leucosticta* (Thuret) Neefus & J.Brodie, *Porphyra linearis* Greville e *P. umbilicalis* Kützing (Kim *et al*. 2007). Essas espécies apresentaram um menor crescimento em temperaturas acima de 15 ºC e em menores concentrações de nitrogênio. Entretanto, *Wildemania amplissima* Foslie teve um maior crescimento em temperatura de 20 ºC nas maiores e menores concentrações de nitrogênio (Kim *et al*. 2007).

Nos últimos anos, houve um aumento da preocupação dos efeitos das mudanças climáticas em organismos marinhos. Ainda assim, são poucos os estudos sobre os efeitos da acidificação dos oceanos, aumento da temperatura da água e os efeitos sinérgicos de concentrações de nutrientes e CO_2 em algas marinhas bentônicas. Foi encontrado um estudo que relata os efeitos dessas váriaveis em *Chnoospora implexa* J. Agardh. Concentrações de amônio (2,54 µM) e de fosfato (1,60 µM), junto a elevada temperatura (29°C) e CO_2 (1000 ppm) diminuiram a biomassa, concentração de clorofila *a* e β-caroteno, e aumentaram a fotossíntese máxima de *C. implexa* (Bender *et al.* 2014).

Apesar do aumento da preocupação a respeito dos efeitos das mudanças climáticas e dos impactos locais em organismos marinhos, existe uma grande lacuna no conhecimento dos efeitos sinérgicos do CO_2, temperatura e nitrogênio no metabolismo e fisiologia de macroalgas marinhas bentônicas, principalmente em espécies de importância econômica e ecológica pouco conhecidas, como *Hypnea aspera* e algas calcárias que, além de serem grandes reservas de carbono, apresentam um alto potencial econômico, sendo utilizadas em indústria farmacêutica e agricultura como fonte de cálcio para nutrição animal e vegetal (Dias 2000). *Dichotomaria marginata* (J. Ellis & Solander) Lamarck apresenta importância na terapia do câncer, atividade neurotóxica e fotoprotetora, sendo utilizada no tratamento de doenças neurodegenerativas, como a Doença de Alzheimer (Zhang *et al.* 2005, Rozaz & Freitas 2008). Extratos obtidos em espécies de *Amphiroa* J.V. Lamouroux apresentaram uma alta atividade antioxidante, podendo ser usados na indústrica alimentícia (Khan *et al.* 2010) e também apresentaram atividade espamogênica e histamínica (Wahidulla *et al.* 1987).

Objetivos

2.1 Objetivo geral

- Avaliar os efeitos do aumento da temperatura e dos níveis de CO_2 na água do mar, considerando o cenário proposto pelo modelo climático CMIP5, RCP 8,5 e cenários atuais do IPCC (2013), e o aumento das concentrações de nitrogênio na água do mar sobre o metabolismo de rodofíceas marinhas bentônicas.

2.2 Objetivos específicos

- Avaliar a captação, remoção e assimilação do nitrogênio e fósforo da água do mar por *Hypnea aspera*, cultivadas em diferentes concentrações de nitrato ou amônio, em tratamentos com relação N:P 10:1 e 100:1.
- Avaliar os efeitos do aumento da temperatura e dos níveis de CO_2 e do nitrogênio da água do mar no crescimento, fotossíntese, conteúdo de pigmentos, proteínas e de C, H e N no talo de *Hypnea aspera*;
- Avaliar os efeitos do aumento da temperatura e dos níveis de CO_2 e do nitrogênio da água do mar no crescimento, fotossíntese, conteúdo de pigmentos, proteínas e de C, H e N, e na calcificação de *Dichotomaria marginata* (J. Ellis & Solander) Lamarck (Nemaliales), que apresenta calcificação na forma de cristais de aragonita;
- Avaliar os efeitos do aumento da temperatura e dos níveis de CO_2 e do nitrogênio da água do mar no crescimento, fotossíntese, conteúdo de pigmentos, proteínas e de C, H e N e na calcificação de *Amphiroa fragilissima* (Linnaeus) J.V. Lamouroux (Corallinales), que apresenta calcificação na forma de cristais de calcita;

- Ampliar o conhecimento sobre os impactos das mudanças climáticas e enriquecimento de nutrientes em ambientes marinhos, sobre as macroalgas marinhas bentônicas.

3.1 Material biológico estudado

3.1.1 Hypnea aspera Kützing

H. aspera pertence à ordem Gigartinales, família Cystocloniaceae e gênero *Hypnea* J.V. Lamouroux (Guiry & Guiry 2016). A ocorrência de *H. aspera* foi recentemente descrita por Nauer *et al*. (2014) para o Atlântico e para o Brasil (estados de São Paulo e do Rio de Janeiro). Essa espécie pode ser considerada críptica, uma vez que se assemelha morfologicamente com as espécies *H. pseudomusciformis* (Nauer *et al*. 2015), *H. valentiae* (Turner) Montagne e *H. flexicaulis* Y.Yamagishi & M. Masuda. A espécie apresenta hábito ereto, medindo de 5 a 7 cm de comprimento, talo com coloração marrom amarelado, verde amarelado ou marrom avermelhado, podendo formar gavinhas nos ápices dos ramos, o que leva a ser confundida com *H. pseudomusciformis* (Figura 8 A-B).

3.1.2 Dichotomaria marginata (J. Ellis & Solander) Lamarck

D. marginata (Figura 9 A-B) pertence à ordem Nemaliales, família Galaxauraceae e gênero *Dichotomaria* Lamarck (Guiry & Guiry 2016). A espécie pode ser encontrada nos estados de Santa Catarina, São Paulo, Rio de Janeiro, Espírito Santo, Bahia, Pernambuco, Rio Grande do Norte, Ceará e Maranhão (Creed *et al.* 2010). As espécies do gênero apresentam deposição de carbonato de cálcio nos espaços intercelulares na forma de finas agulhas de aragonita (Kerkar 1994, Lobban & Harrison 2004).

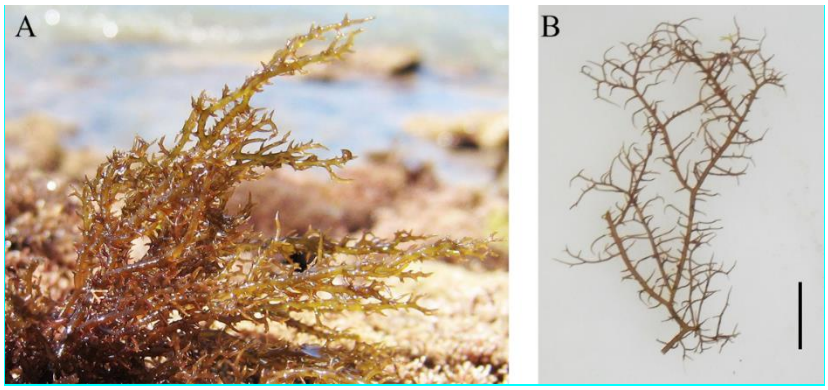

Figura 9: A) Aspecto geral de *Hypnea aspera* no costão rochoso (segundo Nauer 2013) e B) espécime cultivado em laboratório com água do mar esterilizada enriquecida com a solução de von Stosch (VSES/4), temperatura média 23 ± 3 °C, fotoperíodo de 14 h, salinidade de 32, pH 8,0 e densidade de fluxo fotônico de 60 a 90 µmol.fótons.m^{-2}.s^{-1}. Escala = 1 cm.

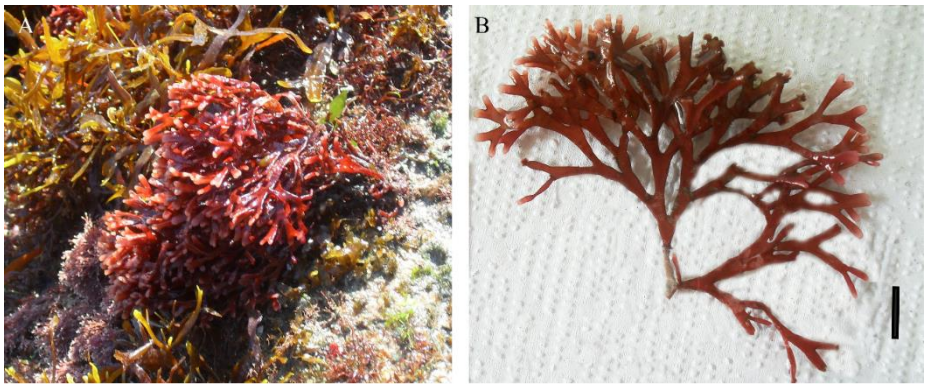

Figura 10: A) Aspecto geral de *Dichotomaria marginata* no costão rochoso da Praia da Fortaleza, Ubatuba e B) após coleta e já limpa de epífitas em laboratório. Escala = 1 cm.

3.1.3 Amphiroa fragilissima (Linnaeus) J.V. Lamouroux

A. fragilissima (Figura 10) pertence à ordem Corallinales, família Corallinaceae e gênero *Amphiroa* J.V. Lamouroux (Guiry & Guiry 2016). A espécie pode ser encontrada nos estados de Santa Catarina, São Paulo, Rio de Janeiro, Espírito Santo, Bahia, Alagoas, Pernambuco, Paraíba, Rio Grande do Norte e Ceará (Creed *et al.* 2010). As espécies do gênero apresenta deposição de carbonato de cálcio na parede celular na forma de cristais de calcita (Lobban & Harrison 2004).

Figura 11: Aspecto de fragmento de *Amphiroa fragilissima* coletada na Praia da Fortaleza, Ubatuba, SP. Escala = 1 cm.

3.1.4 Coleta, transporte e processamento das algas

As algas foram coletadas com auxílio de espátula durante os períodos de maré-baixa, armazenadas em sacos de coleta e mantidas em caixa térmica com gelo durante o transporte até o Laboratório de Cultura de Algas e Cianobactérias "Marilza Cordeiro Marino" do Núcleo de Pesquisa em Ficologia do Instituto de Botânica de São Paulo.

A Tabela 3 apresenta as informações de coleta das espécies estudadas e alguns exemplares foram depositados no Herbário do Instituto de Botânico (SP).

Tabela 3: Dados de coleta e número de depósito no Herbário do Instituto de Botânica (SP) das espécies estudadas.

Espécie	Local de coleta	Data de coleta	Número de depósito
Hypnea aspera	Ilha das Couves, Ubatuba, SP (23° 25' 03" S, 44° 51' 58" W)	07/05/2009	SP 400933
Dichotomaria marginata	Praia da Fortaleza, Ubatuba, SP (23º 50' 15.6"S, 45º 17' 40.5'' W)	12/08/2014 09/09/2014	SP 428540 SP 428429
Amphiroa fragilissima	Praia da Fortaleza, Ubatuba, SP (23º 50' 15.6"S, 45º 17' 40.5'' W)	06/03/2015 02/04/2015	SP 428543 SP 428544

Dados físicos e químicos da água do mar foram obtidos nas coletas realizadas para as espécies de *D. marginata* e *A. fragilissima,* utilizando uma sonda multiparâmetro da marca Horiba (Modelo Horiba W23X) (Tabela 4 e 5). A densidade de fluxo fotônico foi mensurada com o sensor Li-COR (Li-190) acoplado ao Diving-PAM. Dados de calcificação das espécies coletadas em campo foram realizadas segundo a metodologia descrita no item 3.5.9 e são mostrados junto com a tabelas 4 e 5.

Tabela 4: Parâmetros físicos e químicos (pH, cond., O.D., T. e prof., S.) da água do mar, irradiância (PAR), e calcificação dos talos de *Dichotomaria marginata* coletadas em agosto e setembro de 2014 na Praia da Fortaleza, Ubatuba, SP. Os valores correspondem à média (n=3) ± desvio padrão.

Meses/ Parâmetros	pH	Cond. (%)	O.D. (mg/L)	T (°C)	Prof. (m)	S.	PAR (µmol.fótons.m^{-2}.s^{-1})	$CaCO_3$ (%)
Agosto	9,07 ± 0,15	5,69 ± 0,01	7,7 ± 0,02	22,36 ± 0,12	1,26 ± 0,11	34	*N.C	76.86 ± 0.70
Setembro	9,45 ± 0,17	5,81 ± 0,00	8,19 ± 0,25	22,83 ± 0,17	1,26 ± 0,17	34	222,83 ± 58,58	86.68 ± 0.50

Cond. – condutividade, O.D. – oxigênio dissolvido, T. – temperatura, Prof. – profundidade, S. – salinidade.

Tabela 5: Parâmetros físicos e químicos (pH, cond., O.D., T. e prof., S.) da água do mar, irradiância (PAR) e a calcificação ($CaCO_3$) dos talos de *Amphiroa fragilissima* coletados em março e abril de 2015 na Praia da Fortaleza, Ubatuba, SP. Os valores correspondem à média (n=3) ± desvio padrão.

Meses/ Parâmetros	pH	Cond. (%)	O.D. (mg/L)	T (°C)	Prof. (m)	S.	PAR (µmol.fótons.m^{-2}.s^{-1})	$CaCO_3$ (%)
Março	9,36 ± 0,03	5,68 ± 0,01	6,41 ± 0,18	29,17 ± 0,08	0,66 ± 0,05	34	380,33 ± 16,86	88.01 ± 9.70
Abril	9,32 ± 0,17	5,84 ± 0,01	4,98 ± 0,04	26.89 ± 0,04	0.66 ± 0,05	35	428,00 ± 42,15	84.60 ± 13.88

Cond. – condutividade, O.D. – oxigênio dissolvido, T. – temperatura, Prof. – profundidade, S. – salinidade.

3.1.5 Cultivo e propagação de Hypnea aspera

Os procedimentos descritos a seguir foram realizados a fim de se obter culturas unialgáceas de *H. aspera* e sua propagação para produção de biomassa para a realização dos experimentos de captação, remoção e assimilação de nutrientes, bem como para os

experimentos sobre os efeitos do aumento da temperatura e dos níveis de CO_2 e do nitrogênio na água do mar.

As culturas unialgáceas foram obtidas pelo método de isolamento de segmentos apicais (1 cm) de espécimes clones e tetrasporofíticos, que foram limpos semanalmente com auxílio de pincel e lavados com água do mar esterilizada. Os segmentos apicais foram cultivados em meio de cultura preparado com água do mar esterilizada e enriquecida com solução de von Stosch, segundo Oliveira *et al.* (1995) e modificado por Yokoya (2000) (Tabela 6), e com adição de 1 ml de dióxido de germânio (GeO_2) por litro de água do mar esterilizada. Para o cultivo, foram usados frascos tipo "papinha de bebê" com 50 ml de meio de cultura.

A propagação dos espécimes utilizados nos experimentos de captação e remoção de nutrientes da água do mar foi feita após o isolamento das culturas utilizando frascos de vidro com volume total de 230 ml contendo 130 ml de meio de cultura. Em cada frasco, foram cultivados 5 segmentos apicais de 1 cm de comprimento, que foram mantidos por 3 semanas e a troca do meio foi feita semanalmente.

Tabela 6: Composição química da solução von Stosch preparada segundo Oliveira *et al.* (1995) e modificado segundo Yokoya (2000).

Componentes	Concentrações para um litro de meio
$NaNO_3$	0,50 mM
$Na_2HPO_4.12H_2O$	30 µM
$FeSO_4.7H_2O$	1 µM
$MnCl_2.4\ H_2O$	0,1 µM
$Na_2EDTA.2\ H_2O$	10 µM
Tiamina.HCl	0,59 µM*
Biotina	4,10 µM*
Cianocobalamina	1,0 nM*

* concentração equivalente a 50% em relação à composição original proposta por Oliveira *et al.* (1995).

A propagação dos espécimes utilizados nos experimentos para verificar os efeitos do aumento da temperatura e dos níveis de CO_2 e do nitrogênio na água do mar foi feita em Erlenmeyer de 500 ml contendo 300 ml de meio de cultura. Em cada frasco foram cultivados 10 segmentos apicais de 1 cm de comprimento, que foram mantidos por 3 semanas e a troca do meio foi feita semanalmente. Os experimentos foram realizados em Erlenmeyer de 500 ml mantendo uma proporção de 500 mg de biomassa fresca para 400 ml de meio de cultura.

3.1.6 Aclimatação de Dichotomaria marginata

No laboratório, as algas foram limpas com auxílio de pincel e foram cortados segmentos apicais de 2 cm. Em cada Erlenmeyer de 500 ml contendo 300 ml de meio de cultura composto de solução VSES/4 com adição de dióxido de germânio, foram

colocados 10 segmentos apicais de 2 cm de comprimento, que foram aclimatados por uma semana nas condições laboratoriais descritas no item 3.2. Os experimentos foram realizados em Erlenmeyer de 500 ml mantendo uma proporção de 700 mg de biomassa fresca para 400 ml de meio de cultura.

3.1.7 Aclimatação de Amphiroa fragilissima

Em cada Erlenmeyer de 500 ml contendo 300 ml de meio de cultura composto de solução VSES/4 com adição de dióxido de germânio, foram colocados 15 segmentos apicais de 2 cm de comprimento, que foram aclimatados por uma semana nas condições laboratoriais descritas no item 3.2. Os experimentos foram realizados em Erlenmeyer de 500 ml mantendo uma proporção de 300 mg de biomassa fresca para 400 ml de meio de cultura.

3.2 Procedimentos gerais de cultivo em laboratório

Todos os procedimentos de triagem, limpeza, cultivo, aclimatação e propagação das espécies e experimentos foram realizados no laboratório de Cultivo de Algas e Cianobactérias "Marilza Cordeiro Marino", do Núcleo de Pesquisa em Ficologia do Instituto de Botânica de São Paulo.

3.2.1 Esterilização da água do mar

A água do mar utilizada no presente estudo foi coletada na Base Norte do Instituto Oceanográfico da Universidade de São Paulo, na Praia do Lamberto, Ubatuba (SP). A água do mar foi filtrada em pré filtro AP 20 (Millipore) e aquecida em banho-maria durante 1 hora, contado a partir do início da fervura, e este processo foi realizado duas

vezes. Para a realização de cada experimento, um único lote de água do mar foi utilizado para a padronização da composição química da água.

3.2.2 Condições laboratoriais

Toda a vidraria utilizada para a obtenção de culturas unialgáceas, propagação, aclimatação e experimentos foi esterilizada a 121°C por meia hora e os demais instrumentos foram esterilizados em álcool 70% e flambados.

As culturas foram mantidas em temperatura média de 23 ± 3 ºC, fotoperíodo de 14h, salinidade 32 (para *H. aspera*) e 34 (para *D. marginata* e *A. fragilissima*), pH 8,0, densidade de fluxo fotônico de 60 a 90 μmol de fótons m^{-2} s^{-1}, provenientes de duas lâmpadas fluorescentes de 40 W do tipo "luz do dia" dispostas horizontalmente acima dos frascos de cultura. A densidade de fluxo fotônico foi mensurada com um sensor esférico subaquático (Li-COR, LI 192 SA) submerso na água e conectado a um medidor de quanta (Li-COR, LI- 250). Foram feitos rodízios dos frascos diariamente para que todos recebessem a mesma irradiância.

3.3 Experimentos de captação, assimilação e remoção de nitrogênio e fósforo da água do mar por *H. aspera*

3.3.1 Desenho experimental

Para verificar o potencial de *H. aspera* para captar, assimilar e remover nitrogênio e fósforo da água do mar, foram realizados dois experimentos:

1. Adições de nitrato (NO_3^-) na água do mar: para esse experimento, foi utilizada água do mar esterilizada enriquecida com VSES/4 modificado (sem $NaNO_3$ e

$Na_2HPO_4.12H_2O$). A este meio de cultura foi adicionado $NaNO_3$ como fonte de nitrogênio nas concentrações de zero, 25, 50, 100, 150 μM.

2. Adições de amônio (NH_4^+): a fonte de amônio utilizada para os experimentos foi o cloreto de amônio (NH_4Cl) e foram testadas as concentrações de zero, 10, 30, 50 e 70 μM de amônio.

As concentrações de nitrogênio descritas acima foram testadas junto com a adição de fósforo no meio ($Na_2HPO_4.12H_2O$) mantendoa razão de 10:1, simulando um meio eutrófico e 100:1, simulando um meio oligotrófico. Os experimentos foram realizados utilizando Erlenmeyer de 500 ml e foi mantida a proporção de 1,5 g de massa fresca de algas por 400 ml de meio de cultura, nos tratamentos testados.

Os experimentos tiveram a duração de sete dias cada e foram coletadas amostras de água do mar dos tratamentos testados no tempo inicial de incubação (Ti = 0 dia, antes da incubação das algas) e no tempo final (Tf = 7 dias, após 7 dias de incubação das algas). As amostras (n=3) foram filtradas em membrana de acetato de celulose com diâmetro do poro de 0,45 μM (marca Millipore), e em seguida foram congeladas em freezer a -20ºC para análises das concentrações de nitrato, nitrito (NO_2^-), amônio, nitrogênio orgânico dissolvido (NOD) e fosfato. Foram calculados a porcentagem de remoção e captação de cada nutriente na água do mar e a liberação de nitrogênio orgânico dissolvido (NOD) por *H. aspera*. Além disso, foi determinada a taxa de crescimento (TC) da espécie para o período de 7 dias, o conteúdo de pigmentos, proteínas solúveis totais e de C, H, N e P do talo em 11 dias de cultivo, como descritos no item 3.5.

3.4 Experimentos com diferentes temperaturas e concentrações de CO_2 e de nitrogênio da água do mar

A seguir são descritas os métodos utilizados para a realização dos experimentos com diferentes temperaturas e concentrações de CO_2 e nitrogênio na água do mar e seus efeitos nas rodofíceas *H. aspera, D. marginata* e *A. fragilissima*.

3.4.1 Desenho experimental

Os efeitos do aumento da temperatura e das concentrações de CO_2 e de nitrogênio na água do mar na fisiologia e bioquímica das rodofíceas *H. aspera, D. marginata* e *A. fragilissima* foram testados segundo os procecedimentos laboratorias descritos a seguir. Foram testados três concentrações de CO_2, nitrogênio e diferentes temperaturas como descritos na tabela 7.

O meio de cultura utilizado para esses experimentos foi composto por água do mar esterilizada enriquecida com a solução de VSES/4 modificada (solução de von Stoch preparada sem nitato, mas com fosfato, ferro, manganês, EDTA e três vitaminas). Três concentrações de nitrato ($NaNO_3$) ou amônio (NH_4Cl), foram adicionadas: 1) baixa concentração (0 µM de nitrato ou amônio), 2) concentração intermediária (125 ou 50 µM de nitrato ou amônio, respectivamente) e (3) alta concentração (500 ou 100 µM de nitrato ou amônio respectivamente).

Para os testes com diferentes temperaturas, as algas foram cultivadas em câmaras de cultivo do tipo BOD (Demanda Bioquímica de Oxigênico) da Marca Eletrolab. As concentrações de CO_2 e diferentes temperaturas foram testadas de acordo com o Modelo Climático CMIP 5, seguindo as concentrações atuais e as estipuladas pelo RCP 8,5 para 2100. As três concentrações de CO_2 foram obitdas como a seguir: 1) baixa (os Erlenmeyer foram fechados sem adição de CO_2 assumindo a concentraçao de 0 ppm), 2) intermediário

(380 ppm de CO_2 foram injetados na água do mar com o auxílio de bastões de vidro, a partir de um sistema de bombeamento de aquário, Marca Boyu Electromagnetic Air Compressor ACQ-001), e 3) alta (1000 ppm de CO_2 foi injetado com o auxílio de bastões de vidro, usando um cilindro de CO_2 puro, marca P. ONU1013 2.2 – Oxylumen). A aeração e injeção de CO_2 foram filtrados e depois umedecidos, reguladas usando um mainfold, e foram constantes durante todo o período experimental.

Tabela 7: Concentrações baixas, médias e altas de nitrato ou amônio, CO_2 e diferentes temperaturas selecionadas para os experimentos com *Hypnea aspera*, *Dichotomaria marginata* e *Amphiroa fragilissima*. A variação dos níveis de CO_2 e o aumento da temperatura foram propostos de acordo com o CMIP5, cenário atual e cenário RCP 8,5 (IPCC 2013).

Nível	**Variáveis**		
	NO_3^- (µM)	CO_2 (ppm)	Temperatura (ºC)
Baixo	0	0	21
Médio	125	380	25
Alto	500	1000	30
	NH_4^+ (µM)	CO_2 (ppm)	Temperatura (ºC)
Baixo	0	0	21
Médio	50	380	23
Alto	100	1000	30

A partir dessas concentrações, foi feito um desenho amostral fatorial (ANOVA Factorial Design, 3^3), a fim de estipular um número total de experimentos, obtendo-se um cruzamento dos fatores a serem testados (Tabela 8). Através desse desenho experimental, foi identificado qual fator (CO_2, nitrogênio ou temperatura) interfere nas variáveis analisadas (como taxa de crescimento, por exemplo). Cada experimento teve duração de duas semanas e cada tratamento foi testado com 3 repetições (Figura 11 e 12). Foram

determinadas a taxa de crescimento (TC), parâmetros da fotossíntese e parâmetros dos sistema carbonato da água do mar para o período de 14 dias de cultivo. O conteúdo de pigmentos, proteínas solúveis totais e C, H e N do talo das espécies e a calcificação de *D. marginata* e *A. fragilissima* foram determinas em 18 dias de experimento. A metodologia utilizada para ánalise de cada variável está descrita no item 3.5.

Tabela 8: Total de experimentos testados para cada espécie de alga, a partir da ANOVA desenho fatorial (3^3) e as concentrações propostas na tabela 7.

Experimento fatorial (3^3)	**Amônio (µM)**	**Nitrato (µM)**	**CO_2 (ppm)**	**Temperatura (ºC)**
1	0	0	0	21
2	0	0	0	25
3	0	0	0	30
4	0	0	380	21
5	0	0	380	25
6	0	0	380	30
7	0	0	1000	21
8	0	0	1000	25
9	0	0	1000	30
10	50	125	0	21
11	50	125	0	25
12	50	125	0	30
13	50	125	380	21
14	50	125	380	25
15	50	125	380	30
16	50	125	1000	21
17	50	125	1000	25
18	50	125	1000	30
19	100	500	0	21
20	100	500	0	25
21	100	500	0	30
22	100	500	380	21
23	100	500	380	25
24	100	500	380	30
25	100	500	1000	21
26	100	500	1000	25
27	100	500	1000	30

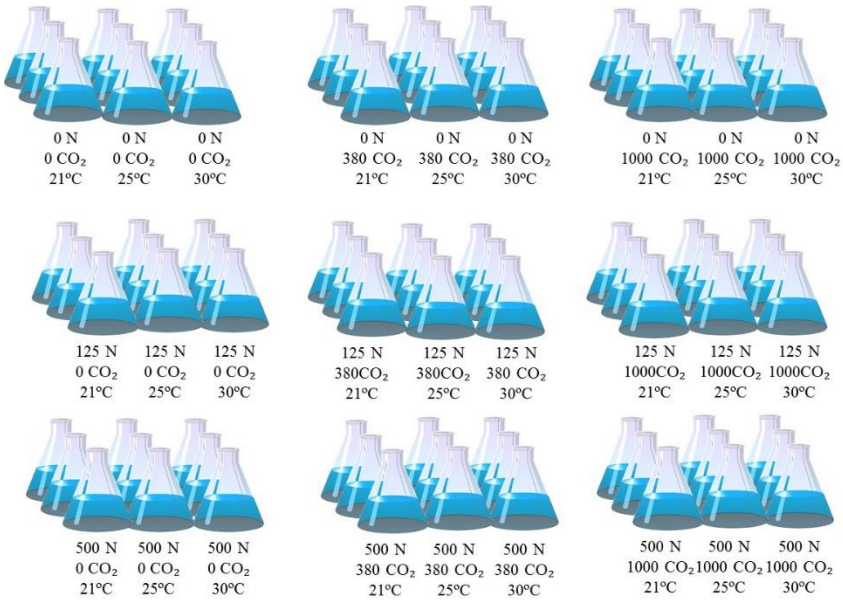

Figura 12: Desenho amostral realizado a partir da ANOVA desenho fatorial com os tratamentos testados nas diferentes concentrações de nitrato (N) em µM, CO_2 (ppm) e em diferentes temperaturas (em ºC), com n=3.

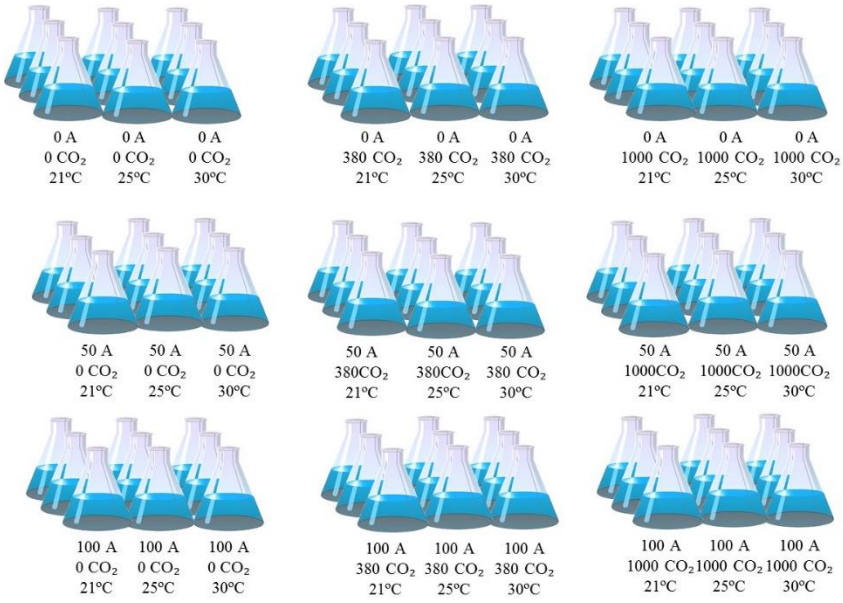

Figura 13: Desenho amostral realizado a partir da ANOVA desenho fatorial com os tratamentos que testados nas diferentes concentrações de amônio (A) em µM, CO_2 (ppm) e em diferentes temperaturas (em ºC), com n=3.

3.5 Variáveis analisadas

3.5.1 Análise de nutrientes da água do mar

As concentrações de nutrientes da água do mar foram analisadas no Laboratório de Biogeoquímica de Nutrientes, Micronutrientes e Traços do Mar do Instituto Oceanográfico da Universidade de São Paulo (IO-USP) com a colaboração da professora Drª Elisabete de Santis Braga da Graça Saraiva e do mestre Vitor Gonsalez Chiozzini.

3.5.1.1 Quantificação de nitrato (NO_3^-) e nitrito (NO_2^-) da água do mar

A quantificação de NO_3^- e NO_2^- dissolvidos foi realizada utilizando o Technicon Auto Analyzer II (marca Technicon ®), seguindo a metodologia descrita por Grasshoff *et al.* (1999), que consiste na redução do NO_3^- à NO_2^- através da passagem das amostras por uma coluna de cádmio. A concentração final de NO_2^- foi determinada por espectrofotometria a 543 nm, após a adição de sulfanilamida e n-(1-naftil) etilenodiamina diidrocloreto (NED).

3.5.1.2 Quantificação de amônio (NH_4^+) da água do mar

A quantificação de NH_4^+ foi realizada com a adição de um reagente contendo hipoclorito e de um reagente contendo fenol. O amônio reage com o hipoclorito formando monocloramina, que na presença de fenol, forma azul de indofenol, que então foi medido por espectrofotometria (espectrofotômetro Milton Roy Genesis II) a 630 nm (Tréguer & Le Corre 1975).

3.5.1.3 Quantificação de fosfato (PO_4^{3-}) da água do mar

A obtenção dos valores de PO_4^{3-} foi feita pela reação do íon com um reagente contendo molibdato, que em meio ácido produz o complexo fosfomolibdato. Essa reação

é catalisada pelo antimônio e forma-se um complexo amarelado que é reduzido por ação do ácido ascórbico a um composto azul. Por fim, foi feita a leitura por espectrofotometria (espectrofotômetro Milton Roy Genesis II) a 880 nm (Grasshoff *et al.* 1999).

3.5.1.4 Quantificação de nitrogênio orgânico dissolvido (NOD) da água do mar

O NOD foi quantificado a partir da foto-oxidação das amostras de água do mar realizadas em tubos de ensaio de quartzo com adição de peróxido de hidrogênio e incubadas por 10 horas em câmara com lâmpada de radiação ultravioleta (UV). A radiação UV quebra moléculas orgânicas e oxida o nitrogênio, que por fim, forma NO_3^- e NO_2^-. O NO_3^- e NO_2^- foram analisados como descrito no item 3.5.1.1. Por fim, obtêm-se o conteúdo de nitrogênio total dissolvido (NTD). Para o cálculo do valor de NOD, o valor de NTD é subtraído pelos valores de NO_3^-, NO_2^- e NH_4^+ das análises anteriores. Essa análise foi realizada de acordo com a metodologia descrita por Armstrong *et al.* (1966), Armstrong & Tibbits (1968) e Saraiva (2003).

3.5.2 Porcentagem de remoção, taxa de captação de nutrientes e liberação de NOD na água do mar

A porcentagem de remoção de nutrientes (%) foi calculada segundo a fórmula:

$$100 \times ([\text{inicial}] - [\text{final}]) \cdot [\text{inicial}]^{-1}$$

Onde, [inicial] = concentração do nutriente no tempo zero e [final] = concentração de nutriente após sete dias de incubação.

O cálculo da taxa de captação dos nutrientes (em $\mu M.g^{-1}.d^{-1}$) seguiu a seguinte fórmula, segundo Kregting *et al.*(2008):

$$T_{captação} = [([incial] - [final]) / (\text{biomassa fresca} \times \text{tempo})] / \text{volume}$$

A liberação de nitrogênio orgânico dissolvido (NOD em µM) foi calculada pela diferença nas concentrações iniciais e finais de NOD na água do mar segundo a fórmula:

$$\text{Liberação de NOD} = [\text{final NOD}] - [\text{inicial NOD}]$$

3.5.3. Taxa de crescimento

O excesso de água sobre o talo das algas foi retirado utilizando papel absorvente e posteriormente a biomassa fresca foi pesada. As taxas de crescimento (TC) das espécies foram calculadas utilizando-se a fórmula (Yokoya *et al.* 2003):

$$TC = (\ln \text{Massa final} - \ln \text{Massa inicial}) \times \text{tempo final}^{-1}$$

3.5.4 Extração e Quantificação de pigmentos

As análises de pigmentos foram realizadas triturando 80mg de massa fresca em nitrogênio líquido. O material triturado foi suspenso em 1 ml de tampão fosfato 50 mM, pH 5,5 e temperatura de 4ºC. A solução foi centrifugada por 20 minutos a 14000 g a 4ºC e o sobrenadante, contendo as ficobiliproteínas, foi mantido no escuro em eppendorfs, para posterior leitura em espectrofotômetro. O sedimento foi ressuspendido em 1 ml de acetona 90% para obter um homogeneizado e foi centrifugado a 12000 g a 4ºC, durante 15 minutos. O sobrenadante (contendo a clorofila *a*) foi transferido para eppendorfs e mantido no escuro até a leitura em espectrofotômetro. As concentrações dos pigmentos foram determinadas pelas equações de Kursar *et al*. (1983) e Jeffrey & Humphrey (1975) (Tabela 9).

Tabela 9: Equações utilizadas para quantificar as concentrações de ficobiliproteínas e clorofila *a* (em mg/mL) segundo Kursar *et al.* (1983) e Jeffrey & Humphrey (1975), respectivamente.

Pigmento	**Equação**
Aloficocianina – AFC	$AFC=181,3.\ A_{651} – 22,3.\ A_{614}$
Ficocianina – FC	$FC=151,1.\ A_{614} – 99,1.\ A_{651}$
Ficoeritrina – FE	$FE=155,8.\ A_{498,\ 5} – 40,0.\ A_{614} – 10,5.\ A_{651}$
Clorofila *a* - Cl *a*	$Cl\ a= 11.85\ A_{664} –1.54\ A_{647} –0.08\ A_{630}$

$A_{498,\ 5}$ = Valor da absorbância no comprimento de onda de 498,5 nm; A_{614} = Valor da absorbância no comprimento de onda de 614 nm; A_{630} = Valor da absorbância no comprimento de onda de 630; A_{647} = Valor da absorbância no comprimento de onda de 647 nm; A_{651} = Valor da absorbância no comprimento de onda de 651nm.

3.5.5 Extração e Quantificação de proteínas solúveis totais

As análises de proteínas foram realizadas através da trituração de 80 mg de biomassa fresca em nitrogênio líquido. O material triturado foi suspenso em tampão de extração (0,2 M tampão fosfato, pH 8, 5 mM EDTA; 1 mM DTT) na proporção de 1g de biomassa fresca por 10 ml de tampão. A solução foi centrifugada por 15 minutos a 12000 *g* e 4 ºC e o conteúdo de proteína solúvel total foi determinado por espectrofotômetro a 595 nm após a adição da solução de Coomassie Blue (Bio-Rad), segundo o método de Bradford (1976). A curva padrão utilizada para a realização dos cálculos foi feita utilizando-se BSA (albumina sérica bovina).

3.5.6 Determinação do conteúdo de carbono (C), hidrogênio (H), nitrogênio (N) e fósforo (P) do talo

Ao final dos experimentos, a biomassa fresca das algas foi seca em estufa a 70ºC por 72 horas. As amostras foram analisadas na Central Analítica do Instituto de Química da Universidade de São Paulo (USP). A análise elementar de C, H e N foi determinada por combustão das amostras à 925 °C na presença de oxigênio puro utilizando o equipamento Perkin Elmer – CHN 2400. A quantificação de P foi feita pelo método de Espectrometria de Emissão Óptica com Plasma Indutivamente Acoplado (ICP-OES).

3.5.7 Fotossíntese e parâmetros da fotossíntese

Esse parâmetro foi determinado pela análise da fluorescência da clorofila *a*, segundo a metodologia descrita por Necchi (2004) e Necchi & Alves (2005). A fluorescência da clorofila *a* foi medida usando um fluorômetro subaquático com pulso de amplitude modulada (PAM) (Diving-PAM, Walz, Effeltrich, Germany). Dois parâmetros principais foram determinados para cada amostra:

1. O rendimento quântico efetivo do fotossistema II (RQE) = ΔF/Fm, onde ΔF = Fm′ − Ft, Fm′ é a fluorescência máxima e Ft é a fluorescência transitória. Esse parâmetro mede a proporção de luz absorvida pela Cl *a* associada ao fotossistema II (PSII) que é usada na fotoquímica.
2. A taxa de transporte de elétrons relativa (ETR) = ΔF/Fm′ × PAR× 0,5; onde PAR é a irradiância em μmol fótons m^{-2} s^{-1} e 0,5 é um fator de multiplicação utilizado considerando que o transporte de um único elétron requer a absorção de dois quantas.

A curva de fotossíntese x irradiância (F x I) foi obtida em 11 níveis de irradiância crescentes e os valores de RQE e ETR foram obtidos em cada ponto de luz. Os parâmetros derivados da curva F x I (eficiência fotossintetizante (α), fotossíntese máxima (ETRmax), irradiância de saturação (Ik) e parâmetro de fotoinibição (β)) foram calculados pela equação de Platt *et al*. (1980):

$$ETRmax = ETR.\{[\alpha/(\alpha + \beta)]\cdot[\beta/(\alpha + \beta)]\}^{\beta/\alpha}$$

3.5.8 Análise da alcalinidade e sistema carbonato da água do mar

As análises da alcalinidade total foram realizadas com as medidas de pH e temperatura da água usando pHmetro com sensor de temperatura acoplado (Marca Jenway 3020). A salinidade da água foi determinada usando um refratômetro (Modelo AO 10440 T/C Hand Refractometer).

As análises do sistema carbonato e alcalinidade foram realizadas através do método de padronização do HCl por titulação utilizando solução de bórax 0,1 N, indicador vermelho de metila e HCl 0.025N (Parson *et al.* 1984). Os cálculos de CO_2, bicarbonato (HCO_3^-), íons cabonato (CO_3^{2-}), carbono inorgânico dissolvido (DIC) e estado de saturação da aragonita (Ωarag, para os experimentos com *D. marginata*) e saturação da calcita (Ωcalc, para os experimentos com *A. fragilissima*) foram realizados segundo Dickson & Millero (1987) e por Dickson *et al*. (2007) usando o programa R, pacote Seacarb.

3.5.9 Calcificação de Dichotomaria marginata e Amphiroa fragilissima

A calcificação das rodofíceas marinhas com deposição de carbonato de cálcio ($CaCO_3$) foi determinada pela quantificação do $CaCO_3$ no final do período experimental (18 dias). As amostras foram secas em papel absorvente, colocadas em sacos de papel e

secas em estufa à 80 °C por 72 horas e após esse período foram pesadas, obtendo-se a massa seca. As amostras foram transferidas para placas de Petri onde foram cobertas com HCl 5% por 48 horas, sendo o ácido renovado por mais 24 horas depois desse período. Após esse procedimento as amostras retornaram à estufa à 80°C por 48 horas, sendo logo após, pesadas, assumido como peso da massa seca não-calcária. Caso houvesse HCl residual nas amostra, essa foi retirada utlizando uma pipeta Pasteur acoplada a bomba à vácuo, tomando cuidado para não remover partes da amostra. Essas amostras voltaram à estufa por mais 24 horas e depois foram pesadas. A quantificação de $CaCO_3$ (em %) foi feita segundo Digby (1977), modificado por Amâncio (2007), pela fórmula:

$$CaCO_3 = [(\text{massa seca} - \text{massa seca não calcária})/ \text{massa seca}] \times 100$$

3.6 Análise estatística dos dados

Os resultados foram submetidos à análise de variância (ANOVA) de um fator, e teste "*a posteriori"* de comparação múltipla de Student-Newman-Keuls para distinguir os tratamentos significativamente diferentes. As análises da curva F x I foram feitas pela análise fatorial ANOVA e a comparação entre os dados foram obtidas como descrito acima, utilizando o programa STATISTICA (versão 9).

A análise de componentes principais (PCA) foi usada para ordenar os efeitos testados em cada experimento (por exemplo, NO_3^-, NH_4^+, CO_2, temperatura) e as variáveis analisadas (como a TC, conteúdo de pigmentos, etc.). A metodologia foi descrita detalhadamente em cada capítulo. Antes da computação dos dados, as variáveis foram transformadas pelo cálculo de "ranging" (Legendre & Legendre 1998). As análises foram feitas usando o programa PC-ORD, versão 6.0 (MjM Software, USA).

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Capítulo 1

Physiological responses and biofilter potential of *Hypnea aspera* (Rhodophyta, Gigartinales) cultivated in different availabilities of nitrate, ammonium and phosphate

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Abstract

Along with the search for new species of seaweeds with biofilter capacity, it is also necessary to understand the physiological and biochemical responses of these seaweeds cultivated in different availabilities of nitrate, ammonium and phosphate. To accomplish this, a laboratory study was performed to evaluate the ability of *Hypnea aspera* Kützing (Gigartinales, Rhodophyta), as a model seaweed, to 1) grow under different concentrations of nitrate, ammonium and phosphate nutrients and 2) uptake, assimilate and remove them from seawater. Treatments were composed of sterilized seawater enriched with quarter-strength von Stosch´s nutrient solution modified (without nitrogen and phosphorus). Nitrate or ammonium, together with phosphate, was added in combined N/P ratios of 100:1 and 10:1. Nitrate concentrations varied from 0 to 150 μM, and ammonium concentrations varied from 0 to 70 μM. Growth rates of *H. aspera* increased linearly with nitrate addition, but the addition of high ammonium concentration in N/P ratio of 10:1 inhibited the growth rates. Excess nutrients were accumulated as proteins and phycobiliproteins, mainly phycoerythrin, at higher phosphate availability (N/P ratio of 10:1) for nitrate addition and lower phosphate availability (N/P ratio of 100:1) for ammonium addition. Nitrogen and phosphate were assimilated into thallus for all nutrient availabilities. *H. aspera* uptake nutrients and showed good removal efficiency of nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+) and phosphate (PO_4^{-3}) present in the seawater. These responses suggest that this species could be used in integrated multitrophic aquaculture systems as a biofilter to reduce nutrient loading in eutrophic seawater.

Keywords: ammonium, assimilation, biofilter, *Hypnea*, nitrate, phosphate, uptake, removal

Introduction

Recent interest has been focused on integrated multi-trophic aquaculture (IMTA) as a tool for aquaculture species from different trophic levels for environmental sustainability and economic stability (Chopin et al. 2012). Coastal mariculture is faced with the problem of inorganic nutrients in excess into local waters, contributing to eutrophication of coastal ecosystems. However, mariculture of both plant and animal species could be utilized in the process of bioremediation in that seaweeds uptake, assimilate and remove nutrients in excess into eutrophic seawater, mainly nitrogen and phosphorus. Such nutrients are used for growth, and nitrogen is assimilated into amino acids, the building blocks for protein synthesis (Lobban and Harrison 2004). As a result, the output of dissolved nutrients by the culture system is decreased, and the production of both seaweeds and cultured aquatic animals is improved (Ridler et al. 2007).

Seaweeds considered suitable for culture in an IMTA system are those having rapid growth, high accumulation of N and P on thallus, high assimilation of nutrients and high economic importance. Studies with different seaweeds showed a decrease of nutrient in excess and high growth rate, e.g., species of *Porphyra* C. Agardh with salmon integrated aquaculture (Chopin et al. 1999) and the cultivation of *Gracilaria gracilis* (Stackhouse) M.Steentoft, L.M. Irvine & W.F. Farnham, *Enteromorpha intestinalis* (Linnaeus) Nees, and *Ulva rotundata* Bliding with sea bass (Dicentrarchus labrax Linnaeus) (Martínez-Aragón et al. 2002), as well as *Ulva lactuca* Linnaeus with the fish *Oreochromis niloticus* Linnaeus (Al- Hafedh et al. 2014) and *Gracilaria chouae* Zhang & B.M.Xia cultivated with the fish *Sparus microcephalus* Bleeker (Wu et al. 2015).

Along with the search for new species of seaweeds with biofilter capacity, it is also necessary to understand the physiological and biochemical responses of these seaweeds cultivated in different availabilities of nitrate, ammonium and phosphate. For

example, the red seaweed *Hypnea musciformis* (Wulfen in Jacqu.) J.V. Lamouroux (Haines and Wheeler 1978) and *H. pseudomusciformis* Nauer, Cassano & M.C. Oliveira (cited as *H. musciformis,* Martins and Yokoya 2010) showed biofilter potential in studies with different nitrogen sources and availabilities, as well as phosphorus when added in an N/P ratio of 4:1 (Martins et al. 2011), and *Hypnea cervicornis* J. Agardh cultivated in N/P ratio of 10:1 and 100:1 (Ribeiro et al. 2013). These species showed high growth rate, indicating equally high N/P assimilation as protein and photosynthetic pigments. Thus, it is plausible that *Hypnea aspera* Kützing could be a good candidate as a biofilter based on its economic importance as a raw material for carrageenan production, an important phycocolloid used in the pharmaceutical and food industries (Neushull 1990; Knutsen et al. 1995; Nagano et al. 2005; Cordeiro et al. 2006; Nascimento et al. 2006; Rodrigues et al. 2011).

Therefore, this study aimed to evaluate the ability of *Hypnea aspera* Kützing (Gigartinales, Rhodophyta), as a model seaweed, to 1) grow under different concentrations of nitrate, ammonium and phosphate nutrients and 2) uptake, assimilate and remove them from seawater.

Material and Methods

Species studied and unialgal cultures

Tetrasporophytes of *H. aspera* were collected at Ilha das Couves, Ubatuba municipality, Sao Paulo State, southeastern Brazil (23° 25’ 03” S and, 44° 51’ 58”W). Voucher specimens were deposited in the Herbarium SP with accession number SP 400933. Unialgal cultures were obtained by vegetative propagation of thallus segments of 1 cm in length. Culture medium was composed by sterilized seawater (salinity of 30) enriched with a quarter-strength of von Stosch’s nutrient solution (VSES/4) following

Oliveira et al. (1995), and modified with the reduction of 50% in vitamin concentrations as described by Yokoya (2000). Thallus propagation was performed in glass bottles with 130 mL of culture medium, and which was replaced every week. The propagation of seaweed lasted three weeks, and cultures were incubated at salinity of 30, temperature of 23±3 °C, photon flux densities of 60 - 90 μmol photons m^{-2} s^{-1}, provided by cool-white fluorescent lamps, and light:dark cycle of 14:10 h. Irradiance was measured with a quantum photometer (LI- 250, Li-COR, Lincoln, NE, USA) equipped with spherical underwater quantum sensor (LI- 250, Li-COR).

Experimental design

Experiments were performed in Erlenmeyer (500 mL) with the ratio of 1.5 g of algal biomass per 400 mL of culture medium. Treatments were composed of sterilized seawater enriched with VSES/4 modified (von Stosch's solution prepared without nitrate and phosphate, but with salts of iron, manganese, EDTA, and three vitamins, as already described above). Nitrogen concentrations and sources were added according to the treatment to be tested. Nitrate ($NaNO_3$) or ammonium (NH_4Cl) and phosphate ($Na_2HPO_4.12H_2O$) were added to the medium in order to obtain nitrogen/phosphorus (N/P) ratios of 10:1 and 100:1. Sterilized seawater was used as the control, and each treatment was tested with three replicates. Nitrate in concentrations of zero, 25, 50, 100, and 150 μM and sodium phosphate dibasic dodecahydrate ($Na_2HPO_4.12H_2O$) were added to culture medium. Ammonium was added in concentrations of zero, 10, 30, 50 and 70 μM with $Na_2PO_4.12H_2O$. Other laboratory conditions were the same as those described for unialgal cultures. At the end of the experimental period (7 days), samples were stored at -20 °C for subsequent analyses.

Hypnea aspera was incubated for 7 days, and water samples were collected before (T0) and after (T7) this period. Water samples were filtered through a cellulose acetate membrane with pore size of 0.45 µm (Millipore) and stored at -20°C. The growth rate (GR) and nutrients analysis of nutrients were analyzed with 7 days of experiments, and pigments, protein and C, H, N e P on thallus of species were analyzed with 11 days. All treatments and analysis were performed in triplicate.

Analysis of nutrients

Nitrate (NO_3^-) and nitrite (NO_2^-) were analyzed according to Grasshoff et al. (1999). Dissolved total nitrogen (DTN) and dissolved organic nitrogen (DON) were determined as photo-oxidation with UV according to Armstrong et al. (1966), Armstrong and Tibbits (1968) and Saraiva (2003). DON was obtained by the difference between DTN and dissolved inorganic nitrogen (DIN) (nitrate + nitrite + ammonium). Ammonium (NH_4^+) was analyzed according the methodology described by Tréguer and Le Corre (1975), and phosphate (PO_4^{3-}) analysis was performed according to Grasshof et al. (1999). Removal efficiency (percentage) was calculated as 100 x ([T0] – [T7])/ [T0] for each nutrient (%), where [T0] is the nutrient concentration before the incubation of seaweed into seawater, and [T7] is the nutrient concentration after the incubation period (7 days). Nutrient uptake was calculated according to Kregting et al. (2008) as ([T0] – [T7]*[FW * time]$^{-1}$)* volume, where FW is the seaweed fresh biomass after 7 days of incubation, time is 7 days and volume is the volume of cultured medium in each Erlenmeyer (400 ml). The release of DON (µM) was calculated as [initial DON] – [final DON].

Growth rates

Fresh biomass was recorded for initial time (T0) and final time (T7), and growth rates (GR) were calculated from three replicates of each treatment and calculated as [ln ($Bf \bullet Bo^{-1}$) • t^{-1}], where Bo is the initial fresh biomass, Bf is the fresh biomass after t days, and t corresponds to the experimental period (Yokoya et al. 2003).

Pigment contents

The algal mass (80 mg of fresh mass for each replicate, n=3) was ground to a powder with liquid nitrogen and mixed with 50 mM phosphate buffer (pH 5.5). The homogenates were centrifuged at 14,000 g for 20 min in order to separate the phycobiliproteins present in the supernatant. Chlorophyll *a* was extracted after dissolving the pellet in 90% acetone and centrifuging at 12,000 g for 15 min., at 4°C. Pigments were quantified by spectrophotometry (Shimadzu-UV 1800). Concentrations of phycobiliproteins (phycoerythrin – PE, phycocyanin – PC and allophycocyanin – APC) were calculated according to Kursar et al. (1983), and the concentration of chlorophyll *a* (Chl *a*) was calculated according to Jeffrey and Humphrey (1975).

Total soluble protein contents

For total soluble protein analysis, 80 mg of algal fresh biomass for each replicate (n=3) were ground with liquid nitrogen, and extractions were carried out at 4 °C using 0.2 M phosphate buffer (pH 8) containing 5 mM of ethylenediaminetetraacetic acid (EDTA) and 1 mM of dithiotreitol (DTT). Buffer was added in the proportion of 10 mL g^{-1} fresh biomass, and the homogenates were centrifuged at 12,000 g for 15 min. Total soluble protein contents were determined according to Bradford (1976), using a Bio-Rad protein assay kit and BSA as standard.

Internal C, H, N and P contents

Analyses of internal C, H and N were determined according to the Pregl – Dumas method using Perkin-Elmer 2400 Series II equipment. Internal P was determined by inductively coupled plasma optical emission spectrometry (ICP- OES), using a SPECTRO ARCOS high-resolution analyzer. Analyses were performed by Central Analítica, Instituto de Química of Universidade de São Paulo.

Statistical Analyses

Data were analyzed by ANOVA (one-way analysis) of variance followed by Student Newman Keuls *a posteriori* test for multiple comparison to distinguish significantly different results ($p < 0.05$) using the STATISTICA software (version 9). For multivariate analysis, the data of growth rates (GR), pigment contents (APC, PC, PE and Chl *a*), soluble protein contents (PT), element content in thallus (C, H, N and P), nutrient removal (NO_3^- rem, NO_2^- rem, NH_4^+ rem and PO_4^{-3} rem), nutrient uptake (NO_3^- up, NO_2^- up, NH_4^+ up and PO_4^{-3} up) and release of DON (DON) of *H. aspera* were used in a covariance matrix for Principal Components Analyses (PCA) performed in PC-ORD 6 software (MjM Software, USA). The variability of the data was adjusted by the method of ranging ($[x-x_{min})/x_{max} - x_{min})]$) (Legendre and Legendre 1998).

Results

High nitrate concentration (150 µM) significantly influenced the growth rate and total protein concentration of *Hypnea aspera* cultivated in N/P ratio of 100:1 (GR of 4.68 ± 0.76 % d^{-1} and PT of 3.40 ± 0.31 mg protein g^{-1} FW), but no significant differences were observed in the other treatments with N/P ratio of 10:1 (Fig. 1).

Nitrogen and phosphorus were mainly stored as APC and PE (Fig. 2). APC, PC and PE contents were all higher with nitrate additions of 50 (0.29 ± 0.08, 0.10 ± 0.02 and 0.34 ± 0.06 mg g^{-1} FW, respectively) and 150 µM (0.31 ± 0.11, 0.10 ± 0.04 and 0.33 ± 0.09 mg g^{-1} FW, respectively) in N/P ratio of 100:1. For 100 µM of nitrate in N/P ratio of 100:1, PE also showed high concentration (0.29 ± 0.06 mg g^{-1} FW), and with 150 µM of nitrate in N/P ratio of 10:1 (0.26 ± 0.07 mg g^{-1}FW). Concentrations of Chl *a* did not differ among nitrate treatments and were lower than 0.1 mg g^{-1} FW.

Contents of C and H in the thallus of *H. aspera* were not significantly affected by the increase of nitrate concentration in the culture medium. N content, which varied linearly with increasing nitrate concentrations, was higher in nitrate additions of 100 (4.27 ± 0.03 and 4.29 ± 0.03 %) and 150 µM (4.00 ± 0.34 and 4.37 ± 0.90 %) in both N/P ratios, respectively (Fig. 3). P content increased with high nitrate concentrations, and was higher with the addition of 100 µM of nitrate in N/P ratio of 10:1 (0.27 ± 0.00 %). On the other hand, C/N ratio decreased with the increase of nitrate concentrations such that C/N ratio was higher when nitrate was limited, i.e., seawater-only treatments and 0 µM (14.32 ± 0.92 and 12.08 ± 0.40 %, respectively).

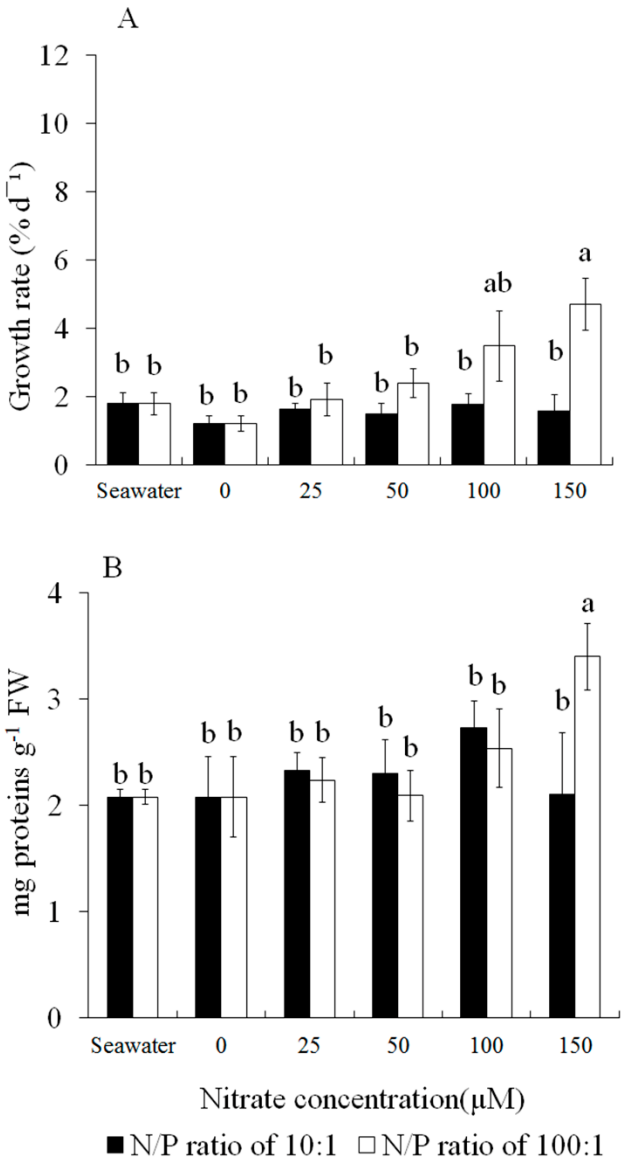

Fig. 1: Growth rate (% d^{-1}) (A) and protein concentration (mg g^{-1} FW) (B) of *Hypnea aspera* cultured for 7 days in VSES/4 modified and enriched with different nitrate concentrations in N/P ratios of 10:1 and 100:1, 23 ± 3°C, photoperiod of 14 h, salinity of 30, pH 8.0 and photon flux densities of 60 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to one-way ANOVA and Student-Newman-Keuls multiple comparison test ($p < 0.05$). ■ N/P 10:1, □ N/P 100:1.

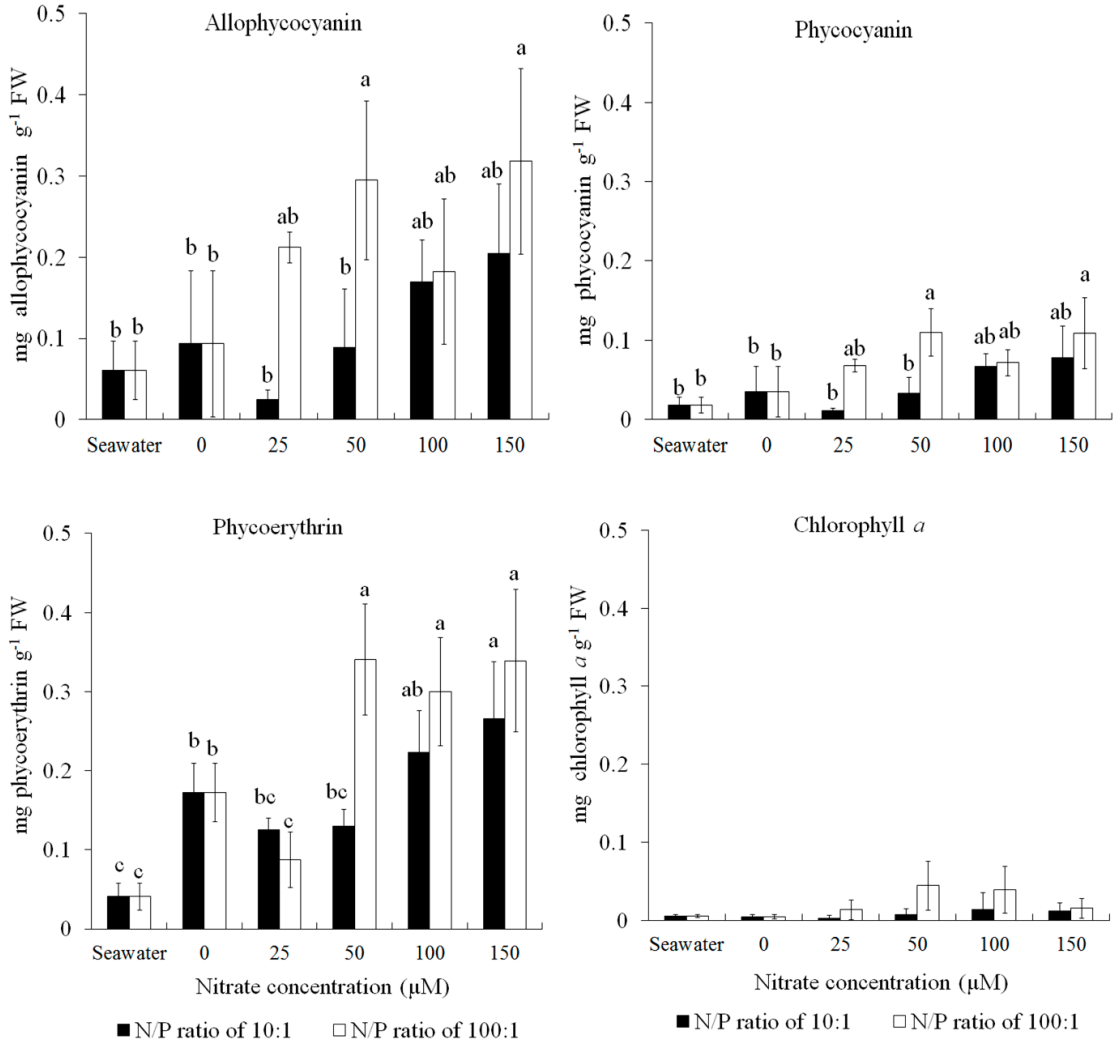

Fig. 2: Concentration (mg g^{-1} FW) of allophycocyanin, phycocyanin, phycoerythrin and chlorophyll *a* of *Hypnea aspera* cultured for 7 days in VSES/4 modified and enriched with different nitrate concentrations in N/P ratios of 10:1 and 100:1, 23 ± 3°C, photoperiod of 14 h, salinity of 30, pH 8.0 and photon flux densities of 60 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to one-way ANOVA and Student- Newman-Keuls multiple comparison test ($p< 0.05$). ■ N/P 10:1, □ N/P 100:1.

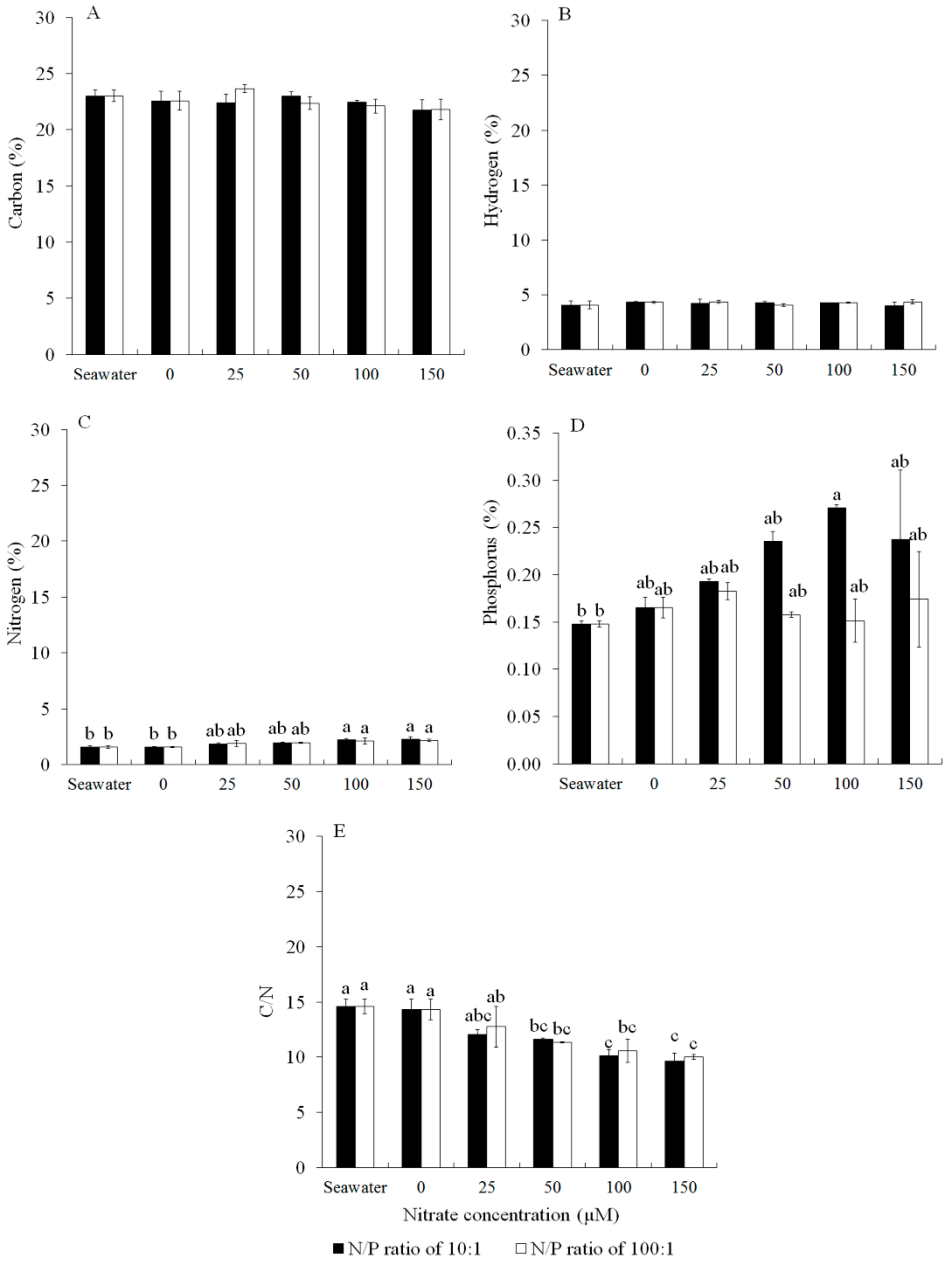

Fig. 3: Internal carbon (A), hydrogen (B), nitrogen (C) and phosphorus (D) content (%) and C/N ratio (E) of *Hypnea aspera* cultured for 7 days in VSES/4 modified enriched with different nitrate concentrations in N/P ratios of 10:1 and 100:1, 23 ± 3°C, photoperiod of 14 h, salinity of 30, pH 8.0 and photon flux densities of 60 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to one-way ANOVA and Student-Newman-Keuls multiple comparison test ($p < 0.05$). ■ N/P 10:1, □ N/P 100:1.

Nutrient uptake by *Hypnea aspera* is shown in Figure 4, and removal efficiency (%) is shown in Table 1. Nitrate uptake increased with increasing nitrate concentrations and was higher for 100 µM in N/P ratio of 10:1 (3.44 ± 0.75 µM g^{-1} FW d^{-1}) and 150 µM in N/P ratio of 100:1 (3.34 ± 0.86 µM g^{-1} FW d^{-1}). Seaweeds had high removal efficiency in all treatments (> 96 %), except 150 µM of nitrate in N/P ratio of 10:1 (47.11 ± 43.16 %). The lower uptake was for nitrite (< 0.01) for all treatments with nitrate addition, except for seawater in which *H. aspera* removed higher nitrite concentration (46.47 ± 10.45 %). Ammonium uptake by *H. aspera* was low (< 0.1 µM g^{-1} FW d^{-1}), with higher uptake and removal efficiency occurring in 25 µM of nitrate in N/P ratio of 100:1 (0.05 ± 0.02 µM g^{-1} FW d^{-1} and 99.03 ± 1.66 %, respectively). This species uptake more phosphate in nitrate additions of 100 and 150 µM in N/P ratio of 10:1 (0.31 ± 0.02 and 0.32 ± 0.03 µM g^{-1} FW d^{-1}, respectively), and removal efficiency did not vary significantly among different nitrate addition and N/P ratios. The amount of DON released by *H. aspera* was high with the addition of 50 µM nitrate in N/P ratio of 10:1 (4.64 ± 2.19 µM). Low release of DON occurred with the addition of 25 and 150 µM of nitrate in both N/P ratios and in 50 and 100 µM in N/P ratio of 100:1 (Fig. 5).

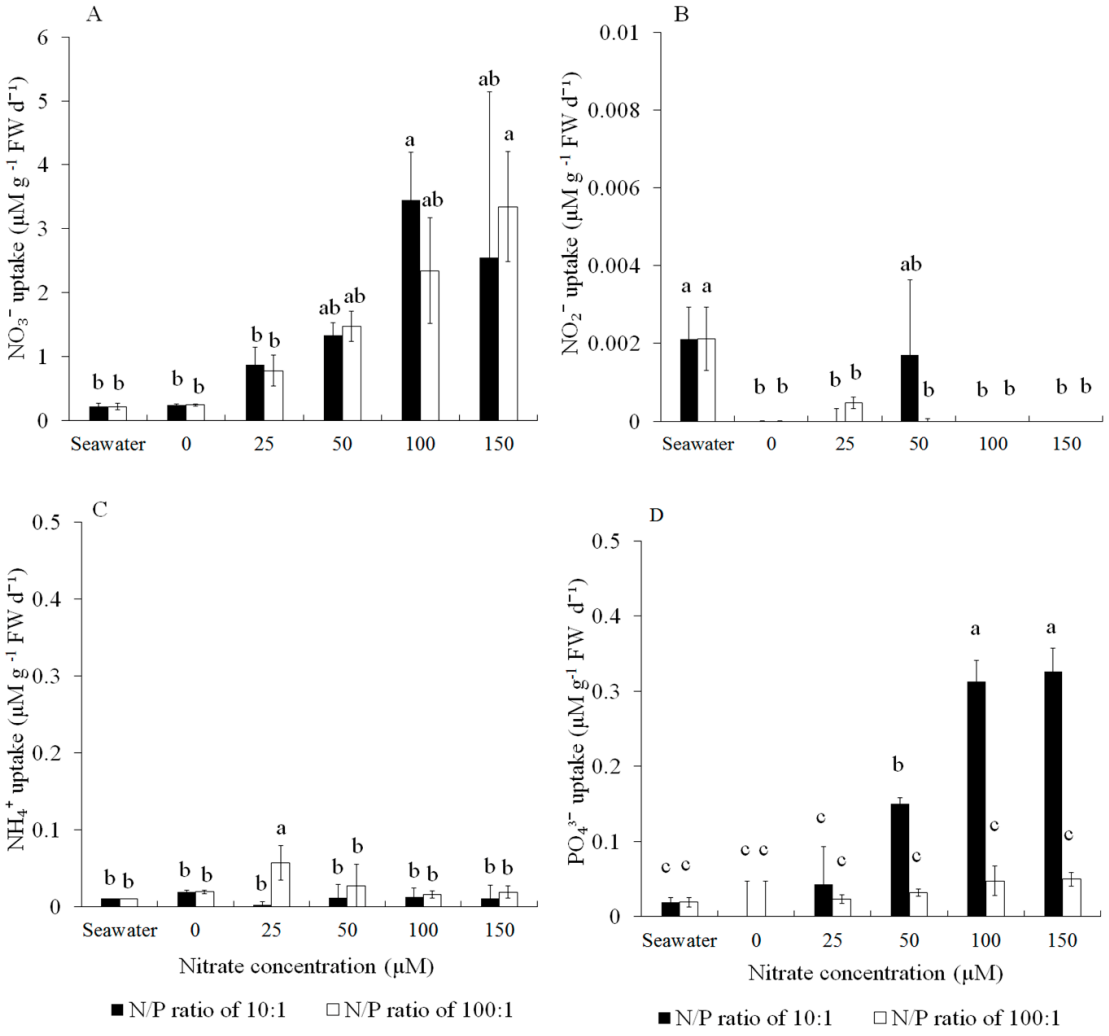

Fig. 4: Uptake (µM gFW^{-1} d^{-1}) of nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+) and phosphate (PO_4^{3-}) of *Hypnea aspera* cultured for 7 days in VSES/4 modified enriched with different nitrate concentrations in N/P ratios of 10:1 and 100:1, 23 ± 3°C, photoperiod of 14 h, salinity of 30, pH 8.0 and photon flux densities of 60 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to one-way ANOVA and Student- Newman-Keuls multiple comparison test ($p < 0.05$). ■ N/P 10:1, □ N/P 100:1.

Table 1: Removal efficiency (%) of *Hypnea aspera* cultured for 7 days in VSES/4 modified enriched with different nitrate/phosphate concentrations in N/P ratios of 10:1 and 100:1, 23 ± 3°C, photoperiod of 14 h, salinity of 30, pH 8.0 and photon flux densities of 60 to 90 µmol of photons m^{-2} s^{-1}. Data are mean of three replicates ± SD. Treatments with distinct letters indicate significant differences according to one-way ANOVA and Student-Newman-Keuls multiple comparison test ($p < 0.05$).

Nitrate concentrations (µM)	Nutrient removal (%)							
	NO_3^- removal		NO_2^- removal		NH_4^+ removal		PO_4^{3-} removal	
	N/P ratio							
	10:1	100:1	10:1	100:1	10:1	100:1	10:1	100:1
Seawater	96.79 ± 2.09[a]	96.79 ± 2.09[a]	46.47 ± 10.45[a]	46.47 ± 10.45[a]	74.56 ± 22.30	74.56 ± 22.30	98.81 ± 1.14	98.81 ± 1.14
0	98.24 ± 1.29[a]	98.24 ± 1.29[a]	0.02 ± 0.04[b]	0.02 ± 0.04[b]	82.36 ± 18.08	82.36 ± 18.08	56.57 ± 49.62	56.57 ± 49.62
25	99.24 ± 0.25[a]	99.43 ± 0.37[a]	5.55 ± 9.62[b]	17.83 ± 5.51 [ab]	35.17 ± 37.12	99.03 ± 1.66	57.89 ± 50.73	89.71 ± 3.87
50	99.53 ± 0.19[a]	99.66 ± 0.08[a]	30.80 ± 25.87[ab]	1.65 ± 2.86[b]	61.53 ± 53.84	92.20 ± 13.50	94.61 ± 4.63	93.49 ± 1.54
100	98.35 ± 2.48[a]	99.81 ± 0.02[a]	0.00 ± 0.00[b]	0.00 ± 0.00[b]	50.00 ± 43.44	97.15 ± 4.92	92.64 ± 4.07	97.12 ± 0.17
150	47.11 ± 43.16[b]	97.99 ± 3.31[a]	0.00 ± 0.00[b]	0.00 ± 0.00[b]	63.33 ± 55.07	91.60 ± 14.53	72.97 ± 10.07	97.39 ± 0.99

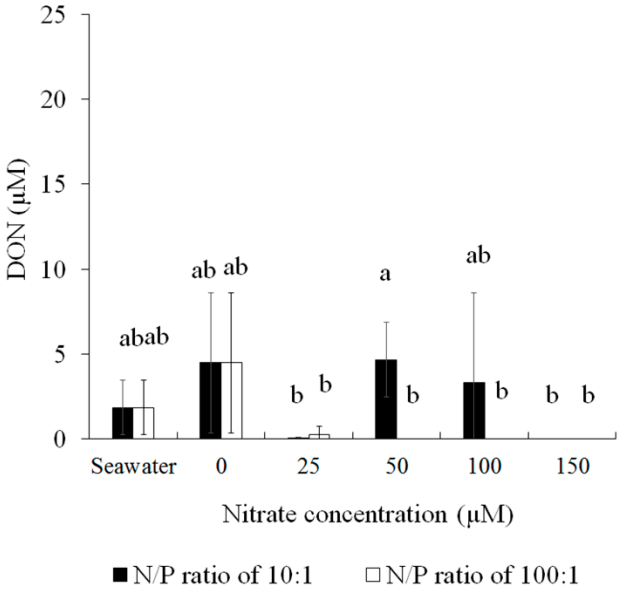

Fig. 5: Release of DON (µM) of *Hypnea aspera* cultured for 7 days in VSES/4 modified enriched with different nitrate concentrations in N/P ratios of 10:1 and 100:1, 23 ± 3°C, photoperiod of 14 h, salinity of 30, pH 8.0 and photon flux densities of 60 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to one-way ANOVA and Student- Newman-Keuls multiple comparison test ($p< 0.05$). ■ N/P 10:1, □ N/P 100:1.

The growth rate of *H. aspera* in seawater with ammonium and phosphate additions showed a linear increase in N/P ratio of 100:1. However, in N/P ratio of 10:1, the seaweed showed negative growth rate (Fig. 6). The highest growth rate was observed in 70 µM of ammonium in N/P of 100:1 (8.88 ± 0.73 % d^{-1}). In treatments with 30, 50 and 70 µM of ammonium in N/P ratio of 10:1, *H. aspera* assimilated nitrogen as pigments and proteins (Fig. 6 and 7).

The highest protein content of *H. aspera* was observed with the addition of 50 and 70 µM of ammonium in N/P ratio of 10:1 (3.14 ± 0.37 and 3.22 ± 0.14 mg protein g^{-}

^{1} FW, respectively), and the lowest protein concentration occurred with the addition of 0 µM of ammonium (1.95 ± 0.10 mg protein g^{-1} FW) (Fig. 6).

H. aspera accumulated pigments mainly like PE form, and Chl *a* was the lower form of pigment (Fig. 7). High APC concentrations occurred with the addition of 30 µM ammonium in N/P ratio of 100:1 (0.13 ± 0.01 mg g^{-1} FW), and 70 µM of ammonium in N/P of 10:1 (0.14 ± 0.02 mg g^{-1} FW). The highest PC contents was observd with 70 µM of ammonium in N/P ratio of 10:1 (0.15 ± 0.02 mg g^{-1} FW), and PE content was observed with the addition of 50 µM of ammonium in both N/P ratios (Fig. 7). Chlorophyll *a* showed high concentration without ammonium addition (0.03 ± 0.01 mg g^{-1} FW), while the lowest concentration for this pigment and PE was observed in 10 µM of ammonium in N/P ratio of 100:1.

Results of internal C, H, N, P contents and C/N ratio of *H. aspera* are shown in Figure 8. The largest reserve was carbon; however, no significant difference was noted between ammonium additions and N/P ratios. The same was observed for nitrogen contents; however, they were found in lower concentrations in seaweed thallus. Hydrogen concentrations varied among treatments, but were higher in 0 µM and lower in 70 µM of ammonium in N/P ratio of 100:1 (4.74 ± 0.12 and 3.82 ± 0.11 %, respectively). P content increased with high ammonium concentrations and was highest with the addition of 70 µM of ammonium in N/P ratio of 10:1 (0.21 ± 0.01 %). The addition of ammonium had no effect on the C/N ratio of *H. aspera*.

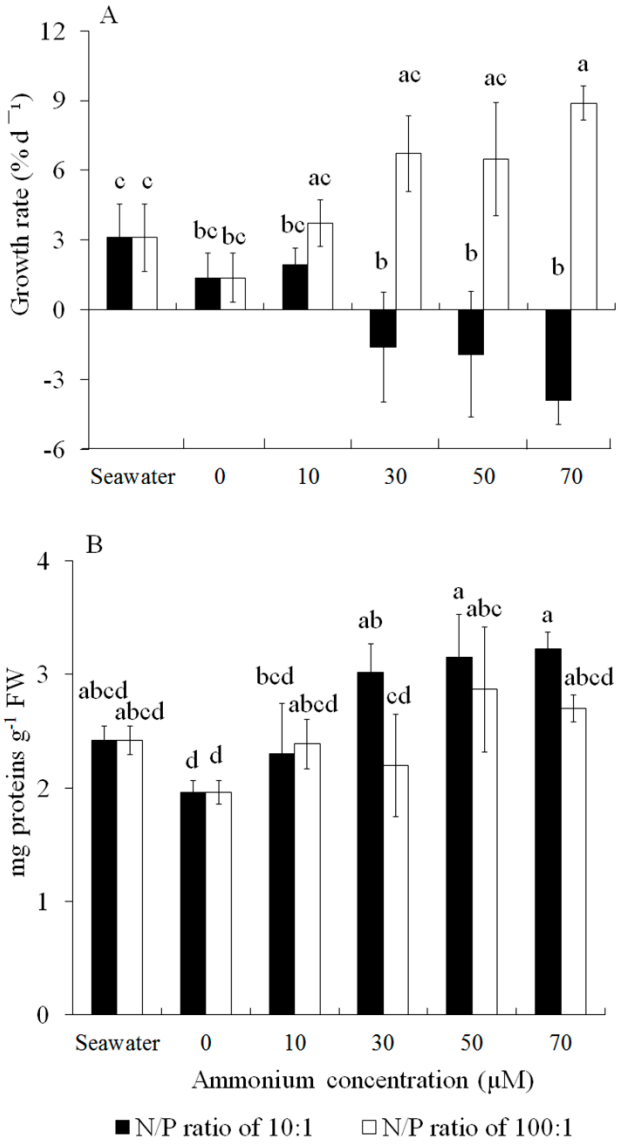

Fig. 6: Growth rate (% d^{-1}) (A) and protein concentration (mg g^{-1} FW) (B) of *Hypnea aspera* cultured for 7 days in VSES/4 modified enriched with different ammonium concentrations in N/P ratios of 10:1 and 100:1, 23 ± 3°C, photoperiod of 14 h, salinity of 30, pH 8.0 and photon flux densities of 60 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to one-way ANOVA and Student-Newman-Keuls multiple comparison test ($p < 0.05$). ■ N/P 10:1, □ N/P 100:1.

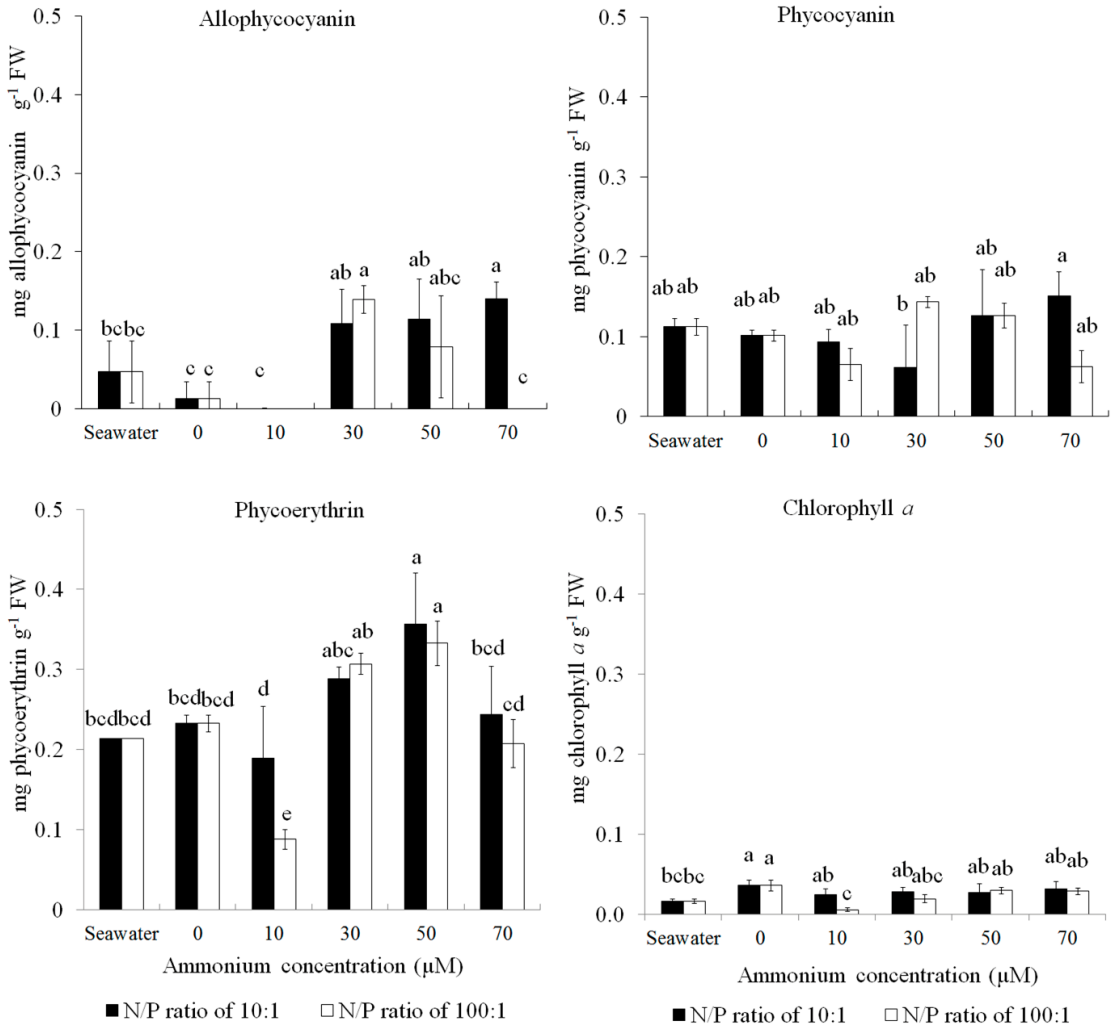

Fig. 7: Concentration (mg g^{-1} FW) of allophycocyanin, phycocyanin, phycoerythrin and chlorophyll *a* of *Hypnea aspera* cultured for 7 days in VSES/4 modified enriched with different ammonium concentrations in N/P ratios of 10:1 and 100:1, 23 ± 3°C, photoperiod of 14 h, salinity of 30, pH 8.0 and photon flux densities of 60 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to one-way ANOVA and Student- Newman-Keuls multiple comparison test ($p< 0.05$). ■ N/P 10:1, □ N/P 100:1.

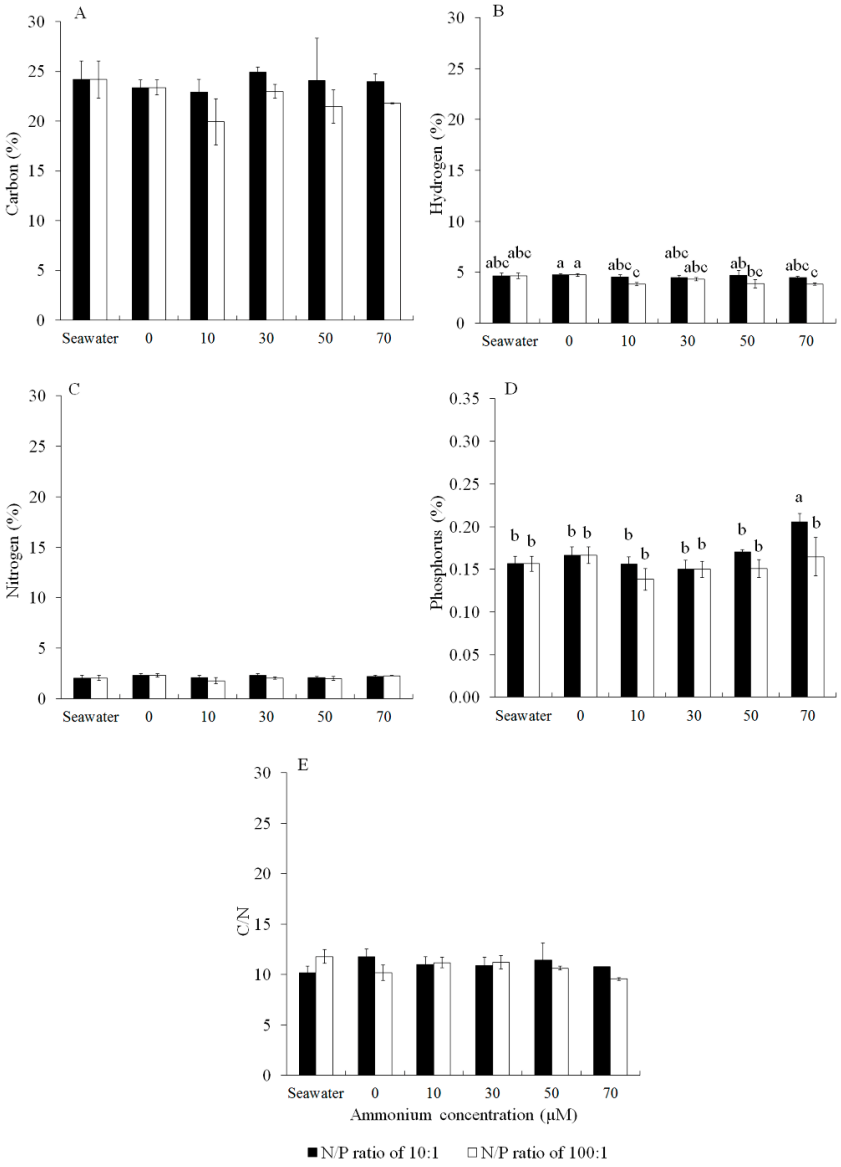

Fig. 8: Internal carbon (A), hydrogen (B), nitrogen (C) and phosphorus (D) content (%) and C/N ratio (E) of *Hypnea aspera* cultured for 7 days in VSES/4 modified enriched with different ammonium concentrations in N/P ratios of 10:1 and 100:1, 23 ± 3°C, photoperiod of 14 h, salinity of 30, pH 8.0 and photon flux densities of 60 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to one-way ANOVA and Student-Newman-Keuls multiple comparison test ($p < 0.05$). ■ N/P 10:1, □ N/P 100:1.

Results of nutrient uptake and removal efficiency are shown in Figure 9 and Table 2, respectively. *Hypnea aspera* removed all nitrate in the seawater in all treatments of ammonium and phosphate (> 97 %), and high uptake occurred in all ammonium additions, except seawater. The uptake and removal of nitrite were lower than those of the other nutrients (< 0.015 µM g^{-1} FW d^{-1} and 65 %). High uptake occurred with 70 µM of ammonium in N/P ratio of 100:1 (0.005 ± 0.003 µM g^{-1} FW d^{-1}), but it was low at 50 µM in N/P ratio of 10:1 (0.000 ± 0.003 µM g^{-1} FW d^{-1}). Ammonium uptake and removal increased linearly with ammonium concentrations, and uptake was higher in 70 µM in N/P ratio of 10:1 (2.85 ± 0.09 µM g^{-1} FW d^{-1}), while removal efficiency did not differ among ammonium additions, except for seawater and 0 µM, in which removal efficiency was lower (26.37 ± 45.68 % and 16.66 ± 28.86 %, respectively). Phosphate uptake was high in N/P ratio of 100:1 with 70 µM of ammonium (0.26 ± 0.00 µM g^{-1} FW d^{-1}), but removal efficiency did not change with the addition of ammonium, except to 50 and 70 µM in N/P ratio of 10:1. The release of DON (µM) was lower in seawater and the addition of 0 µM ammonium, but it was higher in 50 µM of ammonium in N/P ratio of 10:1 (10.29 ± 9.67 µM) (Fig. 10).

Principal components (PCA) analysis explained total variability of 47.24% in the two first axis. The positive side of axis 1 grouped treatments with N limitations with C/N ratio of *H. aspera*, and the negative side grouped all other nitrate and ammonium additions with nitrogen assimilation (PT, PE, PC and N) and nutrient uptake (NO_3^-, NH_4^+ and PO_4^{3-}). The positive side of axis 2 was associated with ammonium/phosphate with NO_2^- and PO_4^{3-} removal, NO_2^- uptake and GR, and the negative side grouped nitrate/phosphate additions with tissue P and APC (Fig. 11 and Table 3).

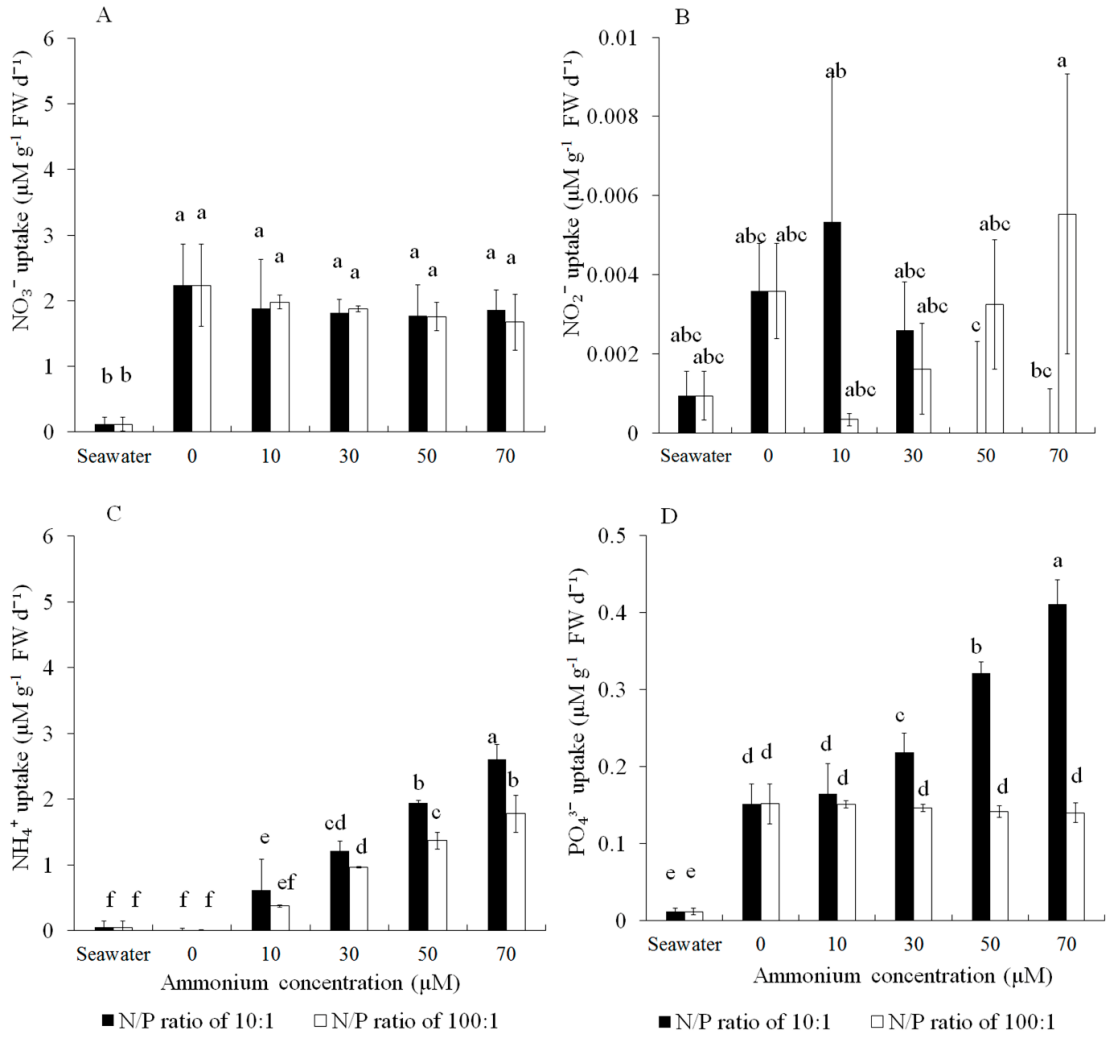

Fig. 9: Uptake (µM gFW^{-1} d^{-1}) of nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+) and phosphate (PO_4^{3-}) of *Hypnea aspera* cultured for 7 days in VSES/4 modified enriched with different ammonium concentrations in N/P ratios of 10:1 and 100:1, 23 ± 3°C, photoperiod of 14 h, salinity of 30, pH 8.0 and photon flux densities of 60 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to one-way ANOVA and Student- Newman-Keuls multiple comparison test ($p < 0.05$). ■ N/P 10:1, □ N/P 100:1.

Table 2: Removal efficiency (%) of *Hypnea aspera* cultured for 7 days in VSES/4 modified enrichedwith different ammonium/phosphate concentrations in N/P ratios of 10:1 and 100:1, 23 ± 3°C, photoperiod of 14 h, salinity of 30, pH 8.0 and photon flux densities of 60 to 90 µmol of photons m^{-2} s^{-1}. Data are mean of three replicates ± SD. Treatments with distinct letters indicate significant differences according to one-way ANOVA and Student-Newman-Keuls multiple comparison test ($p< 0.05$).

Ammonium concentrations (µM)	Nutrient removal (%)							
	NO_3^- removal		NO_2^- removal		NH_4^+ removal		PO_4^{3-} removal	
	N/P ratio							
	10:1	100:1	10:1	100:1	10:1	100:1	10:1	100:1
Seawater	97.10 ± 1.55	97.10 ± 1.55	40.87 ± 13.69^{ab}	40.87 ± 13.69^{ab}	26.37 ± 45.68^{b}	26.37 ± 45.68^{b}	90.22 ± 8.95^{a}	90.22 ± 8.95^{a}
0	99.79 ± 0.17	99.79 ± 0.17	59.06 ± 5.12^{a}	59.06 ± 5.12^{a}	16.66 ± 28.86^{b}	16.66 ± 28.86^{b}	93.55 ± 9.02^{a}	93.55 ± 9.02^{a}
10	99.95 ± 0.08	99.49 ± 0.25	57.22 ± 25.22^{a}	15.38 ± 7.69^{b}	93.11 ± 5.31^{a}	90.87 ± 3.84^{a}	94.98 ± 4.93^{a}	98.90 ± 0.57^{a}
30	99.95 ± 0.08	99.74 ± 0.15	43.08 ± 24.31^{ab}	53.68 ± 29.14^{ab}	98.86 ± 0.53^{a}	96.52 ± 1.61^{a}	80.68 ± 4.59^{a}	99.53 ± 0.17a
50	98.21 ± 2.97	99.79 ± 0.02	11.60 ± 20.09^{b}	62.37 ± 28.38^{a}	98.64 ± 1.00^{a}	97.33 ± 1.48^{a}	59.02 ± 18.72^{b}	99.36 ± 0.46^{a}
70	99.80 ± 0.02	99.51 ± 0.49	13.27 ± 11.57^{b}	65.93 ± 36.11^{a}	99.56 ± 0.15^{a}	96.85 ± 3.51^{a}	61.56 ± 13.56^{b}	98.92 ± 0.69^{a}

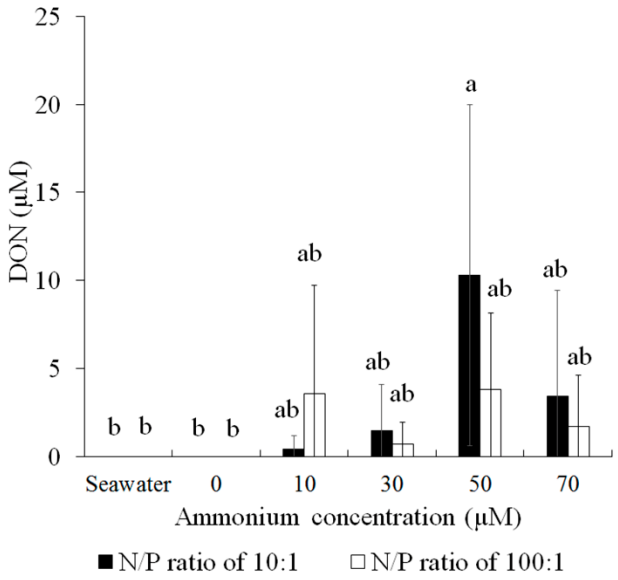

Fig. 10: Release of DON (µM) of *Hypnea aspera* cultured for 7 days in VSES/4 modified enriched with different ammonium concentrations in N/P ratios of 10:1 and 100:1, 23 ± 3°C, photoperiod of 14 h, salinity of 30, pH 8.0 and photon flux densities of 60 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to one-way ANOVA and Student- Newman-Keuls multiple comparison test ($p< 0.05$). ■ N/P 10:1, □ N/P 100:1.

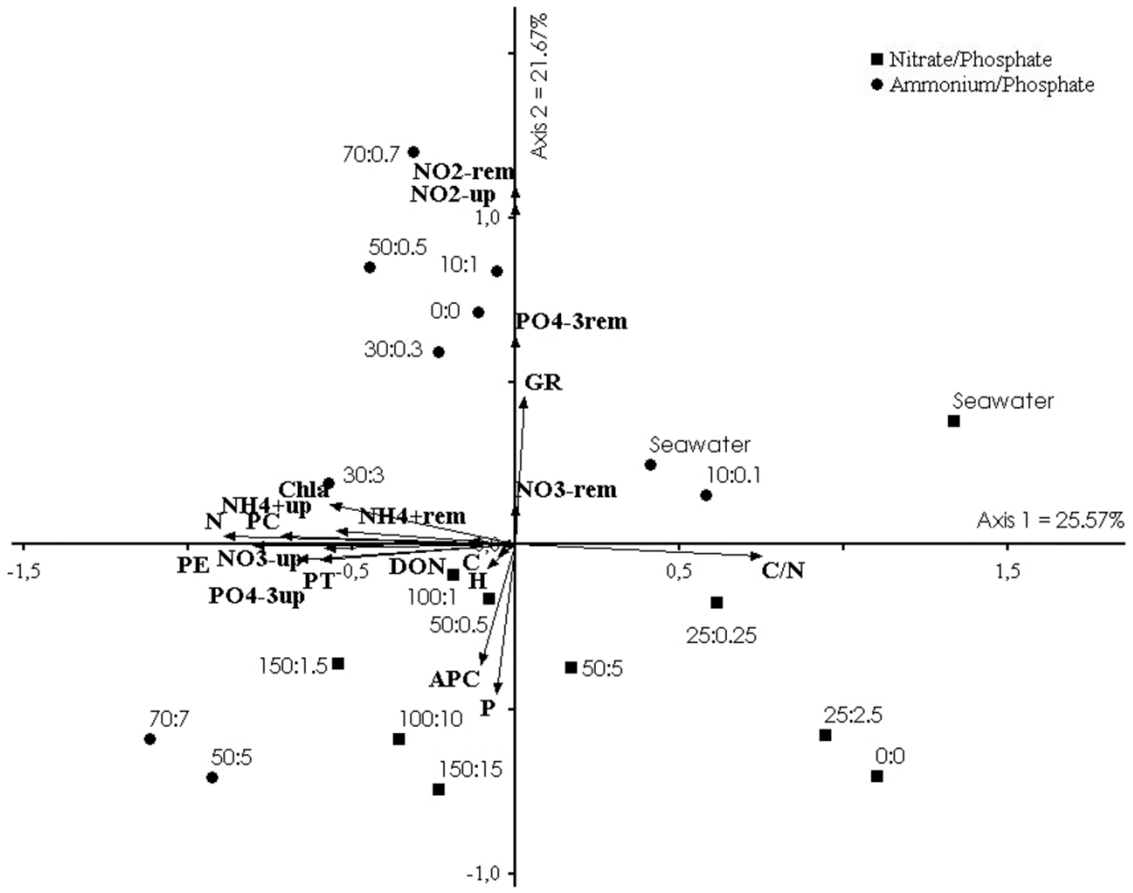

Fig. 11: Scatter diagram of plots of the first two principal component analysis axes of data on the effects of nitrate and ammonium on growth rates, pigment, protein contents, tissue elements (C, H, N, P and C/N ratio), removal efficiency (rem), nutrient uptake (up) and release of DON of *Hypnea aspera* cultured for 7 days in VSES/4 modified enriched with different nitrate/phosphate (black square) or ammonium/phosphate (black circle) concentrations in N/P ratios of 10:1 and 100:1, 23 ± 3°C, photoperiod of 14 h, salinity of 30, pH 8.0 and photon flux densities of 60 to 90 µmol of photons m^{-2} s^{-1}. The first two components accounted for 47.24 % of total variance. Growth rates (GR), pigment contents (APC, PC, PE, and Chl *a*), protein contents (PT), tissue elements (C, H, N, P and C/N ratio), removal efficiency (rem), nutrient uptake (up) and release of DON, respectively.

Table 3: Pearson correlation coefficient among variables analyzed for *Hypnea aspera* to evaluate the effects of nitrate and ammonium on growth rates, pigment, protein contents, element contents in the thallus (C, H, N, P and C/N ratio), removal efficiency (rem), nutrient uptake (up) and release of DON. The scatterplot diagram of PCA is shown in Fig. 11.

Variables	Principal components	
	Axis 1	Axis 2
Growth rate (GR)	0.152	0.591
Total soluble protein (PT)	-0.681	-0.192
Allophycocyanin (APC)	-0.288	-0.535
Phycocyanin (PC)	-0.745	0.136
Phycoerythrin (PE)	-0.789	-0.070
Chlorophyll *a* (Chl *a*)	-0.662	0.309
Carbon (C)	-0.196	-0.080
Hydrogen (H)	-0.210	-0.174
Nitrogen (N)	-0.830	0.141
C/N ratio (C/N)	0.765	-0.169
Phosphorus (P)	-0.217	-0.596
PO_4^{3-} uptake (PO_4^{3-} up)	-0.719	-0.199
NH_4^+ uptake (NH_4^+ up)	-0.650	0.179
NO_2^- uptake (NO_2^- up)	-0.050	0.898
NO_3^- uptake (NO_3^- up)	-0.673	-0.105
PO_4^{3-2} removal (PO_4^{3-} rem)	0.010	0.702
NH_4^+ removal (NH_4^+ rem)	-0.332	0.082
NO_2^- removal (NO_2^- rem)	-0.052	0.920
NO_3^- removal (NO_3^- rem)	0.051	0.313
Release of dissolved organic nitrogen (DON)	-0.257	-0.246
% of variance	25.57	21.67

Discussion

Hypnea aspera showed higher growth rates when cultured with ammonium than when cultured with nitrate as a nitrogen source. However, this effect was lower in N/P ratio of 10:1, mainly for ammonium. Growth rate increased with the enhance of nitrogen concentrations, and *H. aspera* showed higher growth in 150 µM of nitrate and 70 µM of ammonium, both in N/P ratio of 100:1. Similar results were reported for the green

seaweed *Ulva lactuca*, which grew faster with 50 µM of ammonium than with 50 µM of nitrate (Ale et al. 2011), and for *Ulva australis* Areschoug, *Sargassum fulvellum* (Turner) C. Agardh and *Gelidium amansii* (J.V. Lamouroux) J.V. Lamouroux cultivated with 80 and 200 µM of ammonium and nitrate, respectively (Dogyan et al. 2004). In contrast, our results showed that higher phosphate concentrations (N/P ratio of 10:1) inhibited the growth of *H. aspera* cultivated with ammonium concentrations higher than 30 µM. To explain this, high phosphate availability may have increased the inorganic phosphate in intracellular medium causing decreased growth, but increased ability of the seaweed to uptake, assimilate and remove N and P as pigments, proteins and thallus P content. However, ammonium additions higher than 15 µM were toxic and lethal for brown and light green strains of *H. pseudomusciformis* (cited as *H. musciformis* by Martins and Yokoya 2010). These results could be explained by ammonium accumulation into cytoplasm which decreased internal pH, which would be prejudicial to the cells (Turpin 1991).

High nitrate or ammonium concentrations in seawater stimulated total soluble protein accumulation, the largest pool of nitrogen storage. In nitrate additions, *H. aspera* accumulated more protein in N/P ratio of 100:1, while in ammonium concentrations, protein content was high in N/P ratio of 10:1. The same results were observed for phycobiliproteins, mainly PE. Total soluble protein and PE accumulation when cultivated in nitrogen availabilities has been described for other seaweeds, such as *Hypnea cervicornis* cultivated with 0 to 500 µM of nitrate and 0 to 50 µM of ammonium in N/P ratio of 10:1 and 100:1 (Ribeiro et al. 2013) and *Gracilaria tikvahiae* McLachlan cultivated in 1 mM of ammonium and 1 mM of nitrate with 1 mM for phosphate concentration (Bird et al. 1982). These compounds can be considered nitrogen storage

pools under conditions of nutrient limitation and can be used to growth (Andria et al. 2009).

Variation of C/N ratio is a response to carbon and nitrogen storage, resulting from possible nitrogen limitation for growth. The presence of high nitrate concentrations stimulated the assimilation of tissue nitrogen and phosphorus, and C/N ratio was high in N limitation. In tests with ammonium, only tissue phosphorus was high with increased ammonium concentration in N/P ratio of 10:1. On the other hand, hydrogen content was assimilated in greatest amounts in N limitation. Nitrogen limitation increased C/N ratio, and nitrogen availability increased tissue N in some seaweeds, as observed in *Ulva fasciata* Delile (Lapointe and Tenore 1981), *Gracilaria tikvahiae* (Friedlander and Dawes 1985), *Ulva clathrata* (Roth) C. Agardh (Copertino et al. 2009) and *Palmaria palmata* (Linnaeus) F. Weber & D. Mohr (Corey et al. 2013). Some studies with *Chondrus crispus* Stackhouse (Chopin and Wagey 1999), *Pyropia yezoensis* (Ueda) M.S. Hwang & H.G. Choi (Chopin et al. 1999) and *Chaetomorpha linum* (O.F. Müller) Kützing (Menéndez et al. 2002) showed that these species assimilated greatest N and P content with P enrichment, as reported in the present study. The high hydrogen content observed could be explained by the increase of polysaccharide production for energy storage resulting from N limitation (Serra 2013), but the implications of this are beyond the scope of the present work and should, therefore, be investigated in future studies.

A relationship was observed between tissue N and P in *H. aspera* and the uptake of nitrogen and phosphorus from seawater. Nitrate and phosphate additions stimulated high NO_3^- and PO_4^{3-} uptake, and this was improved with high phosphorus availability. The same was observed for NH_4^+ uptake in tests with ammonium and phosphate availabilities. The uptake and removal of NO_2^- were extremely low in both N resources. NO_3^- and NH_4^+ uptake by *Hypnea musciformis* was higher than nutrient uptake by

Gracilaria foliifera (Forsskål) Børgesen and *Agardhiella subulata* (C. Agardh) Kraft & M.J. Wynne cultivated with 0 to 30 µg at N L^{-1} (Haines and Wheeler 1978). *Adamsiella chauvinii* (Harvey) L.E. Phillips & W.A. Nelson showed a NO_3^- uptake maximum around 12 µM g^{-1} DW d^{-1} and NH_4^+ uptake around 79 µM g^{-1} DW d^{-1} when grown in 64 µM of nitrate or ammonium (Kregting et al. 2008). In the color strains of *H. pseudomusciformis*, NO_3^- uptake increased with high nitrate concentrations and showed uptake of about 9 µM g^{-1} FW d^{-1} for nitrate additions of 100 µM, while phosphate additions (N/P ratio of 4:1) did not affect the nitrate uptake for this species (cited as *H. musciformis* by Martins 2007). In the present study, *H. aspera* showed NO_3^- uptake around 5 µM g^{-1} FW d^{-1} and NH_4^+ uptake of about 3 µM g^{-1} FW d^{-1}. Uptake of nutrients was stimulated by increased phosphate concentrations in N/P ratio of 10:1. Moreover, *H. aspera* removed high concentrations of nutrients available in the seawater and when relationship with results for other seaweeds (Table 4).

Release of DON was high in 50 µM of nitrate and 50 µM of ammonium addition in N/P ratio of 10:1 (around 5 and 10 µM of DON, respectively). Thus, although *Hypnea aspera* removed all nitrate and ammonium from seawater, it could not completely assimilate and store them since much of the nitrate or ammonium removed was returned to the environment in the form of DON. The same was observed for color strains of *H. pseudomusciformis* cultivated in 100 µM of nitrate in N/P ratio of 4:1 (cited as *H. musciformis*, Martins 2007). These results could be explained for active release of retained products in excess by carbon fixation in the photosynthesis process.

Table 4: Maximum nutrient removal by seaweed acting as biofilter in laboratory experiments or in integrated systems with marine animals.

Species	Type of cultivation	% of nutrient removal	Reference
Saccharina latissima (Linnaues) C.E. Lane, C. Mayes, Druehl & G.W. Saunders	Salmon farming	40 % of DIN (NO_3^- + NH_4^+)	Subandar et al. (1993)
Ulva rotundata Bliding	Sea bass farming	96.2% of PO_4^{-3}	Martínez-Aragón et al. (2002)
Ulva intestinalis L.Nees		99.2% of PO_4^{-3}	
Gracilaria gracilis (Stack.) M. Steentoft. L.M.Irv. & W.F. Farnham		98% of PO_4^{-3}	
Hypnea pseudomusciformis (cited as *H. musciformis*) Brown strain	Laboratory	99.98% of NO_3^- 94.89 % of NO_2^- 44.16% of NH_4^+ 98.33 % of PO_4^{-3}	Martins (2007)
Green light strain		99.98% of NO_3^- 93.92 % of NO_2^- 38.88% of NH_4^+ 99.31 % of PO_4^{-3}	
Kappaphycus alvarezii (Doty) Doty ex P.C. Silva	Florida pompano farming (Trachinotus carolinus L.)	18.20% of NO_3^- 50.84% of NO_2^- 70.54% of NH_4^+ 26.76% of PO_4^{-3}	Hayashi et al. (2008)
Ulva lactuca	Abalone farming (Haliotis midae L.)	80% of NH_4^+ 77% of NO_3^- 75% of NO_2^-	Robertson-Anderson et al. (2008)
Porphyra dioica J. Brodie & L.M. Irvine	Laboratory	98% of NO_3^- 88% of PO_4^{-3}	Pereira et al. (2008)
Gracilariopsis lemaneiformis (Bory de Saint-Vicent) E.Y. Dawson, Acleto & Foldvik	Scallop farming (Chlamys farreri Jones et Preston**)**	80 % of NH_4^+ 70.4 % of PO_4^{-3}	Mao et al. (2009)
Ulva clathrata (Roth) C. Agardh	Shrimp farming (Litopenaeus vannamei Boonei)	85 % of DIN 44% of PO_4^{-3}	Copertino et al. (2009)
Ulva lactuca	Laboratory	80% of PO_4^{-3}	Tsagkamilis et. al. (2010)
Ulva prolifera O.F. Müller	Laboratory	99.24% of NO_3^- 91.30% of PO_4^{-3}	Fan et al. (2014)
Hypnea aspera	Laboratory (nitrate and phosphate addition)	99.81% of NO_3^- 46.47 % of NO_2^- 99.03 % of NH_4^+ 98.81 % of PO_4^{-3}	Present study
	(ammonium and phosphate addition)	99.95% of NO_3^- 65.93 % of NO_2^- 99.56 % of NH_4^+ 99.53 % of PO_4^{-3}	

Overall, our results showed that *H. aspera* tolerated high nutrient concentrations since it grew and assimilated N and P as proteins, pigments and thallus C, N, H and P in both nitrogen sources. In addition, this species could uptake and remove high nutrient concentrations from seawater, indicating that this species could be cultivated in integrated multitrophic aquaculture systems to reduce nutrient loading in eutrophic seawater.

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Capítulo 2

Effects of increased temperature, CO_2 levels and nitrogen availability on the physiology and biochemistry of the seaweed *Hypnea aspera* (Gigartinales, Rhodophyta)

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Abstract

By the year 2100, the Intergovernmental Panel on Climate Change (IPCC) estimates an increase from 380 to 1000 ppm in atmospheric levels of CO_2, which could result in ocean acidification, and it is also expected to temperature increase by 4.8°C. Besides these factors, the increase of nitrogen concentration due to eutrophication may impact marine ecosystems, in particular seaweeds. Therefore, the effects of CO_2 and temperature variations (as proposed by IPCC for the Coupled Model Intercomparison Project Phase 5, and Representative Concentration Pathways 8.5 for 2100) together with the increase on nitrogen availability in seawater were evaluated on the physiology and biochemistry of *Hypnea aspera* Kützing (Rhodophyta, Gigartinales). Tetrasporophytes of *H. aspera* were cultivated for two weeks under three levels of CO_2 (0, 380 and 1000 ppm), temperature (21, 25 and 30°C) and nitrogen availability (0, 125 and 500 µM of nitrate or 0, 50 and 100 µM of ammonium). Results showed the negative effects of the highest CO_2 and temperature levels, associated with N-limitation condition on growth rate, pigment content, protein concentration and photosynthesis of this species. *H. aspera* showed higher values for these variables when cultivated at temperatures of 21 and 25°C, 0 or 380 ppm of CO_2, and nitrogen (nitrate at 125 and 500 µM or ammonium at 50 and 100 µM). These results suggest that *H. aspera* is highly sensitive to increases in temperature and CO_2 levels. In conclusion, our results may provide information on the impact of global warming, increased CO_2, and eutrophication on physiological responses of *H. aspera*, a benthic marine red alga, which could be used as an experimental model for understanding the effects of global changing on physiological process marine organisms.

Introduction

According to Intergovernmental Panel on Climate Change (IPCC, 2013), the atmospheric level of CO_2 has increased over the last several years. Atmospheric concentrations of CO_2 reached values from 380 to 390 ppm in 2011, an increase of about 40% since the preindustrial period. Estimates suggest that 30 % of emitted CO_2 has been absorbed by the oceans (Caldeira & Wickett, 2003; Feely *et al.*, 2004), and that this absorbed CO_2 reacts with seawater, resulting in an increase of H^+ protons, a condition which, in turn, reduces seawater pH, decreases carbonate ion concentration (CO_3^{2-}), and increases HCO_3^- levels (Hurd *et al.*, 2009). According to the Coupled Model Intercomparison Project Phase 5 (CMIP5) and Representative Concentration Pathways (RCP) 8.5 for 2100 (IPCC, 2013), atmospheric CO_2 levels could likely increase to 1000 ppm and temperature increase could reach 4.8°C on the ocean surface.

This process results in ocean acidification, and when coupled with global warming, the dual effects can have serious consequences for marine ecosystems. Moreover, nitrogen availability plays a critical role in the physiology of benthic marine algae by controlling their growth and metabolism. N-limitation can cause inhibition on photosynthetic process (Turpin, 1991; Russell *et al.*, 2009) and decrease in phycobiliprotein content in seaweeds (Andria *et al.*, 2009; Barufi *et al.*, 2012; Ribeiro *et al.*, 2013). Furthermore, carbon and nitrogen pathways are coupled in seaweed metabolism, while CO_2 fixation and N assimilation compete for assimilatory energy and C skeletons for amino acid synthesis (Turpin, 1991).

Some studies have reported on the synergistic effects of CO_2 and temperature on recruitments of the kelp *Ecklonia radiata* (C.Agardh) J. Agardh, and dry mass, effective quantum yield and percentage of cover of algal turfs (e.g., *Feldmannia* spp.) (Connell & Russell, 2010), on mortality and calcification of coralline alga *Porolithon onkodes* (Heydrich) Foslie (Diaz-Pulido *et al.*, 2012), and on photosynthetic process of *Gracilariopsis*

lemaneiformis (Bory de Saint-Vicent) E.Y. Dawson, Acleto & Foldvik (Zou & Gao, 2014). Other studies reported the effects of CO_2 and nutrient availability on growth, pigment and protein contents, and internal C and N on thallus of *Gracilaria* sp. (Andria *et al.*, 2009), on cover of algal turfs (e.g., *Feldmania* spp and their dry mass ((Falkenberg *et al.*, 2012), on photosynthesis, respiration and growth of *Dictyota* sp. and calcification of *Halimeda opuntia* (Linnaeus) J.V. Lamouroux (Hofmann *et al.*, 2015). Effects of nutrient availability and temperature were evaluated on recruitment and juvenile survival of *Macrocystis pyrifera* (Linnaeus) C. Agardh (Hernández-Carmona *et al.*, 2001) as well as on photosynthetic rate and nutrient uptake capacity (Fan *et al.*, 2014).

Few studies have evaluated the synergism of CO_2, temperature and nutrient availability on seaweed physiology and biochemistry (Bender *et al.*, 2014). To address this gap in the knowledge, the effects of increasing CO_2 and temperature levels, as well as increased nitrogen concentrations were evaluated on the growth, photosynthesis, protein concentration, pigment content, and C, H, N contents on the red alga *Hypnea aspera* Kützing (Gigartinales). Species of *Hypnea* are economically important as raw material for carrageenan production, and this sulphated polysaccharide is widely used in the food and pharmaceutical industries (Neushul, 1990; Knutsen *et al.*, 1995; Rodrigues *et al.*, 2011).

Material and Methods

Unialgal cultures of Hypnea aspera

Tetrasporophytes of *H. aspera* were collected at Ilha das Couves (23° 25’ 03” S, 44° 51’ 58” W), Ubatuba municipality, Sao Paulo State, southeastern Brazil. Voucher specimens were deposited in the herbarium of Institute of Botany with accession numbers SP with accession number SP 400933. Unialgal cultures were obtained by vegetative propagation of

thallus segments of 1 cm in length. Culture medium was composed by sterilized seawater enriched with a quarter-strength of von Stosch's nutrient solution (VSES/4) following Oliveira *et al.* (1995), and modified with the reduction of 50% in vitamin concentrations as described by Yokoya (2000). Thallus propagation was performed in 500-mL Erlenmeyer flasks with 300 ml of culture medium, which was replaced every week. Seaweed propagation lasted three weeks, and cultures were incubated at salinity of 32, temperature of 23 ± 3 °C, under irradiances of 60 - 90 µmol photons m^{-2} s^{-1}, provided by cool-white fluorescent lamps, and light:dark cycle of 14:10h. Irradiance was measured with a quantum photometer (LI- 250, Li-COR, Lincoln, NE, USA) equipped with spherical underwater quantum sensor (LI- 250, Li-COR). All procedures were performed at the Laboratório de Cultura de Algas e Cianobactérias Marilza Cordeiro Marino, Instituto de Botanica, Sao Paulo municipality, Brazil.

Experimental design – CO2, nitrogen and temperature experiments

Apical segments of *H. aspera* were cultivated in 500-ml Erlenmeyer flasks, each one maintaining a ratio of 0.5 g of seaweed per 400 mL of culture medium. Treatments were composed of sterilized seawater enriched with VSES/4 modified (von Stosch´s solution prepared without nitrate, but with salts of phosphate, iron, manganese, EDTA, and three vitamins, as already described above). Nitrogen concentrations and nitrogen sources were added according to the treatment to be tested. Nitrate ($NaNO_3$) or ammonium (NH_4Cl) were added to the medium in three concentrations: (i) low (0 µM of nitrate or ammonium), (ii) intermediate (125 or 50 µM of nitrate or ammonium, respectively) and (iii) high (500 or 100 µM of nitrate or ammonium, respectively).

CO_2 and temperature levels were tested according to conditions predicted by the IPCC for the current scenario and the Model CMIP5 and RCP 8.5 predicted for 2100 (IPCC, 2013).

Three levels of CO_2 were tested: (i) low (without CO_2 addition, assuming the concentration of 0 ppm), (ii) intermediate (ambient air - 380 ppm of CO_2 pumped with an aquarium system (Boyu Electromagnetic Air Compressor ACQ-001), and (iii) high (1000 ppm of CO_2 pumped using a pure CO_2 cylinder (P. ONU1013 2.2 – Oxylumen). Specimens were cultivated in culture chambers (Electrolab, Brazil), and three temperatures were tested: (i) low, 21°C; (ii) intermediate, 25°C; and (iii) high, 30°C.

Based on these nitrogen concentrations, CO_2 levels and temperature, ANOVA Factorial Design (3^3) was performed to obtain a combination of factors to be tested (Table 1). Experiments were carried out at salinity of 32, irradiances of 80 - 90 μmol photons m^{-2} s^{-1}, provided by cool-white fluorescent lamps with a 14:10 h light:dark cycle. Each treatment was tested with three replicates (n=3) in which both CO_2 flux and temperature were controlled daily. Culture medium was replaced each week after determining the variation on fresh biomass (mg), photosynthesis, alkalinity, pH, temperature and salinity of seawater. At the end of the experimental period (14 days), culture medium was renewed, and 4 days thereafter (18^{nd} day), samples of each replicate were frozen with liquid nitrogen and stored at -20 °C for analysis of protein and pigment contents. This procedure was used to standardize the period between culture medium renewal and sample freezing since pigment and protein contents were influenced by this period.

Table 1: Factorial design of treatments performed for *Hypnea aspera* cultivated with different concentrations of nitrogen (nitrate or ammonium), CO_2 and temperature.

Treatments	Factors			
	Nitrate (µM)	**Ammonium (µM)**	**CO_2 (ppm)**	**Temperature (ºC)**
1	0	0	0	21
2	0	0	0	25
3	0	0	0	30
4	0	0	380	21
5	0	0	380	25
6	0	0	380	30
7	0	0	1000	21
8	0	0	1000	25
9	0	0	1000	30
10	125	50	0	21
11	125	50	0	25
12	125	50	0	30
13	125	50	380	21
14	125	50	380	25
15	125	50	380	30
16	125	50	1000	21
17	125	50	1000	25
18	125	50	1000	30
19	500	100	0	21
20	500	100	0	25
21	500	100	0	30
22	500	100	380	21
23	500	100	380	25
24	500	100	380	30
25	500	100	1000	21
26	500	100	1000	25
27	500	100	1000	30

Determination of carbonate chemistry parameters

Total alkalinity (T_A) of the seawater of each treatment (n=3) was measured when the medium was renewed, using the titration method according to Dickson & Millero (1987). Temperature and pH were measured using pH meter coupled with temperature sensor (Jenway 3020). Salinity was determined using a Refractometer (American Opticals, model 10440 T/C).

T_A, pH, salinity and temperature of seawater were used to calculate carbonate chemistry parameters (dissolved CO_2, bicarbonate – HCO_3^-, carbonate – CO_3^{2-} and dissolved inorganic carbon – DIC) (Tables 2 and 3), using the R program and Seacarb package (Dickson *et al*., 2007).

Growth rates

Fresh biomass was recorded weekly for 2 weeks, corresponding to replacement of medium. Growth rates (GR) were calculated from three replicates of each treatment and calculated as $[\ln (Bf \bullet Bo^{-1}) \bullet t^{-1}]$, where Bo is the initial fresh biomass, Bf is the fresh biomass after t days, and t corresponds to the experimental period (Yokoya *et al.*, 2003).

Pigment and protein extraction and quantification

The algal mass (80 mg of fresh biomass for each replicate, n=3) was ground to a powder with liquid nitrogen and mixed with 50 mM phosphate buffer (pH 5.5). The homogenates were centrifuged at 14,000 g for 20 min. at 4ºC in order to separate the phycobiliproteins present in the supernatant. Chlorophyll *a* (Chl *a*) was extracted after dissolving the pellet in 90% acetone and centrifuging at 12,000 *g* for 15 min. at 4ºC. Pigments were quantified by spectrophotometry (Shimadzu-UV 1800). Concentrations of phycobiliproteins (phycoerythrin - PE, phycocyanin - PC and allophycocyanin - APC) were calculated according to Kursar *et al.* (1983), and the concentration of Chl *a* was calculated according to Jeffrey and Humphrey (1975).

For total soluble protein analysis (PT), 80 mg of algal fresh biomass for each replicate (n=3) were ground with liquid nitrogen, and extractions were carried out at 4 °C using 0.2 M phosphate buffer (pH 8) containing 5 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM Dithiothreitol (DTT). Buffer was added in the proportion of 10 mL g^{-1} fresh biomass, and the

homogenates were centrifuged at 12,000 g for 15 min. Total soluble protein contents were determined according to Bradford (1976), using a Bio-Rad protein assay kit and BSA as standard.

Internal C, H and N contents

At the end of the experimental period, samples of each treatment (n=3) were oven dried (70°C for 72 h) for analyses of thallus contents of internal carbon (C), hydrogen (H) and nitrogen (N), as determined by the Pregl-Dumas method with Perkin-Elmer 2400 Series II equipment. These analyses were performed by the Central Analítica core facility at the Instituto de Química, Universidade de São Paulo.

Photosynthetic parameters

Measurements of chlorophyll *a* fluorescence were estimated using a pulse amplitude-modulated (PAM) fluorometer (Diving PAM underwater fluorometer, Walz, Effeltrich, Germany). For each treatment, five apical segments of each replicate (n=3) were placed on the tip of the fiber-optic fluorometer using the magnet sample holder. Photosynthesis x irradiance (PI) curves consisted of the fluorescence responses to eleven increasing irradiance levels within range of 0 - 1564 µmol photons m^{-2} s^{-1}, using the "light curve" option of the Diving PAM. Two parameters were determined for each sample: (i) Effective quantum yield - EQY (II) = ΔF/Fm, where ΔF = Fm′ - Ft, Fm′ is the maximum fluorescence and Ft is the steady state fluorescence; and (ii) relative electron transport rate (ETR) = EQY(II)*PAR*ETR-factor*0.5, where the ETR-factor used was 0.84, and 0.5 is the factor related to proportion of Chl *a* at photosystem II (PSII).

The following photosynthetic parameters were calculated by PI curves according to equation of Platt *et al.* (1980), using KaleidaGraph (Synergy Software): photosyntetic efficiency (α), saturation irradiance (Ik), maximal ETR (Pmax) and photoinhibition parameter (β).

Statistical analyses

Data were analyzed by One-Way and Factorial ANOVA, followed by the Student-Newman-Keuls multiple comparison test, in order to distinguish significantly different results ($p < 0.05$), using STATISTICA software (version 9). For multivariate analysis, the data of growth rates (GR), pigment contents (APC, PC, PE, and Chl *a*), protein contents (PT), tissue elements (C, H, N and C/N ratio), photosynthetic parameters (ETR, EQY (effective quantum yield), α, Ik and Pmax), calcification ($CaCO_3$) and carbonate system of seawater (T_A, pH, CO_2, CO_3^{2-}, HCO_3^- and DIC (dissolved inorganic carbon)) were used in a covariance matrix for principal component analyses (PCA) performed in a PC-ORD 6 software (MjM Software, USA). The variability of the data was adjusted by the method of ranging ($[X-X_{min})/(X_{max}-X_{min})]$) (Legendre & Legendre 1998), using the PC-ORD 6 software.

Table 2: Carbonate system of the seawater medium in which *Hypnea aspera* samples were cultured with different nitrate concentrations, CO_2 levels and temperature for 14 days. Data are the mean of three replicates ± standard deviation. Treatments with distinct letters indicate significant differences according to the one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

T (°C)	CO_2 (ppm)	$[NO_3^-]$ (µM)	pH	T_A (mM)	CO_2 (mM)	HCO_3^- (mM)	CO_3^{2-} (mM)	DIC (mM)
21°C	0	0	8.28 ± 0.56^{ab}	2.27 ± 0.00	0.01 ± 0.01^{c}	1.40 ± 0.62^{b}	0.43 ± 0.31^{c}	1.85 ± 0.32^{d}
		125	9.20 ± 0.56^{a}	2.27 ± 0.00	0.00 ± 0.00^{c}	0.51 ± 0.52^{cd}	0.88 ± 0.26^{ab}	1.39 ± 0.26^{d}
		500	8.73 ± 0.20^{ab}	2.27 ± 0.00	0.00 ± 0.00^{c}	0.90 ± 0.25^{bcd}	0.69 ± 0.13^{abc}	1.59 ± 0.13^{d}
	380	0	6.91 ± 0.06^{cd}	2.27 ± 0.00	0.16 ± 0.02^{bc}	2.22 ± 0.01^{a}	0.03 ± 0.00^{d}	2.41 ± 0.30^{cd}
		125	7.24 ± 0.24^{c}	2.27 ± 0.00	0.08 ± 0.04^{c}	2.15 ± 0.07^{a}	0.06 ± 0.03^{d}	2.29 ± 0.07^{cd}
		500	7.31 ± 0.25^{c}	2.27 ± 0.00	0.07 ± 0.04^{c}	2.13 ± 0.08^{a}	0.07 ± 0.04^{d}	2.27 ± 0.08^{cd}
	1000	0	7.84 ± 0.09^{bc}	2.27 ± 0.00	0.02 ± 0.00^{c}	1.89 ± 0.06^{a}	0.19 ± 0.03^{c}	2.10 ± 0.04^{cd}
		125	7.83 ± 0.03^{bc}	2.27 ± 0.00	0.02 ± 0.00^{c}	1.90 ± 0.02^{a}	0.19 ± 0.01^{c}	2.10 ± 0.01^{cd}
		500	7.76 ± 0.06 ^{bc}	2.27 ± 0.00	0.02 ± 0.00^{c}	1.95 ± 0.04^{a}	0.16 ± 0.02^{c}	2.13 ± 0.02^{cd}
25°C	0	0	8.63 ± 0.10^{ab}	2.27 ± 0.00	0.00 ± 0.00^{c}	0.82 ± 0.12^{bcd}	0.72 ± 0.06^{abc}	1.55 ± 0.06^{d}
		125	8.91 ± 0.23^{a}	2.27 ± 0.00	0.00 ± 0.00^{c}	0.54 ± 0.19^{cd}	0.87 ± 0.10^{ab}	1.41 ± 0.10^{d}
		500	9.30 ± 0.23^{a}	2.27 ± 0.00	0.00 ± 0.00^{c}	0.34 ± 0.13^{d}	0.97 ± 0.07^{a}	1.30 ± 0.07^{d}
	380	0	8.56 ± 0.18^{ab}	2.27 ± 0.00	0.00 ± 0.00^{c}	1.07 ± 0.23^{bc}	0.60 ± 0.11^{bc}	1.67 ± 0.12^{d}
		125	8.66 ± 0.41^{ab}	2.27 ± 0.00	0.00 ± 0.00^{c}	0.97 ± 0.48^{bc}	0.65 ± 0.24^{bc}	1.62 ± 0.24^{d}
		500	9.21 ± 0.44^{a}	2.27 ± 0.00	0.00 ± 0.00^{c}	0.45 ± 0.33^{cd}	0.91 ± 0.17^{ab}	1.36 ± 0.17^{d}
	1000	0	8.51 ± 0.04^{ab}	2.27 ± 0.00	0.00 ± 0.00^{c}	1.14 ± 0.05^{b}	0.57 ± 0.03^{c}	1.71 ± 0.03^{d}
		125	8.35 ± 0.31^{ab}	2.27 ± 0.00	0.00 ± 0.00^{c}	1.33 ± 0.37^{b}	0.47 ± 0.18^{c}	1.81 ± 0.19^{d}
		500	8.47 ± 0.30^{ab}	2.27 ± 0.00	0.00 ± 0.00^{c}	1.19 ± 0.37^{b}	0.54 ± 0.19^{c}	1.73 ± 0.19^{d}
30°C	0	0	6.17 ± 1.06^{def}	2.27 ± 0.00	2.55 ± 3.40^{abc}	2.24 ± 0.04^{a}	0.01 ± 0.02^{d}	4.81 ± 3.41^{ac}
		125	5.77 ± 0.25^{ef}	2.27 ± 0.00	2.44 ± 1.15^{ab}	2.27 ± 0.00^{a}	0.00 ± 0.00^{d}	4.71 ± 1.15^{abc}
		500	5.63 ± 0.63^{ef}	2.27 ± 0.00	4.86 ± 3.73^{a}	2.26 ± 0.00^{a}	0.00 ± 0.00^{d}	7.12 ± 3.73^{a}
	380	0	6.25 ± 0.37^{def}	2.27 ± 0.00	0.91 ± 0.58^{bc}	2.26 ± 0.01^{a}	0.01 ± 0.01^{d}	3.17 ± 0.59^{bc}
		125	5.65 ± 0.14^{f}	2.27 ± 0.00	2.99 ± 0.89^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{d}	5.26 ± 0.89^{ab}
		500	5.45 ± 0.18^{f}	2.27 ± 0.00	4.91 ± 1.75^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{d}	7.17 ± 1.75^{a}
	1000	0	7.39 ± 0.58^{c}	2.27 ± 0.00	0.10 ± 0.13^{c}	2.05 ± 0.16^{a}	0.11 ± 0.08^{d}	2.26 ± 0.21^{cd}
		125	6.53 ± 0.81^{def}	2.27 ± 0.00	0.81 ± 0.78^{bc}	2.21 ± 0.10^{a}	0.03 ± 0.05^{d}	3.05 ± 0.82^{bcd}
		500	5.71 ± 0.11^{ef}	2.27 ± 0.00	2.58 ± 0.61^{ab}	2.27 ± 0.00^{a}	0.00 ± 0.00^{d}	4.85 ± 0.61^{abc}

T_A = total alkalinity, CO_2 = dissolved CO_2, HCO_3^- = bicarbonate concentration, CO_3^{2-} = carbonate concentration and DIC = dissolved inorganic carbon.

Table 3: Carbonate system of the seawater medium in which *Hypnea aspera* samples were cultured with different ammonium concentrations, CO_2 levels and temperature for 14 days. Data are the mean of three replicates ± standard deviation. Treatments with distinct letters indicate significant differences according to the one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

T (°C)	CO_2 (ppm)	$[NH_4^+]$ (µM)	pH	T_A (µM)	CO_2 (mM)	HCO_3^- (mM)	CO_3^{2-} (mM)	DIC (mM)
21°C	0	0	8.58 ± 0.24^{b}	2.27 ± 0.00^{a}	0.00 ± 0.00^{g}	1.35 ± 0.29^{b}	0.46 ± 0.15^{b}	1.81 ± 0.15^{de}
		50	9.36 ± 0.06^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{g}	0.45 ± 0.05^{c}	0.91 ± 0.02^{a}	1.36 ± 0.02^{e}
		100	9.35 ± 0.09^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{g}	0.46 ± 0.08^{c}	0.90 ± 0.04^{a}	1.37 ± 0.04^{e}
	380	0	7.74 ± 0.03^{bc}	2.27 ± 0.00^{a}	0.03 ± 0.00^{f}	2.07 ± 0.01^{a}	0.10 ± 0.01^{c}	2.20 ± 0.01^{cde}
		50	7.95 ± 0.17^{b}	2.27 ± 0.00^{a}	0.05 ± 0.04^{defg}	2.03 ± 0.19^{a}	0.12 ± 0.09^{c}	2.19 ± 0.13^{cde}
		100	8.18 ± 0.48^{b}	2.27 ± 0.00^{a}	0.07 ± 0.04^{def}	2.13 ± 0.08^{a}	0.07 ± 0.04^{c}	2.27 ± 0.08^{cde}
	1000	0	5.25 ± 0.06^{e}	2.26 ± 0.00^{b}	0.02 ± 0.00^{g}	1.89 ± 0.06^{a}	0.19 ± 0.03^{c}	2.10 ± 0.04^{cde}
		50	5.38 ± 0.05^{e}	2.26 ± 0.00^{b}	0.02 ± 0.00^{g}	1.90 ± 0.02^{a}	0.19 ± 0.01^{c}	2.10 ± 0.01^{cde}
		100	5.33 ± 0.04^{e}	2.26 ± 0.00^{b}	0.02 ± 0.00^{g}	1.95 ± 0.04^{a}	0.16 ± 0.02^{c}	2.13 ± 0.02^{cde}
25°C	0	0	7.36 ± 0.05^{cd}	2.27 ± 0.00^{a}	0.07 ± 0.01^{e}	2.17 ± 0.01^{a}	0.05 ± 0.00^{c}	2.29 ± 0.01^{cde}
		50	7.43 ± 0.03^{cd}	2.27 ± 0.00^{a}	0.06 ± 0.00^{e}	2.16 ± 0.01^{a}	0.06 ± 0.00^{c}	2.27 ± 0.01^{cde}
		100	7.14 ± 0.12^{d}	2.27 ± 0.00^{a}	0.12 ± 0.04^{d}	2.21 ± 0.02^{a}	0.03 ± 0.01^{c}	2.36 ± 0.04^{cd}
	380	0	8.10 ± 0.02^{b}	2.27 ± 0.00^{a}	0.00 ± 0.00^{g}	1.07 ± 0.23^{b}	0.60 ± 0.11^{b}	1.67 ± 0.12^{e}
		50	8.27 ± 0.03^{b}	2.27 ± 0.00^{a}	0.00 ± 0.00^{g}	0.97 ± 0.48^{bc}	0.65 ± 0.24^{b}	1.62 ± 0.24^{e}
		100	8.17 ± 0.11^{b}	2.27 ± 0.00^{a}	0.00 ± 0.00^{g}	0.45 ± 0.33^{c}	0.91 ± 0.17^{a}	1.36 ± 0.17^{e}
	1000	0	5.77 ± 0.68^{e}	2.26 ± 0.00^{b}	0.00 ± 0.00^{g}	1.14 ± 0.05^{b}	0.57 ± 0.03^{b}	1.71 ± 0.03^{de}
		50	5.51 ± 0.14^{e}	2.26 ± 0.00^{b}	0.00 ± 0.00^{g}	1.33 ± 0.37^{b}	0.47 ± 0.18^{b}	1.81 ± 0.19^{de}
		100	5.37 ± 0.09^{e}	2.26 ± 0.00^{b}	0.00 ± 0.00^{g}	1.19 ± 0.37^{b}	0.54 ± 0,19^{b}	1.73 ± 0.19^{de}
30°C	0	0	7.01 ± 0.00^{d}	2.27 ± 0.00^{a}	0.15 ± 0.00^{d}	2.23 ± 0.00^{a}	0.02 ± 0.00^{c}	2.40 ± 0.00^{cd}
		50	6.93 ± 0.04^{d}	2.27 ± 0.00^{a}	0.18 ± 0.02^{d}	2.23 ± 0.00^{a}	0.02 ± 0.00^{c}	2.44 ± 0.02^{cd}
		100	6.92 ± 0.02^{d}	2.27 ± 0.00^{a}	0.19 ± 0.01^{d}	2.23 ± 0.00^{a}	0.02 ± 0.00^{c}	2.44 ± 0.01^{c}
	380	0	6.96 ± 0.45^{d}	2.27 ± 0.00^{a}	0.91 ± 0.58^{c}	2.26 ± 0.01^{a}	0.01 ± 0.01^{c}	3.17 ± 0.59^{c}
		50	7.14 ± 0.17^{d}	2.27 ± 0.00^{a}	2.99 ± 0.89^{b}	2.27 ± 0.00^{a}	0.00 ± 0.00^{c}	5.26 ± 0.89^{b}
		100	7.18 ± 0.34^{d}	2.27 ± 0.00^{a}	4.91 ± 1.75^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{c}	7.17 ± 1.75^{a}
	1000	0	5.43 ± 0.05^{e}	2.26 ± 0.00^{b}	0.10 ± 0.13^{defg}	2.05 ± 0.16^{a}	0.11 ± 0.08^{c}	2.26 ± 0.21^{cde}
		50	5.70 ± 0.62^{e}	2.26 ± 0.00^{b}	0.81 ± 0.13^{c}	2.21 ± 0.10^{a}	0.03 ± 0.05^{c}	3.05 ± 0.82^{cd}
		100	5.66 ± 0.22^{e}	2.26 ± 0.00^{b}	2.58 ± 0.61^{b}	2.27 ± 0.00^{a}	0.00 ± 0.00^{c}	4.85 ± 0.61^{b}

T_A = total alkalinity, CO_2 = dissolved CO_2, HCO_3^- = bicarbonate concentration, CO_3^{2-} = carbonate concentration and DIC = dissolved inorganic carbon.

Results

Tables 1 and 2 showed the variation of carbonate system in seawater due the increase temperature, CO_2 levels and nitrogen concentration. In nitrate tests, the pH and CO_3^{2-} decreased, while HCO_3^- and DIC increased mainly in high temperature, could be worsened by the increased CO_2 (Table 1). In contrast, in tests with ammonium availability, the CO_2 and CO_3^{2-} availabilities in seawater, pH and T_A, decreased and HCO_3^- increased mainly with high CO_2 (Table 2).

Treatments with high CO_2 and 30°C that no show indicate that no fresh biomass could be obtained for the analyses because the alga died. The highest CO_2 level (1000 ppm) and temperature (30°C) had negative effects on the growth rates of *Hypnea aspera*, independently of the nitrate or ammonium addition (Fig. 1a, b), while the highest growth rates were observed in treatments with the addition of nitrate at 500 µM in 380 ppm of CO_2 at 21 and 25°C (Fig. 1a). High growth rates occurred in *H. aspera* cultured with the addition of ammonium at 100 µM in 380 ppm of CO_2 at 21°C (Fig. 1b).

Treatments with high CO_2 and temperature caused a decrease in *H. aspera* biomass, preventing the extraction of proteins (Fig. 2a, b). *H. aspera* showed high concentrations of total soluble proteins (PT) when cultured with the addition of 500 µM nitrate in 380 ppm of CO_2 at 25°C (Fig. 2a), and with the addition of 100 µM ammonium, but without CO_2 addition, at 25°C (Fig. 2b). Low values for PT content were observed in *H. aspera* cultured without nitrate addition, and in 0 and 380 ppm of CO_2 at 21 and 25°C (Fig. 2a), while low values for PT content were observed for 0 µM ammonium and 0 ppm of CO_2 at 25°C (Fig. 2b).

Phycobiliprotein contents in *H. aspera* did not vary with nitrate concentrations, CO_2 levels or temperatures tested (Fig. 3a, c). Treatments with a) 125 µM of nitrate in 380 ppm of CO_2 at 21 and 25°C and b) 500 µM of nitrate in 380 ppm of CO_2 at 25°C stimulated high Chl *a* content (Fig. 3d). Low Chl *a* content occurred in treatments without nitrate additions (0 µM)

in 0 and 380 ppm of CO_2 at 21 and 25 °C (Fig. 3d). On the other hand, the content of phycobiliproteins varied with the addition of ammonium (Fig. 4a,c). Specifically, allophycocyanin (APC) (Fig. 4a) and Chl *a* (Fig. 4d) were high upon addition of 100 µM ammonium, without CO_2 (0 ppm), at 25°C. Phycocyanin content (PC) was high for the same values of ammonium and CO_2 (Fig. 4b) as those noted for APC, but at 21°C. Phycoerythrin (PE) was higher, again for the same values of ammonium and CO_2 (Fig. 4c) as those noted for APC, but at both 21 and 25°C.

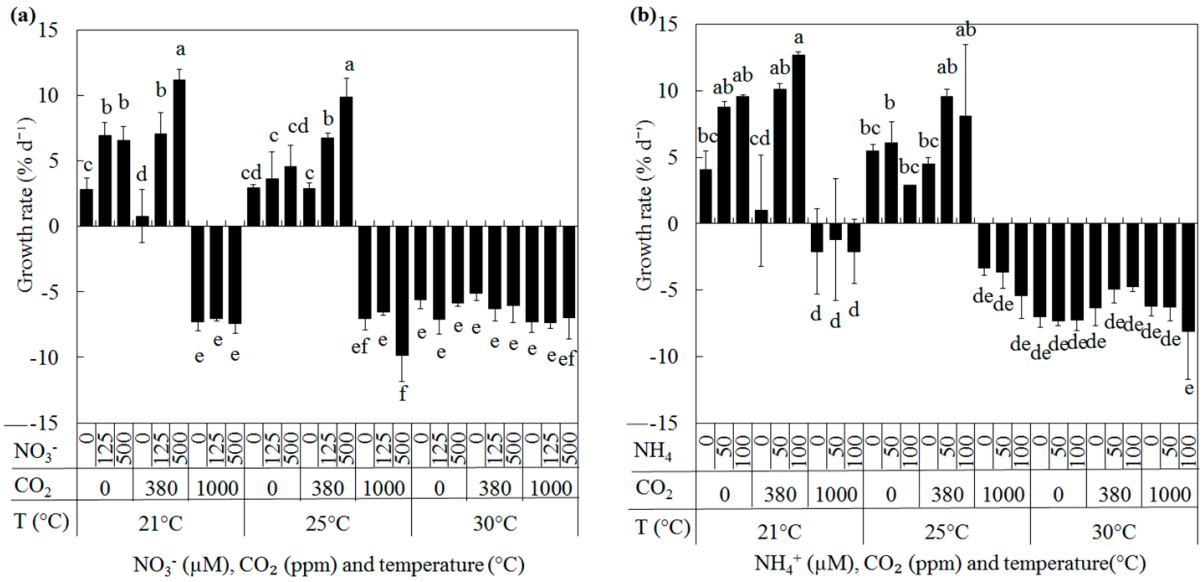

Fig. 1: Growth rate (% d^{-1}) of *Hypnea aspera* cultivated for 14 days in VSES/4 modified enriched with different (a) nitrate (NO_3^-) and (b) ammonium (NH_4^+) concentrations, different CO_2 levels and temperatures, photoperiod of 14 h, salinity of 32 and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to the one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

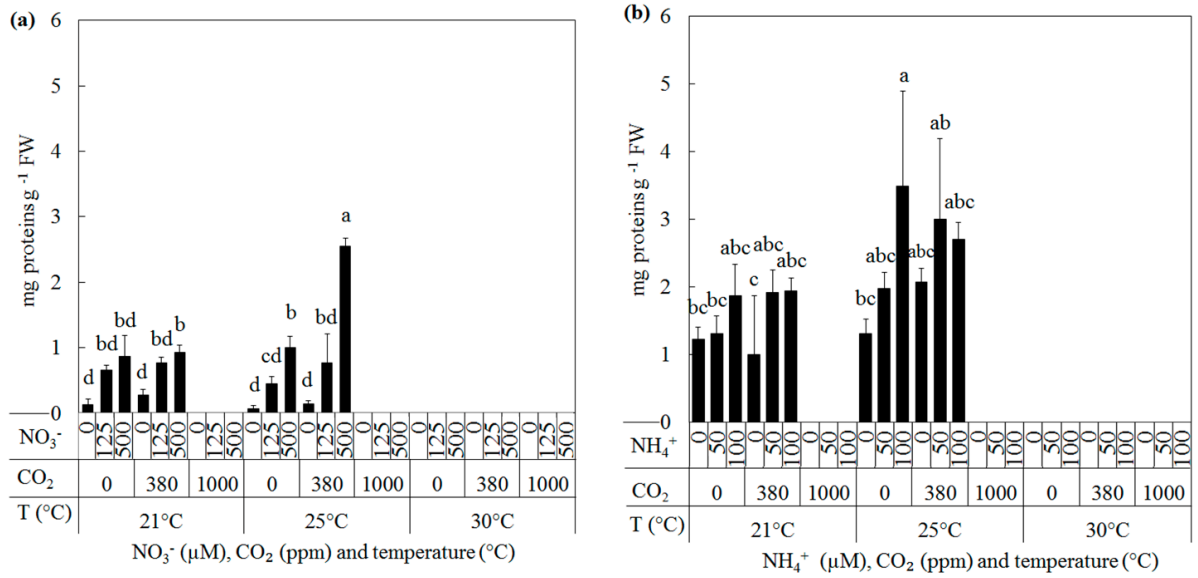

Fig. 2: Concentration of total soluble protein of *Hypnea aspera* cultivated for 18 days in VSES/4 modified enriched with different (a) nitrate (NO_3^-) and (b) ammonium (NH_4^+) concentrations, different CO_2 levels and temperatures, photoperiod of 14 h, salinity of 32 and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to the one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

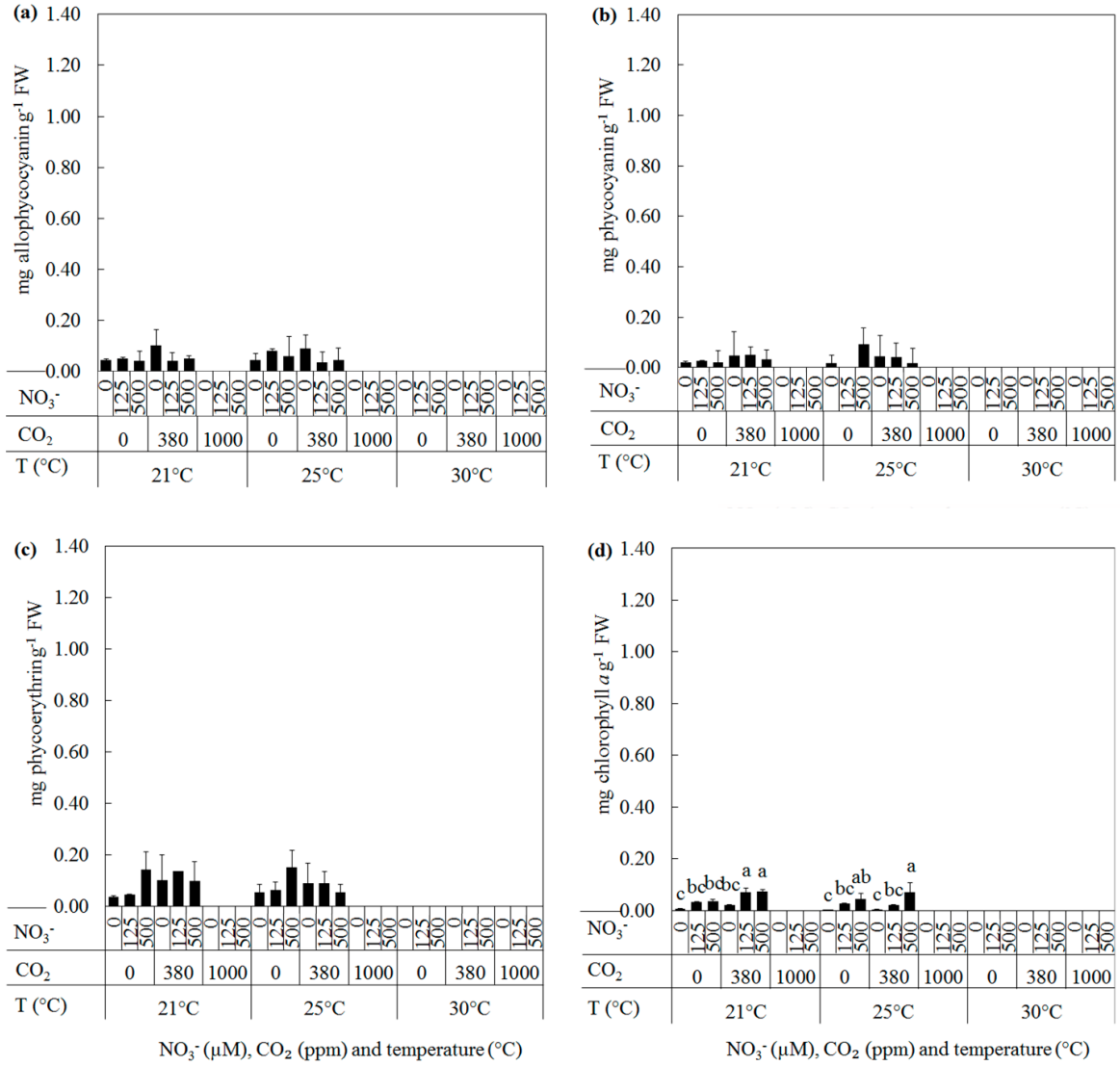

Fig. 3: Concentration of (a) allophycocyanin, (b) phycocyanin, (c) phycoerythrin and (d) chlorophyll *a* of *Hypnea aspera* cultivated for 18 days in VSES/4 modified enriched with different nitrate (NO_3^-) concentrations, CO_2 levels and temperatures, photoperiod of 14 h, salinity of 32 and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to the one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

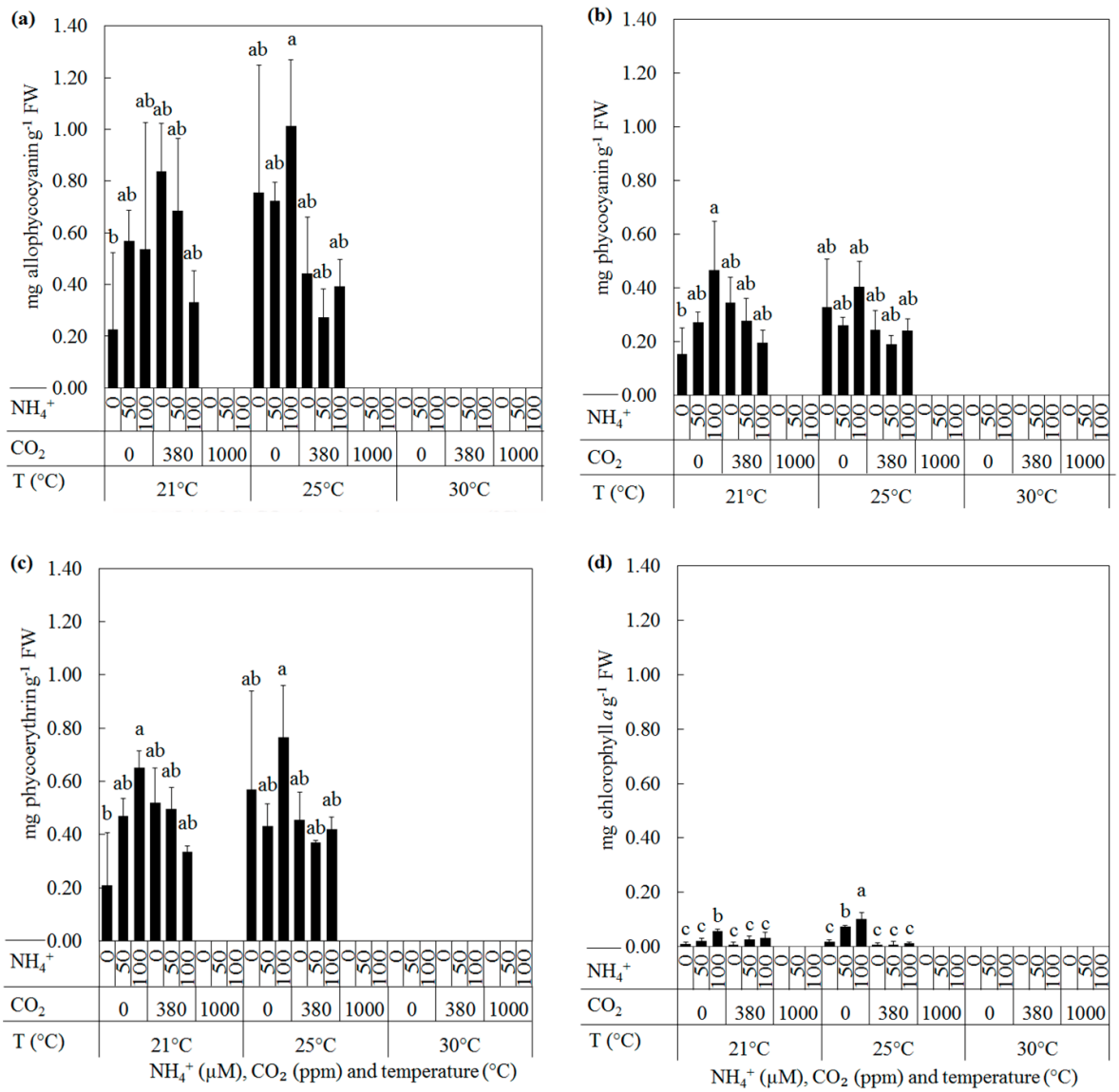

Fig. 4: Concentration of (a) allophycocyanin, (b) phycocyanin, (c) phycoerythrin and (d) chlorophyll *a* of *Hypnea aspera* cultivated for 18 days in VSES/4 modified enriched with different ammonium (NH_4^+) concentrations, CO_2 levels and temperatures, photoperiod of 14 h, salinity of 32 and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to the one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

The C, H, N contents and C/N ratio observed in *H. aspera* thallus cultured with different nitrate availability, CO_2 levels and temperatures were presented in Fig. 5a,d. C content was higher in algae cultured with 0 µM nitrate in 0 and 380 ppm of CO_2 at 25°C, as well as with 125 µM nitrate in 0 ppm of CO_2 at 25°C (Fig. 5a), while low C content was observed in treatments with 125 and 500 µM nitrate in 0 and 380 ppm of CO_2, respectively, at 30°C. H content was higher when *H. aspera* was cultivated with 125 µM nitrate in 1000 ppm of CO_2 at 25°C, but lower when samples were cultivated with 500 µM nitrate in 380 ppm of CO_2 at 21°C (Fig. 5b). N content increased linearly with nitrate additions and was high in 500 µM of nitrate, without CO_2 addition, at 21 and 25°C, or with the addition of 380 ppm of CO_2 at 25°C (Fig. 5c). High C/N ratio was observed in *H. aspera* cultured without nitrate addition in 380 ppm of CO_2 at 25°C, whereas low C/N ratio occurred with high nitrate addition (500 µM), without CO_2, at 21 and 25°C, as well as 380 ppm of CO_2 at 25°C (Fig. 5d).

When *H. aspera* was cultivated with the addition of ammonium, different CO_2 levels and different temperatures, high C and H content could be observed in 100 µM of ammonium and 1000 ppm of CO_2 at of 21°C (Fig. 6a, b). Low C and H contents were observed with 50 µM of ammonium, without CO_2, at 21°C. However, N content was stimulated with high ammonium concentration (100 µM), without CO_2 addition, at 25°C (Fig. 6c). The lowest N content occurred in algae cultured without ammonium and CO_2 addition at 21°C. C/N ratio was high without ammonium concentrations, in both 0 and 380 ppm of CO_2 at 21 and 25 °C, respectively. The species showed decreased C/N ratio with 100 µM of ammonium in 0 and 380 ppm of CO_2 at 21 and 25 °C, respectively (Fig. 6d).

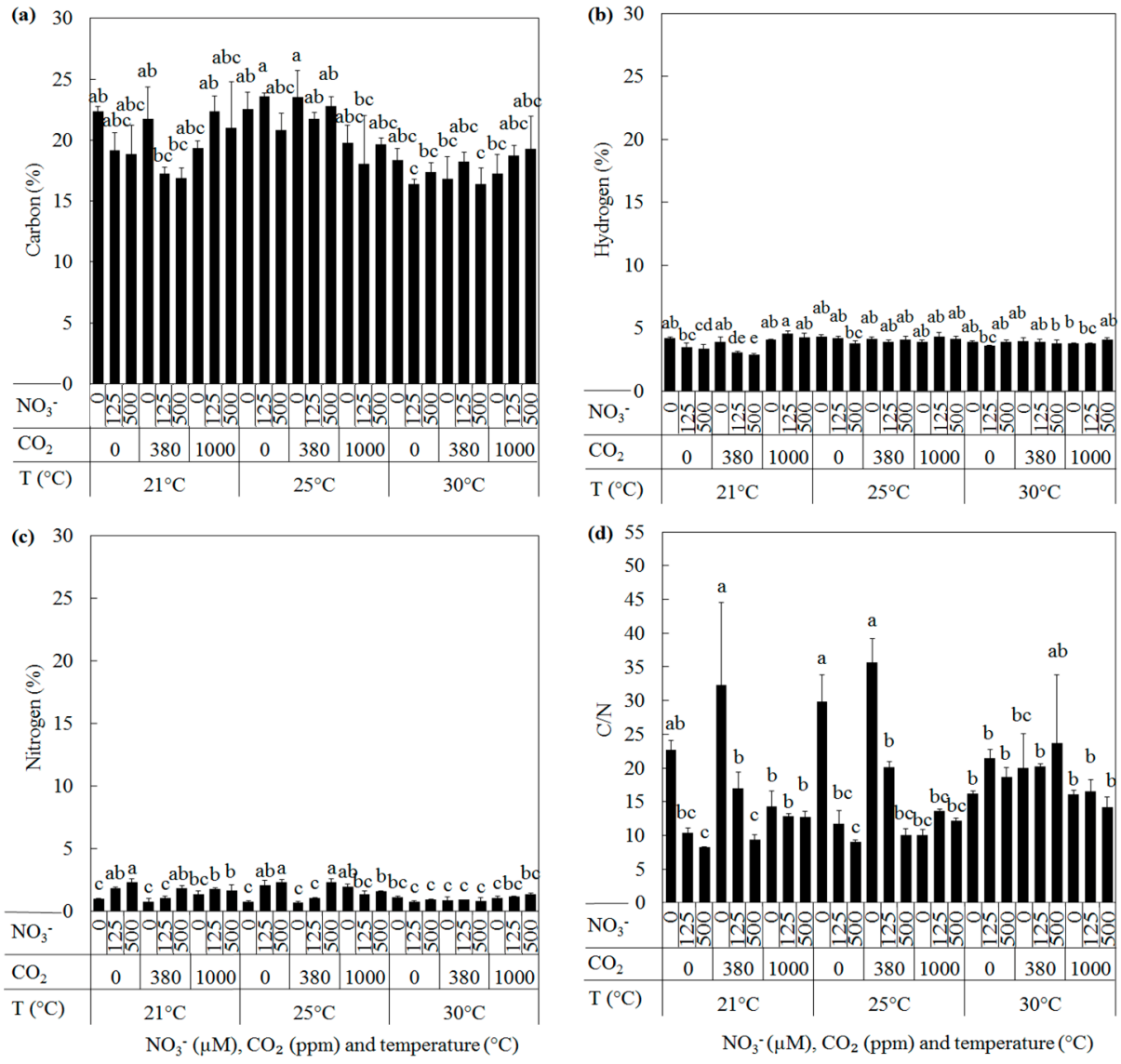

Fig. 5: Concentration of internal carbon (a), hydrogen (b), nitrogen (c), and C/N ratio of *Hypnea aspera* cultivated for 18 days in VSES/4 modified enriched with different nitrate (NO_3^-) concentrations, CO_2 levels and temperatures, photoperiod of 14 h, salinity of 32 and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to the one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

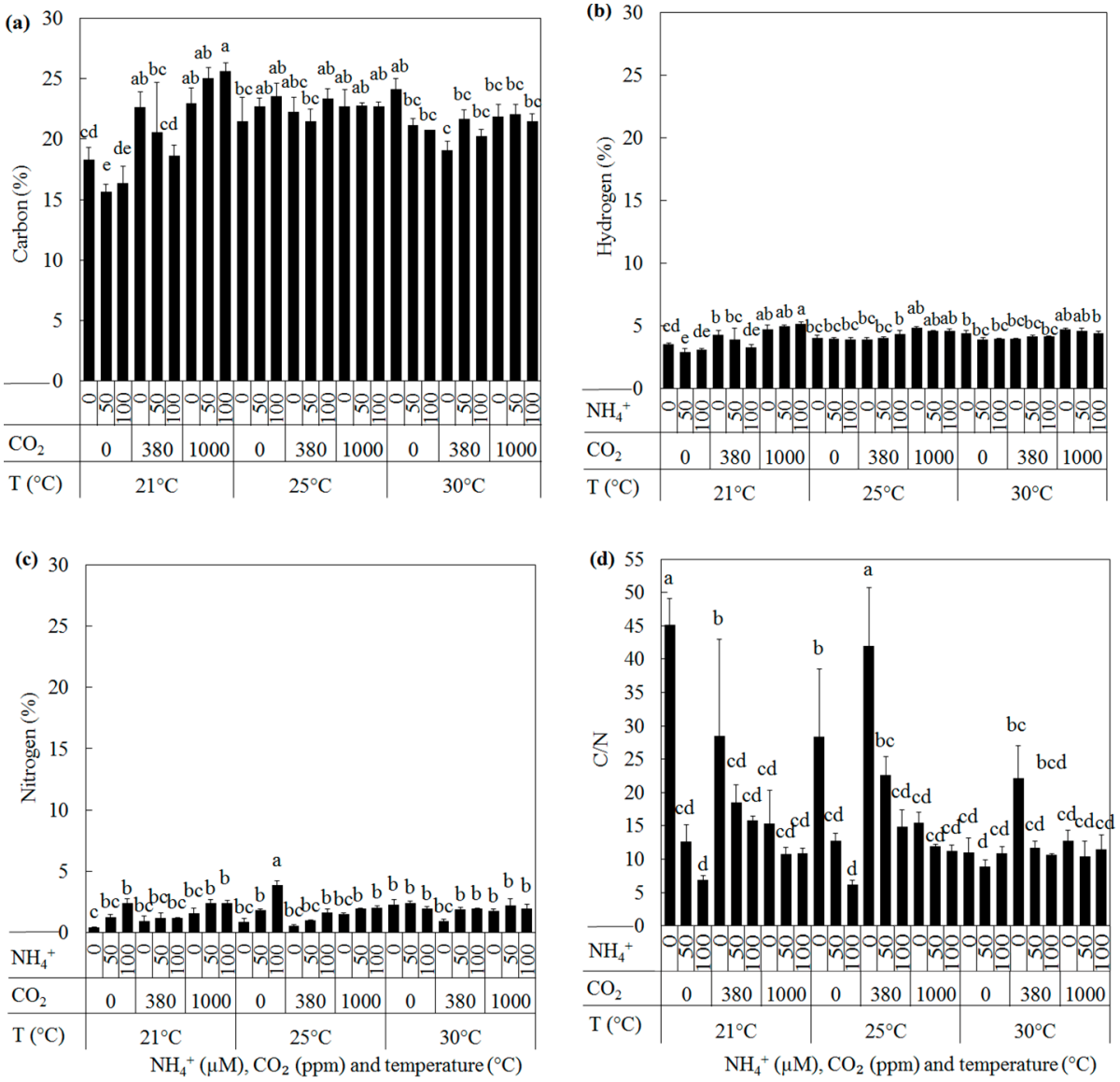

Fig. 6: Concentration of internal carbon (a), hydrogen (b), nitrogen (c) and C/N ratio (d) of *Hypnea aspera* cultivated for 18 days in VSES/4 modified enriched with different ammonium (NH_4^+) concentrations, CO_2 levels and temperatures, photoperiod of 14 h, salinity of 32 and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to the one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

The differences observed in photosynthetic responses of *H. aspera* to increased nitrogen, CO_2 and temperature are presented in PI curves shown in Figure 7 a, f. PI curves were influenced by nitrate concentrations, CO_2 and temperatures (*F= 361.79* and *p= 0.00*) and by irradiance (*F= 245.14* and *p= 0.00*). When cultured with nitrate, *H. aspera* showed high ETR values at a temperature of 21°C and in all CO_2 levels and nitrate concentrations (Fig. 7 a, c). A higher ETR value was observed when *H. aspera* was cultivated without nitrate in 380 ppm of CO_2 at 25°C and in 174 µmol photons m^{-2} s^{-1}. With increased temperature, the ETR values decreased, but they were higher in treatments without CO_2, independent of nitrate concentrations. Increased CO_2 levels were lethal. At all temperatures, lower ETR values occurred in treatments with high CO_2 (Fig. 7a, b, c). Moreover, PI curves of *H. aspera* showed saturation with increased irradiance, and higher Ik occurred at 21°C without the addition of nitrate or CO_2 (Table 4). The highest Pmax, α and β values were observed in treatments without nitrate and CO_2 at 25°C, and high EQY occurred at the same temperature, but in 380 ppm of CO_2 without nitrate (Table 4).

PI curves showed the influence of ammonium, CO_2 concentrations, different temperatures (*F= 121.16* and *p=0.00*) and increase of irradiance (*F= 225.09* and *p= 0.00*) in the photosynthetic responses of *H. aspera* (Fig. 7d, e, f). A high ETR value was observed with the addition of 50 µM of ammonium, without CO_2, at 21°C and 130 µmol photons m^{-2} s^{-1}, but a low ETR value occurred for samples cultivated without ammonium concentration in 1000 ppm of CO_2 at 21°C and 130 µmol photons m^{-2} s^{-1}. Treatments with high CO_2, all ammonium concentrations, and temperatures of 25 and 30°C were lethal for *H. aspera* and showed no values for photosynthesis. Table 5 shows the photosynthetic parameters obtained from PI curves. EQY was highest in all ammonium concentrations, without CO_2, at 21°C and also without ammonium in 380 ppm of CO_2 at 25°C. Low EQY and Pmax values occurred in all

ammonium treatments with 1000 ppm of CO_2 at 21°C. Addition of 50 µM ammonium without CO_2 at 21°C showed high Pmax values. The highest α was observed with 100 µM ammonium without CO_2 at 21°C and without ammonium and CO_2 at 30°C, but the lowest value occurred with 50 µM ammonium in 1000 ppm of CO_2 at 21°C. Ik was higher without ammonium, but with 380 ppm of CO_2 at 21°C, while the lower values occurred in treatments with 50 and 100 µM ammonium in 1000 ppm of CO_2 at 21°C. High β was observed in 50 µM of ammonium in 1000 ppm of CO_2 at 21°C.

Treatments with high CO_2 levels and all nitrate availabilities were lethal for *H. aspera*, therefore, ETR values were not showed (Fig. 7a, b, c) and photosynthetic parameters (Table 4), and high CO_2 and all ammonium concentrations at 25° and 30°C were also lethal for the species. In contrast, *H. aspera* cultured with ammonium addition at 21°C, showed ETR and photosynthetic parameters values (Fig. 7d, e, f, and Table 5).

The principal components analysis (PCA) showed the explicability of the variability of 37.64% for axis 1, 20.09% for axis 2, and total variability of 57.74%, in two axis (Fig. 8). The positive side of axis 1 grouped treatments with low and intermediate CO_2 concentrations and temperatures independent of nitrate or ammonium concentrations with GR, PT, all pigments, Pmax, Ik, pH, T_A and CO_3^{2-} of seawater. The negative side of axis 1 grouped treatments with high CO_2 and temperature with CO_2, HCO_3^- and DIC of seawater. The positive side of axis 2 associated the concentration of ammonium with APC. The negative side did not group with any variables (Table 6).

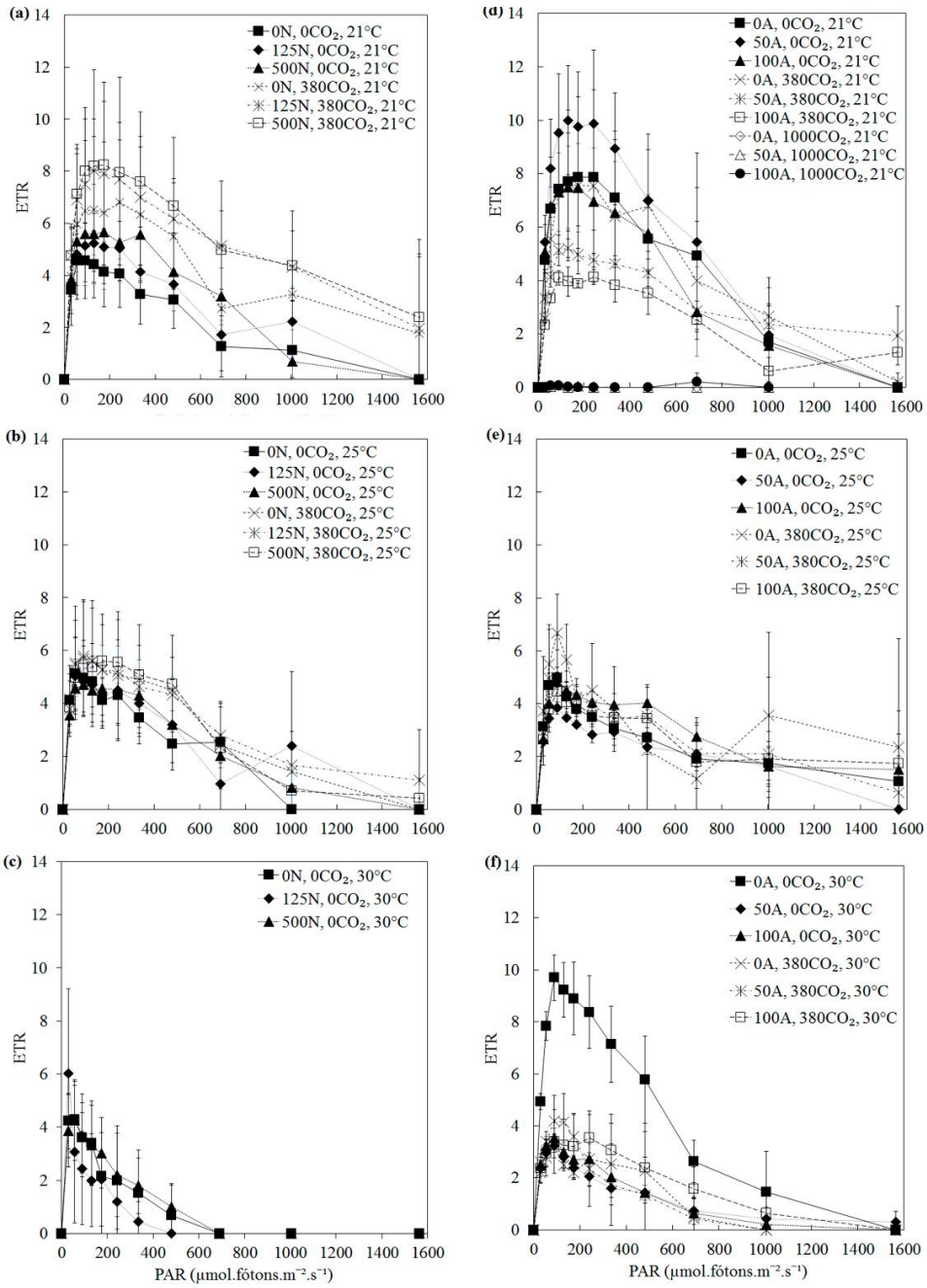

Fig. 7: Photosynthesis X irradiance curves of *Hypnea aspera* cultivated for 14 days in VSES/4 modified enriched with different nitrate (NO_3^-) (a, b and c) and ammonium (NH_4^+) (d, e and f) concentrations, CO_2 levels and temperatures, photoperiod of 14 h, salinity of 32 and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation.

Table 4: Photosynthetic parameters (EQY (effective quantum yield); Pmax, α (photosynthetic efficiency); Ik (saturation irradiance); and β (photoinhibition)) of *Hypnea aspera* cultivated for 14 days in VSES/4 modified and enriched with different nitrate (NO_3^-) concentrations, CO_2 levels and temperatures (T), photoperiod of 14 h, salinity of 32 and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to the factoral ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$). - = algae died; treatments with algal fresh biomass below the was obtained.

TREATMENTS			PHOTOSYNTHETIC PARAMETERS				
T (°C)	CO_2 (ppm)	NO_3^- (µM)	EQY	Pmax	α	Ik	β
21°	0	0	0.13 ± 0.03^{ab}	8.53 ± 3.38^{ab}	0.15 ± 0.10^{b}	75.96 ± 50.69^{a}	0.00 ± 0.00^{b}
		125	0.12 ± 0.03^{ab}	3.50 ± 0.64^{ab}	0.10 ± 0.07^{b}	42.21 ±16.84^{ab}	0.04 ±0.03^{ab}
		500	0.10 ± 0.01^{ab}	4.42 ± 1.94^{ab}	0.16 ± 0.02^{b}	29.65 ±15.48^{ab}	0.03 ± 0.03^{b}
	380	0	0.15 ± 0.05^{a}	7.62 ± 3.91^{ab}	0.21 ± 0.02^{b}	35.71 ± 8.23^{ab}	0.00 ± 0.00^{b}
		125	0.13 ± 0.00^{ab}	7.33 ± 0.32^{ab}	0.15 ± 0.02^{b}	49.21 ± 9.79^{ab}	0.02 ± 0.01^{b}
		500	0.11 ± 0.02^{ab}	5.85 ± 0.65^{ab}	0.10 ± 0.01^{b}	57.20 ± 0.51^{ab}	0.02 ± 0.00^{b}
	1000	0	-	-	-	-	-
		125	-	-	-	-	-
		500	-	-	-	-	-
25°	0	0	0.09 ± 0.02^{bc}	9.86 ± 5.85^{a}	3.66 ± 2.94^{a}	5.46 ± 5.18^{b}	0.15 ± 0.12^{a}
		125	0.11 ± 0.01^{ab}	8.06 ± 4.17^{ab}	1.96 ± 3.09^{ab}	22.70 ± 17.66^{b}	0.13 ±0.11^{ab}
		500	0.08 ± 0.03^{bc}	3.00 ± 0.32^{ab}	0.10 ± 0.03^{b}	30.77 ± 9.65^{ab}	0.00 ± 0.00^{b}
	380	0	0.15 ± 0.02^{a}	3.15 ± 1.50^{ab}	0.06 ± 0.04^{b}	54.96 ± 6.74^{ab}	0.06 ±0.00^{ab}
		125	0.11 ± 0.00^{ab}	3.90 ± 1.51^{ab}	0.11 ± 0.07^{b}	41.03 ± 9.93^{ab}	0.04 ± 0.03^{b}
		500	0.09 ± 0.01^{bc}	4.94 ± 1.00^{ab}	0.10 ± 0.03^{b}	52.95 ±25.27^{ab}	0.01 ± 0.00^{b}
	1000	0	-	-	-	-	-
		125	-	-	-	-	-
		500	-	-	-	-	-
30°	0	0	0.09 ± 0.03^{bc}	3.61 ± 1.31^{ab}	0.20 ± 0.05^{b}	18.34 ± 5.73^{b}	0.01 ± 0.00^{b}
		125	0.05 ± 0.00^{c}	1.67 ± 0.15^{b}	0.17 ± 0.05^{b}	10.13 ± 2.55^{b}	0.01 ± 0.00^{b}
		500	0.04 ± 0.01^{c}	1.40 ± 0.20^{b}	0.16 ± 0.05^{b}	9.65 ± 3.99^{b}	0.01 ± 0.00^{b}
	380	0	-	-	-	-	-
		125	-	-	-	-	-
		500	-	-	-	-	-
	1000	0	-	-	-	-	-
		125	-	-	-	-	-
		500	-	-	-	-	-

Table 5: Photosynthetic parameters (EQY (effective quantum yield); Pmax; α (photosynthetic efficiency); Ik (saturation irradiance); and β (photoinhibition)) of *Hypnea aspera* cultivated for 14 days in VSES/4 modified and enriched with different ammonium (NH_4^+) concentrations, CO_2 levels and temperatures (T), photoperiod of 14 h, salinity of 32 and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to the factoral ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$). - = algae died; treatments for which no fresh biomass was obtained.

TREATMENTS			PHOTOSYNTHETIC PARAMETERS				
T (°C)	CO_2 (ppm)	NH_4^+ (µM)	EQY	Pmax	α	Ik	β
21°	0	0	0.14 ± 0.03^{a}	6.16 ±1.89^{abc}	0.19 ±0.06^{ab}	37.08±19.46^{ab}	0.02 ± 0.01^{c}
		50	0.17 ± 0.03^{a}	10.53 ± 2.39^{a}	0.22 ±0.05^{ab}	47.41±4.75^{ab}	0.03 ± 0.01^{c}
		100	0.15 ± 0.01^{a}	8.01 ± 1.61^{abc}	0.23 ±0.04^{a}	35.63±12.07^{ab}	0.01 ± 0.00^{c}
	380	0	0.12 ± 0.02^{b}	7.97 ± 3.31^{abc}	0.15 ±0.02^{ab}	51.19±13.61^{a}	0.02 ± 0.01^{c}
		50	0.08 ± 0.02^{bc}	5.14 ±0.33^{abc}	0.13 ±0.02^{ab}	38.95±2.77^{ab}	0.00 ± 0.00^{bc}
		100	0.07 ± 0.02^{bc}	4.28 ± 0.35^{c}	0.10 ±0.00^{bc}	43.15±3.61^{ab}	0.00 ± 0.00^{bc}
	1000	0	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}	0.00 ±0.00^{cd}	0.42 ± 0.36^{bc}	0.75 ±1.29^{abc}
		50	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}	0.00 ±0.01^{d}	0.43 ± 0.23^{c}	1.58 ± 1.36^{a}
		100	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}	0.00 ±0.00^{cd}	0.30 ± 0.28^{c}	1.42 ± 1.22^{ab}
25°	0	0	0.09 ± 0.01^{bc}	4.47 ± 0.30^{bc}	0.21 ±0.01^{ab}	21.48±0.78^{abc}	0.00 ± 0.00^{bc}
		50	0.07 ± 0.00^{bc}	3.60 ± 0.17^{c}	0.15 ±0.01^{ab}	23.90±1.27^{abc}	0.05 ± 0.01^{bc}
		100	0.08 ± 0.00^{bc}	4.61 ± 0.21^{bc}	0.14 ±0.03^{ab}	33.01±4.89^{ab}	0.00 ± 0.00^{bc}
	380	0	0.10 ± 0.01^{bc}	5.15 ±0.28^{bc}	0.20 ±0.10^{ab}	30.13±14.05^{ab}	0.01 ± 0.02^{c}
		50	0.08 ± 0.02^{bc}	4.75 ± 0.61^{bc}	0.17 ± 0.07^{ab}	31.14±8.15^{ab}	0.00 ± 0.00^{c}
		100	0.08 ± 0.03^{bc}	4.12 ± 0.65^{c}	0.15 ± 0.04^{ab}	28.39±6.92^{ab}	0.00 ± 0.00^{bc}
	1000	0	-	-	-	-	-
		50	-	-	-	-	-
		100	-	-	-	-	-
30°	0	0	0.15 ± 0.00^{a}	9.67 ± 1.10^{ab}	0.23 ± 0.03^{a}	41.58±7.15^{ab}	0.03 ± 0.00^{c}
		50	0.06 ± 0.01^{c}	3.02 ± 0.47^{cd}	0.15 ±0.02^{ab}	20.55±0.95^{bc}	0.00 ± 0.00^{c}
		100	0.07 ± 0.00^{bc}	3.40 ± 0.37^{c}	0.14 ±0.02^{ab}	25.08±2.06^{abc}	0.01 ± 0.00^{c}
	380	0	0.06 ± 0.02^{bc}	3.01 ± 0.24^{d}	0.11 ± .06^{ab}	21.45±0.05^{bc}	0.02 ± 0.01^{c}
		50	0.07 ± 0.00^{bc}	2.90 ± 1.64^{cd}	0.15 ±0.00^{cd}	19.96±11.36^{bc}	0.07 ± 0.11^{c}
		100	0.07 ± 0.01^{bc}	3.70 ±1.06^{cd}	0.09 ± 0.04^{b}	45.16±22.83^{ab}	0.01 ± 0.00^{c}
	1000	0	-	-	-	-	-
		50	-	-	-	-	-
		100	-	-	-	-	-

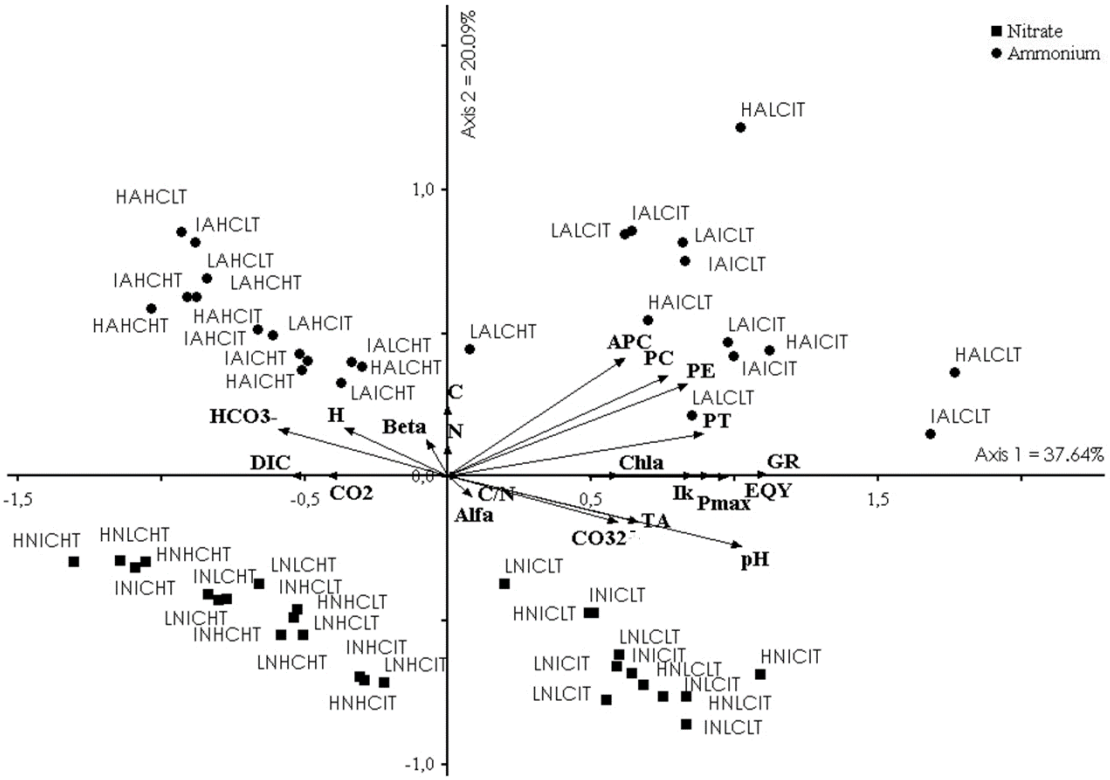

Fig. 8: Scatter diagram of plots of the first two principal component analysis axes of data on the effects of nitrate or ammonium, CO_2 levels and temperatures on growth rates (GR), pigment contents (APC, PC, PE and Chl *a*), protein contents (PT), tissue elements (C, H, N and C/N ratio), photosynthetic parameters (Pmax, alfa, beta and Ik) and carbonate system of seawater (pH, T_A, CO_2, HCO_3^-, CO_3^{2-} and DIC) in *Hypnea aspera* cultured in VSES/4 modified and enriched with different nitrate (black square) or ammonium (black circle) concentrations, photoperiod of 14 h, salinity of 32 and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1} . The first two components accounted for 57.74 % of total variance. LA – low ammonium, IA – intermediate ammonium, HA- high ammonium, LN- low nitrate, IN- intermediate nitrate, HN – high nitrate, LC – low CO_2, IC – intermediate CO_2, HC- high CO_2, LT- low temperature, IT- intermediate temperature and HT - high temperature.

Table 6: Pearson correlation coefficient among variables analyzed for *Hypnea aspera* to evaluate the effects of increased temperature, CO_2 levels and nitrogen availability (nitrate and ammonium) on growth rates, pigment, protein contents, calcification (%), element contents in the thallus (C, H, N, P and C/N ratio), photosynthetic parameters and carbonate system of seawater. The scatterplot diagram of PCA is shown in Fig. 8.

Variables	Principal components	
	Axis 1	Axis 2
Growth rate (GR)	0.861	0.060
Total soluble protein (PT)	0.771	0.311
Allophycocyanin (APC)	0.643	0.521
Phycocyanin (PC)	0.713	0.448
Phycoerythrin (PE)	0.746	0.461
Chlorophyll *a* (Chl *a*)	0.632	0.036
Carbon (C)	0.042	0.404
Hydrogen (H)	-0.490	0.331
Nitrogen (N)	0.039	0.273
C/N ratio (C/N)	0.166	-0.047
Effective quantum yield (EQY)	0.807	-0.062
Maximal ETR (Pmax)	0.782	-0.003
Photosynthetic efficiency (α)	0.240	-0.221
Saturation irradiance (Ik)	0.755	-0.034
Photoinhibition (β)	-0.228	0.292
pH	0.826	-0.406
Total alkalinity (T_A)	0.665	-0.329
CO_2	-0.530	-0.038
Bicarbonate (HCO_3^-)	-0.627	0.329
Carbonate (CO_3^{2-})	0.627	-0.328
Dissolved inorganic carbon (DIC)	-0,604	-0.328
% of variance	37.64	20.09

Discussion

The results of the present study demonstrate that *Hypnea aspera* is highly sensitive to the increased CO_2 and temperature at the scenario proposed by CMIP5 and RCP 8.5

predicted for 2100 (IPCC, 2013). High CO_2 levels decrease pH which, in turn, causes the acidification of seawater in the context of the higher temperatures that result from global warming (Tables 2, 3). Growth, protein concentration, pigment contents, and photosynthesis were all inhibited by high CO_2 and temperature. On the other hand, the availability of either nitrate or ammonium stimulated these factors when cultivated without CO_2 or in ambient CO_2 level (380 ppm) and at temperatures of 21 and 25°C. However, an inverse relationship among these variables was also demonstrated.

The negative effects of increased CO_2 on growth of *Hypnea aspera* were observed in the present study, and similar results were reported in the other seaweed species. For example, *Hypnea musciformis* (Wulfen) J.V. Lamouroux showed negative growth and *Hypnea cornuta* (Kützing) J. Agardh grew only slightly with addition of 750 ppm of CO_2 (Israel & Hophy, 2002). Growth inhibition was observed in the brown alga *Fucus visiculosus* Linnaeus when cultivated with 1615 ppm of CO_2 (Gutow *et al.*, 2014), and *Saccharina latissimi* (Linnaeus) C.E. Lane, C. Mayes, Druehl, & G.W. Saunders when cultivated with 3000 ppm of CO_2 (Swanson & Fox, 2007). The growth of *Chnoospora implexa* J. Agardh decreased when cultivated in conditions predicted by RCP 8.5 for 2100 (Bender *et al.*, 2014). In contrast, the highest growth of *Gracilaria* sp. and *Gracilaria chilensis* C.J. Bird, McLachlan & E.C. Oliveira was observed in the presence of 1250 ppm of CO_2 (Gao *et al.*, 1993), and the growth of *Hypnea spinella* (C. Agardh) Kützing increased with 700 and 1600 ppm of CO_2 (Suárez-Álvarez *et al.*, 2012).

Protein concentration and pigment contents of *Hypnea aspera* were stimulated with high nitrogen concentrations, temperatures of 21 and 25°C, and CO_2 levels of 0 and 380 ppm, demonstrating that nitrogen availability control the biosynthesis of these compounds, as noted in previous studies with *Gracilaria tikvahiae* McLachlan (Bird *et*

al., 1982), *Gracilaria* sp. (Andria *et al.*, 2009), *Hypnea pseudomusciformis* (cited as *H. musciformis*, Martins *et al.*, 2011) and *Hypnea cervicornis* J. Agardh (Ribeiro *et al.*, 2013). Furthermore, enrichment with either nitrate or ammonium contributed to the increase of C and N contents in *H. aspera* thallus, and C content was also stimulated by CO_2. On the other hand, this enrichment contributed to low C/N ratio detected in *H. aspera* thallus, as reported by Andria *et al.* (2009) to *Gracilaria* sp. and Mayakun *et al.* (2013) to *Halimeda macroloba* Decaisne and *Turbinaria conoides* (J.Agardh) Kützing. Although the growth of *H. aspera* was inhibited in treatments with high CO_2 resulting in low fresh biomass, which prevented the analyses of pigments and proteins, C content was present in the thallus.

The lethality of high CO_2 level for *H. aspera* could have occurred by the simple diffusion of inorganic C from the media to the chloroplasts in the absence of carbon concentrating mechanism (CCM). Most seaweeds have mechanisms which allow them to utilize HCO_3^- as a source of CO_2 for Rubisco in photosynthesis. In species without CCM, increased HCO_3^- in seawater can result in saturation of carbon fixation, thus limiting photosynthesis (Kübler *et al.*, 1999; Hepburn *et al.*, 2011). Such limited photosynthesis in the presence of high CO_2 levels was observed in *Halimeda opuntia* and *Dictyota* sp. (Hofmann *et al.*, 2015), in *Lithophyllum* sp. and *Feldmannia* spp. (Russell *et al.*, 2009), and also in our study. Treatment with high ammonium concentration and CO_2 at 21°C, and treatment with CO_2, without nitrate addition, at 25°C led to high photoinhibition in *H. aspera*, which could have occurred by the increase of respiration caused by high temperature. Nutrient limitation affects the enzymes of photosynthetic carbon metabolism, reducing Rubisco concentration per cell, corresponding to decreased photosynthetic capacity. This decrease may be related to the increased susceptibility of

N-limitation to photoinhibition (Turpin 1991). Therefore, in our study, the photoinhibition was stimulated in the presence of high ammonium concentration.

Growth, pigment contents, and photosynthesis of *Hypnea aspera* were all negatively affected by high CO_2 concentrations. However, increase in temperature could also affect these same variables. For example, the growth of *Gracilaria vermiculophylla* (Ohmi) Papenfuss tetrasporophytes decreased with increasing temperature up to 30°C (Yokoya *et al.,* 1999). Brown and green strains of *Hypnea pseudomusciformis* presented low growth rates in temperatures of 30°C, and with photoperiod of 10h (cited as *H. musciformis*, Yokoya *et al*., 2006). High temperatures (32°C) negatively affected the growth and pigment contents of *Kappaphyccus alvarezii* (Doty) Doty ex P.C. Silva (Araújo *et al*., 2014). The negative effects of temperature on photosynthetic responses were observed in the red algae *H. musciformis*, *Gracilaria debilis* (Forsskål) Børgesen and *Eucheuma gelidium* J. Agardh, and all of which showed optimal photosynthesis at temperatures of 24°C (Mathieson & Dawes 1986). In contrast, *H. musciformis* showed optimal photosynthesis at temperatures of 28-32°C (Dawes *et al.* 1976). Temperature also plays a key role in chemical reaction rates and can cause protein and enzyme denaturation. Furthermore, high temperatures can affect the rates of diffusion, the carbonic anhydrase activity and transport of CO_2 and HCO_3^- (Lobban & Harrison 2004).

In conclusion, our results suggest that *Hypnea aspera* is highly sensitive to increases of CO_2 and temperature at levels predicted by CMIP5 and RCP 8.5 for 2100 (IPCC, 2013). Furthermore, the increase of nitrogen availability as a result of seawater eutrophication could worsen the impact of these effects on the metabolism and biochemistry of *H. aspera*, a benthic marine red alga, which could be used as an

experimental model for understanding the effects of global changing on physiological process of marine organisms.

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Capítulo 3

TOLERANCE AND PHYSIOLOGICAL RESPONSES OF *Dichotomaria marginata* (RHODOPHYTA, NEMALIALES) TO INCREASES IN TEMPERATURE, NITROGEN AND CO_2 AT LEVELS EXPECTED BY GLOBAL CLIMATE CHANGES

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Abstract

Studies reporting the effects of climate change on calcified macroalgae have generally focused on the impact of elevated CO_2 on marine calcifiers with skeletons of high-magnesium calcite and little is known about the physiology and metabolism of aragonite-calcified species, such as the red macroalga *Dichotomaria marginata* (J.Ellis & Solander) Lamarck (Rhodophyta, Nemaliales), under the same environmental pressures. Therefore, our study aimed to assess the combined effect of three temperatures (21, 25 and 30°C), three CO_2 levels (0, 380 and 1000 ppm) and three nitrogen availabilities (nitrate at 0, 125 and 500 µM or ammonium at 0, 50 and 100 µM) in seawater on the growth, contents of total soluble protein, photosynthetic pigments, thallus contents of C, H and N, calcification and photosynthesis in *D. marginata* cultured in laboratory controlled conditions. The temperature and CO_2 levels tested were those predicted by Intergovernmental Panel on Climate Change, Coupled Model Intercomparison Project Phase 5 and Representative Concentration Pathways 8.5 to 2100 compared to the current scenario. In general, optimal values of variables were observed in 125 and 500 µM of nitrate or 50 µM of ammonium, and 0 and 380 ppm of CO_2 at all temperatures. However, high CO_2 and temperature negatively affected growth, photosynthesis and calcification, while pigment, C and H contents increased. In conclusion, the present study is the first to evaluate the physiological and biochemical responses of *D. marginata* exposed to environmental stressors, indicating that this species has survival strategies to tolerate the increase of CO_2 and temperature levels caused by global climate changes.

Keywords: aragonite, climate change, CMIP5 modeling, CO_2, *Dichotomaria marginata*, growth, nitrogen, photosynthesis, temperature

Introduction

Over the last decade, rising temperature coupled with ocean acidification, resulting from reduced pH in seawater by absorbed CO_2, has received considerable attention in the context of the effects on marine ecosystems (Orr et al. 2005, Kelly and Hofmann 2013). Atmospheric CO_2 level has recently increased from pre-industrial levels of 280 to 380 ppm, and it is predicted to rise to about 1000 ppm in 2100 (IPCC 2013). Oceans absorb approximately 30% of atmospheric carbon dioxide produced by anthropogenic activities (Feely et al. 2004, Jiang et al. 2014). Along with increased CO_2 levels, a rise in global temperature of 4.8℃ is expected by 2100 (IPCC 2013). Moreover, increased local impacts of eutrophication and nitrogen input can influence the physiology of marine organisms (Cloern 2001, Russell et al. 2009).

Carbon (C) acquisition plays an important role in the metabolism of marine macroalgae, including, for example uptake and fixation of carbon in photosynthesis and calcification in seaweeds by calcium carbonate ($CaCO_3$) deposition. Seaweeds have different strategies in terms of acquiring inorganic C, e.g., using carbonate (HCO_3^-) for growth and/or taking up CO_2 from seawater by the carbon-concentrating mechanism (CCM). When exposed to high CO_2 concentrations, species with CCM demonstrate high photosynthesis (Zou and Gao 2005, Xu et al. 2015), Rubisco activity (Raven et al. 2012), anhydrase carbonic activity (Gordillo et al. 2016) and growth (Gao et al. 1993, Liu and Zou 2015). In contrast, studies showed that calcifying seaweeds were highly sensitive to increased CO_2, decreasing growth, photosynthesis and calcification, as observed in *Halimeda macroloba* Descaine (Sinutok et al. 2011), *Padina pavonica* (Linnaeus) Thivy and *P. australis* Hauck (Johnson et al. 2012), *Lithothamnion corallioides* (P.Crouan & H. Crouan) P. Crouan & H. Crouan (Noisette et al. 2013), and *Porolithon onkodes* (Heydrich) Foslie (Diaz-Pulido et al. 2012).

Temperature plays a key role in chemical reactions, and several studies have investigated the effects of temperature on growth (Yokoya et al. 1999, 2006; Ramlov et al. 2012), photosynthesis (Nishihara et al. 2004; Webster et al. 2011), proteomic profile (Xu et al. 2014) and photosynthetic pigments (Araujo et al. 2014; Zanolla et al. 2014) of red seaweeds. On the other hand, few studies have reported the effects of temperature in combination with variations in CO_2 levels on the metabolism and physiology of these organisms. For example, the calcification of *Lithothamnion corallioides* was low in temperature up to 19°C and CO_2 increased to 1000 ppm (Noisette et al. 2013). In contrast, *Gracilariopsis lemaneiformis* (Bory de Saint-Vicent) E.Y. Dawson, Acleto & Foldvik had high photosynthesis at 26°C and 4 mM of inorganic carbon (Ci) (Zou and Gao 2014). *Phycodrys rubens* (Linnaeus) Batters and *Desmarestia aculeata* (Linnaeus) J.V. Lamouroux showed high growth in 390 and 1000 ppm of CO_2 at 10°C (Gordillo et al. 2016).

Besides the importance of CO_2 and temperature, nitrogen is an essencial element to the metabolism and development of marine macroalgae. Carbon and nitrogen metabolic pathways in benthic marine algae are integrally coupled, and compete for assimilatory energy and for C skeletons to N assimilation into amino acids (Turpin 1991). The potential synergistic effects of these three factors (temperature, CO_2 and nitrogen availability) have received little attention over the last few years. Bender et al. (2014) verified the effects of rising temperature, CO_2 and ammonium (NH_4^+) on the growth, photosynthesis and pigments of *Chnoospora implexa* J. Agardh, and observed a decrease of these variables when the species was exposed to 990 ppm of CO_2, 2.49 µM of NH_4^+ and 26.2°C. Notwithstanding this alarming report, knowledge about the effects of ocean acidification, warming and eutrophication on calcified macroalgae is scarce in the literature, with most studies focusing on the impact of elevated CO_2 on marine calcifiers

with skeletons of high-magnesium calcite (e.g., Corallinaceae) (Kuffner et al. 2008, Russell et al. 2009, Büdenbender et al. 2011, Diaz-Pulido et al. 2012, Ragazzola et al. 2012). These organisms experience more difficulty producing their $CaCO_3$ skeletons under increasing CO_2 than aragonite calcified species because the saturation state of aragonite (Ω_{arag} 3-4) is higher than that of high-magnesium calcite (Ω_{hmcal} 2-3) (Kleypas et al. 1999, Johnson et al. 2012).

However, the red macroalga *Dichotomaria marginata* (J. Ellis & Solander) Lamarck, an aragonite species which has intercellular crystals of aragonite that form small bundles (Lobban and Harrison 2004), is understudied as a calcified marine organism exposed to local and global environmental stressors. Studying the physiology and metabolism of this species under environmental variations would provide particularly important data about acclimation to future conditions, such as those proposed by the Coupled Model Intercomparison Project Phase 5 (CMIP5), Representative Concentration Pathways (RCP) 8.5 predicted to the year 2100 (IPCC 2013) (Kelly and Hofmann 2013). Therefore, our study aimed to evaluate the responses of *D. marginata* to combined ocean warming and acidification, as well as rising nitrogen concentration, such as nitrate (NO_3^-) or ammonium (NH_4^+), which could be caused, for example, by eutrophication. To accomplish this, the effects of increased CO_2, temperature and nitrogen on growth, total soluble protein, phycobiliproteins and chlorophyll *a* contents, biochemical composition of C, H and N, calcification and photosynthesis on this aragonite species were analyzed in *D. marginata* in laboratory controlled conditions.

Materials and Methods

Collection site and cultivation in laboratory

Dichotomaria marginata was collected in August (for nitrate experiments) and September 2014 (for ammonium experiments) at Fortaleza Beach, Ubatuba municipality, Sao Paulo State, southeastern Brazil (23º 50' 15.6"S, 45º 17' 40.5'' W). Voucher specimens were deposited in the herbarium of Institute of Botany with accession numbers SP 428540 and SP 428429, for samples collected in August and September, respectively. Surface seawater temperature, pH and salinity were determined during sample collection using a multi-parameter water probe (Horiba W23X). In August, the seawater temperature was 22.36 ± 0.12 ºC, pH 9.07 ± 0.15 and salinity of 34. In September, seawater temperature was 22.83 ± 0.17 ºC, pH 9.45 ± 0.17 and salinity of 34.

After collection, algal samples were washed thoroughly with seawater to remove sand particles and epiphytes, stored in plastic bags, and transported to the laboratory in thermally insulated box at temperature of approximately 15-20ºC. In laboratory, the selected individuals were cleaned by washing with sterilized seawater, and epiphytic organisms were removed under stereomicroscopy. Apical segments (2 cm) were excised from cleaned individuals and acclimated for one week in culture medium composed of sterilized seawater enriched with quarter-strength of von Stosch's nutrient solution (VSES/4) following Oliveira *et al.* (1995), and modified with the reduction of 50% in vitamin concentrations, as described by Yokoya (2000), and addition of 1 $mg.L^{-1}$ of germanium dioxide to inhibit the growth of diatoms. Specimens were cultured in 500-mL Erlenmeyer with 400 ml of VSES/4 culture medium, salinity of 34 and temperature of 23 ± 3 ºC under irradiances of 60 - 90 μmol photons m^{-2} s^{-1}, provided by cool-white fluorescent lamps with a 14:10 h light:dark cycle. Irradiance was measured with a quantum photometer (LI- 250, Li-COR, Lincoln, NE, USA) equipped with spherical

underwater quantum sensor (LI- 250, Li-COR). All procedures were performed at the Laboratório de Cultura de Algas e Cianobactérias Marilza Cordeiro Marino, Instituto de Botanica, Sao Paulo municipality, Brazil.

CO_2, nitrogen and temperature experiments

D. marginata samples were cultivated in 500-ml Erlenmeyer flasks, each one maintaining a ratio of 0.7 g of seaweed per 400 mL of culture medium. Treatments were composed of sterilized seawater enriched with VSES/4 modified (von Stosch´s solution prepared without nitrate, but with salts of phosphate, iron, manganese, EDTA, and three vitamins with concentration reduced to 50%, as already described above). Nitrogen concentrations and nitrogen sources were added to the VSES/4 modified medium according to the treatment to be tested. Nitrate ($NaNO_3$) or ammonium (NH_4Cl) were added to the medium in three concentrations: (i) low (0 µM of nitrate or ammonium), (ii) intermediate (125 or 50 µM of nitrate or ammonium, respectively) and (iii) high (500 or 100 µM of nitrate or ammonium, respectively).

CO_2 and temperature levels were tested according to different scenarios predicted by the IPCC for current scenario, and Model CMIP5 and RCP 8.5 predicted for 2100 (IPCC, 2013). Three levels of CO_2 were tested: (i) low (without CO_2 addition, assuming the concentration of 0 ppm), (ii) intermediate (ambient air - 380 ppm of CO_2 pumped with an aquarium system (Boyu Electromagnetic Air Compressor ACQ-001), and (iii) high (1000 ppm of CO_2 pumped using a pure CO_2 cylinder (P. ONU1013 2.2 – Oxylumen). Specimens were cultivated in culture chambers (Electrolab, Brazil), and three temperatures were tested: (i) low, 21°C; (ii) intermediate, 25°C; and (iii) high, 30°C.

Based on these nitrogen concentrations, CO_2 levels and temperature, ANOVA Factorial Design (3^3) was performed to obtain a combination of factors to be tested (Table

1). Experiments were carried out at salinity of 34, irradiances of 80 - 90 μmol photons m^{-2} s^{-1}, provided by cool-white fluorescent lamps with a 14:10 h light:dark cycle. Each treatment was tested with three replicates (n=3) in which both CO_2 flux and temperature were controlled daily. Culture medium was replaced each week after determining the variation on fresh biomass (mg), photosynthesis, alkalinity, pH, temperature and salinity of seawater. At the end of the experimental period (14 days), culture medium was renewed, and 4 days thereafter (18^{nd} day), samples of each replicate were frozen with liquid nitrogen and stored at -20 °C for analysis of protein and pigment contents. This procedure was used to standardize the period between culture medium renewal and sample freezing since pigment and protein contents were influenced by this period.

TABLE 1. Factorial design of treatments performed for *Dichotomaria marginata* cultivated with different concentrations of nitrogen (nitrate or ammonium), CO_2 and temperature.

Treatments	Factors			
	Nitrate (µM)	**Ammonium (µM)**	**CO_2 (ppm)**	**Temperature (ºC)**
1	0	0	0	21
2	0	0	0	25
3	0	0	0	30
4	0	0	380	21
5	0	0	380	25
6	0	0	380	30
7	0	0	1000	21
8	0	0	1000	25
9	0	0	1000	30
10	125	50	0	21
11	125	50	0	25
12	125	50	0	30
13	125	50	380	21
14	125	50	380	25
15	125	50	380	30
16	125	50	1000	21
17	125	50	1000	25
18	125	50	1000	30
19	500	100	0	21
20	500	100	0	25
21	500	100	0	30
22	500	100	380	21
23	500	100	380	25
24	500	100	380	30
25	500	100	1000	21
26	500	100	1000	25
27	500	100	1000	30

Determination of carbonate chemistry parameters

Total alkalinity (T_A) of the seawater of each treatment (n=3) was measured when the medium was renewed, using the titration method according to Dickson & Millero (1987). Temperature and pH were measured using pH meter coupled with temperature sensor (Jenway 3020). Salinity was determined using a Refractometer (American Opticals, model 10440 T/C). T_A, pH, salinity and temperature of seawater were used to

calculate carbonate chemistry parameters (dissolved CO_2, bicarbonate – HCO_3^-, carbonate – CO_3^{2-}, dissolved inorganic carbon – DIC, and aragonite saturation state - Ωarag of seawater) (Tables 2 and 3), using the R program and Seacarb package (Dickson et al., 2007).

Growth rates

Fresh biomass was recorded weekly for 2 weeks, corresponding to replacement of medium. Growth rates (GR) were calculated from three replicates of each treatment and calculated as $[\ln (Bf \bullet Bo^{-1}) \bullet t^{-1}]$, where Bo is the initial fresh biomass, Bf is the fresh biomass after t days, and t corresponds to the experimental period (Yokoya et al., 2003).

Pigment and protein extraction and quantification

The algal mass (80 mg of fresh biomass for each replicate, n=3) was ground to a powder with liquid nitrogen and mixed with 50 mM phosphate buffer (pH 5.5). The homogenates were centrifuged at 14,000 g for 20 min. at 4ºC in order to separate the phycobiliproteins present in the supernatant. Chlorophyll *a* (Chl *a*) was extracted after dissolving the pellet in 90% acetone and centrifuging at 12,000 *g* for 15 min. at 4ºC. Pigments were quantified by spectrophotometry (Shimadzu-UV 1800). Concentrations of phycobiliproteins (phycoerythrin - PE, phycocyanin - PC and allophycocyanin - APC) were calculated according to Kursar et al. (1983), and the concentration of Chl *a* was calculated according to Jeffrey and Humphrey (1975).

For total soluble protein analysis, 80 mg of algal fresh biomass for each replicate (n=3) were ground with liquid nitrogen, and extractions were carried out at 4 °C using 0.2 M phosphate buffer (pH 8) containing 5 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM Dithiothreitol (DTT). Buffer was added in the proportion of 10 mL g^{-1} fresh

biomass, and the homogenates were centrifuged at 12,000 g for 15 min. Total soluble protein contents were determined according to Bradford (1976), using a Bio-Rad protein assay kit and BSA as standard.

Calcification

The proportion of calcium carbonate ($CaCO_3$) was determined by weighing the fresh biomass of *D. marginata* samples of each treatment (n=3) tested in laboratory, and in algal samples collected in the field. Afterwards, the biomass was dried at 80 ° C for 48 to 72 h until reaching constant weight. Dry biomass was determined, and the samples transferred to Petri dishes were covered with hydrochloric acid (HCl) 5% for 48 hours, with renewal of acid after 24 hours. The sample returned to dry at 80 ° C for 48 to 72 hours and after this procedure, was immediately determined the weight of non-calcareous dry mass. The quantification of $CaCO_3$ (%) was calculated according to Digby (1977) modified by Amancio (2007), as:

$$CaCO_3 = [(\text{dry biomass} - \text{non-calcareous dry biomass}) / \text{dry biomass}] \bullet 100.$$

Internal C, H and N contents

At the end of the experimental period, samples of each treatment (n=3) were oven dried (60°C for 72 h) for analyses of thallus contents of internal carbon (C), hydrogen (H) and nitrogen (N), as determined by the Pregl-Dumas method with Perkin-Elmer 2400 Series II equipment. These analyses were performed by the Central Analítica core facility at the Instituto de Química, Universidade de São Paulo.

Photosynthetic parameters

Measurements of chlorophyll *a* fluorescence were estimated using a pulse amplitude-modulated (PAM) fluorometer (Diving PAM underwater fluorometer, Walz, Effeltrich, Germany). For each treatment, three apical segments of each replicate (n=3) were placed on the tip of the fiber-optic fluorometer using the magnet sample holder. Photosynthesis x irradiance (PI) curves consisted of the fluorescence responses to eleven increasing irradiance levels within range of 0 - 1564 µmol photons m^{-2} s^{-1}, using the "light curve" option of the Diving PAM. Two parameters were determined for each sample: (i) Effective quantum yield - EQY (II) = ΔF/Fm, where ΔF = Fm′ - Ft, Fm′ is the maximum fluorescence and Ft is the steady state fluorescence; and (ii) relative electron transport rate (ETR) = EQY(II)*PAR*ETR-factor*0.5, where the ETR-factor used was 0.84, and 0.5 is the factor related to proportion of Chl *a* at photosystem II (PSII).

The following photosynthetic parameters were calculated by PI curves according to equation of Platt et al. (1980), using KaleidaGraph (Synergy Software): photosyntetic efficiency (α), saturation irradiance (Ik), maximal ETR (Pmax) and photinhibition parameter (β).

Statistical analyses

Data were analyzed by One-Way and Factorial ANOVA, followed by the Student-Newman-Keuls multiple comparison test, in order to distinguish significantly different results ($p < 0.05$), using STATISTICA software (version 9). For multivariate analysis, the data of growth rates (GR), pigment contents (APC, PC, PE, and Chl *a*), protein contents (PT), tissue elements (C, H, N and C/N ratio), photosynthetic parameters (ETR, EQY (effective quantum yield), α, Ik and Pmax), calcification ($CaCO_3$) and carbonate system of seawater (T_A, pH, CO_2, CO_3^{2-}, HCO_3^- , DIC (dissolved inorganic carbon) and Ωarag

(aragonite saturation state)) were used in a covariance matrix for principal component analyses (PCA) performed in a PC-ORD 6 software (MjM Software, USA). The variability of the data was adjusted by the method of ranging ($[X-X_{min})/(X_{max}-X_{min})]$) (Legendre & Legendre 1998), using the PC-ORD 6 software.

TABLE 2. Carbonate system of the seawater medium in which *Dichotomaria marginata* samples were cultured with different nitrate concentrations, CO_2 levels and temperature for 14 days. Data are the mean of three replicates ± standard deviation. Treatments with distinct letters indicate significant differences according to the one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

T (°C)	CO_2 (ppm)	$[NO_3^-]$ (µM)	pH	T_A (mM)	CO_2 (mM)	HCO_3^- (mM)	CO_3^{2-} (mM)	DIC (mM)	Ωarag
21°C	0	0	7.24 ± 0.07^{b}	2.27 ± 0.00^{a}	0.10 ± 0.02^{a}	2.20 ± 0.01^{a}	0.04 ± 0.01^{c}	2.33 ± 0.02^{c}	0.56 ± 0.09^{ab}
		125	7.39 ± 0.16^{ab}	2.27 ± 0.00^{a}	0.07 ± 0.02^{bc}	2.17 ± 0.04^{a}	0.05 ± 0.02^{c}	2.29 ± 0.04^{c}	0.82 ± 0.30^{ab}
		500	7.58 ± 0.01^{ab}	2.27 ± 0.00^{a}	0.04 ± 0.00^{bc}	2.12 ± 0.00^{a}	0.08 ± 0.00^{c}	2.24 ± 0.00^{bc}	1.18 ± 0.02^{ab}
	380	0	7.52 ± 0.57^{ab}	2.27 ± 0.00^{a}	0.08 ± 0.07^{c}	2.07 ± 0.23^{a}	0.10 ± 0.12^{c}	2.25 ± 0.18^{c}	1.63 ± 1.85^{ab}
		125	7.44 ± 0.12^{ab}	2.27 ± 0.00^{a}	0.06 ± 0.02^{bc}	2.16 ± 0.03^{a}	0.06 ± 0.01^{c}	2.28 ± 0.03^{c}	0.90 ± 0.21^{ab}
		500	7.59 ± 0.65^{ab}	2.27 ± 0.00^{a}	0.08 ± 0.09^{c}	2.02 ± 0.30^{a}	0.13 ± 0.15^{c}	2.22 ± 0.23^{bc}	2.00 ± 2.33^{ab}
	1000	0	5.33 ± 0.05^{d}	2.26 ± 0.00^{b}	8.01 ± 0.96^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{c}	10.28 ± 0.96^{a}	0.01 ± 0.00^{c}
		125	5.77 ± 0.80^{d}	2.26 ± 0.00^{b}	5.70 ± 4.83^{ab}	2.27 ± 0.01^{a}	0.00 ± 0.01^{c}	7.97 ± 4.83^{ab}	0.06 ± 0.09^{c}
		500	5.74 ± 0.65^{cd}	2.26 ± 0.00^{b}	5.21 ± 4.58^{ab}	2.26 ± 0.00^{a}	0.00 ± 0.00^{c}	7.47 ± 4.58^{ab}	0.04 ± 0.05^{c}
25°C	0	0	6.68 ± 0.05^{bc}	2.27 ± 0.00^{a}	0.33 ± 0.04^{b}	2.25 ± 0.00^{a}	0.01 ± 0.00^{c}	2.59 ± 0.04^{c}	0.18 ± 0.02^{b}
		125	6.71 ± 0.06^{bc}	2.27 ± 0.00^{a}	0.31 ± 0.04^{b}	2.25 ± 0.00^{a}	0.01 ± 0.00^{c}	2.57 ± 0.04^{c}	0.19 ± 0.03^{b}
		500	6.76 ± 0.03^{bc}	2.27 ± 0.00^{a}	0.28 ± 0.02^{b}	2.25 ± 0.00^{a}	0.01 ± 0.00^{c}	2.54 ± 0.02^{c}	0.21 ± 0.01^{b}
	380	0	8.07 ± 0.04^{ab}	2.27 ± 0.00	0.01 ± 0.00^{c}	1.83 ± 0.04^{b}	0.22 ± 0.02^{b}	2.06 ± 0.02^{c}	3.50 ± 0.30^{a}
		125	8.38 ± 0.11^{a}	2.27 ± 0.00	0.00 ± 0.00^{c}	1.52 ± 0.13^{c}	0.38 ± 0.07^{a}	1.90 ± 0.07^{c}	6.01 ± 1.07^{a}
		500	8.33 ± 0.33^{a}	2.27 ± 0.00	0.01 ± 0.00^{c}	1.55 ± 0.38^{c}	0.36 ± 0.19^{a}	1.92 ± 0.19^{c}	5.78 ± 3.03^{a}
	1000	0	5.77 ± 0.60^{cd}	2.27 ± 0.00^{a}	4.25 ± 3.26^{ab}	2.26 ± 0.00^{a}	0.00 ± 0.00^{c}	6.51 ± 3.26^{abc}	0.04 ± 0.06^{c}
		125	5.45 ± 0.03^{d}	2.27 ± 0.00^{a}	5.72 ± 0.48^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{c}	7.99 ± 0.48^{ab}	0.01 ± 0.00^{c}
		500	5.76 ± 0.57^{cd}	2.27 ± 0.00^{a}	4.21 ± 3.14^{ab}	2.26 ± 0.00^{a}	0.00 ± 0.00^{c}	6.48 ± 3.14^{abc}	0.04 ± 0.05^{c}
30°C	0	0	6.88 ± 0.01^{b}	2.27 ± 0.00^{a}	0.21 ± 0.01^{b}	2.24 ± 0.00^{a}	0.02 ± 0.00^{c}	2.46 ± 0.01^{c}	0.28 ± 0.02^{b}
		125	6.89 ± 0.08^{b}	2.27 ± 0.00^{a}	0.21 ± 0.04^{b}	2.24 ± 0.01^{a}	0.02 ± 0.00^{c}	2.46 ± 0.04^{c}	0.29 ± 0.05^{b}
		500	6.88 ± 0.01^{b}	2.27 ± 0.00^{a}	0.21 ± 0.01^{b}	2.24 ± 0.00^{a}	0.02 ± 0.00^{c}	2.46 ± 0.01^{c}	0.29 ± 0.01^{b}
	380	0	7.49 ± 0.33^{ab}	2.27 ± 0.00^{a}	0.06 ± 0.05^{c}	2.11 ± 0.09^{a}	0.08 ± 0.04^{c}	2.25 ± 0.10^{c}	1.29 ± 0.71^{ab}
		125	7.53 ± 0.15^{ab}	2.27 ± 0.00^{a}	0.05 ± 0.02^{c}	2.12 ± 0.05^{a}	0.08 ± 0.03^{c}	2.24 ± 0.04^{c}	1.26 ± 0.41^{ab}
		500	7.38 ± 0.18^{ab}	2.27 ± 0.00^{a}	0.07 ± 0.03^{c}	2.16 ± 0.05^{a}	0.06 ± 0.02^{c}	2.28 ± 0.05^{c}	0.92 ± 0.39^{ab}
	1000	0	6.13 ± 0.42^{bcd}	2.27 ± 0.01^{ab}	1.56 ± 1.21^{bc}	2.26 ± 0.01^{a}	0.00 ± 0.00^{c}	3.82 ± 1.21^{bc}	0.07 ± 0.07^{bc}
		125	5.92 ± 0.72^{cd}	2.26 ± 0.00^{b}	3.48 ± 2.78^{bc}	2.26 ± 0.01^{a}	0.00 ± 0.01^{c}	5.74 ± 2.78^{bc}	0.08 ± 0.11^{bc}
		500	6.04 ± 0.61^{bcd}	2.27 ± 0.01^{ab}	2.40 ± 2.29^{bc}	2.26 ± 0.01^{a}	0.00 ± 0.01^{c}	4.67 ± 2.29^{bc}	0.08 ± 0.10^{bc}

T_A = total alkalinity, CO_2 = dissolved CO_2, HCO_3^- = bicarbonate concentration, CO_3^{2-} = carbonate concentration, DIC = dissolved inorganic carbon, Ωarag = aragonite saturation state in seawater.

TABLE 3. Carbonate system of the seawater medium in which *Dichotomaria marginata* samples were cultured with different ammonium concentrations, CO_2 levels and temperature for 14 days. Data are the mean of three replicates ± standard deviation. Treatments with distinct letters indicate significant differences according to the one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

T (°C)	CO_2 (ppm)	$[NH_4^+]$ (µM)	pH	T_A (µM)	CO_2 (mM)	HCO_3^- (mM)	CO_3^{2-} (mM)	DIC (mM)	Ωarag
21°C	0	0	8.85 ± 0.29^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{b}	0.96 ± 0.37^{c}	0.65 ± 0.19^{a}	1.62 ± 0.19^{c}	10.35 ± 2.96^{a}
		50	8.87 ± 0.01^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{b}	0.93 ± 0.0^{c}	0.67 ± 0.00^{a}	1.60 ± 0.00^{c}	10.60 ± 0.05^{a}
		100	8.81 ± 0.05^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{b}	1.01 ± 0.06^{c}	0.63 ± 0.03^{a}	1.64 ± 0.03^{c}	9.98 ± 0.46^{a}
	380	0	7.55 ± 0.04^{bc}	2.27 ± 0.00^{a}	0.04 ± 0.00^{b}	2.13 ± 0.01^{a}	0.07 ± 0.01^{cd}	2.24 ± 0.01^{c}	1.15 ± 0.11^{c}
		50	7.53 ± 0.04^{bc}	2.27 ± 0.00^{a}	0.05 ± 0.00^{b}	2.13 ± 0.01^{a}	0.07 ± 0.01^{cd}	2.25 ± 0.01^{c}	1.12 ± 0.10^{c}
		100	7.57 ± 0.07^{bc}	2.27 ± 0.00^{a}	0.04 ± 0.01^{b}	2.12 ± 0.02^{a}	0.08 ± 0.01^{cd}	2.24 ± 0.02^{c}	1.21 ± 0.18^{c}
	1000	0	5.27 ± 0.16^{d}	2.26 ± 0.02^{b}	9.32 ± 3.06^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{d}	11.58 ± 3.06^{ab}	0.01 ± 0.00^{d}
		50	5.19 ± 0.02^{d}	2.26 ± 0.00^{b}	10.71 ± 0.41^{a}	2.26 ± 0.00^{a}	0.00 ± 0.00^{d}	12.97 ± 0.41^{a}	0.01 ± 0.00^{d}
		100	5.76 ± 0.98^{d}	2.26 ± 0.04^{b}	7.20 ± 6.08^{a}	2.26 ± 0.02^{a}	0.01 ± 0.01^{d}	9.46 ± 6.09^{ab}	0.09 ± 0.15^{d}
25°C	0	0	7.52 ± 0.25^{bc}	2.27 ± 0.00^{a}	0.05 ± 0.03^{b}	2.10 ± 0.10^{a}	0.09 ± 0.05^{cd}	2.23 ± 0.07^{c}	1.43 ± 0.79^{c}
		50	7.40 ± 0.06^{bc}	2.27 ± 0.00^{a}	0.06 ± 0.01^{b}	2.15 ± 0.02^{a}	0.06 ± 0.01^{cd}	2.27 ± 0.02^{c}	1.00 ± 0.14^{c}
		100	7.44 ± 0.16^{bc}	2.27 ± 0.00^{a}	0.06 ± 0.02^{b}	2.13 ± 0.05^{a}	0.07 ± 0.02^{cd}	2.26 ± 0.04^{c}	1.11 ± 0.38^{c}
	380	0	8.12 ± 0.70^{ab}	2.27 ± 0.00^{a}	0.03 ± 0.04^{b}	1.58 ± 0.52^{b}	0.35 ± 0.26^{b}	1.95 ± 0.30^{c}	5.64 ± 4.17^{b}
		50	7.90 ± 0.83^{b}	2.27 ± 0.00^{a}	0.06 ± 0.09^{b}	1.71 ± 0.47^{b}	0.28 ± 0.23^{bc}	2.05 ± 0.32^{c}	4.56 ± 3.78^{bc}
		100	8.10 ± 0.66^{ab}	2.27 ± 0.00^{a}	0.02 ± 0.04^{b}	1.60 ± 0.49^{b}	0.34 ± 0.25^{b}	1.96 ± 0.28^{c}	5.44 ± 3.99^{b}
	1000	0	5.75 ± 0.79^{d}	2.26 ± 0.03^{b}	5.27 ± 4.28^{a}	2.26 ± 0.01^{a}	0.00 ± 0.01^{d}	7.53 ± 4.28^{b}	0.07 ± 0.11^{d}
		50	5.33 ± 0.02^{d}	2.26 ± 0.00^{b}	7.20 ± 0.36^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{d}	9.47 ± 0.36^{ab}	0.01 ± 0.00^{d}
		100	5.34 ± 0.01^{d}	2.26 ± 0.00^{b}	6.92 ± 0.16^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{d}	9.19 ± 0.16^{ab}	0.01 ± 0.00^{d}
30°C	0	0	6.77 ± 0.05^{c}	2.27 ± 0.00^{a}	0.25 ± 0.03^{b}	2.24 ± 0.00^{a}	0.02 ± 0.00^{d}	2.51 ± 0.03^{c}	0.25 ± 0.03^{d}
		50	6.74 ± 0.06^{c}	2.27 ± 0.00^{a}	0.27 ± 0.04^{b}	2.24 ± 0.00^{a}	0.01 ± 0.00^{d}	2.53 ± 0.04^{c}	0.24 ± 0.03^{d}
		100	6.65 ± 0.10^{c}	2.27 ± 0.00^{a}	0.34 ± 0.08^{b}	2.25 ± 0.01^{a}	0.01 ± 0.00^{d}	2.60 ± 0.08^{c}	0.20 ± 0.04^{d}
	380	0	7.56 ± 0.04^{bc}	2.27 ± 0.00^{a}	0.04 ± 0.00^{b}	2.09 ± 0.01^{a}	0.09 ± 0.01^{cd}	2.22 ± 0.01^{c}	1.45 ± 0.11^{c}
		50	7.57 ± 0.04^{bc}	2.27 ± 0.00^{a}	0.04 ± 0.00^{b}	2.09 ± 0.02^{a}	0.09 ± 0.01^{d}	2.22 ± 0.01^{c}	1.49 ± 0.12^{c}
		100	7.83 ± 0.40^{b}	2.27 ± 0.00^{a}	0.02 ± 0.02^{b}	1.90 ± 0.30^{ab}	0.18 ± 0.15^{bcd}	2.11 ± 0.17^{c}	2.98 ± 2.43^{bcd}
	1000	0	5.32 ± 0.08^{d}	2.26 ± 0.01^{b}	7.47 ± 1.34^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{d}	9.73 ± 1.34^{ab}	0.01 ± 0.00^{d}
		50	5.29 ± 0.02^{d}	2.26 ± 0.00^{b}	7.79 ± 0.32^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{d}	10.06 ± 0.32^{ab}	0.01 ± 0.00^{d}
		100	5.28 ± 0.02^{d}	2.26 ± 0.00^{b}	8.04 ± 0.29^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{d}	10.31 ± 0.29^{ab}	0.01 ± 0.00^{d}

T_A = total alkalinity, CO_2 = dissolved CO_2, HCO_3^- = bicarbonate concentration, CO_3^{2-} = carbonate concentration, DIC = dissolved inorganic carbon, Ωarag = aragonite saturation state in seawater.

Results

Tables 1 and 2 showed that the pH, T_A and CO_3^{2-} concentrations decreased mainly with the increase of CO_2. In tests with nitrate availabilities, the Ωarag (aragonite saturation state) decreased with rising CO_2, while in ammonium concentrations the saturation decreased with high CO_2 and with increase of temperature.

The growth rate (GR) of *Dichotomaria marginata* was influenced by 1) 125 and 500 µM of NO_3^-, 0 ppm CO_2 at 21°C; 2) all additions of NO_3^- in 380 ppm of CO_2 at 21°C; 3) 125 µM of NO_3^- in 380 ppm of CO_2 at 25°C; and 4) without addition of NO_3^- and 380 ppm of CO_2 at 30°C (Fig. 1a). GR of *D. marginata* was stimulated in all ammonium availabilities in 380 ppm of CO_2 at 25 and 30°C (Fig. 1b). High CO_2 level inhibited the GR of species with all nitrogen sources and concentrations at all temperatures (Fig. 1a, b).

For nitrate, the highest total soluble protein (PT) content was observed in *D. marginata* cultured with 1) NO_3^- additions of 125 and 500 µM in 380 ppm of CO_2 at 21°C, and 2) 500 µM of NO_3^- in 380 ppm of CO_2 at 30°C. The lowest PT concentration occurred in algae treated with 500 µM of NO_3^- in 1000 ppm of CO_2 at 25°C (Fig. 2a). For ammonium, the highest PT concentration occurred in algae cultured with 50 and 100 µM of NH_4^+ in 380 ppm of CO_2 at 21°C and lowest with 50 µM of NH_4^+ in 1000 ppm of CO_2 at 30°C (Fig. 2b).

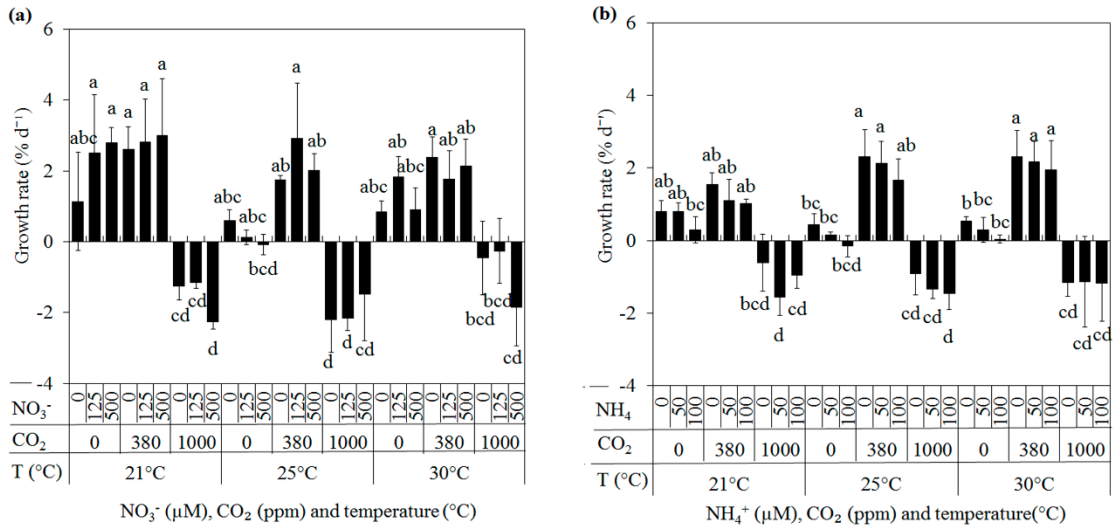

FIG. 1. Growth rate (% d^{-1}) of *Dichotomaria marginata* cultivated for 14 days in VSES/4 modified and with different (a) nitrate (NO_3^-) and (b) ammonium (NH_4^+) concentrations, different CO_2 levels and temperatures, photoperiod of 14 h, salinity of 34 and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to the one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

APC contents of *D. marginata* did not vary with NO_3^- availabilities, CO_2 levels or temperatures tested (Fig. 3a). PC concentrations of species increased with 1) addition of 125 µM of NO_3^-, without CO_2 addition, at 25°C; 2) 500 µM of NO_3^-, without CO_2, at 30°C; and 3) with 500 µM of NO_3^- in 380 ppm of CO_2 at 30°C. The addition of 125 and 500 µM of NO_3^- , without CO_2 addition, at 25°C stimulated a high PE content of *D. marginata* (Fig. 3c). Lower PC and PE contents occurred during cultivation without NO_3^- in 380 ppm of CO_2 at 30°C (Fig. 3b, c). Optimal values of Chl *a* were observed with 1) 125 and 500 µM of NO_3^-, without CO_2 addition, at 25°C and 2) 500 µM of NO_3^- in 380 ppm of CO_2 at 30°C. In contrast, low values of this pigment occurred in *D. marginata*

cultured with 1) 1000 ppm of CO_2 with all NO_3^- concentrations at 25°C and 2) 125 µM of NO_3^- in 1000 ppm of CO_2 at 21°C (Fig. 3d).

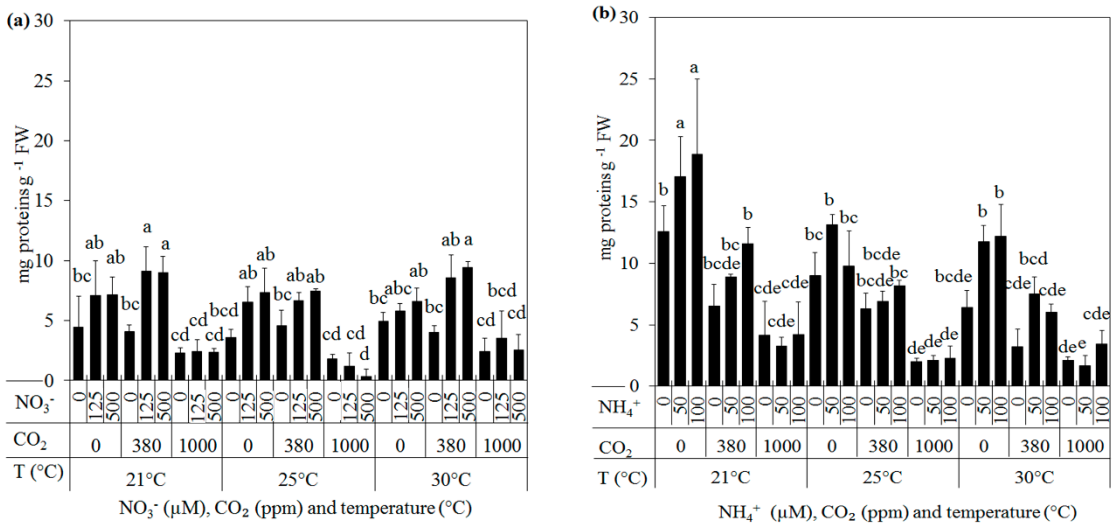

FIG. 2. Concentration of total soluble protein of *Dichotomaria marginata* cultivated for 18 days in VSES/4 modified enriched with different (a) nitrate (NO_3^-) or (b) ammonium (NH_4^+) concentrations, different CO_2 levels and temperatures, photoperiod of 14 h, salinity of 34, and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to the one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

In treatments with different concentrations of NH_4^+, high APC content of *D. marginata* was observed in 50 µM, without CO_2, at 25°C, and low values of APC were seen with the same concentrations of NH_4^+ and CO_2, but at 21°C (Fig. 4a). Concentrations of PC, PE and Chl *a* were all stimulated by treatments with 50 µM of NH_4^+, without CO_2 addition, at 30°C (Fig. 4b, c, d). The lower values of PC and PE occurred with 100 µM of NH_4^+ in 1000 ppm of CO_2 at 30°C (Fig. 4b, c). Chl *a* was low in 1) 0 and 50 µM of

NH_4^+ in 1000 ppm of CO_2 at 25°C and 2) all NH_4^+ concentrations in 1000 ppm of CO_2 at 30°C (Fig. 4d).

Algal samples collected in the field showed 76.86 ± 0.70 % and 86.68 ± 0.50 of calcification on thallus for August and September months, respectively. The calcification was high in *D. marginata* samples cultured with 1) NO_3^- additions of 125 and 500 µM, without CO_2, at 21°C; 2) 500 µM of NO_3^- in 380 ppm of CO_2 at 21°C; and 3) 0 and 500 µM of NO_3^- in 380 ppm of CO_2 at 25°C (Fig. 5a). In general, the thallus calcification decreased in treatments with high CO_2 concentrations and high temperature independent of NO_3^- addition. In contrast, treatments with NH_4^+ stimulated high calcification when samples were cultured with 1) all NH_4^+ concentrations in 380 ppm of CO_2 at 21 and 25°C and 2) 0 and 100 µM of NH_4^+, without CO_2, at 25°C (Fig. 5b). Lower calcification values were observed in algae cultured with 100 µM of NH_4^+ in 1000 ppm of CO_2 at 30°C.

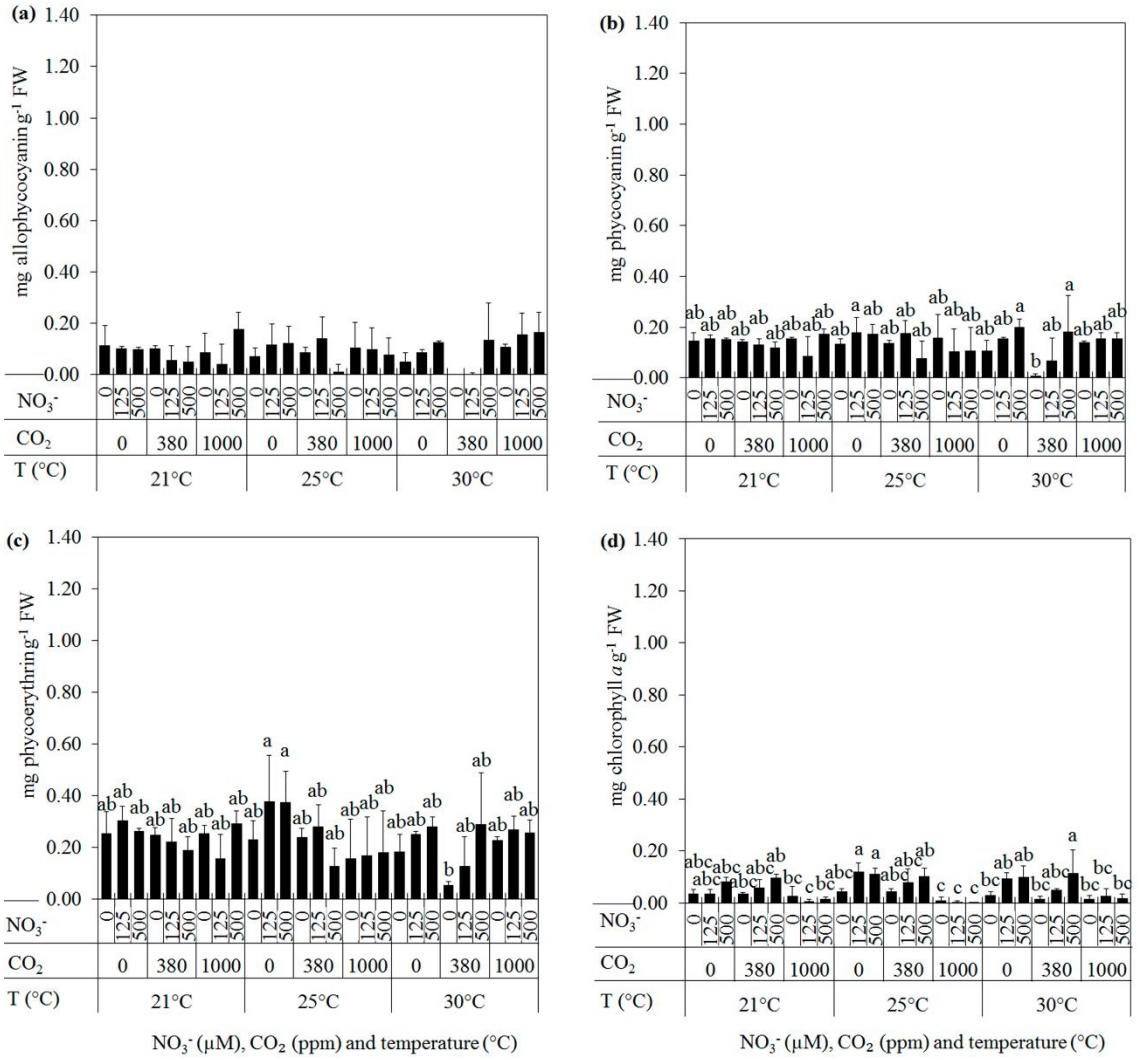

FIG. 3. Concentration of (a) allophycocyanin, (b) phycocyanin, (c) phycoerythrin and (d) chlorophyll *a* of *Dichotomaria marginata* cultivated for 18 days in VSES/4 modified enriched with different nitrate (NO_3^-) concentrations, CO_2 levels and temperatures, photoperiod of 14 h, salinity of 34, and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

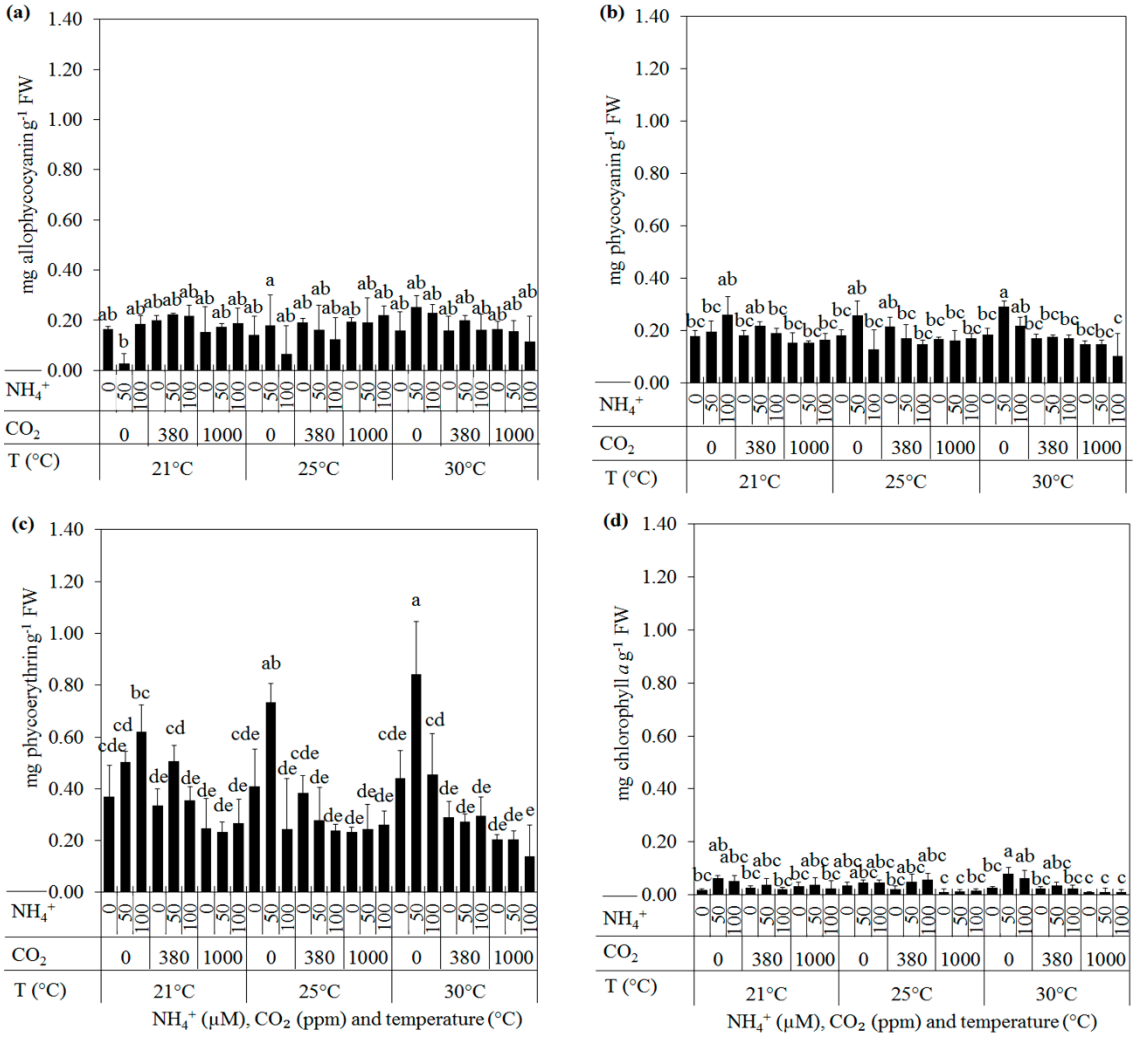

FIG. 4. Concentration of (a) allophycocyanin, (b) phycocyanin, (c) phycoerythrin and (d) chlorophyll *a* of *Dichotomaria marginata* cultivated for 18 days in VSES/4 modified enriched with different ammonium (NH_4^+) concentrations, CO_2 levels and temperatures, photoperiod of 14 h, salinity of 34, and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

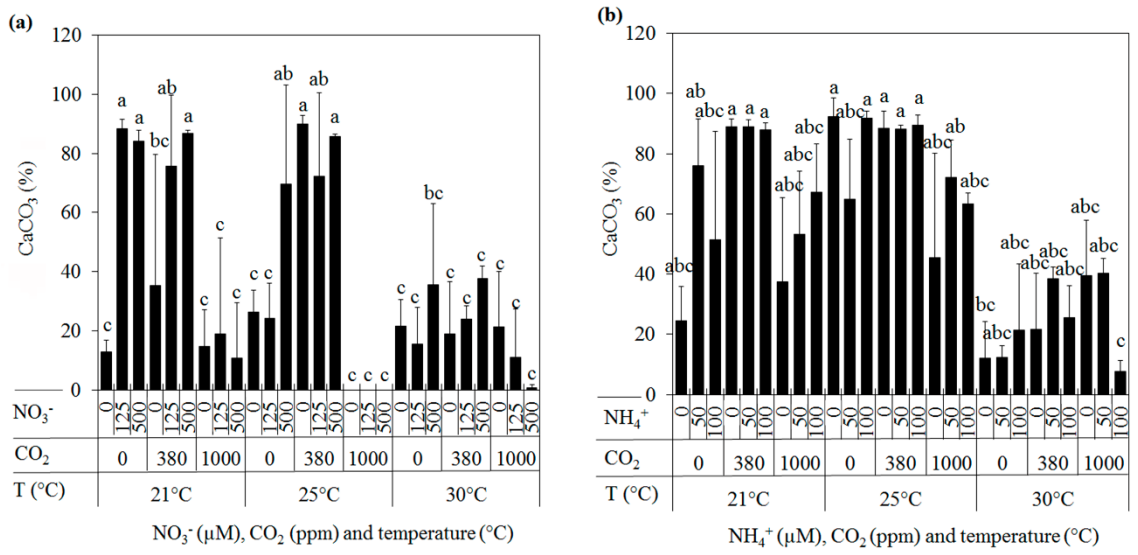

FIG. 5. Calcification of *Dichotomaria marginata* cultivated for 14 days in VSES/4 modified enriched with different (a) nitrate (NO_3^-) or (b) ammonium (NH_4^+) concentrations, different CO_2 levels and temperatures, photoperiod of 14 h, salinity of 34, and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

High C content of *D. marginata* thallus occurred in all treatments tested, except for 1000 ppm of CO_2 at 21° and 25°C, and lower values were observed in 1) 125 and 500 µM of NO_3^- in 1000 ppm of CO_2 at 21°C and 2) 500 µM of NO_3^- in 1000 ppm of CO_2 at 25°C (Fig. 6a). Lower H and N content and C/N ratio occurred in the same treatments that C content was low (Fig. 6b, c, d). High H content was observed in 1) 500 µM of NO_3^- in 380 ppm of CO_2 at 21°C; 2) all NO_3^- concentrations, without additions of CO_2, at 25°C; 3) 125 and 500 µM of NO_3^- in 380 ppm of CO_2 at 25°C; 4) without NO_3^-, but with CO_2 additions, at 30°C; 5) without NO_3^- in 380 ppm of CO_2 at 30°C; and 6) all NO_3^- additions in 1000 ppm of CO_2 at 30°C (Fig. 6b). Tissue N was high with 1) 125 and 500 µM of

NO_3^-, without CO_2, at 25℃ and 2) 125 µM of NO_3^- in 1000 ppm of CO_2 at 30℃ (Fig. 6c). C/N ratio was highest without NO_3^- availability in 380 ppm of CO_2 at 25°C (Fig. 6d).

In treatments with NH_4^+ availabilities, C content in *D. marginata* thallus was stimulated with high NH_4^+ concentrations in 1000 ppm of CO_2 in all temperatures, as well as 50 µM of NH_4^+ in 1000 ppm of CO_2 at 30℃. Low C and H contents in algal thallus occurred without NH_4^+ in 380 ppm of CO_2 at 30℃ (Fig. 7a, b). H content was higher in 100 µM of NH_4^+ in 1000 ppm pf CO_2 at 21℃ (Fig. 7b). Thallus N content increased in samples cultured with additions of 1) 50 and 100 µM of NH_4^+, without CO_2, at 25℃ and 2) 100 µM of NH_4^+, without CO_2 addition, at 30℃. Lower N content of *D. marginata* was observed without NH_4^+ additions in 380 ppm of CO_2 at 25°C (Fig. 7c). C/N ratio was stimulated by treatments without NH_4^+ addition in 380 ppm of CO_2 at 25°C, and lower C/N ratio occurred in samples cultured with high NH_4^+ concentrations without CO_2 at 21, 25 and 30°C (Fig. 7d).

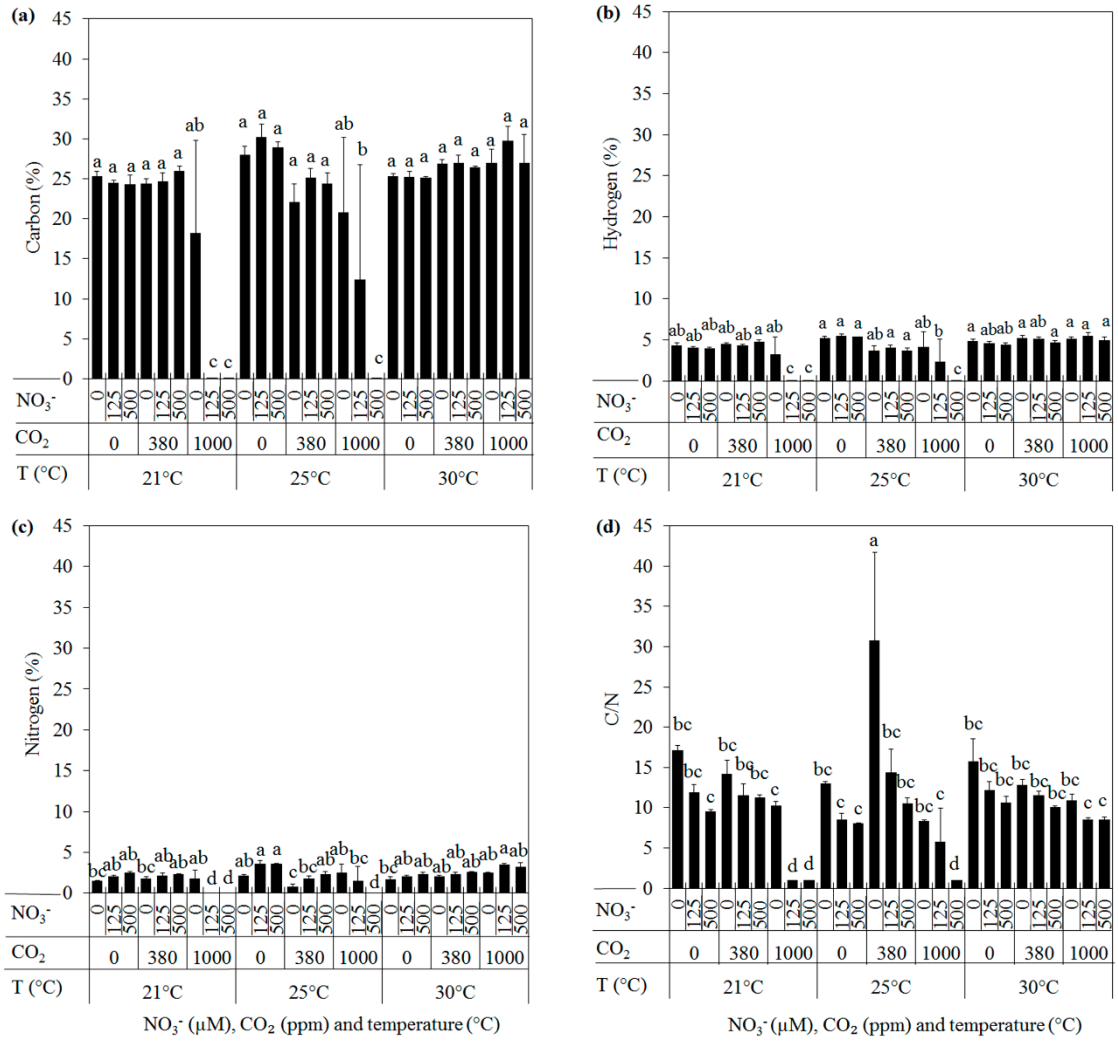

FIG. 6. Concentration of internal carbon (a), hydrogen (b), nitrogen (c), and C/N ratio of *Dichotomaria marginata* cultivated for 18 days in VSES/4 modified enriched with different nitrate (NO_3^-) concentrations, CO_2 levels and temperatures, photoperiod of 14 h, salinity of 34, and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

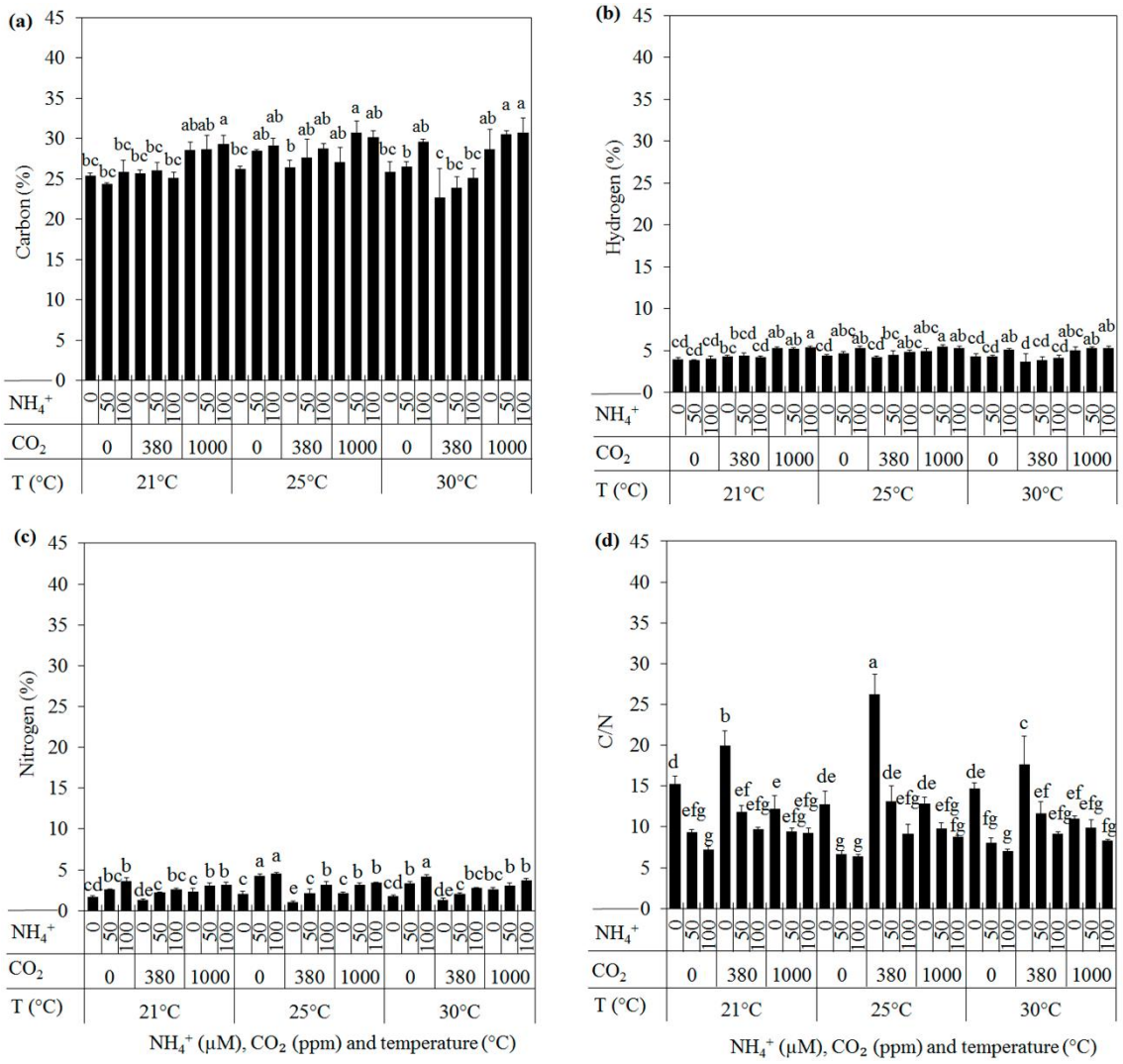

FIG. 7. Concentration of internal carbon (a), hydrogen (b), nitrogen (c) and C/N ratio (d) of *Dichotomaria marginata* cultivated for 18 days in VSES/4 modified enriched with different ammonium (NH_4^+) concentrations, CO_2 levels and temperatures, photoperiod of 14 h, salinity of 34, and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

The differences observed in photosynthetic responses of *D. marginata* to increased nitrogen, CO_2 and temperature are presented in PI curves presented in Fig. 8. PI curve

was influenced by NO_3^- concentration, CO_2 and temperature (*F= 72.91* and *p= 0.00*), as well as irradiance (*F= 59.76* and *p= 0.00*). ETR values were high at 500 µM of NO_3^-, without CO_2 addition, at 21°C and in 335 µmol photons m^{-2} s^{-1} (Fig. 8a). As temperature increased, ETR values decreased, and at all temperatures, lower ETR values occurred in treatments with high CO_2 (Fig. 8a, b, c). Moreover, PI curves showed saturation with increased of irradiance, and higher Ik occurred in *D. marginata* cultures with 1) 125 and 500 µM of NO_3^-, without CO_2 addition, at 21°C; 2) 500 µM of NO_3^- in 380 ppm of CO_2 at 21°C; and 3) 125 µM of NO_3^- in 380 ppm of CO_2 at 25°C (Table 4). The highest EQY occurred in samples treated with 125 µM of NO_3^-, without CO_2, at 25°C, and the highest Pmax was observed in 500 µM of NO_3^-, without CO_2, at 21°C. β was higher in 500 µM of $NO3^-$ in 380 ppm of CO_2 at 25°C. The values of α did not vary between treatments tested (Table 4).

PI curves showed the influence of NH_4^+, CO_2 concentrations, different temperatures (*F= 52.66* and *p=0.00*) and increase of irradiance (*F= 141.33* and *p= 0.00*) on the photosynthesis of *D. marginata* (Fig. 8d, e, f). A high ETR value was observed with the addition of 100 µM of ammonium, without CO_2 addition, at 21°C and 335 µmol photons m^{-2} s^{-1}, but a low ETR value occurred for samples cultivated without ammonium concentration in 1000 ppm of CO_2 at 21°C. Table 5 shows the photosynthetic parameters obtained from PI curves. EQY was highest in 1) all NH_4^+ concentrations, without and with 380 ppm of CO_2, at 21°C and 2) 50 and 100 µM of NH_4^+ in 380 ppm of CO_2 at 25 and 30°C. The highest Pmax occurred in 100 µM of NH_4^+, without CO_2, at 21°C. Low EQY, Pmax and α values occurred without NH_4^+ concentration, but with 380 of CO_2, at 30°C. EQY also was low in all ammonium concentrations and temperatures with 1000 ppm of CO_2. The α was higher in treatment without NH_4^+, but with 380 ppm of CO_2, at

21°C. Low Ik was observed in all ammonium concentrations and temperatures with 1000 ppm of CO_2 and β did not vary among treatments tested.

Principal components (PCA) analysis explained variability of 41.41% in the first axis, 19.15% in the second axis and total variability of 60.56% in the first two axes (Fig. 9). The positive side of axis 1 grouped treatments with low and intermediate CO_2 concentrations and temperatures independent of nitrate or ammonium concentrations with GR, PT, Chl *a*, $CaCO_3$, EQY, Pmax, Ik, pH, T_A, CO_3^{2-} and Ω_{arag} of seawater. The negative side of axis 1 grouped together high CO_2 and temperature with β, CO_2, HCO_3^- and DIC of seawater. The negative side of axis 2 associated the concentration of ammonium with APC and PC. The positive side did not group any variables (Table 6).

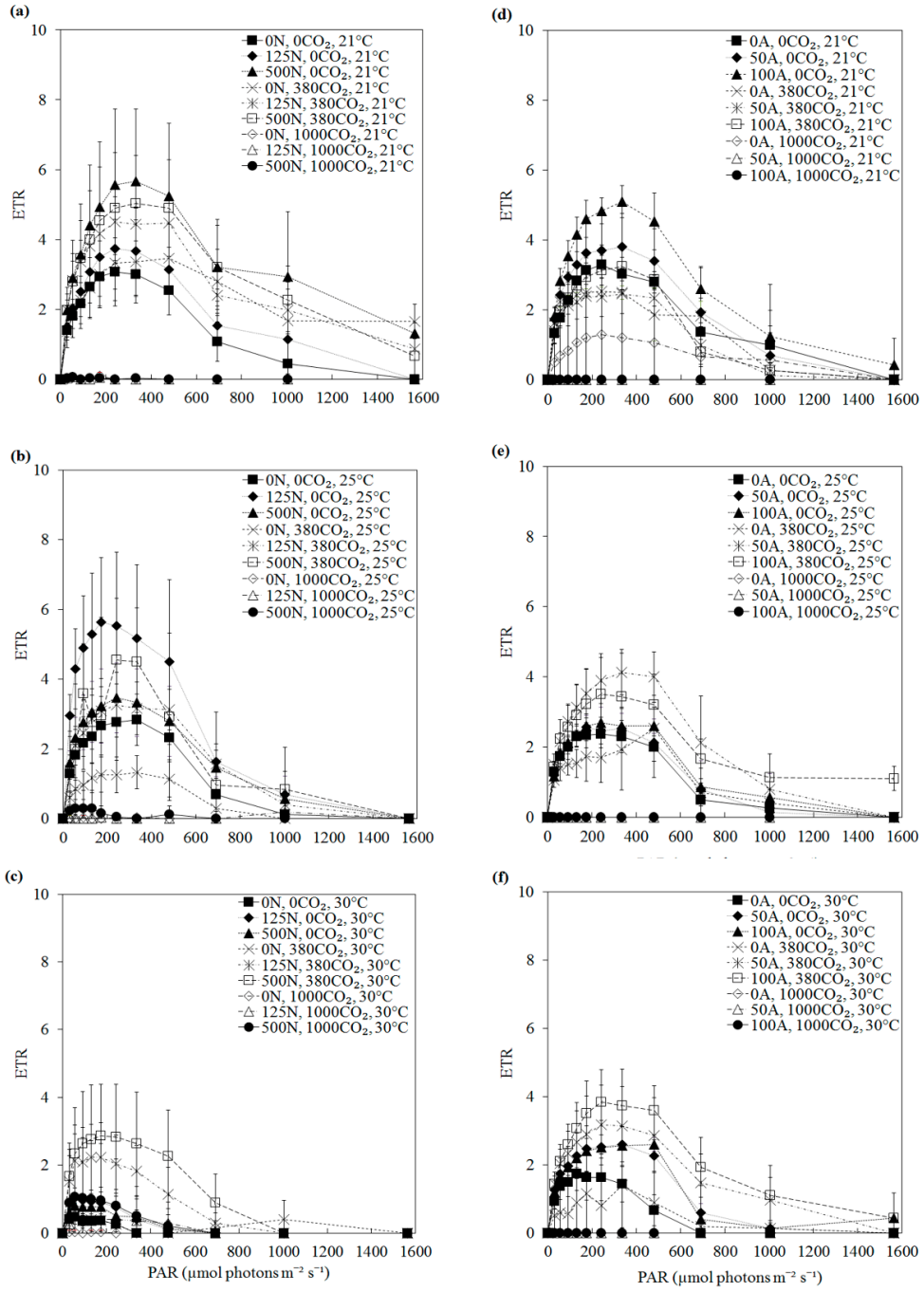

FIG. 8. Photosynthesis x irradiance (PI) curve of *Dichotomaria marginata* cultivated for 14 days in VSES/4 modified enriched with different nitrate (NO_3^-) (a, b, c) or ammonium (NH_4^+) (d, e, f) concentrations, CO_2 levels and temperatures, photoperiod of 14 h, salinity of 34, and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation.

TABLE 4. Photosynthetic parameters (EQY (effective quantum yield); Pmax, α (photosynthetic efficiency), Ik (saturation irradiance); and β (photoinhibition)) of *Dichotomaria marginata* cultivated for 14 days in VSES/4 modified enriched with different nitrate (NO_3^-) concentrations, CO_2 levels and temperatures (T), photoperiod of 14 h, salinity of 34 and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to the factorial ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

TREATMENTS			PHOTOSYNTHETIC PARAMETERS				
T (°C)	CO_2 (ppm)	$[NO_3^-]$ (µM)	EQY	Pmax	α	Ik	β
21°	0	0	0.05±0.00^{abd}	3.15±0.48^{abc}	0.04±0.01	83.00±7.78^{ab}	-0.10±0.79^{b}
		125	0.06±0.01^{abd}	3.74±1.47^{abc}	0.04±0.02	86.48±3.09^{a}	-0.57±1.04^{b}
		500	0.08±0.01^{ab}	5.60±2.09^{a}	0.06±0.02	94.82±4.75^{a}	-0.59±1.05^{b}
	380	0	0.05±0.00^{abcd}	3.49±1.22^{abc}	0.04±0.01	92.47±15.34^{a}	0.00±0.02^{ab}
		125	0.07±0.00^{ab}	4.61±0.48^{ab}	0.06±0.02	79.18±19.87^{ab}	0.01±0.00^{ab}
		500	0.07±0.01^{ab}	5.13±1.53^{ab}	0.06±0.02	93.79±6.49^{a}	0.02±0.00^{ab}
	1000	0	0.00±0.00^{f}	0.01±0.02^{f}	0.00±0.00	11.61±17.82^{d}	1.23±0.92^{ab}
		125	0.00±0.00^{f}	0.05±0.05^{f}	0.01±0.01	8.33±6.15^{d}	0.67±0.67^{ab}
		500	0.00±0.00^{f}	0.04±0.07^{f}	0.00±0.00	5.57±7.62^{d}	0.62±0.74^{ab}
25°	0	0	0.05±0.01^{abd}	2.92± 0.98^{abcd}	0.04±0.01	79.68±5.77^{ab}	1.44±1.05^{b}
		125	0.10±0.01^{a}	3.82±2.39^{abc}	0.10±0.02	41.98±27.70^{bcd}	0.20±0.30^{ab}
		500	0.06±0.00^{abd}	5.06±2.81^{ab}	0.21±0.29	56.49±36.34^{abc}	0.02±0.02^{ab}
	380	0	0.03±0.00^{cdef}	1.37±0.52^{cdef}	0.02±0.01	77.61±7.19^{ab}	-0.14±0.77^{b}
		125	0.05±0.00^{abd}	3.47±0.91^{abc}	0.04±0.01	87.81±3.36^{a}	-0.19±0.51^{b}
		500	0.05±0.03^{bcde}	2.14±3.41^{bcdef}	0.04±0.04	29.46±42.93^{cd}	-2.23±5.16^{a}
	1000	0	0.00±0.00^{f}	0.00±0.00^{f}	0.00±0.00	0.84±0.90^{d}	1.25±1.18^{ab}
		125	0.00±0.00^{f}	0.40±0.69^{ef}	0.19±0.34	1.94±0.11^{d}	1.35±0.12^{ab}
		500	0.01±0.00^{ef}	0.31±0.54^{ef}	0.01±0.02	9.16±13.82^{d}	0.72±0.72^{ab}
30°	0	0	0.01±0.00^{cdef}	0.47±0.25^{def}	0.02±0.01	24.43±6.78^{cd}	0.38±0.07^{ab}
		125	0.02±0.00^{bcdef}	1.09±0.15^{bcdef}	0.04±0.01	27.38±2.99^{cd}	-0.01±0.03^{b}
		500	0.02±0.00^{cdef}	0.85±0.18^{cdef}	0.03±0.00	30.99±6.97^{cd}	0.00±0.00^{ab}
	380	0	0.01±0.00^{cdef}	0.48±0.12^{def}	0.02±0.01	28.85±2.16^{cd}	0.39±0.10^{ab}
		125	0.04±0.01^{abd}	2.43±1.06^{abcde}	0.06±0.03	43.66±14.66^{bcd}	0.01±0.00^{ab}
		500	0.05±0.02^{abd}	3.12±1.72^{abcd}	0.05±0.03	61.12±5.67^{abc}	0.20±0.30^{ab}
	1000	0	0.00±0.00^{cdef}	0.03±0.06^{f}	0.00±0.01	3.81±5.51^{d}	0.92±1.01^{ab}
		125	0.01±0.02^{ef}	0.43±0.76^{ef}	0.01±0.01	18.52±31.22^{d}	0.00±0.01^{ab}
		500	0.02±0.04^{cdef}	1.13±1.97^{cdef}	0.04±0.07	9.17±15.03^{d}	0.00±0.01^{ab}

TABLE 5. Photosynthetic parameters (EQY (effective quantum yield); Pmax; α (photosynthetic efficiency); Ik (saturatuion parameter); and β (photoinhibition)) of *Dichotomaria marginata* cultivated for 14 days in VSES/4 modified enriched with different ammonium (NH_4^+) concentrations, CO_2 levels and temperatures (T), photoperiod of 14 h, salinity of 34 and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to the factorial ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

TREATMENTS			PHOTOSYNTHETIC PARAMETERS				
T (°C)	CO_2 (ppm)	$[NH_4^+]$ (µM)	EQY	Pmax	α	Ik	β
21°	0	0	0,05±0.00^{a}	3.35±0.33^{abc}	0.04±0.00^{abcd}	84.31±11.46^{a}	-1.43±3.06
		50	0.06±0.00^{a}	4.00±0.97^{ab}	0.05±0.01^{abc}	81.86±8.77^{a}	0.03±0.01
		100	0.07±0.00^{a}	5.10±0.50^{a}	0.06±0.01^{ab}	88.61±9.62^{a}	-1.84±3.82
	380	0	0.05±0.00^{a}	2.72±0.03^{bc}	0.06±0.01^{a}	44.21±9.19^{a}	0.00±0.00
		50	0.05±0.01^{a}	2.68±0.54^{bc}	0.04±0.02^{abcd}	70.52±16.42^{a}	0.20±0.33
		100	0.05±0.00^{a}	3.65±0.12^{ab}	0.04±0.00^{abc}	82.79±1.98^{a}	0.73±0.04
	1000	0	0.02±0.03^{bc}	1.69±1.92^{bc}	0.02±0.02^{cd}	56.66±47.46^{a}	0.47±0.80
		50	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{d}	0.63±0.00^{b}	0.00±0.00
		100	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{d}	0.63±0.00^{b}	0.00±0.00
25°	0	0	0.04±0.00^{abc}	2.56±0.39^{bc}	0.04±0.00^{abcd}	67.96±8.13^{a}	0.26±0.43
		50	0.04±0.00^{abc}	2.73±0.52^{bc}	0.04±0.00^{bcd}	77.62±7.97^{a}	0.49±0.41
		100	0.04±0.00^{abc}	2.85±0.29^{bc}	0.04±0.00^{bcd}	80.83±2.12^{a}	0.70±0.79
	380	0	0.04±0.00^{abc}	2.06±0.87^{bc}	0.03±0.01^{bc}	72.41±43.04^{a}	1.31±3.30
		50	0.06±0.00^{a}	4.16±0.78^{ab}	0.04±0.01^{abc}	100.27±7.77^{a}	-0.29±3.50
		100	0.05±0.00^{a}	3.46 ±1.14^{abc}	0.05±0.04^{abc}	68.35±14.27^{a}	0.00±0.00
	1000	0	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{c}	0.00±0.00
		50	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{c}	0.00±0.00
		100	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{c}	0.00±0.00
30°	0	0	0.03±0.00^{abc}	1.80±0.60^{bc}	0.03±0.01^{bcd}	53.73±8.62^{a}	0.30±0.25
		50	0.04±0.00^{abc}	2.71±0.73^{bc}	0.03±0.01^{bcd}	78.13±4.93^{a}	0.50±0.42
		100	0.04±0.00^{ab}	2.65±0.03^{bc}	0.03±0.00^{bcd}	76.75±1.69^{a}	-0.28±1.12
	380	0	0.01±0.02^{c}	1.47±1.37^{c}	0.02±0.02^{d}	85.56±6.88^{a}	0.53±0.11
		50	0.05±0.00^{a}	3.25±1.16^{abc}	0.05±0.01^{abc}	72.08±30.50^{a}	0.16±0.27
		100	0.05±0.00^{a}	3.87±1.07^{ab}	0.04±0.01^{ab}	89.15±9.70^{a}	0.18±0.29
	1000	0	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{c}	0.00±0.00
		50	0.00±0.00^{c}	0.00±0.00^{c}	0.01±0.01^{d}	0.51±0.20^{b}	0.69±1.19
		100	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{c}	0.00±0.00

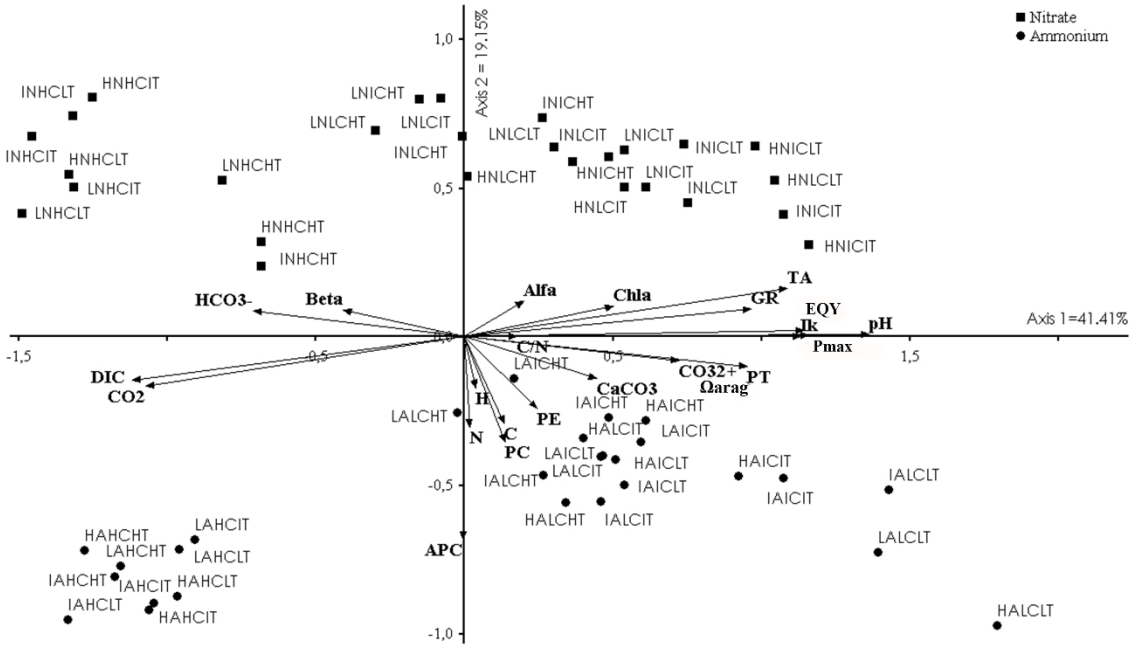

FIG. 9. Scatter diagram of plots of the first two principal component analysis axes of data on the effects of nitrate or ammonium, CO_2 levels and temperatures on growth rates (GR), pigment contents (APC, PC, PE and Chl*a*), protein contents (PT), calcification ($CaCO_3$), tissue elements (C, H, N and C/N ratio), photosynthetic parameters (EQY, Pmax, alpha, beta and Ik) and carbonate system of seawater (pH, T_A, CO_2, HCO_3^-, CO_3^{2-}, DIC and Ωarag) in *Dichotomaria marginata* cultured in VSES/4 modified enriched with different nitrate (black square) or ammonium (black circle) concentrations, photoperiod of 14 h, salinity of 34, and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. The first two components accounted for 60.56 % of total variance. LA – low ammonium, IA – intermediate ammonium, HA- high ammonium, LN- low nitrate, IN- intermediate nitrate, HN – high nitrate, LC – low CO_2, IC – intermediate CO_2, HC- high CO_2, LT- low temperature, IT- intermediate temperature and HT-high temperature.

TABLE 6. Pearson correlation coefficient among variables analyzed for *Dichotomaria marginata* to evaluate the effects of increased temperature, CO_2 levels and nitrogen availability (nitrate and ammonium) on growth rates, pigment, protein contents, calcification (%), element contents in the thallus (C, H, N, P and C/N ratio), photosynthetic parameters, carbonate system of seawater, and Ωarag (aragonite saturation state). The scatterplot diagram of PCA is shown in Fig. 9.

Variables	Principal components	
	Axis 1	Axis 2
Growth rate (GR)	0.803	0.251
Total soluble protein (PT)	0.797	-0.262
Allophycocyanin (APC)	-0.067	-0.674
Phycocyanin (PC)	0.305	-0.486
Phycoerythrin (PE)	0.407	0.164
Chlorophyll *a* (Chl *a*)	0.578	0.265
Calcification ($CaCO_3$)	0.547	-0.308
Carbon (C)	0.299	-0.443
Hydrogen (H)	0.163	-0.342
Nitrogen (N)	0.122	-0.451
C/N ratio (C/N)	0.345	-0.012
EQY (efficiency quantum yield)	0.872	0.126
Pmax (maximal ETR)	0.878	0.068
α (photosynthetic efficiency)	0.368	0.283
Ik (saturation irradiance)	0.868	0.015
β (photoinhibition)	-0.522	0.244
pH	0.952	0.070
T_A (total alkalinity)	0.851	0.329
CO_2	-0.845	-0.335
HCO_3^- (bicarbonate)	-0.689	0.240
CO_3^{2-} (carbonate)	0.693	-0.237
DIC (dissolved inorganic carbon)	-0.862	-0.316
Ωarag (aragonite saturation state)	0.693	-0.238
% of variance	41.41	19.15

Discussion

Our results showed that *Dichotomaria marginata* tolerated all treatments tested in the present study, including the increase of CO_2 levels (up to 1000 ppm), high temperatures (up to 30°C) and high nitrogen availabilities (500 or 100 µM of nitrate or ammonium, respectively). This species grew in both NO_3^- and NH_4^+ and the range of temperature variation of 9 °C (21 to 30°C). Similar results was observed in *Tricleocarpa fragilis* (Linnaues) Huisman & R.A Townsend, which had optimal growth in 25°C with 4 µM of NH_4^+. In temperatures of 30°C, however, growth of *T. fragilis* decreased (Su et al. 2009), as also observed in the present study for *D. marginata*.

Increase of CO_2 inhibited the growth and calcification in any nitrogen concentration or temperature, but *D. marginata* still survived in these conditions and continued to assimilate proteins, pigments, C, H, N, and performing photosynthesis. Negative effects of CO_2 on seaweed growth were reported in *Hypnea musciformis* (Wulfen) J.V. Lamouroux (Israel and Hophy 2002), *Saccharina latissima* (Linnaeus) C.E. Lane, C. Mayes, Druehl, & G.W. Saunders (Swanson and Fox 2007), *Lithophyllum* cf. *pallescens* (Foslie) Foslie, *Hydrolithon* sp. and *Porolithon* sp. (Jokiel et al. 2008), and *Fucus vesiculosus* Linnaeus (Gutow et al. 2014). These responses of different algal species can be explained by the existence of an enzymatic carbon concentrating mechanism (CCM) that allows for the acquisition of carbon from the pool of HCO_3^- in seawater (Israel and Hophy 2002, Gutow et al. 2014), and this effectiveness still depends on both the availability of CO_2 and other variables, such as nutrient availabilities and variations of temperature, which influence physiological responses (Xu et al. 2010, Diaz-Pulido et al. 2012).

N assimilated as proteins and pigments occurred in two forms in *Dichotomaria marginata*. In treatments with NO_3^-, the species accumulated more nitrogenous

compounds when cultivated with high N availability and with ambient CO_2, independent of temperature. In treatments with NH_4^+, accumulation occurred in high N concentrations with both low CO_2 and temperature. In general, the presence of high CO_2 concentrations and high temperature decreased the assimilation of these compounds and photosynthesis. Nitrogen metabolism and photosynthesis are integrally coupled, and treatments that inhibit photosynthetic carbon fixation limit the provision of carbon skeletons and decrease N assimilation into amino acids (Turpin 1991). In addition, high temperature may cause downregulation of phycobiliproteins, that influence light absorbance and energy transfer in photosynthesis (Xu et al. 2014), or initiate protein denaturation (Lobban and Harrison 2004).

C, N, and H contents of the thallus of *D. marginata* increased by enrichment with either NO_3^- or NH_4^+, and also stimulated by CO_2 availability. Therefore, C/N ratio decreases with high nutrient concentrations. Similar results were observed by Andria et al. (2009) when *Gracilaria* sp. was cultivated with high CO_2 (3.7 mM of DIC) and 75 µM of NO_3^-. High C and H contents in thallus of algae may be attributed to the accumulation of polysaccharides as storage energy (Serra 2013, Pacoda et al. 2014).

The percentage of calcification of *D. marginata* was stimulated by treatments with nitrate addition at 125 and 500 µM, and all NH_4^+ additions with 0 and 380 ppm of CO_2 at 21° and 25°C. High CO_2 availability and temperature decrease the percentage of calcification, and can reduce growth, photosynthesis and photosynthetic pigments.

Decreased carbonate concentration (CO_3^{2-}) in seawater caused by ocean acidification reduces the saturation state of aragonite and calcite, in turn reducing the deposition of $CaCO_3$ in the skeleton (Morse et al. 2006). This decreased calcification can result from increased dissolution of Ωaragonite at high temperatures and CO_2 (Table 2 and 3). Moreover, calcification of *D. marginata* thallus decreased with the reduction of

pH, which is consistent with results with other aragonite calcified species, including *Halimeda opuntia* (Linnaeus) J.V. Lamouroux, *H. taenicola* W.R. Taylor (Price et al. 2011), *Padina pavonica* (Linnaeus) Thivy, and *Padina australis* Hauck (Johnson et al. 2012).

Some studies have suggested that photosynthesis and calcification are directly related (Borowitzka and Larkum 1976, Martin et al. 2006). Laboratory studies with *Corallina sessilis* Yendo (Gao and Zheng 2010), *Halimeda macroloba* Decaisne and *H. cylindracea* Decaisne (Sinutok et al. 2011) have demonstrated reductions of pigment contents in high CO_2 level, a condition which is indicative of Chl *a* degradation, reduced photosynthesis and reduced photosystem II (PSII) reaction centers. The same results were observed in *Pyropia haitanensis* (T.J. Chang & B.F. Zheng) N. Kikuni & M. Miyata cultivated in 1000 ppm of CO_2 at 18 and 22℃ (Liu and Zou 2015). These results are in accordance with observations in the present study since the photosynthetic capacity of *D. marginata* decreased with reduced pH owing to high temperature and CO_2.

In summary, our results showed that *D. marginata* was able to survive climate changes at CO_2 and temperature levels predicted by CMIP5 and RCP 8.5 for 2100 (IPCC, 2013), as well as the increases of nitrogen availability owing to local impacts as eutrophication. At the same time, however, high CO_2 and temperature negatively affected growth, photosynthesis and calcification, while pigment and C/H contents increased. Thus, the present study is the first to evaluate the physiological and biochemical responses of *D. marginata* exposed to environmental stressors indicating that this species has survival strategies to tolerate the increase of CO_2 and temperature levels caused by global climate changes.

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Capítulo 4

Combined effects of rising temperature, CO_2 and nitrogen concentrations on the physiology and biochemistry of the coralline alga *Amphiroa fragilissima* (Corallinaceae, Rhodophyta)

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Abstract

The combined effects of increased CO_2, temperature and nitrogen availability on the metabolism and physiology of coralline alga are little studied. The coralline red alga *Amphiroa fragilissima* (L.) J.V. Lamouroux was cultured in laboratory conditions to assess the tolerance to increase of temperature and CO_2, at levels predicted by the Coupled Model Intercomparison Project Phase 5, Representative Concentration Pathways 8.5 to the year 2100, and current scenario according to Intergovernmental Panel on Climate Change, as well as nitrate or ammonium availabilities. The effects of temperatures (21, 25 and 30°C), CO_2 (0, 380 and 1000 ppm) and nitrogen availabilities (nitrate at 0, 125 and 500 µM or ammonium at 0, 50 and 100 µM) on the growth, protein, pigments and C, H and N contents, calcification and photosynthesis of were evaluated. The increased CO_2 affected negatively the physiology and metabolism of this species, however, the assimilation of nitrogen compounds, photosynthesis and calcification were highest with 500µM of nitrate in 1000 ppm of CO_2 at 25°C, and 50 µM of ammonium at 30°C. These results showed that *A. fragilissima* tolerate the highest level of CO_2, and temperature suggesting that this species could acclimate to the global climate changes.

Keywords: *Amphiroa fragilissima,* calcite, climate change, CO_2, nitrogen, temperature

Introduction

Red coralline algae (Corallinales, Rhodophyta) are the most abundant and important components of benthic marine communities, mainly in coral reef ecosystems, serving as support for the settlement and resilience of coral reefs and metamorphosis of coral larvae (Diaz – Pulido et al. 2012). Coralline algae deposit calcium carbonate in the form of high-magnesium calcite, and have global importance with the respect to coastal calcium carbonate ($CaCO_3$) deposition, carbon cycling and ecosystem engineering. (Semesi et al. 2009; Büdenbender et al. 2011; Egilsdottir et al. 2016). Calcifier organisms may be considered as an important carbon reserve (Blue Carbon) on oceans, and these organisms can convert and fix atmospheric CO_2 and stored it in their tissue (e.g., calcification, photosynthetic carbon fixation) (Trevathan-Tackett et al. 2015). Calcareous seaweeds have been threatened by increased atmospheric CO_2 levels from combustion of fossil fuels, which causes ocean acidification and global warming, along with decrease on calcium carbonate saturation levels of seawater (Egilsdottir et al. 2016).

The atmospheric CO_2 has increased from 280 ppm in the pre-industrial period to nearly 380 ppm in present day, and the Intergovernmental Panel on Climate Change (IPCC) emission scenarios suggest an increase to 1000 ppm of CO_2 by the year 2100 (IPCC, 2013). Estimates suggest that 30% of emitted CO_2 have been absorbed by the ocean and this absorbed CO_2 reacts with seawater, increasing H^+ protons, thereby reducing pH which causes ocean acidification, and decreased the calcite saturation state in seawater and the carbonate ion (CO_3^{2-}) levels which is used in calcareous algae calcification (Feely et al. 2004; Orr et al. 2005; Dickson et al. 2007). Furthermore, rising atmospheric CO_2 increases global temperature by the greenhouse effect of CO_2, and it is estimated that temperature will rise 4.8°C by 2100 (IPCC, 2013). If these changes occur,

they can have significant impacts on the biological systems and marine carbonates (Feely et al. 2004).

Mechanisms of acclimation to changes in water chemistry have been observed for coralline macroalgae, mainly involving calcification, as observed in the species of *Amphiroa foliacea* J.V. Lamouroux (Borowitzka 1981), *Neogoniolithon* sp. (Ries et al 2009) and *Ellisolandia elongata* (J.Ellis & Solander) K.R. Hind & G.W. Saunders (Egilsdottir et al. 2016). Most studies have only confirmed the effects of increasing CO_2 on physiology and metabolism of coralline seaweeds, for example, on calcification of *Lithothamnion glaciale* Kjellman (Büdenbender et al. 2011) and on photosynthesis of *Corallina pilulifera* Postels & Ruprecht (Gao et al. 1993), *Porolithon onkodes* (Anthony et al. 2008), and *Neogoniolithon* sp. (Ries et al. 2009).

Despite increasing concern over climate change in marine organisms, few studies have investigated the integrated effects of ocean acidification and ocean warming on coralline algae. Martin & Gattuso (2009) observed increased thallus necrosis and mortality, along with decreased calcification, of *Lithophyllum cabiochae* (Boudouresque & Verlaque) Athanasiadis exposed to 16.3℃ and 700 ppm of CO_2. Diaz-Pulido et al. (2012) reported bleaching, an increase of dead and pale tissue area, and decreased calcification of *Porolithon onkodes* (Heydrich) Foslie in 1000 to 1300 ppm of CO_2 at 29℃. Noisette et al. (2013) showed that primary production and respiration of *Lithothamnion corallioides* (P. Crouan & H. Crouan) P. Crouan & H. Crouan were not affected by a temperature increase from 10℃ to 19℃ and CO_2 increase of 380 ppm to 1000 ppm. However, contents of chlorophyll *a*, carotene, and zeaxanthin, as well as calcification, did decrease, mainly with the increase of temperature.

The effects of global climate change, together with the increase of nitrogen (N) caused by human activities (e.g., changing landscape, increasing concentration and

production of effluents), may change the metabolism and growth of seaweeds, and nitrogen is essential for growth and metabolism of this organisms (Lobban & Harrison 2004). High availability of nitrogen may decrease the occurrence of coralline algae in reef coral increasing the bleaching of thallus (Littler et al. 2006; 2009), and increase the soluble protein, phycocyanin and phycoerythrin contents of coralline alga, as observed in *Corallina elongata* J. Ellis & Solander (Vergara & Niell 1993).

The integrated effects of ocean acidification resulting from increased CO_2, in addition to enhanced temperature and nitrogen availability, on the metabolism and physiology of coralline algae are little known. Thus, the present study aimed to evaluate the effects of increased temperature and CO_2 concentration at the levels proposed by the Coupled Model Intercomparison Project Phase 5 (CMIP5), Representative Concentration Pathways (RCP) 8.5 to the year 2100, and current scenario according to IPCC (2013), as well as rising nitrate (NO_3^-) or ammonium (NH_4^+) concentration, on physiological and biochemical responses of the coralline red alga *Amphiroa fragilissima* (Linnaeus) J.V. Lamouroux cultured in laboratory conditions.

Material and Methods

Collection site and cultivation

Amphiroa fragilissima was collected in March (for nitrate experiments) and April 2015 (for ammonium experiments) at Fortaleza Beach, Ubatuba municipality, Sao Paulo State, southeastern Brazil (23º 50’ 15.6”S, 45º 17’ 40.5’’ W). Voucher specimens were deposited in the herbarium of Institute of Botany with accession numbers SP 428543 and SP 428544, for samples collected in March and April, respectively. Surface water temperature, pH and salinity were determined using a multi-parameter water probe (Horiba W23X). In March, the water temperature was 29.17 ± 0.08, pH 9.36 ± 0.03 and

salinity of 34. In April, water temperature was 26.89 ± 0.04, pH 9.32 ± 0.03 and salinity of 35.

After collection, algal samples were washed thoroughly with seawater to remove sand particles and epiphytes, stored in plastic bags, and transported to the laboratory in thermally insulated box at temperature of approximately 15-20ºC. In laboratory, the selected individuals were cleaned by washing with sterilized seawater, and epiphytic organisms were removed under stereomicroscopy. Apical segments (2 cm) were excised from cleaned individuals and acclimated for one week in culture medium composed of sterilized seawater enriched with quarter-strength of von Stosch's nutrient solution (VSES/4) following Oliveira *et al.* (1995), and modified with the reduction of 50% in vitamin concentrations, as described by Yokoya (2000), and addition of 1 $mg.L^{-1}$ of germanium dioxide to inhibit the growth of diatoms. Specimens were cultured in 500-mL Erlenmeyer with 400 ml of VSES/4 culture medium, salinity of 34 and temperature of 23 ± 3 °C under irradiances of 60 - 90 μmol photons m^{-2} s^{-1}, provided by cool-white fluorescent lamps with a 14:10 h light:dark cycle. Irradiance was measured with a quantum photometer (LI- 250, Li-COR, Lincoln, NE, USA) equipped with spherical underwater quantum sensor (LI- 250, Li-COR). All procedures were performed at the Laboratório de Cultura de Algas e Cianobactérias Marilza Cordeiro Marino, Instituto de Botanica, Sao Paulo municipality, Brazil.

Experiments of CO_2, nitrogen and temperature

A. fragilissima samples were cultivated in 500-ml Erlenmeyer flasks, each one maintaining a ratio of 0.3 g of seaweed per 400 mL of culture medium. Treatments were composed of sterilized seawater enriched with VSES/4 modified (von Stosch´s solution prepared without nitrate, but with salts of phosphate, iron, manganese, EDTA, and three

vitamins with concentration reduced to 50%, as already described above). Nitrogen concentrations and nitrogen sources were added to the VSES/4 modified medium according to the treatment to be tested. Nitrate ($NaNO_3$) or ammonium (NH_4Cl) were added to the medium in three concentrations: (1) low (0 µM of nitrate or ammonium), (2) intermediate (125 or 50 µM of nitrate or ammonium, respectively) and (3) high (500 or 100 µM of nitrate or ammonium, respectively).

CO_2 and temperature levels were tested according to different scenarios predicted by the IPCC for current scenario, and Model CMIP5 and RCP 8.5 predicted for 2100 (IPCC, 2013). Three levels of CO_2 were tested: (1) low (without CO_2 addition, assuming the concentration of 0 ppm), (2) intermediate (ambient air - 380 ppm of CO_2 pumped with an aquarium system (Boyu Electromagnetic Air Compressor ACQ-001), and (3) high (1000 ppm of CO_2 pumped using a pure CO_2 cylinder (P. ONU1013 2.2 – Oxylumen). Specimens were cultivated in culture chambers (Electrolab, Brazil), and three temperatures were tested: (1) low, 21°C; (2) intermediate, 25°C; and (3) high, 30°C.

Based on these nitrogen concentrations, CO_2 levels and temperature, ANOVA Factorial Design (3^3) was performed to obtain a combination of factors to be tested (Table 1). Experiments were carried out at salinity of 34, irradiances of 80 - 90 µmol photons m^{-2} s^{-1}, provided by cool-white fluorescent lamps with a 14:10 h light:dark cycle. Each treatment was tested with three replicates (n=3) in which both CO_2 flux and temperature were controlled daily. Culture medium was replaced each week after determining the variation on fresh biomass (mg), photosynthesis, alkalinity, pH, temperature and salinity of seawater. At the end of the experimental period (14 days), culture medium was renewed, and 4 days thereafter (18^{nd} day), samples of each replicate were frozen with liquid nitrogen and stored at -20 °C for analysis of protein and pigment contents. This

procedure was used to standardize the period between culture medium renewal and sample freezing since pigment and protein contents were influenced by this period.

Table 1: Factorial design of treatments performed for *Amphiroa fragilissima* cultivated with different concentrations of nitrogen (nitrate or ammonium), CO_2 and temperature.

Treatments	Factors			
	Nitrate (µM)	**Ammonium (µM)**	**CO_2 (ppm)**	**Temperature (ºC)**
1	0	0	0	21
2	0	0	0	25
3	0	0	0	30
4	0	0	380	21
5	0	0	380	25
6	0	0	380	30
7	0	0	1000	21
8	0	0	1000	25
9	0	0	1000	30
10	125	50	0	21
11	125	50	0	25
12	125	50	0	30
13	125	50	380	21
14	125	50	380	25
15	125	50	380	30
16	125	50	1000	21
17	125	50	1000	25
18	125	50	1000	30
19	500	100	0	21
20	500	100	0	25
21	500	100	0	30
22	500	100	380	21
23	500	100	380	25
24	500	100	380	30
25	500	100	1000	21
26	500	100	1000	25
27	500	100	1000	30

Determination of carbonate chemistry parameters

Total alkalinity (T_A) of the seawater of each treatment (n=3) was measured when the medium was renewed, using the titration method according to Dickson & Millero (1987). Temperature and pH were measured using pH meter coupled with temperature

sensor (Jenway 3020). Salinity was determined using a Refractometer (American Opticals, model 10440 T/C). $T_{A,}$ pH, salinity and temperature of seawater were used to calculate carbonate chemistry parameters (dissolved CO_2, bicarbonate – HCO_3^-, carbonate – CO_3^{2-}, dissolved inorganic carbon – DIC, and calcite saturation state - Ωcalc of seawater) (Tables 2 and 3), using the R program and Seacarb package (Dickson et al., 2007).

Growth rates

Fresh biomass was recorded weekly for 2 weeks, corresponding to replacement of medium. Growth rates (GR) were calculated from three replicates of each treatment and calculated as [ln (Bf • Bo^{-1}) • t^{-1}], where Bo is the initial fresh biomass, Bf is the fresh biomass after t days, and t corresponds to the experimental period (Yokoya et al., 2003).

Pigment and protein extraction and quantification

The algal mass (80 mg of fresh biomass for each replicate, n=3) was ground to a powder with liquid nitrogen and mixed with 50 mM phosphate buffer (pH 5.5). The homogenates were centrifuged at 14,000 g for 20 min. at 4ºC in order to separate the phycobiliproteins present in the supernatant. Chlorophyll *a* (Chl *a*) was extracted after dissolving the pellet in 90% acetone and centrifuging at 12,000 *g* for 15 min. at 4ºC. Pigments were quantified by spectrophotometry (Shimadzu-UV 1800). Concentrations of phycobiliproteins (phycoerythrin - PE, phycocyanin - PC and allophycocyanin - APC) were calculated according to Kursar et al. (1983), and the concentration of Chl *a* was calculated according to Jeffrey and Humphrey (1975).

For total soluble protein analysis, 80 mg of algal fresh biomass for each replicate (n=3) were ground with liquid nitrogen, and extractions were carried out at 4 °C using

0.2 M phosphate buffer (pH 8) containing 5 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM Dithiothreitol (DTT). Buffer was added in the proportion of 10 mL g^{-1} fresh biomass, and the homogenates were centrifuged at 12,000 g for 15 min. Total soluble protein contents were determined according to Bradford (1976), using a Bio-Rad protein assay kit and BSA as standard.

Calcification

The proportion of calcium carbonate ($CaCO_3$) was determined by weighing the fresh biomass of *A. fragilissima* samples of each treatment (n=3) tested in laboratory, and in algal samples collected in the field. Afterwards, the biomass was dried at 80 ° C for 48 to 72 h until reaching constant weight. Dry biomass was determined, and the samples transferred to Petri dishes were covered with hydrochloric acid (HCl) 5% for 48 hours, with renewal of acid after 24 hours. The sample returned to dry at 80 ° C for 48 to 72 hours and after this procedure, was immediately determined the weight of non-calcareous dry mass. The quantification of $CaCO_3$ (%) was calculated according to Digby (1977) modified by Amancio (2007), as:

$$CaCO_3 = [(\text{dry biomass} - \text{non-calcareous dry biomass}) / \text{dry biomass}] \bullet 100.$$

Internal C, H and N contents

At the end of the experimental period, samples of each treatment (n=3) were oven dried (60°C for 72 h) for analyses of thallus contents of internal carbon (C), hydrogen (H) and nitrogen (N), as determined by the Pregl-Dumas method with Perkin-Elmer 2400 Series II equipment. These analyses were performed by the Central Analítica core facility at the Instituto de Química, Universidade de São Paulo.

Photosynthetic parameters

Measurements of chlorophyll *a* fluorescence were estimated using a pulse amplitude-modulated (PAM) fluorometer (Diving PAM underwater fluorometer, Walz, Effeltrich, Germany). For each treatment, five apical segments of each replicate (n=3) were placed on the tip of the fiber-optic fluorometer using the magnet sample holder. Photosynthesis x irradiance (PI) curves consisted of the fluorescence responses to eleven increasing irradiance levels within range of 0 - 1564 µmol photons m^{-2} s^{-1}, using the "light curve" option of the Diving PAM. Two parameters were determined for each sample: (i) Effective quantum yield - EQY (II) = ΔF/Fm, where ΔF = Fm′ - Ft, Fm′ is the maximum fluorescence and Ft is the steady state fluorescence; and (ii) relative electron transport rate (ETR) = EQY(II)*PAR*ETR-factor*0.5, where the ETR-factor used was 0.84, and 0.5 is the factor related to proportion of Chl *a* at photosystem II (PSII).

The following photosynthetic parameters were calculated by PI curves according to equation of Platt et al. (1980), using KaleidaGraph (Synergy Software): photosyntetic efficiency (α), saturation irradiance (Ik), maximal ETR (Pmax) and photoinhibition parameter (β).

Statistical analyses

Data were analyzed by One-Way and Factorial ANOVA, followed by the Student-Newman-Keuls multiple comparison test, in order to distinguish significantly different results ($p < 0.05$), using STATISTICA software (version 9). For multivariate analysis, the data of growth rates (GR), pigment contents (APC, PC, PE, and Chl *a*), protein contents (PT), tissue elements (C, H, N and C/N ratio), photosynthetic parameters (ETR, EQY (effective quantum yield), α, Ik and Pmax), calcification ($CaCO_3$) and carbonate system of seawater (T_A, pH, CO_2, CO_3^{2-}, HCO_3^- , DIC (dissolved inorganic carbon) and Ωcalc

(calcite saturation state)) were used in a covariance matrix for principal component analyses (PCA) performed in a PC-ORD 6 software (MjM Software, USA). The variability of the data was adjusted by the method of ranging ($[X-X_{min})/(X_{max}-X_{min})]$) (Legendre & Legendre 1998), using the PC-ORD 6 software.

Table 2: Carbonate system of the seawater medium in which *Amphiroa fragilissima* samples were cultured with different nitrate concentrations, CO_2 levels and temperature for 14 days. Data are the mean of three replicates ± standard deviation. Treatments with distinct letters indicate significant differences according to the one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

T (°C)	CO_2 (ppm)	$[NO_3^-]$ (µM)	pH	T_A (mM)	CO_2 (mM)	HCO_3^- (mM)	CO_3^{2-} (mM)	DIC (mM)	Ωcalc
21°C	0	0	7.43 ± 0.05^{a}	2.27 ± 0.00	0.06 ± 0.01^{c}	2.16 ± 0.01^{ab}	0.06 ± 0.01^{bc}	2.27 ± 0.01^{c}	1.40 ± 0.15^{bc}
		125	7.46 ± 0.05^{a}	2.27 ± 0.00	0.05 ± 0.01^{c}	2.15 ± 0.01^{ab}	0.06 ± 0.01^{bc}	2.26 ± 0.01^{c}	1.53 ± 0.17^{bc}
		500	7.44 ± 0.06^{a}	2.27 ± 0.00	0.06 ± 0.01^{c}	2.15 ± 0.02^{ab}	0.06 ± 0.01^{bc}	2.27 ± 0.02^{c}	1.45 ± 0.19^{bc}
	380	0	7.97 ± 0.09^{a}	2.27 ± 0.00	0.02 ± 0.00^{c}	1.92 ± 0.06^{bc}	0.18 ± 0.03^{ab}	2.11 ± 0.04^{c}	4.28 ± 0.77^{a}
		125	8.05 ± 0.09^{a}	2.27 ± 0.00	0.01 ± 0.00^{c}	1.85 ± 0.07^{bc}	0.21 ± 0.03^{a}	2.08 ± 0.04^{c}	5.03 ± 0.80^{a}
		500	8.02 ± 0.02^{a}	2.27 ± 0.00	0.01 ± 0.00^{c}	1.88 ± 0.01^{bc}	0.20 ± 0.01^{a}	2.09 ± 0.01^{c}	4.72 ± 0.14^{a}
	1000	0	5.51 ± 0.27^{d}	2.27 ± 0.00	5.75 ± 3.92^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{c}	8.02 ± 3.92^{a}	0.02 ± 0.01^{c}
		125	5.53 ± 0.08^{d}	2.27 ± 0.00	4.86 ± 0.89^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{c}	7.13 ± 0.89^{a}	0.02 ± 0.00^{c}
		500	6.05 ± 0.56^{cd}	2.27 ± 0.00	2.35 ± 2.47^{bc}	2.26 ± 0.01^{a}	0.00 ± 0.00^{c}	4.62 ± 2.47^{bc}	0.10 ± 0.11^{c}
25°C	0	0	7.20 ± 1.03^{ab}	2.27 ± 0.00	0.02 ± 0.00^{c}	2.00 ± 0.03^{bc}	0.14 ± 0.02^{b}	2.16 ± 0.02^{c}	3.27 ± 0.40^{ab}
		125	7.84 ± 0.13^{a}	2.27 ± 0.00	0.02 ± 0.00^{c}	1.94 ± 0.07^{ab}	0.16 ± 0.04^{ab}	2.13 ± 0.04^{c}	3.97 ± 0.86^{a}
		500	7.79 ± 0.02^{a}	2.27 ± 0.00	0.02 ± 0.00^{c}	1.99 ± 0.01^{ab}	0.14 ± 0.01^{ab}	2.15 ± 0.01^{c}	3.43 ± 0.15^{ab}
	380	0	7.90 ± 0.08^{a}	2.27 ± 0.00	0.02 ± 0.00^{c}	1.92 ± 0.05^{bc}	0.18 ± 0.03^{ab}	2.11 ± 0.03^{c}	4.25 ± 0.62^{a}
		125	8.03 ± 0.15^{a}	2.27 ± 0.00	0.01 ± 0.00^{c}	1.82 ± 0.13^{c}	0.23 ± 0.07^{a}	2.06 ± 0.07^{c}	5.49 ± 1.58^{a}
		500	8.01 ± 0.13^{a}	2.27 ± 0.00	0.01 ± 0.00^{c}	1.84 ± 0.12^{c}	0.22 ± 0.06^{ab}	2.07 ± 0.06^{c}	5.25 ± 1.44^{a}
	1000	0	7.14 ± 0.82^{ab}	2.27 ± 0.00	0.76 ± 0.67^{c}	2.24 ± 0.04^{a}	0.02 ± 0.02^{c}	3.02 ± 0.69^{c}	0.39 ± 0.51^{c}
		125	5.94 ± 0.10^{cd}	2.27 ± 0.00	1.79 ± 0.37^{bc}	2.27 ± 0.00^{a}	0.00 ± 0.00^{c}	4.06 ± 0.37^{bc}	0.06 ± 0.01^{c}
		500	7.14 ± 1.01^{ab}	2.27 ± 0.00	0.02 ± 0.01^{c}	1.91 ± 0.20^{bc}	0.18 ± 0.10^{ab}	2.11 ± 0.11^{c}	4.44 ± 2.44^{a}
30°C	0	0	7.87 ± 0.43^{a}	2.27 ± 0.00	0.03 ± 0.00^{c}	2.07 ± 0.00^{ab}	0.10 ± 0.00^{b}	2.20 ± 0.00^{c}	2.50 ± 0.06^{ab}
		125	7.65 ± 0.03^{a}	2.27 ± 0.00	0.03 ± 0.00^{c}	2.05 ± 0.01^{b}	0.11 ± 0.01^{ab}	2.19 ± 0.01^{c}	2.75 ± 0.13^{ab}
		500	7.73 ± 0.04^{a}	2.27 ± 0.00	0.02 ± 0.00^{c}	2.01 ± 0.01^{bc}	0.13 ± 0.01^{ab}	2.16 ± 0.01^{c}	3.20 ± 0.14^{ab}
	380	0	7.78 ± 0.13^{a}	2.27 ± 0.00	0.02 ± 0.01^{c}	1.94 ± 0.09^{bc}	0.17 ± 0.04^{ab}	2.12 ± 0.05^{c}	4.08 ± 1.05^{a}
		125	7.87 ± 0.10^{a}	2.27 ± 0.00	0.02 ± 0.01^{c}	1.85 ± 0.17^{bc}	0.21 ± 0.09^{a}	2.08 ± 0.09^{c}	5.07 ± 2.08^{a}
		500	8.00 ± 0.15^{a}	2.27 ± 0.00	0.02 ± 0.00^{c}	1.90 ± 0.01^{bc}	0.18 ± 0.01^{ab}	2.10 ± 0.01^{c}	4.47 ± 0.22^{a}
	1000	0	5.76 ± 0.50^{cd}	2.27 ± 0.00	3.63 ± 2.61^{ab}	2.27 ± 0.01^{a}	0.00 ± 0.00^{c}	5.90 ± 2.61^{ab}	0.06 ± 0.07^{c}
		125	6.37 ± 0.01^{cd}	2.27 ± 0.00	0.63 ± 2.61^{c}	2.26 ± 0.00^{a}	0.01 ± 0.00^{c}	2.90 ± 0.01^{c}	0.15 ± 0.00^{c}
		500	6.33 ± 0.01^{cd}	2.27 ± 0.00	0.69 ± 0.01^{c}	2.26 ± 0.00^{a}	0.01 ± 0.00^{c}	2.95 ± 0.01^{c}	0.14 ± 0.00^{c}

T_A = total alkalinity, CO_2 = dissolved CO_2, HCO_3^- = bicarbonate concentration, CO_3^{2-} = carbonate concentration, DIC = dissolved inorganic carbon, Ωcalc = calcite saturation state in seawater.

Table 3: Carbonate system of the seawater medium in which *Amphiroa fragilissima* samples were cultured with different ammonium concentrations, CO_2 levels and temperature for 14 days. Data are the mean of three replicates ± standard deviation. Treatments with distinct letters indicate significant differences according to the one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

T (°C)	CO_2 (ppm)	$[NH_4^+]$ (µM)	pH	T_A (mM)	CO_2 (mM)	HCO_3^- (mM)	CO_3^{2-} (mM)	DIC (mM)	Ωcalc
21°C	0	0	7.01 ± 0.05^{bc}	2.27 ± 0.00^{a}	0.16 ± 0.02^{b}	2.23 ± 0.00^{a}	0.02 ± 0.00^{bc}	2.41 ± 0.02^{bc}	0.56 ± 0.06^{b}
		50	6.99 ± 0.06^{bc}	2.27 ± 0.00^{a}	0.16 ± 0.02^{b}	2.23 ± 0.01^{a}	0.02 ± 0.00^{bc}	2.41 ± 0.02^{c}	0.55 ± 0.07^{b}
		100	7.02 ± 0.08^{bc}	2.27 ± 0.00^{a}	0.15 ± 0.03^{b}	2.22 ± 0.01^{a}	0.02 ± 0.00^{bc}	2.40 ± 0.03^{bc}	0.58 ± 0.10^{b}
	380	0	8.32 ± 0.03^{a}	2.27 ± 0.00^{a}	0.01 ± 0.00^{c}	1.62 ± 0.03^{cd}	0.33 ± 0.02^{a}	1.95 ± 0.02^{c}	7.94 ± 0.41^{a}
		50	8.33 ± 0.08^{a}	2.27 ± 0.00^{a}	0.01 ±0.00^{c}	1.60 ± 0.09^{cd}	0.34 ± 0.05^{a}	1.94 ± 0.05^{c}	8.15 ± 1.15^{a}
		100	8.41 ± 0.07^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{c}	1.51 ± 0.08^{de}	0.38 ± 0.04^{a}	1.89 ± 0.04^{c}	9.28 ±0.91^{a}
	1000	0	6.00 ± 0.80^{d}	2.27 ± 0.00^{a}	3.48 ± 3.39^{a}	2.26 ± 0.02^{a}	0.01 ± 0.01^{d}	5.74 ± 3.40^{b}	0.15 ±0.23^{cd}
		50	5.35 ± 0.11^{d}	2.27 ± 0.00^{a}	7.54 ± 1.86^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{d}	9.81 ± 1.86^{a}	0.01 ± 0.00^{d}
		100	5,96 ± 0.60^{d}	2.27 ± 0.00^{a}	3.17 ± 3.60^{a}	2.27 ± 0.01^{a}	0.00 ± 0.00^{d}	5.44 ± 3.60^{b}	0.08 ±0.09^{cd}
25°C	0	0	8.32 ± 0.03^{ab}	2.27 ± 0.00^{a}	0.03 ± 0.00^{bc}	2.05 ± 0.01^{ab}	0.11 ± 0.01^{ab}	2.19 ± 0.01^{c}	2.71 ± 0.15^{ab}
		50	8.33 ± 0.08^{ab}	2.27 ± 0.00^{a}	0.03 ± 0.00^{bc}	2.03 ± 0.02^{ab}	0.12 ± 0.01^{ab}	2.18 ± 0.01^{c}	2.93 ± 0.26^{ab}
		100	8.41 ± 0.07^{ab}	2.27 ± 0.00^{a}	0.03 ± 0.00^{bc}	2.04 ± 0.00^{ab}	0.12 ± 0.00^{ab}	2.18 ± 0.00^{c}	2.82 ± 0.04^{ab}
	380	0	8.34 ± 0.04^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{c}	1.53 ± 0.04^{de}	0.37 ± 0.02^{a}	1.91 ± 0.02^{c}	9.01 ± 0.45^{a}
		50	8.57 ± 0.25^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{c}	1.25 ± 0.33^{e}	0.51 ± 0.16^{a}	1.76 ± 0.16^{c}	12.45 ± 3.96^{a}
		100	8.55 ± 0.13^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{c}	1.29 ± 0.16^{de}	0.49 ± 0.08^{a}	1.78 ± 0.08^{c}	11.97 ± 2.00^{a}
	1000	0	5.97 ± 0.36^{d}	2.27 ± 0.00^{a}	2.12 ± 1.76^{a}	2.27 ± 0.00^{a}	0.00 ± 0.00^{d}	4.39 ± 1.76^{b}	0.07 ± 0.05^{cd}
		50	5.97 ± 0.26^{d}	2.27 ± 0.00^{a}	1.89 ± 1.05^{a}	2.27 ± 0.00a	0.00 ± 0.00^{d}	4.16 ± 1.05^{b}	0.06 ± 0.04^{cd}
		100	7.14 ± 0.72^{bc}	2.27 ± 0.00^{a}	0.28 ± 0.42^{b}	2.16 ± 0.10^{a}	0.06 ± 0.05^{bc}	2.50 ± 0.46^{c}	1.39 ± 1.18^{b}
30°C	0	0	7.90 ± 0.08^{ab}	2.26 ± 0.00^{b}	0.02 ± 0.00^{bc}	1.92 ± 0.05^{abc}	0.17 ± 0.03^{ab}	2.11 ± 0.03^{c}	4.17 ± 0.64^{ab}
		50	8.13 ± 0.03^{a}	2.26 ± 0.00^{b}	0.01 ± 0.00^{c}	1.75 ± 0.03^{bcd}	0.26 ± 0.01^{ab}	2.01 ± 0.02^{c}	6.33 ± 0.35^{a}
		100	8.25 ± 0.23^{a}	2.26 ± 0.00^{b}	0.01 ± 0.00^{c}	1.61 ± 0.25^{cd}	0.33 ± 0.13^{a}	1.95 ± 0.13^{c}	7.98 ±3.07^{a}
	380	0	8.24 ± 0.21^{a}	2.26 ± 0.00^{b}	0.01 ± 0.00^{c}	1.62 ± 0.23^{d}	0.33 ± 0.12^{a}	1.95 ± 0.12^{c}	7.95 ± 2.81^{a}
		50	8.40 ± 0.19^{a}	2.26 ± 0.00^{b}	0.00 ± 0.00^{c}	1.44 ± 0.22^{de}	0.41 ± 0.11^{a}	1.86 ± 0.11^{c}	10.10 ± 2.68^{a}
		100	8.59 ± 0.01^{a}	2.26 ± 0.00^{b}	0.00 ± 0.00^{c}	1.21 ± 0.02^{e}	0.53 ± 0.01^{a}	1.74 ± 0.01^{c}	12.82 ± 0.18^{a}
	1000	0	5.79 ± 0.24^{d}	2.26 ± 0.00^{b}	2.74 ± 1.30^{a}	2.26 ± 0.00^{a}	0.00 ± 0.00^{d}	5.01 ± 1.30^{b}	0.04 ± 0.02^{cd}
		50	7.75 ± 0.52^{ab}	2.26 ± 0.00^{b}	0.04 ± 0.03^{bc}	1.93 ± 0.35^{abc}	0.17 ± 0.18^{ab}	2.13 ± 0.21^{c}	4.09 ± 4.31^{ab}
		100	6.57 ± 1.26^{cd}	2.26 ± 0.00^{b}	1.60 ± 1.69^{ab}	2.13 ± 0.24^{a}	0.07 ± 0.12^{cd}	3.79 ± 1.79^{bc}	1.71 ± 2.88^{bc}

T_A = total alkalinity, CO_2 = dissolved CO_2, HCO_3^- = bicarbonate concentration, CO_3^{2-} = carbonate concentration, DIC = dissolved inorganic carbon, Ωcalc = calcite saturation state in seawater**.**

Results

Tables 2 and 3 showed the variation of carbonate system of seawater with the variations of temperature, CO_2 levels and nitrogen concentrations. The increased CO_2 decreased the CO_3^{2-} availability, $\Omega_{calcite}$ and pH of seawater, while the HCO_3^- concentrations increased. However, exceptions were observed in nitrate concentrations of 500 µM, 1000 ppm of CO_2 at 25°C, and ammonium concentrations of 50 µM in 1000 ppm of CO_2 at 30°C. In these treatments, the pH, CO_3^{2-} concentration and $\Omega_{calcite}$ increased and HCO_3^- decreased.

In experiments with nitrate (NO_3^-), the growth rate (GR) of *A. fragilissima* was influenced by 125 and 500 µM of NO_3^- with 380 ppm of CO_2 at 21°C (Fig 1a). In contrast, GR was stimulated in 1) all additions of ammonium (NH_4^+) without CO_2 at 21°C; 2) 50 µM of NH_4^+ in 380 ppm of CO_2 at 21°C; and 3) 50 and 100 µM of NH_4^+ in 380 ppm of CO_2 at 25°C (Fig 1b). High CO_2 concentration inhibited the GR of this species with all nitrogen sources and concentrations at all temperatures, except 500 µM of NO_3^- at 25°C, conditions in which this species showed positive GR (Fig 1a, b).

The highest protein (PT) concentrations of *A. fragilissima* were observed with 1) NO_3^- additions of 125 and 500 µM in 0 and 380 ppm of CO_2 at 21°C; 2) all NO_3^- concentrations without CO_2 at 25° and 30°C; and 3) 125 and 500 µM of NO_3^- in 380 ppm of CO_2 at 25° and 30°C. The lowest PT concentrations occurred with all treatments with NO_3^- additions in 1000 ppm of CO_2 at 21°C, and with 0 and 125µM of NO_3^- in 1000 ppm of CO_2 at 25°C (Fig 2a). For ammonium, the highest PT contents of this species occurred with 100 µM of NH_4^+ in 380 ppm of CO_2 at 25°C and lowest PT concentration with 1) all NH_4^+ concentrations in 1000 ppm of CO_2 at 21 and 25°C and 2) without NH_4^+ additions in 1000 ppm of CO_2 at 30°C (Fig 2b).

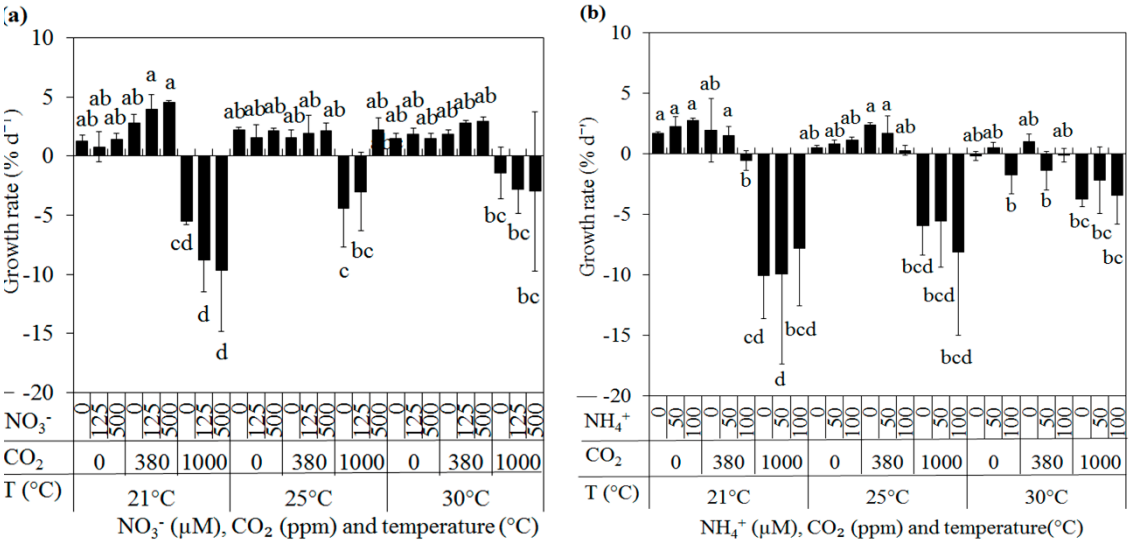

Fig 1: Growth rate (% d^{-1}) of *Amphiroa fragilissima* cultivated for 14 days in VSES/4 modified enriched with different (a) nitrate (NO_3^-) and (b) ammonium (NH_4^+) concentrations, different CO_2 levels and temperatures, photoperiod of 14 h, salinity of 34 and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to the one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

APC contents of *Amphiroa fragilissima* increased with 125 µM of NO_3^- in 380 ppm of CO_2 at 30°C. The same was observed for PE content, which was also high with 125 µM of NO_3^- in 380 ppm of CO_2 at 21°C. Lower APC and PE contents occurred in individuals cultured with 1000 ppm of CO_2 with all NO_3^- concentrations at 21°C and 0 and 125 µM of NO_3^- at 25°C (Fig 3a, c). PC concentrations were highest in samples cultured in all treatments, except 1000 ppm of CO_2 with 1) all NO_3^- additions at 21°C and 2) 0 and 125 µM of NO_3^- at 25 and 30°C, treatments which showed lower content of this pigment (Fig 3b). Optimal values of Chl *a* were observed in samples cultured with 500 µM of NO_3^- in 1000 ppm of CO_2 at 25°C. In contrast, low values of this pigment occurred

in algae treated with 1) 1000 ppm of CO_2 with all NO_3^- concentrations at 21°C; 2) 0 and 125 µM of NO_3^- in 1000 ppm of CO_2 at 25°C; and 3) 125 µM of NO_3^- in 1000 ppm of CO_2 at 30°C (Fig 3d).

In treatments with different concentrations of NH_4^+, low pigment contents of *A. fragilissima* were observed in 1000 ppm of CO_2 with 1) all NH_4^+ concentrations at 21 and 25°C and 2) without NH_4^+ addition at 30°C (Fig 4a, b, c, d). With addition of 100 µM of NH_4^+, high APC content was observed in treatments with 1) 0 ppm of CO_2 at 21 and 25°C and 2) with 380 ppm of CO_2 at 25°C. High APC content also occurred during cultivation without NH_4^+ and 1) without CO_2 addition at 25 and 30°C, and 2) 380 ppm of CO_2 at 21°C. High APC content was also observed in 50 µM of NH_4^+ and 380 ppm of CO_2 at 30°C (Fig 4a). PC content was high in samples cultured with 50 and 100 µM of NH_4^+ in 1) 0 and 380 ppm of CO_2 at 21 and 25°C and 2) without CO_2 at 30°C (Fig 4b). PC was increased in treatments with 1) all NH_4^+ additions in 0 and 380 ppm of CO_2 at 25 and 30°C and 2) 50 and 100 µM of NH_4^+ in 0 and 380 ppm of CO_2 at 21°C (Fig 4c). Concentrations of Chl *a* were all stimulated with 50 µM of NH_4^+ in 380 ppm of CO_2 at 25°C (Fig 4d).

Algal samples collected in the field showed 88.01 ± 9.70 % and 84.60 ± 13.88 of calcification on thallus for March and April months, respectively The calcification of this species was high in 1) all NO_3^- additions in 0 and 380 ppm CO_2 at 21 and 25°C; 2) 125 and 500 µM of NO_3^- without CO_2 at 30°C; and 3) without NO_3^- in 380 ppm of CO_2 at 30°C (Fig 5a). Lower calcification values were observed in 1000 ppm of CO_2 with 1) all treatments with NO_3^- additions at 21°C; 2) without NO_3^- at 25°C; and 3) 125 µM of NO_3^- at 30°C (Fig 5a). Tests with NH_4^+ stimulated high calcification with 1) 50 µM without CO_2 at 21°C; 2) all NH_4^+ concentrations in 380 ppm of CO_2 at 21°C; 3) all NH_4^+ concentrations without CO_2 at 25°C; 4) 0 and 50 µM in 380 ppm of CO_2 at 25°C; 5) 0

and 50 µM without CO_2 at 30°C; and 6) without NH_4^+ in 380 ppm of CO_2 at 30°C (Fig 5b). Lower calcification values were observed in 1) all NH_4^+ additions at 21°C; 2) 0 and 100 µM of NH_4^+ at 25°C; and 3) 100 µM of NH_4^+ at 30°C (Fig 5b).

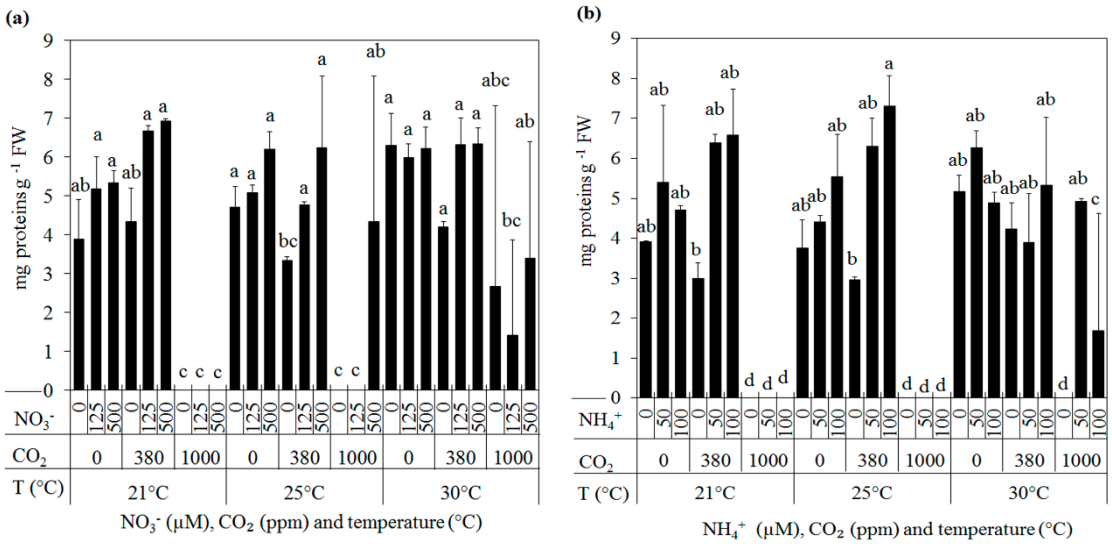

Fig 2: Concentration of total soluble protein of *Amphiroa fragilissima* cultivated for 18 days in VSES/4 modified enriched with different (a) nitrate (NO_3^-) and (b) ammonium (NH_4^+) concentrations, different CO_2 levels and temperatures, photoperiod of 14 h, salinity of 34, and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to the one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$). Treatments with no letter indicate that no fresh biomass could be obtained for the analyses.

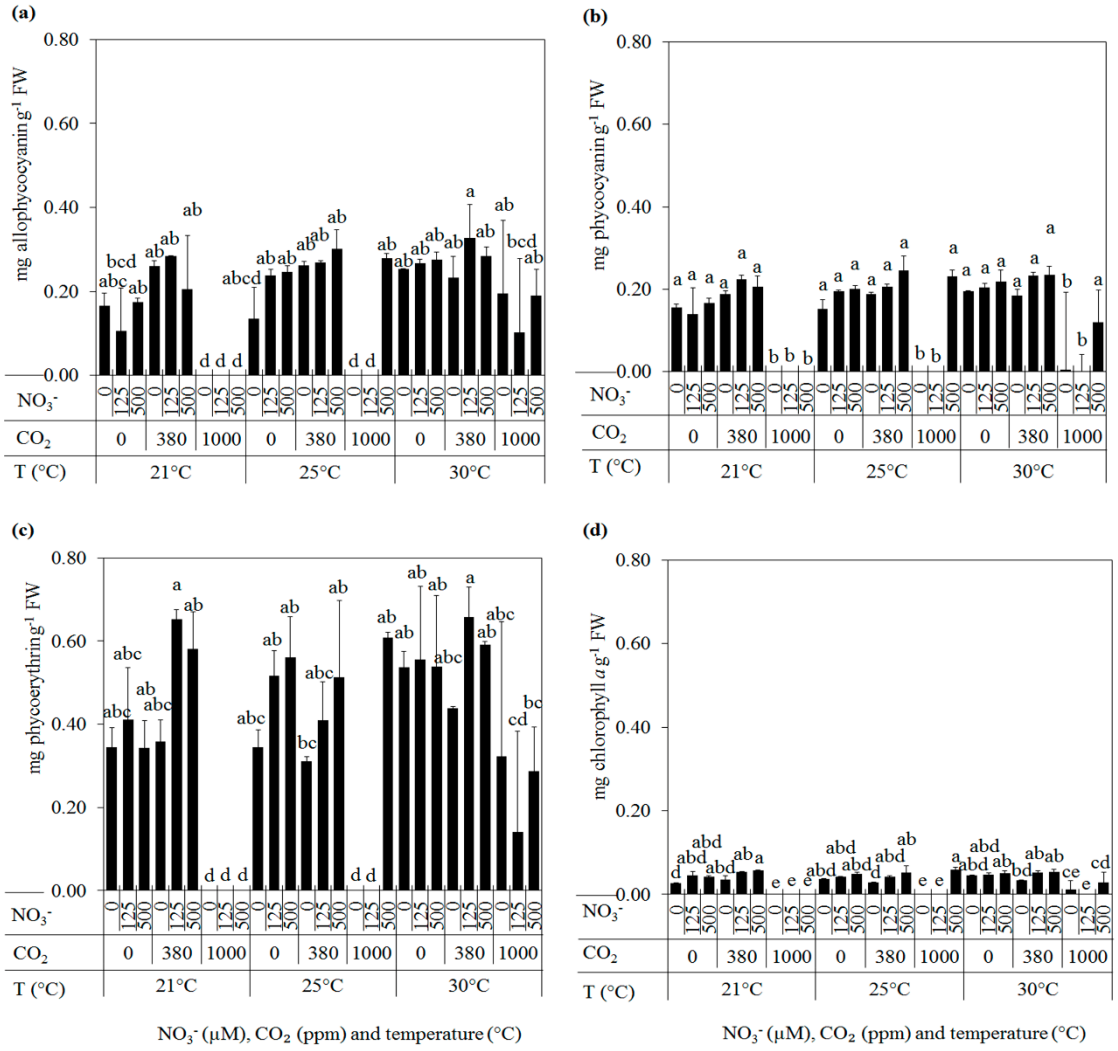

Fig 3: Concentration of (a) allophycocyanin, (b) phycocyanin, (c) phycoerythrin and (d) chlorophyll *a* of *Amphiroa fragilissima* cultivated for 18 days in VSES/4 modified enriched with different nitrate (NO_3^-) concentrations, CO_2 levels and temperatures, photoperiod of 14 h, salinity of 34, and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$). Treatments with no letter indicate that no fresh biomass could be obtained for the analyses.

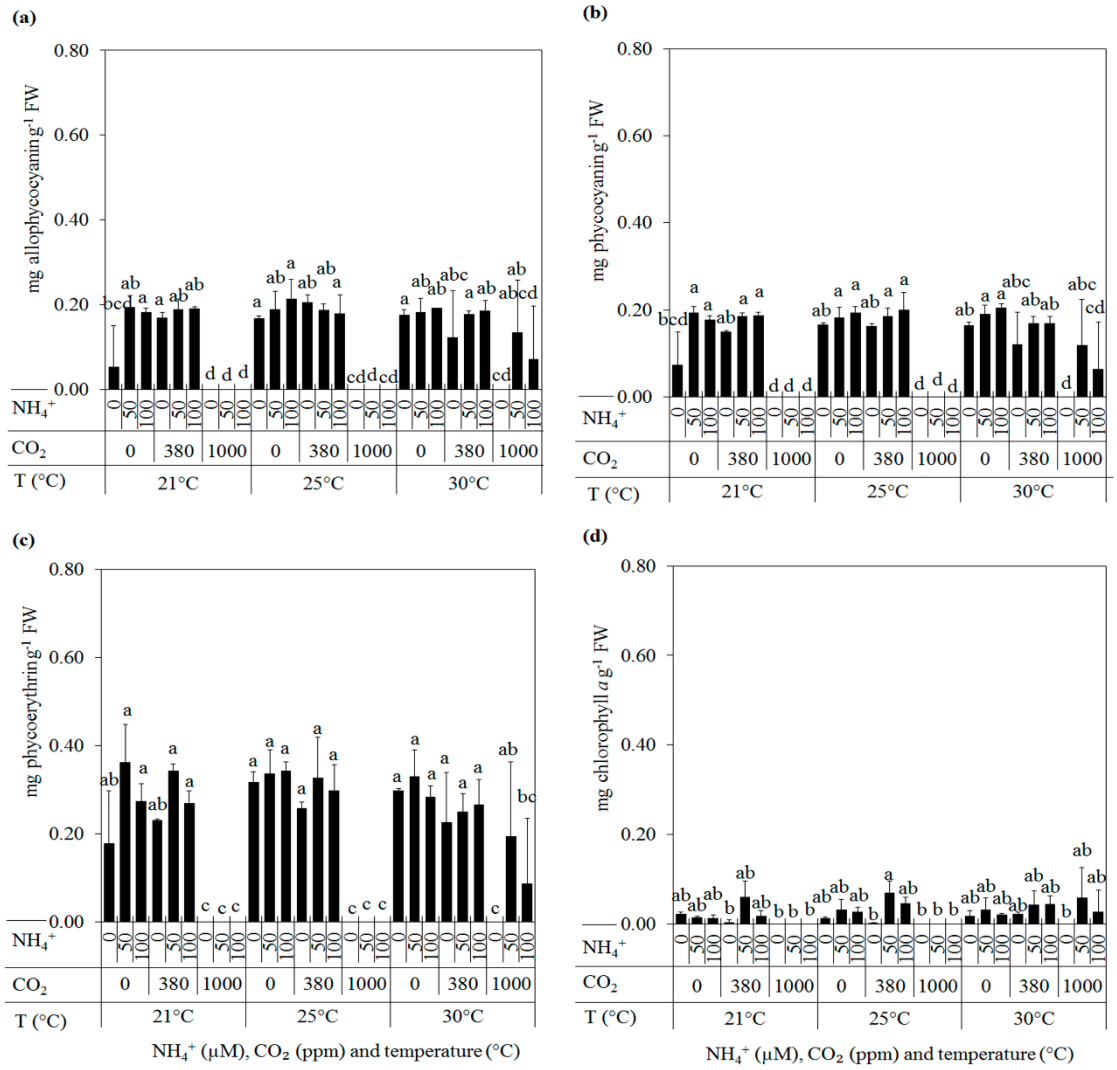

Fig 4: Concentration of (a) allophycocyanin, (b) phycocyanin, (c) phycoerythrin and (d) chlorophyll *a* of *Amphiroa fragilissima* cultivated for 18 days in VSES/4 modified enriched with different ammonium (NH_4^+) concentrations, CO_2 levels and temperatures, photoperiod of 14 h, salinity of 34, and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$). Treatments with no letter indicate that no fresh biomass could be obtained for the analyses.

High C content on thallus of *A. fragilissima* occurred in all NO_3^- concentrations with 0 and 380 ppm of CO_2 at 21 and 30℃. At 25℃, the high C content was observed with 1) 500 µM of NO_3^- without CO_2 and 2) 0 and 500 µM of NO_3^- in 380 ppm of CO_2 (Fig 6a). H concentration was high with 1) 125 and 500 µM of NO_3^- in 380 ppm of CO_2 at 21℃ and 2) 500 µM of NO_3^- in 1000 ppm of CO_2 at 25℃ (Fig 6b). High N content was observed with 500 µM of NO_3^- in 380 ppm of CO_2 at 21℃ and high C/N ratio occurred without NO_3^- and CO_2 at 25℃ (Fig 6c, d). Lower C, H and N content and C/N ratio occurred in 125 and 500 µM of NO_3^- in 1000 ppm of CO_2 at 21 and 21°C. C and H content was also lower in 125 µM of NO_3^- in 1000 ppm of CO_2 at 30℃, and C/N ratio was also lower in 0 and 125 µM of NO_3^- in 1000 ppm of CO_2 at 30℃ (Fig 6).

In treatments with NH_4^+ availabilities, C and H content on thallus of *A. fragilissima* were stimulated with 1) 0 and 50 µM of NH_4^+ in 380 ppm of CO_2 at 21°C; 2) all NH_4^+ concentrations in 380 ppm of CO_2 at 25 °C; 3) 50 µM of NH_4^+ without CO_2 at 25 and 30℃; and 4) 0 and 100 µM of NH_4^+ in 380 ppm of CO_2 at 30°C (Fig 7a, b). Low C and H concentrations occurred in 1000 ppm of CO_2 with 1) 50 and 100 µM of NH_4^+ at 21°C; 2) all NH_4^+ concentrations at 25℃; and 3) 0 and 100 µM of NH_4^+ at 30°C (Fig 7a, b). N content on thallus increased with the addition of 50 µM of NH_4^+ in 380 ppm of CO_2 at 21℃ and decreased in 1000 ppm of CO_2 with 1) all additions of NH_4^+ at 21 and 25℃ and 2) 0 and 100 µM of NH_4^+ at 30°C (Fig 7c). C/N ratio was stimulated without NH_4^+ addition in 380 ppm of CO_2 at 25°C, and lower C/N ratio occurred in 1) all treatments with high CO_2 levels and temperatures; 2) 0 and 100 µM of NH_4^+ without CO_2 at 21 and 25℃; and 3) 100 100 µM of NH_4^+ without CO_2 at 30°C (Fig 7d).

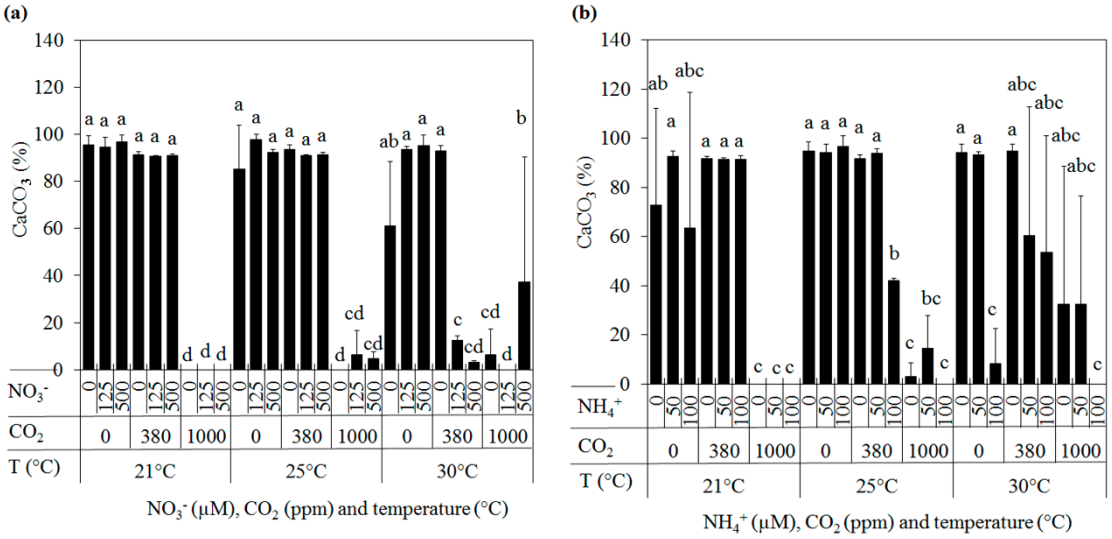

Fig 5: Calcification of *Amphiroa fragilissima* cultivated for 14 days in VSES/4 modified enriched with different (a) nitrate (NO_3^-) and (b) ammonium (NH_4^+) concentrations, different CO_2 levels and temperatures, photoperiod of 14 h, salinity of 34, and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

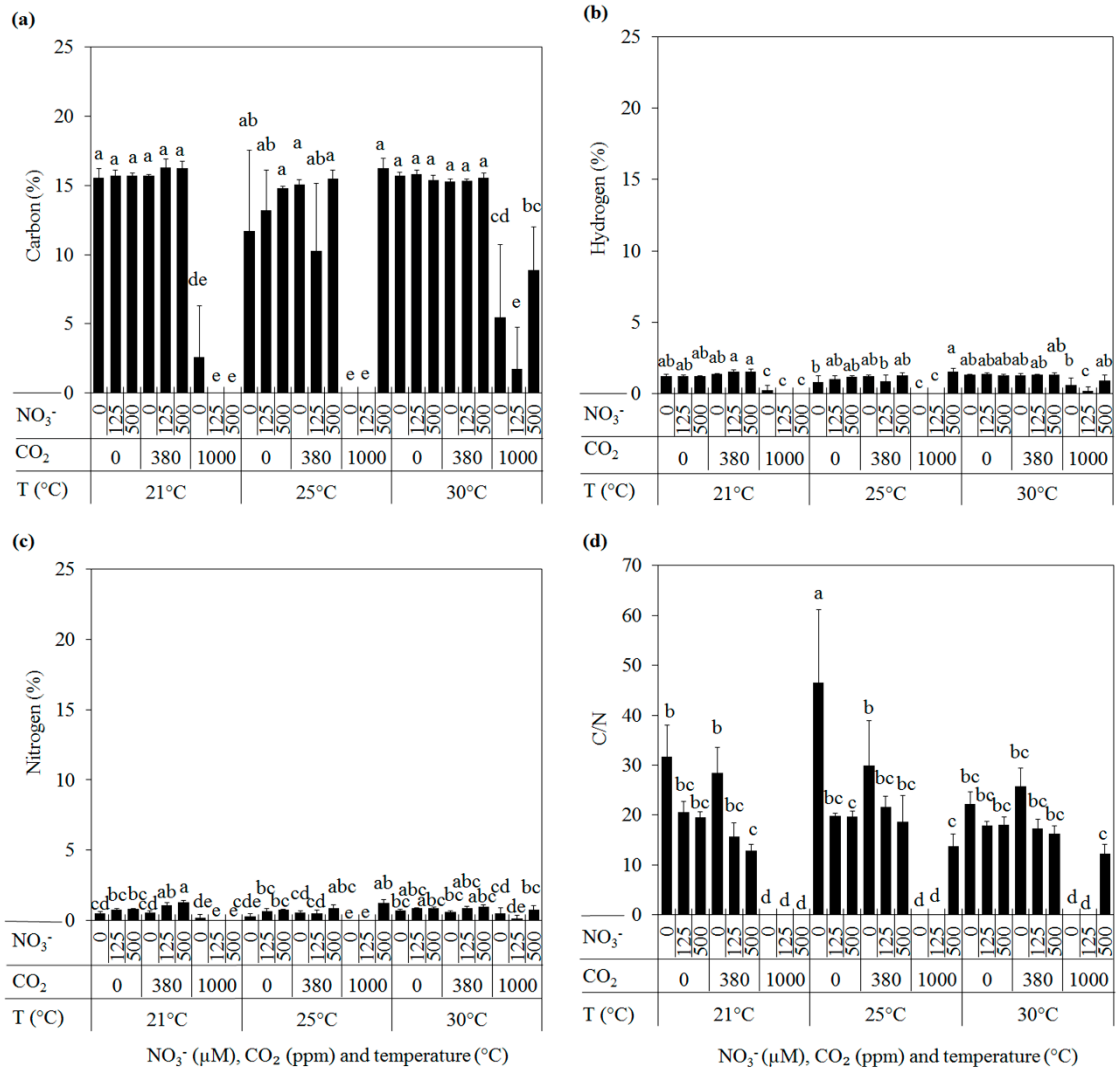

Fig 6: Concentration of internal carbon (a), hydrogen (b), nitrogen (c), and C/N ratio of *Amphiroa fragilissima* cultivated for 18 days in VSES/4 modified enriched with different nitrate (NO_3^-) concentrations, CO_2 levels and temperatures, photoperiod of 14 h, salinity of 34, and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

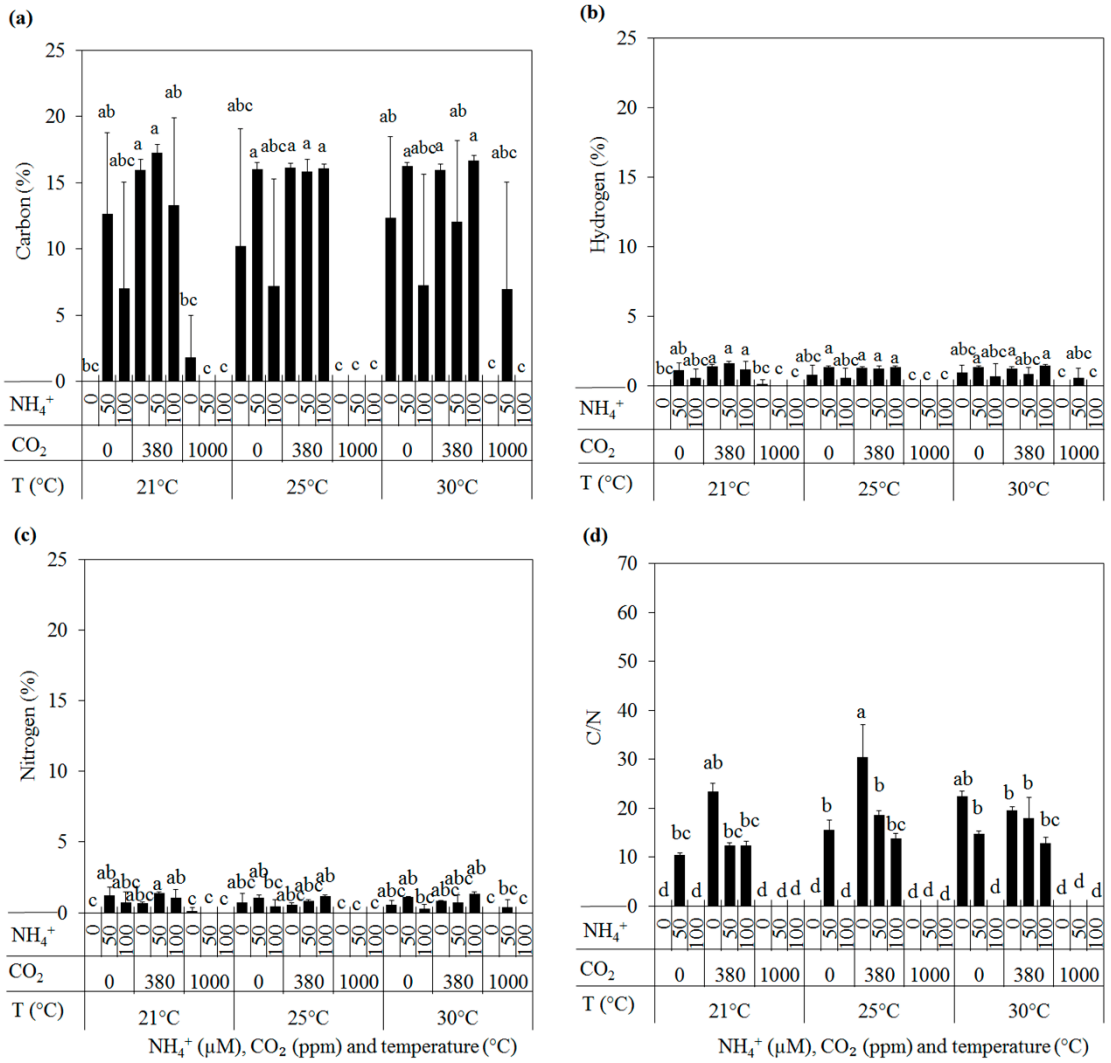

Fig 7: Concentration of internal carbon (a), hydrogen (b), nitrogen (c) and C/N ratio (d) of *Amphiroa fragilissima* cultivated for 18 days in VSES/4 modified enriched with different ammonium (NH_4^+) concentrations, CO_2 levels and temperatures, photoperiod of 14 h, salinity of 34, and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to one-way ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

Photosynthetic responses of *A. fragilissima* to increased nitrogen, CO_2 and temperature are presented in PI curves in Figure 8. PI curves was influenced by NO_3^- concentration, CO_2 and temperature (*F= 36.69* and *p= 0.00*), as well as irradiance (*F= 63.46* and *p= 0.00*). ETR values were high at 500 µM of NO_3^- in 1000 ppm of CO_2 at 25°C and in 480 µmol photons m^{-2} s^{-1} (Fig 8c). Furthermore, this treatment increased values of EQY, Pmax and α (Table 4). ETR values decreased mainly at 21°C, and at all temperatures and lower ETR values occurred in tests with 0 and 125 µM of NO_3^- in 1000 ppm of CO_2 (Fig 8a, b, c). Moreover, PI curves showed saturation with increased irradiance, and higher Ik occurred in 500 µM of NO_3^- in 380 ppm of CO_2 at 25°C (Table 4). Low EQY and Ik values occurred in 1000 ppm of CO_2 with 1) 0 and 125 µM of NO_3^- at 21°C; 2) 125 µM of NO_3^- at 25°C; and 3) without NO_3^- at 30°C (Table 4). The values of β did not vary between treatments tested (Table 4).

PI curves showed the influence of NH_4^+, CO_2 concentrations, different temperatures (*F= 59.67* and *p=0.00*) and increase of irradiance (*F= 96.65* and *p= 0.00*) on the photosynthesis of *A. fragilissima* (Fig 8d, e, f). A high ETR value was observed with the addition of 100 µM in 380 ppm of CO_2 at 21°C and 335 µmol photons m^{-2} s^{-1}, but a low ETR value occurred for samples cultivated in all NH_4^+ concentrations in 1000 ppm of CO_2 at 25 and 30°C. Table 5 shows the photosynthetic parameters obtained from PI curves. EQY, Pmax and IK were highest in 100 µM of NH_4^+ without CO_2 at 21°C, but they decreased in 1000 ppm of CO_2 with 1) all NH_4^+ additions at 21°C and 2) 100 µM at 30°C (Table 5). High α value occurred in 100 µM of NH_4^+ in without CO_2 at 30 °C and decreased in 1) all NH_4^+ concentrations, without and with 380 ppm of CO_2, at 21°C and 2) 100 µM of NH_4^+ with 380 ppm of CO_2 at 30°C. The value of β was highest without NH_4^+ and CO_2 additions at 30°C.

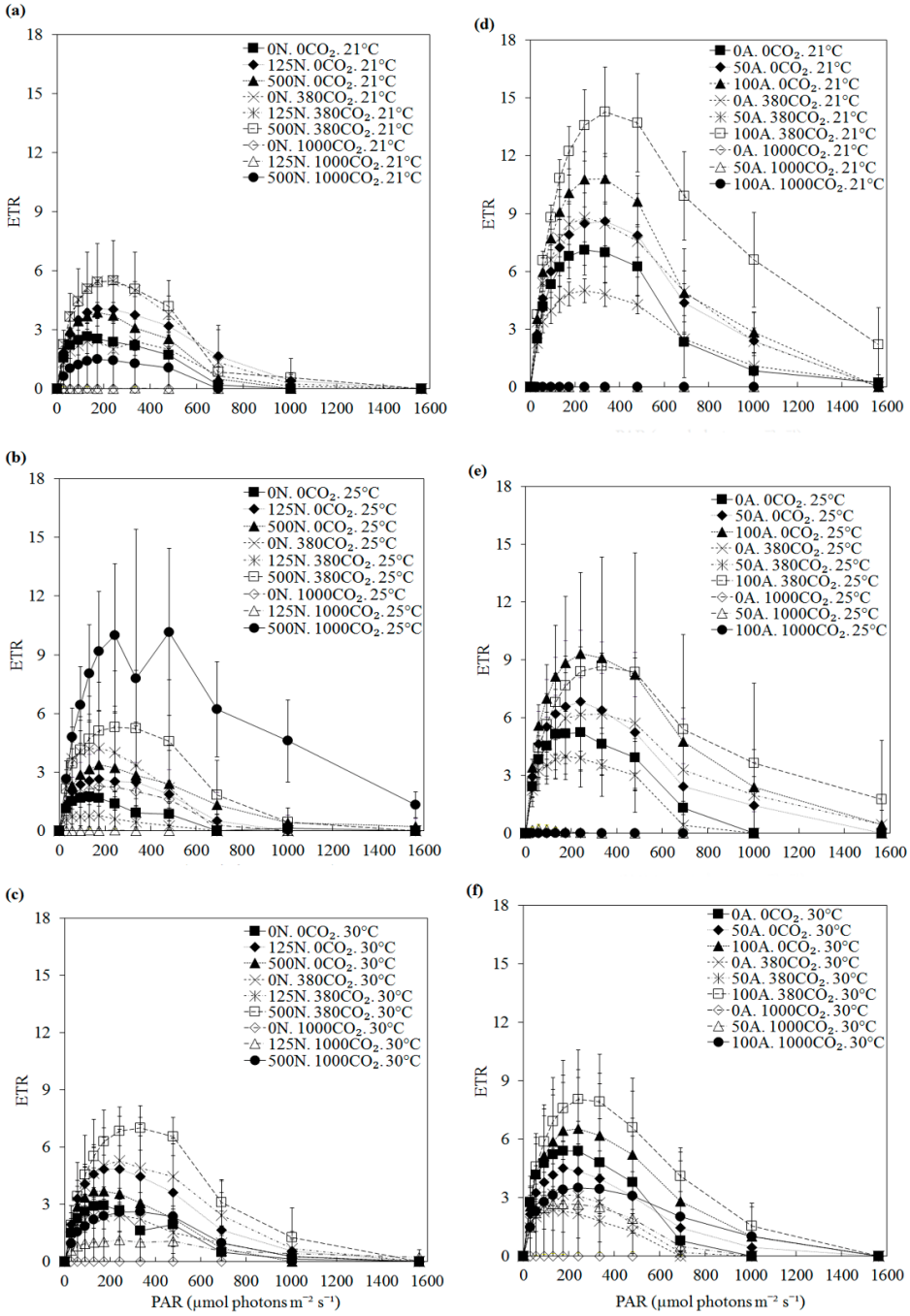

Fig 8: Photosynthesis x irradiance (PI) curve of *Amphiroa fragilissima* cultivated for 14 days in VSES/4 modified enriched with different nitrate (NO_3^-) (a, b, c) or ammonium (NH_4^+) (d, e, f) concentrations, CO_2 levels and temperatures, photoperiod of 14 h, salinity of 34, and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation.

Table 4: Photosynthetic parameters (EQY (effective quantum yield); Pmax, α (photosynthetic efficiency), Ik (saturation irradiance); and β (photoinhibition)) of *Amphiroa fragilissima* cultivated for 14 days in VSES/4 modified enriched with different nitrate (NO_3^-) concentrations, CO_2 levels and temperatures (T), photoperiod of 14 h, salinity of 34 and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to the factorial ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

TREATMENTS			PHOTOSYNTHETIC PARAMETERS				
T (°C)	CO_2 (ppm)	$[NO_3^-]$ (µM)	EQY	Pmax	α	Ik	β
21°C	0	0	0.05±0.00^{ab}	2.42±0.99^{b}	0.06±0.01^{ab}	43.92±20.80^{ab}	0.03 ± 0.02
		125	0.06±0.00^{abc}	2.86±2.57^{b}	0.06±0.01^{ab}	43.83±39.35^{ab}	-0.74 ± 1.32
		500	0.06±0.00^{abc}	4.05±0.36^{ab}	0.07±0.00^{ab}	60.75±4.79^{ab}	0.46 ± 0.66
	380	0	0.04±0.00^{abc}	2.59±0.55^{b}	0.04±0.01^{ab}	58.84±3.30^{ab}	0.02 ± 0.02
		125	0.08±0.01^{ab}	5.73±2.06^{ab}	0.08±0.02^{ab}	69.57±5.32^{ab}	1.69 ± 2.27
		500	0.08±0.00^{ab}	5.72±0.15^{b}	0.08±0.01^{ab}	72.10±6.48^{ab}	0.04 ± 1.42
	1000	0	0.00±0.00^{c}	0.00±0.00^{b}	0.00±0.00^{ab}	0.00±0.00^{b}	0.00 ± 0.00
		125	0.00±0.00^{c}	0.43±0.98^{b}	0.17±0.59^{ab}	1.12±0.68^{b}	1.07 ± 1.00
		500	0.02±0.03^{bc}	1.54±2.67^{b}	0.02±0.04^{ab}	22.71±38.24^{ab}	0.41 ± 0.72
25°C	0	0	0.03±0.00^{bc}	1.79±0.27^{b}	0.05±0.01^{ab}	37.49±0.73^{ab}	0.02 ± 0.02
		125	0.04±0.00^{abc}	2.86±0.61^{b}	0.04±0.01^{ab}	64.64±12.04^{ab}	0.20 ± 0.29
		500	0.05±0.00^{abc}	3.42±0.77^{b}	0.05±0.01^{ab}	62.83±2.32^{ab}	0.02 ± 0.00
	380	0	0.02±0.01^{bc}	0.88±0.48^{b}	0.02±0.00^{ab}	35.10±18.52^{ab}	0.15 ± 0.22
		125	0.07±0.02^{ab}	4.62±1.95^{ab}	0.09±0.03 ^{ab}	51.75±14.30^{ab}	0.97 ± 0.78
		500	0.07±0.01^{ab}	5.64±2.83^{ab}	0.07±0.02^{ab}	77.23±18.08^{a}	0.91 ± 0.75
	1000	0	0.03±0.03^{bc}	2.38±4.00^{b}	0.04±0.06^{ab}	30.93±35.77^{ab}	0.52 ± 0.64
		125	0.00±0.00^{c}	0.00±0.00^{b}	0.00±0.00^{ab}	1.08±0.68^{b}	1.13 ± 1.02
		500	0.11±0.01^{a}	12.04±5.08^{a}	0.38±0.45^{a}	61.46±50.86^{ab}	0.06 ± 0.06
30°C	0	0	0.05±0.00^{abc}	2.98±0.50^{b}	0.06±0.00^{ab}	50.83±5.51^{ab}	0.31 ± 0.49
		125	0.07±0.02^{ab}	5.20±3.24^{ab}	0.07±0.04^{ab}	70.00±8.49^{ab}	0.31 ± 0.44
		500	0.06±0.00^{abc}	3.95±0.25^{ab}	0.07±0.01^{ab}	61.34±9.54^{ab}	0.11 ± 0.11
	380	0	0.04±0.01^{abc}	2.68±0.61^{b}	0.05±0.02^{ab}	59.20±27.13^{ab}	0.03 ± 0.03
		125	0.07±0.01^{abc}	5.46±1.74^{ab}	0.06±0.01^{ab}	84.21±8.98^{a}	-0.11 ± 0.85
		500	0.08±0.00^{ab}	4.79±4.26^{ab}	0.07±0.00^{ab}	65.40±57.77^{ab}	0.00 ± 0.00
	1000	0	0.00±0.00^{c}	0.00±0.00^{b}	0.00±0.00^{ab}	0.00±0.00^{b}	0.00 ± 0.00
		125	0.02±0.03^{bc}	1.11±1.93^{b}	0.02±0.04^{ab}	17.69±29.54^{ab}	0.00 ± 0.00
		500	0.03±0.04^{bc}	2.69±4.67^{b}	0.03±0.05^{ab}	33.67±47.35^{ab}	0.38 ± 0.66

Table 5: Photosynthetic parameters (EQY (effective quantum yield); Pmax; α (photosynthetic efficiency); Ik (saturatuion parameter); and β (photoinhibition)) of *Amphiroa fragilissima* cultivated for 14 days in VSES/4 modified enriched with different ammonium (NH_4^+) concentrations, CO_2 levels and temperatures (T), photoperiod of 14 h, salinity of 34 and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1}. Each data point is the mean of three replicates, and bars are standard deviation. Treatments with distinct letters indicate significant differences according to the factorial ANOVA and Student-Newman-Keuls multiple comparison tests ($p < 0.05$).

TREATMENTS			PHOTOSYNTHETIC PARAMETERS				
T (°C)	CO_2 (ppm)	$[NH_4^+]$ (µM)	EQY	Pmax	α	Ik	β
21°C	0	0	0.09±0.00^{ab}	7.37±1.37^{bc}	0.09±0.01^{b}	83.54±9.59^{abc}	0.92±0.83^{ab}
		50	0.10±0.00^{ab}	8.86±2.14^{ab}	0.09±0.01^{b}	93.89±9.35^{ab}	1.05±0.94^{ab}
		100	0.13±0.01^{ab}	11.14±1.42^{ab}	0.12±0.01^{b}	92.82±2.76^{ab}	0.75±0.98^{ab}
	380	0	0.08±0.01^{abc}	5.20±0.74^{bc}	0.07±0.01^{ab}	72.35±4.22^{bc}	0.02±0.01^{ab}
		50	0.12±0.00^{ab}	9.21±1.09^{ab}	0.11±0.01^{b}	84.34±4.30^{abc}	0.50±0.80^{ab}
		100	0.15±0.00^{a}	14.21±2.76^{a}	0.13±0.01^{b}	107.12±12.19^{a}	0.07±0.03^{ab}
	1000	0	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{ab}	0.63±0.00^{d}	0.00±0.00^{ab}
		50	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{ab}	0.32±0.30^{d}	0.00±0.00^{ab}
		100	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{ab}	0.63±0.00^{d}	0.00±0.00^{ab}
25°C	0	0	0.08±0.01^{ab}	5.61±1.17^{bc}	0.08±0.02^{b}	69.63±3.21^{abc}	0.13±0.08^{ab}
		50	0.10±0.01^{ab}	7.11±2.76^{bc}	0.10±0.02^{b}	69.18±13.90^{abc}	0.06±0.04^{ab}
		100	0.12±0.01^{ab}	9.68±1.08^{ab}	0.11±0.02^{b}	86.00±5.38^{abc}	0.09±0.06^{ab}
	380	0	0.07±0.01^{abc}	4.34±1.32^{bc}	0.08±0.01^{ab}	56.19±20.60^{bc}	0.46±0.76^{ab}
		50	0.09±0.01^{ab}	6.56±3.03^{bc}	0.08±0.02^{ab}	81.92±15.64^{abc}	0.31±0.49^{ab}
		100	0.10±0.02^{ab}	8.79±5.48^{ab}	0.09±0.05^{b}	97.59±9.61^{ab}	-2.27±4.04^{ab}
	1000	0	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{ab}	0.67±0.07^{d}	0.66±1.15^{ab}
		50	0.00±0.00^{c}	0.22±0.20^{c}	0.01±0.01^{ab}	13.53±11.88^{d}	0.17±0.15^{ab}
		100	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{ab}	0.63±0.00^{d}	0.00±0.00^{ab}
30°C	0	0	0.09±0.01^{ab}	5.82±0.50^{bc}	0.09±0.01^{b}	65.57±6.58^{bc}	13.03±20.53^{a}
		50	0.07±0.01^{abc}	4.68±1.37^{bc}	0.07±0.02^{ab}	64.89±1.63^{bc}	1.29±2.19^{b}
		100	0.09±0.02^{ab}	4.54±4.51^{bc}	9.32±16.01^{a}	43.93±44.61^{c}	9.29±16.02^{ab}
	380	0	0.04±0.01^{bc}	2.43±1.55^{bc}	0.05±0.02^{ab}	45.39±15.89^{c}	0.04±0.01^{ab}
		50	0.05±0.01^{bc}	3.10±0.45^{bc}	0.06±0.01^{ab}	50.41±6.88^{bc}	-1.73±1.53^{ab}
		100	0.10±0.02^{ab}	8.33±1.13^{ab}	0.09±0.03^{b}	91.93±15.37^{ab}	-2.32±6.45^{ab}
	1000	0	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{ab}	0.63±0.00^{d}	0.00±0.00^{ab}
		50	0.04±0.04^{bc}	2.97±2.80^{bc}	0.05±0.05^{ab}	42.95±37.72^{c}	-0.98±1.75^{ab}
		100	0.05±0.07^{bc}	4.30±7.45^{bc}	0.02±0.05^{ab}	0.42±0.36^{d}	0.21±0.37^{ab}

Principal components (PCA) analysis explained total variability of 75.83% in the first two axes, variability of 58.82% in the first axis and 17.01% in the second axis (Fig 9). The positive sides of axis 1 grouped treatments with high CO_2 concentrations independent temperatures and nitrate or ammonium concentrations tested with CO_2, HCO_3^- and DIC of seawater. The negative sides of axis 1 grouped mainly low and intermediate CO_2 with GR, PT, APC, PC, PE, Chl *a*, $CaCO_3$, C, H, N, C/N ratio, EQY, Pmax, Ik, pH, CO_3^{2-} and Ωcalc of seawater. The positive and negative sides of axis 2 did not group together any variables (Table 6).

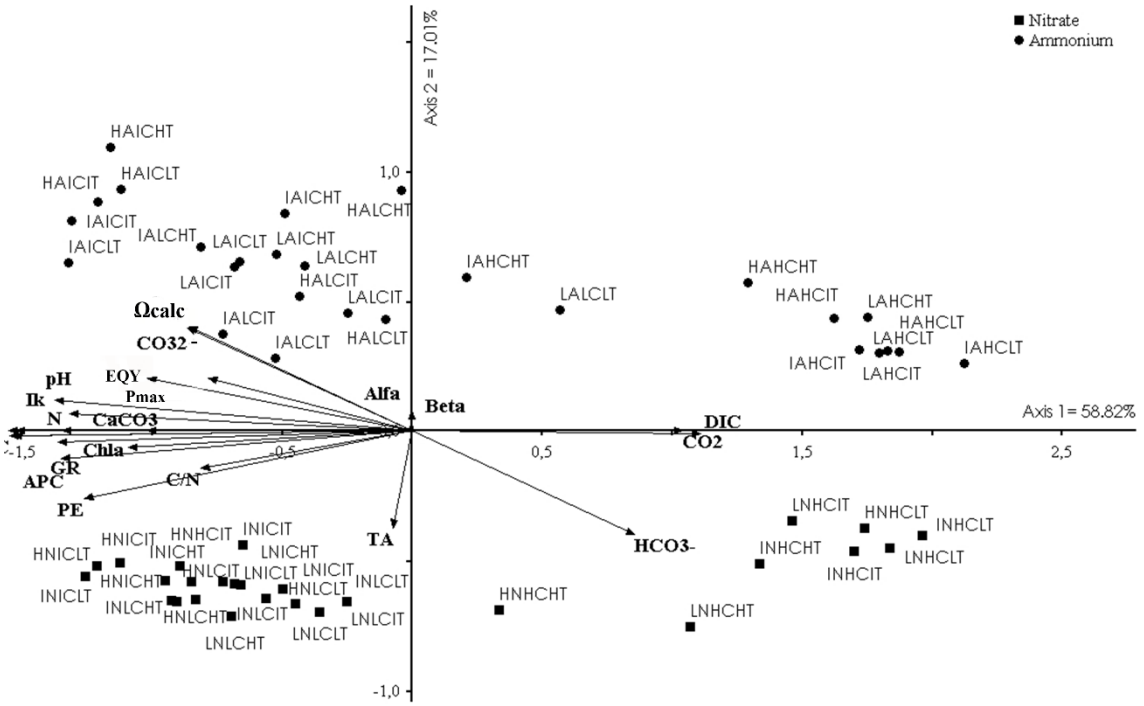

Fig 9: Scatter diagram of plots of the first two principal component analysis axes of data on the effects of nitrate or ammonium, CO_2 levels and temperatures on growth rates (GR), pigment contents (APC, PC, PE and Chl*a*), protein contents (PT), calcification ($CaCO_3$), tissue elements (C, H, N and C/N ratio), photosynthetic parameters (EQY, Pmax, alpha, beta and Ik) and carbonate system of seawater (pH, T_A, CO_2, HCO_3^-, CO_3^{2-}, DIC and Ωcalc) in *Amphiroa fragilissima* cultured in VSES/4 modified enriched with different nitrate (black square) or ammonium (black circle) concentrations, photoperiod of 14 h, salinity of 34, and photon flux densities of 80 to 90 µmol of photons m^{-2} s^{-1} . The first two components accounted for 75.83 % of total variance. LA – low ammonium, IA – intermediate ammonium, HA- high ammonium, LN- low nitrate, IN- intermediate nitrate, HN – high nitrate, LC – low CO_2, IC – intermediate CO_2, HC- high CO_2, LT- low temperature, IT- intermediate temperature and HT-high temperature.

Table 6: Pearson correlation coefficient among variables analyzed for *Amphiroa fragilissima* to evaluate the effects of increased temperature, CO_2 levels and nitrogen availability (nitrate and ammonium) on growth rates, pigment, protein contents, calcification (%), element contents in the thallus (C, H, N, P and C/N ratio), photosynthetic parameters, carbonate system of seawater, and Ωcalc (calcite saturation state). The scatterplot diagram of PCA is shown in Fig. 9.

Variables	Principal components	
	Axis 1	Axis 2
Growth rate (GR)	-0.891	-0.163
Total soluble protein (PT)	-0.941	-0.020
Allophycocyanin (APC)	-0.886	-0.254
Phycocyanin (PC)	-0.950	-0.48
Phycoerythrin (PE)	-0.856	-0.392
Chlorophyll *a* (Chl *a*)	-0.796	-0.194
$CaCO_3$	-0.771	0.004
Carbon (C)	-0.950	-0.107
Hydrogen (H)	-0.946	-0.112
Nitrogen (N)	-0.885	0.040
C/N ratio (C/N)	-0.688	-0.293
EQY (efficiency quantum yield)	-0.771	0.345
Pmax (ETRmax)	-0.675	0.345
α (photosynthetic efficiency)	-0.027	0.214
Ik (saturation irradiance)	-0.876	0.198
β (photoinhibition)	0.003	0.135
pH	-0.895	0.260
T_A (total alkalinity)	-0.210	-0.469
CO_2	0.779	-0.051
HCO_3^- (bicarbonate)	0.706	-0.483
CO_3^{2-} (carboante)	-0.708	0.481
DIC (dissolved inorganic carbon)	0.804	-0.090
Ωcalc (calcite saturation state)	-0.707	-0.090
% of variance	58.82%	17.01%

Discussion

The increase of atmospheric CO_2 changes the chemical composition of seawater, reducing pH, CO_3^{2-} availability and Ωcalc, as showed in the Tables 2 and 3. In high CO_2 availability, *Amphiroa fragilissima* decreased its metabolism, reducing growth, photosynthesis, as well as contents of pigments, proteins and elements (C, N and H) on the thallus. Moreover, temperature of 21°C caused bleaching in *A. fragilissima* in high CO_2 concentration, independent of nitrogen resources or concentrations tested. The negative effects of CO_2 on marine macroalgae have been reported in many studies, mainly on calcified algae. The growth, photosynthesis, Chl *a* and PE contents, and calcification of *Corallina pilulifera* Postels & Ruprecht decreased with 1000 ppm of CO_2 (Gao & Zheng 2010). At the same CO_2 level, the calcification and photosynthetic parameters (Pmax, α and β) decreased in *Halimeda opuntia* (Linnaeus) J.V. Lamouroux and *H. taenicola* W.R. Taylor (Price et al. 2011). The negative growth rates and decreased of photosynthesis were observed for *Corallina officinalis* Linnaeus cultivated with 1313 and 1939 ppm of CO_2 (Hofmann et al. 2012).

However, our study showed that the highest values of all variables analyzed for *A. fragilissima* occurred with 500µM of NO_3^- in 1000 ppm of CO_2 and temperature of 25°C. In these conditions, the chemical parameters of seawater was altered in relation to the other treatments with high CO_2, maintaining the pH of seawater above 7 and elevating the CO_3^{2-} concentration and the calcite saturation state (Ωcalc). The same pattern was observed in treatments with 50 µM of NH_4^+ at 30°C. However, under these conditions, *A. fragilissima* showed negative growth rate. Supersaturation of calcite in these conditions is controversial in nearly all studies, which showed that the saturation state of calcite decreases with rising CO_2 (Morse et al. 2006; Russell et al. 2009; Semesi et al. 2009; Gao & Zheng 2010; Büdenbender et al. 2011; Ragazzola et al. 2012). The calcification of *A.*

fragilissima in these treatments was lower based on the dissolution of calcium carbonate in high CO_2. The high Ωcalc in the seawater could be explained by the short duration of the experimental period which did not allow enough time to balance the amount of $CaCO_3$ dissolved against dissolution capacity, in turn causing supersaturation. However, further investigations should be made to explain these phenomena.

High nitrogen availability contributes to the increase in photosynthesis and assimilation of compounds, such as pigments and proteins in *A. fragilissima,* as observed for other macroalgae. For example, photosynthesis, pigments and mycosporine-like amino acids content of *Pyropia leucosticta* (Thuret) Neefus & J. Brodie and *P. umbilicalis* Kützing increased with 100 and 300 µM of NH_4^+ (Korbee et al. 2005). The photosynthesis and phenolic compounds of *Ulva rigida* C. Agardh were stimulated with NO_3^- concentrations of 50 µM (Cabello-Pasini et al. 2010). The photosynthesis and mycosporine-like amino acids content of *Gracilaria tenuistipitata* C. F. Chang & B.M. Xia were high with 2 mM of NO_3^- (Barufi et al. 2011). In contrast, low nitrogen availability results in a decline in phycobiliproteins, Chl *a*, PT, C, N and H content. N-limitation can affect the enzymes of photosynthetic carbon metabolism and decrease Rubisco into cells, reducing photosynthetic capacity and increasing the photoinhibition (Turpin 1991). Accordingly, the present study showed an increase of photoinhibition when the species was cultivated with low NH_4^+ without CO_2 and in high temperature.

In general, the increase of CO_2 combined with rising temperature decreased growth, pigment contents, photosynthesis and calcification of *A. fragilissima*. Temperature determines the processes and metabolic function of seaweeds (Lobban & Harrison 2004). Calcified macroalgae showed sensitivity to high temperatures, as observed in *Corallina officinalis* when exposed at 35°C. This species showed bleaching, decreased phycobiliprotein content and increased bromoperoxidase activity at high

temperatures (Latham 2008). *Neogoniolithon fosliei* (Heydrich) Setchell & L.R. Mason showed bleaching and a reduction in maximum quantum yield at 32°C (Webster et al. 2011). Temperature of 29°C and high CO_2 levels (1010 and 1350 ppm) increased necrotic and depigmented thallus area, and decreased calcification of *Porolithon onkodes* (Heydrich) Foslie (Diaz-Pulido et al. 2012).

Amphiroa spp. can use bicarbonate availability in seawater, demonstrating a carbon concentrating mechanism (CCM), as observed by Borowitzka (1981) in studies on calcification of *Amphiroa anceps* (Lamarck) Decaisne and *A. foliacea* J.V. Lamouroux, findings that corroborate with results obtained in the present study to *A. fragilissima*, and this ability increased with high nitrogen concentrations in the seawater.

N metabolism and C fixation in the photosynthesis process are coupled. The assimilation of N into amino acids requires the provision of carbon skeletons produced during photosynthetic carbon fixation (Turpin 1991). *A. fragilissima* increased its assimilation of proteins, phycobiliproteins, Chl *a*, C, N and H into thallus, in addition to increased growth rates and photosynthesis when cultivated with high NO_3^- and CO_2 concentrations. The increase in these nutrients may have stimulated the metabolic activities of *A. fragilissima*, but without reaching a saturation state for these nutrients. The same was observed for intermediate NH_4^+ availability; however, growth rate was negative in high CO_2. In this case, the species inhibited growth rate, instead using N in the assimilation of phycobiliproteins, proteins, Chl *a*, C, N and H into thallus. Furthermore, the species increased photosynthesis in order to maintain the production of carbon skeletons as substrate for N assimilation. The rising accumulation of compounds (e.g., C and H in thallus) in high CO_2 levels may have occurred as storage energy and increased production of polysaccharides in stress situations (Pacoda et al. 2014), however more investigations should be conducted in order to clarify these observations.

Conclusions

This study demonstrated that the effects of warming and increased CO_2 at levels proposed by CMIP5 and RCP 8.5 for 2100 (IPCC, 2013), together with impacts of increased nitrogen (e.g., eutrophication), on the growth, metabolism and assimilation of compounds of the coralline alga *Amphiroa fragilissima* are complex. In general, a rise in CO_2 levels negatively affects growth, photosynthesis and assimilation of proteins, pigments and elements into seaweed thallus. However, the enhance of nitrogen content increased the metabolism of this species, suggesting that it could acclimate to global change and eutrophication under some conditions. Furthermore, the species acclimated to increase of temperature. Thus, this study contributes to the knowledge of the synergistic effects of global environmental changes on the calcareous red algae.

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Considerações gerais

O presente estudo verificou o potencial como biofiltro da espécie *Hypnea aspera* e os efeitos das mudanças climáticas e eutrofização no metabolismo e fisiologia dessa alga. Por ser uma espécie cuja ocorrência foi recentemente descrita para o Brasil e por ser confundida morfologicamente com uma espécie de grande importância econômica, *H. pseudomusciformis* (uma espécie nova recentemente descrita para o Brasil por Nauer et al. (2015) e referida anteriormente como *H. musciformis*), os resultados obtidos no presente estudo foram importantes para conhecer a fisiologia e o metabolismo da espécie. *H. aspera* mostrou potencial como biofiltro quando cultivada em diferentes concentrações de nitrato:fosfato e amônio:fosfato como vericado no capítulo 1. A espécie removeu mais de 99% do nitrato, amônio e fosfato disponíveis na água do mar, além de ter captado altas concentrações desses nutrientes. O aumento da disponibilidade de nitrato (150 µM) e do amônio (70 µM) na relação de N:P de 100:1 aumentou o crescimento e concentrações de proteínas solúveis totais e pigmentos fotossintetizantes de *H. aspera*. Além disso, o aumento das concentrações de amônio causaram efeitos negativos no crescimento na relação de N:P de 10:1, entretanto foi observado um aumento da assimilação de N na forma de pigmentos e proteínas nessas condições. A razão de 10:1 de N:P aumentou a liberação de nitrogênio orgânico dissolvido, mostrando que *H. aspera* não conseguiu armazenar e converter o N em produtos de reserva em adições de 0, 50 e 100 µM de nitrato e em 50 µM de amônio.

Dessa forma, os resultados obtidos no capítulo 1 mostraram diferenças entre o metabolismo do nitrato e do amônio em *Hypnea aspera* e como o fósforo contribui na fisiologia e metabolismo da espécie. Estudos mais amplos, como o cultivo da espécie em sistemas multi-tróficos em campo ou em tanques, são importantes para se confirmar os

resultados obtidos no presente estudo e avaliar o potencial da espécie como biofiltro. Além disso, por ser uma espécie pouco conhecida e pertencente a um gênero importante economicamente, seria interessante quantificar o conteúdo e a qualidade da carragenana a fim de verificar o potencial uso econômico da espécie.

O estudo sobre os efeitos do aumento da temperatura e variação dos níveis dos CO_2 e concentrações de nitrogênio na água do mar nas rodofíceas *Hypnea aspera*, *Dichotomaria marginata* e *Amphiroa fragilissima* mostraram diferenças na resposta fisiológicas e metabólicas desses organimos sobre diferentes condições de estresse. No capítulo 2, verificamos que *H. aspera* é altamente sensível aos efeitos das mudanças climáticas. Nesse estudo, observamos que o aumento da concentração de CO_2 e temperatura proposto pelos cenários climáticos (CMIP5; RCP 8,5) do IPCC para o ano de 2100 podem ser letais para a espécie. Em relação ao aumento das concentrações de nitrogênio na forma de nitrato ou amônio, a espécie mostrou aumento de compostos nitrogenados em altas concentrações desses nutrientes quando cultivada sem adição de CO_2 e em ar ambiente e em temperaturas baixas e médias. Esse resultado evidenciou que em condições ambientais atuais, a espécie mantêm seu metabolismo. Assim, é importante a realização de estudos que verifiquem os efeitos sinérgicos de fatores bióticos e abióticos na fisiologia e metabolismo dessa espécie e estudos futuros incluindo novos fatores como irradiância e concentrações de fósforo na água do mar e testes em campo ou em mesocosmos (tanques).

O capítulo 3 mostrou como *D. marginata* respondeu ao aumento das temperaturas, do níveis de CO_2 e disponibilidade de nitrogênio na forma de amônio ou nitrato. Apesar do crescimento negativo nas altas concentrações de CO_2, *D. marginata* manteve as suas atividades metabólicas incorporando proteínas e pigmentos independente das concentrações de nitrogênio, e assimilou C, H e N no talo, principalmente quando

cultivada com disponibilidade de amônio. Essa espécie mostrou diferenças no metabolismo do N, tendo maior afinidade ao amônio do que ao nitrato. O aumento da temperatura diminuiu a calcificação e a fotossíntese da espécie, entretanto o principal fator limitante para o crescimento de *D. marginata* foi o aumento dos níveis de CO_2. Sob altos níveis de CO_2, observou-se uma descalcificação dos talos de *D. marginata* e ocorreu o branqueamento do talo devido à degradação de pigmentos fotossintetizantes. Como era esperado, o aumento das concentrações de CO_2 na água, diminuiu o pH, a disponibilidade de CO_3^{2-} e o estado de saturação da aragonita da água do mar, fatores importantes para os processos de calcificação e fotossíntese, e que influenciaram negativamente os processos metabólicos da espécie. Nas condições de baixo CO_2 e temperatura e no cenário atual segundo o IPCC (380 ppm e 25°C), a espécie apresentou alto crescimento e incorporou C, H e N no talo, pigmentos fotossintetizantes e proteínas solúveis totais. Esse capítulo é de grande importância científica e vem contribuir para o conhecimento referente a fisiologia de organimos com deposição de carbonato de cálcio na forma de aragonita, fornecendo informações para que novos estudos sejam realizados e para o preenchimento na lacuna de conhecimento sobre a fisiologia de espécies com calcificação na forma de aragonita.

O capítulo 4 mostrou resultados inesperados sobre os efeitos dos cenários climáticos do IPCC e o aumento do nitrogênio na água do mar na espécie coralinácea *A. fragilissima*. No geral, altas concentrações de CO_2 causaram o branqueamento do talo da espécie, diminuindo a concentração de pigmentos, proteínas e elementos (C, H, e N). Além disso, ocorreu a diminuição da calcificação, do crescimento e da fotossíntese da espécie, assim como foi observado para *H. aspera* e *D. marginata*. Entretanto, os resultados para os seguintes tratamentos foram inesperados : 1°) quando *A. fragilissima* foi cultivada com 500 µM de nitrato, 1000 ppm de CO_2 à 25°C e 2°) quando a espécie foi

cultivada com 50 µM de amônio, 1000 ppm de CO_2 à 30°C. Nesses tratamentos, ocorreu alta fotossíntese, concentrações de pigmentos, proteínas, C e H no talo. O aumento das concentrações de C e H, pode estar relacionado com o aumento de produção de polissacarídeos em situações de estresse, entretanto esses dados merecem ser melhor investigados em estudos futuros. Outro importante resultado observado e não esperado foi que, nesses tratamentos, não ocorreu a queda do pH, da disponibilidade CO_3^{2-} e do estado de saturação da calcita na água do mar. Nesse caso, pode ter ocorrido um desequilíbrio entre as concentrações de $CaCO_3$ na água do mar e a capacidade de dissolução da calcita, ocasionando uma supersaturação na água. Uma investigação mais minuciosa sobre o sistema carbonato nestes casos, bem como do processo de calcificação de *A. fragilissima* são necessários para uma melhor compreensão do que pode ter ocorrido.

A partir dos resultados obtidos nos quatro capítulos apresentados, observamos questões importantes sobre os efeitos dos impactos das mudanças climáticas e enriquecimento de nutrientes na água do mar em rodofíceas marinhas bentônicas, contribuindo assim para um maior conhecimento da fisiologia e do metabolismo das espécies e fornecendo suporte para pesquisas futuras.