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MÄERL CALCIFICATION, PHOTOSYNTHESIS AND RESPIRATION IN AN ACIDIFIED OCEAN

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Declaração de autoria do trabalho.

Declaro ser a autora deste trabalho que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Laura Sordo De las Nieves

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This work is dedicated to my family,
My safe harbor and the most important people in my world
To my father, Manuel Sordo, who has always supported me and encouraged me to follow my dreams and to my mother Mila De las Nieves and brother Alvaro Sordo for always being there for me. To my true "amores" and everyday companions, Piergiorgio Pucci and Rubi

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“Valeu a pena? Tudo vale a pena

Se a alma não é pequena.

Quem quere passar além do Bojador

Tem que passar além da dor.

Deus ao mar o perigo e o abismo deu,

Mas nele é que espelhou o céu.”

Fernando Pessoa

Mar Português, in Mensagem (1934)

Abstract

With the increase of atmospheric CO_2 and the associated acidification of the oceans, rhodolith (mäerl) beds are under severe threat. The general lack of consensus regarding the foreseeable effects of Ocean Acidification (OA) on coralline algae is largely due to the divergences of results obtained in different scientific experiments. These divergences may be related to differences in temperature, irradiance, CO_2 levels, time of exposure and also on technical difficulties concerning the experimental methodologies used. This thesis aimed to determine the photosynthesis, calcification and respiration rates of the mäerl species *Phymatolithon lusitanicum* under natural conditions in Southern Portugal and to assess the effect that ocean acidification will have on these processes under different irradiances, temperatures, CO_2 concentrations and times of exposure. Dark respiration and photosynthesis increased with temperature in summer and spring and decreased in winter and autumn while calcification rates did not change seasonally. A direct CO_2 control system was developed and found to be reliable to assess the short and long term effect of OA on coralline algae. In the short term, photosynthesis and calcification increased with CO_2 and temperature, but after prolonged exposure this pattern was reversed and algae exposed to high CO_2 showed lower photosynthetic and calcification rates and accumulated growth with respect to control algae, effects that were enhanced with increasing irradiances. Dark respiration was unaffected by CO_2 but increased with temperature. The results suggest that temperature, irradiance, CO_2 level and time of exposure are determinant factors in ocean acidification experiments with coralline algae. Both temperature and high light intensified the effect of high CO_2 on *Phymatolithon lusitanicum* and these will be determinant factors on the long-term resilience of Lusitanian rhodolith beds to OA.

Key-words: Coralline algae, mäerl, Ocean Acidification (OA), respiration, photosynthesis, calcification

Resumo

Face ao aumento do CO_2 atmosférico e consequente acidificação dos oceanos os bancos de rodolitos (mäerl) estão severamente ameaçados. A falta de consenso relativamente aos efeitos previsíveis da acidificação do oceano nas algas calcárias deve-se sobretudo às muitas divergências encontradas entre as diferentes experiências científicas conduzidas ao longo dos últimos anos. Estas divergências estarão provavelmente relacionadas com diferenças na temperatura, irradiância, nível de CO_2, tempo de exposição e metodologias utilizadas. Esta tese teve como objectivo principal determinar as taxas de fotossíntese, respiração e calcificação da espécie de mäerl mais comum nos bancos de rodolitos do Sul de Portugal, *Phymatolithon lusitanicum*, nas suas condições naturais e também investigar os efeitos que a acidificação oceânica terá nestes processos sob diferentes condições de irradiância, temperatura, concentração de CO_2 e tempo de exposição. A respiração e a fotossíntese aumentaram com a temperatura no verão e na primavera, e diminuíram no inverno e no outono, embora a calcificação não tenha apresentado variações sazonais. Um sistema experimental de mesocosmos com controlo direto de CO_2 foi desenvolvido e validado para avaliar o efeito da acidificação oceânica nas algas calcárias a curto e longo prazo. A curto prazo, a fotossíntese e a calcificação aumentaram tanto com o CO_2 como com a temperatura, mas a longo prazo este padrão inverteu-se e as algas expostas a CO_2 elevado mostraram menores taxas fotossintéticas e de calcificação e também menor crescimento acumulado do que as algas controlo, tendo o efeito do CO_2 sido amplificado pela irradiância elevada. A respiração no escuro não foi afectada pelo CO_2 mas aumentou com a temperatura. Os resultados sugerem que a temperatura, irradiância, o nível de CO_2 e o tempo de exposição são fatores determinantes em experiências de acidificação do oceano com algas calcárias. Tanto a temperatura como a irradiância amplificam o efeito do CO_2 em *P. lusitanicum* e estes serão factores determinantes na resiliência a longo prazo dos bancos de rodolitos lusitanos à acidificação oceânica.

Palavras-chave: Algas calcárias, mäerl, acidificação do oceano, respiração, fotossíntese, calcificação

Resumo estendido

Face ao aumento do CO_2 atmosférico e consequente acidificação dos oceanos os bancos de rodolitos (mäerl) estão severamente ameaçados. A falta de consenso relativamente aos efeitos previsíveis da acidificação do oceano nas algas calcárias deve-se sobretudo às muitas divergências encontradas entre as diferentes experiências científicas conduzidas ao longo dos últimos anos. Estas divergências estarão provavelmente relacionadas com diferenças na temperatura, irradiância, nível de CO_2, tempo de exposição e metodologias utilizadas. Esta tese teve como objectivo principal determinar as taxas de fotossíntese, respiração e calcificação da espécie de mäerl mais comum nos bancos de rodolitos do Sul de Portugal, *Phymatolithon lusitanicum*, nas suas condições naturais e também investigar os efeitos que a acidificação oceânica terá nestes processos sob diferentes condições de irradiância, temperatura, concentração de CO_2 e tempo de exposição.

A temperatura, a irradiância e o oxigénio dissolvido foram medidos em contínuo num banco de mäerl situado a 25 metros de profundidade na baía de Armação de Pêra (sul de Portugal) durante aproximadamente dois anos. O crescimento de algas marcadas no campo foi também acompanhado ao longo deste período e os pigmentos fotossintéticos analisados sazonalmente. A actividade fotossintética das algas foi medida sazonalmente *in situ* (fluorescência de pulso de luz de amplitude modulada ou PAM) e no laboratório (eléctrodo de oxigénio tipo Clark). O efeito da temperatura na fotossíntese e respiração das algas também foi investigado.

Apesar das poucas flutuações na temperatura e irradiância observadas a 25 m de profundidade ao longo do ano, a fotossíntese e a respiração de *P. lusitanicum* aumentaram com a temperatura no verão e na primavera, e diminuíram no inverno e no outono. A respiração foi duas vezes maior no verão do que no inverno. Relativamente à atividade fotossintética, padrões idênticos foram obtidos *in situ* através da fluorescência da clorofila (PAM) e no laboratório com as curvas de fotossintese-irradiância convencionais (elétrodo de oxigénio tipo Clark). No laboratório, tanto a fotossíntese como a respiração no escuro aumentaram com a temperatura, tendo ficado claramente ilustrado o papel central deste factor na respiração e, a par com a irradiância, na actividade fotossintética da alga coralina *P. lusitanicum*.

A concentração de ficobilinas nas algas aumentou no verão e no outono e diminuíu no inverno e na primavera. Estes pigmentos podem ser usados como reservas de azoto em períodos de

baixa disponibilidade de nutrientes na coluna de água. Por outro lado, a concentração em clorofila *a* e em carotenóides aumentou no inverno e na primavera e diminuiu no outono e no verão. As algas terão provavelmente compensado os baixos níveis de luz durante o inverno de 2014 e 2015 através do aumento da concentração desses pigmentos fotossintéticos.

A calcificação não apresentou um padrão sazonal claro, embora as maiores taxas de calcificação tenham coincidido com o período em que foram registados os maiores valores de temperatura e irradiância (verão de 2013). Estes resultados sugerem que, mesmo que a calcificação das algas aumente com a temperatura e irradiância, será provavelmente mais dependente da temperatura do que da irradiância. Dentro do seu intervalo ótimo de temperatura, as algas conseguem manter um crescimento continuado apesar dos baixos níveis de luz.

Um sistema de mesocosmos com controlo direto de CO_2 foi desenvolvido e validado para avaliar o efeito da acidificação do oceano nas algas calcárias a curto e longo prazo. Neste sistema, a variável controlo (pCO_2) é medida directamente através de um analisador de gases por infravermelho (IRGA), e a injecção de CO_2 é regulada por um sistema de controlo digital avançado que permite um ajuste fino do gás necessário ao sistema para atingir e manter a pressão parcial desejada. Este sistema pode também ser adaptado para incorporar controlo de outras variáveis relevantes em simultâneo com o CO_2, como diferentes níveis de temperatura, luz e nutrientes.

O efeito do enriquecimento de CO_2 e do aumento da temperatura e irradiância na fotossíntese, respiração e calcificação de *P. lusitanicum* foi investigado a curto e a longo prazo. A curto prazo, foi conduzida uma experiência onde as algas foram expostas durante 1 mês a três temperaturas e dois níveis de pCO_2. Para avaliar os efeitos a longo prazo, as algas foram expostas durante 20 meses a três níveis de pCO_2 (400 (controlo), 550 e 750µatm). Nesta experiência, as medidas de fotossíntese e calcificação foram efetuadas ao fim de 11 meses também ao fim de 20 meses. Nesta experiência foram também determinadas as taxas de crescimento de algas marcadas individualmente, ao longo dos 20 meses que durou a experiência.

A curto prazo, a respiração no escuro aumentou com a temperatura, mas não foram encontradas alterações ou foi verificada apenas uma leve diminuição com o CO_2, enquanto

que a fotossíntese e a calcificação aumentaram tanto com o CO_2, como com a temperatura. Ao fim de duas semanas de aclimatação a diferentes temperaturas, as taxas fotossintéticas e de respiração das algas aumentaram, mas diminuiram as taxas de calcificação. Por outro lado, depois de um mês de aclimatação às diferentes temperaturas e duas semanas de aclimatação a diferentes níveis de CO_2 (400 e 1000 µatm), as taxas de fotossíntese, respiração e calcificação de *Phymatolithon lusitanicum* aumentaram com o CO_2 e com a temperatura. Estes resultados sugerem que tanto o tempo de aclimatação como a temperatura são fatores determinantes em experiências sobre o efeito da acidificação oceânica. É também sugerido que o aquecimento global amplifique o efeito da acidificação oceânica nas algas calcárias.

Após 11 meses de exposição a diferentes níveis de CO_2, a respiração não foi afectada pelo CO_2 mas aumentou proporcionalmente com temperatura atingindo os maiores valores a 26 °C. Por outro lado, a fotossíntese e calcificação, aumentaram com o CO_2 e com a irradiância. No entanto, a longo prazo (12-20 meses) este padrão reverteu-se e as algas controlo apresentaram maiores taxas fotossintéticas e de calcificação e maior crescimento acumulado, por comparação com as algas acidificadas. As diferenças entre os tratamentos foram sobretudo visíveis quando as medidas pontuais foram efectuadas a irradiâncias elevadas.

Globalmente, os resultados sugerem que a temperatura, irradiância, nível de CO_2 e tempo de exposição e aclimatação são fatores determinantes em experiências de acidificação oceânica com algas calcárias. A curto prazo, as taxas metabólicas das algas aumentaram em resposta às condições potencialmente stressantes, mas a longo prazo o custo metabólico terá eventualmente sido demasiado elevado e as algas não tiveram capacidade de compensar as condições corrosivas da água do mar. As algas cultivadas a baixa irradiância e temperatura mostraram uma maior resiliência à acidificação.

Tanto a temperatura como a irradiância amplificam o efeito do aumento de CO_2 em *P. lusitanicum* e estes serão factores determinantes na resiliência a longo prazo dos bancos lusitanos de rodolitos à acidificação do oceano. Num futuro cenário de mudança global, acima de 26°C, a respiração e a calcificação (ambas fontes de CO_2) poderiam superar a fotossíntese com repercussões negativas para todo o ecossistema e importantes perdas a longo prazo na capacidade de armazenamento de carbono. Por outro lado, abaixo de 26 °C e sob irradiâncias moderadas, os bancos de mäerl do Sul de Portugal podem tornar-se refúgios naturais da acidificação oceânica.

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General introduction

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Ocean acidification (OA)

Oceans are important carbon sinks that between 1750 and 2000 have absorbed about one-third of the CO_2 emitted by humans (Zeebe and Ridgwell, 2011). Nonetheless, the rapid increase of anthropogenic CO_2 emissions is changing the global marine environment as a consequence of the acidification and warming of the world's oceans (van der Heijden and Kamenos, 2015) with risks of collapse of many critical ocean and coastal ecosystem services and important environmental and economic repercussions (Talberth and Niemi, 2017).

At an unprecedented rate in geological history, the concentration of atmospheric carbon dioxide (CO_2) has risen from 316 ppm in 1959 to more than 400 ppm in 2016 (NOAA, 2017). Between 2015 and 2016 the highest CO_2 growth rate in human's history was recorded and for the first time the concentration of CO_2 in the atmosphere stood above 400 ppm during the entire year (Betts et al., 2016).

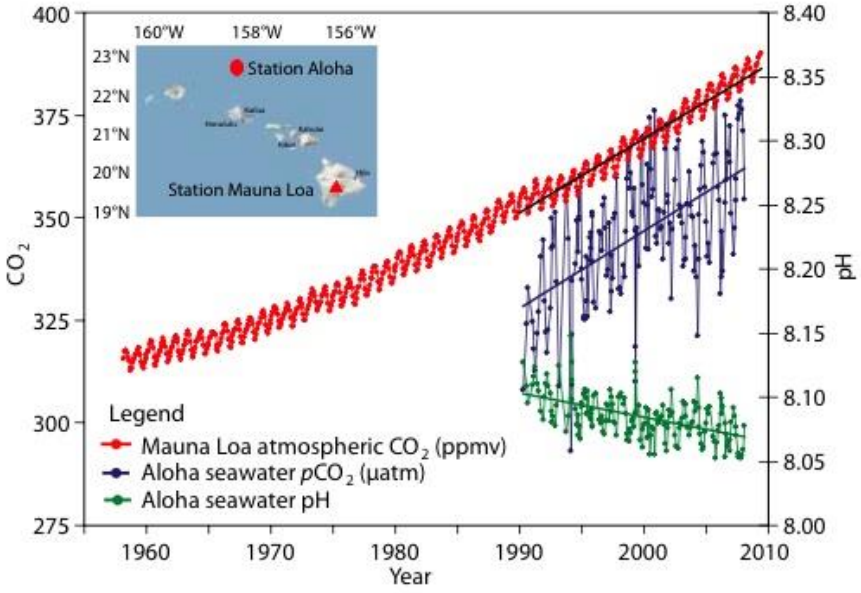

Figure1.1. Time series of atmospheric CO_2 at Mauna Loa (ppmv) and surface ocean pH and *p*CO2 (µatm) at Ocean Station Aloha in the subtropical North Pacific Ocean (Doney et al., 2009).

Figure 1.1. displays the longest time series of ocean measurements of (top) atmospheric CO_2 and surface ocean pCO_2 and (bottom) pH at the atmospheric Mauna Loa Observatory (MLO)

on the island of Hawaii and Station ALOHA in the subtropical North Pacific (Doney et al., 2009).

Rising atmospheric CO_2 causes a net air-to-sea flux of CO_2 that dissolves in surface seawater as inorganic carbon through well-known physical-chemical reactions. The first main reaction occurs when CO_2 gas from the atmosphere dissolves into seawater (Feely et al., 2009):

$$CO_2\,(atm) \leftrightarrow CO_2\,(aq) \qquad (1)$$

The ocean's buffering capacity to hold additional CO_2 from the atmosphere is inversely proportional to the Revelle factor (R, inversely related to $[CO_3^{2-}]$), which is the ratio of the relative change in pCO_2 (or CO_2) to the relative change in total Dissolved Inorganic Carbon (DIC) (Orr, 2011):

$$R = (\partial pCO_2 / pCO_2) / (\partial\,[DIC] / [DIC]) \qquad (2)$$

One of the most important consequences of OA is the increase of the Revelle buffer factor, causing a drastic decrease in the rate and capacity of ocean water to take up CO_2 from the atmosphere, leading to a temporary accumulation of CO_2 in the atmosphere. CO_2 is a gas that dissolves in water forming carbonic acid (H_2CO_3), which in turn dissociates and releases protons (H^+) to the water through a series of reversible reactions (Dore et al., 2009)

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-} \qquad (3)$$

The concentration of hydrogen ions is what determines the ocean's acidity, commonly expressed on a logarithmic scale as pH:

$$pH = -\log_{10}[H^+] \qquad (4)$$

Therefore, the rise in atmospheric CO_2 acidifies seawater by lowering its pH (Dore et al., 2009). Since the beginning of the industrial period, average oceanic pH dropped from 8.2 to 8.1, with a 30% increase in the H^+ concentration, and a 16 % drop in the carbonate ion concentration. The IPCC projections for the end of this century (2100) predicts that pH may drop a further 0.3 to 0.4 units what would be a 100-150% increase of acidity and a 50%

decrease of carbonate ion concentration (IPCC, 2000). This predicted pH reduction will cause major shifts in seawater chemistry with the increase in the concentration of bicarbonate ions (HCO_3^-) and the decrease in the concentration of carbonate ions (CO_3^{2-}) and saturation state of calcium carbonate ($CaCO_3$) (Doney et al., 2009).

Marine ecosystems and their role on the mitigation of CO_2 emissions

Seawater carbonate chemistry is determined by a series of abiotic chemical reactions (acid/base chemistry, CO_2 dissolution) and biological reactions (photosynthesis, respiration, calcification and dissolution) (Feely et al., 2009). The contribution of an ecosystem to the global carbon cycle is driven by the balance between carbon production and consumption and between calcium carbonate production and dissolution (Gattuso et al., 1995). Changes in the water chemistry as a consequence of pH decrease are expected to disrupt the ocean's carbon sequestration capacity and affect photosynthesis and calcification, both of which use DIC as substrate (Martin and Hall-Spencer, 2017). However, information on the potential of marine ecosystems to mitigate the effect of increasing anthropogenic CO_2 and the carbon storage capacity of photosynthetic calcifiers such as coralline algae is yet scarce (van der Heijden and Kamenos, 2015).

The carbonate production of benthic communities has a global significance since it may affect the Earth's climate (Barberá et al., 2003). Coralline algae are expected to be one of the most vulnerable groups of species to ocean acidification (Martin and Hall Spencer, 2017), but the effects of the future global change on their carbon storage capacity is still uncertain (van der Heijden and Kamenos, 2015). In the short term, coralline algae, as well as other photosynthetic calcifiers, can act both as CO_2 sinks via photosynthesis and as CO_2 sources through respiration and calcification. The long-term balance between these processes will determine the carbon storage potential of the coralline algae deposits over geological time scales (van der Heijden and Kamenos, 2015) and their role on the global CO_2 sequestration. Thus, rhodolith/mäerl beds are of special interest to ocean acidification research because they perform both photosynthesis and calcification (Martin et al., 2013), have a worldwide distribution and are composed of high Mg-calcite, one of the most soluble forms of calcium carbonate (van der Heijden and Kamenos, 2015).

Despite their importance, there is relatively few information on the response of rhodolith/mäerl beds to OA in comparison to other abundant nearshore communities such as coral reefs, seagrass beds and kelp forests (Barberá et al., 2003; Foster et al., 2013; Attard et al., 2015).

Calcification, dissolution, photosynthesis and respiration

Calcification is the precipitation of calcium carbonate crystals by living organisms. In algae, this process can occur in intercellular spaces (e.g. in the genus *Halimeda*), within the cell walls, (e.g. the genus *Corallines*) or on the surface of the thalli (e.g. genus *Padina)* (Koch et al., 2013). Calcification can be generically described by equation 5;

$$Ca^{2+} + 2HCO_3^- \leftrightarrow CO_2 + CaCO_3 + H_2O \qquad (5)$$

The final reaction at the site where $CaCO_3$ is precipitated is controlled by the concentration of carbonate ions (Eq. 6) and hence the saturation state (Gattuso and Hansson, 2011)

$$Ca^{2+} + CO_3^{2-} \leftrightarrow CaCO_3 \qquad (6)$$

Equation 5 may give the erroneous impression that ocean acidification increases calcification. However, Eq. 6 shows that the precipitation of $CaCO_3$ is controlled by the concentration of carbonate ions (CO_3^{2-}) and hence the $CaCO_3$ saturation state, both of which decrease with increasing ocean acidification (Gattuso and Hansson, 2011). Reaction 6 contributes to the formations of $CaCO_3$ skeletons and shells of many marine organisms like shellfish or calcareous algae. If H^+ is in excess, CO_3^{2-} concentration decreases, bicarbonate (HCO_3^-) concentration increases, calcification rates usually decline and it becomes more difficult for all calcifiers to form their calcium carbonate skeletons or shells (Feely et al., 2009).

The saturation state of the calcium carbonate mineral phases (Ω) is a geochemical indicator defined by equation 7. When $\Omega > 1$, seawater is $CaCO_3$ super-saturated and precipitation occurs. When $\Omega < 1$ seawater is under-saturated and dissolution is favoured. At equilibrium ($\Omega = 1$), the calcium concentration times the carbonate concentration is equal to a constant called the apparent solubility product defined by K'sp (Feely et al., 2009). K'sp depends on temperature, salinity, and pressure, and differs among calcium carbonate mineral forms.

$$\Omega = [Ca^{2+}]\,[CO_3^{2-}] / K'sp \qquad (7)$$

$$K'sp = [Ca^{2+}] \times [CO_3^{2-}] \qquad (8)$$

Aragonite is a more soluble $CaCO_3$ form than calcite. However, when magnesium is associated with calcite, this $CaCO_3$ mineral form becomes more soluble than aragonite. Calcification and inorganic precipitation is favoured in relation to dissolution at the ocean surface where aragonite has a saturation state of about 2 to 6 and calcite 4 to 8 ($\Omega \geq 1$) promoting precipitation. The saturation states of carbonate minerals naturally decrease with depth as total dissolved CO_2 increases because of biological respiration and cold temperatures. Thus, in oceanic deep cold waters and in the high arctic regions Ω be close to 1, where it is very difficult to form calcareous structures, or be less than 1 where dissolution takes place (Feely et al., 2009).

Photosynthesis (Equations 9 and 10, left to right) is expected to increase under an ocean acidification scenario, especially for those primary producers that rely on CO_2 diffusion. Ocean acidification may also benefit those algae that use carbon concentrating mechanisms which take HCO_3^- and convert it into CO_2 in the cells. In contrast, respiration (right to left) in calcareous algae is apparently unaffected by ocean acidification although previous studies have found its increase with the increase on temperature (reviewed in Martin and Hall-Spencer, 2017).

$$CO_2 + H_2O \leftrightarrow CH_2O + O_2 \qquad (9)$$

$$HCO_3^- + H_2O \leftrightarrow CH_2O + O_2 + OH^- \qquad (10)$$

Photosynthetically driven changes in water chemistry by surrounding plants can affect calcification rates in calcareous algae and other calcifiers (Semesi et al., 2009). Highly productive tropical seagrasses can raise the external pH during the day to ~9, due to the uptake of CO_2 for photosynthesis, thus elevating two to six-fold the calcification rate of the calcifying algae growing in their vicinity. This suggests that in coastal areas the increase in pH caused by an increase in the photosynthesis of calcareous algae and the surrounding vegetation may counteract the negative effects of OA and rising temperature on calcification (Semesi et al., 2009). In a similar way, coral reefs, which are sources of CO_2 to the atmosphere, can act as carbon sinks when dominated by non-calcareous algae or macrophytes (Gattuso et al., 1999).

Calcification in coralline algae is an extracellular chemical process largely controlled by photosynthesis, respiration and proton pumping (Borowitzka, 1981; de Beer and Larkum, 2001). Photosynthesis stimulates calcification by providing an organic matrix in the cell walls where the nucleation of calcite crystals is thought to occur, and by increasing pH and consequently the $CaCO_3$ saturation state (Borowitzka, 1981; Gao et al. 1993). In contrast, respiration decreases pH and acts in the opposite direction by hindering calcification (de Beer and Larkum, 2001).

Rhodolith (mäerl) beds importance and the predicted effect of ocean acidification

Rhodolith beds are aggregations of living unattached coralline algae. A type of rhodolith known as "mäerl" in the North East Atlantic refers to a free-living non-geniculate (lacking decalcified joints) coralline algae without a shell or pebble core (reviewed in Hernandez and Kantun et al., 2017). Rhodolith beds are globally distributed from the poles to the tropics (Barberá et al., 2003; Foster et al., 2013), but the most extensive rhodolith bed of the world is located in the mesophotic zone of the Brazilian continental shelf, covering about 20,900 km^2 (Amado-filho et al., 2012). The three-dimensional matrix and high organic matter that this ecosystem accumulates (Attard et al., 2015) makes rhodolith beds biodiversity hotspots that sustain fisheries as nursery and juvenile feeding or refuge areas for commercial species (Barberá et al., 2003; Martin and Hall-Spencer, 2017; Hernandez-Kantun et al., 2017) like, for e.g., cod (Hall-Spencer et al., 2003) or scallops (Foster et al., 2013).

In the context of ocean acidification, coralline algae are receiving renewed attention due to their vulnerability because of their high-Mg calcite skeletons and ecological importance (McCoy and Kamenos, 2015). The response of calcareous algae to ocean acidification varies, but are mostly negatively affected (Anthony et al. 2008; Gao and Zheng 2010), with a larger impact under elevated temperature (Anthony et al. 2008) and irradiance (Gao and Zheng, 2010). Conversely, some authors found positive (Borowitzka, 1981; Semesi et al., 2009) or parabolic (Borowitzka, 1981) responses (Martin et al., 2013 and Martin and Hall-Spencer, 2017 for review). The considerable divergences among experimental studies on the photosynthetic and calcification response of coralline algae to OA might be related to different factors, some of which are dealt with in this thesis, such as temperature, irradiance, level of CO_2 and time of exposure to high CO_2 conditions.

Most OA experiments fail to monitor the seasonality of the natural communities on the field. This is mandatory in order to apply realistic conditions during the experiments and to record any interannual variability. In a long-term experiment (one year), Martin et al. (2013) found that the effect of OA on the crustose coralline algae *Lithophyllum cabiochae* changed seasonally. These results suggested that both temperature and irradiance modify the effect of OA on coralline algae and agree with other studies where the response of coralline algae to OA changed with temperature (e.g. Martin and Gattuso, 2009; Martin et al., 2013; Vásquez-Elizondo and Enríquez, 2016) and irradiance levels (e.g. Gao and Zheng, 2010; Yildiz et al., 2013).

Another important factor to consider is the length of the experiment (see McCoy and Kamenos, 2015). OA experimental studies with coralline algae have been restricted to a maximum duration of 1 year (see Martin et al., 2013). This is partly due to the difficulties to maintain constant CO_2 levels for a long period of time using pH stats to control CO_2 concentrations in the seawater. In this work, a novel autonomous CO_2 control system suitable for running long-term experiments was developed.

Short-term studies indicate that under elevated CO_2 levels the photosynthesis and growth of macroalgal species increase (Koch et al., 2013). In contrast, in 82% of the long-term studies reviewed by Koch et al. (2013) there was a decrease in calcification, and an enhanced dissolution in calcifying species with accentuated negative effects under elevated temperatures. Other results suggest that photosynthesis in calcifying algae is not likely to increase under OA, and a greater CO_2 and HCO_3^- availability may uncouple photosynthesis-calcification reactions and subsequently impede calcification and growth (Koch et al., 2013).

In the North Atlantic Ocean the number of coralline algae species that form rhodolith/mäerl beds is higher in the overlapping Boreal/ Lusitanian regions than in the Subarctic Coasts (Hernandez-Kantun et al., 2017). Recent efforts to understand the biodiversity of rhodolith/mäerl forming species through genetic analysis revealed that some species are changing their distribution (Carro et al., 2014). *Lithothamnion corallioides* and *Phymatolithon calcareum*, the most common species in the British Isles and Brittany have been gradually replaced in Galicia by the recently identified as new species *Phymatolithon lusitanicum* (Peña et al., 2015) and have become extremely rare in Algarve, southern Portugal (Carro et al., 2014). The replacement of mäerl species at northern latitudes increases further south along the

coasts of the Iberian Peninsula, and *Phymatolithon lusitanicum* forms the largest rhodoliths with thickest branches in Algarve (Carro et al., 2014; referred as *Phymatolithon* sp3. see Peña et al., 2015). These changes in distribution are probably due to global warming and are expected to shift as temperatures continue to rise (Brodie et al., 2014). As a consequence of ocean acidification and warming, calcium carbonate saturation will decrease and rhodolith/mäerl beds from northern latitudes will be lost while Lusitanian beds are expected to persist (Brodie et al., 2014). However, there are no studies on the effect of OA on rhodolith/mäerl beds from southern Portugal, and the available information is restricted to the species composition of the natural beds (Carro et al., 2014; Peña et al., 2015), and their associated flora (Peña and Barbara, 2013).

Main objectives

Taking into account the important gap on information on the natural seasonality of mäerl beds from Southern Portugal and the impact that global climate changes will have on these communities in the North Atlantic Ocean, the main objectives of this thesis were:

i) To determine the seasonal and interannual evolution patterns of key abiotic parameters and biological indicators in the most important mäerl bed from Southern Portugal. Temperature, photosynthetic active radiation (PAR) and oxygen evolution were monitored continuously in situ, while photosynthesis, calcification, respiration and photosynthetic pigment content of *P. lusitanicum* were determined seasonally. The effect of increasing temperature on the photosynthetic performance and respiration was also investigated.

ii) To develop a direct CO_2 control system to set and control pCO_2 levels in a open-circuit mesocosm, using CO_2 as the control variable, in order to obtain a stable and robust experimental system to evaluate the long- and short-term effects of high CO_2 on *Phymatolithon lusitanicum.*

iii) To investigate the short-term effects of temperature and high CO_2 on the photosynthesis, respiration and calcification of the free living coralline algae *Phymatolithon lusitanicum*.

iv) To investigate the long-term effect of high CO_2 on the respiration and the effect of CO_2 and irradiance on the photosynthesis and calcification of *Phymatolithon lusitanicum*.

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Chapter 2

Seasonal respiration, photosynthesis and calcification of a temperate mäerl bed in Southern Portugal

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(To be submitted)

Seasonal respiration, photosynthesis and calcification of a temperate mäerl bed in Southern Portugal

Abstract

Rhodolith (mäerl) beds are biodiversity hotspots with a worldwide distribution. There is already information on the seasonality of northeastern Atlantic beds. However there is no information on the key environmental variables that regulate the seasonal metabolism of rhodoliths beds from southern Portugal. These beds, are mainly composed by the unknown and recently identified as a new species *Phymatolithon lusitanicum*. To determine the seasonal and interannual evolution patterns of key abiotic parameters, temperature, irradiance and dissolved oxygen were measured on a continuous basis in the field for almost two years. Calcification, photosynthesis, respiration and pigment content of algae were measured seasonally. The effects of increasing temperature on the photosynthetic and respiration rates of individual thalli were assessed in the laboratory under different temperatures. Despite relatively low thermal and irradiance fluctuations, an important interannual variability and metabolic seasonality was observed. Photosynthesis and respiration increased in summer and decreased in autumn and winter while calcification did not change seasonally. Phycobilins increased in summer and autumn and decreased in winter and spring as opposed to chlorophyll *a* and carotenoids. Under experimental conditions, respiration and photosynthesis increased with temperature. Both temperature and irradiance regulate the photosynthesis and pigment concentrations of algae, and temperature the respiration of *P. Lusitanicum*. Calcification also increased with temperature and irradiance but algae were able to keep constant growth rates despite low light temperature conditions. The results suggest that the metabolic rates of *P. lusitanicum* are strongly dependant on small temperature and irradiance changes and Lusitanian rhodolith beds are important calcifiers in the Northeastern Atlantic.

Key-words: non-geniculate coralline algae, photosynthesis, respiration, calcification, photosynthetic pigments, temperature, irradiance

Introduction

Rhodolith (mäerl) beds are composed by free living non-geniculate coralline algae and are biodiversity spots globally distributed from the poles to the tropics (Foster et al., 2013). In the Northeastern Atlantic, rhodolith beds are often a key component of the ocean floor and support complex trophic chains (Barberá et al., 2003; Hernandez-Kantun et al., 2017). Mäerl beds are protected under the European Directive 92/43/EEC on the conservation of natural habitats and of wild fauna and flora, and species such as *Phymatholithon calcareum* and *Lithothamnion corallioides* are priority species for conservation (OSPAR, 2008). However, further research on mäerl ecosystems is needed and the full range of mäerl forming species should be listed so the rarest types of mäerl beds can also be protected (Barberá et al., 2003). While information on the primary production, respiration and calcification of northern rhodolith beds already exists (e.g. Martin et al., 2006; Martin et al., 2007; Martin et al., 2013a), there is no information on the key environmental parameters that regulate the metabolism of Lusitanian mäerl beds.

Temperature and irradiance are important factors in mäerl community energetics, primary production and calcification (Martin et al., 2006; Peña and Barbara, 2010). However, there are few studies on the field that relate primary production and calcification of free-living non-geniculate coralline algae to these variables (e.g. Martin et al., 2006; Martin et al., 2007). Previous studies have found that seasonal changes in temperature and irradiance regulate the photosynthesis, respiration and calcification of coralline algae (Martin et al., 2006; Martin et al., 2007; Martin et al 2013a; Egilsdottir et al., 2016). However, there is no information on the environmental interannual variability or any information on biological indicators such as seasonal photosynthetic pigment concentrations.

Under a future increasing acidity and temperature of the ocean this ecosystem is expected to suffer severe impacts worldwide (Foster et al., 2013). However, the diversity in results among different experimental studies with positive, negative and parabolic metabolic responses to ocean acidification (OA) and warming (Martin et al., 2013b) make difficult to predict the future outcome of this ecosystem. Divergences in results may be related to the interaction with other environmental variables such as temperature (Vásquez-Elizondo and Enríquez, 2016) and irradiance (Gao and Zheng, 2010) which modify the effect of OA on coralline algae. Even if it is essential to apply realistic environmental conditions, experimental studies

are not usually complemented by a monitoring of the seasonality of the natural communities and there is an important gap of information on field and community-scale studies (McCoy and Kamenos, 2015).

In the northeastern Atlantic, the mäerl habitat is expected to be firstly lost at high latitudes, although Lusitanian mäerl should persist longer (Brodie et al., 2014). Mäerl beds in Algarve (southern Portugal) are mainly composed of unattached, monospecific branches of the non-geniculate coralline red algae *Phymatolithon lusitanicum*, which has recently been described as a new species (Peña et al., 2015). The species *Lithothamnion corallioides* and *Phymatolithon calcareum* are also present but their occurrence is rare (Carro et al., 2014*;* Peña et al., 2015). These two species are especially abundant in the British Isles and Brittany but they are gradually replaced by *Phymatolithon lusitanicum* in Galicia (NW Spain) (Carro et al., 2014). *P. lusitanicum* is particularly abundant in subtidal mäerl beds of the Atlantic Iberian Peninsula, in Galicia (4-13 m) and Algarve (15-25 m) but it has also been detected in Northern Ireland (from the intertidal to 4 m), in the Alborán Sea (40-48 m) and the Balearic Islands (54-64 m) (Peña et al., 2015). The largest rhodoliths of *P. lusitanicum* in the Iberian Peninsula have been found in the southernmost locations and show the thickest morphology in Algarve (Carro et al., 2014; *P lusitanicum* referred as *Phymatolithon* sp3 see Peña et al., 2015).

So far, the information available on *P. lusitanicum* is restricted to descriptive studies on the morphology of the species and composition of the beds (Carro et al., 2014; Peña et al., 2015), associated flora (Peña and Barbara, 2013) and the effect of high CO_2 and warming on the photosynthesis and respiration of *P. lusitanicum* (Sordo et al., 2016). Despite the importance of this unknown ecosystem, there is no information on the environmental conditions under which this species lives or on the key abiotic variables that regulate the metabolic rates of *P. lusitanicum* under natural conditions.The objectives of this study are; 1) To investigate the seasonal and interannual evolution patterns of key abiotic parameters under natural conditions on the field, in order 2) to determine which parameters regulate the seasonal photosynthesis, calcification, respiration and pigment content of *P. lusitanicum*; and also 3) to assess the effect of increasing temperature on the photosynthetic performance and respiration of the algae.

Methods

Environmental description

The studied mäerl bed is located at 4.7 nautical miles offshore in Armação de Pêra, in the region of Algarve, Southern Portugal (N 37°011'.650"/W -8° 19'.034") (Figure 2.1.). This rhodolith bed covers an extension of about 3 km^2, from 13 to 25 m depth. The study site has a sandy bottom where thalli get accumulated on the depressions of the ripple marks. The thickness of the mäerl can reach 5 cm and most thalli present a discoidal shape. In summer of 2013 an area of the mäerl bed at 20-23 m depth was delimited to install environmental sensors and a translucent box with tagged algae for growth measurements.

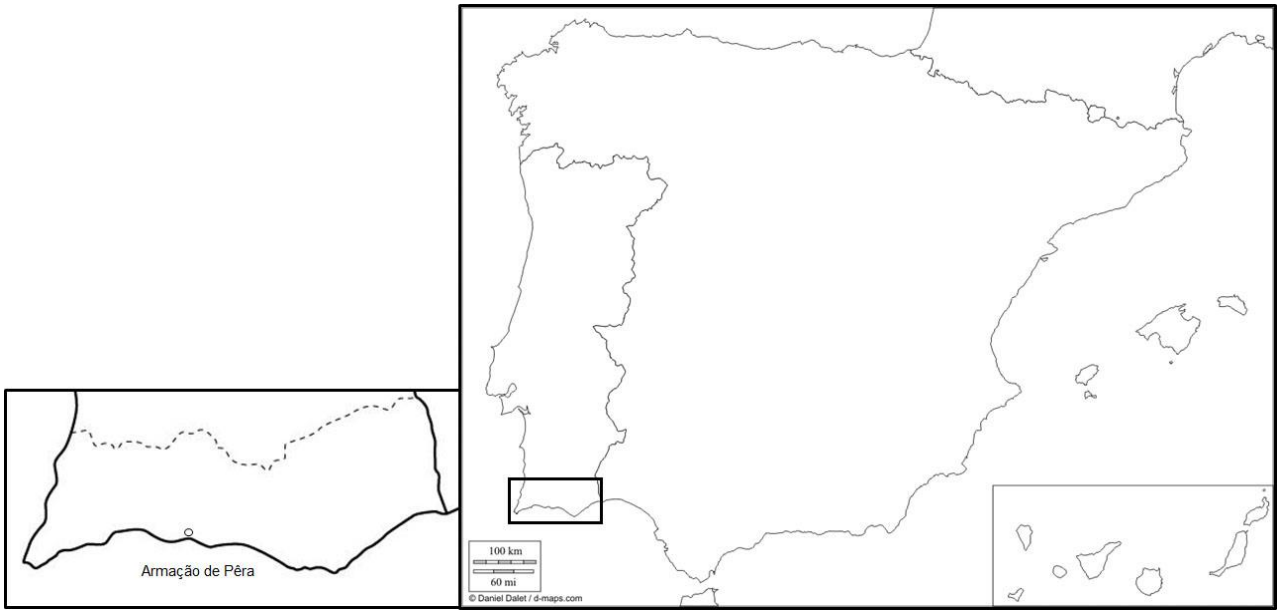

Figure 2.1. Study site location (SW Iberian Peninsula) in Armação de Pêra, southern Portugal. The mäerl bed (20-23 m depth) is located 4.7 nautical miles offshore.

Sensors for temperature (HOBO temperature loggers Onset company, USA), PAR (Odyssey Integrating PAR Sensor, Dataflow Systems PTY Limited, UK), and dissolved oxygen (miniDOT oxygen logger, Precision Measurement Engineering, Inc., PME, USA) were attached to a concrete block at 22 m depth, ~30 cm above the seabed and left to record in a continuous basis (every 15 minutes) from July 2013 to May 2015. Every three to four months the sensors were removed from the field and brought back to the lab to download the data.

Due to adverse weather conditions, it was not possible to deploy sensors in the field from December 2013 to February 2014 nor from October to November of 2014.

Photosynthesis and dark-respiration

Effective quantum yield (Y) and relative electron transport rates (rETR) were determined *in situ* using a pulse-amplitude modulated (PAM) fluorometer (Diving-PAM, Heinz Walz, Effeltrich, Germany).

$$Y= (F'm-F't)/F'm$$

Where F'm is the maximum chlorophyll fluorescence in the light-adapted thalli, Ft is the steady-state level of fluorescence under non-saturating illumination (Genty et al., 1989). The relative electron transport rate (rETR) was calculated for each irradiance level (Schreiber et al., 1995) as;

$$rETR= Y \times Irradiance \times AF \times 0.5$$

Where AF is the absorption factor (AF is the instrument's pre-defined value of 0.84), and 0.5 is a constant assuming that both PSI and PSII absorb equal amounts of the incoming photons (Beer et al., 1998). Rapid light curves (RLCs) were generated (n=9) *in situ*.

P-I curves and dark-respiration rates of *P. lusitanicum* samples collected every season were also determined in the laboratory. Rhodoliths were collected by SCUBA diving (n=10), cleaned for epiphytes and transferred to a walk-in chamber. Algae were kept under the same light and temperature conditions than those found in the field for 1 day prior to measurements.

In a separate experiment, P-I curves and dark-respiration rates of *P. lusitanicum* were also determined at eight different temperatures (n=6): 12ºC, 14ºC, 16ºC, 18ºC, 20ºC, 22ºC, 24ºC and 26ºC using a Clark-type oxygen electrode. In December 2011, algae were collected and maintained under the conditions found in the field (16°C) for two weeks. Before the measurements, thalli were left to acclimate for 48 hours, at each tested temperature in a walk-in chamber. All the measurements were carried out during four weeks.

P-I curves and dark-respiration were measured using a Clark-type oxygen electrode. For each measurement, an individual rhodolith was held vertically inside an incubation chamber (15 ml volume) coupled to a Clark-type oxygen electrode (DW3/CB1, Hansatech, Norfolk, UK) and filled with GF/F filtered seawater which was renovated every three measurements. Actinic light was provided by a slide projector (Pradovit 150, Leica, Germany) equipped with a halogen lamp (Osram Xenophot 150W). P-I curves were obtained by sequentially applying thirteen different light levels, increasing from 6 to 615 μmol photons m^{-2} s^{-1}; a series of neutral density filters mounted on slide frames were used to achieve the different light intensities.

Dark -respiration was measured after turning off the light source and covering the incubation chamber with a black opaque plastic. A magnetic stirrer was used for water homogenization.
A thermostatic exterior bath with a recirculation system was used to maintain the desired temperatures at the incubation chamber (Julabo HC, Julabo LabortechnIk, Seelbach, Germany). Oxygen evolution/consumption was recorded and photosynthetic and dark-respiration rates were calculated on a dry-weight or area basis using the following formula:

$$\mathrm{A} = \frac{\frac{[O_2](\mathrm{mg/l})}{1000\ \mathrm{ml}} \times 15\ \mathrm{ml}}{C_1\ (\mathrm{cm})} \times tg\alpha \times \frac{30\ (\mathrm{cm})}{3600\ (\mathrm{s})} \times \frac{1}{\mathrm{U}\ (\mathrm{cm}^2\ \mathrm{ou\ g})} \times \frac{1}{Exp}$$

Where A is the oxygen evolution/ consumption rate per unit of area or weight ($mgO_2.s^{-1}.cm^{-2}$ or g^{-1}); $[O_2]$ is the oxygen concentration (mgL^{-1}); C_1 is the calibration height (cm); α is the angle of the slope of the registered line of oxygen evolution/consumption; 30/3600 is the paper speed of the recording device ($cm.s^{-1}$); U is the area (cm^2) or weight (g) of the sample; *Exp* is the signal amplification of the recording device. At the end of the measurements, the fresh weight, maximum width and length of each rhodolith were recorded. Then, each thalli was partially covered with aluminum foil, left to dry in the oven at 60°C and weighted one week later (dry weight).

Simultaneously to the oxygen electrode measurements, chlorophyll fluorescence measurements were carried out using a pulse-amplitude modulated (PAM) fluorometer (Diving-PAM, Heinz Walz, Effeltrich, Germany) coupled to the oxygen electrode (n=8). The

simultaneous use of both methods allowed us to establish the relationship between the relative electron transport rates (rETR) (µmol $e^-.m^{-2}.s^{-1}$) and gross photosynthesis (µmol $O_2.m^{-2}.s^{-1}$).

Both the oxygen evolution and the relative electron transport rate light response curves were fitted with the models of Smith (1936) and Talling (1957) (see Silva and Santos, 2004 for further details) and the maximum electron-transport rate (ETR_m) maximum photosynthetic rate (P_{max}), quantum yield (α) and the half-saturation irradiation (E_k) were calculated. Curve fitting was performed using the SigmaPlot software package (Systat Software 2008, Inc. Germany)

Photosynthetic pigments

Photosynthetic pigment content was analyzed in autumn and summer 2013, winter and spring 2014 and summer 2015. Rhodoliths were collected and stored at -80°C until analyses. About 1.5 g of thalli were ground in liquid nitrogen, extracted in 5 mL of acetone and centrifuged (Heraeus Megafuge 16R, Thermo scientific, MA, USA) for 5 minutes at 2000xg and 4°C.
The samples for chlorophyll *d* were extracted in 90% acetone. The extracts were read at 630 nm, 647 nm, 664 nm and 691 nm, and the concentrations calculated according to Ritchie (2008) using the equation;

$$\text{Chlorophyll } d \text{ (g/m}^3\text{)} = -0.5881\ A_{630} + 0.0902\ A_{647} - 0.1564\ A_{664} - 11.0473\ A_{691}$$

Carotenoids and chlorophyll *a* were extracted in 100 % acetone, the extract was read at 470 nm and 661.6 nm and the concentration of the pigments was calculated using the equations of Torres et al. (2014) adapted from Lichtenthaler and Buschmann (2001) for red algae;

$$\text{Chlorophyll } a \text{ (µg. mL}^{-1}\text{)} = 10.82 \times A_{661.6}$$
$$\text{Carotenoids (µg. mL}^{-1}\text{)} = (1000 \times A_{470} - 1.90 \times \text{Chl } a)/214$$

Phycoerythrin (PE) and phycocyanin (PC) were quantified from 1 g of fresh thalli that was ground in liquid nitrogen. PE and PC were extracted in 2 ml phosphate buffer (pH 6.5) at 4°C. The extract was then centrifuged (Heraeus Megafuge 16R, Thermo scientific, USA) at 4696xg for 40 minutes. The extracts were read at 564 nm, 618 nm and 730 nm and PE and PC concentrations were determined using the equations of Sampath-Wiley and Neefus (2007);

$$\text{Phycocyanin (PC) (mg/ml)} = 0.154\ (A_{618}\text{-}A_{730})$$

$$\text{Phycoerythrin (PE) (mg/ml)} = 0.1247\ [(A_{564}\text{-}A_{730}) - 0.4583\ (A_{618}\text{-}A_{730})]$$

The absorbances of all pigment extracts were measured with a spectrophotometer (Beckman coulter, DU-650, USA) and the concentrations were calculated per sample fresh weight.

Calcification

The growth of thalli was followed *in situ* under natural conditions from summer 2013 to spring 2016. A total of 160 algae were tagged with small plastic labels attached with nylon wire and placed in a translucent plastic box. The box was fixed with chains and weights to a concrete block at 22 m depth. Every two months the algae were taken to the laboratory, gently cleaned to remove the excess of epiphytes and their growth was measured using the buoyant weight (BW) technique. This technique was first proposed by Jokiel et al. (1978) for coral growth and is described in detail by Steller et al. (2007).

The buoyant weight technique assumes that the entire calcareous skeleton of the algae is composed of carbonates. Because the density of the organic tissue is equivalent to that of seawater, any increment on the buoyant weight is an increment of $CaCO_3$ deposition or calcification (Steller et al., 2007). $CaCO_3$ composition in the coralline algae was determined by eliminating $CaCO_3$ through acidification according to Steller et al. (2007). Samples were dried and pre-weighed. Then, 24 rhodoliths were placed individually in 50 ml falcon flasks and 25 ml of 5% HCl was added in each flask. The solution was changed every 24h until all $CaCO_3$ was dissolved, when no bubbles were observed. Samples were rinsed with distilled water and dried to constant weight in an oven at 60°C. $CaCO_3$ was determined by subtracting from the pre-acidified weight.

The individual weight of the thalli was determined by suspending the sample in a beaker filled with filtered seawater by a nylon string attached to an electronic balance (Sartorius 0.1 mg, Germany). Water temperature (Roth digital thermometer, Hanna, EU) and salinity (CO310 conductivity meter, VWR, USA) were measured each period the algae were weighted in order to calculate the seawater density. The buoyant weight of the algae was then calculated using the following equation according to Steller et al. (2007);

$$Wcc=Wb\ [Dcc.\ (Dcc-Dw)^{-1}]$$

Where Wcc is the dry weight of the $CaCO_3$, Wb is the buoyant weight of the replicate, Dcc is the density of $CaCO_3$ (2.71 $g.cm^{-3}$), and Dw is the density of seawater displaced by the sample (1.03 $g.cm^{-3}$). Substituting the density of seawater and $CaCO_3$, the following equation is obtained

$$Wcc=1.61Wb$$

The results are expressed in relative growth rate (RGR) as it allows more equitable comparisons than an absolute growth rate (Hunt, 1990). The mean value of the RGR expresses the increase of weight per unit of pre-existing weight (W) over the interval t_1 to t_2 and is given by;

$$RGR= (logW_2-logW_1).\ (t_2-t_1)^{-1}$$

Statistical analyses

All statistical analyses were performed using the software package SigmaPlot version 11.0 (Systat Software 2008, Inc. Germany). Monthly or seasonal differences among the abiotic variables (temperature, dissolved oxygen and PAR), respiration, calcification, photosynthesis and pigment content were analysed using one-way ANOVA tests. When significant differences were found, a *post hoc test* (Student–Newman–Keuls, SNK) was applied to explore differences between treatments. When data presented equal variance, but did not meet the assumption of normal distribution (Kolmogorov-Smirnov test) even after square root or log transformation, an ANOVA on ranks test (Kruskal-Wallis analyses of variance) was applied.

Results

Environmental variables

Mäerl beds from southern Portugal are exposed to low light and relatively stable temperatures (Figure 2.2.). However, we recorded important inter-annual variability (P=<0.001). Maximum

temperatures in summer 2013 (24 ºC) were eight degrees higher than those observed in summer of 2014 (16 ºC).

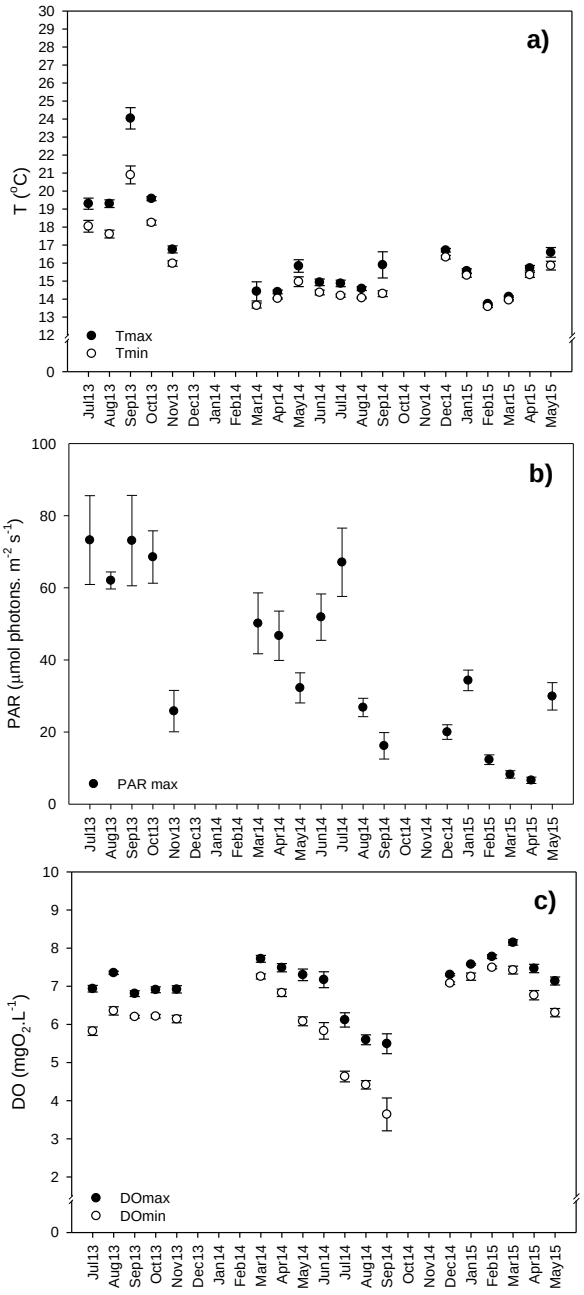

Figure 2.2. Monthly maximum and minimum mean daily values of temperature (a; ºC), maximum photosynthetically active radiation (PAR) (b; µmol photon.$m^{-2}s^{-1}$), maximum and minimum dissolved oxygen (DO) concentrations (c; mg.L^{-1}). Values are depicted as mean ± standard error.

In autumn and summer of 2013 we recorded the highest temperatures (17-24 ºC), PAR values (62 to 73 µmol photons.m^{-2}.s^{-1}) and high dissolved oxygen concentrations (6.8-7.3 $mgO_2.L^{-1}$).

In spring and the beginning of summer 2014 the PAR irradiances were high (32-67 µmol photons.$m^{-2}.s^{-1}$) and also the dissolved oxygen (7.5-7.7 $mgO_2.L^{-1}$). But by the end of summer of 2014, irradiance decreased (~20 PAR) and dissolved oxygen reached the minimum values (3.6-4.6 $mgO_2.L^{-1}$) while temperature remained unaltered (~15 ºC). The minimum temperatures (13.6-13.9 ºC) and irradiances (6.6-12 µmol photons.$m^{-2}.s^{-1}$) were recorded from winter to spring of 2015 and also the maximum DO concentrations (7.7-8.1 $mgO_2.L^{-1}$). The low PAR and temperature values and the increase of DO during this period was related to a higher agitation of the water due to rough weather conditions. The rest of the periods maximum and minimum temperatures ranged from 15 to 16ºC, PAR from 16 to 34 µmol photons.$m^{-2}.s^{-1}$ and DO from 5.8 to 7.25 $mgO_2.L^{-1}$ (Figure 2.2.a, b, c)

Photosynthesis and respiration

During the RLCs measurements on the field the temperature recorded went from 14°C in winter to 17°C in summer and 15°C in spring and autumn. Also, the PAR irradiances were higher in summer and spring (32 and 29 µmol.photons.$m^{-2}s^{-1}$) than in winter and autumn (23 and 26 µmol.photons.$m^{-2}s^{-1}$).

Maximum relative electron transport rates ($rETR_{max}$) were recorded in summer and the lowest in winter (Figure 2.3; Appendix 2.1.). There were significant differences between seasons (P=<0.001) but no differences between spring and autumn (P=0.797). The slope (α) also changed seasonally (P=<0.001), being higher in winter and autumn and lower in spring and summer, but with no differences between autumn and summer (P=0.507). The E_k was lower in the winter, and did not differ between summer and autumn (P=0.180) or spring (P=0.187).

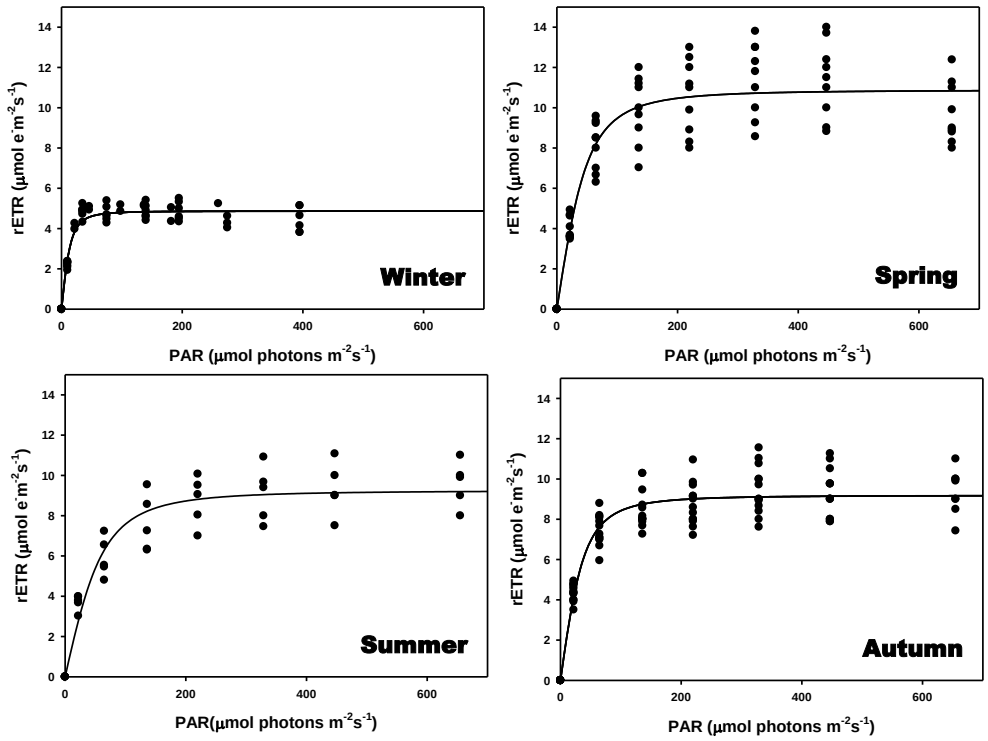

Figure 2.3. Seasonal Rapid Light Curves (RLC). Data generated *in situ* at a mäerl bed of *P. lusitanicum* (~22 m depth) using a Diving PAM underwater fluorometer. Curves represent the adjustment of the model equation of Smith (1936) and Talling (1957) to the observed values (n=9).

The maximum photosynthetic rates (P_{max}) and slope (α) of the oxygen evolution curve also changed seasonally (P=<0.001) with the highest values in summer and spring and the lowest in autumn and winter (Figure 2.4., Appendix 2.2.). In contrast, there were no significant seasonal differences for E_k (P= 0.681). When comparing both methods, we found that the slope (α) of the oxygen evolution curve was lower than the slope obtained during the RLCs. While the E_k values from the O_2 evolution were higher than those values obtained during the seasonal RLCs (Appendix 2.1.). Probably because algae under experimental conditions were acclimated to higher light conditions than those found in the field.

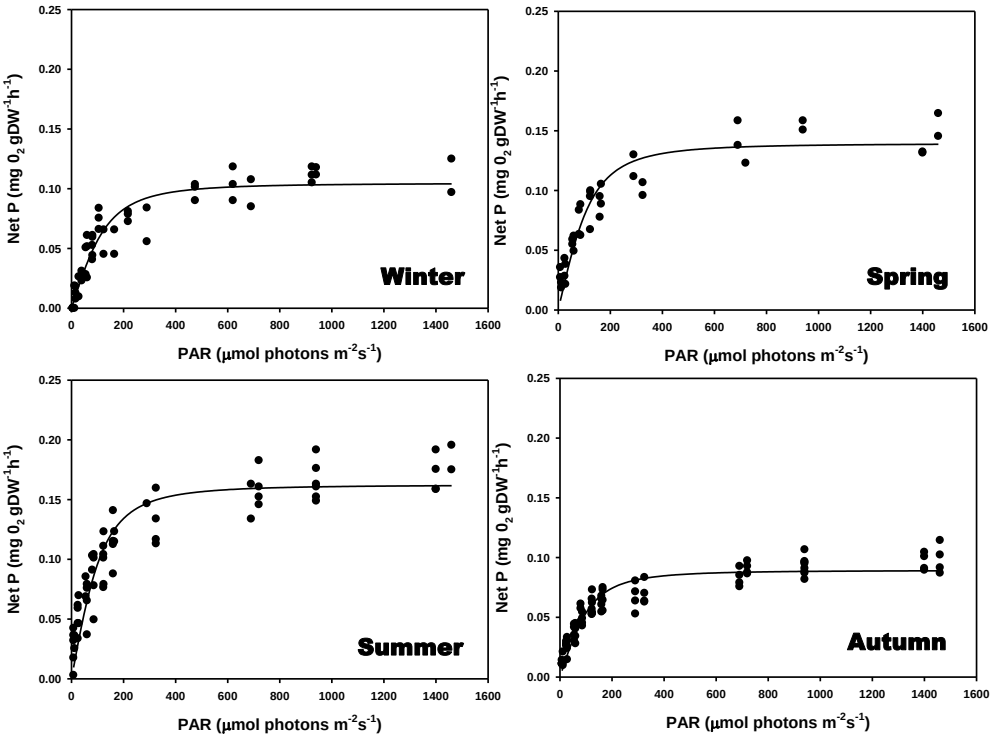

Figure 2.4. Seasonal photosynthesis-irradiance (P-I) curves, determined on individual thalli of *P. lusitanicum* in the laboratory using an oxygen Clark-type electrode (n=5). Curves represent the adjustment of the model equation of Smith (1936) and Talling (1957) to the observed values.

Dark respiration also changed seasonally with the highest respiration rates in summer and spring and the lowest in winter and autumn (Figure 2.5.). Significant differences were observed only between winter and summer (P=0.023).

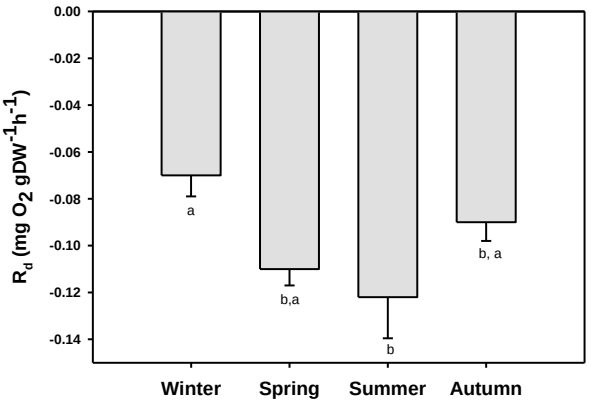

Figure 2.5. Seasonal dark respiration rates of *P. lusitanicum* (R_d; $mgO_2.gDW^{-1}h^{-1}$) obtained in the laboratory. Different letters indicate significant differences. Mean values ± standard error (n=10).

The simultaneous use of both methods (Figure 2.6.a) allowed us to establish the coupling degree between oxygen production and rETR, expressed by the molar ratio rETR/O_2 (Silva and Santos 2004). We found a linear relationship (r^2=0.95) between gross primary production (GPP) and relative electron transport rates (rETR) with an average molar ratio of 0.24 (~1/4) (Figure 2.6.b).

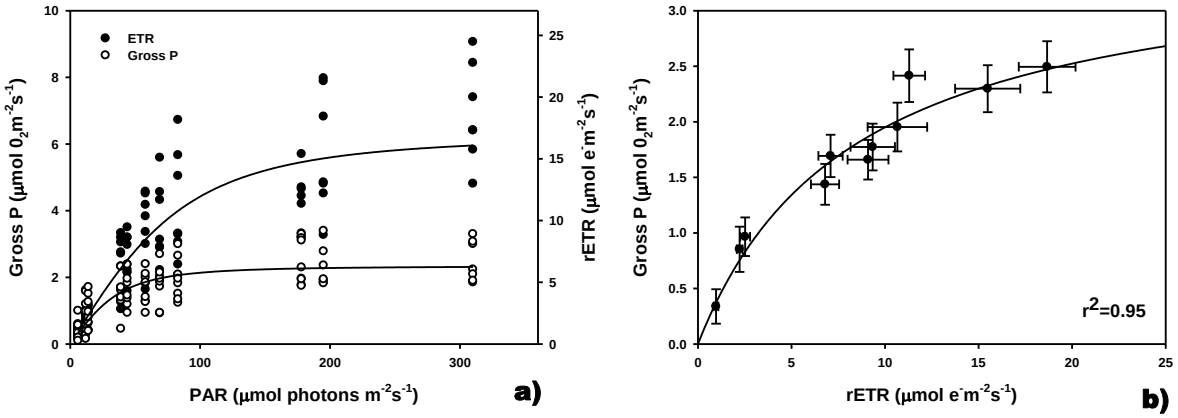

Figure 2.6. Simultaneous measurements of oxygen evolution (Gross P.; µmolO_2.m^{-2}s^{-1}) and relative electron transport rates (rETR; µmol e$^-$.m^{-2}s^{-1}) in *P. lusitanicum* (a). Gross Photosynthesis vs. rETR relationship (b). Mean values (± standard error) of Gross P and rETR measured on individual thalli (n=8).

Seasonal photosynthetic pigment concentration

Chlorophyll *d*, a pigment present in some red algae adapted to low light conditions, was undetected. Chlorophyll *a* changed seasonally (P = <0.001) increasing in winter and spring of 2014 and decreasing in autumn and summer of 2013 (Figure 2.7.a). However the differences were due to the high values observed in winter 2014 with respect to summer 2013 (P<0.001), summer 2015 (P<0.004), autumn 2013(P<0.001) and spring 2014 (P=0.021). Carotenoids also changed seasonally (P = 0.003) increasing in winter and spring and decreasing in autumn and summer (Figure 2.7.a). But once again, these differences were due to the high values observed in winter of 2014 with respect to autumn 2013 (P=0.003), summer 2013 (P=0.008), summer 2015 (P=0.013) and spring 2014 (P=0.043).

During all periods *Phymatolithon lusitanicum* presented higher concentrations (mg.gFW^{-1}) of phycoerythrin (0.188±0.067) than phycocyanin (0.01±0.004). Phycocyanin (P = 0.003) and phycoerythrin (P = <0.001) changed seasonally increasing in autumn and summer and

decreasing in winter and spring (Figure 2.7.b). Phycocyanin concentrations were significantly lower in winter 2014 with respect to autumn 2013 (P=0.003) and summer 2015 (P=0.007). While the phycoerythrin concentrations in winter 2014 were significantly lower with respect to autumn 2013 (P<0.001), summer 2013 (P=0.002) and also spring of 2014 (P=0.014).

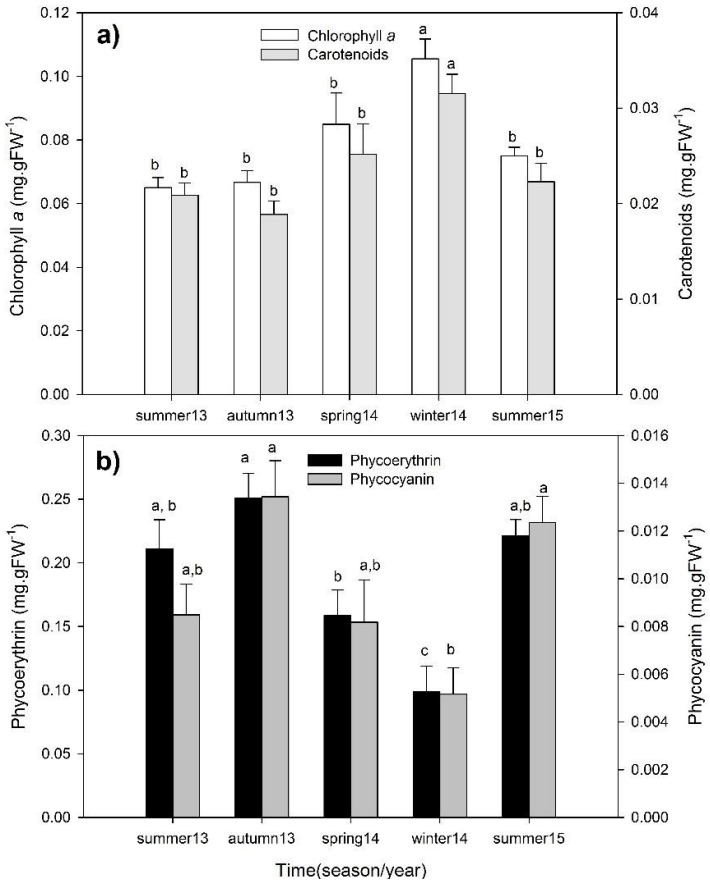

Figure 2.7. Seasonal chlorophyll *a* and carotenoid pigment content (a), and phycoerythrin and phycocyanin pigment content (mg.gFW^{-1}) (b) of *Phymatolithon lusitanicum*. Different letters indicate significant differences between seasons for each photosynthetic pigment. Mean values ± standard error (n=5).

Relative growth rate (RGR) of algae at the field (Buoyant weight technique)

In this study we estimated, through HCl acidification, that *P. lusitanicum* is composed by ~96 % of $CaCO_3$. After more than 695 days in the field the relative growth rate changed with time (P=<0.001) (Figure 2.8.). These differences were due to the high RGR observed from September to November of 2013 (1.34 µmol CaCO3.g^{-1}.day^{-1}). The rest of the periods the growth rates were constant and from November of 2014 to May 2016 went from 0.77-0.66

µmol $CaCO_3.g^{-1}day^{-1}$. While the lowest RGR was observed from November 2013 to November of 2014 (0.23-0.28 µmol $CaCO3.g^{-1}.day^{-1}$).

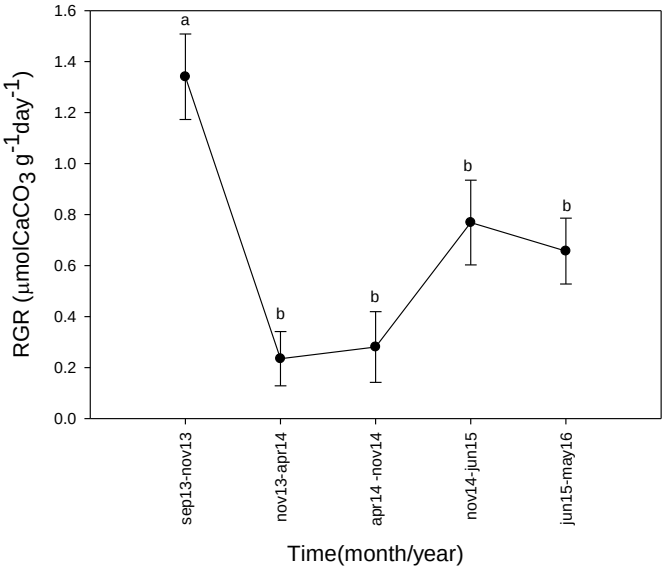

Figure 2.8. Relative growth rate of *P. lusitanicum* (RGR) from September 2013 (sep13) to May 2016 (may16) determined on marked individuals on the field. Different letters indicate significant differences. Mean values ± standard error (n=160).

Effects of temperature on respiration and photosynthesis

The results show that the dark respiration rates in *P. lusitanicum* increased with temperature (Figure 2.9.), and were significantly higher at 24°C and 26°C (P=<0.001).

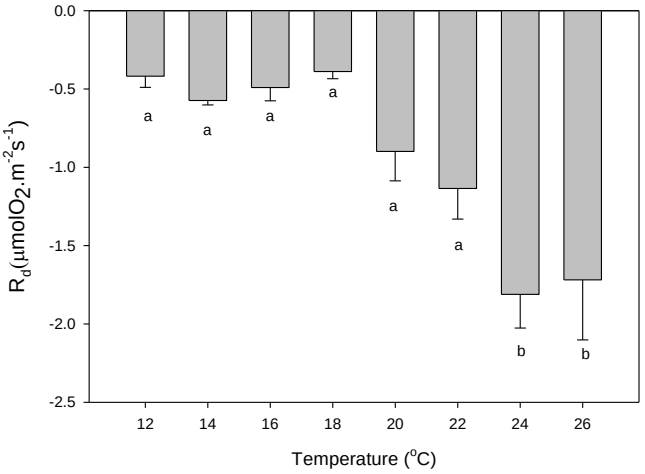

Figure 2.9. Dark respiration rates of *P. lusitanicum* (R_d; $\mu molO_2.m^{-2}.s^{-1}$) as a function of temperature. Different letters indicate significant differences. Mean values ± standard error (n=6).

The maximum photosynthetic rates (P_{max}) also increased with temperature (Figure 2.10.) (P = <0.001).

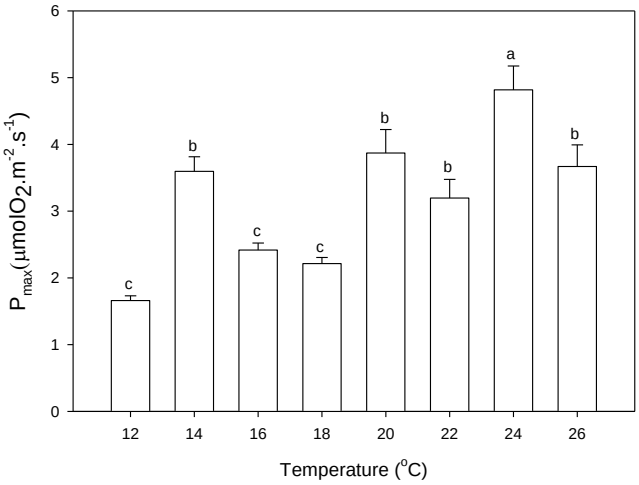

Figure 2.10. Maximum photosynthetic rates of *P. lusitanicum* (P_{max}; µmolO_2.m^{-2}.s^{-1}) as a function of temperature. Different letters indicate significant differences. Mean values ± standard error (n=6).

From 12 to 18ºC there were no differences, with the exception of the 14ºC treatment. But from 20 to 26ºC, P_{max} increased reaching the highest value at 24 ºC but with a significant decrease at 26°C (P=0.007). The E_k values (µmol photons m^{-2} s^{-1}) had a tendency to increase from 18 to 20ºC but decreased under high temperatures (24-26ºC) (Table 2.1.). However, there were no statistical differences for any of the temperatures tested (P=0.450) nor on the ascending slope at limiting irradiances (α) obtained at each temperature (P=0.271).

Table 2. 1. Parameters from Photosynthesis-Irradiance (P-I) curves at different temperatures (12-26ºC): maximum photosynthetic rates (P_{max}), ascending slope at limiting irradiances (α), and half-saturation irradiation (E_k). Different letters indicate significant differences. Mean values ± standard error.

T	**P_{max}**	**α**	**E_k**
(ºC)	*(µmolO_2.$m^{-2}s^{-1}$)*	*(µmolO_2.µmol photons^{-1})*	*(µmol photons.$m^{-2}s^{-1}$)*
12	1.66±0.07*c*	0.06±0.01	26.84±5.37
14	3.60±0.22*b*	0.10±0.03	35.81±9.18
16	2.42±0.11*c*	0.06±0.01	39.64±7.60
18	2.22±0.09*c*	0.03±001	64.19±9.67
20	3.87±0.35*b*	0.06±0.02	67.44±22.47
22	3.20±0.28*b*	0.05±0.02	64.57±21.50
24	4.82±0.26*a*	0.09±0.03	53.70±15.38
26	3.67±0.33*b*	0.07±0.02	52.25±18.31

Discussion

Seasonal photosynthesis and effect of temperature and irradiance

Both the photosynthetic efficiency of the mäerl community measured on the field and the gross photosynthesis of *P. lusitanicum* measured on the laboratory increased in summer and spring and decreased in autumn and winter. This agrees with previous studies where the net production of *Lithothamnion corallioides* (Martin et al., 2006) and the gross production of *Ellisolandia elongata* (Egilsdottir et al., 2016) was two to three-fold higher in summer than in winter. Martin et al. (2007) also found that the gross community production of mäerl beds in the Bay of Brest displayed a seasonal variability with a factor of 3.5 between winter and summer.

There are few studies where the non-invasive chlorophyll-a fluorometry technique is used to assess the photosynthetic efficiency of non-geniculate coralline algae (e.g. Wilson et al., 2004; Burdett et al., 2012). This technique is relatively simple and can be used to assess the stress level in mäerl coralline algae (Wilson et al., 2004). In this study, the seasonal measurements carried out *in situ* (PAM fluorescence) and in the laboratory (O_2 Clark electrode) revealed that despite relatively low thermal and irradiance fluctuations there is a strong seasonality of the photosynthetic activity of mäerl beds from southern Portugal. Both methods led to similar results and an average molar ratio close to ¼ between gross primary production (GPP) and rETR was verified in this study. However, a more extensive study is needed to verify if it is possible to make an extrapolation between GPP and rETR in rhodolith beds from Southern Portugal.

The saturating irradiance for *P. lusitanicum* on the field was high when compared with other temperate species. The E_k values from the RLC on the field ranged from 17 to 44 µmol photons.$m^{-2}s^{-1}$ in winter and autumn, and from 56-70 µmol photons.$m^{-2}.s^{-1}$ in summer and spring. These values are higher than the E_k values observed in May and March for the low light adapted mäerl species *Lithothamnion glaciale* (4.45-54.6 µmol photons.$m^{-2}.s^{-1}$) (Burdett et al., 2012) or the crustose Arctic species *Phymatolithon foecundum* (40 µmol photons. $m^{-2}.s^{-1}$) (Kuhl et al., 2001). The high E_k values observed with respect to other species suggest that *Phymatolithon lusitanicum*, as *Phymatolithon calcareum*, can tolerate high temperatures

better than most subtidal red algae species from the temperate zone which explains the wide temperature range distribution of these two species (Wilson et al., 2004).

Gross photosynthesis increased with temperature from 20 to 24°C but started to decrease at 26°C. The highest P_{max} values were also observed to increase with temperature, from 20°C and above, this could be a way to compensate, at least partially, the increase in the respiratory rates of algae. When we compare the values obtained at 12 and 24 ºC, we verify that the respiration rates increase by a factor of four and P_{max} increased by a factor of three (1.2431 at 12 ºC and 3.006 a 24 ºC). Between 12 and 18 ºC, with the exception of 14ºC, P_{max} was not higher than 2.5 µmol O_2 m^{-2} s^{-1}, even if we found the lowest respiration rates at these same temperatures. In contrast, between 20-24ºC the algae reached their maximum net photosynthetic rates (>2.5 µmol O_2 m^{-2} s^{-1}) with lower respiration rates at those same temperatures.

Even though there were no statistical differences, we observed the highest E_k values at 18°C and above. This shows that the maximum net photosynthetic rates will be reached only if the light intensity is superior to 50 µmol photons m^{-2} s^{-1}. This is opposite to what happened at 14ºC, where the maximum net photosynthetic rates were reached at lower irradiances of about 35.8 µmoles photons m^{-2} s^{-1}, resulting in an α slightly higher than the α obtained at 24ºC. At low light intensities and temperatures (12 and 16ºC), the P_{max} observed was inferior to 3 µmoles O_2 m^{-2} s^{-1} with α values of 0.06 µmoles O_2.µmoles photons^{-1}. These results suggest that even with lower photosynthetic rates *P. lusitanicum* will be able to maintain some growth at low temperatures if the light intensity does not exceed much the E_k for that temperature (at low temperatures algae are more prone to photoinhibition).

Under natural conditions, P_{max} or photosynthesis at light saturation, is mainly limited by the rate of carbon acquisition (Henley, 1993) to a point where the physiological limitations do not allow any increment on the photosynthetic performance, not even with any increment of the light intensity. Under high irradiances and low or high temperatures photoprotection mechanisms may be activated, consuming extra energy and, therefore, decreasing the photosynthetic rates of algae leading to photoinhibition (Taiz and Zeiger, 2002; Necchi, 2004).

Coutinho et al. (1989) registered E_k values of 98 μmol photons m^{-2} s^{-1} in the intertidal red algae *Corallina officinalis*, higher to those found in this study (26-67 μmol photons m^{-2} s^{-1}) , while in deeper rhodolith communities Figueiredo et al. (2012) found E_k values from 12 to 30 μmol photons m^{-2} s^{-1}. When irradiance increases, this adaptation to low light makes these algae more susceptible to photoinhibition especially if this increase is accompanied by lower or higher temperatures than those optimum for the organism (Taylor and Rowley, 1971; Kalituho et al., 2003).

The observed P_{max} and α decrease in *P. lusitanicum* at 26°C could be related to an eventual increase of the photorespiration and possible direct O_2 reduction in the chloroplast. One of the consequences to high temperatures is the increase on the photorespiration rates (oxygen fixing catalyzed by the enzyme RuBisCO, the same that catalyzes the primary fixation of CO_2 during the Calvin cycle in the photosynthesis). Both photosynthesis and photorespiration occur in the presence of light and both depend on RuBisco for the fixation of CO_2 or O_2, respectively. This dispute between CO_2 and O_2 for the active center of RuBisCO makes photosynthesis and photorespiration competitive processes in which the O_2 fixation results in a loss of CO_2 from the cells. Anterior studies have shown that the efficiency of the carbon fixation in photosynthesis decrease by 90% to approximately 50% when photorespiration occurs even in the absence of stress (Sharkey, 2005). It is also known that when temperature increases, photorespiration rates increase relatively to photosynthetic rates. The advantage associated to this process under high irradiances has to do with the removal of excess of excitation energy relatively to the amount that can be used in the photosynthetic electron transport chain. This removal of excess energy is fundamental to avoid damages in the photosynthetic apparatus (for e.g. in the components of the electron transport chain (Taiz and Zeiger, 2002).

On the other hand, the alterations on the biomolecules properties and cellular membranes is frequently associated to high temperatures and could cause a decrease on the transport capacity of electrons in the thylakoid membranes. This, associated to high light intensities could led to an excessive capture of light more than what can actually be processed. In these conditions, the electrons can abandon the transport chain and reduce the O_2 directly, with the consequent formation of reactive oxygen species (ROS) (Logan, 2005).

Low temperature can also provoke alterations to the photosynthetic apparatus that could lead to a decrease in the photosynthetic rates. Low temperature or "chilling" may decrease the fluidity of the membrane, negatively interfering with the electron transport chain which, in these conditions, lose electrons for O_2 leading to the formation of ROS (Reis et al. 2011; Goh et al., 2012). In this way, the low temperatures associated to high irradiance levels may lead to the decrease in the electron transport rates, photoinhibition and, ultimately to the formation of ROS (Reis et al. 2011; Goh et al., 2012). These processes could explain the low P_{max} values observed in this study for *P. lusitanicum* at 12ºC.

Even if temperature was the main factor regulating the photosynthetic activity of algae, irradiance intensified the negative effects on the net production at 26°C. Our results suggest that a decrease in the net production at a high temperature is more evident under a high irradiance. Therefore, both temperature and irradiance have a main role on the regulation of the photosynthetic rates in the red algae *P. lusitanicum*. From 20 to 26°C the algae reached the highest P_{max}, but also at 14°C (a temperature close to the values observed at the field). Even with low P_{max} values these algae will be able to keep some growth at low temperatures. But this will be possible only if the irradiance level does not exceed the saturation light intensity for that temperature.

The fact that primary production increased in summer and spring despite the small seasonal differences in temperature and irradiance may also be related to the peaks of maximum abundance of the algal community associated to mäerl beds. Previous studies on more northern rhodolith beds in the NE Atlantic have also found a strong seasonality on the associated flora, increasing in summer and spring (see Hernandez-Kantun et al., 2017 for review). The non-coralline crustose algae associated with mäerl beds in southern Portugal has been studied by Peña and Barbara (2013) but not its seasonality. However, many of the species described for southern Portugal in Peña and Barbara (2013) are also present in other Atlantic mäerl beds (*see* Peña and Barbara 2010).

In more northern mäerl beds of the Iberian Peninsula, the mäerl community is tightly correlated with temperature and irradiance increases (Peña and Barbara, 2010) and seasonality on the flora associated was observed during this study but not quantified. Therefore, the seasonal flora associated to the mäerl bed is an important factor to consider because it plays a major role in the gross community production (Martin et al., 2007). Under a global change

scenario, the increase of the abundance of the epiflora during high temperature and irradiance periods could favour calcification by increasing the pH as a consequence of the photosynthetic activity of the non-calcareous algae (Short et al., 2015). Algal cover can also protect the mäerl beds from the detrimental effect of high irradiance (Figueiredo et al., 2000). Since the community response to global change is complex, to be able predict the future response of marine communities it is important to understand the effect of environmental changes on species interactions (Short et al., 2015).

Seasonal respiration and effect of temperature

Respiration was almost two-fold higher in summer than in winter. This agrees with previous studies where the respiration rates of the non-geniculate *Lithothamnion corallioides* (Martin et al., 2006) and the geniculate *Ellisolandia elongata* (Egilsdottir et al., 2016) were three-fold and ten-fold higher respectively in summer than in winter. Also, Martin et al. (2007) found that in in Bay of Brest (France) the mäerl community respiration exhibited a strong seasonality with the greatest rates in August and the lowest in February.

The measurements at different temperatures, confirmed that respiration of *P. lusitanicum* increased gradually with temperature. It is widely known that respiration of coralline algae increase with temperature until the temperature exceeds their thermal tolerance limit (see Martin et al., 2013b for review). In the only experiment done on the effect of temperature and high CO_2 on the respiration rates of *P. lusitanicum*, the authors found that respiration was unaffected by CO_2 but positively affected by temperature (Sordo et al., 2016). This increase was significant only from 24°C but decreased under 26°C. Even if not significant, this might indicate us that the thermal limit for *P. lusitanicum* is above 26°C, a higher value than the temperatures observed under natural conditions. In contrast, the lowest respiration rates were observed at the lowest temperatures. This might constitute an algae's mechanism to avoid excessive loss of carbon through the liberation of CO_2, the main product of the mitochondrial respiration (Martin et al., 2006).

Seasonal pigment content and growth

Currently, there is no information on how environmental factors affect the pigment content in *Phymatolithon lusitanicum* on the field. Chlorophyll *a* and carotenoids increased in winter of

2014-2015 and decreased in summer of 2013 and 2015. The increase of temperature could have a negative effect on the chlorophyll *a* and carotenoid concentrations decreasing the photosynthetic efficiency with temperature. This would explain the high concentrations found in winter of 2014-2015 when the temperatures decreased. During winter of 2014-2015, we recorded the lowest PAR and temperature values and algae compensated the light deprivation by increasing their photosynthetic pigments.

Also in winter of 2014-2015, we found the lowest phycocyanin and phycoerythrin concentrations, and in spring of 2014 when the temperatures were low, but these pigments increased during the warmer seasons (summer and autumn of 2013 and summer of 2015). The phycobilins phycoerythrin and phycocyanin are the principal light-harvesting accessory pigments in the plastids of marine red algae (Beer and Eshel, 1985). Also, phycobilin pigments may be used as nitrogen reserves for growth and other necessary physiological processes when nitrogen supply becomes limiting (Lin and Stekoll, 2011). During the warmer months, one can expect a higher availability of nutrients and, because there are no light limitations, the increase of phycobilin concentrations might be related to a high nutrient availability. In contrast, during low light and temperature periods when there is less primary production and less nutrients available, *P. lusitanicum* might be using the stored nitrogen in their phycobilin pigments.

Even if calcification increases with irradiance, it is more temperature controlled and not as dependent on light as photosynthesis. The highest relative growth rates were observed from September to November of 2013 when we recorded the highest temperature and irradiance conditions. These results suggest that during this period, calcification in the field increased with temperature and irradiance. This agrees with previous studies where the calcification of *Lithothamnion corallioides* (Martin et al., 2006) and *Ellisolandia elongata* (Egilsdottir et al., 2016) increased with temperature in summer and decreased in winter. Also, Martin et al. (2007) found that mäerl community calcification decreased seasonally, being 6-fold higher in summer than in winter. However, the rest of the periods the environmental conditions were relatively constant and algae did not change their calcification rates. From November of 2013 to April of 2014, the temperatures went down and also did the calcification rates of algae. While from November 2014 to June 2015 the temperatures increased and despite the low light conditions, algae increased their calcification rates. Also, in the period where we registered the lowest PAR irradiances and temperatures (February to March of 2015) algae did not

decreased their growth rates. During this period algae were probably buried at some points or at very low light intensities.

Many coralline algae species are low light adapted and can survive to sporadic burial events. Wilson et al. (2004) found that the non-geniculate species *Phymatolithon calcareum* showed little stress after being in the dark for 4 weeks. Even if this was a short period of time, the authors suggested that is very likely that mäerl can survive several months of darkness without deleterious effects. We found that *P. lusitanicum* is able to keep relatively constant growth rates despite low light and temperature conditions.

Conclusions

Despite the stable environmental conditions recorded in the studied area, the metabolic rates of *Phymatolithon lusitanicum* are strongly dependant on small temperature and irradiance changes. The photosynthetic rates of algae and pigment concentrations are regulated by temperature and irradiance and respiration by temperature. Calcification is influenced by irradiance but more dependent on temperature changes. Both photosynthesis and respiration increased in summer and decreased in autumn and winter while calcification did not change seasonally but increased with temperature. Under a global warming scenario, temperature and irradiance will have a positive effect on the photosynthetic rates of mäerl beds from southern Portugal and respiration and calcification will increase with temperature. In a drastic scenario, above 26°C, respiration and calcification (both CO_2 sources) could end up surpassing photosynthetic rates. In this case, dissolution could surpass calcification, and photorespiration be higher than net photosynthesis, with an important negative effect on the mäerl community and on the carbon cycle. However, the mäerl bed studied is located at 20-23 m depth, under relatively stable and low PAR and temperature conditions and could become a natural refuge from global warming. The fact that algae kept constant growth rates during two years and were able to calcify despite low light and temperature conditions suggests that Lusitanian mäerl beds are important calcifiers in the Northeastern Atlantic.

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Appendix Chapter 2

Appendix 2.1. Maximum electron transport rates (ETR $_{max}$), ascending slope at limiting irradiances (α), and half-saturation irradiation (E_k) calculated from seasonal Rapid Light Curves (RLC). Different letters indicate significant differences. Mean values ± standard error.

Season	**rETR$_{max}$**	**α**	**E$_k$**
	(mmol e^-. m^{-2}. s^{-1})	*(µmolO_2.µmol photons^{-1})*	*(µmol photons.$m^{-2}s^{-1}$)*
Winter	4.87±0.07*c*	0.29±0.02*a*	16.97±1.49*c*
Spring	9.25±0.26*b*	0.13±0.02*c*	69.92±10.05*a*
Summer	10.88±0.25*a*	0.19±0.02*b*	56.46±7.10*a,b*
Autumn	9.19±0.13*b*	0.21±0.01*b*	43.57±3.40*b*

Appendix 2.2. Maximum photosynthetic rate (P_{max}), ascending slope at limiting irradiances (α) and half-saturation irradiation (E_k) calculated from seasonal P-I curves. Different letters indicate significant differences. Mean values ± standard error.

Season	**P$_{max}$**	**α**	**E$_k$**
	(mgO_2.gDW^{-1}.h^{-1})	*(µmolO_2.µmol photons^{-1})*	*(µmol photons.$m^{-2}s^{-1}$)*
Winter	0.10±0.00*c*	0.0007±0.00*c*	154.95±17.56
Spring	0.14±0.00*b*	0.0009±0.00*b*	145.51±16.52
Summer	0.16±0.00*a*	0.0012±0.00*a*	136.36±13.71
Autumn	0.09±0.00*d*	0.0007±0.00*c*	133.13±10.18

Chapter 3

A direct CO_2 control system for ocean acidification experiments: testing effects on the coralline red algae *Phymatolithon lusitanicum*

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PeerJ 4:e2503

A direct CO_2 control system for ocean acidification experiments: testing effects on the coralline red algae *Phymatolithon lusitanicum*

Abstract

Most ocean acidification (OA) experimental systems rely on pH as an indirect way to control CO_2. However, accurate pH measurements are difficult to obtain and shifts in temperature and/or salinity alter the relationship between pH and pCO_2. Here we describe a system in which the target pCO_2 is controlled via direct analysis of pCO_2 in seawater. This direct type of control accommodates potential temperature and salinity shifts, as the target variable is directly measured instead of being estimated. Water in a header tank is permanently re-circulated through an air-water equilibrator. The equilibrated air is then routed to an infrared gas analyzer (IRGA) that measures pCO_2 and conveys this value to a PID (Proportional-Integral-Derivative) controller. The controller commands a solenoid valve that opens and closes the CO_2 flush that is bubbled into the header tank. This low-cost control system allows the maintenance of stabilized levels of pCO_2 for extended periods of time ensuring accurate experimental conditions. This system was used to study the long term effect of OA on the coralline red algae *Phymatolithon lusitanicum*. We found that after 11 months of high CO_2 exposure, photosynthesis increased with CO_2 as opposed to respiration, which was positively affected by temperature. Results showed that this system is adequate to run long-term OA experiments and can be easily adapted to test other relevant variables simultaneously with CO_2, such as temperature, irradiance and nutrients.

Key-words: Ocean Acidification (OA); control system; CO_2 bubbling; coralline algae

Introduction

Mesocosm systems are widely used in ocean acidification research, allowing the simulation of predicted ocean conditions for the near future. While important information has been obtained regarding the response of different kinds of marine organisms to increased CO_2 and lower pH levels, inconsistencies and uncertainties in results are common, even among similar experiments with related taxa (Gattuso et al., 2012). Some of these discrepancies may be related to the control mechanisms used to maintain the desired experimental levels (typically pH levels). A greater understanding of the complex responses of marine organisms and ecosystems to high CO_2 requires accurate carbonate system manipulations and well-controlled experimental setups (Schulz et al., 2009).

Four parameters are often used to describe the seawater carbonate system in OA experiments: pH, CO_2 partial pressure (pCO_2), dissolved inorganic carbon (DIC) and total alkalinity (TA). From any two of these parameters it is possible to calculate the other two as well as a few other parameters of the system. However, such calculations rely on empirically derived apparent dissociation constants, which are functions of temperature and salinity (Dore et al., 2009). Because there is no consensus on which two parameters should preferentially be measured, carbonate system calculations in different OA studies are often based on different input parameters. The direct measurement of pCO_2 in seawater is considered to be difficult in small volumes, and so this variable is usually calculated from pH, TA or DIC data. Therefore, real uncertainties in both measured and calculated values persist and are often unknown, especially under high pCO_2 (Hoppe et al., 2012). For example, in the few datasets of OA studies where all parameters were measured, Hoppe et al. (2012) found that pCO_2 values calculated from TA and DIC were about 30% lower than those calculated from TA and pH or from DIC and pH. Because of this, the calculated parameters of the carbonate system (e.g. pCO_2, calcite, saturation state) are not always comparable between CO_2 perturbation studies (see Hoppe et al., 2012).

The most used control variable in OA experiments is pH. After a long debate on the use of different calibration scales and protocols (Dickson, 1993; Rérolle et al., 2012) the total scale has been established as the most appropriate and recommended scale for pH determinations in seawater (Rérolle et al., 2012). The determination of seawater pH is intrinsically difficult, it is temperature and salinity dependant and only for solutions whose pH values closely match that

of a standard can the pH be regarded as an approximate measure of the hydrogen ion activity of the solution (Gieskes, 1969). Most chemical sensors have inherent drifts and unpredictable behaviors, requiring frequent recalibrations to improve accuracy. In addition, accuracy is also affected by the type of electrode, calibration type, electrode filling solution, measurement conditions and susceptibility to electromagnetic interferences (Seiter and DeGrandpre, 2001; Dickson 1993).

The spectrophotometric pH measurement is more accurate and precise than the potentiometric method and is being established as the reference method (see Dickson, 1993; Hoppe et al., 2010; Rérolle et al., 2012). There are already semi-automated systems that can be used to implement the standard spectrophotometric approach (Carter et al., 2013; McGraw et al., 2010). However, their accuracy is still dependent on the inherent uncertainties of spectrophotometric pH measurements such as dye impurities (see Carter et al., 2013 and McGraw et al., 2010). The accuracy of the pH measurements could be improved by purifying the dye and determining the molar absorptivity ratios for the dye and spectrometer (McGraw et al., 2010), or adequately addressing the dye impurities and the indicator's temperature and salinity (Carter et al., 2013). Even if the spectrophotometric measurement is the ideal technique to measure seawater pH, there are still difficulties to apply it to real-time pH determinations or monitoring/control systems, partially because this technique cannot be applied for high frequency (e.g. 1 minute interval) underway measurements (Frankignoulle and Borges, 2001).

In addition, the calculation of pCO_2 from pH is influenced by other variables such as salinity and, even more critically, temperature. Particularly in systems with temperature fluctuations, the necessary pH to reach a certain target pCO_2 will be different for every temperature, which is naturally incompatible with the conventional pH-stats control systems. Hence, even considering that the pH readings can be accurate, these systems will always have variations of pCO_2 as a function of temperature. Because ocean's pH is expected to decrease as a consequence of the increase in atmospheric CO_2, it is logical that ocean acidification experiments should target the future projections of pCO_2 concentrations in seawater.

The partial pressure of CO_2 (pCO_2) is a parameter commonly used in oceanography, mostly for underway measurements on board research vessels. In these systems, water is continuously pumped through a water-gas equilibrator (Frankignoulle et al., 2001), where the

partial pressure of CO_2 in the water phase is equilibrated with the air and CO_2 is then read in the gas phase by an infrared gas analyser (IRGA). These systems provide very accurate and robust measurements of seawater pCO_2, which makes them particularly adequate for long-term or continuous data-acquisition setups.

In the context of high-CO_2 research, either on terrestrial, freshwater or marine (ocean acidification) environments, pCO_2 is the obvious variable used to describe present conditions and future scenarios. Abril et al. (2015) recently pointed out that regional and global estimates of CO_2 outgassing from freshwater based on pH and TA are most likely overestimated and direct pCO_2 measurements are recommended in inland waters. Therefore, it appears only logical that CO_2 could be used not only as a simple descriptor but also as the control variable in experimental systems. If such is already happening in terrestrial high-CO_2 research (e.g. Lin et al., 1999) and a recommendation for its use in freshwater has recently been published (*see* Abril et al., 2015), such is not the case yet for ocean acidification experimental systems, where pH-stats remain the most common control method. Here we describe a technical approach to set and control pCO_2 levels in an open-circuit mesocosm system using CO_2 as the control variable. Seawater in header tanks is equilibrated with air, and CO_2 is analyzed with a non-dispersive infrared gas analyzer (IRGA). This information is then transmitted to a PID (Proportional-Integrative-Derivative) digital controller that in turn regulates pure CO_2 injection in the header tanks through an algorithm-regulated process, thus avoiding fluctuations of the target pCO_2 that are characteristic of all on-off processes. This semi-automated system can maintain continuous and stable pCO_2 setpoints and does not require frequent calibrations. It is more autonomous than previous CO_2 control systems and is suitable for running long-term experiments. The system was developed with the objective of assessing the long-term effects of ocean acidification on the coralline algae *Phymatolithon lusitanicum,* recently described as a new species for science and the most common in the mäerl beds from Southern Portugal (*see* Peña et al., 2015). Here we evaluated the long-term effects (11 months) of high CO_2 in the photosynthetic rates of the algae. As well, the effects of temperature on the respiration rates of *P. lusitanicum,* at different CO_2 levels, was assessed.

Several authors have highlighted the need for more long-term experiments to evaluate the response of coralline algae to ocean acidification (e.g. Martin et al., 2013; Ragazzola et al., 2013; McCoy and Kamenos, 2015). Long-term studies can reveal very different results with respect to short-term experiments (McCoy and Kamenos, 2015) and give important

information on the potential for physiological acclimation (see Hurd et al., 2009; Martin et al., 2013; Ragazzola et al., 2013). Long-term experiments, where the synergistic effect of light, temperature and other factors is also investigated, are essential to understand how ecosystems will respond to global change.

Methods

Mesocosm layout

The whole circuit was assembled indoors, in a temperature-controlled room. Following decantation, sand- and cartridge-filtering (10 to 20 µm and 5 µm), running seawater enters a preliminary 2000L tank, where it is strongly bubbled with compressed air, to insure full pCO_2 equilibration with the atmosphere (Figure 3.1.). It is then UV-filtered (16 and 8 W) and pumped to the three 200L header tanks of the circuit, one per pCO_2 level, where CO_2 is injected. From each header tank, water is pumped to six 25L individual experimental aquaria, in a continuous 0.05 L min^{-1} flow. This is a flow-through open circuit. The temperature is largely maintained by the room's air-conditioning system, with the assistance of dedicated water-chillers, when deemed necessary. While pCO_2 is continuously measured in the header tanks, temperature, pH, dissolved oxygen and salinity are monitored regularly in the experimental aquaria.

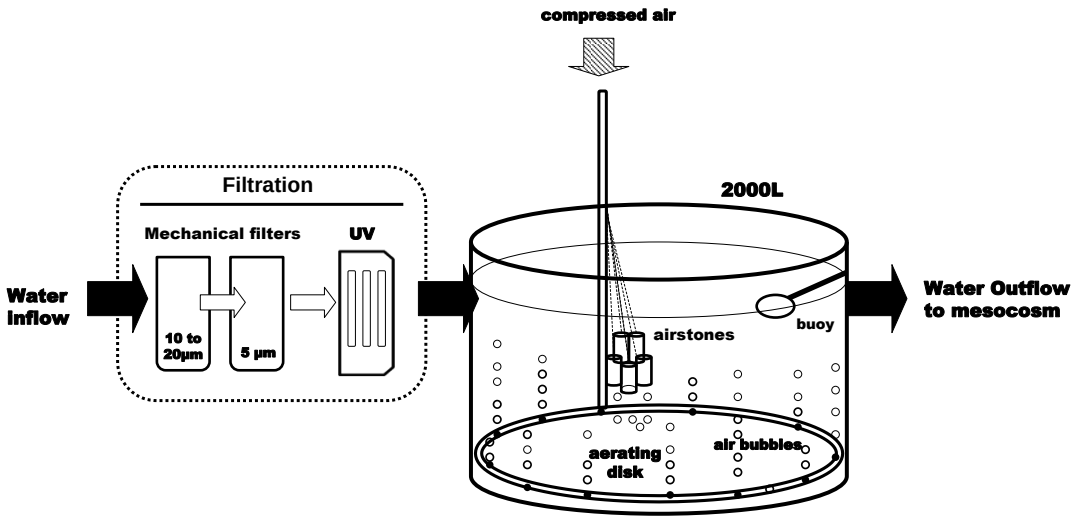

Figure 3.1. Diagram of the water supply system. The seawater is filtered before it enters the 2000L open tank. A large aerating ring and air stones maintain a continuous air bubbling to equilibrate seawater with air before it gets distributed into the head tanks (*see Figure 3.2*)

pCO_2 control system

The CO_2 control system is depicted in Figure 3.2. At the 200L header tank (1) water is flushed with industry-grade commercial CO_2. From the header tanks water is pumped (2) through an 8W UV filter (3) and is distributed to the experimental aquaria (4) Part of the water is directly channeled to a gas flushing equilibrator (5). The air-flushing equilibrator is used to equilibrate gas partial pressures between water and air, and was built according to Frankignoulle, Borges & Biondo (2001). It consists of a vertical Plexiglas tube (height: 80cm, diameter: 10 cm), sealed at both ends and filled with glass marbles to increase the exchange surface and reduce air volume. Seawater enters the equilibrator from the top (at 3 L min^{-1}) and percolates downwards, being returned to the header tank. The air coming from the IRGA (7) is injected at the bottom and aspired at the top of the equilibrator, returning to the IRGA for analysis, after passing through a Drierite® column (6) for humidity scrubbing. The air that circulates between the IRGA and the equilibrator is therefore a closed circuit of low volume (~785 mL). The CO_2 dissolved in the water equilibrates with the air (wet air) and is directly read by the IRGA (WMA-4, PP Systems, USA). All air-carrying tubing is made out Tygon® to minimize CO_2 losses.

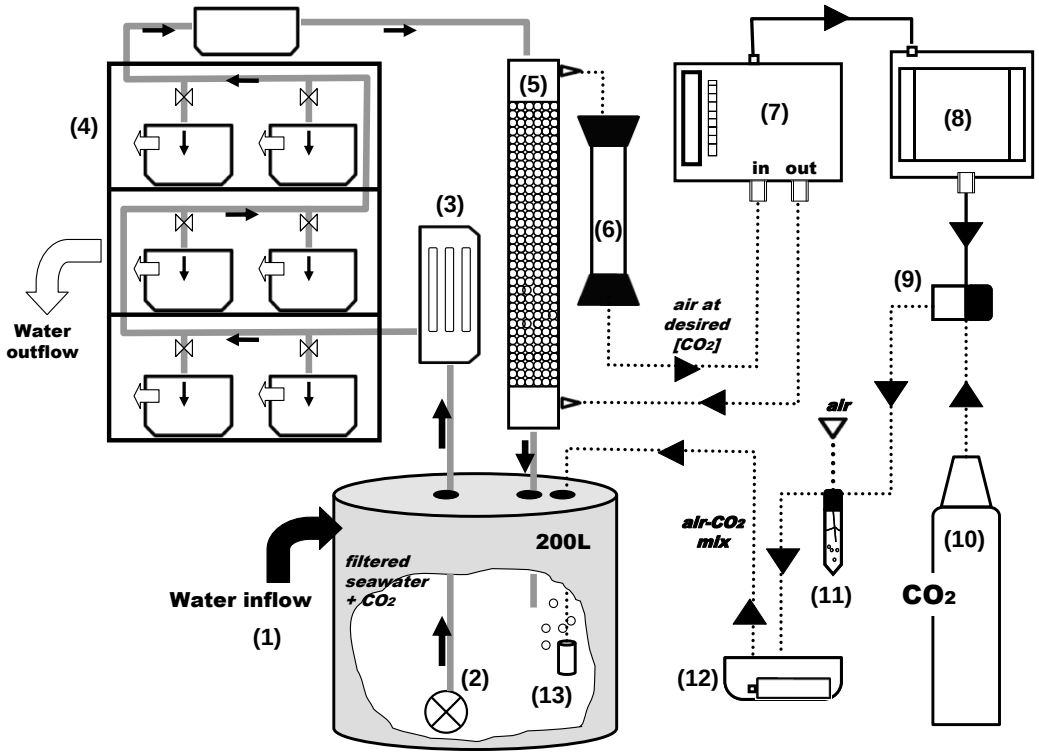

Figure 3.2. General scheme of the experimental system. Grey and black solid arrows indicate water flow and electrical connections respectively and dotted arrows indicate CO_2-air flow. From the 2000L tank (*see Figure 1*), water goes into a second reservoir of 200-L(1) and with a water pump (2) is pushed through an UV filter of 8 W (3) and distributed to the aquariums

and a sealed box located on the top of the shelves (4). From the sealed box, seawater reaches the top of the equilibrator with a close air circuit (5) and goes through a Drierite syringe (6) before entering the analyzer. The rate of CO_2 injection into the system is controlled by a CO_2 gas analyzer (7) coupled to a PID controller (8) and a solenoid valve (9) which is connected to the CO_2 bottle (10). Air and pCO_2 are mixed in a falcon flask (11) and injected into the head tank using an air pump on a sealed box (12) with aquarium air stones (13).

The Proportional-Integral-Derivative (PID) controller (8) (TEMPATRON PID 330, Farnell, UK) is an advanced controller, originally designed for the control of processes in a wide range of industrial applications. In this system, the PID controller receives the CO_2 readings from the IRGA and controls the operation of an in-line solenoid valve (9) that opens and closes CO_2 injection (10). The controller determines the difference between the measured CO_2 and the programmed set point and attempts to minimize it by adjusting the process control input (CO_2 injection). The PID controller also comprises an auto-tune feature that improves accuracy and stability. It is applied in cases when the control result is unsatisfactory or for an initial set up of a new process. This function allows the controller to "learn" the process characteristics and automatically set the necessary control coefficients to minimize deviations of the measured values relative to the set point. In practical terms, this means that CO_2 is injected in very short bursts and the controller waits for the system's response (the evolution pattern of the measured CO_2) before injecting more CO_2. The CO_2 is mixed with air (11) and injected into the header tank with an air pump (12) through air-stones (13) that create fine bubbles and allow a faster equilibration.

Biological material

The thalli were collected by SCUBA diving in Armação de Pêra, Southern Portugal (N 37° 011'.650"/W -8° 19'.034") in a mäerl bed located at 20-25 m depth and 4.7 nautical miles from the coast. The algae were immediately transferred to a cool box and transported to the CCMAR's (Centre for Marine Sciences) marine station in Ria Formosa coastal lagoon, southern Portugal. The thalli were gently cleaned to remove excess of epiphytes and kept in aquaria with filtered seawater under controlled conditions. The seawater was filtered through two in-line and two UV filters before entering the aquariums.

Experimental conditions

Based on the International Panel on Climate Change (IPCC IS92) scenarios for atmospheric pCO_2 increase (IPCC, 2000), three treatments were selected for this particular experiment: a control level running at current pCO_2 (400 µatm) and two high CO_2 levels (550 µatm and 750 µatm). Six independent 25-L aquaria were used per treatment.

A hard plastic mesh platform was placed in the bottom of each aquarium, on top of which a ca. 5 cm-high layer of mäerl thalli was installed, mimicking the natural assemblage pattern observed in natural beds. The water entry tubing for each aquarium was placed beneath the mesh platform to guarantee circulation and prevent anoxic conditions. The thalli were regularly manually revolved in the aquaria, to emulate the natural movements of the algae in their seafloor beds. The aquaria were cleaned regularly to control the growth of turf algae.

In this particular experiment, the daily variability was minimal, in order to properly mimic the natural conditions of the natural mäerl beds where algae were collected. Temperature and irradiance levels were set to emulate as much as possible the prevailing conditions at the natural beds (where a long-term monitoring program is in place). Irradiance in the aquaria was set to mimic average irradiance values measured at 25 m depth in the mäerl bed where algae were collected, in Armação de Pêra, southern Portugal. The light source consisted of two green and white led strips of 24W placed above each aquarium delivering a PAR of 8 µmol photons m^{-2} s^{-1}. Photoperiod was adjusted seasonally using a timer to the desired L: D (light and dark, h) according to natural fluctuations. It varied from 10:14 in December to 15:9 in June. Temperature was allowed to follow the natural seasonal variation (14-20°C).

Seawater parameters

The pCO_2 was continuously measured in the header tanks using an IRGA analyzer (WMA-4, PPSystems, USA). Data was downloaded into a computer every 15 days. Salinity (CO310 conductivity meter, VWR, USA), pH (Orion 8103SC pH meter, Thermo scientific, USA), temperature (Roth digital thermometer, Hanna, EU) and dissolved oxygen (Symphony SB90M5, VWR, USA, accuracy ± 0.2 mg/L; ± 2%) were regularly monitored in the experimental aquaria.

Total alkalinity (TA) was periodically measured at different points of the system; the 2000 L source tank, the 200 L header tanks and the 25 L aquaria where the algae were kept. Water samples were poisoned with 20 µl of $HgCl_2$ and sealed without bubbles in borosilicate Winkler bottles until analysis. Total alkalinity (TA) was determined using the Gran titration method, as in Lewis and Wallace (1998). Sub-samples of 80 ml were titrated with HCl 0.5M using an open cell automatic titrator (Metrohm 794, Switzlerland). Alkalinity values were corrected using Certified Reference Materials (CRMs, Batches Nos. 121 and 126) supplied by A. Dickson (Scripps Institution of Oceanography, USA). The carbonate chemistry of all water samples was determined from pH (measured with an Orion 8103SC pH electrode calibrated on the National Bureau of Standards (NBS) scale), TA, temperature and salinity using the CO_2SYS software (Lewis and Wallace, 1998) with the constants of Mehrbach et al. (1973) (refitted by Dickson and Millero, 1987).

Photosynthesis and respiration measurements

After 11 months of treatment with different pCO_2 levels, algae were collected from the mesocosm for photosynthesis and respiration measurements. All measurements were conducted using water collected in the header tanks during three successive days, one day per CO_2 level. Algae were maintained at controlled temperature at its respective CO_2 level in a "walk in" chamber during the analyses.

A square section incubation chamber (15 ml volume) coupled to a Clark-type oxygen electrode (DW3/CB1, Hansatech, Norfolk, UK) was used for measurements of oxygen evolution (µmol O_2. g FW^{-1}. h^{-1}). Photosynthesis-irradiance (P-I) response curves were built for all three pCO_2 levels (400 µatm, 550 µatm and 750 µatm) with 6 replicates per CO_2 level. For each curve, 8 light levels increasing from 6 to 860 µmol photons m^{-2} s^{-1} (PAR) were applied sequentially. The water temperature of the incubation chamber was set and maintained at 14°C using a thermostatic bath with outer recirculation system (Julabo HC, Julabo Labortechnik, Seelbach, Germany). The maximum rate of photosynthesis (P_{max}) at saturating irradiances was calculated for each curve using the model equation of Smith (1936).

For respiration rate measurements, six replicates were used per CO_2 level (400 µatm, 550 µatm and 750 µatm) and per each of 8 temperatures (12°C, 14°C, 16°C, 18°C, 20°C, 22°C, 24°C and 26°C). Prior to the measurements, algae were acclimated at the tested temperature for two days in a "walk in" chamber where light and temperature are controlled. Measurements for each temperature were carried out the same day.

Statistics

The software package SigmaPlot version 11.0 was used to perform the statistical analyses. Differences in respiration with pCO_2 and temperature, and on photosynthesis with pCO_2 and light were tested using two-way ANOVA tests. Normal distribution (Kolmogorov-Smirnov test) and equal variance (Levene's test) were verified. When differences were significant (P<0.05), ANOVA was followed by a *post hoc* test for multiple comparisons (Tukey's HSD). Photosynthesis-irradiance curve fitting was performed using the SigmaPlot software package. Differences in P_{max} were tested using a one-way ANOVA test.

Results

CO_2 control system assessment

The response time and performance of the system was evaluated at three different pCO_2 levels. Each level took between two to five hours to reach steady-state (Figure 3.3).

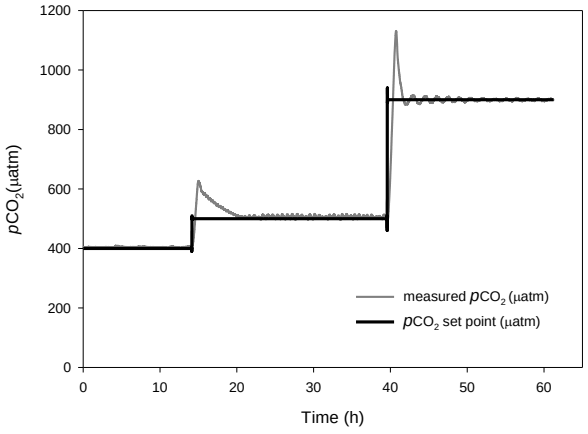

Figure 3.3. Response time (h) and stabilization of pCO_2 (µatm). Preliminary tests at three different pCO_2 levels.

At the higher pCO_2 levels the system took longer to stabilize and experienced higher amplitude oscillations, requiring a frequent use of the auto-tune mode.

The mean pCO_2 values under control conditions ranged from 397 to 470 µatm of CO_2. The mean values for the intermediate CO_2 treatment went from 539 to 577 µatm of CO_2, and the mean values for the high CO_2 treatment went from 740 to 804 µatm of. The control and the two enriched pCO_2 levels in the system were consistent throughout the experimental period and reflected the daily variations observed under natural conditions (Figure 3.4.). Total alkalinity ranged from 2491.5 to 2520.4µmol. $kgSW^{-1}$ and there were no significant differences between the sampled points of the experimental system and CO_2 treatments.

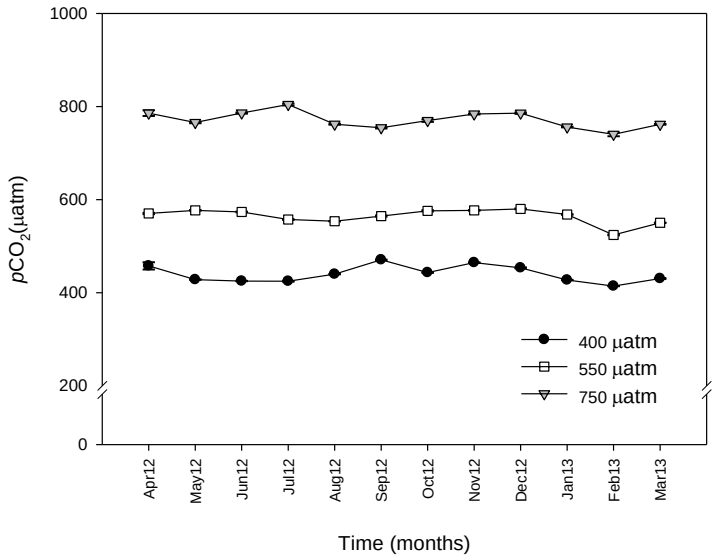

Figure 3.4. Monthly pCO_2 values (µatm) from April 2012 (Apr12) to March 2013 (Mar13) for control (~400µatm), 550µatm and 750µatm pCO_2 conditions. The values were measured with an IRGA analyzer (WMA-4, PP-systems, USA) every 20 minutes and are expressed as mean ± SE

Table 3.1. shows the carbonate system after 11 months at the three different CO_2 concentrations.The pH at the acidified treatments decreased gradually allowing for a proper acclimation of the algae. Temperatures in the aquaria during the experiment ranged from 13 to 19 ºC, salinity ranged from 34 to 38 psu and dissolved oxygen was always close to 100% of saturation (~6.7 mgO_2/L).

Table 3.1. Carbonate chemistry for each pCO_2 level (400, 550 and 750 µatm) after 11 months of experimental treatment. Total alkalinity (TA), salinity, temperature and pH were measured while dissolved inorganic carbon (DIC), aragonite saturation state (Ωarag) and pCO_2 were calculated using the CO_2SYS software. Values expressed as means ± SE (n=11).

CO_2 treatments *(µatm)*	TA *(µmol/kgSW)*	S *(psu)*	T *(°C)*	pH	DIC *(µmol/kgSW)*	Ωarag.	indirect pCO_2 *(µatm)*
400	2461.76 ± 4.08	34.87 ± 0.03	15.65 ± 0.19	8.18 ± 0.01	2183.93 ± 7.48	3.09 ± 0.05	345.62 ± 7.31
550	2449.17 ± 2.67	34.85 ± 0.02	15.93 ± 0.12	8.10 ± 0.00	2215.27 ± 2.84	2.65 ± 0.03	430.60 ± 4.69
750	2468.32 ± 2.84	34.63 ± 0.16	15.75 ± 0.14	7.90 ± 0.03	2326.21 ± 11.02	1.81 ± 0.11	755.75 ± 56.41

Long term effect of OA on coralline algae

After 11 months of high pCO_2 exposure, net photosynthesis was positively affected by elevated pCO_2 and light (P=<0.001). Results suggest that these algae have an irradiance threshold of 200 µmol photons.$m^{-2}.s^{-1}$(PAR), above which photosynthetic rates saturate (Figure 3.5.).

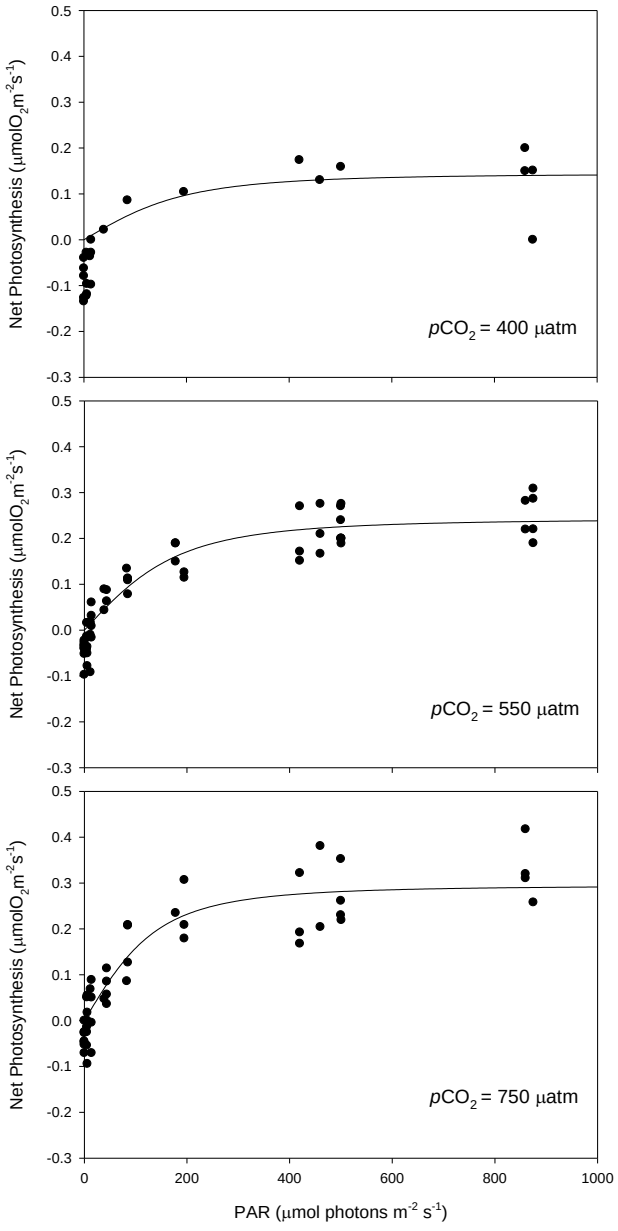

Figure 3.5. Light response curves of oxygen evolution (µmol $O_2.m^{-2}.s^{-1}$) of *Phymatolithon lusitanicum,* under control (~400 µatm), 550 µatm and 750 µatm conditions, determined on individual thalli (n=6) and fitted with the model equation of Smith (1936).

The maximum photosynthetic rates (P_{max}) increased with pCO_2 (P=0.003) reaching the maximum values at 750 µatm of CO_2 (0.29 µmol O_2. $m^{-2}.s^{-1}$) with respect to 550 µatm (0.24 µmol O_2. $m^{-2}.s^{-1}$) and control conditions (400 µatm) (0.14 µmol O_2. $m^{-2}.s^{-1}$) (Figure 3.6.).

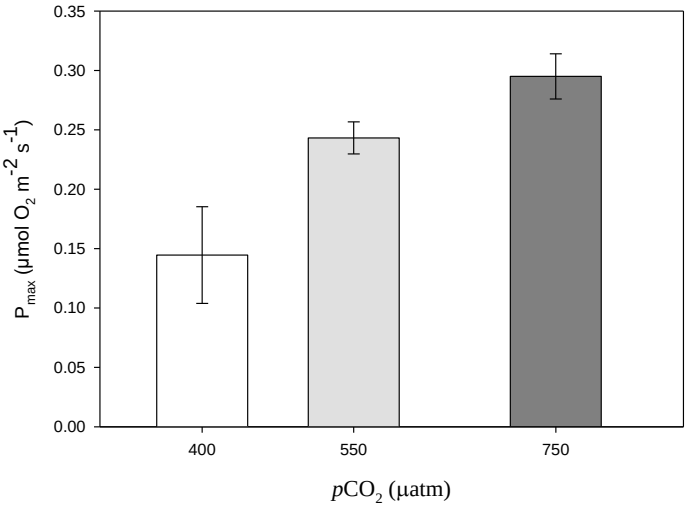

Figure 3.6. Maximum photosynthesis (P_{max}) of *Phymatolithon lusitanicum* after 11 months at different CO_2 levels (control~400 µatm, 550 µatm and 750 µatm). Values expressed as mean ± SE.

Respiration increased with temperature (P=<0.001) but it was unaffected by CO_2 (P=0.965). The highest respiration rates (µmol O_2. $m^{-2}.s^{-1}$) were observed at 26°C and the lowest at 12°C (Figure 3.7.).

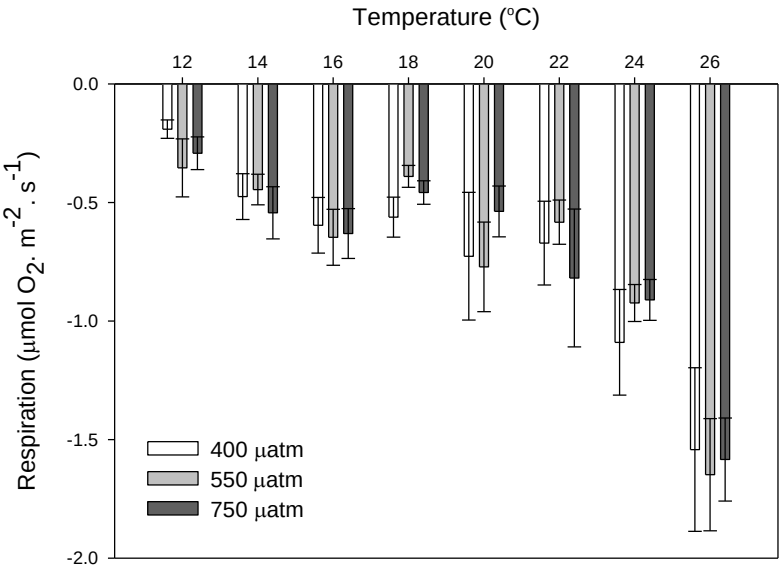

Figure 3.7. Respiration rates (µmol O_2.$m^{-2}.s^{-1}$) of coralline algae at different pCO_2 (control ~400 µatm, 550 µatm and 750 µatm) measured in individual thalli using the Clark-type

oxygen electrode at eight different temperatures from 12 to 26 °C with water exchange between replicates. Values expressed as mean ±SE (n=6).

Discussion and conclusions

This mesocosm is equipped with a simple, accurate and reliable control system that operates almost unattended apart from the regular maintenance and data download routines. Instead of using pH, the system uses CO_2 as a control variable, measured with an accurate CO_2 analyzer coupled to a PID controller. This combination circumvents the problems and uncertainties associated with pH control and allows the maintenance of long-term stability in the set pCO_2 values. This allows a proper acclimation of the organisms to different CO_2 levels and a more realistic response of their metabolic rates. It is also possible to test other relevant variables simultaneously with CO_2, such as temperature, irradiance, nutrients etc.

The combination of the gas-flushing equilibrator and the air pump results in an improved gas mixing, taking the system only 2 to 5 hours to reach a steady state. The gas-flushing equilibrator is a simple structure, easy to build and with a short response time, that can be used in highly turbid waters (Frankignoulle Borges & Biondo, 2001) and CO_2 oversaturated water (Frankignoulle and Borges, 2001). Even if an air pump to bubble the experimental water it is not commonly used in OA studies, it has been suggested by Gattuso and Lavigne (2009) as a simple alternative to inject the CO_2-air mix. In this experiment this combination has proven to be efficient.

In addition, this gas bubbling system is more economical than previous designs that use pre-mixed gasses, which are expensive in long-term experiments and only suitable for small volumes (Gattuso et al., 2010). Once the system is stabilized, the consumption of food-grade 100% CO_2 is largely minimized by the reduction of gas waste obtained with the PID control, making the system not only suitable but also affordable for long-term experiments (months to years).

This control system can also be adapted to other types of organisms, namely phytoplankton, since there is no direct bubbling into the experimental tanks. Seawater aeration by bubbling might lead to difficulties in phytoplankton cultures because it may enhance the coagulation of organic matter (Gattuso and Lavigne, 2009) or damage the calcareous structures.

It is now widely accepted that OA experiments should incorporate the natural patterns of daily fluctuations in pH. This is particularly relevant when studying species that live in environments with considerable variability, such as shallow marine habitats or coastal upwelling systems (see Eriander et al., 2015, Reum et al., 2015). With this control system, the habitat-specific pCO_2 variability can be easily incorporated in the control routine, in a similar way than with pH stats (*see* Eriander et al., 2015). The PID controller can be programmed to adjust the CO_2 injection along the day, as a function of both the target value and its desired degree of daily fluctuation.

Following the recent recommendations of Cornwall and Hurd (2015) we have in the meantime adapted the experimental design depicted in Figure 2. Instead of using the header tanks to mix the CO_2 directly with seawater, CO_2 is injected into a large air tank (4000L), where it is mixed with air to obtain the target pCO_2 value. This target value is set and controlled by a PID controller coupled to a gas analyser (IRGA). The pre-prepared mixture is then pumped by an air compressor and injected in the header tanks, one per experimental tank. The CO_2-enriched air is equilibrated with seawater in the header tanks using air stones. In this way, all the experimental tanks are true replicates. This experimental design has already been used effectively in an experiment with the seagrass species *Cymodocea nodosa* (Ruocco et al., in prep.).

The photosynthetic and respiration results after 11 months of high CO_2 exposure showed that this system is adequate to run long-term experiments with coralline algae and can be easily adapted to be used with other organisms. The increased rates of maximum photosynthesis (P_{max}) under elevated pCO_2 indicate higher carbon uptake via photosynthesis with the increase of CO_2 concentration. After 11 months of high pCO_2 exposure, *P. lusitanicum* revealed physiological capacity to acclimate to high pCO_2. This agrees with the results from Noisette et al. (2013a, b), in which the photosynthetic rates of the mäerl species *Lithothamnium corallioides* increased with CO_2. However, Martin et al. (2013) and Martin and Gattuso (2009) found decreasing or unaffected photosynthetic rates of the crustose coralline algae *Lithophyllum cabiochae* after 1 year of exposure to high CO_2. Time of acclimation and exposure is an important factor which could partially explain the differences found among OA experimental studies where coralline algae increase, decrease or have a parabolic photosynthetic response to OA (reviewed in Martin et al., 2013). The results from this study suggest that more long-term studies with coralline algae are needed. It appears important to

gradually acclimate the algae to acidified conditions and compare the results after at least two different time periods. However, it is also important to consider that the responses in long-term experiments might not be necessarily indicative of those that will occur in the future. Martin and Gattuso (2009) found that after one year in the laboratory the calcification rates of the crustose coralline alga *Lithophyllum cabiochae* decreased by several orders of magnitude. The authors attributed this decrease to the stress caused by changing environmental conditions from natural to artificial ones. The interaction with natural seasonal fluctuations of environmental parameters such as irradiance and temperature are determinant factors in OA experiments. To properly mimic the natural conditions we suggest that experimental studies should be accompanied by monitoring programs in the field.

Respiration rates responded to temperature but not to CO_2, suggesting that in a future ocean, the respiration rates of these coralline algae from southern Portugal will increase because of ocean warming, independently of ocean acidification. This agrees with previous OA studies where respiration in coralline algae was unaffected by CO_2 (Noisette et al., 2013a) but positively affected by temperature (Noisette et al., 2013b; Martin et al., 2013).

Photosynthesis and respiration results suggest that temperature and irradiance have an important role on the metabolism of coralline algae (*see* Vásquez-Elizondo and Enríquez, 2016; Martin et al., 2013) and should be carefully controlled and considered in OA experiments. Moreover, the effect of temperature on respiration is key to assess the effects of climate change in these organisms, as it will be reflected on the global carbon budgets of mäerl beds. The direct control of pCO_2 is probably the best way to standardize comparisons among high-CO_2 experiments (see Reum et al., 2015). Since similar control systems are already being used in terrestrial and freshwater ecosystems, the use of a unique control system not only facilitate the inter-comparisons but would also allow more reliable estimates of the impacts of high pCO_2 in the global carbon fluxes.

Many questions remain unanswered in OA research and the inconsistencies and differences in results due to technical problems must be minimized namely through the improvement of experimental setups and standard protocols. We demonstrated that a direct control of CO_2 can be used in long term OA experiments and propose this simple and affordable control system that is also flexible enough to be adapted and customized according to specific requirements.

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Chapter 4

High CO_2 amplifies the effect of temperature on photosynthesis, respiration and calcification of the rhodolith *Phymatolithon lusitanicum*

Laura Sordo, Rui Santos, Isabel Barrote, João Silva

(To be submitted)

High CO_2 amplifies the effect of temperature on photosynthesis, respiration and calcification of the rhodolith *Phymatolithon lusitanicum*

Abstract

The combination of ocean acidification (OA) and global warming is expected to have a profound effect on the diversity and functioning of marine ecosystems, particularly on calcifying algae such as rhodoliths (mäerl), which form extensive beds worldwide from the polar to tropical regions. However, the future of these habitats is uncertain as the few studies where the effects of both factors were investigated simultaneously have revealed contradictory results, probably due to differences in the setup of experiments, particularly their duration. Adding to the breadth of knowledge, we tested the effects of temperature and CO_2 on the net photosynthesis, respiration and calcification of the recently described species *Phymatolithon lusitanicum*, the most common mäerl species of southern Portugal. After 2 weeks of low light acclimation to temperature, calcification increased significantly whereas no effects were found on net photosynthesis and respiration. After 1 month at different temperatures and 15 days of high CO_2, photosynthesis, light calcification and respiration increased with temperature, and the differences among treatments were enhanced under high CO_2. The results suggest that temperature and time of acclimation are determinant factors in ocean acidification experiments. In a short term, the positive effect of global warming on the metabolic rates of algae will be accentuated with increasing CO_2. But in a long term *P. lusitanicum* might not be able to cope with the high energetic demands and corrosive conditions of seawater, with negative repercussions for the whole ecosystem.

Key-words: Coralline algae, mäerl, photosynthesis, respiration, calcification, temperature, ocean acidification (OA), CO_2

Introduction

Over the period from 1750 to 2000 oceans have absorbed about one-third of the carbon dioxide (CO_2) emitted by humans (Zeebe and Ridgwell, 2011). At an unprecedented rate in geological history, atmospheric CO_2 has risen from 280 ppm in 1750 to 400 ppm in 2014 (reviewed in van der Heijden and Kamenos, 2015). The CO_2 absorbed by the oceans from 1750 to 2000 has led to a pH drop from ~8.2 to 8.1, a decrease of the carbonate ion concentration (CO_3^{2-}) and $CaCO_3$ saturation state of seawater and an increase of bicarbonate ions (HCO_3^-) (Zeebe and Ridgwell, 2011). As a consequence of increasing anthropogenic emissions, the oceans have become warmer and more acidic with unknown consequences for calcifying organisms and serious implications for marine ecosystems (van der Heijden and Kamenos, 2015; Johnson and Carpenter, 2012).

Rhodolith (mäerl) beds are worldwide distributed aggregations of free living coralline algae especially sensitive to ocean acidification due to the high solubility of their high-Mg calcite skeletons (Martin et al., 2013). This is the most soluble polymorph of $CaCO_3$, a 50 % more soluble than calcite and a 20 % more soluble than aragonite (Ragazzola et al., 2013). Different studies on the influence of ocean acidification, rise of temperature and burial have confirmed a negative effect, suggesting that a combination of physical stressors can affect coralline algae and the fauna and flora associated to them (reviewed in Hernandez-Kantun et al., 2017). However, previous studies have found that calcification of coralline algae decreases (Noisette et al., 2013ab) increases (Kamenos et al., 2013) or does not change with high CO_2 (Martin et al., 2013). Also, the photosynthetic response of coralline algae to high CO_2 varies with positive, negative and parabolic responses (see Martin et al., 2013; Martin and Hall-Spencer, 2017 for review). While respiration appears to be unaffected by high CO_2 (e.g. Noisette et al., 2013ab, Martin et al., 2013) but positively affected by temperature (Martin et al., 2013; see Martin and Hall Spencer 2017 for review). Because of the great variability in results patent among different published studies (McCoy and Kamenos, 2015; Martin and Hall Spencer, 2017) the future response and resilience of coralline algae to global change is yet poorly understood (Kroeker et al., 2010; Martin et al., 2013).

Some of divergences observed among OA experiments with coralline algae might be related to multiple factors, such as the methodology used, algal physiology, time of acclimation, experiment duration (Hurd et al., 2009) or/and the light levels applied (Gao and Zheng, 2010)

and temperature used during the experiments (Vasquez-Elizondo and Enriquez, 2016; Martin and Gattuso, 2009). Seasonality based on irradiance and temperature changes is particularly important because these physical factors regulate the photosynthesis and calcification of algae (Martin et al., 2013). Light modifies the response of coralline algae to OA (Gao and Zheng, 2010) and some authors have found a significant effect of CO_2 on the calcification of algae only when in combination with high temperature (Martin and Gattuso, 2009). The effect of ocean acidification is exacerbated with warming (Martin and Hall-Spencer, 2017), and according to Vásquez-Elizondo and Enríquez (2016), coralline algae physiology is more adversely affected by temperature than high CO_2. However, few studies have investigated the simultaneous effect of temperature and high CO_2 on the photosynthesis, respiration and calcification of coralline algae (e.g. Martin et al., 2013; Noisette et al., 2013a) There is a lack of studies on how these processes are related and how these factors interact (Martin and Hall-Spencer, 2017)

Photosynthesis, calcification and respiration alter the pH in seawater (Hurd et al., 2009). Both photosynthesis and respiration are inter-connected and suggested to control the formation of $CaCO_3$ crystals on the cell walls of coralline algae. Photosynthesis increases pH while respiration decreases pH and the saturation state of SW thus hindering calcification (Hurd et al., 2009; Martin et al., 2013; Kamenos et al., 2013). In the short term, calcifying photosynthetic organisms can act as a CO_2 source through calcification and respiration and a CO_2 sink through photosynthesis. On the other hand, in the long term, the accumulation of coralline algae over geological timescales has a carbon storage potential (van der Heijden and Kamenos, 2015) and might mitigate the effect of anthropogenic emissions. Nonetheless, under OA, photosynthesis in calcifying algae is not likely to increase, and a greater CO_2 and HCO_3^- availability may uncouple photosynthesis-calcification reactions (Koch et al., 2013) with unknown repercussions for the whole ecosystem.

Global warming and OA are projected to impact the benthic flora and coastal ecosystems of the northeast Atlantic. Brodie et al. (2014) predict that global warming will kill off the kelp forests from the south and ocean acidification will remove the mäerl beds from the north. As calcium carbonate saturation falls and the sea surface isotherms are moving polewards, waters corrosive to carbonate are now presents in the Arctic and spreading south. Because of this, in the near future northern mäerl beds are expected to be lost, while mäerl beds from Southern Portugal are expected to persist (Brodie et al., 2014). However, the information available on

this geographical area is restricted to descriptive studies on the morphology and composition of mäerl beds (Carro et al., 2014; Peña et al., 2015) and associated flora (Peña and Barbara, 2013). So far, only one study (see Sordo et al., 2016) has addressed the effect of high CO_2 on the recently identified new species *Phymatolithon lusitanicum*, the most common species found in the mäerl beds from Southern Portugal (*see* Peña et al., 2015). The objective of this work is to investigate the effect of temperature and high CO_2 on the photosynthesis, respiration and calcification of the free-living coralline algae *Phymatolithon lusitanicum*.

Material and methods

Biological material

Rhodolith beds in Southern Portugal are mainly composed by non-geniculate free-living red coralline algae or mäerl without a shell or pebble core. *Phymatolithon lusitanicum* forms the largest rhodoliths with the thickest branches in Algarve (Carro et al., 2014) and has been recently identified as a new species by Peña et al. (2015). Algae were collected by SCUBA diving in Armação de Pêra, (N 37°011'.650"/W -8° 19'.034"), immediately transferred to a cool box maintained at *in situ* temperature and transported to CCMAR field station. Prior to the initial measurements, thalli of similar size and morphology were selected, gently cleaned of epiphytes and distributed in a thin layer among the experimental aquaria.

Experimental setup and design

The experimental setup used in this experiment is largely based on the one described in Sordo et al. (2016) with significant upgrades, namely to avoid pseudoreplication. Seawater is pumped from an adjacent coastal lagoon in front of CCMAR field station and goes through a preliminary mechanical filtration, two in-line cartridge filters of 10-20 and 5µm and two UV filters of 16 and 8W UV. The experimental system is located on an isolated room under controlled light and temperature conditions. For this experiment, a series of 3L experimental aquaria were connected to individual 5L header tanks (one header tank per aquarium, n=5 sets per experimental treatment). This is an open system where seawater is distributed from the header tanks into the aquariums at a flow rate of 150 mL/min. The aquariums were cleaned regularly to control the growth of turf algae.

High pCO_2 air was prepared in a large volume premix tank (4000L) where industrial grade CO_2 was mixed with air to obtain the target pCO_2 value. This mixture was continuously prepared and injected in the header tanks. pCO_2 in the premix tank was controlled via direct analysis of CO_2 using a gas analyser (IRGA) (WMA-4, PPSystems, USA) coupled to a PID controller (PID330, TEMPATRON, UK) that operated a solenoid valve to regulate the CO_2 flush into the premix tank.

Photoperiod was adjusted to 10:14 (light:dark, h). Ambient light was provided by two fluorescent tubes (Osram Luminux Plus Daylight L18W/11-860, Munich, Germany) installed above the aquariums. Irradiance was adjusted to 50 µmol photons m^{-2} s^{-1} (PAR) using a quantum sensor (Li-192SA connected to a Li-1000 Data Logger, Li-Cor, Lincoln, Nebraska, USA). Air temperature inside the container was homogenized with an AC apparatus. Water temperature was controlled using thermostatic water chillers (Sunsun HYH-0.5 D-C, China) plus additional water heaters when required.

Algae were exposed to three different temperatures (16, 19 and 23 °C) for an initial period of 15 days, where algae were under low light conditions for a week (8 µmol photons m^{-2} s^{-1}), followed by another 15 days where two pCO_2 levels were also imposed, a control one at 400 µatm and a high CO_2 level at 1000 µatm.

Seawater parameters

Temperature in the aquariums was monitored using HOBO temperature loggers (Onset Corp.). Salinity (CO310 conductivity meter, VWR, USA), pH (Orion 8103SC pH meter, Thermo scientific, USA) temperature (Roth digital thermometer, Hanna, EU) were measured regularly in each aquarium. The pCO_2 at each treatment was recorded in a continuous basis using an IRGA (WMA-4, PPSystems, USA). The Photosynthetic Active Radiation (PAR) in the aquariums and during the incubations was recorded using a PAR measuring device (Li-192SA quantum sensor connected to a Li-1000 Data Logger, Li-Cor, Lincoln, Nebraska, USA).

Photosynthesis, respiration and calcification

Net photosynthesis, respiration and calcification rates were determined through short-time thalli incubations of pre-weighted samples (ca. 20g FW and 30-60 min for photosynthesis and calcification in the light, ca. 40g FW and 75-120 min for respiration and calcification in the dark). Algae were placed in 250 mL Erlenmeyer flasks filled to the top and sealed to avoid gas diffusion. The Erlenmeyer flasks were placed in an agitation platform at the lowest speed to promote water mixture inside the flasks and avoid the formation of gas bubbles due to the irregular forms of the thalli. To control the temperature during the incubations, the flasks with algae were partially immersed in water in a 30L aquarium at the desired temperature using a thermostatic bath with outer recirculation system (Julabo HC, Julabo Labortechnik, Seelbach, Germany). Two fluorescent tubes were placed above the 30 L aquarium supplying an approximate PAR irradiance of ~ 60 μmol photons m^{-2} s^{-1}.

Water samples for dissolved oxygen and total alkalinity (TA) were collected at the beginning and end of the incubation periods, and the temperature, pH and salinity measured. From these variables the net community production was estimated both as oxygen production and inorganic carbon uptake. The modified Winkler method was used to determine the dissolved oxygen concentration by direct spectrophotometry (DU 650, beckman coulter) of total iodine following the protocol described in Labasque et al. (2004).

The net community photosynthesis or net primary production (NPP) and the dark respiration (R_d) ($\mu molO_2.gFW^{-1}. h^{-1}$) were calculated from the difference between initial and final concentrations of oxygen, normalized by the incubation time, the volume of the chamber and the fresh weight of the incubated thalli.

Gross Photosynthesis (GP) was calculated as the sum of the Net Photosynthesis (NP) and Respiration (R);

$$GP=NP + R$$

Total alkalinity (TA) was determined using the Gran titration method, as in Lewis and Wallace (1998). Sub-samples of 80 ml were titrated using an open cell automatic titration system comprising an Orion 8103SC pH electrode calibrated in the National Bureau of

Standards (NBS) scale, and a computer-driven basic titrator (Metrohm 794 Dosimat, Switzlerland, EU) using HCl 0.5 M as acid titrant. The obtained alkalinity values were corrected using Certified Reference Materials (CRMs, Batch No. 129) supplied by A. Dickson (Scripps Institution of Oceanography, USA). The carbonate chemistry of seawater was determined from measured pH, TA, temperature and salinity using the software CO_2SYS (Lewis and Wallace, 1998) with the constants of Mehrbach et al. (1973) (refitted by Dickson and Millero, 1987). The total alkalinity anomaly technique (Smith and Key, 1975) was used to determine the community's calcification rates. Light and dark calcification rates ($\mu molCaCO_3.g\ FW^{-1}.\ h^{-1}$) were calculated as:

$$G = -(\Delta TA.\ v)/(2\ .\ \Delta t\ .\ FW)$$

where calcification (G) is equal to the difference between initial and final TA multiplied by the incubation volume (v) and divided by two, the incubation time (Δt) and the fresh weight of the sample (FW).

Statistical analyses

The software package SigmaPlot version 11.0 was used to perform the statistical analysis. Differences in photosynthesis, respiration, dark and light calcification and DIC with temperature were assessed using one-way ANOVA. The combined effect of pCO_2 and temperature was tested with two-way ANOVA. Normal distribution (Shapiro-Wilk) and equal variance (Levene's test) were verified. When P was significant ($P<0.05$), ANOVA was followed by a *post hoc* test for multiple comparisons (Holm-Sidak). When data did not pass the normality test even after log and square root transformation an ANOVA on ranks was applied. The Pearson correlation coefficient was used to assess the linear dependence between photosynthesis and calcification during the initial and final incubations.

Results

Net and gross photosynthesis and dark respiration did not change significantly with temperature after two weeks of acclimation under control CO_2 conditions, despite decreasing and increasing trends, respectively (Figure 4.1.a, b and c). After 1 month of incubation, respiration, net and gross photosynthesis increased with temperature (Figure 4.1.d, e and f).

At all temperatures, net photosynthesis was negatively correlated with calcification (Pearson correlation; R = -0.582, *P*=0.037) (Figure 4.2.a) in the first 15 days of incubation. This pattern was reversed after 1 month of incubation both under control and high CO_2 conditions (Fig 4.2.b). Calcification in the light (G_L) was positively affected by temperature (*P*=0.025) (Figure 4.3.a). Algae exposed to high temperature calcified the most but there were no significant differences between the low and mid temperature treatments (*P*=0.767). This pattern was maintained after 1 month of incubation, but at 16º C, calcification turned negative under control conditions, Figure 4.3.c. Under dark conditions dissolution surpassed calcification at all temperatures but there was a high variability in the results (Figure 4.3.b) and no differences between treatments were found (*P*=0.565). After 1 month of incubation, the response variability decreased and dark calcification became positive at 16º and 23º C (control, Fig 4.3.d).

Algae took up dissolved inorganic carbon in the light at the highest rate at 16º C and no significant differences were observed between mid and high temperatures. After 1 month, DIC consumption increased with temperature. Under dark conditions, there was DIC production and no differences between temperatures was observed. High variability was observed at 23º C. This pattern was not maintained after 1 month as DIC production was only observed at 19º C.

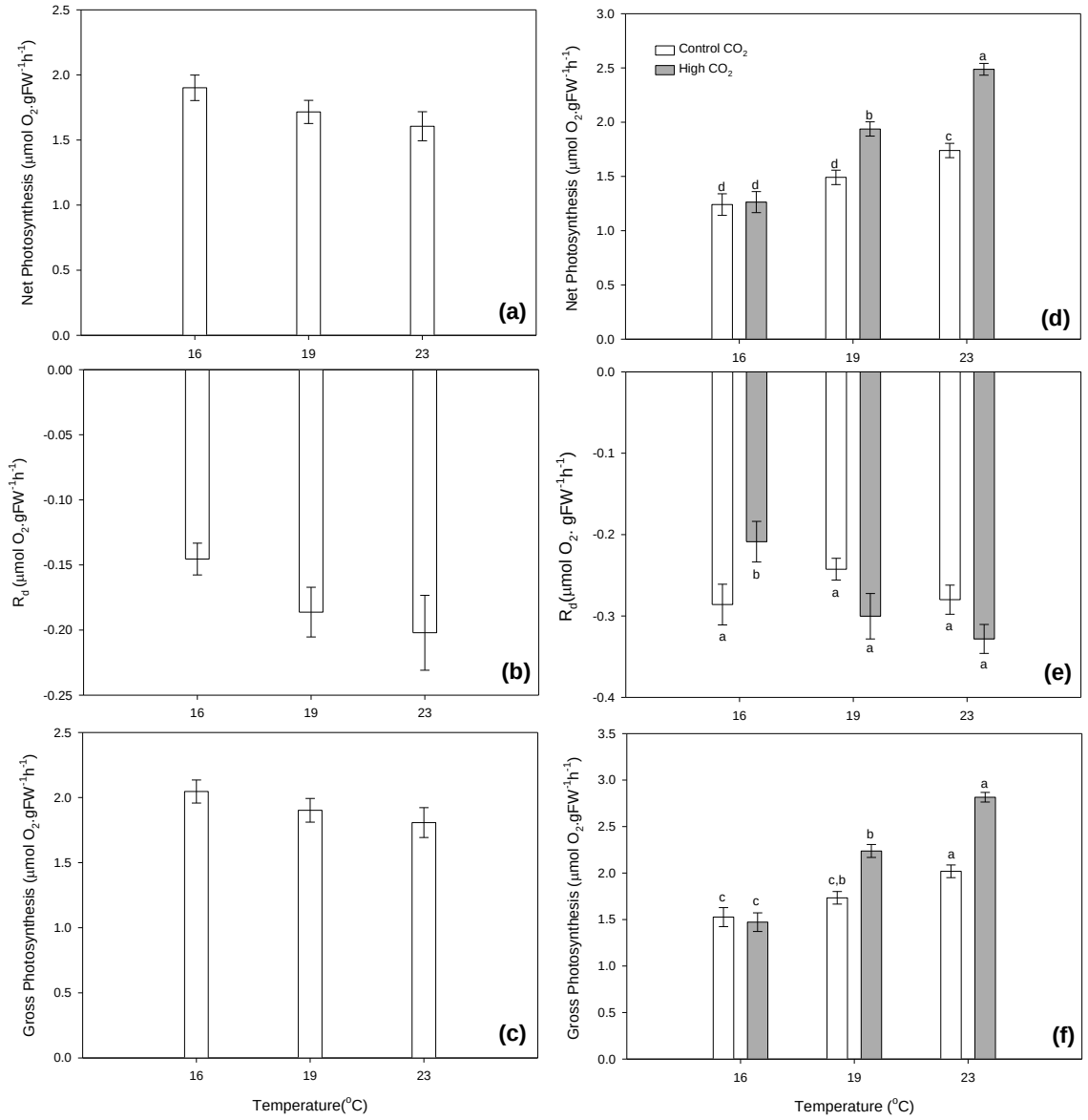

Figure 4.1. Net photosynthesis, dark respiration (R_d) and gross photosynthesis ($\mu molO_2.g\ FW^{-1}.\ h^{-1}$), after 2 weeks (a, b and c) of acclimation to low (16°C), mid (19°C) and high (23°C) temperatures and 1 month (d, e and f) of acclimation to temperature and 15 days of high CO_2. Mean ± SE (n=5), different letters indicate significant differences.

Effects of CO_2 and temperature on photosynthesis, respiration and calcification

Net photosynthesis increased with temperature and CO_2 (Figure 4.1.d) with a significant interaction between both (Two-way ANOVA; *P*=<0.001). Net photosynthesis increased with CO_2 at all temperatures, except at 16°C, where net photosynthesis did not vary with CO_2 concentration (*P*=0.836).

The effect of increasing temperature on the dark respiration of *P. lusitanicum* was differently influenced by the level of CO_2 (Figure 4.1.e).The differences observed between treatments were due to the significant interaction between temperature and CO_2 (Two-way ANOVA, P=0.007). Under normal CO_2 conditions dark respiration was unaffected by temperature but under high CO_2 respiration increased at 19 and 23°C. In contrast, the only treatment where respiration decreased with CO_2 was the low temperature one (16°C) (P=0.016).

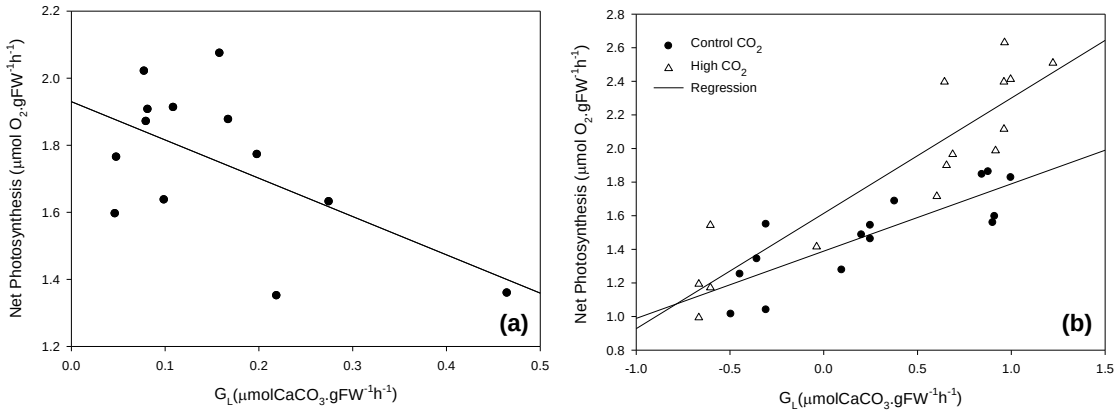

Figure 4. 2. Correlation of net photosynthesis and light calcification (G_L; µmol$CaCO_3$.g FW^{-1}. h^{-1}) of *Phymatolithon lusitanicum* after 2 weeks (a) of acclimation to low (16°C), mid (19°C) and high (23°C) temperatures and 1 month (b) of acclimation to temperature and 15 days of high CO_2. Mean ± SE (n=5), different letters indicate significant differences.

Gross photosynthesis (GP) was not affected by CO_2 (Two way ANOVA, P=0.238). On the contrary, there was a positive effect of temperature on GP (Figure 4.1.f). Net photosynthesis increased significantly with calcification (P>0.05) both under control (Pearson correlation; R=0.827; P=0.000142) and high CO_2 conditions (R=0.910; P=0.00000248). This positive correlation was intensified under high CO_2 conditions (Figure 4.2.b).

Both temperature and CO_2 had a significant, positive effect on light calcification (Figure 4.3.c), but temperature was the factor that most contributed to the differences observed (P=<0.001 versus P=0.014 of CO_2). Calcification was negative under 16°C. There was a significant interaction between temperature and CO_2 (Two way ANOVA; P=0.006), as the CO_2 positive effect was only significant at 19º C. Both temperature and CO_2 had significant effects on dark calcification (G_D) of *P. lusitanicum* (Figure 3d) and their interaction was also significant (P=<0.001). Under control conditions there was no clear pattern of the temperature effect on dark calcification. Calcification exceeded dissolution under 16 and 23°C while

under 19°C, dissolution exceeded calcification. In contrast, under high CO_2 conditions dissolution exceeded calcification at all treatments and decreased with temperature.

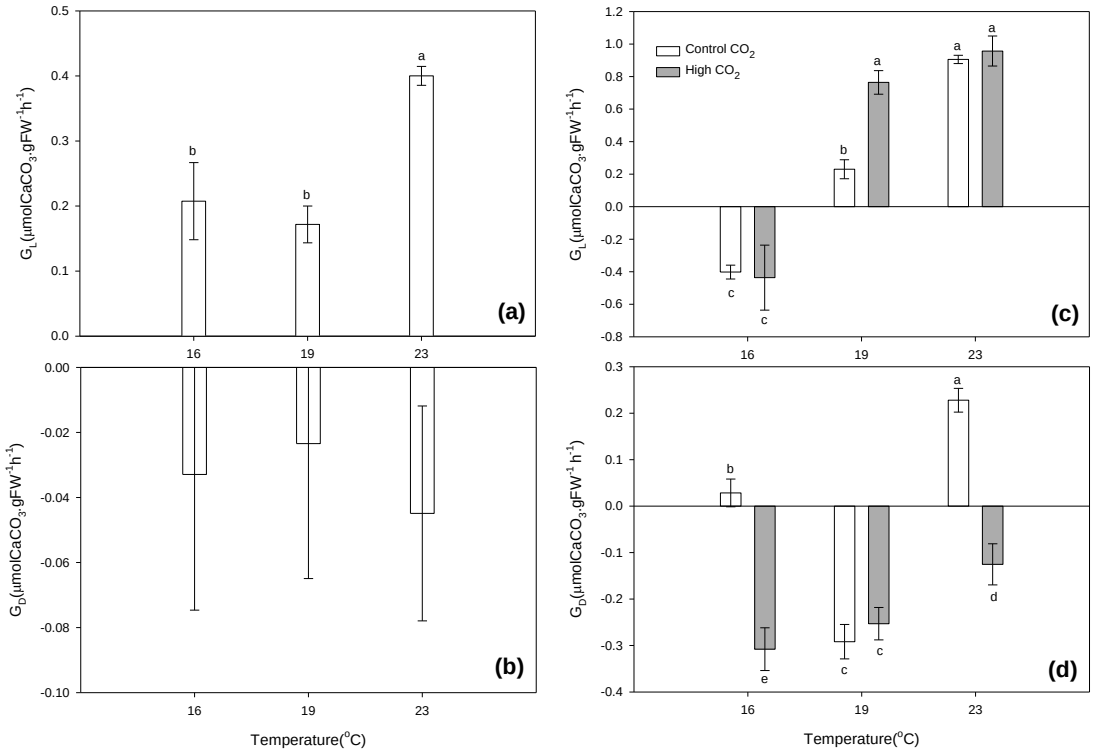

Figure 4.3. Light (G_L) and dark (G_D) calcification rate ($\mu molCaCO_3.gFW^{-1}h^{-1}$) of *Phymatolithon lusitanicum* after 2 weeks of acclimation to low (16°C), mid (19°C) and high (23°C) temperatures (a, b) and 1 month at different temperatures and 15 days of high CO_2 (c, d). Mean ± SE (n=5), different letters indicate significant differences.

Discussion

Effect of temperature and high CO_2 on photosynthesis

Both net and gross photosynthesis of *P. lusitanicum* increased with temperature and this increase was amplified under high CO_2 conditions. At the mid and high temperature treatments, photosynthetic rates increased under high CO_2 conditions and only at 16°C the effect of high CO_2 was not visible. The clear results obtained after one month were not visible after the initial two weeks of temperature-only treatment. This indicates that the algae were not fully acclimated to the different temperatures and light conditions after the first two weeks, probably because of their inherent slow metabolic response. Time of acclimation of

coralline algae is slow and a very important factor to consider in the setup of the experiments. As these algae are commonly found under low temperature and light conditions, the response of algae after two weeks of acclimation to temperature under low and high irradiance conditions can be attributed to increasing stressful conditions. The temperature values on the natural bed (14-19°C) where algae were collected are usually closer to the low and mid treatment values and rarely reach 23 to 25 °C and as such, it makes sense that during the initial incubations algae presented their highest net and gross photosynthetic rates at these temperatures. Our results show that in a scenario of global warming combined with rising atmospheric CO_2, the photosynthetic rates of these algae will be affected by a double enhancement effect.

The physiological response to altered environmental conditions is strongly influenced by the time of acclimation and length of the experiments, and could partially explain the differences observed among studies (Hurd et al., 2009; Ragazzola et al., 2013). Some short term experiments do not allow enough time for proper acclimation and may overestimate the longer term effect of increasing CO_2 on the metabolic rates of algae (Hurd et al., 2009). While short term experiments indicate an increase of the metabolic rates under elevated CO_2, the 82% of long term experiments reviewed by Koch et al. (2013) indicate a decrease of the calcification rates of algae. The importance of acclimation time was confirmed on the experiment of Ragazzola et al.(2013) with the coralline algae *Lithothamnion glaciale*, where the response of algae were compared after 3 and 10 months of high CO_2 exposure and acclimation differed over time. After 3 months, at high CO_2 algae reduced their wall thickness but maintained their growth rate. While after 10 months, the wall thickness was maintained but there was a reduction in their growth rates.

The responses of coralline algae to temperature and CO_2 have shown to be highly variable, depending on the duration and conditions of the experiment and the species studied. For the same species, *P. lusitanicum*, Sordo et al. (2016) found that after 11 months net photosynthetic rates increased with CO_2 but the differences between treatments increased under high light irradiances. In a one-month experiment Noisette et al., (2013a) confirmed that the gross production of the mäerl species *Lithothamnion corallioides* slightly increased with CO_2 while the gross photosynthesis of the geniculate species *Corallina elongata* and the crustose *Lithophyllum incrustans* were unaffected. In contrast, in a three month experiment the same authors found that the gross photosynthesis of the rhodolith *L. corallioides* increased

with CO_2 but it was unaffected by temperature. In a 10 days experiment, Vásquez-Elizondo and Enríquez (2016) found that the photosynthesis of three coralline algae species was unaffected by high CO_2 and concluded that elevated temperature is a stronger threat to coralline algae than ocean acidification (OA). In a long-term experiment (one year), Martin et al. (2013) found that under ambient irradiance the net and gross photosynthesis of the crustose *Lithophyllum cabiochae* was unaffected by temperature in most seasons but strongly affected by pCO_2 in summer autumn and winter. However, under different irradiances the authors found a significant interaction between pCO_2 and temperature with contrasting responses depending on the pCO_2 level. The authors concluded that the effect of high CO_2 on the photosynthesis of coralline algae depends on the irradiance level used in the experiments and that it is critical to consider the interaction of seasonal changes of temperature and irradiance during OA experiments.

We suggest that the diversity of results among studies, apart from the interspecific differences, is related to the temperatures and CO_2 levels, but also with the length of the experiment/acclimation and the light intensity used during the experimental/acclimation periods. It appears natural that different environmental conditions or treatments during indoor experiments can lead to different results (Gao and Zheng, 2010). For example, Noisette et al., (2013b) found no differences in photosynthesis with temperature, but the high temperature used in their experiment (19°C) was a much lower temperature than the high temperature used in this study (23°C). As Noisette et al (2013b) we also did not find any differences between the 16 and 19°C treatments under control conditions, and the effect of temperature was only evident at 23 °C. Even if the temperatures under natural conditions on the field usually go from 14 to 17°C in summer of 2013 were recorded temperatures from 17 to 24°C (data not shown). These high temperatures were probably due to a punctual sporadic event. However, under a future global warming scenario sporadic events like this, are expected to become frequent. Vásquez-Elizondo and Enríquez (2016) also suggested that the undetected effect of temperature on the photosynthesis of algae may be related to the low light levels used during experiments to properly mimic natural conditions. In a previous long-term experiment with *P. lusitanicum* (Sordo et al., 2016), we also observed that under low irradiance (8 μmol photons m^{-2} s^{-1}) there was no effect of high CO_2. However, when irradiance was increased (to a maximum of 200 μmol photons m^{-2} s^{-1}), the differences among treatments increased proportionally to CO_2 concentration. Because of this previous result and in order to observe

the effect of temperature and high CO_2 during this short-term experiment, we decided to incubate the algae under an intermediate PAR irradiance (50 μmol photons m^{-2} s^{-1}).

Even if the effect of irradiance was not tested in this experiment, at lower irradiances we will expect different results than the ones obtained in this study. In a 31-day experiment with the geniculate coralline algae *Corallina sessilis,* Gao and Zheng (2010) found that solar UV irradiance exacerbates the negative effect of high CO_2 on the calcification rates of algae. Elevated PAR irradiance may harm the cells and alter the response of marine calcifiers to ocean acidification (Gao and Zheng, 2010). Patterns of light tolerance might be important to coastal carbon dynamics and should be addressed across a range of environments and species (McCoy and Kamenos, 2015). Under the current low PAR irradiances (8-40 μmol photons m^{-2} s^{-1}) and temperatures (13-17°C) observed in the field at southern Portugal photosynthetic rates of *P. lusitanicum* could be unaffected by increasing high CO_2. However, further studies where time of exposure, irradiance, temperature and high CO_2 are addressed simultaneously are necessary to unravel the complex relationship between these factors. A further study of these interactions will help us explain the high variability in results in OA experiments with coralline algae.

Effect of temperature and high CO_2 on respiration

Under high CO_2 conditions respiration increased at 19 and 23 °C. After 2 weeks of acclimation, dark respiration had a tendency to increase with temperature, but with no significant differences between treatments. After 1 month, dark respiration under control conditions was also unaffected by temperature. However, under high CO_2 conditions algae exposed to mid and high temperatures increased their respiration rates and only algae under low temperature decreased their rates with CO_2.

Most studies have found that respiration of coralline algae increases with temperature but it is usually unaffected by CO_2 (*see* Martin et al., 2013 for a review). However there are few studies that investigate the simultaneous effect of both factors on the respiration rates of coralline algae (e.g. Martin et al., 2013; Noisette et al., 2013a; Sordo et al., 2016). In previous studies, Sordo et al. (2016) and Noisette et al. (2013a) found that the respiration rates of the rhodolith species *P. lusitanicum* and *L. corallioides*, respectively, were unaffected by high CO_2 but increased with temperature. Also, Martin et al. (2013) found that the respiration rates

of the crustose *L. cabiochae* were unaffected by elevated CO_2 but showed a trend to increase with temperature except in summer where R_d decreased with increasing temperature and CO_2. Due to the few experiments that consider these two variables together and the multiple factors that may be interacting, further research on this topic is necessary.

Effect of temperature and high CO_2 on calcification

The positive effect of temperature and high CO_2 on the light calcification rates of algae only at 19°C and negative effect on dark dissolution with positive values under control conditions at 16 and 23 °C were unexpected results. After 2 weeks of acclimation, the significant increase of calcification in the light with temperature suggests that calcification responds faster to changes in temperature than photosynthesis or respiration. After 1 month of acclimation, calcification also increased with temperature. Even if there was a significant interaction between temperature and CO_2, the positive effect of high CO_2 on calcification was observed only at 19°C. The increase of the calcification with temperature during the initial incubations and at the end, especially under high CO_2 conditions, can be seen as a mechanism to compensate the dissolution at night (Kamenos et al., 2013)

At low temperature calcification in the light was negative both under control and high CO_2 conditions and did not change with CO_2 under 23°C. In a previous 21-days study with the crustose coralline algae *Hydrolithon onkodes*, Johnson and Carpenter (2012) also found that temperature and pCO_2 had a significant interactive effect on the algae's net calcification. Even if *H. onkodes* responded under moderate elevated pCO_2 by increasing its calcification rates, this response was variable and non-linear with the highest calcification at ambient temperature (26°C). The authors also found that under high CO_2 and temperature algae reduced the protective function of the calcified thallus becoming more susceptible to grazing with important cascading community effects on the stability of coral reefs and their associated biodiversity (Johnson and Carpenter, 2012).

Calcification in the dark presented a high variability and no clear pattern could be observed after 2 weeks of acclimation to temperature. After 1 month, calcification in the dark under control conditions changed with temperature, with positive values at 16 and 23°C and negative values under 19°C. Dark calcification is likely sourced by an accumulation of energy during periods of light and there is evidence that some coralline algae can grow in the dark

(reviewed in McCoy and Kamenos, 2015). However, this mechanism could also explain the growth decrease and dissolution under stressful conditions (McCoy and Kamenos, 2015). This agrees with the results from this study where dissolution surpassed calcification in the dark under stressful conditions at the beginning, during the acclimation to temperature, and at the end of the experiment, where algae where exposed to high CO_2 conditions.

Under high CO_2 conditions, dissolution surpassed calcification in all treatments and decreased with temperature. This agrees with previous studies (Martin and Gattuso, 2009; Martin et al., 2013; Noisette et al., 2013a, b; Kamenos et al., 2013) where dark calcification of coralline algae was modified with temperature or/and CO_2. Kamenos et al. (2013) observed that under dark conditions the rhodolith *Lithothamnion glaciale* was able to calcify under control conditions but dissolved under high CO_2 conditions. Also, Noisette et al. (2013b) found that algae dissolved only under dark conditions and elevated CO_2. In other studies, under different temperatures and CO_2 concentrations, interactions between both factors have been found, like in Noisette et al. (2013a) where dark calcification increased with temperature (from 10 to 16°C) but decreased with pCO_2 and in Martin et al. (2013) where dark calcification of *L. cabiochae* in a long-term experiment was unaffected by temperature and the detrimental effect of increasing temperature was only observable under high CO_2 conditions. Also, Martin and Gattuso (2009) found a higher sensitivity of *L. cabiochae* to warming under elevated CO_2.

Both respiration and calcification release CO_2, thus decreasing the pH of seawater. The inhibition of calcification in the dark can be further amplified by the release of respiratory CO_2 (see Hurd et al., 2009 and McCoy and Kamenos, 2015 for a review). Respiration rates increased with temperature, particularly under high CO_2. The combination of all these factors plus the fact that calcification is a CO_2 source itself can explain why dissolution overwhelmed calcification in the dark under high CO_2.

As in this study, Noisette et al. (2013a) found that after 3 months of exposure to high CO_2 the calcification rates of *L. corallioides* were higher in the light than in the dark. However, the authors also found that calcification decreased with CO_2 but not with temperature while we found that light calcification tended to increase both with temperature and CO_2. However, the effect of temperature was greater than the effect of high CO_2, which agrees with the results obtained by Vásquez-Elizondo and Enríquez (2016). Martin and Gattuso (2009) and Martin et

al. (2013) found that the calcification response of the crustose algae *Lithophyllum cabiochae* to temperature and CO_2 also varied with the seasons. Light calcification increased with temperature and ambient CO_2 but decreased under high CO_2 in the summer, while throughout the rest of the year calcification showed a positive or neutral response to temperature and was maintained and even enhanced under high CO_2 (Martin et al., 2013). Thus, seasonality appears to be also an important factor influencing the diversity of results and should be addressed in future studies.

Photosynthesis and calcification

Even if it is widely known that calcification and photosynthesis are inter-connected (see Hurd et al., 2009), the details of this coupling are not totally understood yet (Johnson and Carpenter, 2012). In this study we observe that the relationship between photosynthesis and light calcification changed over time and when exposed to different stressors. After 2 week of acclimation to temperature, calcification increased and photosynthesis decreased with temperature. Both processes were negatively correlated and calcification tended to increase with decreasing photosynthesis. Algae under 16 and 19 °C used the dissolved inorganic carbon available to increase their photosynthetic rates but presented low calcification rates in the light. In contrast, algae under 23°C used the DIC available to increase their calcification rates while slightly decreasing their photosynthetic rates. Because algae were not fully acclimated after 2 weeks, under stressful conditions it appears *P. lusitanicum* favored calcification over photosynthesis.

Once the algae were fully acclimated the situation changed. After one month, net photosynthesis was positively correlated with calcification and both processes increased with temperature and high CO_2 conditions. In fact, algae under higher temperatures (19 and 23°C) increased both their calcification and photosynthetic rates and this increase was accentuated under high CO_2 conditions.

Conclusions

The response of rhodolith beds to global warming will be amplified by increasing CO_2. Temperature strongly regulates the photosynthesis, respiration and calcification *of P. lusitanicum* and high CO_2 intensifies the effect of increasing temperature. The results from

this study suggest that it is important to consider the interactive effects of temperature and ocean acidification on the main physiological processes as already suggested by Johnson and Carpenter (2012). This interaction could partially explain the divergences observed among OA experimental studies with coralline algae. The integration of additional abiotic stressors, such as light and temperature in OA experiments with coralline algae will increase our understanding of the basic relationship of photosynthesis and calcification as a function of environmental parameters (McCoy and Kamenos, 2015).

Under the current temperature and irradiance levels, mäerl beds from Southern Portugal are expected to persist. Under a global change scenario, the results from this experiment suggest that *P. lusitanicum* will increase their photosynthetic, calcification and respiration rates. The increase on the respiration rates under these conditions is expected to increase the dissolution of algae in the dark and probably their susceptibility to grazing. Hence, in the long term the high energetic costs are expected to have a negative effect on the growth of algae. However, the resilience of these algae will be determined not only by temperature and CO_2 changes but will also probably be influenced by irradiance. Further research where these three physical variables are considered and where photosynthesis, calcification and respiration are addressed simultaneously is mandatory to elucidate the future resilience of mäerl beds under a global change scenario.

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Chapter 5

Long-term effects of ocean acidification and light intensity on photosynthesis, calcification and growth of the free-living coralline algae *Phymatolithon lusitanicum*

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(To be submitted)

Long-term effects of ocean acidification and light intensity on photosynthesis, calcification and growth of the free-living coralline algae *Phymatolithon lusitanicum*

Abstract

Mäerl/rhodolith beds are protected habitats that may be affected by ocean acidification (OA), but it is still unclear how a decrease on the ocean's pH will affect this ecosystem. Some of the inconsistencies found among OA experimental studies may be related to experimental exposure time and synergetic effects with other stressors, such as high light intensity. The objective of this study was to measure the synergistic effect of OA and light on a time scale of 11 to 20 months, on the metabolism and calcification of the most common mäerl species of southern Portugal, *Phymatolithon lusitanicum.* The photosynthetic and calcification responses to elevated pCO_2 were investigated after 11 months, at different light intensities, and after 20 months of high CO_2, at ~200 μmol photons $m^{-2}s^{-1}$. The photosynthetic pigments were analysed at the end of the experiment and were unaffected by pCO_2. Photosynthetic and calcification rates increased both with CO_2 and light the first 11 months of the experiment whereas respiration slightly decreased with CO_2. After 20 months the pattern reversed and acidified algae showed lower photosynthetic and calcification rates, and lower accumulated growth than control algae, suggesting that a threshold of rhodolith resilience to OA was exceeded.

Key-words; ocean acidification (OA), *Phymatolithon lusitanicum*, coralline algae, irradiance, time, photosynthesis, respiration, calcification, photosynthetic pigments, southern Portugal.

Introduction

The expected increase of atmospheric CO_2 and consequent ocean acidification (OA) will cause major shifts on seawater chemistry. The lowering of the saturation state and carbonate concentration on seawater is expected to affect the ability of marine calcifiers to form their carbonate skeletons or shells. OA is also likely to affect photosynthesis due to the changes of the relative proportions of CO_2 and HCO_3^-, the two possible substrates for photosynthesis (Hurd et al., 2009; Martin et al., 2013). However, divergences in results among studies have prevented the scientific community to deliver a clear message on how OA will affect calcifiers in a near future (Gattuso et al., 2012). Coralline algae are of particular interest to investigate as they conduct both photosynthesis and calcification simultaneously (Martin et al., 2013).

Beds of unattached coralline algae are important carbon sinks and nursery grounds for commercial species, being also major players in the global carbon cycle through the production of $CaCO_3$ sediment (McCoy and Kamenos, 2015). These habitats are found from the poles to the tropics, and can form extensive beds of 20,900 km^2 in the tropical South West Atlantic (Amado-Filho et al., 2012). In spite of its global distribution, importance and sensitivity to OA, rhodolith beds have received less attention with respect to other more geographically restricted ecosystems such coral reefs. These globally distributed habitats are especially sensitive to Ocean Acidification (OA) and global warming because of their slow growth rates and increasing solubility of their high-Mg calcite skeletons (Williamson et al., 2014; McCoy and Kamenos, 2015). In a future ocean, filamentous algae are expected to be fitter competitors than coralline algae (Short et al., 2014). The degradation and replacement of mäerl beds by fleshy algae communities will affect the world's carbon cycle, with wide repercussions, including the fishery industry (Brodie et al., 2014).

Laboratory experiments have revealed mixed responses of the photosynthesis, calcification and respiration of coralline algae to OA. These vary among species, with positive, negative and parabolic responses (Martin et al., 2013), and because of this, the future consequences from OA on coralline algae are still unclear. Divergences in results have been attributed to differences in algal physiology, methodologies used (gas bubbling vs. acid-base additions) and/or timescale and period of acclimation (see Hurd et al., 2009; Ragazzola et al., 2013).

The current challenge in OA research is to understand how whole ecosystems react to a range of climate-related stressors (Dupont and Pörtner, 2013a). Most experimental studies do not consider the interactions with environmental factors such as irradiance, temperature or nutrients, which in many cases are determinant factors on the algae's respiration, photosynthesis and calcification. The synergistic effects of global warming (see Martin and Gattuso, 2009) and an increase in the irradiance (Gao and Zheng, 2010) are expected to aggravate and/or accelerate the detrimental effect of OA on this ecosystem. The increments of UVB irradiance as a result of the ozone reduction may bring additional damage to calcifying algae resulting in excessive energy harming the cells and altering the responses of marine calcifiers to OA (Gao and Zheng, 2010). The light quality used in ocean acidification experiments also affects the predictions of how calcified macroalgae will respond to elevated CO_2 (Yildiz et al., 2013). Because responses among living organisms are so variable, applying similar abiotic conditions is important to compare species-specific responses (Noisette et al., 2013a).

Several authors have highlighted the importance of long-term experiments in evaluating the responses of coralline algae (e.g. Martin et al., 2013; Ragazzola et al., 2013; McCoy and Kamenos, 2015). Long-term studies can reveal very different results with respect to short-term ones (McCoy and Kamenos, 2015) and give important information on the potential for physiological acclimation (see Hurd et al., 2009; Martin et al., 2013; Ragazzola et al., 2013). Nevertheless, the response of coralline algae to OA has mainly been investigated through short-term experiments (Martin et al., 2013). The longest OA experimental studies done with coralline algae lasted for a year (Martin and Gattuso, 2009; Martin et al., 2013), using the crustose species *Lythophyllum cabiochae*. In contrast, the longest experiments with mäerl species ranged from 1-3 months (e.g. Ragazzola et al., 2012; Noisette et al., 2013a; Noisette et al., 2013b; Kamenos et al., 2013) to a maximum of ten months (see Ragazzola et al., 2013). Ragazzola et al. (2013) compared the responses of the mäerl species *Lithothamnion glaciale* after 3 and 10 months of OA and obtained different results with time. After 3 months, algae cultured under high CO_2 maintained their growth rate but reduced their cell (inter and intra) wall thickness. While, after 10 months, the cell wall thickness was maintained but there was a reduction in their growth rates. The authors concluded that long-term experiments provide a better analog for understanding the organism's response to OA and a more accurate projection of future impact. Long-term experiments, where the synergistic effect of light, temperature

and other factors is also investigated, are essential to understand how ecosystems will respond to global change.

Few studies have investigated the complex and tightly linked processes of photosynthesis, calcification and respiration on coralline algae (e.g. Martin et al., 2013; Noisette et al., 2013a; Noisette et al., 2013b). These three metabolic processes are able to alter the pH of seawater, thus modifying the carbon speciation (Hurd et al., 2009). The increase of pH during photosynthesis increases the $CaCO_3$ saturation state and consequently fosters calcification, while respiration decreases pH, hindering calcification (see Gao et al., 1993; Martin et al., 2013). A better understanding of how these processes are inter-related under an OA scenario is required to elucidate how mäerl species will respond to global environmental changes (Hurd et al., 2009; Martin et al., 2013).

The effect of OA on the mäerl species from Southern Portugal is so far unknown. The objective of this study was to investigate the effect of OA and light on photosynthesis, and calcification of *Phymatolithon lusitanicum*. The effects of OA on respiration was also assessed. We conducted a long-term experiment and compared the responses at 11 and 20 months to assess whether the algae responses change with the time of exposure. This study provides new information on the synergistic effects of OA with light on coralline algae. This is also the first long term OA experiment with the mäerl species *Phymatolithon lusitanicum*, recently described as a new species (Peña et al., 2015) and the main component on some of the most southwestern mäerl/rhodolith beds in Europe.

Material and methods

Biological material

Rhodolith beds from Southern Portugal are mainly composed by non-geniculate red coralline algae or mäerl. *Phymatolithon lusitanicum* forms the largest rhodoliths with thickest branches in these beds (Peña et al., 2015). The thalli were collected by SCUBA diving in Armação de Pêra, Faro (N 37°011'.650"/W -8° 19'.034"). The algae were immediately transferred to a cool box maintained at *in situ* temperature and transported to CCMAR field biological station at the Formosa lagoon natural park. The thalli were gently cleaned to remove the excess of

epiphytes and put in aquaria with filtered seawater, where they were kept under controlled conditions during all the experiment.

Experimental system

The experimental system used in this study is described in detail in Sordo et al. (2016). Briefly, seawater is pumped from an adjacent coastal lagoon in front of CCMAR field station and passes through a preliminary mechanical filtration and through two in-line filters of 10-20 and 5µm before entering a preliminary 2000L reservoir. Here, seawater is continuously bubbled (compressed air at ambient conditions) with air stones to reach equilibrium with the atmosphere and further routed through two 16 and 8W UV filters before being distributed into three 200L head tanks.

The CO_2-air mix bubbling system is located in an isolated container where photoperiod and temperature are adjusted seasonally. This system works in an open circuit with low water flow rates. CO_2 injection is controlled by a solenoid valve coupled to an Infrared Gas Analyzer (WMA-4 IRGA, PPSystems, USA), a PID controller (PID330, TEMPATRON, UK) and an air-flushing equilibrator (Frankignoulle et al., 2001). A mix of food-grade CO_2 and ambient air is injected into the three 200L head tanks using an air pump. This is a novel OA experimental design, conceived to run long-term experiments (Sordo et al., 2016).

Experimental design

Photoperiod was adjusted using a timer to the desired L:D (light and dark, h) according to natural fluctuations. It varied from 10:14 in December to 15:9 in June. The ambient light source consisted of two 12W led strings, green and white, above the aquariums. The photosynthetic photon flux density was kept at ca. 8 µmol photons m^{-2} s^{-1}. These values were calculated based on field-collected data from an annual sampling cycle. Air and water temperatures were controlled with an AC apparatus and water chillers (Sunsun HYH-0.5 D-C, China). It varied from 14 °C in December to 21 °C in June.

Based on the CO_2 emission perspectives of the International Panel on Climate Change (IPCC IS92) (IPCC, 2000), two enrichment levels of pCO_2 = 550µatm (2050 predicted scenario) and pCO_2 = 750µatm (2100 predicted scenario) were simultaneously tested together with a control

level without enrichment (pCO_2 ~390-435 µatm). A total of 270 thalli (90 per pCO_2 level) were labeled with numbered plastic tags attached with nylon fishing wire and randomly distributed by the eighteen 25-L aquaria and maintained at the different CO_2 levels for 20 months. The aquaria were regularly cleaned to control the growth of turf algae. The growth of the thalli was followed during the experiment using the non-destructive buoyant weight technique. In addition, unlabeled thalli were kept in the aquaria for metabolic measurements.

Seawater parameters

The seawater and air temperature were monitored every five minutes using HOBO temperature loggers (Onset Corp.). Salinity (CO310 conductivity meter, VWR, USA), pH (Orion 8103SC pH meter, Thermo scientific, USA), temperature (Roth digital thermometer, Hanna, EU) and dissolved oxygen (Symphony SB90M5, VWR, *USA*, accuracy ± 0.2 mg/L; ± 2%) were measured in each aquarium on a bi-weekly basis. Total alkalinity (TA) was measured at different points of the system: the source 2000 L tank, the three head 200 L tanks and the 25L aquariums where the algae were kept. The pCO_2 at each treatment was recorded every 20 minutes by the gas analyzer (WMA-4, PPSystems, USA).

Photosynthesis, respiration and calcification

Net photosynthesis and calcification rates of algae exposed to the different pCO_2 treatments were determined three times during the experiment (at 0, 11 and 20 months of treatment) through short time incubations. After 11 months, incubations were made at different irradiance levels (8, 45, 195 and 450 µmol photons $m^{-2}s^{-1}$) and the effect of high CO_2 on the respiration and photosynthetic rates of algae was investigated. At 0 and 20 months light incubations were done at the approximated saturating irradiance of ~200 µmol photons $m^{-2}s^{-1}$. Pre-weighted algae individuals were placed in 500 mL Erlenmeyer flasks (n=4) filled to the top and sealed to prevent gas loss by leakage. For the photosynthetic and calcification measurements, 20 g of mäerl were incubated for one hour per light intensity and CO_2 level. Dark respiration (Rd) and calcification (Gd) were measured in 40 g of mäerl incubated for two and a half hours per CO_2 levels. To guarantee robust respiration results, the algae were left in the dark for 30 minutes prior to the incubations.

Samples for dissolved oxygen and total alkalinity (TA) were collected at the beginning and at the end of the incubation periods, and temperature, pH and salinity were measured. Dissolved oxygen was analyzed by direct spectrophotometry as described in Labasque et al. (2004).Briefly, a calibration curve was done with six different concentrations of KIO_3 (0, 40, 80, 160, 240 and 320 μM). Cl_2Mn 3M, NaOH 8M and NaI 4M were added to the calibration curve as well as to the water samples right after collection. After acidification with H_2SO_4 10M, the iodometric reaction was measured by direct spectrophotometry at 466 nm (Beckman Coulter DU-650).

The net photosynthesis (NP) and dark respiration (R_d) ($\mu molO_2\ gFW^{-1}\ h^{-1}$) were calculated from the O_2 change over time as follows:

$$NP \text{ or } R_d = sO_2 \times v/FW$$

Where sO_2 is the O_2 difference between the final and initial measurements with time ($\mu mol\ O_2.gFW^{-1}h^{-1}$), v is the volume of the chamber (L), and FW is the fresh weight (g) of the incubated thalli.

The total alkalinity (TA) anomaly technique (see Smith and Key, 1975) was used to determine the community's calcification rates. When 1 mole of $CaCO_3$ precipitates TA decreases in the ratio of 1:2 (see Wolf-Gladrow et al., 2007). Total alkalinity was determined using the Gran titration method as in Lewis and Wallace (1998). Seawater was sampled at the beginning and end of the incubations and at different points of the system in 100 mL Winkler bottles and immediately poisoned with 20μl mercuric chloride. TA was measured in sub-samples of 80 ml which were titrated using an open cell automatic titration system comprising an Orion 8103SC pH electrode calibrated on the National Bureau of Standards (NBS) scale, and a computer-driven basic titrator (Metrohm 794 dosimat titrator, Switzlerland, EU) using an acid titrant of HCl 0.5 M. The values were corrected using Certified Reference Materials (CRMs, Batch No. 121 and 126) supplied by A. Dickson (Scripps Institution of Oceanography, USA). The carbonate chemistry of seawater samples was determined from measured pH, TA, temperature and salinity using the software CO_2SYS (Lewis and Wallace, 1998) with the constants of Mehrbach et al. (1973) (refitted by Dickson and Millero, 1987). Light and dark calcification rates were calculated as:

$$G = - (\Delta TA).\ v / (2\ .\ \Delta t.\ FW)$$

Where calcification (G) is equal to the difference between initial and final total alkalinity (ΔTA) multiplied by the incubation volume (v) and divided by two, the incubation time (Δt) and the fresh weight of the sample (FW).

Calcium carbonate and organic matter content (HCl acidification)

Organic matter and $CaCO_3$ composition in the algae were determined by eliminating $CaCO_3$ through acidification (see Steller et al., 2007). 24 individual rhodoliths per CO_2 level were weighed and then dried to constant weight. After this step, each rhodolith was placed in 25 ml of 5% HCl which was changed every 24h until no bubbles were observed and all $CaCO_3$ was dissolved. Samples were rinsed with distilled water and dried to constant weight in a drying oven at 60°C. Organic matter and $CaCO_3$ were determined by subtraction from the pre-acidified dry weight.

Calcification/growth (buoyant weight technique)

The calcification/growth of algae was determined using the buoyant weight (BW) technique, firstly proposed for coral growth by Jokiel et al. (1978) and also used in coralline algae (e.g. Steller et al., 2007 and Short et al., 2014). A total of 270 thalli (90 per CO_2 level) were tagged at the beginning of the experiment. The individual weight of each thallus was determined every two months, for a total of 23 months growth, by suspending the sample in a beaker filled with filtered seawater by a nylon string attached to an electronic balance (Sartorius 0.1 mg).

The weight of $CaCO_3$ was calculated using the following equation, the derivation of which is explained in detail in Steller et al. (2007);

$$Wcc=Wb\ (Dcc/Dcc-Dw)$$

Where Wcc is the dry weight of the $CaCO_3$, Wb is the buoyant weight of the algae, Dcc is the density of $CaCO_3$ (2.71 $g.cm^{-3}$), and Dw is the density of seawater displaced by the sample (1.03 $g.cm^{-3}$). Replacing the densities of seawater and $CaCO_3$, the following equation is obtained;

$$Wcc=1.61Wb$$

The total growth was calculated by dividing the difference between the initial and the final buoyant weight by the initial weight of each replicate. The calcification rate was calculated by dividing the total growth by the number of days between measurements.

Determination of photosynthetic pigments

Samples for pigments were collected at the end of the experiment. Algae were frozen in liquid nitrogen and stored at -80°C until analysis. Approximately 1.5 g FW was ground in liquid nitrogen, extracted in 5 mL of acetone and centrifuged for 5 minutes at 2000xg and 4°C. Chlorophylls *a* and *d* were extracted in 90% acetone, and their concentrations were calculated according to Ritchie (2008);

$$\text{Chl } a \text{ (g.m}^{-3}) = -0.3319\ A_{630} - 1.7485\ A_{647} + 11.9442\ A_{664} - 1.4306\ A_{691}$$
$$\text{Chl } d \text{ (g.m}^{-3}) = -0.5881\ A_{630} + 0.0902\ A_{647} - 0.1564\ A_{664} - 11.0473\ A_{691}$$

Where:

A_{630} - absorbance at 630 nm, A_{647} - absorbance at 647nm, A_{664} - absorbance at 664 nm, A_{691} - absorbance at 691 nm.

Carotenoids were extracted in 100 % acetone and their concentration was calculated according to Torres et al. (2014);

$$\text{Carotenoids } (\mu\text{g. mL}^{-1}) = (1000 \times A_{470} - 1.90 \times \text{Chl } a)/214$$

Where:

A_{470} – absorbance at 470 nm

Phycoerythrin (PE) and phycocyanin (PC) content were determined from 1 g of fresh thalli ground in liquid nitrogen and extracted in 2 ml of 0.1M phosphate buffer (pH 6.5) at 4°C after centrifugation (Heraeus Megafuge 16R, Thermo scientific, USA) at 4696xg for 40 minutes. PE and PC concentrations were determined according to Sampath-Wiley and Neefus (2007);

$$\text{Phycocyanin (PC) (mg.mL}^{-1}) = 0.154\ (A_{618} - A_{730})$$
$$\text{Phycoerythrin (PE) (mg.mL}^{-1}) = 0.1247\ [(A_{564} - A_{730}) - 0.4583\ (A_{618} - A_{730})]$$

Where:

A_{618} - absorbance at 618 nm, A_{730} - absorbance at 730nm, A_{564} - absorbance at 564 nm.

All absorbances were measured in a Beckman Coulter DU-650 spectrophotometer.

Statistical analyses

SigmaPlot (version 11.0) software package was used to perform all statistical analysis. The effects of pCO_2 on net O_2 evolution under dark and ambient conditions were assessed with a Kruskal-Wallis analyses of variance (ANOVA on ranks) since data presented equal variance but did not meet the assumptions of normal distribution (Kolmogorov-Smirnov test) after a log and square root transformation. When significant differences were found, a *post hoc* test (Student–Newman–Keuls, SNK) was applied to explore differences between treatments. The effect of pCO_2 and light, and pCO_2 with time of exposure on photosynthesis were assessed with separate two-way ANOVA tests where normal distribution (Kolmogorov-Smirnov test) and equal variance (Levene's test) were verified. When *P* was significant, ANOVA was followed by a *post hoc* test for multiple comparisons (Tukey's HSD).

The effects of pCO_2 on the calcification of the algae under dark and ambient conditions were assessed with a two-way ANOVA when normal distribution (Kolmogorov-Smirnov test) and equal variance (Levene's test) were verified after a log transformation of the data. When *P* was significant, ANOVA was followed by a *post hoc* test for multiple comparisons (Tukey's HSD). The effect of pCO_2 on the calcification with light and time, and the calcium carbonate content with pCO_2 were assessed with separate Kruskal-Wallis ANOVA on ranks since data presented an equal variance but did not meet the assumptions of normal distribution (Kolmogorov-Smirnov test) after a log and square root transformation. When significant differences were found, a *post hoc* test (Student–Newman–Keuls, SNK) was applied to explore differences between treatments.

The results for the pigments were analysed using a one-way ANOVA. When significant differences were found, a *post hoc* test (Student–Newman–Keuls, SNK) was applied to explore differences between treatments. Since carotenoid data presented equal variance but did not meet the assumptions of normal distribution (Shapiro-Wilk test), data went through a square root transformation prior to analysis.

Results

Salinity, temperature and dissolved oxygen did not differ among CO_2 treatments. During the 20 months of the experiment, salinity ranged from 34 to 38 psu and temperature fluctuated in a similar way as the natural beds (14 to 20 °C). Dissolved oxygen at the mesocosms remained constant (~6.7 mgO_2/l). Total alkalinity (TA) was identical at different points of the experimental system both in CO_2 and control treatments and ranged between 2449 and 2536 µmol $kgSW^{-1}$ (Table 5.1.).

The mean CO_2 values under control conditions ranged from 397 to 470 µatm of CO_2, while the mean values for the intermediate CO_2 treatment ranged from 539 to 577 µatm of CO_2 and the mean values for the high CO_2 treatment ranged from 740 to 804 µatm of CO_2 (Figure 5.1.).

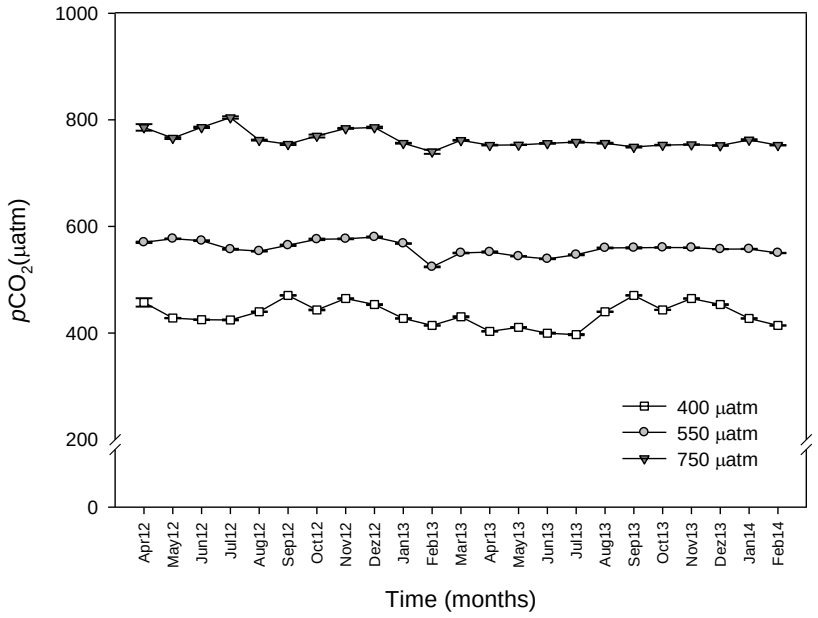

Figure 5.1. Monthly pCO_2 average values (µatm ± standard error) from April 2012 (Apr12) to February 2014 (Feb14) at the mesocosms, under control ~400, 550 and 750µatm pCO_2 conditions. The values were measured with an IRGA analyzer (WMA-4, PP Systems, USA) every 20 minutes.

Table 5.1. shows the parameters of the carbonate system after 11 and 20 months of high CO_2 exposure

Table 5.1. Carbonate chemistry for each CO_2 concentrations (control ~400, 550 and 750 µatm) after 11 and 20 months of high CO_2. Total alkalinity (TA), salinity (Sal.), temperature (T) and pH were measured while dissolved inorganic carbon (DIC) and aragonite saturation state (Ω_{arag}) were calculated using CO_2SYS software. Values expressed as means ± standard error (n=11).

Time	***p*CO$_2$** *(µatm)*	**TA** *(µmol/kgSW)*	**Sal.** *(psu)*	**T** *(°C)*	**pH**	**DIC** *(µmol/kgSW)*	**Ω_{arag}**
11 months	400	2461.76 ± 4.07	34.87 ± 0.02	15.64 ± 0.19	8.18 ± 0.01	2183.93 ± 7.48	3.09 ± 0.05
	550	2449.17 ± 2.67	34.84 ± 0.02	15.92 ± 0.12	8.10 ± 0.00	2215.27 ± 2.84	2.65 ± 0.03
	750	2468.31± 2.84	34.63 ± 0.16	15.74 ± 0.14	7.90 ± 0.03	2326.21 ± 11.02	1.81 ± 0.11
20 months	400	2513.72 ± 3.52	36.30 ± 0.00	17.00 ± 0.00	8.06 ± 0.00	2292.98 ± 3.48	2.53 ± 0.01
	550	2517.91 ± 3.99	36.40 ± 0.00	16.80 ± 0.00	8.01 ± 0.01	2323.36 ± 4.19	2.28 ± 0.01
	750	2536.66 ± 9.11	36.40 ± 0.00	17.05 ± 0.01	7.87 ± 0.01	2401.50 ± 9.10	1.76 ± 0.02

Effects of light and CO_2 on photosynthesis and of CO_2 on respiration

Dark respiration decreased significantly with pCO_2 (P=<0.001) even though the differences between treatments were small (Figure 5.2.). Control algae presented the highest respiration rates (0.1 $\mu molO_2\ gFW^{-1}\ h^{-1}$) with respect to 550 µatm (0.08 $\mu molO_2\ gFW^{-1}\ h^{-1}$) and 750 µatm of CO_2 (0.07$\mu molO_2\ gFW^{-1}\ h^{-1}$). Photosynthetic rates increased significantly with both pCO_2 and light. At 195 and 450 µmol photons $m^{-2}\ s^{-1}$ net photosynthesis was always significantly higher at 750 µatm (P=<0.001), but was saturated by light at 195 µmol photons $m^{-2}\ s^{-1}$, since no significant differences were observed between 195 and 450 µmol photons $m^{-2}\ s^{-1}$ (P=0.478) at any pCO_2 level (Figure 5.2.).

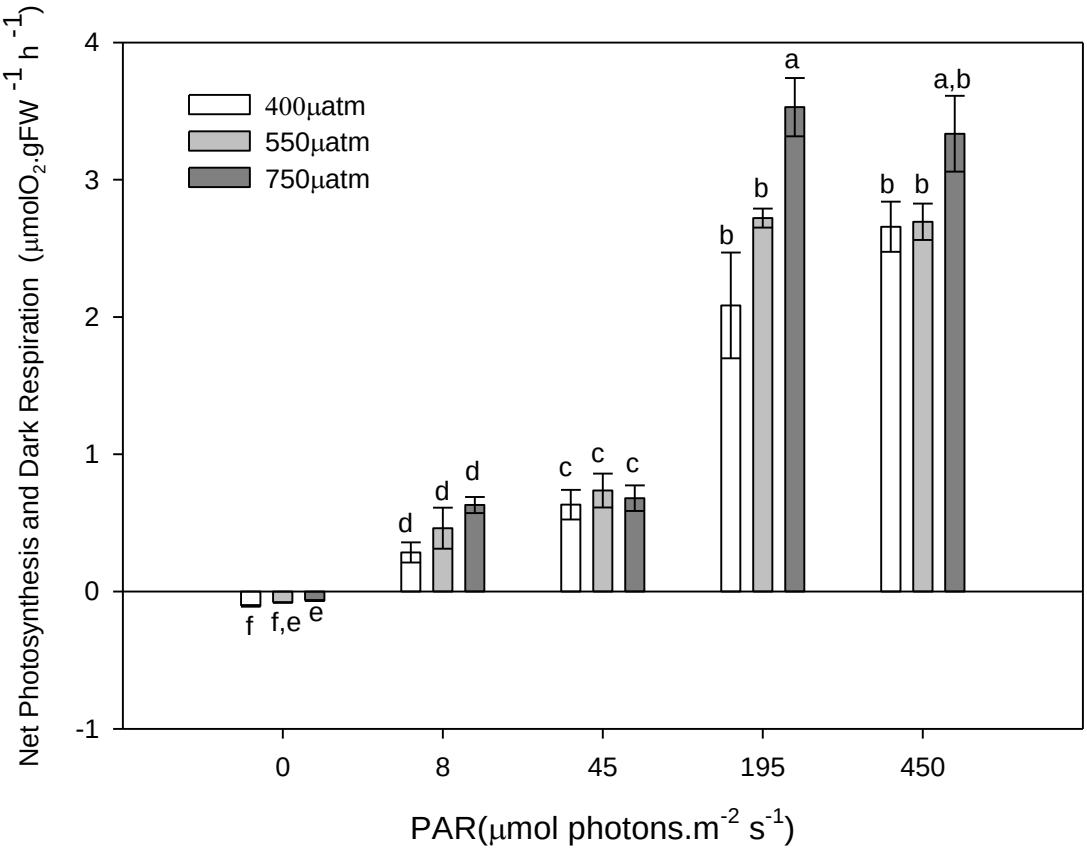

Figure 5.2. Dark respiration and Net Photosynthesis ($\mu molO_2.gFW^{-1}.\ h^{-1}$) of *Phymatolithon lusitanicum* at three different CO_2 levels (control~400, 550 and 750µatm) as a function of PAR irradiances (8, 45, 195 and 450 µmol photons.$m^{-2}.s^{-1}$) after ~ 11 months. Different letters indicate significant differences with CO_2 and irradiance. Values are expressed as mean ± standard error (n=4).

The net photosynthesis of acidified algae changed significantly with time of exposure to high CO_2 (P=0.004) (Figure 5.3.). After 11 months of treatment the net photosynthetic rates were higher at both elevated CO_2 levels. However, after 20 months this pattern was reversed and algae from the intermediate (550 µatm) and high (750 µatm) pCO_2 treatments showed a significantly decrease of photosynthetic rates. In contrast, algae kept at control conditions presented unaltered rates through time (P=0.998) (Figure 5.3.).

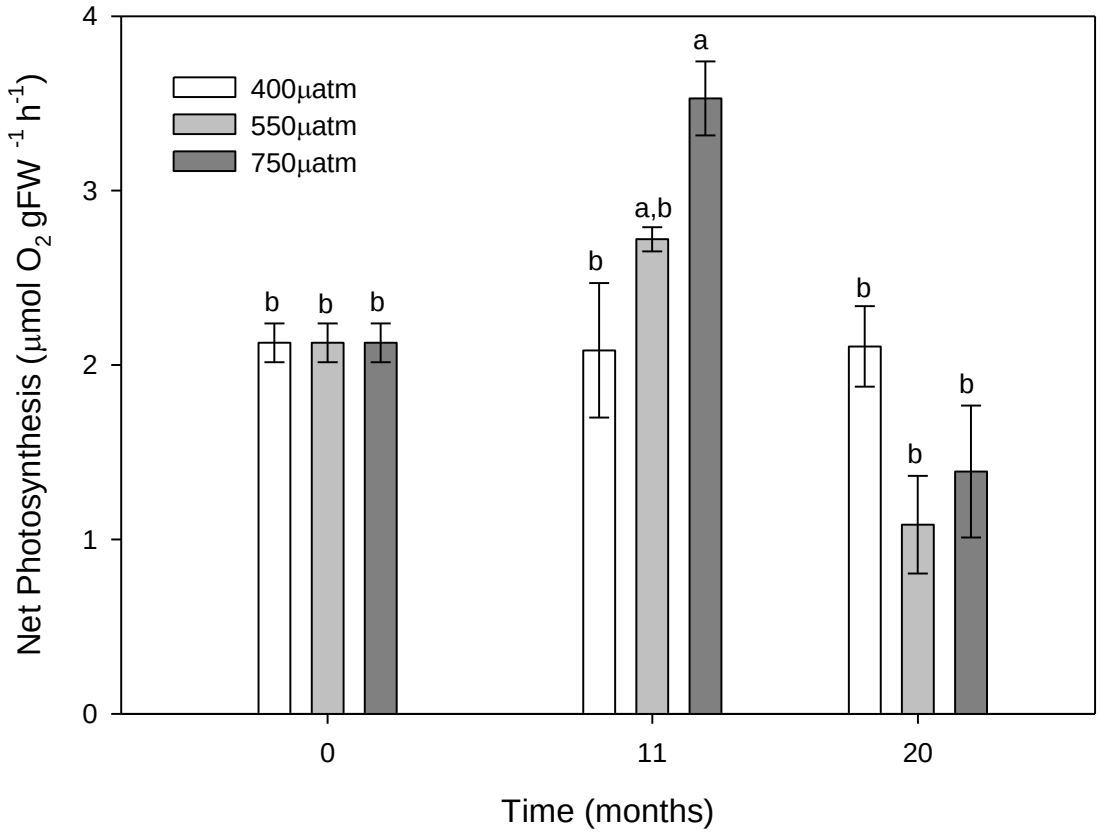

Figure 5.3. Net photosynthesis (µmolO_2.gFW^{-1}. h^{-1}) of *Phymatolithon lusitanicum* at ~ 200 µmol photons.m^{-2}.s^{-1} (PAR), incubated for 0, 11 and 20 months under three different CO_2 levels (control~400, 550 and 750µatm). Different letters indicate significant differences with CO_2 and time. Values expressed as mean ± standard error (n=4).

Effects of light and CO_2 on calcification

The calcification rates of *Phymatolithon lusitanicum* increased with light intensity saturating at 45 µmol photons m^{-2} s^{-1}. Algae increased their rates with CO_2 at irradiances of and above 45 µmol photons m^{-2} s^{-1} (Figure 5.4.).

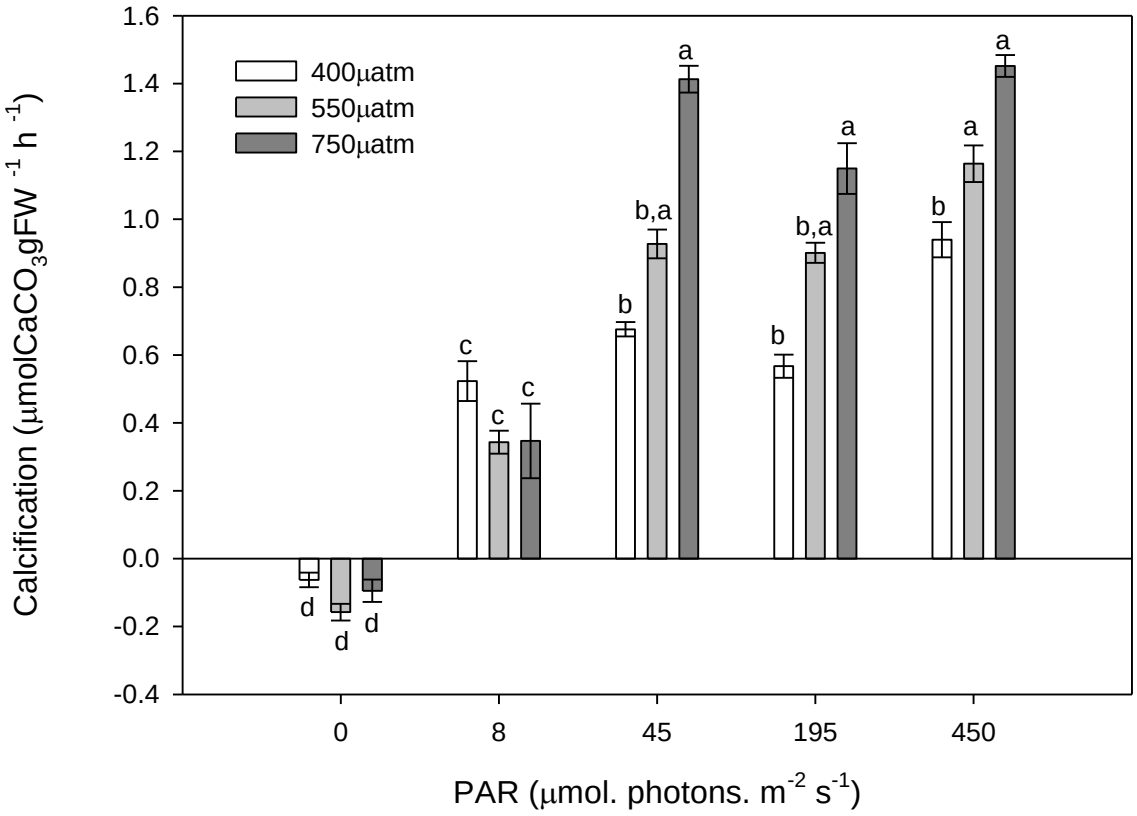

Figure 5.4. Dark and light calcification rates (µmol$CaCO_3$.gFW^{-1}. h^{-1}) of *Phymatolithon lusitanicum* at three different CO_2 levels (control ~400µatm, 550 µatm and 750µatm) and PAR irradiances (8, 45, 195 and 450 µmol photos.m^{-2}.s^{-1}) after ~ 11 months of high CO_2. Results calculated using the total alkalinity anomaly technique. Different letters indicate significant differences with CO_2 and irradiance. Values expressed as mean ± standard error (n=4).

Calcification in the dark was always negative (net dissolution), especially under intermediate CO_2 conditions. After 12 months of exposure, the effect of CO2 on calcification rates became negative (Figure 5.5.) in both high-CO_2 treatments (550 and 750 µatm). In contrast, control algae presented unaltered calcification rates.

After nearly two years at the mesocosm, control algae presented the highest accumulated growth (1771.62 µmol$CaCO_3$ gFW^{-1}). In the long term (after 12 months) CO_2 had a negative effect on growth both under 550 and 750 µatm (1249.73 and 1016.34 µmol$CaCO_3$ gFW^{-1}, respectively) (Figure 5.6.).

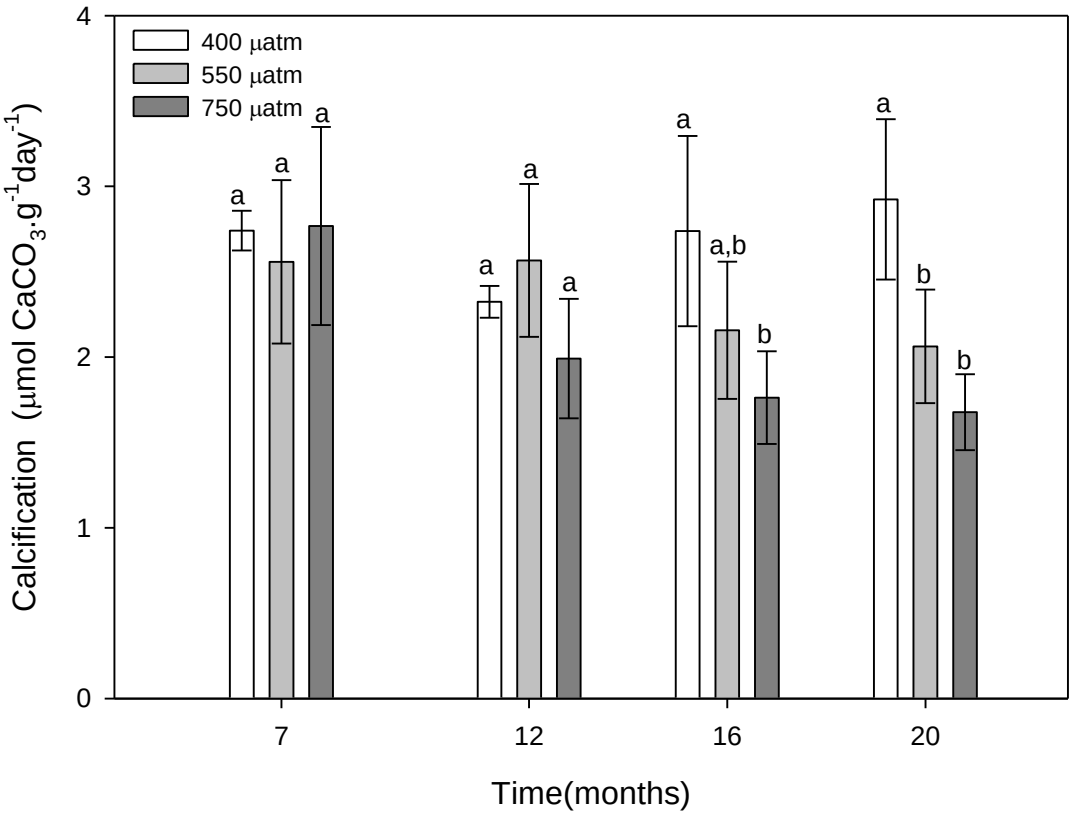

Figure 5.5. Calcification rates (µmol$CaCO_3$.gFW^{-1}. day^{-1}) of *Phymatolithon lusitanicum* thalli cultivated under low light ambient irradiance (8 µmol photos.m^{-2}.s^{-1})at three different CO_2 levels (control~400 µatm, 550 µatm and 750 µatm) after 7, 12, 16 and 20 months of high CO_2. Results calculated using the buoyant weight technique. Different letters indicate significant differences with CO_2 and time. Values expressed as mean ± standard error (n=90).

In contrast, the instant calcification rates calculated using the alkalinity anomaly technique, after 20 months at 200 µmol photons m^{-2} s^{-1}, were not significantly affected by CO_2 ($P = 0.380$), even though they increased. The results (±SE) ranged from 0.73±0.10 µmol$CaCO_3$.$gFW^{-1}h^{-1}$ under control conditions, 0.86±0.09 µmol$CaCO_3$.$gFW^{-1}h^{-1}$ under 550 µatm, to 0.94±0.11 µmol$CaCO_3$.$gFW^{-1}h^{-1}$ under high CO_2 conditions. However, from 11 (see Figure 5.4. results after 11 months at 195 PAR) to 20 months, the calcification rates of algae decreased both under 750 µatm (from 1.15 to 0.94 µmol$CaCO_3$.$gFW^{-1}h^{-1}$) and 550 µatm (from 0.90 to 0.86 µmol$CaCO_3$.$gFW^{-1}h^{-1}$) but increased under control conditions (from 0.57 to 0.86 µmol$CaCO_3$.$gFW^{-1}h^{-1}$).

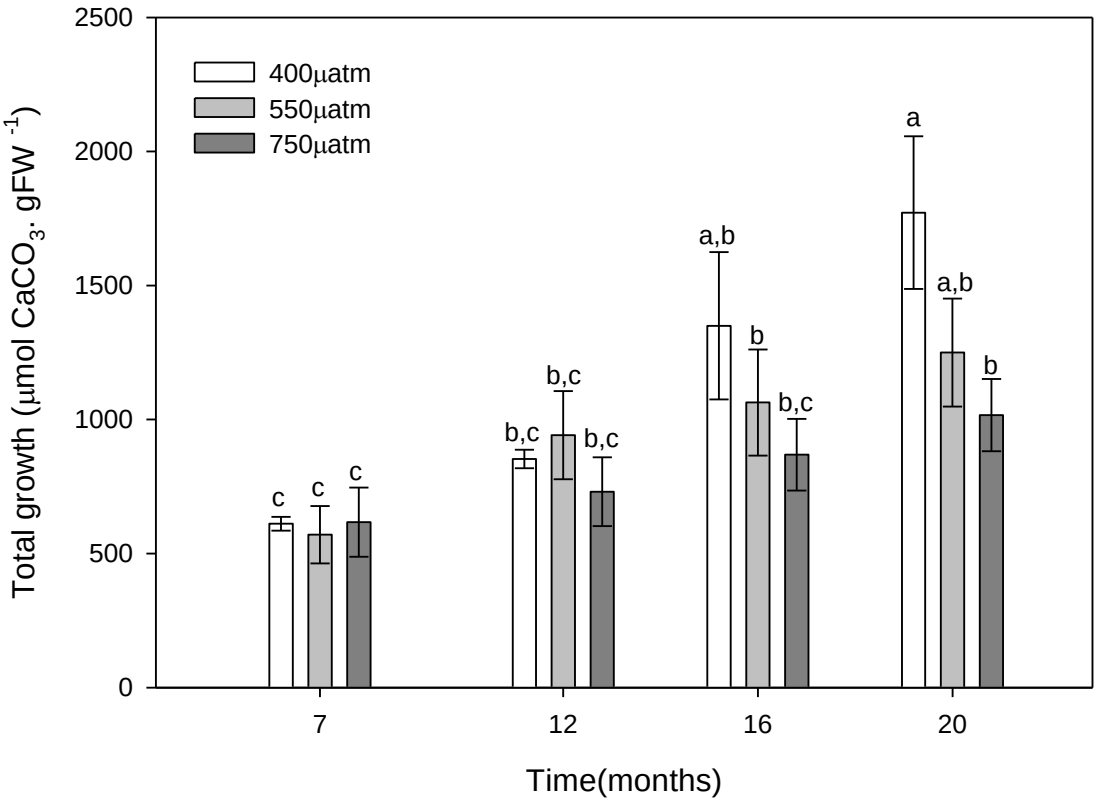

Figure 5.6. Total cumulative growth (µmol$CaCO_3$.gFW^{-1}) of *Phymatolithon lusitanicum* thalli cultivated under low light ambient irradiance (8 µmol photos.m^{-2}.s^{-1}) under control ~ 400, 550 and 750 µatm of pCO_2, after 7, 12, 16 and 20 months at the mesocosm. Different letters indicate significant differences with CO_2 and time. Values are expressed as mean ± standard error (n=90).

Calcium carbonate and organic matter content

The results showed that the ~96% of these algae weight is composed of calcium carbonate. The percentage of $CaCO_3$ content of the dry weight of the thalli was similar at the three pCO_2 concentrations (Kruskal-Wallis one-way; P=0.101). The mean values (±SE) were 95.93%±0.31 for control (400 µatm), 96.09 %±0.18 for the 550 µatm treatment and 96.66%±0.13 for the 750 µatm pCO_2 treatment.

Effect of pCO_2 on the photosynthetic pigment concentration

None of the detected photosynthetic pigments was affected by CO_2 concentration (Table 5.2.). *Phymatolithon lusitanicum* presented higher concentrations of phycoerythrin than

phycocyanin. Chlorophyll *d*, a pigment present in some red algae adapted to low light conditions, was not detected.

Chlorophyll *a* and phycobiliproteins had a tendency to increase under 550 µatm conditions, but neither chlorophyll *a* ($P = 0.901$), phycocyanin ($P = 0.446$), phycoerythrin ($P = 0.105$) nor carotenoids ($P = 0.887$), were significantly affected by CO_2.

Table 5.2. Chlorophyll *a*, carotenoids, phycocyanin and phycoerythrin pigment concentrations ($mg.gFW^{-1}$) of *Phymatolithon lusitanicum* under control 400, 550 and 750 µatm conditions after 20 months of high CO_2. Values expressed as mean ± standard error (n=5).

***p*CO₂** *(µatm)*	**Chlorophyll *a*** *(mg.gFW^{-1})*	**Carotenoids** *(mg.gFW^{-1})*	**Phycocyanin** *(mg.gFW^{-1})*	**Phycoerythrin** *(mg.gFW^{-1})*
400	0.040±0.006	0.016±0.004	0.004±0.000	0.122±0.019
550	0.048±0.004	0.014±0.002	0.004±0.000	0.153±0.015
750	0.039±0.002	0.016±0.001	0.003±0.000	0.097±0.016

Discussion

This study illustrates the importance of the time of exposure and light on the predictions of how calcified macroalgae will respond to elevated CO_2. Some of the divergences in results among OA experiments with coralline algae may be related to light intensity, time for acclimation, length of the experiment, the studied species, and the pCO_2 levels used (reviewed in Martin et al., 2013). To our knowledge, this is the first OA experiment with mäerl coralline algae of 20 months of duration. Previous OA short-time experiments with coralline algae have shown that growth rates decrease with high CO_2, after 20 hours (Gao et al., 1993) or 1 month (Hofmann et al., 2012; Büdenbender et al., 2011), and that this decrease is intensified under high light irradiance (Gao and Zheng, 2010). However, other short time experiments of 1 month have found an increase in the coralline algae's growth rate (Ragazzola et al., 2013) patent only under increasing temperature (Büdenbender et al., 2011). On the other hand, longer experiments have shown sustained growth rates or no effect after 1 year (Martin et al., 2013), increases after 80 days (Kamenos et al., 2013) or decreases after 3 months (Ragazzola et al., 2013; Noisette et al., 2013) and 1 year (Martin and Gattuso, 2009) of high CO_2 exposure. Because of the great variability in results, long-term experiments where variables such as irradiance and temperature are tested simultaneously may provide the most realistic

insights. In addition, a regular monitoring of the natural populations is essential in establishing the appropriate experimental conditions.

Phymatolithon lusitanicum became more vulnerable to increased CO_2 after one year of the experiment, suggesting that a threshold of resilience to OA was exceeded. During the first 11 months both photosynthetic and calcification rates increased with CO_2 and light irradiance (saturating at 200 PAR). However, after 20 months of treatment, photosynthesis and calcification decreased at both concentrations of 550 µatm and 750 µatm and 200 PAR. Most authors have found that coralline algae have a CO_2 threshold of 700-750 µatm after 1 year (Martin et al., 2013; Martin and Gattuso, 2009) and three months (Noisette et al., 2013b) of high CO_2 exposure. In this study, we found that in a long-term exposure of approximate 1 year algae were able to sustain their growth, but after almost two years, both calcification and photosynthetic rates decreased for acidified algae. When Ragazzola et al. (2013) compared the results after 3 and 10 months of high CO_2 the authors obtained different results with time and suggested that acclimation differs over time and there is a high plasticity present in these algae. Even if coralline algae are plastic enough to sustain high rates in a long time, a loss of structural integrity is inherent (Kamenos et al., 2013, Martin et al., 2013; Ragazzola et al., 2012). The lowering of the saturation state of water is expected to have an additional energetic cost and these algae might not be able to compensate the acidified conditions. In the long term this ability could impact the general resistance of the organism, facilitating disease development and bleaching. This will reduce the algae fitness and its ability to resist boring by predators (Ragazzola et al., 2012) or compete with fleshy algae (Short et al., 2014).

Irradiance intensified the effect of OA on *P. lusitanicum*, but under low ambient light and in dark conditions there was no effect of OA on photosynthesis and calcification after 11 months of high CO_2 exposure. In a previous study of 1 year of duration, Martin et al. (2013) found that the crustose coralline alga *Lithophyllum cabiochae* decreased its photosynthesis under ambient light conditions and increased pCO_2. However, the ambient light the authors used was adjusted seasonally and most of the time was higher than the ambient irradiance used in this study. The use of low irradiance, similar to field conditions proved critical in the maintenance of well-adapted organisms for long periods of time. Control algae maintained their metabolic rates unaltered during the whole experiment, which clearly showed that algae were in good conditions throughout.

Many published studies have assumed that most calcifiers rely on carbonate to form their skeletons whereas some are able to use either bicarbonate or metabolically produced CO_2 as a substrate for calcification (Roleda et al., 2012). In this study we found that *Phymatolithon lusitanicum* uses bicarbonate (HCO_3^-) as a substrate for calcification. Because both photosynthesis and calcification use the same substrate, calcification, a less light limited process, was favored over photosynthesis under intermediate CO_2 (550µatm).

After 11 months of OA, the increase of both calcification and photosynthesis with CO_2 was a mechanism to compensate the lowering of pH and carbonate saturation state of the treated seawater. With the lowering of the calcium carbonate saturation state the water became more corrosive and this was accentuated with the light increment. Even if conditions never got corrosive enough for algae to dissolve ($\Omega ar<1$), the initial saturation values for 750 µatm were close to 1 where aragonite does not dissolve or precipitate. Kamenos et al. (2013) demonstrated that at low pH coralline algae survived by increasing their calcification rates. However, even if the acidified algae calcified more to compensate OA, they were under a constant dissolution/calcification process and in the long term grew less (see Dupont and Pörtner, 2013b). Kamenos et al. (2013) showed that coralline algal structure is more sensitive to rate rather than magnitude of acidification. Even if coralline algae can survive to abrupt pH decreases by increasing their calcification rates, the authors detected weakness in the calcite skeletons. The results from this study suggest that more long term studies with coralline algae are needed. It is important to gradually acclimatize the algae to acidified conditions and compare the results after at least two different time periods (like for e.g. Ragazzola et al., 2013).

Even if respiration decreased with higher CO_2, the results were not conclusive. Most authors have found that coralline algae's respiration rates do not change with high CO_2 but increase with temperature (Noisette 2013a, 2013b; Martin et al., 2013). In an anterior study with *P. lusitanicum* we also found that respiration was unaffected by CO_2 but positively affected by temperature (Sordo et al., 2016). Further research on the effect of OA and temperature on the respiration of coralline algae is necessary.

Pigment concentrations were unaffected by CO_2. Different authors have found that chlorophyll *a* (Egilsdottir et al., 2012; Martin et al., 2013; Noisette et al., 2013b; Yildiz et al., 2013), phycobiliprotein (Yildiz et al., 2013) and carotenoid concentrations (Gao and Zheng,

2010; Noisette at al., 2013b) are unaffected by high pCO_2, what agrees with our results. However, factors such as temperature (Noisette et al., 2013b), light (Gao and Zheng, 2010; Yildiz et al., 2013) and nutrients (Lin and Stekoll, 2011) have a strong effect on pigment content. Noisette et al. (2013b) found that pCO_2 had no effect on pigment content but temperature impacted all pigments with the exception of Chl *a*. Also, Yildiz et al. (2013) found that pigment content was unaffected by pCO_2 but phycocyanin decreased with increasing light. In this study, pigments were apparently unaffected by high CO_2, with the algae being maintained at low irradiance and temperature, similar to the normal conditions found in the field. Considering that we found that light intensity accentuated the differences among pCO_2 treatments, we could expect different pigments results if algae were incubated at different irradiances and temperatures.

The future outcome of mäerl beds from Southern Portugal to OA will depend on other factors involved that are not easy to predict. In the short term algae will increase their photosynthetic and calcification rates to compensate the low saturation state of the seawater. But in the long term algae will not be able to keep up with the high energetic costs and will decrease both their photosynthetic and calcification rates. *Phymatolithon lusitanicum* could survive with minor production losses until an intermediate pCO_2 concentration (550 µatm, IPCC projections for 2050), but in the next century (750 µatm, IPCC projection for 2100) the situation could be irreversible. Deeper areas where rhodolith beds are less exposed to light changes could become ocean acidification refuges for numerous calcifiers that live associated to these algae. Mäerl beds from Southern Portugal are biodiversity oasis. Despite the low light conditions experienced by the algae at c. 25 m depth, *Phymatolithon lusitanicum* can be considered a major contributor to primary productivity and calcium carbonate deposition in the Iberian Peninsula.

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Final synthesis

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1. Both photosynthesis and dark respiration of *P. lusitanicum* collected in its natural habitat were highest in summer and spring and lowest in winter and autumn, despite the relatively low thermal and irradiance fluctuations at 25 m depth along the year and the absence of a clear seasonal pattern in these abiotic variables. Respiration was almost two-fold higher in summer than in winter. Regarding photosynthetic activity, identical patterns were obtained with in situ measurements of chlorophyll fluorescence (Rapid Light Curves) and with conventional P-E curves determined in the laboratory (Clark type oxygen electrodes).

2. A strong relationship (r^2=0.95) was found between gross primary production (GPP) and relative electron transport rates (rETR). Both methods led to similar results and an average molar ratio close to ¼ between GPP and rETR was verified in this study. However, a more extensive study is still needed to verify if it is possible to make an extrapolation between GPP and rETR in rhodolith beds from Southern Portugal.

3. Alongside with the lack of a clear seasonal pattern, a considerable interannual variability was also observed on the abiotic variables recorded continuously in the natural mäerl bed between 2013 and 2014. Average temperatures in the autumn and summer of 2013 were almost four degrees higher than those observed for the same periods in 2014.

4. Calcification in the natural community increased in the period with the highest temperature and irradiance values (summer 2013), but with the exception of this period calcification rates were fairly constant, with no clear seasonal pattern. These results suggest that even if calcification increases with both irradiance and temperature, it is probably more dependent on temperature than on light. This hypothesis is verified in several calcareous algae known to be able to calcify despite low light conditions if inside their optimal temperature range.

5. Phycobilins were higher in summer and autumn and lower in winter and spring. This decrease in their concentration during winter and spring might indicate that *P. lusitanicum*

uses the nitrogen stored in the phycobilins in periods when there are less nutrients available in the water column. Chlorophyll *a* and carotenoids increased in winter and spring and decreased in autumn and summer. Algae compensated the low light availability recorded during winter 2014-2015 by increasing these photosynthetic pigments.

6. In the laboratory, photosynthesis and dark respiration increased with temperature reaching the highest values at 24°C. At 26 °C net photosynthesis decreased but respiration did not change. Under a global warming scenario photosynthesis is thus likely to increase with temperature, within its optimum range (below 26°C), but above 26°C respiration may surpass photosynthesis. In addition, the decrease in the net production at high temperatures is accentuated under high irradiance. Therefore, both temperature and irradiance have a main role on the regulation of the photosynthetic rates in the red coralline algae *P. lusitanicum*.

7. A direct control system was developed for mesocosm experiments, where the control variable is pCO_2 itself, measured, with a non-dispersive infrared gas analyzer (IRGA) coupled to a PID controller, in a setup that proved to be a reliable, simple and accurate control design that operates almost unattended apart from the regular maintenance and data download routines. This system circumvents the problems and uncertainties associated with pH control and is adequate to run short or long-term OA experiments. It can also be easily adapted to test other relevant variables simultaneously with CO_2, such as temperature, irradiance and nutrients.

8. Net photosynthesis tended to decrease while respiration and calcification increased with temperature. In a short term experiment, after only two weeks of acclimation, *Phymatolithon lusitanicum* increased its photosynthetic, and respiration rates but decreased its calcification rates with temperature. These results are attributed to increasing stressful light and temperature conditions and an insufficient period of acclimation. Once properly acclimated, after one month at different temperatures and 15 days of high CO_2algae increased also their calcification rates and this increase was accentuated under high CO_2 conditions. These results suggest that both time of acclimation and temperature are

determinant factors in OA experiments and that global warming will intensify the effect of ocean acidification on coralline alga in the short-term.

9. In a long-term (11 months) experiment, respiration was unaffected (O_2 electrode results) or decreased with CO_2 (incubation results). When temperature and CO_2 were tested simultaneously, respiration did not change with CO_2 but increased proportionally with temperature, reaching the highest values at 26°C. Therefore, temperature, and not CO_2, was the main factor that contributed to the differences observed.

10. Also in the long-term (11 months) algae compensated the OA conditions by increasing their photosynthetic and calcification rates proportionally to CO_2. Results changed with time tough, and after 20 months both calcification and photosynthetic rates decreased for acidified algae while control algae presented the highest rates and accumulated growth. The differences between CO_2 treatments were only visible when punctual measurements were done under high irradiances (≥45 PAR) and no differences were detected under low ambient irradiance (8 PAR). Photosynthetic pigments were unaffected by high CO_2 but differences are expected if algae were cultivated under high irradiances (≥45 PAR). The results suggest that light intensity and time of exposure are determinant factors in OA experiments and in a long term intensify the negative effect of ocean acidification on coralline algae.

11. The time of acclimation and exposure to high CO_2, the temperature, the level of CO_2 used, the irradiance at which algae are incubated and the irradiance used during the punctual measurements are determinant factors in ocean acidification experiments with coralline algae. In the short term algae increase its metabolic rates to compensate the stressful conditions, but in a long term the metabolic costs were too high and the algae were not able to compensate the corrosive conditions of seawater. Algae cultivated under low light irradiance and temperature showed a higher long term resilience to OA than if cultivated under intermediate irradiance and high temperature conditions. Both irradiance and temperature intensified the effect of OA on coralline algae and this effect changed with time of exposure.

12. The long-term resilience of Lusitanian rhodolith beds to OA will be dependent on future temperature and irradiance changes. In a worst-case scenario, the effect of high CO_2 on *P. lusitanicum* will be accentuated under global warming and increasing irradiance. In a drastic scenario, above 26°C, respiration and calcification (both CO_2 sources) could surpass photosynthesis, with negative repercussions for the whole community and implying an important loss of oceanic carbon storage capacity in the long term. However, under a global change scenario, below 26 °C and moderate light irradiances, mäerl beds from Southern Portugal could become a natural refuge to OA and even extent its distribution further north.