Molecular and cellular investigations of photosynthesis driven carbon fixation in the seagrass *Zostera muelleri*

Mikael Kim

PhD by Research

University of Technology Sydney

School of Life Sciences, Climate Change Cluster (C3)

March 2020

Supervisors: Professor Peter J. Ralph (primary), Dr Mathieu Pernice and

Professor John A. Raven

CERTIFICATE OF ORIGINAL AUTHORSHIP

I, Mikael Kim declare that this thesis is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Life Science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

This research is supported by an Australian Government Research Training Program.

Signature:

Production Note:
Signature removed prior to publication.

Date: 25/03/2020

Acknowledgements

I'd like to thank my supervisors: Peter J. Ralph, John A. Raven and Mathieu Pernice for their guidance, support, patience and criticisms. I would also like to thank my co-authors: Kasper Elgetti Brodersen, Paul Guagliardo, Matt R. Kilburn, Anthony W. D. Larkum, Alexander Watson-Lazowski, and Milán Szabó for their contributions, without which the published work contained in this thesis would not have been possible. I'd also like to thank Paul Brooks and UTS technical staff for helping me with the various training and inductions, your help in preparing for each experiment has not been taken for granted. To all the people I worked with along the way, thank you all for your help throughout my PhD. Finally, thank you to my friends and family, the completion of this thesis was only possible as a result of your continued support.

Preface

This thesis has been prepared for submission as a thesis by compilation, whereby the thesis contains a combination of published and publishable work. As a result, there is a degree of repetition across chapters, particularly within the introductions and materials and methods sections of Chapter 2, 3 and 4. Published works have been incorporated into this thesis and appear as they were presented to the journal immediately prior to publication with the following alterations: i) the font and format was changed so as to maintain consistency across the thesis, ii) figures and tables were re-numbered to reflect the chapter numbering and iii) supplementary information for each chapter appear in the appendix and have been re-numbered accordingly. The referencing format used throughout this thesis conforms to the requirements of the journal; Photosynthesis Research.

List of publications included in the thesis:

Chapter 2:

Low oxygen affects photophysiology and the level of expression of two-carbon metabolism genes in the seagrass *Zostera muelleri*

Mikael Kim, Kasper Elgetti Brodersen, Milán Szabó, Anthony W.D. Larkum, John A. Raven, Peter J. Ralph and Mathieu Pernice

Published on the 4th of October, 2017 in the journal 'Photosynthesis Research', volume: 136, issue 2, page: 147-160

Contributions

MK, KEB, MS, AWDL, JAR, PJR and MP designed the experiment; MK and MP carried out the experiment; MK, KEB and MS performed the microsensor analysis; MK and MP performed the RTqPCR analysis; MK wrote the manuscript with contributions from all authors.

Chapter 3:

Effect of reduced irradiance on ^{13}C uptake, gene expression and protein activity of the seagrass *Zostera muelleri*

Mikael Kim, Mathieu Pernice, Alexander Watson-Lazowski, Paul Guagliardo, Matt R. Killburn, Anthony W.D. Larkum, John A. Raven and Peter J. Ralph

Published on the 6th of June, 2019 in the journal 'Marine Environmental Research', volume: 149, page: 80-89

Contributions

MK, MP, AWDL, JAR and PJR designed the experiment; MK and MP carried out the experiment; MK and MP performed the nanoSTRING analysis; MK and AWL performed the enzyme assays; PG and MRK performed the nanoSIMS analysis; MK wrote the manuscript with contributions from all authors.

Another article was published in association with my PhD, however it does not form a part of this thesis. The title page of this publication is included in the appendix of this thesis:

Development of an efficient protein extraction method compatible with LC-MS/MS for proteome mapping in two Australian seagrasses *Zostera muelleri* and *Posidonia australis*

Zhijian Jiang, Manoj Kumar, Matthew P. Padula, Mathieu Pernice, Tim Kahlke, Mikael Kim and Peter J. Ralph

Published on the 15th of August, 2017 in the journal 'Frontiers in Plant Science', volume: 8, article no. 1416

Contributions

ZJ, MaK, MP, and PR conceived and designed research. ZJ, MaK, and MPP performed 2D-IEF and protein identification using LC-MS/MS and analyzed the data. PD and MiK performed Western Blot analysis while kindly providing the primary and secondary antibodies, and standard for PEPC enzyme. MaK, ZJ, and MPP, wrote manuscript. TK assisted in bioinformatics analysis. PR revised and edit the manuscript. All authors read and approved the manuscript.

Table of Contents

List of Figures and Tables

Thesis abstract

Seagrasses are marine foundation species that have evolved to live in shallow coastal waters, an environment regularly subjected to changes through tidal patterns and anthropogenic activity. Global seagrass coverage is declining worldwide, mainly driven by decreasing water quality due to anthropogenic activity. Therefore, there is an urgent need to better understand seagrass biology, particularly the way their growth will be impacted by changing environments in order to assist their preservation. Through the use of emerging techniques such as gene expression analyses, the underlying mechanism driving photosynthesis can now be explored at the molecular level and is an emerging field in seagrass research.

The overarching aim of my thesis was to identify the molecular mechanisms driving carbon metabolism in the seagrass *Zostera muelleri.* To accomplish this aim, a combination of physiological and molecular analytical tools were used to measure changes in photosynthesis in response to decreased O_2 concentrations in the water column, decreased irradiance and chemical inhibition of carbonic anhydrase (CA).

The results presented in this thesis describe the ways in which genes associated with carbon metabolism were regulated in response to the different environmental factors as listed above. Specifically, we found that in low O_2 conditions, photosynthetic efficiency was enhanced while genes involved in the photorespiratory and recycling of TCA cycle intermediates were down-regulated. In addition, we found that under reduced irradiance conditions, ^{13}C uptake was reduced and this correlated with a down-regulation of genes involved in photosynthetic, photorespiratory and recycling of TCA cycle intermediates pathways. Finally, when exposed to inhibition of CA, *Z. muelleri* plants displayed a decrease in photosynthetic rate concomitant with up-regulation of photosynthetic and photorespiratory genes, suggesting the presence of a compensatory mechanism in this seagrass specie in order to overcome CA inhibition. In view of these results, we provide further details to the

carbon metabolism pathways in *Z. muelleri* and presents new techniques in molecular analysis that can be applied to different areas of seagrass research.

Chapter 1: Thesis introduction

Seagrasses are a polyphyletic group of marine angiosperms that evolved from terrestrial plants between 70 million and 100 million years ago (McRoy and Helfferich 1977; Larkum and den Hartog 1989; Les et al. 1997). With 72 identified species across 6 families; *Cymodoceaceae*, *Hydrocharitaceae*, *Posidonia*, *Ruppiaceae*, *Zannichelliaceae* and *Zosteraceae* (Short et al. 2011), seagrasses make up less than 0.1% of all flowering species (Les et al. 1997). Seagrasses are distributed globally (Figure 1. 1), typically inhabiting coastlines at depths between 0 to 70 m in meadows and have low taxonomic diversity (Short et al. 2011).

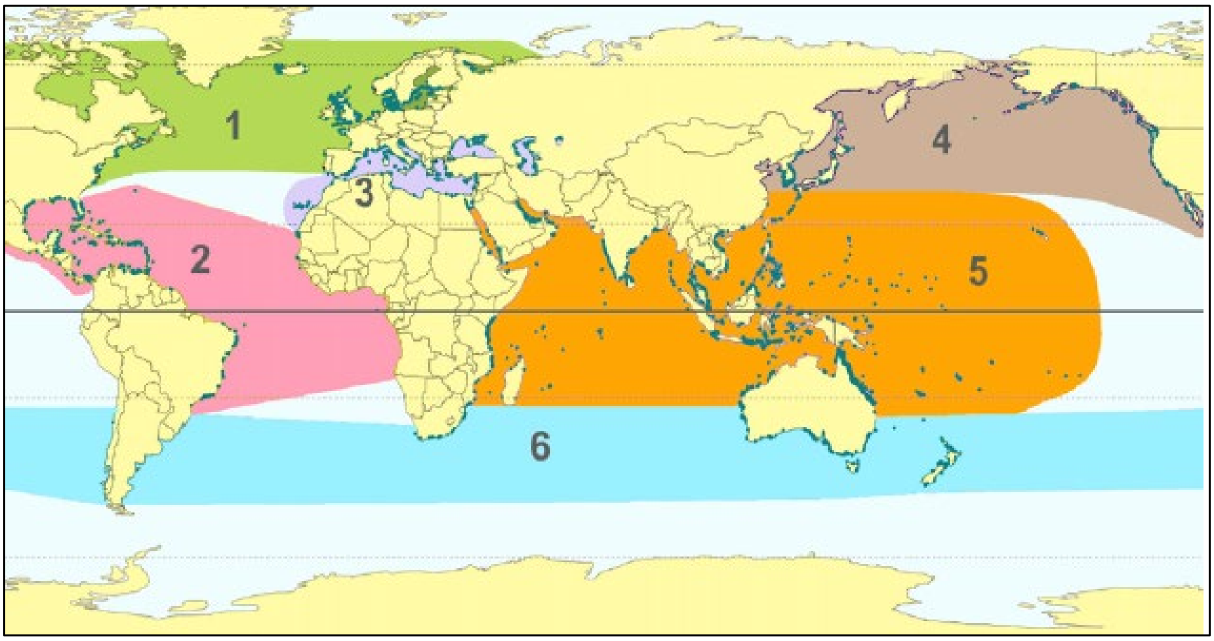


Figure 1. 1. Global distribution of seagrasses (blue points) relative to geographic bioregions 1; Temperate North Atlantic, 2; Tropical Atlantic, 3; Mediterranean, 4; Temperate North Pacific, 5; Tropical Indo-Pacific and 6; Temperate Southern Oceans (Short et al. 2007).

Seagrass adaptations to the marine environment

The coastal environment that seagrasses inhabit is highly variable and is characterised by periodic light limitation due to varying turbidity from sediment runoff/resuspension, fluctuating water temperature and flow, tidal depth and changing salinity. Seagrass habitats are also CO_2 limited as the diffusion of CO_2 in seawater is approximately 10, 000 fold slower than in air (Stumm and Morgan 2012). Furthermore, some seagrasses are exposed to atmospheric conditions at low tide for hours at a time, adding periodic desiccation and increased exposure to ultraviolet radiation as further stressors (Trocine et al. 1981; Kuo et al. 1990; Leuschner et al. 1998). These environmental conditions have led to emergent properties such as anatomical reinforcement of leaves that aid in resisting wave action (Cooper and McRoy 1988; Kuo et al. 1990; Kuo and Den Hartog 2007), the ability to photosynthesise in the marine environment (Kuo and Den Hartog 2007), internal transport and storage of gases via air lacunae to assist in metabolic functions (Andrews and Abel 1979; Beer and Wetzel 1981; Kuo et al. 1990), modified roots and rhizomes to survive in an anoxic environment, (Barnabas and Arnott 1987; Kuo and Den Hartog 2007; Brodersen et al. 2015) and the ability to propagate via hydrophilous pollination (den Hartog 1970; Cox 1993; Olsen et al. 2016).

Ecological roles and ecosystem services provided by seagrass

These adaptations enable seagrasses to thrive in the coastal environments where they have become a keystone species, supporting higher trophic levels in a variety of ways. These include but are not limited to; protecting coastlines from wave energy (Manca et al. 2012), reducing sediment re-suspension (Widdows et al. 2008), contributing to nutrient cycling (Susan and Ronald 1999), filtering sediment out of the water column (Hendriks et al. 2008; Kennedy et al. 2010) and providing habitats for finfish, shellfish and herbivorous mammals (Ford et al. 2010; Mizerek et al. 2011; Ray et al. 2014). Furthermore, they are one of the most productive marine ecosystems with a productivity rate exceeding 15 g C m^{-2} d^{-1} (Phillips and McRoy 1980; Hillman et al. 1989), and are responsible for approximately 10-18% of the oceans carbon accumulation despite only covering 0.1% of the sea floor (Duarte et al. 2005; Kennedy et al. 2010). In addition, they are able to trap allochthonous carbon at a rate of 48-112 Tg a^{-1} (Kennedy et al. 2010), and sequester this carbon for millennia (Macreadie et al. 2012; Greiner et al. 2013), making seagrass meadow restoration an effective method of enhancing coastal carbon sequestration (Kennedy et al. 2010; Greiner et al. 2013; Marbà et al. 2015). Due to this effectiveness of seagrasses to sequester carbon, seagrass ecosystems are an important component of blue carbon (carbon stored in the ocean) research and management (Fourqurean et al. 2012).

The economic benefits seagrasses provide globally are also considerable. The benefits people derive from the worlds ecosystems have been investigated in the past and include: regulation of atmospheric chemical composition, water regulation, soil formation, nutrient cycling and food production (Costanza et al. 1997). These services, along with others not listed here, has been termed 'ecosystem services' and when the contribution of each type of ecosystem or biome was combined, the value of the planets ecosystem services was estimated at US$125 trillion a^{-1} in 2011 (Costanza et al. 2014). Of the marine biome, seagrasses and algae beds were the 2nd highest contributors, with Costanza et al. (2014) evaluating their ecosystem services worldwide as approximately US$28,916 h^{-1} a^{-1}, making seagrasses and algae beds more valuable than forests (US$3,800 h^{-1} a^{-1}) and cropland (US$5,567 h^{-1} a^{-1}).

Seagrass decline

Despite their global importance, seagrass meadows face continued threats from anthropogenic and environmental impacts. Although natural disturbances such as geological events, meteorological events and biological interactions can negatively affect seagrass coverage, anthropogenic and other environmental factors have been identified as the primary cause of recent seagrass loss (Short and Wyllie-Echeverria 1996; Orth et al. 2006; Waycott et al. 2009).

Human activity which leads to reduced water quality and clarity, is considered to be the most significant threat to global seagrass survival (Short and Wyllie-Echeverria 1996; Kemp et al. 2005; Burkholder et al. 2007). This is unsurprising as seagrasses require an unusually high amount of light, ranging from 5% to 37% of surface irradiance compared to the light requirements of most marine macrophytes, which range from 0.1% to 1% of surface irradiance (Duarte 1991; Olesen and Sand-Jensen 1993; Kenworthy and Fonseca 1996; Sharon et al. 2011). The high light requirements of seagrasses is due to the relatively high demand for photosynthate for the below ground tissue such as the roots and rhizomes and has been partly attributed to a CO_2 concentrating mechanism (CCM; Durako 1993; Beer and Rehnberg 1997; Zimmerman et al. 1997; Invers et al. 2001), hence making them particularly vulnerable to reduced water quality and clarity. For example, increased sediment loading and eutrophication were found to be the primary factors responsible for the decline of *Zostera noltii* in the Dutch Wadden Sea (Giesen et al. 1990; Philippart et al. 1992) and Chesapeake Bay (Kemp et al. 1983; Orth and Moore 1983; Dennison et al. 1993; Kemp et al. 2005). Similar declines in seagrass coverage as a result of increased turbidity due to dredging have also been documented in Pensacola Bay, Florida (Livingston 1987) and Laguna Madre, Texas (Quammen and Onuf 1993), while direct mechanical damage caused from cutting by propellers, propeller wash (Zieman 1976; Walker et al. 1989) and boat mooring (Walker et al. 1989; Short et al. 1991) have also contributed to the decline.

Another, less reported, anthropogenic impact is introduced or invasive species (Orth et al. 2006; Williams 2007). A review conducted by Williams (2007) found that between 1990 and 2007, there were at least 56 reported non-native species introduced into seagrass meadows, primarily as a result of shipping activity and aquaculture. Largely invertebrates and seaweeds, of the introduced species studied (less than half) there was a predominantly negative effect as they were responsible for reduced macroalgal diversity (Piazzi and Cinelli 2003), reduced seagrass growth or density (Davis et al. 1998; Eklöf et al. 2005; Eklöf et al. 2006) and increased competition between native and non-native seagrass species (Wonham et al. 2005).

These threats combined with natural disturbances have contributed to a global decline in seagrass coverage. In a meta-analysis by Waycott et al. (2009), the estimated rate of decline is 110 km^2 a^{-1} since 1980, with an overall seagrass coverage decline of 29% since 1879 despite local, occasional increases (Kendrick et al. 2000; Cardoso et al. 2005; Short et al. 2007). This equates to an increase in the rate of decline of 0.9% a^{-1} from before 1940 to 7% a^{-1} since 1990 (Waycott et al. 2009), which compared to tropical rainforest decline (0.5% a-1, Achard et al. 2002) and mangrove forest decline (1.8% a-1, Valiela et al. 2001), places seagrass meadows as one of the most threatened ecosystems on earth.

Therefore, conservation efforts have focused primarily on improving water quality, leading to agreements such as the Chesapeake Bay Watershed Agreement 2014, Florida's State Wildlife Action Plan and the establishment of Commonwealth Marine Reserves across Australia. Although conservation efforts have been successful in partially restoring seagrass coverage in some impacted areas such as Tampa Bay and Sarastosa Bay, Florida (Tomasko et al. 2005), seagrass coverage in other areas such as Cockburn Sound, Western Australia and the southern regions of the Dutch Wadden Sea, are still in decline (Kendrick et al. 2002) or have seen no change in coverage (Reise et al. 2005) despite improvements in water quality. This has led to the suggestion that the use of molecular profiling, genetics and omics techniques could be used as an additional tool to assist in combating seagrass decline (Procaccini et al. 2007; Macreadie et al. 2014; Davey et al. 2016).

Molecular biology of seagrasses

Macreadie et al. (2014) argues that the identification of molecular indicators and the use of molecular analytical techniques such as real-time quantitative polymerase chain reaction (RT-qPCR) would be an effective tool in seagrass conservation as molecular indicators provide the earliest detectable evidence of stress and impending mortality in organisms (Hoffmann and Daborn 2007). Subsequently, there has been a recent push to link physiological pathways and the regulation of gene expression in response to stress in seagrasses (Procaccini et al. 2007; Papenbrock 2012; Procaccini et al. 2012; Mazzuca et al. 2013; Macreadie et al. 2014; Davey et al. 2016).

Although some advancements have been made in various fields of seagrass molecular biology such as metabolomics (Hasler-Sheetal et al. 2015), gene expression analysis (Salo et al. 2015; Kim et al. 2018; Buapet et al. 2019; Kim et al. 2019), proteomics (Mazzuca et al. 2009; Jiang et al. 2017; Kumar et al. 2017), transcriptomics (Franssen et al. 2011; Davey et al. 2018), the creation of an online data repository of expressed sequence tags of *Zostera marina*, *Posidonia oceanica* (Wissler et al. 2009) and the creation of an open-source transcriptomics database for eight species of aquatic plants, including *Zostera muelleri* (Sablok et al. 2018), the overall contributions across these fields remains limited. At the time of publication, a review of the available literature by Davey et al. (2016) found that since 2006, there have only been 31 research studies that integrate these various techniques, while an earlier review by Procaccini et al. (2007) identified less than 100 studies between 1980 to 2006 with ties to seagrass genetics. Furthermore, these studies have primarily been restricted to Northern Hemisphere seagrass species such as: *Z. marina, Zostera noltii, P. oceanica* and *Cymodocea nodosa* (Procaccini et al. 2007; Davey et al. 2016), with the only complete genome sequence available being that of *Z. marina (Olsen et al. 2016)*. By comparison, the genomes of 49 terrestrial plant species have been sequenced since 2013 (Michael and Jackson 2013). Hence there is much more research to be done using these genetic tools to understand the underlying molecular mechanisms controlling seagrass biology.

Photosynthetic biochemistry of seagrass

One such research area of interest is the photosynthetic biochemistry of seagrass. As mentioned earlier, seagrass are particularly vulnerable to decreased light availability due to their high demand for light (Durako 1993; Beer and Rehnberg 1997; Zimmerman et al. 1997; Invers et al. 2001); therefore there is an urgent need to better understand seagrass photobiology. In the past, pulse amplitude modulated (PAM) fluorometry based research has been partially successful in characterising the photosynthetic efficiency of seagrass (Ralph et al. 2002); providing insight into the biochemical mechanisms driving their photosynthesis. Sharing similarities in the basic photosynthetic biochemistry as other angiosperms (Goodwin and Mercer 1972; Beer et al. 1998), the pigment composition of seagrass chloroplasts include (i) chlorophylls *a* and *b,* which are directly involved in photosynthesis, and (ii) carotenoids, which assist in absorbing UV light, contributes to light harvesting and protects against photodamage (Beer and Waisel 1979; Beer 1989; Berera et al.

2010). These pigments act to convert light energy into chemical energy (NADPH and ATP) via a process called Forster transfer (Taiz and Zeiger 2003), whereby excitation energy from one pigment is transferred to another. This antenna system contains a highly conserved central reaction site, containing the protein complexes P680 and P700 (Wales et al. 1989) which is typically depicted as a Z scheme (Figure 1. 2).

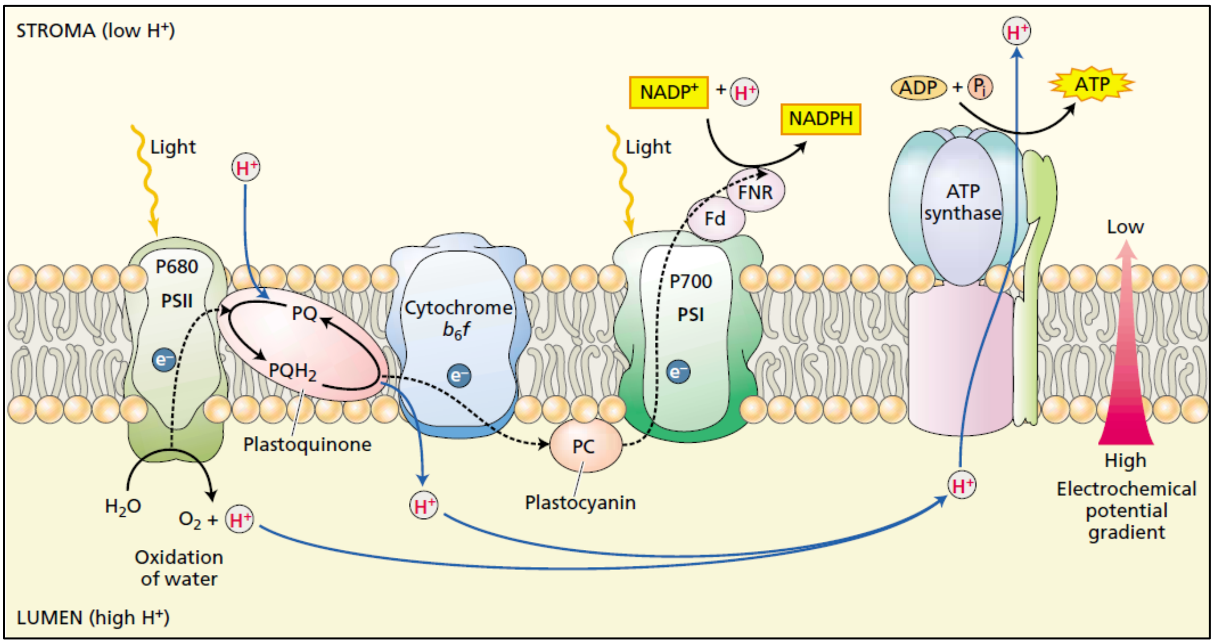


Figure 1. 2. Conceptual diagram of the photosynthesis light reactions. Photosynthetically active radiation (PAR) of wavelengths 680 nm excites the protein complex P680 in photosystem II producing a strong oxidant that oxidises water, exciting electrons. These electrons are released and transported down the electron transport chain. PAR of wavelengths greater than 680 nm excites the protein complex P700 in photosystem I producing a strong reductant which reduces $NADP^+$ to produce NADPH. This system forms the basis of photosynthetic electron transport (Taiz and Zeiger 2003).

CO_2 concentrating mechanisms in seagrasses

The primary form of dissolved inorganic carbon (DIC) in seawater is HCO_3^- at 85.8%, followed by CO_3^{2-} at 13.7%, and CO_2 at a mere 0.4% (at pH 8.16, salinity 31ppt and 22°C; Pierrot et al. 2006). As a result, there is much less CO_2 available for photosynthesis in seawater than in air, especially when factoring in the reduced diffusion rate of CO_2 in seawater. This supports the argument that seagrasses require a CO_2 concentrating mechanism (CCM) or the ability to utilize HCO_3^-, which has hence been a source of debate when classifying these angiosperms as either C_3 or C_4 plants (Koch et al. 2013). A proposed biophysical CCM model by Larkum et al. (2006) and reviewed recently (Larkum et al. 2017) is located on the outer surface of the epidermal cell wall and involves: i) production of carbonic anhydrase which is responsible for catalyzing the interconversion of HCO_3^- to CO_2 ($HCO_3^- + H^+ = CO_2 + H_2O$), ii) localized acidification of the diffusive boundary layer (DBL) and acidification of the cell wall via a proton pump to concentrate CO_2 by shifting the HCO_3^-/CO_2 equilibrium towards CO_2, and iii) at least one system for the active uptake of HCO_3^- (Figure 1. 3). This was derived from experiments utilising carbonic anhydrase (CA) inhibitors including the cell impermeable inhibitor acetazolamide (AZ), the cell permeable CA inhibitor ethoxyzolamide (EZ) and tris(hydroxymethyl)aminomethane (TRIS) buffer (Table 1. 1). It is important to note that there is a difference between a biophysical CCM and a biochemical CCM. Biophyiscal CCMs are typically characterised by energy dependent Ci uptake, CA catalysed dehydration of HCO_3^-, localisation of Rubisco in subcellular compartments and mechanisms to minimise CO_2 diffusion away from carboxylation sites, where as biochemical CCMs are characterised by an additional carboxylation enzyme (typically phosphoenolpyruvate carboxylase) which is carboxylated via HCO_3^- to form a 4 C molecule (Rae et al. 2017).

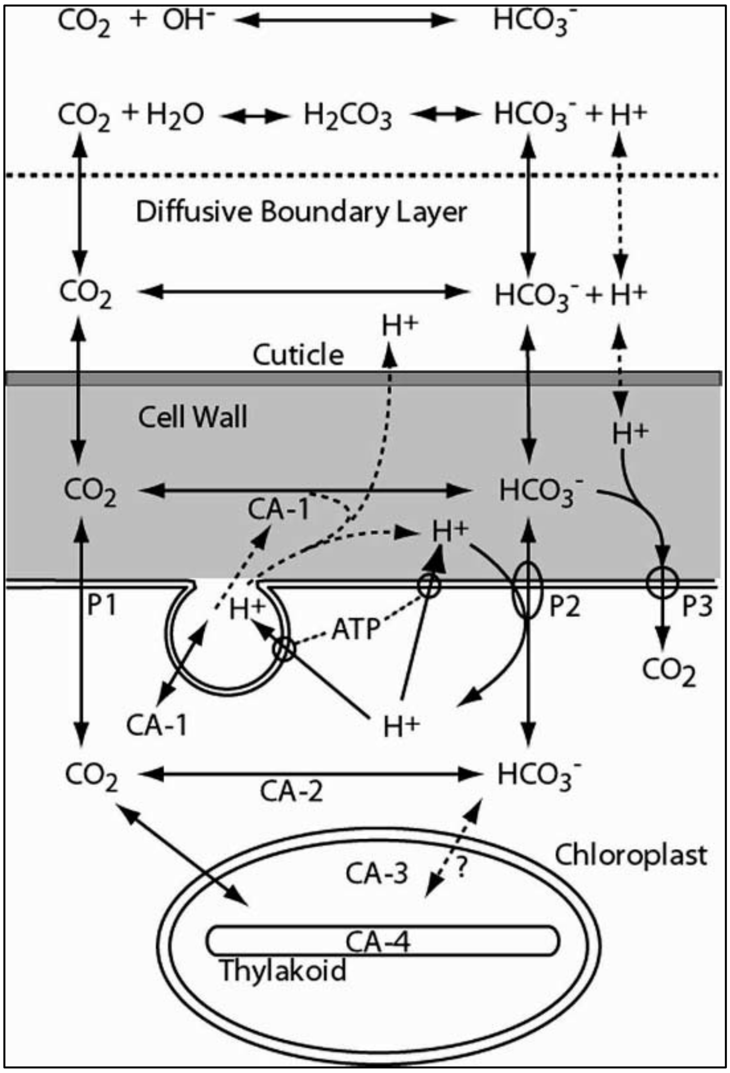


Figure 1. 3. Conceptual diagram illustrating a potential CCM located on the outer surface of the epidermal cell wall or cuticle. P1 represents a CA dependent CO_2 acquisition mechanism, P2 represents an H^+/HCO_3^- symport mechanism and P3 represents a CA linked HCO_3^- mechanism carrying CO_2 into the cytoplasm. CA-1, CA-2, CA-3 and CA-4 are carbonic anhydrases (Larkum et al. 2006).

Table 1. 1. Studies on seagrass photosynthesis in response to cell impermeable/cell permeable CA inhibitors: AZ and EZ respectively and TRIS buffer. Presence or absence of AZ, EZ or TRIS use in references cited are indicated by Y or – respectively.

Species	**AZ**	**EZ**	**TRIS**	**Reference**
Cymodocea nodosa	Y	-	Y	Invers et al. (1999), Invers et al. (2001)
Cymodocea rotundata	Y	-	Y	Björk et al. (1997), Uku et al. (2005)
Cymodocea serrulata	Y	-	Y	Björk et al. (1997)
Enhalus acoroides	Y	-	Y	Björk et al. (1997)
Halodule wrightii	Y	-	Y	Björk et al. (1997)
Halophila johnsonii	Y	-	Y	Gavin and Durako (2019)
Halophila ovalis	Y	-	Y	Björk et al. (1997), Uku et al. (2005)
Phyllospadix torreyi	Y	-	Y	Invers et al. (2001)
Posidonia oceanica	Y	Y	Y	Invers et al. (1999), Invers et al. (2001), Rubio et al. (2017)
Ruppia cirrhosa	Y	-	Y	Hellblom and Axelsson (2003)
Syringodium isoetifolium	Y	-	Y	Uku et al. (2005)
Thalassia hemprichii	Y	-	Y	Uku et al. (2005)
Thalassia testudinum	Y	-	Y	Gavin and Durako (2019)
Thalassodendron cilliatum	Y	-	Y	Uku et al. (2005)
Zostera marina	y	-	Y	Beer and Rehnberg (1997), Beer et al. (2002), Invers et al. (2001)
Zostera muelleri	Y	-	-	Millhouse and Strother (1986)

C_3 and C_4 photosynthesis

Plants that utilize the C_3 carbon fixation process (C_3 plants) are generally characterised by the initial carbon product from photosynthesis being a 3 carbon molecule; 3-phosphoglycerate, following carboxylation of ribulose-1, 5-bisphosphate (RuBP). Alternatively, RuBP can be oxygenated in situations where O_2 outcompetes CO_2 for the active site of RuBP, resulting in photorespiration. Photorespiration is possible as RuBP can undergo oxidation, resulting in the net loss of C. Most plants that utilise C_4 carbon fixation (C_4 plants) on the other hand, prevent the loss of C due to photorespiration via anatomical and biochemical adaptations. In addition, most C_4 plants possess specialized anatomy referred to as Kranz anatomy, whereby there is cellular separation between the site of CO_2 uptake and the location of RuBP. This eliminates photorespiration as when CO_2 is taken up by a C_4 plant, it is hydrated to HCO_3^- which then carboxylates phosphoenolpyruvate (PEP) via the oxygen insensitive enzyme PEP carboxylase (PEPC), forming oxaloacetate (OAA) in the mesophyll cells. OAA is then transported to the bundle shealth cells as malate or aspartate where it is decarboxylated and free to carboxylate RuBP. Since PEPC can not be oxygenated, C_4 plants are able to concentrate CO_2 around the active site of RuBP, hence increasing their photosynthetic efficiency over C_3 plants (Taiz and Zeiger 2003). In this way, C_4 plants are able to concentrate CO2

However, Kranz anatomy, which has previously been the defining attribute in C_4 plants, has been shown not to be an essential feature for C_4 carbon fixation (Shomer-Ilan et al. 1975; Voznesenskaya et al. 2001). Based on a study involving the immunolocalization of photosynthetic enzymes, the terrestrial plant *Borszczowia aralocaspica* was shown to spatially compartmentalise photosynthetic enzymes, thus achieving C_4 carbon fixation despite lacking Kranz anatomy (Voznesenskaya et al. 2001). Similarly, other methods to determine C_3 or C_4 carbon fixation in seagrasses have been explored. Based on the rate of O_2 evolved per absorbed photon during light-limited photosynthesis (ϕ_a), Frost-Christensen and Sand-Jensen (1992) reported values of ϕ_a for *Z. marina* that were comparable to terrestrial C_4 plants. Direct measurements of $\delta^{13}C$ were also used, whereby *Thalassia* *testudinum* were classified as C_4 plants when compared to the $\delta^{13}C$ values of terrestrial C_4 plants (Benedict and Scott 1976). Further, *Cymodocea nodosa* was found to convert nearly 50% of labeled C (from $H^{14}CO_3^-$) after a 5 s pulse into malate, indicating C_4 metabolism (Beer et al. 1980).

Despite these lines of evidence, there still remains some scepticism regarding the true nature of seagrass carbon metabolism. In the case of ϕ_a and $\delta^{13}C$ measurements, the classification of C_4 or C_3 was solely reliant on the comparison to terrestrial plants. Critics suggest that the environment in which seagrasses inhabit along with differences in their anatomy to terrestrial plants could influence these results as: diffusion of CO_2 is much slower through water than air (Raven 1984; Stumm and Morgan 2012) and the presence of air lacunae containing O_2 and potentially CO_2 in the leaves could also impact the photosynthetic processes (Andrews and Abel 1979; Kuo et al. 1990), thus accounting for the similarities of ϕ_a and $\delta^{13}C$ of these seagrass with terrestrial C_4 plants (Benedict and Scott 1976; Kuo et al. 1990; Durako 1993). In addition, plants with biophysical CCMs can also have the same $\delta^{13}C$ signature as C_4 plants (Raven et al. 2014). Furthermore, in a similar ^{14}C pulse experiment, *Halophila stipulacea* was also found to initially convert labeled C to malate and other organic acids; however, there was no decline in their respective concentrations as would have been expected in C_4 plants (Beer et al. 1980). This led to the notion that some seagrasses could be classified as a C_3-C_4 intermediate (Beer et al. 1980; Touchette and Burkholder 2000; Papenbrock 2012).

Molecular investigation of photosynthesis in seagrass

With advancements in omics and molecular profiling in seagrass biology, there are new methods in which carbon metabolism in seagrasses can be further investigated. For example, genes for carbonic anhydrase and PEPC have been identified in the genome of *Z. muelleri* (Kim et al. 2018). In addition, changes in irradiance have also been shown to affect methylation activity of PEPC in *P. oceanica* (Greco et al. 2013). Although these enzymes are related to C_4 carbon metabolism, they have also been shown to be involved in anaplerotic processes (Norici et al. 2002; Giordano et al. 2003); a process that resupplies intermediates for the TCA cycle. Therefore, to develop a more holistic understanding of seagrass carbon metabolism, a combination of methodologies need to be utilized. A review by Rawsthorne (1992) of what is currently known about C_3-C_4 intermediate photosynthesis found that the classification of C_3-C_4 intermediate photosynthesis relied on: leaf gas exchange measurements, leaf anatomy, biochemical mechanisms and cell specific expression of genes. Indeed, this has been done successfully in the classification of the terrestrial plant; *B. aralocaspica* whereby a combination of enzyme activity analysis, immunolocalization of these enzymes coupled with measurements of photosynthetic rates proved that it was possible for C_4 carbon metabolism to occur in plants that lacked Kranz anatomy (Voznesenskaya et al. 2001).

Zostera muelleri subsp. capricorni (*Z. muelleri*) as an Australian model organism

The current thesis will focus on the seagrass species *Zostera muelleri* ssp. capricorni (Asch) S. W. L. Jacobs, formally known as *Zostera muelleri* Irmisch ex Ashers (Jacobs et al. 2006). Endemic to Australia, New Zealand and Papua New Guinea, *Z. muelleri* is classified as a species of ‘Least Concern’ by The IUCN Red List of Threatened Species (Figure 1. 4), with local threats due to coastal development not contributing to any major declines throughout its range (Short et al. 2011). This species of seagrass is present in two morphologically distinct forms, an intertidal and a subtidal form (Kuo et al. 1990). In a comprehensive morphology and ultrastructure study conducted by Kuo et al. (1990), the subtidal form is characterised as having a larger number of leaves; 4-5 leaves per branch of the rhizome that extends above the sediment, a larger average leaf volume (58.3 mm^3), a larger average leaf air lacunae volume (19.5 mm^3) and more chloroplasts in the epidermal cells, as compared to the intertidal form. The subtidal form also contains reticulated fungal hyphae in the intercellular spaces which potentially enhances the transfer of solutes in the leaves (Kuo 1984). The intertidal form is characterised by having fewer leaves; 3 leaves per branch of the rhizome that extends above the sediment, a lower average leaf volume (6.7 mm^3), a lower average leaf air lacunae volume (1.3 mm^3), fewer chloroplasts in the epidermal cells and does not contain any reticulated fungal hyphae. The productivity of meadows of subtidal *Z. muelleri* has been suggested to be higher than that of meadows of intertidal *Z. muelleri* due to the larger leaf area, greater number of shoots, increased chloroplast content within the epidermal cells, presence of apoplastic fungal hyphae and larger air lacunae of the subtidal form (Kuo et al. 1990). These differences in morphology and potential productivity have been attributed to the different tidal zones that the 2 forms inhabit. The intertidal form as the name indicates, inhabits the intertidal zone which is closer to the shore and as such, is exposed to air 2-4 h daily (Kuo et al. 1990), whereas the subtidal form inhabits the deeper waters further from the shore and is thus not exposed to air. Despite these differences, the 2 forms of *Z. muelleri* share similar general anatomical appearance as other members of the Zosteraceae family (Barnabas et al. 1977, 1980; Kuo et al. 1988) and is characterised by the lack of visible erect stems, strap shaped leaves originating from a rhizome node and with leaves of lengths up to 76.8 mm and 201.6 mm for the intertidal and subtidal forms of *Z. muelleri* respectively (Larkum et al. 1989; Kuo and Den Hartog 2007).

Figure 1. 4. Geographical distribution of *Z. muelleri*. Extant populations (indicated in orange) have been located in Papua New Guinea, New Zealand and Australia. Image taken from https://www.iucnredlist.org/

Aims of the thesis

The aim of the present thesis is to investigate and characterise the molecular mechanisms driving carbon metabolism in *Z. muelleri* by combining molecular analytical tools with physiological measurements. In this way, the photobiology of *Z. muelleri* can be examined from a molecular perspective, particularly the way in which gene expression and physiology react in response to changes in abiotic factors.

Specifically, the aims of the following chapters were to:

1. Address how the photosynthetic and respiratory rates are affected by experimentally decreased O_2 concentration in the water column using electrochemical microsensors and how this decreased O_2 concentration affects the expression of phosphoenolpyruvate carboxylase and carbonic anhydrase using real time reverse transcription quantitative polymerase chain reaction (RT-qPCR).
2. Investigate the effect of low light on a combination of (i) photosynthetic carbon assimilation measured via stable isotope (^{13}C) probing and mass spectrometry analysis, (ii) level of expression of photosynthetic, photorespiratory and intermediates recycling genes using a new gene expression analytical technique; NanoString and (iii) the enzymatic content and activity via activity bioassays for the Rubisco complex and phosphoenolpyruvate carboxylase.
3. Identify the effect of cell impermeable and cell permeable carbonic anhydrase inhibitors on the molecular physiology of *Z. muelleri* at three different levels (i) photosynthetic rate measured via O_2 electrochemical microsensors, (ii) ^{13}C uptake measured via mass spectrometry and (iii) level of expression of photosynthetic and photorespiratory genes.

References

Achard F, Eva HD, Stibig H-J, Mayaux P, Gallego J, Richards T, Malingreau J-P (2002) Determination of deforestation rates of the world's humid tropical forests. Science 297 (5583):999-1002

Andrews TJ, Abel KM (1979) Photosynthetic carbon metabolism in seagrasses ^{14}C-labeling evidence for the C3 pathway. Plant Physiology 63 (4):650-656

Barnabas AD, Arnott HJ (1987) *Zostera capensis* Setchell: root structure in relation to function. Aquatic botany 27 (4):309-322

Barnabas AD, Butler V, Steinke TD (1977) *Zostera capensis* Setchell. I. Observations of the fine structure of the leaf epidermis. Zeitschrift für Pflanzenphysiologie 85 (5):417-427

Barnabas AD, Butler V, Steinke TD (1980) *Zostera capensis* Setchell II. Fine structure of the cavities in the wall of leaf blade epidermal cells. Zeitschrift für Pflanzenphysiologie 99 (2):95-103

Beer S (1989) Photosynthesis and photorespiration of marine angiosperms. Aquatic Botany 34 (1-3):153-166

Beer S, Bjork M, Hellblom F, Axelsson L (2002) Inorganic carbon utilization in marine angiosperms (seagrasses). Functional Plant Biology 29 (3):349-354

Beer S, Rehnberg J (1997) The acquisition of inorganic carbon by the seagrass *Zostera marina*. Aquatic Botany 56 (3):277-283

Beer S, Shomer-Ilan A, Waisel Y (1980) Carbon Metabolism in Seagrasses II. Patterns of photosynthetic CO_2 incorporation. Journal of Experimental Botany 31 (4):1019-1026

Beer S, Vilenkin B, Weil A, Veste M, Susel L, Eshel A (1998) Measuring photosynthetic rates in seagrasses by pulse amplitude modulated (PAM) fluorometry. Marine Ecology Progress Series 174:293-300

Beer S, Waisel Y (1979) Some photosynthetic carbon fixation properties of seagrasses. Aquatic Botany 7:129-138

Beer S, Wetzel RG (1981) Photosynthetic carbon metabolism in the submerged aquatic angiosperm *Scirpus subterminalis*. Plant Science Letters 21 (3):199-207

Benedict CR, Scott JR (1976) Photosynthetic carbon metabolism of a marine grass. Plant physiology 57 (6):876-880

Berera R, van Stokkum IHM, Kennis JTM, Grondelle Rv, Dekker JP (2010) The light-harvesting function of carotenoids in the cyanobacterial stress-inducible IsiA complex. Chemical Physics 373 (1):65-70. doi:https://doi.org/10.1016/j.chemphys.2010.01.011

Björk M, Weil A, Semesi S, Beer S (1997) Photosynthetic utilisation of inorganic carbon by seagrasses from Zanzibar, East Africa. Marine Biology 129 (2):363-366

Brodersen KE, Lichtenberg M, Paz L-C, Kühl M (2015) Epiphyte-cover on seagrass (*Zostera marina* L.) leaves impedes plant performance and radial O_2 loss from the below-ground tissue. Frontiers in Marine Science 2:58

Buapet P, Mohammadi NS, Pernice M, Kumar M, Kuzhiumparambil U, Ralph PJ (2019) Excess copper promotes photoinhibition and modulates the expression of antioxidant-related genes in *Zostera muelleri*. Aquatic Toxicology 207:91-100. doi:https://doi.org/10.1016/j.aquatox.2018.12.005

Burkholder JM, Tomasko DA, Touchette BW (2007) Seagrasses and eutrophication. Journal of Experimental Marine Biology and Ecology 350 (1–2):46-72

Cardoso P, Brandão A, Pardal M, Raffaelli D, Marques J (2005) Resilience of *Hydrobia ulvae* populations to anthropogenic and natural disturbances.

Cooper LW, McRoy CP (1988) Anatomical adaptations to rocky substrates and surf exposure by the seagrass genus *Phyllospadix*. Aquatic Botany 32 (4):365-381

Costanza R, d'Arge R, de Groot R, Farber S, Grasso M, Hannon B, Limburg K, Naeem S, O'Neill RV, Paruelo J, Raskin RG, Sutton P, van den Belt M (1997) The value of the world's ecosystem services and natural capital. Nature 387 (6630):253-260

Costanza R, de Groot R, Sutton P, van der Ploeg S, Anderson SJ, Kubiszewski I, Farber S, Turner RK (2014) Changes in the global value of ecosystem services. Global Environmental Change 26:152-158. doi:https://doi.org/10.1016/j.gloenvcha.2014.04.002

Cox PA (1993) Water-Pollinated Plants. Scientific American 269 (4):68-74

Davey PA, Pernice M, Ashworth J, Kuzhiumparambil U, Szabó M, Dolferus R, Ralph PJ (2018) A new mechanistic understanding of light-limitation in the seagrass *Zostera muelleri*. Marine Environmental Research 134:55-67. doi:https://doi.org/10.1016/j.marenvres.2017.12.012

Davey PA, Pernice M, Sablok G, Larkum A, Lee HT, Golicz A, Edwards D, Dolferus R, Ralph P (2016) The emergence of molecular profiling and omics techniques in seagrass biology; furthering our understanding of seagrasses. Functional & Integrative Genomics:1-16. doi:10.1007/s10142-016-0501-4

Davis RC, Short FT, Burdick DM (1998) Quantifying the effects of green crab damage to eelgrass transplants. Restoration Ecology 6 (3):297-302

den Hartog C (1970) The seagrasses of the world, vol 59. North-Holland Pub. Co., Amsterdam

Dennison WC, Orth RJ, Moore KA, Stevenson JC, Carter V, Kollar S, Bergstrom PW, Batiuk RA (1993) Assessing water quality with submersed aquatic vegetation. BioScience 43 (2):86-94

Duarte CM (1991) Seagrass depth limits. Aquatic botany 40 (4):363-377

Duarte CM, Middelburg JJ, Caraco N (2005) Major role of marine vegetation on the oceanic carbon cycle. Biogeosciences 2 (1):1-8

Durako MJ (1993) Photosynthetic utilization of $CO_{2\,(aq)}$ and HCO_3^- in *Thalassia testudinum* (*Hydrocharitaceae*). Marine Biology 115 (3):373-380

Eklöf JS, de la Torre Castro M, Adelsköld L, Jiddawi NS, Kautsky N (2005) Differences in macrofaunal and seagrass assemblages in seagrass beds with and without seaweed farms. Estuarine, Coastal and Shelf Science 63 (3):385-396

Eklöf JS, Henriksson R, Kautsky N (2006) Effects of tropical open-water seaweed farming on seagrass ecosystem structure and function. Marine Ecology Progress Series 325:73-84

Ford JR, Williams RJ, Fowler AM, Cox DR, Suthers IM (2010) Identifying critical estuarine seagrass habitat for settlement of coastally spawned fish. Marine Ecology Progress Series 408:181-193

Fourqurean JW, Duarte CM, Kennedy H, Marbà N, Holmer M, Mateo MA, Apostolaki ET, Kendrick GA, Krause-Jensen D, McGlathery KJ (2012) Seagrass ecosystems as a globally significant carbon stock. Nature Geoscience 5 (7):505-509

Franssen SU, Gu J, Bergmann N, Winters G, Klostermeier UC, Rosenstiel P, Bornberg-Bauer E, Reusch TBH (2011) Transcriptomic resilience to global warming in the seagrass *Zostera marina*, a marine foundation species. Proceedings of the National Academy of Sciences of the United States of America 108 (48):19276-19281

Frost-Christensen H, Sand-Jensen K (1992) The quantum efficiency of photosynthesis in macroalgae and submerged angiosperms. Oecologia 91 (3):377-384

Gavin NM, Durako MJ (2019) Carbon acquisition mechanisms in *Halophila johnsonii* and *Thalassia testudinum*. Aquatic Botany 152:64-69. doi:https://doi.org/10.1016/j.aquabot.2018.11.001

Giesen WBJT, Van Katwijk MM, den Hartog C (1990) Temperature, salinity, insolation and wasting disease of eelgrass (*Zostera marina* L.) in the Dutch Wadden Sea in the 1930's. Netherlands Journal of Sea Research 25 (3):395-404

Giordano M, Norici A, Forssen M, Eriksson M, Raven JA (2003) An anaplerotic role for mitochondrial carbonic anhydrase in *Chlamydomonas reinhardtii*. Plant Physiology 132 (4):2126-2134. doi:10.1104/pp.103.023424

Goodwin TW, Mercer EI (1972) Introduction to plant biochemistry. Pergamon Press, Oxford, New York

Greco M, Chiappetta A, Bruno L, Bitonti MB (2013) Effects of light deficiency on genome methylation in *Posidonia oceanica*. Marine Ecology Progress Series 473:103-114

Greiner JT, McGlathery KJ, Gunnell J, McKee BA (2013) Seagrass restoration enhances “Blue Carbon” sequestration in coastal waters. PLoS ONE 8 (8):e72469

Hasler-Sheetal H, Fragner L, Holmer M, Weckwerth W (2015) Diurnal effects of anoxia on the metabolome of the seagrass *Zostera marina*. Metabolomics 11 (5):1208-1218

Hellblom F, Axelsson L (2003) External HCO_3^- dehydration maintained by acid zones in the plasma membrane is an important component of the photosynthetic carbon uptake in *Ruppia cirrhosa*. Photosynthesis Research 77 (2-3):173-181

Hendriks IE, Sintes T, Bouma TJ, Duarte CM (2008) Experimental assessment and modeling evaluation of the effects of the seagrass *Posidonia oceanica* on flow and particle trapping. Marine Ecology Progress Series 356:163-173

Hillman K, Walker D, Larkum A, McComb AJ (1989) Productivity and nutrient limitation. In: Larkum A, W, D, McComb A, J, Shepherd S, A (eds) Biology of seagrasses: A treatise on the biology of seagrasses with special reference to the Australian region. Elsevier Science Publishers, Amsterdam, The Netherlands, pp 635-685

Hoffmann AA, Daborn PJ (2007) Towards genetic markers in animal populations as biomonitors for human-induced environmental change. Ecology Letters 10 (1):63-76

Invers O, Pérez M, Romero J (1999) Bicarbonate utilization in seagrass photosynthesis: role of carbonic anhydrase in *Posidonia oceanica* (L.) Delile and *Cymodocea nodosa* (Ucria) Ascherson. Journal of Experimental Marine Biology and Ecology 235 (1):125-133. doi:https://doi.org/10.1016/S0022-0981(98)00172-5

Invers O, Zimmerman RC, Alberte RS, Perez M, Romero J (2001) Inorganic carbon sources for seagrass photosynthesis: an experimental evaluation of bicarbonate use in species inhabiting temperate waters. Journal of Experimental Marine Biology and Ecology 265 (2):203-217

Jacobs SW, Les DH, Moody ML (2006) New combinations in Australasian *Zostera* (Zosteraceae). Telopea 11 (2):127-128

Jiang Z, Kumar M, Padula MP, Pernice M, Kahlke T, Kim M, Ralph PJ (2017) Development of an efficient protein extraction method compatible with LC-MS/MS for proteome mapping in two Australian seagrasses *Zostera muelleri* and *Posidonia australis*. Front Plant Sci 8:1416. doi:10.3389/fpls.2017.01416

Kemp WM, Boynton WR, Adolf JE, Boesch DF, Boicourt WC, Brush G, Cornwell JC, Fisher TR, Glibert PM, Hagy JD, Harding LW, Houde ED, Kimmel DG, Miller WD, Newell RIE, Roman MR, Smith EM, Stevenson JC (2005) Eutrophication of Chesapeake Bay: historical trends and ecological interactions. Marine Ecology Progress Series 303:1-29

Kemp WM, Twilley RR, Stevenson J, Boynton W, Means J (1983) The decline of submerged vascular plants in upper Chesapeake Bay: Summary of results concerning possible causes. Marine Technology Society Journal 17 (2):78-89

Kendrick G, Hegge B, Wyllie A, Davidson A, Lord D (2000) Changes in seagrass cover on Success and Parmelia Banks, Western Australia between 1965 and 1995. Estuarine, Coastal and Shelf Science 50 (3):341-353

Kendrick GA, Aylward MJ, Hegge BJ, Cambridge ML, Hillman K, Wyllie A, Lord DA (2002) Changes in seagrass coverage in Cockburn Sound, Western Australia between 1967 and 1999. Aquatic Botany 73 (1):75-87

Kennedy H, Beggins J, Duarte CM, Fourqurean JW, Holmer M, Marbà N, Middelburg JJ (2010) Seagrass sediments as a global carbon sink: Isotopic constraints. Global Biogeochemical Cycles 24 (4)

Kenworthy WJ, Fonseca MS (1996) Light requirements of seagrasses *Halodule wrightii* and *Syringodium filiforme* derived from the relationship between diffuse light attenuation and maximum depth distribution. Estuaries 19 (3):740-750

Kim M, Brodersen KE, Szabo M, Larkum AWD, Raven JA, Ralph PJ, Pernice M (2018) Low oxygen affects photophysiology and the level of expression of two-carbon metabolism genes in the seagrass *Zostera muelleri*. Photosynth Res 136 (2):147-160. doi:10.1007/s11120-017-0452-1

Kim M, Pernice M, Watson-Lazowski A, Guagliardo P, Kilburn MR, Larkum AWD, Raven JA, Ralph PJ (2019) Effect of reduced irradiance on ^{13}C uptake, gene expression and protein activity of the seagrass *Zostera muelleri*. Marine Environmental Research 149:80-89. doi:https://doi.org/10.1016/j.marenvres.2019.06.004

Koch M, Bowes G, Ross C, Zhang XH (2013) Climate change and ocean acidification effects on seagrasses and marine macroalgae. Global Change Biology 19 (1):103-132

Kumar M, Padula MP, Davey P, Pernice M, Jiang Z, Sablok G, Contreras-Porcia L, Ralph PJ (2017) Proteome analysis reveals extensive light stress-response reprogramming in the seagrass *Zostera muelleri* (Alismatales, Zosteraceae) metabolism. Frontiers in Plant Science 7 (2023). doi:10.3389/fpls.2016.02023

Kuo J (1984) Structural aspects of apoplast fungal hyphae in a marine angiosperm, *Zostera muelleri* Irmisch ex Aschers. (Zosteraceae). Protoplasma 121 (1):1-7. doi:10.1007/bf01279746

Kuo J, Aioi K, Iizumi H (1988) Comparative leaf structure and its functional significance in *Phyllospadix iwatensis* Makino and *Phyllospadix japonicus* Makino (Zosteraceae). Aquatic botany 30 (3):169-187

Kuo J, Den Hartog C (2007) Seagrass morphology, anatomy, and ultrastructure. In: Larkum A, W, D, Orth RJ, Duarte CM (eds) Seagrasses: Biology, Ecology and Conservation. Springer Netherlands, Dordrecht, The Netherlands, pp 51-87

Kuo J, Ridge RW, Lewis SV (1990) The leaf internal morphology and ultrastructure of *Zostera muelleri* Irmisch ex Aschers.(Zosteraceae): a comparative study of the intertidal and subtidal forms. Aquatic botany 36 (3):217-236

Larkum A, den Hartog C (1989) Evolution and biogeography of seagrasses. In: Larkum AWD, McComb AJ, Shepherd SA (eds) Biology of Seagrasses. 1 edn. Elservier Pub Co, Amsterdam, pp 112-156

Larkum AW, Drew EA, Ralph PJ (2006) Photosynthesis and metabolism in seagrasses at the cellular level. Seagrasses: biology, ecology and conservation. Springer, New York

Larkum AWD, Comb AJM, Shepherd SA (1989) Biology of seagrasses: a treatise on the biology of seagrasses with special reference to the Australian region. Elsevier Science Limited, Amsterdam, The Netherlands

Larkum AWD, Davey PA, Kuo J, Ralph PJ, Raven JA (2017) Carbon-concentrating mechanisms in seagrasses. Journal of Experimental Botany 68 (14):3773-3784. doi:10.1093/jxb/erx206

Les DH, Cleland MA, Waycott M (1997) Phylogenetic Studies in Alismatidae, II: Evolution of Marine Angiosperms (Seagrasses) and Hydrophily. Systematic Botany 22 (3):443-463. doi:10.2307/2419820

Leuschner C, Landwehr S, Mehlig U (1998) Limitation of carbon assimilation of intertidal *Zostera noltii* and *Z. marina* by desiccation at low tide. Aquatic Botany 62 (3):171-176

Livingston RJ Historic trends of human impacts on seagrass meadows in Florida. In: Proceedings of the symposium on subtropical-tropical Seagrasses of the Southeastern United States. Florida Marine Research Publications, 1987. vol 42. pp 139-151

Macreadie PI, Allen K, Kelaher BP, Ralph PJ, Skilbeck CG (2012) Paleoreconstruction of estuarine sediments reveal human-induced weakening of coastal carbon sinks. Global Change Biology 18 (3):891-901. doi:10.1111/j.1365-2486.2011.02582.x

Macreadie PI, Schliep MT, Rasheed MA, Chartrand KM, Ralph PJ (2014) Molecular indicators of chronic seagrass stress: A new era in the management of seagrass ecosystems? Ecological Indicators 38:279-281. doi:https://doi.org/10.1016/j.ecolind.2013.11.017

Manca E, Cáceres I, Alsina JM, Stratigaki V, Townend I, Amos CL (2012) Wave energy and wave-induced flow reduction by full-scale model seagrass. Continental Shelf Research 50–51:100-116

Marbà N, Arias-Ortiz A, Masqué P, Kendrick GA, Mazarrasa I, Bastyan GR, Garcia-Orellana J, Duarte CM (2015) Impact of seagrass loss and subsequent revegetation on carbon sequestration and stocks. Journal of Ecology 103 (2):296-302

Mazzuca S, Björk M, Beer S, Felisberto P, Gobert S, Procaccini G, Runcie J, Silva J, Borges AV, Brunet C, Buapet P, Champenois W, Costa MM, D'Esposito D, Gullström M, Lejeune P, Lepoint G, Olivé I, Rasmusson LM, Richir J, Ruocco M, Serra IA, Spadafora A, Santos R (2013) Establishing research strategies, methodologies and technologies to link genomics and proteomics to seagrass productivity, community metabolism, and ecosystem carbon fluxes. Frontiers in Plant Science 4:38

Mazzuca S, Spadafora A, Filadoro D, Vannini C, Marsoni M, Cozza R, Bracale M, Pangaro T, Innocenti AM (2009) Seagrass light acclimation: 2-DE protein analysis in *Posidonia* leaves grown in chronic low light conditions. Journal of Experimental Marine Biology and Ecology 374 (2):113-122

McRoy CP, Helfferich C (1977) Seagrass ecosystems: a scientific perspective. M. Dekker,

Michael TP, Jackson S (2013) The First 50 Plant Genomes. The Plant Genome 6 (2)

Millhouse J, Strother S (1986) Salt-stimulated bicarbonate-dependent photosynthesis in the marine angiosperm *Zostera muelleri*. Journal of Experimental Botany 37 (7):965-976

Mizerek T, Regan HM, Hovel KA (2011) Seagrass habitat loss and fragmentation influence management strategies for a blue crab *Callinectes sapidus* fishery. Marine Ecology Progress Series 427:247-257

Norici A, Dalsass A, Giordano M (2002) Role of phosphoenolpyruvate carboxylase in anaplerosis in the green microalga *Dunaliella salina* cultured under different nitrogen regimes. Physiologia Plantarum 116 (2):186-191

Olesen B, Sand-Jensen K (1993) Seasonal acclimatization of eelgrass *Zostera marina* growth to light. Marine Ecology Progress Series 94 (9)

Olsen JL, Rouzé P, Verhelst B, Lin Y-C, Bayer T, Collen J, Dattolo E, De Paoli E, Dittami S, Maumus F (2016) The genome of the seagrass *Zostera marina* reveals angiosperm adaptation to the sea. Nature 530 (7590):331-335

Orth RJ, Carruthers TJB, Dennison WC, Duarte CM, Fourqurean JW, Heck Jr KL, Hughes AR, Olyarnik S, Williams SL, Kendrick GA, Kenworthy WJ, Short FT, Waycott M (2006) A global crisis for seagrass ecosystems. BioScience 56 (12):987-996

Orth RJ, Moore KA (1983) Chesapeake Bay: An unprecedented decline in submerged aquatic vegetation. Science 22:51-52

Papenbrock J (2012) Highlights in seagrasses' phylogeny, physiology, and metabolism: what makes them special? ISRN Botany 2012. doi:10.5402/2012/103892

Philippart CJM, Dikkema KS, Van Der Meer J (1992) Wadden Sea Seagrasses: Where and Why? Netherlands Institute of Sea Research Publication 20:177-191

Phillips RC, McRoy CP (1980) Handbook of seagrass biology: an ecosystem perspective. Garland STPM Press,

Piazzi L, Cinelli F (2003) Evaluation of benthic macroalgal invasion in a harbour area of the western Mediterranean Sea. European Journal of Phycology 38 (3):223-231

Pierrot D, Lewis E, Wallace D (2006) MS Excel program developed for CO_2 system calculations: ORNL/CDIAC-105a. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, US Department of Energy, Oak Ridge, Tennessee

Procaccini G, Beer S, Björk M, Olsen J, Mazzuca S, Santos R (2012) Seagrass ecophysiology meets ecological genomics: are we ready? Marine Ecology 33 (4):522-527

Procaccini G, Olsen JL, Reusch TBH (2007) Contribution of genetics and genomics to seagrass biology and conservation. Journal of Experimental Marine Biology and Ecology 350 (1–2):234-259

Quammen ML, Onuf CP (1993) Laguna Madre: seagrass changes continue decades after salinity reduction. Estuaries 16 (2):302-310

Rae BD, Long BM, Förster B, Nguyen ND, Velanis CN, Atkinson N, Hee WY, Mukherjee B, Price GD, McCormick AJ (2017) Progress and challenges of engineering a biophysical CO_2-concentrating mechanism into higher plants. Journal of Experimental Botany 68 (14):3717-3737. doi:10.1093/jxb/erx133

Ralph P, Polk S, Moore K, Orth R, Smith W (2002) Operation of the xanthophyll cycle in the seagrass *Zostera marina* in response to variable irradiance. Journal of Experimental Marine Biology and Ecology 271 (2):189-207

Raven JA (1984) Energetics and transport in aquatic plants. A.R. Liss, New York, USA

Raven JA, Beardall J, Giordano M (2014) Energy costs of carbon dioxide concentrating mechanisms in aquatic organisms. Photosynthesis Research 121 (2):111-124. doi:10.1007/s11120-013-9962-7

Rawsthorne S (1992) C_3–C_4 intermediate photosynthesis: linking physiology to gene expression. The Plant Journal 2 (3):267-274

Ray BR, Johnson MW, Cammarata K, Smee DL (2014) Changes in seagrass species composition in northwestern Gulf of Mexico estuaries: Effects on associated seagrass fauna. PLoS ONE 9 (9):e107751

Reise K, Jager Z, Jong D, Katwijk M, Schanz A (2005) Seagrass. In: Essink K (ed) Wadden sea quality status report 2004. Wadden Sea ecosystem 19. Common Wadden Sea Secretariat, Wilhelmshaven,

Rubio L, Garcia D, Garcia-Sanchez MJ, Niell FX, Felle HH, Fernandez JA (2017) Direct uptake of HCO_3^- in the marine angiosperm *Posidonia oceanica* (L.) Delile driven by a plasma membrane H^+ economy. Plant Cell Environ 40 (11):2820-2830. doi:10.1111/pce.13057

Sablok G, Hayward RJ, Davey PA, Santos RP, Schliep M, Larkum A, Pernice M, Dolferus R, Ralph PJ (2018) SeagrassDB: An open-source transcriptomics landscape for phylogenetically profiled seagrasses and aquatic plants. Scientific Reports 8 (1):2749. doi:10.1038/s41598-017-18782-0

Salo T, Reusch TBH, Boström C (2015) Genotype-specific responses to light stress in eelgrass *Zostera marina*, a marine foundation plant. Marine Ecology Progress Series 519:129-140

Sharon Y, Levitan O, Spungin D, Berman-Frank I, Beer S (2011) Photoacclimation of the seagrass *Halophila stipulacea* to the dim irradiance at its 48-meter depth limit. Limnology and Oceanography 56 (1):357-362. doi:10.4319/lo.2011.56.1.0357

Shomer-Ilan A, Beer S, Waisel Y (1975) *Suaeda monoica*, a C_4 Plant without Typical Bundle Sheaths. Plant Physiology 56 (5):676. doi:10.1104/pp.56.5.676

Short F, Carruthers T, Dennison W, Waycott M (2007) Global seagrass distribution and diversity: a bioregional model. Journal of Experimental Marine Biology and Ecology 350 (1-2):3-20. doi:10.1016/j.jembe.2007.06.012

Short FT, Jones GE, Burdick DM Seagrass decline: problems and solutions. In: Coastal Wetlands, 1991. ASCE, pp 439-453

Short FT, Polidoro B, Livingstone SR, Carpenter KE, Bandeira S, Bujang JS, Calumpong HP, Carruthers TJB, Coles RG, Dennison WC, Erftemeijer PLA, Fortes MD, Freeman AS, Jagtap TG, Kamal AHM, Kendrick GA, Judson Kenworthy W, La Nafie YA, Nasution IM, Orth RJ, Prathep A, Sanciangco JC, Tussenbroek Bv, Vergara SG, Waycott M, Zieman JC (2011) Extinction risk assessment of the world's seagrass species. Biological Conservation 144 (7):1961-1971. doi:http://dx.doi.org/10.1016/j.biocon.2011.04.010

Short FT, Wyllie-Echeverria S (1996) Natural and human-induced disturbance of seagrasses. Environmental Conservation 23 (01):17-27

Stumm W, Morgan JJ (2012) Aquatic chemistry: chemical equilibria and rates in natural waters. 3 edn. John Wiley & Sons, Inc, Canada

Susan Z, Ronald B (1999) Nutrient cycling in the water column of a subtropical seagrass meadow. Marine Ecology Progress Series 188:51-62

Taiz L, Zeiger E (2003) Plant physiology. Annals of Botany

Tomasko DA, Corbett CA, Greening HS, Raulerson GE (2005) Spatial and temporal variation in seagrass coverage in Southwest Florida: assessing the relative effects of anthropogenic nutrient load reductions and rainfall in four contiguous estuaries. Marine Pollution Bulletin 50 (8):797-805

Touchette BW, Burkholder JM (2000) Overview of the physiological ecology of carbon metabolism in seagrasses. Journal of Experimental Marine Biology and Ecology 250 (1):169-205

Trocine RP, Rice JD, Wells GN (1981) Inhibition of seagrass photosynthesis by ultraviolet-B radiation. Plant physiology 68 (1):74-81

Uku J, Beer S, Björk M (2005) Buffer sensitivity of photosynthetic carbon utilisation in eight tropical seagrasses. Marine Biology 147 (5):1085. doi:10.1007/s00227-005-0019-0

Valiela I, Bowen JL, York JK (2001) Mangrove forests: One of the world’s threatened major tropical environments. BioScience 51:807-815

Voznesenskaya EV, Franceschi VR, Kiirats O, Freitag H, Edwards GE (2001) Kranz anatomy is not essential for terrestrial C_4 plant photosynthesis. Nature 414 (6863):543-546

Wales R, Newman BJ, Rose SA, Pappin D, Gray JC (1989) Characterization of cDNA clones encoding the extrinsic 23 kDa polypeptide of the oxygen-evolving complex of photosystem II in pea. Plant molecular biology 13 (5):573-582

Walker D, Lukatelich R, Bastyan G, McComb A (1989) Effect of boat moorings on seagrass beds near Perth, Western Australia. Aquatic Botany 36 (1):69-77

Waycott M, Duarte CM, Carruthers TJB, Orth RJ, Dennison WC, Olyarnik S, Calladine A, Fourqurean JW, Heck KL, Hughes AR, Kendrick GA, Kenworthy WJ, Short FT, Williams SL (2009) Accelerating loss of seagrasses across the globe threatens coastal ecosystems. Proceedings of the National Academy of Sciences of the United States of America 106 (30):12377-12381

Widdows J, Pope ND, Brinsley MD, Asmus H, Asmus RM (2008) Effects of seagrass beds (*Zostera noltii* and *Zostera marina*) on near-bed hydrodynamics and sediment resuspension. Marine Ecology Progress Series 358:125-136

Williams SL (2007) Introduced species in seagrass ecosystems: Status and concerns. Journal of Experimental Marine Biology and Ecology 350 (1–2):89-110

Wissler L, Dattolo E, Moore AD, Reusch TBH, Olsen JL, Migliaccio M, Bornberg-Bauer E, Procaccini G (2009) Dr. Zompo: an online data repository for *Zostera marina* and *Posidonia oceanica* ESTs. Database 2009

Wonham MJ, O’Connor M, Harley CD (2005) Positive effects of a dominant invader on introduced and native mudflat species. Marine Ecology Progress Series 289:109-116

Zieman JC (1976) The ecological effects of physical damage from motor boats on turtle grass beds in southern Florida. Aquatic Botany 2:127-139

Zimmerman RC, Kohrs DG, Steller DL, Alberte RS (1997) Impacts of CO_2 enrichment on productivity and light requirements of eelgrass. Plant Physiology 115 (2):599-607

Chapter 2: Low oxygen affects photophysiology and the level of expression of two carbon metabolism genes in the seagrass *Zostera muelleri*.

Mikael Kim[1], Kasper Elgetti Brodersen[1], Milan Szabo[1], Anthony W. D. Larkum[1], John A. Raven[1,2], Peter J. Ralph[1] and Mathieu Pernice[1].

AFFILIATIONS

[1]Climate Change Cluster, University of Technology Sydney, New South Wales 2007, Australia.

[2]Division of Plant Science, University of Dundee at the James Hutton Institute, Invergowrie, Dundee DD2 5DA, UK

CORRESPONDING AUTHOR:

Mathieu Pernice

[1]Climate Change Cluster, University of Technology Sydney, Sydney, NSW 2007, Australia.

Email: mathieu.pernice@uts.edu.au

Phone: +61295144162

Fax: +61295144079

RUNNING TITLE: LOW O_2 EFFECTS ON *ZOSTERA MUELLERI* PHOTOBIOLOGY

KEYWORDS: Diffusive boundary layer; Photosynthesis; Respiration; RT-qPCR; Seagrass

ACKOWLEDGMENTS

The authors would like to thank UTS and C3 for strategic research support as well as Dr Audrey Commault, Dr Sutinee Sinutok and Paul Brooks for technical assistance. We also like to thank the anonymous reviewers for their comments which contributed in improving the quality of this article. The University of Dundee is a registered Scottish charity, No. 015096.

Compliance with Ethical Standards:

Funding: This study was funded by an Australian Research Council Linkage Grant (LP11020045), Climate Change Cluster Honours Scholarship, University of Technology Sydney (MK) and the Augustinus Foundation (KEB).

Conflicts of Interest: The authors declare that they have no conflict of interest.

Ethical approval: All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

This article was published on the 4th of October, 2017 in the journal ‘Photosynthesis Research’, volume: 136, issue 2, page: 147-160

Contributions

MK, KEB, MS, AWDL, JAR, PJR and MP designed the experiment; MK and MP carried out the experiment; MK, KEB and MS performed the microsensor analysis; MK and MP performed the RTqPCR analysis; MK wrote the manuscript with contributions from all authors.

Abstract

Seagrasses are a diverse group of angiosperms that evolved to live in shallow coastal waters, an environment regularly subjected to changes in oxygen, carbon dioxide and irradiance. *Zostera muelleri* is the dominant species in south-eastern Australia, and is critical for healthy coastal ecosystems. Despite its ecological importance, little is known about the pathways of carbon fixation in *Z. muelleri* and their regulation in response to environmental changes. In this study, the response of *Z. muelleri* exposed to control and very low oxygen conditions was investigated by using (i) oxygen microsensors combined with a custom-made flow chamber to measure changes in photosynthesis and respiration, and (ii) Reverse Transcription quantitative real-time PCR (RT-qPCR) to measure changes in expression levels of key genes involved in C_4 metabolism. We found that very low levels of oxygen (i) altered the photophysiology of *Z. muelleri*, a characteristic of C_3 mechanism of carbon assimilation, and (ii) decreased the expression levels of phosphoenolpyruvate carboxylase (PEPC) and carbonic anhydrase (CA). These molecular-physiological results suggest that regulation of the photophysiology of *Z. muelleri* might involve a close integration between the C_3 and C_4, or other CO_2 concentrating mechanisms metabolic pathways. Overall, this study highlights that the photophysiological response of *Z. muelleri* to changing oxygen in water is capable of rapid acclimation and the dynamic modulation of pathways should be considered when assessing seagrass primary production.

Introduction

Seagrasses are a diverse group of monocotyledonous angiosperms that evolved to live in the marine environment during the Cretaceous period, approximately 100 million years ago (Larkum and den Hartog 1989). There are approximately 72 seagrass species in 12 genera worldwide (Short et al. 2011) playing an important role in coastal ecosystems (Costanza et al. 1997). Indeed, highly productive seagrass ecosystems provide food and shelter for commercially important fish (Beck et al. 2001) with temperate seagrass meadows in southern Australia estimated to supply onshore fisheries valued at $A 230 000 ha^{-1} y^{-1} (Blandon and Zu Ermgassen 2014), and enhance sediment accretion (Koch et al. 2013). Seagrasses have also recently been identified as a major carbon sink, responsible for 10-18% of the Ocean's carbon accumulation (McLeod et al. 2011; Fourqurean et al. 2012; Greiner et al. 2013).

Many seagrasses are intertidal species which grow in shallow coastal lagoons and are therefore exposed to large variations in light and sediment loading/resuspension (Harlin 1995). Additionally, seagrasses are exposed to large fluctuations in oxygen levels, ranging from 71 to 311 µmol L^{-1} under normal conditions (Brodersen et al. 2017) and as low as 10% (approx. 20 µmol L^{-1}) air saturation during night time in areas where seagrass die-offs were observed (Borum et al. 2005). They also have anatomical adaptations such as the absence of stomata and the development of extensive aerenchyma (Penhale and Wetzel 1983) along with physiological adaptations such as the ability to tolerate hypoxic and anoxic conditions especially in the roots and rhizomes (Pregnall et al. 1984; Papenbrock 2012) which they possibly inherited from submerged freshwater ancestors (Les et al. 1997). As seagrass persistence generally require a large flux of photosynthetically active radiation, roughly 10% of surface irradiance (Papenbrock 2012), the effects of light on seagrass ecology have been extensively studied (Ralph et al. 2007; Staehr and Borum 2011; Brodersen et al. 2015; Chartrand et al. 2016). However, less attention has been given to the effects of photosynthetic gases and associated metabolic pathways in seagrasses, e.g., the effects of low O_2 conditions have only been reported in a few papers to our knowledge (e.g. Black et al. 1976; Downton et al. 1976; Beer et al. 2002; Greve et al. 2003; Buapet et al. 2013).

The photosynthetic processes of seagrasses are very similar to that of other angiosperms (Beer et al. 1998). Most seagrasses were classified biochemically as C_3 plants on the biochemical criteria of short-term inorganic ^{14}C incorporation products and the ratio of Ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) to phosphoenolpyruvate carboxylase (PEPC) activities, although *Thalassia testudinum* has C_4 metabolism (Benedict and Scott 1976) and *Halophila stipulacea* has C_3-C_4 intermediate metabolism by these criteria (Beer et al. 1980; Beer et al. 2002; Koch et al. 2013). The C_3 CO_2 fixation process begins with RuBisCO which catalyses the carboxylation using CO_2 (and H_2O) of ribulose 1, 5-bisphosphate (RuBP), producing two of the 3 carbon molecules; 3-phosphoglycerate (PGA) as the initial stable product. In parallel there is the oxygenation of RuBP with O_2 to yield one PGA and one 2-phosphoglycolate. The ratio of carboxylase to oxygenase activity is determined by the kinetic properties of the molecular form of RuBisCO involved and the CO_2:O_2 ratio at the active site of RuBisCO.

The C_4 carbon fixation process on the other hand, begins with the carboxylation (using HCO_3^- as the immediate inorganic C substrate) of phosphoenolpyruvate (PEP) in the RuBisCO-free cytosol, forming the 4-carbon acid oxaloacetate as the initial stable product. Oxaloacetate is subsequently converted to malate and/or aspartate which are moved to, and decarboxylated in, the compartment containing RuBisCO, generating CO_2 which is used in the carboxylation of RuBisCO, and a 3-carbon acid that returns to the cytosol, regenerating PEP. C_4 photosynthesis involves a higher steady-state CO_2 concentration than that available to RuBisCO in biochemically and physiologically defined C_3 photosynthesis with diffusive entry of CO_2 from the bulk external medium to RuBisCO, i.e., C_4 photosynthesis acts as a CO_2 concentrating mechanism (CCM). The regeneration of PEP to allow further PEPC activity is an energy (as ATP) requiring process. The C_4 mechanism has an advantage over the C_3 mechanism in low CO_2 and/or high O_2 environments as PEPC reacts specifically with CO_2

(after its conversion to HCO_3^-) with no interference from O_2 and with accumulation of CO_2 around RuBisCO that largely suppresses RuBisCO oxygenase activity. In C_3 plants, to a much smaller extent, the 2-phosphoglycolate from the oxygenase reaction is metabolised to PGA and then sugar phosphates in energy-requiring photorespiratory carbon oxidation cycle (PCOC); whether the oxygenase-PCOC combination physiologically decreases the energetic efficiency of C_3 plants relative to C_4 plants depends on the energy cost of oxygenase-PCOC relative to the cost of operating the C_4 pathway with its inevitable leakage of CO_2 from the pool accumulated around RuBisCO (Raven 2014; Raven and Beardall 2016; Larkum et al. 2017). It is important to note that the occurrence of any CCM gives physiological (not biochemical) characteristics similar to those of a C_4 plant (Raven 2014; Raven and Beardall 2016, and references therein; Larkum et al. 2017). Thus, photosynthesis that is relatively insensitive to O_2, has a high affinity for CO_2 and can deplete the CO_2 in a closed system to very low concentrations and is not diagnostic of C_4 photosynthesis since it could also result from any CCM activity.

In marine plants, inorganic carbon species diffusion to the leaf surface has a greater potential to restrict the rate of photosynthesis due to the slower diffusion rates in water. Further, the primary form of dissolved inorganic carbon in seawater is HCO_3^- (90%) with CO_2 making up about 1% (see below). Hence a CCM would be advantageous for marine plants, for example, physiologically “C_4-like” inorganic carbon acquisition has been observed in the seagrass *Zostera noltii* based on high light saturation values and a lack of observable photorespiration (Raven 1984; Jiménez et al. 1987); however, as noted by Raven (1984), this is not diagnostic of C_4 rather than some other CCMs. By contrast Waghmode and Joshi (1983), using *Halophila beccarii* (as *H. beccaeii*), showed short-term inorganic ^{14}C labelling of aspartate, and also alanine, i.e. features of C_4 photosynthesis, although critics could say that the labelling period was not short enough to show the real initial product of inorganic C assimilation. Despite this, short-term inorganic ^{14}C labelling is the most conclusive methods to indicate C_4 photosynthesis in seagrasses. The high ratio of PGA phosphatase to 2-phosphoglycolate phosphatase activity in *H. beccarii* (Waghmode and Joshi 1983) is consistent with a decreased RuBisCO oxygenase generating 2-phosphoglycolate, and hence decreased flux through the PCOC producing glycine and serine, and a requirement for the non-phosphorylated pathway from photosynthetic or glycolytic PGA to glycerate and hence to serine and glycine. However, the decreased RuBisCO oxygenase and concomitant requirement for the PGA to glycerate pathway to serine and glycine could occur in any organism with a CCM. Colman and Norman (1997) showed the occurrence of a phosphorylated pathway, not involving PGA phosphatase, from PGA to serine in cyanobacteria with CCMs and minimal 2-phosphoglycolate synthesis. The occurrence of PEPC and aspartate aminotransferase in *H. beccarii* does not signify a C_4 pathway, since these enzymes are ubiquitous in plants (Aubry et al. 2011). HCO_3^- is the predominant inorganic C species in seawater (at pH 8.16: 85.8% HCO_3^-, 0.4% CO_2 and 13.7% CO_3^{2-}, Pierrot et al. 2006) with CO_2 at about the same concentration (mol $m^{-3}$of fluid medium) as in air. However, the diffusion coefficient for CO_2 in water is about 10^{-4} that in air (Raven 1984) so, despite the usually thinner diffusion boundary layer in water (~0.01-0.1 mm) than in air (~1 mm) under ecologically relevant conditions (Vogel 1994), CO_2 diffusion to the leaf surface, and O_2 diffusion from the leaf surface (Mass et al. 2010) may limit photosynthesis in marine plants more than in land plants. Although the diffusion coefficient for HCO_3^- is lower than that of CO_2 (Raven 1984), the quantitative predominance of HCO_3^- in seawater means that it can support a larger flux to the leaf surface in response to a given potential for CO_2 assimilation in the leaf, provided the leaf can use HCO_3^-. Some seagrasses can utilise HCO_3^- either directly via active transport into epidermal cells (Beer and Rehnberg 1997), or more commonly, indirectly by dehydrating HCO_3^- to CO_2 via the enzyme carbonic anhydrase (CA) in the epidermal cell wall (Beer et al. 1980) usually interacting with co-localised leaf surface acidification by energy-requiring H^+ efflux (Hellblom et al. 2001; Hellblom and Axelsson 2003; Borum et al. 2016). Such indirect methods for enhancing inorganic C (Ci) uptake make predictions of C_4 mechanisms in seagrasses doubtful unless supported by strong evidence.

The suggestion that C4 photosynthesis does not occur in seagrasses because of the absence of bundle sheath cells containing chloroplasts and the lack of true Kranz anatomy has subsequently been shown to be invalid (Beer et al. 1980). Some freshwater submerged aquatic plants and some terrestrial C_4 members of the Chenopodiaceae utilise C_4 photosynthesis via the fixation of external inorganic C by PEPC, and the fixation of CO_2 (regenerated from C_4 acid decarboxylation) by RuBisCO, the carboxylases occurring in the cytosol and the chloroplasts respectively of a single cell (Voznesenskaya et al. 2001; references in Raven and Beardall 2016).

With recent advances in genomics and transcriptomics, researchers have the capacity to examine molecular mechanisms which drive seagrass photosynthesis to an extent that was unimaginable just a decade ago. In this context, the presence of genes encoding enzymes characteristic of the C_4 carbon fixation pathway in seagrass transcriptome could provide evidence relevant to the argument that seagrasses are not strictly C_3 plants.

For instance, several genes coding for PEPC, a cytosolic enzyme essential for the C_4 carbon fixation pathway in higher plants (Chollet et al. 1996), have been identified in the transcriptome of *Zostera muelleri* (unpublished data). This enzyme catalyses the irreversible β-carboxylation of phosphoenolpyruvate (PEP) by HCO_3^- to produce oxaloacetate (as described previously), a key intermediate in the C_4 carbon fixation pathway. However, PEPC has a ubiquitous anaplerotic role in plants and algae (excluding dinoflagellates where PEPC is replaced by pyruvate carboxylase) in replenishing the intermediates of the Krebs cycle depleted by the use of oxaloacetate and 2-oxoglutarate in the synthesis of some amino acids and of pyrimidines haems and chlorins (Raven 1984; Raven and Farquhar 1990; Aubry et al. 2011; Raven 2014). Additional PEPC expression is needed in the roots of seagrasses growing on carbonate substrata in the production of organic acids that release phosphate from apatite in the carbonate sediment (Long et al. 2008; Raven 2014). Chi et al. (2014) show that there is at least 1 copy of each of 8 genes related to C_4 photosynthesis and also to other aspects of metabolism in the 4 completely sequenced tracheophytes (2 with C_3 photosynthesis, 2 with C_4 photosynthesis) and 1 completely sequenced C_3 photosynthesis bryophyte. For PEPC, the 2 C_4 plants had 3 or 4 copies of the gene, while the 2 C_3 plants have 4 or 6 copies.

Therefore, the presence of the PEPC gene in the transcriptome of *Zostera muelleri* (unpublished data) does not show that *Z. muelleri* is other than, biochemically, a C_3 plant.

Furthermore, genes encoding CA were also detected in the *Z. muelleri* transcriptome (unpublished data). CA catalyses the reversible interconversion of HCO_3^- to CO_2 ($HCO_3^- + H^+ = CO_2 + H_2O$). CAs are also involved in several non-photosynthetic reactions in plants (Raven 2014 and references therein), possibly including provision of respiratory CO_2 to HCO_3^- for the PEPC activity (Raven 2014) required for synthesis of the organic acids used, after secretion, in phosphate release from carbonate substrata (Long et al. 2008; Raven 2014) in seagrass roots. One or more CAs are components of C_4-based and other CCMs, as well as in C_3 photosynthesis (Aubry et al. 2011; Raven 2014; Raven and Beardall 2016).

However, expression of C_4 photosynthetic metabolism in some submerged freshwater relatives of seagrasses is a function of environmental conditions (low CO_2, high O_2), unlike terrestrial C_4 plants where it is constitutive (references in Raven and Beardall 2016). Therefore it is possible that the expression of some PEPC and CA genes in seagrasses varies with the O_2 concentration and hence the potential for RuBisCO oxygenase activity, noting that the other light dependent O_2 consuming reactions, i.e. the water-water (or oxygen-oxygen) cycles of the Mehler Peroxidase reaction and of the oxidation by the plastid terminal oxidase of plastoquinone reduced by PSII, are minimal in the only seagrass (*Zostera marina*) investigated: Buapet and Björk (2016).

The aim of this molecular-physiological study was to address the following: (i) how the photosynthetic and respiratory rates are affected by experimentally reduced O_2 concentration in the water column using electrochemical microsensors and (ii) how this reduced O_2 concentration affects the expression levels of PEPC and CA using Reverse Transcription quantitative real-time PCR (RT-qPCR).

Material and methods

Seagrass collection and experimental setup

Specimens of *Zostera muelleri* ssp. *capricorni* (Asch) S. W. L. Jacobs and attached marine sediment were collected from Pittwater, NSW, Australia (33° 38′ 45.6″S, 151° 17′ 12.8″E) on the 14th of May 2015. In order to mimic the conditions of Pittwater at the University of Technology Sydney (UTS) aquarium facility, salinity and temperature of the water were measured in the field; ambient salinity: 31 and water temperature: 22°C, along with rapid light curves of *Z. muelleri* to determine suitable light conditions. Rapid light curves measured in the field on 3 *Z. muelleri* plants using a Diving-Pulse Amplitude Modulated (PAM) fluorimeter (DIVING-PAM, Heinz Walz GmbH, Eichenring, Germany) indicated that photosynthetic saturating light was approximately 230 µmol photons m^{-2} s^{-1}, which is consistent with saturating light levels previously found for this seagrass species in temperate regions (Schwarz 2004; Bulmer et al. 2016). Collection was performed at low tide in shallow water (~1 m) and plants were transported immediately to an aquarium facility at the University of Technology Sydney. Before further handling, the specimens and sediment were placed into aquaria for 48 hours, after which they were separated into individual ramets/shoots (see Procaccini et al. 2007). These samples were then acclimated for 2 months in 40 L glass aquaria to conditions mirroring that of the sampling site e.g.: a salinity of 31, temperature of 22±1°C and illumination with an incident photon irradiance of ~230 µmol photons m^{-2} s^{-1} (12 h : 12 h light : dark cycle). Sixteen individual shoots of *Z. muelleri* were then transplanted in pairs into 8 plastic tanks (~150 mm in diameter, 4 tank replicates per treatment) with ~30 mm of sediment and acclimated at the same environmental conditions as above for 2 weeks.

The experiment was initiated in the middle of the photoperiod. Each of the treatments (i.e. control and low O_2) had one 100 L sump (100 L plastic bin) underneath a 40 L aquaria/table that held experimental plastic containers (Supplementary Figure S1. 1). From each sump, water with corresponding O_2 level was pumped into four replicate plastic containers resulting in a total of 8 containers (4 replicate containers x 2 sumps/O_2 treatments = 8 containers in total). Each container contained 2 individual shoots of *Z. muelleri* (8 tanks x 2 shoots = 16 shoots). For the low O_2 treatment, the dissolved O_2 was lowered in the sump over an interval of 1 h via flushing with nitrogen gas (CO_2-free) to an average O_2 concentration of ~9 µmol O_2 L^{-1} as measured by a calibrated dissolved O_2 probe (FDO 925, WTW GmbH, Germany). The dissolved O_2 concentration within the aquaria was maintained between 9 and 16 µmol O_2 L^{-1} for the duration of the experimental period of 24 h (Supplementary Figure S1. 2). Deviations from the initial pH of 8.16 (+/-0.01 pH) was controlled via bubbling of 99.9% pure CO_2, which was automatically controlled by a calibrated pH/CO_2 controller (7074/2, TUNZE Aquarientechnik GmbH, Germany, Supplementary Figure S1. 3). The control tank set up was the same except for bubbling air instead of N_2 and CO_2.

Oxygen measurement setup

The lower half of leaf 2 (~20 mm) were cut from 3 individual ramets/shoots (see Procaccini et al. 2007) randomly picked from a pool of untreated samples which were previously subjected to the same acclimation procedure. The leaf sections were cleaned of any epiphytes and then fixed in place with fine pins on a piece of styrofoam in a custom-made flow-chamber (see Brodersen et al. 2014) . The sections were angled in such a way as to allow for unobstructed flow over each of the sampling areas of the leaves. Illumination of the leaves to the desired light levels was achieved via a fibre-optic tungsten halogen lamp (KL-2500LCD, Schott GmbH, Germany) with the irradiance measured at the leaf surface using a 4π quantum sensor (US-SQS/L, Walz GmbH, Germany) connected to a calibrated light meter (LI-250A, LI-COR Inc., USA). Seawater was pumped through the flow chamber at a constant rate of ~5 mm s^{-1} for the duration of the experiment. Atmospheric air was bubbled during the control phase of the experiment, while nitrogen gas was bubbled during the treatment phase of the experiment, lowering the O_2 concentration from ~231 µmol O_2 L^{-1} to ~9 µmol O_2 L^{-1} (as described above). Salinity, temperature and pH of the seawater were kept constant throughout the experiment.

Vertical O_2 concentration micro-profiles towards the leaf tissue surface (approx. 0.031 cm^2) and thus across the diffusive boundary layer (DBL) were recorded using a Clark-type O_2 microsensor (OX-50, tip diameter approx. 50 µm; Unisense A/S Aarhus, Denmark; Revsbech, 1989) with a fast response time (<0.5 s) mounted on a motorized micromanipulator (Unisense A/S, Aarhus, Denmark). The microsensor was connected to a multimeter (Unisense Microsensor Multimeter A/S. Aarhus, Denmark) and interfaced with a PC running dedicated data acquisition and positioning software (SensorTrace PRO; Unisense A/S, Aarhus, Denmark). The microsensor was positioned at the leaf tissue surface (defined as 0 µm) manually by observing the microsensor tip and leaf tissue surface through a stereo-microscope mounted on an articulating arm. Subsequent measurements of vertical O_2 concentrations for micro-profiles were measured at 100 µm increments using the motorised micromanipulator (Unisense Motorised Micromanipulator A/S, Aarhus, Denmark) controlled by dedicated positioning software (SensorTrace PRO). Linear calibration of the O_2 microsensor was obtained from signal readings in 100% air-saturated seawater and anoxic seawater (seawater amended via N_2 bubbling and the O_2 scavenger sodium dithionite) at experimental salinity, pH and temperature.

Seagrass maximum quantum efficiency of photosystem II (Fv/Fm; Baker 2008) values were measured regularly on 3 biological replicates using a Pulse Amplitude Modulated (PAM) fluorimeter (Pocket PAM, Gademann Instruments, Wuerzburg, Germany, see Figueroa et al. 2013) after dark-adaptation for ~10 min. Minimal fluorescence (F_0) was recorded using a weak measuring light, which was then followed by a saturating pulse (irradiance of 3,500 µmol photons m^{-2} s^{-1} for 0.8 s) to measure maximal fluorescence (Fm). Under these conditions, Fv/Fm ratios provides a measure of maximal PSII photochemical efficiency ($Fv=Fm-F_0$) and were, in this experiment, used as an indicator of seagrass maximum quantum efficiency of PSII during experimentation.

Photosynthesis-Irradiance (P-I) curves

Established methods for determining rates of photosynthesis in marine plants via O_2 microsensors were used in this study (see Jørgensen and Revsbech 1985; Kühl et al. 1995; Lichtenberg and Kühl 2015; Pedersen et al. 2016). The effective DBL thickness was estimated by extrapolating the linear O_2 concentration gradient until it intersects with the constant O_2 concentration in the overlaying water. These O_2 micro-profiles determined at the leaf tissue surface in the flow chamber were measured at incident photon irradiances of 0, 25, 50, 100, 200, 500 and 700 µmol photons m^{-2} s^{-1}, first under control conditions (aerated seawater, 31 salinity, 22±1°C and pH 8.16). Leaves were then exposed to treatment conditions (~9 µmol O_2 L^{-1}, 31 salinity, 22±1°C and pH 8.16) at the beginning of the subsequent light cycle for 3 h before measurements were taken at incident photon irradiances in the following order: 25, 50, 100, 200, 500, 700 and 0 µmol photons m^{-2} s^{-1}. Leaves were allowed to equilibrate to each of the incident photon irradiances for 30 mins before O_2 micro-profiles were recorded.

Based on the measured O_2 concentrations around the leaf tissue surface, O_2 fluxes were calculated using Fick's first law of diffusion:

$$(1)\ J_{O_2} = -D_0 \frac{\Delta C}{\Delta z}$$

where D_0 is the diffusion coefficient of O_2 in seawater at the experimental salinity and temperature (2.2088×10^{-5} cm^{-2} s^{-1}; tabulated values taken from www.unisense.com) and $\frac{\Delta C}{\Delta z}$ is the gradient of the linear O_2 concentration within the DBL.

The measured O_2 fluxes across the leaf surface, which are the equivalent of rates of net photosynthesis, were then fitted with an exponential saturation model (Webb et al. 1974; Lichtenberg and Kühl 2015) using OriginPro (OriginLab, USA) with the added respiration term, *R*, to account for O_2 consumption (Spilling et al. 2010):

$$(2)\ P(E) = P_{max}\left(1 - exp^{\frac{-\alpha}{P_{max}}}\right) + R$$

where α is the initial slope of the P-I curve in the light-limiting phase, P_{max} is the maximum net photosynthetic rate and *R* is the respiration term.

This allowed for calculations of the minimum photosynthetic saturation irradiance (E_k), which gives an indication of the onset of photosynthesis saturation, and the compensation irradiance (E_c), that is, where the O_2 produced via photosynthesis equals the respiratory demands, using the following equations (e.g. Lichtenberg and Kühl 2015):

$$(3)\quad E_k = \frac{P_{max}}{\alpha}$$

$$(4)\quad E_c = \frac{P_{max} log_{10}(\frac{R}{P_{max}}+1)}{-\alpha}$$

Sample collection, RNA extraction and cDNA synthesis

Four biological replicate samples of *Z. muelleri* were randomly collected for each time point (0 and 24 h) and for each treatment (control and low O_2). Samples included above-ground tissue (i.e. leaf biomass) only, as this part of the plant is likely to respond more immediately, being photosynthetically active and in direct contact with molecular O_2 in the water-column. Samples were packed in aluminium foil envelopes and snap-frozen directly in liquid nitrogen. Samples were stored at -80°C for 15 days prior to further RNA extraction and RT-qPCR analysis. Briefly, for each sample, ~70 mg of freeze-dried leaf biomass was grounded into powder using a mortar and pestle in liquid nitrogen. RNA was then extracted using the PureLink RNA Mini Kit (Ambion) following manufacturer's instructions. Column purification DNAse digestion was carried out using PureLink DNase Set (Ambion) following the manufacturer's instructions. The RNA quantity and quality was assessed using a spectrophotometer (NanoDrop 2000) and absorbance at 260/280 nm. Good quality RNA samples were stored at -80°C for further RT-qPCR experiments. A total of 500 ng of good quality RNA was used for each sample for cDNA synthesis using the High-Capacity cDNA Reverse

Transcription Kit (Applied Biosystems) following manufacturer’s instructions. The resulting cDNA samples were diluted 1:20 for use in RT-qPCR analysis.

Primer design

The present study conforms to the Minimum Information for Publication of Quantitative Real-Time PCR guidelines (Bustin et al. 2009). In this section, we indicate the essential information, *sensu* Bustin et al. (2009), required to allow reliable interpretation of the corresponding RT-qPCR results.

In-depth analysis of *Zostera muelleri* Transcriptomics Database (Hayward et al, in prep), revealed transcripts encoding proteins with high similarities to the domains of PEPC and CA proteins already identified in the seagrass *Zostera marina* (Olsen et al. 2016). It is interesting to note that the genome of *Z. muelleri*, which was not available at the time of this study, has been published since (Lee et al. 2016). The functional domains of three of these sequences, coding for PEPC1 (KMZ56135), PEPC2 (KMZ58048) and γ-CA (KMZ56166) respectively, were used as a template to design sequence-specific primers for RT-qPCR using the software, Primer3 0.4.0 (Koressaar and Remm 2007; Untergasser et al. 2012) with default settings. The sizes of the resulting amplicons were kept from 79 to 195 bp (Table 2. 1) ensuring similar PCR efficiencies and facilitating cross comparison of assays. The specificity of each selected primer pair was observed by PCR amplification as single bands at the expected size resolved via agarose gel electrophoresis.

Table 2. 1. Reference genes and target genes investigated in *Zostera muelleri* by using RT-qPCR. Accession numbers of the closest sequence matches available online in the data repository for *Zostera marina* EST (*http://drzompo.uni-muenster.de/*) primers sequences, amplicon length, melting temperature, geometric mean of cycle threshold (CT) and RT-qPCR efficiency are indicated. GADPH: Glyceraldehyde 3-phosphate dehydrogenase; EloF: Translation initiation factor 1 subunit beta; Calmodulin; TubB: Tubulin beta-1 chain; Actin; PolyA: Poly(A) RNA polymerase; S4: 30S ribosomal protein S4; PEPC-1: Phosphoenolpyruvate carboxylase – isoform 1; PEPC-2: Phosphoenolpyruvate carboxylase – isoform 2 and γ-CA: γ Carbonic anhydrase.

Name	Accession number	Forward primer	Reverse primer	Length (bp)	T_m	C_T	Efficiency (%)
GAPDH	Zoma_C_c6252	CGGTTACTGTAGCCCCACTC	CAAAGGCTGGGATTGGTTTA	79	59.9	25	88
EloF	Zoma_C_c59090	AAGCAAAGGCGTCACTTGAT	TCTGCTGCCTTCTTCTCCTC	82	59.9	24	104
Calmodulin	Zoma_B_i07192	ATCCATCCTGGTCTTTGTCG	CACTGTGATCCACTCGTTGG	197	60.1	23	114
TubB	Zoma_Contig120	GGACAAATCTTCCGTCCAGA	TCCAGATCCAGTTCCACCTC	195	60	24	88
Actin	Zoma_ZMF02257	TAAGGTCGTTGCTCCTCCTG	ACTCTGCCTTTGCAATCCAC	104	60.4	26	110
PolyA	Zoma_C_c36619	GCTGCTCGTTCAAATTCCTC	ATGACCGCCATTTAATCTGC	112	59.9	29	93
S4	Zoma_Contig219	ATGGTCTGACAGAGCGACAA	TGTTATCCAAACGCATCTCG	108	59.7	29	114
PEPC-1	KMZ56135	AGGCAAAATTCGGACTTCCT	GAGGACGCAGTGTTGACAGA	84	60.1	29	99
PEPC-2	KMZ58048	TGGCTGTTGTAGCCACTGAG	TCTGTCTCTGGTGTGGCAAG	91	60	28	97
γ-CA	KMZ56166	AGGTCATGGTGCTGTCCTTC	CAGCAACCATTCCGTTCTTT	110	60.1	28	104

Reverse Transcription Quantitative Real Time - PCR and Gene Expression Analysis

SYBR green PCR master mix (Warrington, Cheshire, UK) was used for RT-qPCR assays in 96-wells plates in a Step One Plus^{TM} Real-Time PCR System (Applied Biosystems, USA). PCR conditions were: initial denaturation of 10 min at 95°C, followed by 50 cycles of 95°C for 30 s, 60°C for 30 s and 68°C for 30 s. A dissociation step was included at the end: 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. The final reaction volume was 10 µL, including 0.8 µL of primers (Table 2. 1) and all reactions were conducted in technical triplicates. The RT-qPCR efficiency for each gene and each treatment was determined from a cDNA dilution gradient of 27, 9, 3 and 1 ng and a linear regression model (Pfaffl 2001). The corresponding RT-qPCR efficiencies were calculated according to the equation below (Radonić et al. 2004):

$$(5)\quad PCR\ efficiency = (10^{\left[-\frac{1}{slope}\right]} - 1) \times 100$$

All the RT-qPCR efficiencies obtained with the different primers were between 96-104%, with a calibration coefficients >0.969 (Table 2. 1, see Supplementary Figure S1. 4). A no template control, as well as a no reverse transcription control was generated for each gene and each treatment to ensure that the PCR reactions were free of DNA contamination.

Data from RT-qPCR was analysed using the Step One Plus^{TM} Software (Ver. 2.3; Applied Biosystems). Expression levels were determined as the number of cycles needed for the amplification to reach a fixed threshold in the exponential phase of the RT-qPCR reaction. The cycle threshold (C_T) was set at 0.03 for all genes. To quantify changes in target genes expression, C_T were imported then transformed into quantities using corresponding RT-qPCR efficiency to obtain Normalized Relative Quantities.

Selection of reference genes

In order to select the best reference genes for the experimental conditions, expression stability was analysed using NormFinder (Andersen et al. 2004). The corresponding C_T values were used directly in the software package NormFinder (Andersen et al. 2004) to rank and select the most stable reference genes. Candidate reference genes and corresponding primers used in this study were identified previously (Schliep et al. 2015). Because these candidates reference genes were initially validated under low light stress conditions, we used NormFinder to measure their stability value during low O_2 exposure (i.e. direct measure for the estimated expression variation) as previously described by (Andersen et al. 2004). We also ran complementary analysis using a second software (GeNorm, Vandesompele et al. 2002) which led to similar results as for NormFinder. According to Normfinder, the most stable genes under our experimental conditions were GADPH, Actin and S4 and the best combination of two reference genes: S4 and GADPH (see Supplementary Figure S1. 5) was then used to normalize target gene expression profile in *Z. muelleri* under low O_2.

Statistical analyses

Statistical analyses were performed using a Repeated Measures Analyses of Variance with PERMANOVA+ software in PRIMER v6 (Anderson et al. 2008). The analyses tested the null hypothesis that there is no difference in the α, P_{max}, R, E_k and E_c values derived from the fitted P-I curves of the control and low O_2 treated leaves. The RT qPCR data was analysed in the same way to test the null hypothesis that there is no difference in the normalized relative quantities of PEPC1, PEPC2 and γ CA in control and low O_2 treated plants. We randomized our sampling within each treatment to minimize lack of independence and to separate the two O_2 levels from other potential effects originating from containers location on the table. Throughout this paper, values given for microsensor data are the mean of 3 biological replicates, while RT-qPCR data are the mean of 4 biological replicates, including technical triplicates. Results were considered significant at 5%.

Results

Rates of net photosynthesis and P-I curves

The vertical O_2 concentration micro-profiles showed a ~0.02 cm thick DBL at the leaf surface of *Z. muelleri* at all irradiances tested in both the control and low O_2 conditions (Figure 2. 1). The average O_2 concentration at the leaf tissue surface of the control plants increased from 203 to 352 µmol O_2 L^{-1} as a response to an increasing incident irradiance from 0 to 100 µmol photons m^{-2} s^{-1}, as compared to an increase from 3.6 to 187 µmol O_2 L^{-1} in the low O_2-treated plants. This translated to statistically different O_2 flux values between the control and low O_2 treated plants at incident photon irradiances of 0, 25 and 50 µmol photons m^{-2} s^{-1} (Permutational t-test, *t*4= 4.7575, *P*= 0.0026; *t*4= 12.526, *P*= 0.0002; *t*4= 5.4299, *P*= 0.0032 respectively, Figure 2. 2). Comparison between the dark respiration rates (R) and the initial slope of the P-I curve (α), which gives an indication of photosynthetic activity, of the control and low O_2 treated plants also indicated statistical difference (Permutation t-test between control and low O_2 treated plants for R and α; *t*4= 6.8879, *P*=0.0018; *t*4= 5.2964, *P*= 0.0064 respectively, Table 2. 2); with a decrease in R but an increase in α.

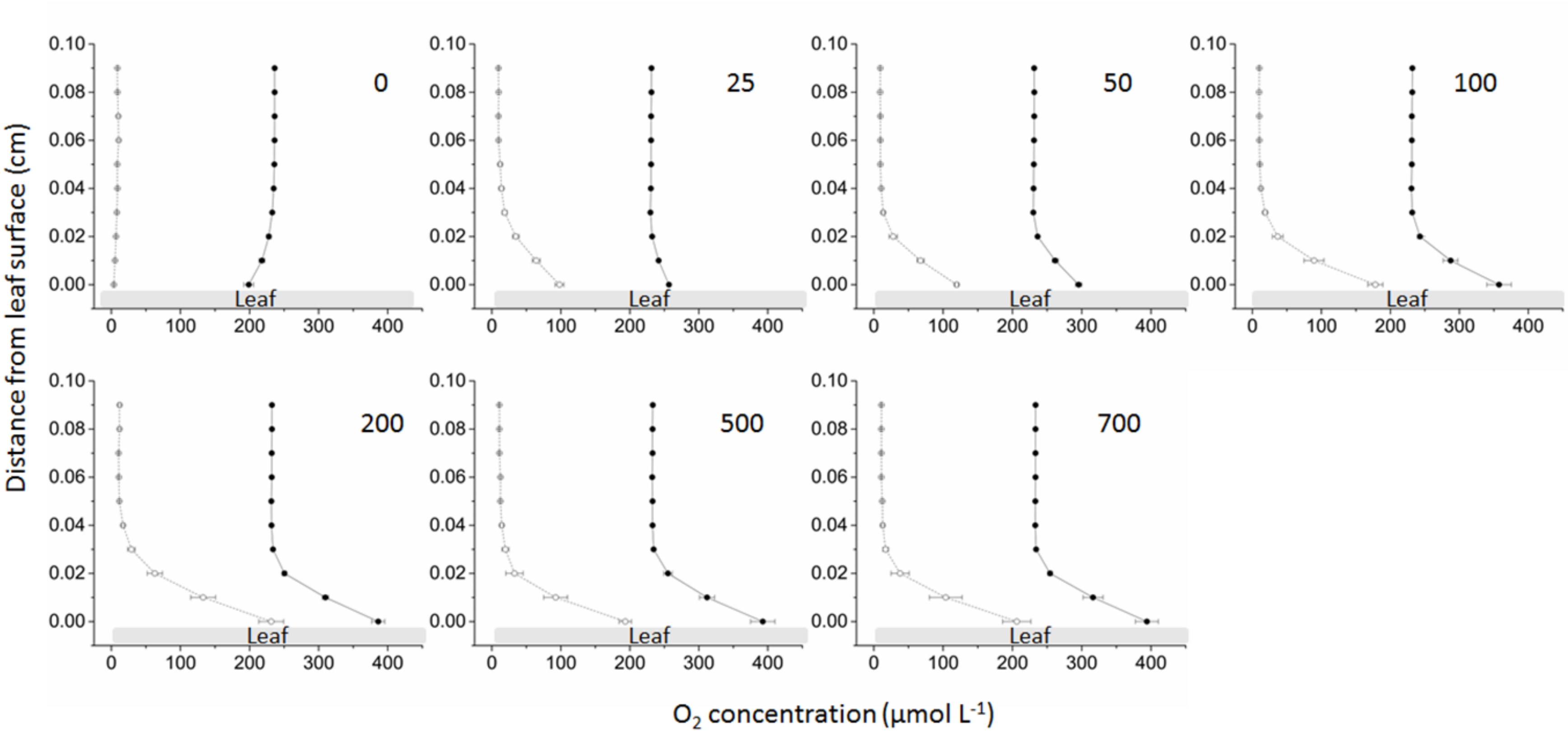


Figure 2. 1. Vertical O_2 concentration micro-profiles measured at the surface of *Zostera muelleri* leaves. Filled circles and solid lines represent data from the control leaves (i.e. leaves exposed to a water-column O_2 concentration of ~231 µmol O_2 L^{-1}), while open circles and dashed lines represent data from leaves exposed to low O_2 conditions (i.e. ~8 µmol O_2 L^{-1}). Incident photon irradiances are indicated by the figure legend (i.e. 0, 25, 50, 100, 200, 500 and 700 µmol photons m^{-2} s^{-1}). Error bars are ± standard error of the mean (SEM). Y = 0 indicate the leaf tissue surface. n=3.

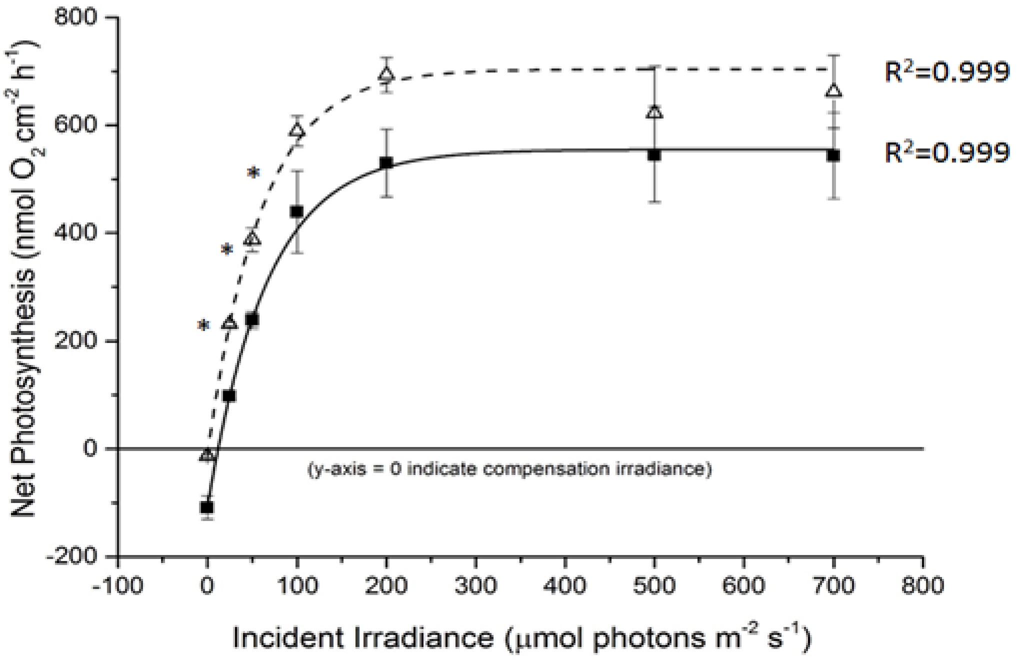


Figure 2. 2. Net photosynthesis of *Zostera muelleri* leaves at an incident photon irradiance of 0, 25, 50, 100, 200, 500 and 700 µmol photons m^{-2} s^{-1}. Data points were fitted with an exponential saturation function (Webb et al., 1974) with an added respiration term, R, to account for respiration (Spilling et al., 2010). Black squares and line represent data of leaves kept in ~231 µmol O_2 L^{-1} (i.e. control plants), while open triangles and dashed line represent data of leaves kept in ~8 µmol O_2 L^{-1} (i.e. low O_2 treatment). Error bars are ±SEM; while statistically different values are indicated by * (Permutation t-test, $P < 0.05$). n=3.

Table 2. 2. The initial slope of the P-I curve in the light-limiting phase (α), maximum net photosynthetic rate (P_{max}), dark respiration rate (R), compensation irradiance (E_c) and minimum saturating irradiance (E_k) in *Zostera muelleri* leaves exposed to water-column O_2 levels of ~231 µmol O_2 L^{-1} (control) and ~8 µmol O_2 L^{-1} (low O_2). Values are given as a mean ±SEM (n=3); with their corresponding P values (Permutation t-test), where * indicate significant difference between treatments on a 5% level.

	α	**P_{max} (nmol O_2 cm^{-2} h^{-1})**	**R (nmol O_2 cm^{-2} h^{-1})**	**E_k (µmol photons m^{-2} s^{-1})**	**E_c (µmol photons m^{-2} s^{-1})**
Control O_2	10.95±0.4	668.14±80.3	-117.09±12.5	60.82±6.0	5.19±0.8
Low O_2	13.34±0.1	687.85±49.3	-21.42±3.9	51.66±4.2	0.71±0.1
P	0.0064*	0.7974	0.0018*	0.2892	0.0012*

In contrast, between incident irradiances of 200 to 700 µmol photons m^{-2} s^{-1}, there was only a slight increase of ~2 µmol O_2 L^{-1} in the control plants, from 386 to 388 µmol O_2 L^{-1}, and an increase of ~50 µmol O_2 L^{-1} in the low O_2 treated plants from 194 to 246 µmol O_2 L^{-1}. This translated to net oxygen flux values that were not statistically different among the 200, 500 and 700 µmol photons m^{-2} s^{-1} irradiance treatments (Permutational t-test, *t*4= 2.2527, *P*= 0.088; *t*4= 0.65804, *P*= 0.5314; *t*4= 1.147, *P*= 0.306 respectively, Figure 2. 2). The calculated P_{max} values in the control and low O_2 treated plants, derived from the fitted P-I curves, were also not statistically different (Permutational t-test, *t*4= 0.2956, *P*= 0.7974, Table 2. 2).

Calculations of the E_k of *Z. muelleri* plants, in the control and low O_2 conditions, yielded an average saturation irradiance of ~60 and 51 µmol photons m^{-2} s^{-1} respectively (Table 2. 2), values that are not statistically different (Permutational t-test, *t*4= 1.2571, *P*= 0.2892, Table 2. 2); however, there was a statistically significant decrease in the E_c between the control plants and low O_2 treated plants (Permutational t-test, *t*4= 6.5624, *P*= 0.0012, Table 2. 2) which were calculated to be at incident irradiances of ~5.19 and 0.71 µmol photons m^{-2} s^{-1}, respectively (Table 2. 2).

Expression levels of target genes

Among the 7 candidate reference genes tested, the best combination of two reference genes included S4 and GADPH gene (M=0.185; see Supplementary Figure S1. 5). These two reference genes were then used to evaluate target gene expression profile in *Z. muelleri* under low O_2. The relative quantification demonstrated a significant down-regulation of the PEPC-1 and γ-CA genes for seagrass incubated in low O_2 when compared to control (Figure 2. 3). Specifically, there was a ~2.2 fold decrease in PEPC-1 gene expression in low O_2 treated samples relative to control (Permutational t-test, *t*6= 2.9916, *P*= 0.0188, Figure 2. 3). Similarly, a ~2.8 fold decrease was observed in γ-CA (Permutational t-test, *t*6= 3.3414, *P*= 0.0072: Figure 2. 3). No statistical difference was observed in the expression level of the PEPC-2 gene (Permutational t-test, *t*6, *P*= 0.0664, Figure 2. 3), although a decreasing trend was observed in low O_2 treated samples when compared to the controls.

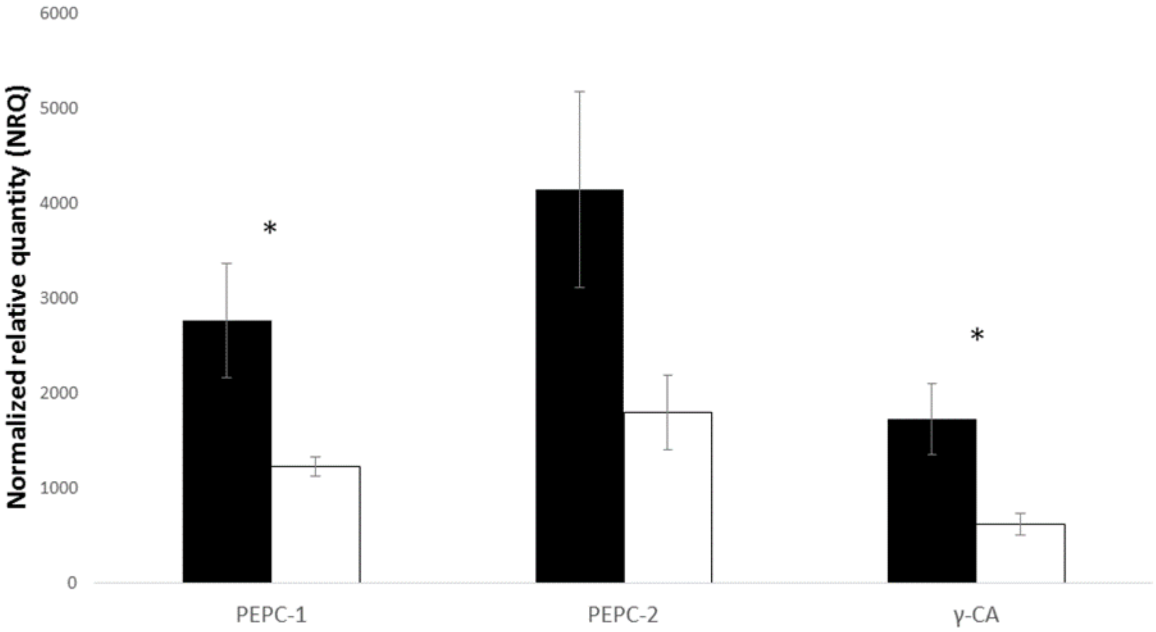


Figure 2. 3. Normalized relative quantity (NRQ) of Phosphoenolpyruvate carboxylase (PEPC-1 isoform 1 and PEPC-2 isoform 2) and γ Carbonic anhydrase (γ-CA) in *Zostera muelleri* under control (solid bars) and low O_2 conditions (open bars) relative to the two most stable reference genes: S4 and GADPH. Statistical differences in the mean are indicated with * (Permutation t-test, $P< 0.05$) and error bars are ±SEM. n=4.

Discussion

Photosynthetic parameters

We observed that by lowering the O_2 concentration in the water-column, the net photosynthetic rates of *Zostera muelleri* were enhanced in the light limited region of the P-I curve (Figure 2. 2). Further, we confirmed that these results were not due to impacts on the health of the *Z. muelleri* photosystems as shown by the Fv/Fm which remained ≥0.7 for the duration of the experimental period (as measured via PAM-fluorometry, see Supplementary Figure S1. 6). This shows that *Z. muelleri* has a higher photosynthetic activity with an increased CO_2:O_2 ratio; conditions which potentially could lead to a decreased oxygenase activity of RuBisCO and decreased flux through the PCOC, while increasing carboxylation. Similar studies involving other marine angiosperms such as *Cymodocea rotundata*, *Zostera marina* and *Ruppia maritima* support our findings since the net photosynthetic rate in these plants also increased in response to reduced ambient O_2 concentrations (Black et al. 1976; Downton et al. 1976; Beer et al. 2002; Buapet et al. 2013) whereas low O_2 conditions had no effect on the gross photosynthetic rate of a green alga *Ulva intestinalis* due to suppressed photorespiration (Drechsler and Beer 1991; Beer et al. 2000) via the maintenance of a CO_2 concentrating mechanism (Björk et al. 1993; Larsson et al. 1997). In addition, within the lacunae of seagrass leaves in light conditions, Carlson et al. (1988) and Roberts and Moriarty (1987) found that O_2 accounted for 38% of the gas within these lacunae, leading to the hypothesis that seagrass leaves may possess a mechanism to minimise photorespiration by inhibiting the accumulation of intracellular O_2. These data show the photosynthetic behaviour of *Z. muelleri* is typical of a plant with C_3 biochemistry and physiology in low O_2 conditions.

We also found a statistically significant decrease in the dark respiration and the subsequent compensation irradiance of the low O_2 treated leaves (Table 2. 2), which was not so surprising owing to the low ambient O_2 availability. A similar effect has been observed in the dark respiration of *Z. marina* when exposed to low O_2 conditions (Buapet et al. 2013). This is believed to be the result of the reduced O_2 availability in the surrounding environment and since O_2 transport to the lacunae ceases after 15 to 30 mins following the onset of darkness (Smith et al. 1984) and the O_2 concentration of the air in the lacunae drops down to <1% of the air around the leaf (Carlson et al. 1988), respiration is decreased. Hence, respiration is an important factor to take into account when interpreting photosynthetic activity and efficiency responses to changing environmental conditions. Indeed, the effect of low O_2 levels on net photosynthetic rates seen in this study could be solely due to a decreased rate of respiration, particularly as the only statistically significant differences between control and low O_2 treatments were in the 0, 25 and 50 µmol photons m^{-2} s^{-1} irradiances where respiration strongly affects the rate of net photosynthesis, while no statistical difference is seen at the higher irradiances where photorespiration would be expected to have stronger effects (Beardall et al. 2003).

While there appears to be a slight increasing trend in the rate of photosynthesis in the light saturated section of the P-I curve, there was no statistical difference in the saturating irradiance and maximum net photosynthetic rate between the low O_2 treated and control leaf fragments (Figure 2. 2; Table 2. 2). As the pH (and subsequently dissolved inorganic carbon; DIC) was maintained at 8.16 throughout the experiment, our results suggest that the growth of *Z. muelleri* was C-limited and this is consistent with previous findings on other seagrass species (Björk et al. 1997; Zimmerman et al. 1997). With regards to the DIC in seawater, speciation depends on the salinity and temperature, but the main form present at pH 8.16 is HCO_3^- (Pierrot et al. 2006), and HCO_3^- is expected to be the major inorganic C source for photosynthesis in seagrasses. Additional experimentation have shown seagrass to be capable of utilizing HCO_3^- by means other than uncatalysed conversion of HCO_3^- to CO_2 in the DBL (Larkum et al. 2017), such as *Halophila stipulacea*, *Thalassodendron ciliatum*, *Halodule uninervis* and *Syringodium isoetifolium* (Beer et al. 1977; Koch et al. 2013; Borum et al. 2016), however the exact method of HCO_3^- uptake remains unclear (Larkum et al. 2017). Moreover, at high photon irradiances O_2 produced via photosynthesis results in similar O_2 microclimates in and around leaves within both treatments, owing to an internal and external build-up of O_2 as a result of the leaf

DBLs impeding gas exchange with the surrounding water column (Brodersen et al. 2015). This may therefore explain the similar maximum net photosynthesis rates measured in the low O_2 and control treatment at photon irradiances ≥100 µmol photons m^{-2} s^{-1} (Figure 2. 1, 2. 2; Table 2. 2). One further point that should not be overlooked is that seagrasses, and many submerged freshwater flowering plants, have photosynthesis almost entirely confined to the epidermis (Larkum et al. 2017). How this anatomical feature affects photosynthesis has been little explored, but its presence in a group of fairly diverse organisms that span several families suggests that it may be important and may affect photosynthesis. Therefore before accepting that seagrasses possess a C_4 metabolism, other explanations should be sought and in this search, gene expression is an important tool.

Gene expression

Within the transcriptome of *Z. muelleri*, we discovered the presence of two different isoforms of PEPC; PEPC-1 and PEPC-2. Molecular differences coupled with differences in phylogenetic relations and gene structure between the two isoforms (Sánchez and Cejudo 2003) have suggested functional differences between the different isoforms in terrestrial plants. However the operation of these isoforms remains to be explored in marine angiosperms. When *Z. muelleri* plants were exposed to low O_2 conditions, we found a significant 2.2-fold decrease in PEPC-1 gene expression (Figure 2. 3). In the terrestrial plant *Arabidopsis*, suppression of an isoform of PEPC via artificial microRNA (amiRNA) impaired root elongation and improved salt tolerance via increasing total PEPC activity (Wang et al. 2012). Little is known about the functionality of this isoform, while we recommend examining the effect of down regulation of PEPC-1 in *Z. muelleri*, the technique for genetic manipulation has not been established in *Z. muelleri* so far. In addition, although there was no statistical difference found in the expression of PEPC-2 in this study (Figure 2. 3); there was a decline in response to low O_2 conditions. As mentioned in the introduction, although PEPC activity has been widely invoked as evidence of C_4 metabolism in aquatic autotrophs, it is also used to feed anaplerotic pathways that produce essential growth compounds such as amino acids (Aubry et al. 2011). Therefore, it is possible that the down- regulation of PEPC under low O_2 reflects a decreased rate of Krebs cycle throughput however, as we only investigated the expression levels of PEPC as opposed to the activity level of the enzyme in response to low O_2, it is important that the implications of these results are not overly extrapolated. Overall, our results indicate that, when exposed to low water-column O_2, *Z. muelleri* plants (i) increase their photosynthetic activity, a characteristic of C_3 plant photosynthesis and (ii) down-regulate genes coding for PEPC, suggesting that these low O_2 conditions yield lower energy costs of photosynthesis.

HCO_3^- can be utilised through extracellular dehydration via CA (Millhouse and Strother 1986a; Beer and Rehnberg 1997; Invers et al. 2001), although this alone does not constitute a CCM (Larkum et al. 2017). In the case of *Z. muelleri*, inhibition of CA activity via acetazolamide (a membrane-impermeant CA inhibitor, so only inhibiting extracellular CA) has been shown to inhibit photosynthetic use of HCO_3^- (Millhouse and Strother 1986b; Koch et al. 2013; Borum et al. 2016). Of the 5 known independently evolved classes of CA (α, β, γ, δ and ζ; Tripp et al. 2001) and the recently described η class (Del Prete et al. 2014), we investigated the expression levels of γ-CA. For this, γ-CA was selected as the sub-complexes are contained in the respiratory complex 1 (NADH:ubiquinone oxidoreductase) of plants and algae and in the mitochondrial respiratory electron transport chain, with sub-complexes serving as the entry point of electrons, potentially playing a role in photorespiration, probably as a HCO_3^- transporter rather than as a normal CA (Braun and Zabaleta 2007; Martin et al. 2009). In agreement with photosynthetic response and regulation in PEPC genes, the significant down regulation of γ-CA genes (2.8-fold decrease: Figure 2. 3) observed in *Z. muelleri* plants exposed to low O_2 suggests that this enzyme might be more critical for photosynthesis under ambient O_2 levels (i.e. lower DIC:O_2) then under low O_2 levels.

In the freshwater aquatic monocot *Hydrilla verticillata*, C_4-type photosynthesis is induced in C_3-type photosynthesising leaves under warmer temperatures, limited CO_2, increased O_2 and high photon irradiances (Bowes and Salvucci 1989). In this way, aquatic plants could have the capacity to acclimate to a changing climate, therefore highlighting the need to better understand these mechanisms especially in keystone seagrass species such as *Z. muelleri*.

Experimental manipulation of photosynthesis in aquatic organism can be complex as several factors need to be carefully considered. Firstly, avoiding pseudo-replication at the chamber/aquarium level is certainly desirable. In this respect, we recognize the limitations of our experimental design as each of the treatments (i.e. control and low O_2) had one sump (100L plastic bin) feeding plastic container replicates, which is not ideal for full replication. However, we have used 4 container replicates for each treatment and we have randomized our sampling within each treatment to ameliorate some of the risks (Hurlbert 1984) and to separate the two O_2 levels from other potential effects originating from container replicates location on the table. This type of design is commonly used in experiments simulating ocean acidification (Sinutok et al. 2011; Sinutok et al. 2012; Sinutok et al. 2014). Secondly, it is also important to make sure that the experimental procedure does not affect multiple components of water chemistry, particularly inorganic carbon levels within different treatment tanks. While we did not perform any alkalinity measurements during the experiment, we can be confident that the various forms of inorganic carbon were in equilibrium during our experiment for the following reasons:

i) The O_2 level in both the treatment and control tanks was stable throughout the experimental period (Supplementary Figure S1. 2).

ii) 99.9% pure CO_2 gas was used in the treatment tank to control the pH which was done via a pH controller connected to a pH/CO_2 controller (7074/2, TUNZE Aquarientechnik GmbH, Germany), to maintain the same pH of 8.16 in both tanks (Supplementary Figure S1. 3). This calibrated pH probe constantly measures the pH of the aquaria whereupon as pH starts to increase due to the flushing of nitrogen gas displacing the dissolved CO_2, subsequently reducing the concentration of HCO_3^-, the controller immediately switches on the CO_2 gas to stop the deviation and return the pH back to the experimental level (display accuracy of +/- 0.01 pH). Since this process occurs continuously, the pH deviates between 0.01-0.05 throughout the experimental period and these deviations were rectified automatically over several seconds.

iii) The tanks were kept in a temperature controlled room and salinity was kept constant throughout the experimental period.

iv) The time required for the various forms of inorganic carbon to reach equilibrium in seawater is at most 10s (see Zeebe and Wolf-Gladrow 2001).

This is the first study to combine microsensors and gene expression analyses to investigate responses to low O_2 in *Z. muelleri* and further studies with more sophisticated experimental set up are clearly needed to give more informative results.

Conclusion

Ambiguous metabolic properties such as the ability to use HCO_3^- and a C_4-type photosynthetic quantum efficiency have led to some seagrass species being classified as C_3-C_4 intermediate plants (Beer et al. 1980; Beer and Wetzel 1981; Bowes and Salvucci 1989). We suggest that the photosynthetic classification of *Z. muelleri* should also be carefully considered, as our results indicate that (i) ambient levels of O_2 affect the photophysiology of this seagrass, a characteristic of C_3 plants, and (ii) low O_2 levels induce the down-regulation of PEPC and γ-CA genes. While regulation of these genes might not be strictly associated with a C_4 biochemistry, our data suggests that when the conditions are favourable for the carboxylation reaction of RuBisCO, *Z. muelleri* down-regulates its CCM(s), thus altering its photophysiology. Future work involving the precise measurement of photorespiration and respiration is needed to show how photorespiration and respiration affect the photosynthetic response of seagrass to low O_2. Furthermore, localization and activity of PEPCs, γ-CA and Rubisco and measurements of short-term (2-5 seconds) inorganic ^{14}C labelling products, is needed to show some type of compartmentalization between initial HCO_3^- incorporation via PEPC and the final fixation of CO_2 via Rubisco, thereby supporting or not the role these enzymes play in the physiology of seagrasses. This research is not only needed to enable development of testable hypotheses to better direct future research, but also to improve the management and protection of these environmentally important marine angiosperms.

References

Andersen CL, Jensen JL, Ørntoft TF (2004) Normalization of Real-Time Quantitative Reverse Transcription-PCR Data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Research 64 (15):5245-5250. doi:10.1158/0008-5472.can-04-0496

Anderson M, Gorley RN, Clarke RK (2008) Permanova+ for Primer: Guide to Software and Statistical Methods. PRIMER-E, Plymouth, UK

Aubry S, Brown NJ, Hibberd JM (2011) The role of proteins in C_3 plants prior to their recruitment into the C_4 pathway. Journal of Experimental Botany 62 (9):3049-3059. doi:10.1093/jxb/err012

Baker NR (2008) Chlorophyll Fluorescence: A Probe of Photosynthesis In Vivo. Annual Review of Plant Biology 59 (1):89-113. doi:10.1146/annurev.arplant.59.032607.092759

Beardall J, Quigg A, Raven JA (2003) Oxygen consumption: photorespiration and chlororespiration. In: Photosynthesis in algae. Springer, pp 157-181

Beck MW, Heck Jr KL, Able KW, Childers DL, Eggleston DB, Gillanders BM, Halpern B, Hays CG, Hoshino K, Minello TJ (2001) The identification, conservation, and management of estuarine and marine nurseries for fish and invertebrates: a better understanding of the habitats that serve as nurseries for marine species and the factors that create site-specific variability in nursery quality will improve conservation and management of these areas. Bioscience 51 (8):633-641

Beer S, Bjork M, Hellblom F, Axelsson L (2002) Inorganic carbon utilization in marine angiosperms (seagrasses). Functional Plant Biology 29 (3):349-354

Beer S, Eshel A, Waisel Y (1977) Carbon metabolism in seagrasses I. The utilization of exogenous inorganic carbon species in photosynthesis. Journal of Experimental Botany 28 (5):1180-1189

Beer S, Larsson C, Poryan O, Axelsson L (2000) Photosynthetic rates of *Ulva* (Chlorophyta) measured by pulse amplitude modulated (PAM) fluorometry. European Journal of Phycology 35 (1):69-74. doi:10.1080/09670260010001735641

Beer S, Rehnberg J (1997) The acquisition of inorganic carbon by the seagrass *Zostera marina*. Aquatic Botany 56 (3):277-283

Beer S, Shomer-Ilan A, Waisel Y (1980) Carbon Metabolism in Seagrasses II. Patterns of photosynthetic CO_2 incorporation. Journal of Experimental Botany 31 (4):1019-1026

Beer S, Vilenkin B, Weil A, Veste M, Susel L, Eshel A (1998) Measuring photosynthetic rates in seagrasses by pulse amplitude modulated (PAM) fluorometry. Marine Ecology Progress Series 174:293-300

Beer S, Wetzel RG (1981) Photosynthetic carbon metabolism in the submerged aquatic angiosperm *Scirpus subterminalis*. Plant Science Letters 21 (3):199-207

Benedict CR, Scott JR (1976) Photosynthetic carbon metabolism of a marine grass. Plant physiology 57 (6):876-880

Björk M, Haglund K, Ramazanov Z, Pedersén M (1993) Inducible mechanisms for HCO_3^- utilization and repression of photorespiration in protoplasts and thalli of three species of *Ulva* (Chlorophyta). Journal of Phycology 29 (2):166-173. doi:10.1111/j.0022-3646.1993.00166.x

Björk M, Weil A, Semesi S, Beer S (1997) Photosynthetic utilisation of inorganic carbon by seagrasses from Zanzibar, East Africa. Marine Biology 129 (2):363-366

Black C, Burris J, Everson R (1976) Influence of oxygen concentration on photosynthesis in marine plants. Functional Plant Biology 3 (1):81-86. doi:http://dx.doi.org/10.1071/PP9760081

Blandon A, Zu Ermgassen PS (2014) Quantitative estimate of commercial fish enhancement by seagrass habitat in southern Australia. Estuarine, Coastal and Shelf Science 141:1-8

Borum J, Pedersen O, Greve TM, Frankovich TA, Zieman JC, Fourqurean JW, Madden CJ (2005) The potential role of plant oxygen and sulphide dynamics in die-off events of the tropical seagrass, *Thalassia testudinum*. Journal of Ecology 93 (1):148-158. doi:10.1111/j.1365-2745.2004.00943.x

Borum J, Pedersen O, Kotula L, Fraser MW, Statton J, Colmer TD, Kendrick GA (2016) Photosynthetic response to globally increasing CO_2 of co-occurring temperate seagrass species. Plant, Cell & Environment 39 (6):1240-1250. doi:10.1111/pce.12658

Bowes G, Salvucci ME (1989) Plasticity in the photosynthetic carbon metabolism of submersed aquatic macrophytes. Aquatic Botany 34 (1):233-266

Braun HP, Zabaleta E (2007) Carbonic anhydrase subunits of the mitochondrial NADH dehydrogenase complex (complex I) in plants. Physiologia Plantarum 129 (1):114-122

Brodersen KE, Hammer KJ, Schrameyer V, Floytrup A, Rasheed MA, Ralph PJ, Kühl M, Pedersen O (2017) Sediment resuspension and deposition on seagrass leaves impedes internal plant aeration and promotes phytotoxic H_2S intrusion. Frontiers in Plant Science 8 (657). doi:10.3389/fpls.2017.00657

Brodersen KE, Lichtenberg M, Paz L-C, Kühl M (2015) Epiphyte-cover on seagrass (*Zostera marina* L.) leaves impedes plant performance and radial O_2 loss from the below-ground tissue. Frontiers in Marine Science 2:58

Brodersen KE, Nielsen DA, Ralph PJ, Kühl M (2014) A split flow chamber with artificial sediment to examine the below-ground microenvironment of aquatic macrophytes. Marine Biology 161 (12):2921-2930. doi:10.1007/s00227-014-2542-3

Buapet P, Björk M (2016) The role of O_2 as an electron acceptor alternative to CO_2 in photosynthesis of the common marine angiosperm *Zostera marina* L. Photosynthesis research:1-11

Buapet P, Rasmusson LM, Gullström M, Björk M (2013) Photorespiration and carbon limitation determine productivity in temperate seagrasses. PloS one 8 (12):e83804

Bulmer R, Kelly S, Jeffs A (2016) Light requirements of the seagrass, *Zostera muelleri*, determined by observations at the maximum depth limit in a temperate estuary, New Zealand. New Zealand Journal of Marine and Freshwater Research:1-12

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry 55 (4):611-622

Carlson P, Yarbro L, Sargent W, Arnold H (1988) Hypoxic stress in *Thalassia testudinum*: Evidence from diurnal changes in rhizome gas composition. Eos 69 (1115):733-746

Chartrand KM, Bryant CV, Carter AB, Ralph PJ, Rasheed MA (2016) Light thresholds to prevent dredging impacts on the Great Barrier Reef seagrass, *Zostera muelleri* ssp. capricorni. Frontiers in Marine Science 3:106

Chi S, Wu S, Yu J, Wang X, Tang X, Liu T (2014) Phylogeny of C_4-photosynthesis enzymes based on algal transcriptomic and genomic data supports an archaeal/proteobacterial origin and multiple duplication for most C_4-related genes. PloS one 9 (10):e110154

Chollet R, Vidal J, O'Leary MH (1996) Phosphoenol pyruvate carboxylase: a ubiquitous, highly regulated enzyme in plants. Annual Review of Plant Biology 47 (1):273-298

Colman B, Norman EG (1997) Serine synthesis in cyanobacteria by a nonphotorespiratory pathway. Physiologia Plantarum 100 (1):133-136

Costanza R, d'Arge R, de Groot R, Farber S, Grasso M, Hannon B, Limburg K, Naeem S, O'Neill RV, Paruelo J, Raskin RG, Sutton P, van den Belt M (1997) The value of the world's ecosystem services and natural capital. Nature 387 (6630):253-260

Del Prete S, Vullo D, Fisher GM, Andrews KT, Poulsen S-A, Capasso C, Supuran CT (2014) Discovery of a new family of carbonic anhydrases in the malaria pathogen *Plasmodium falciparum*—The η-carbonic anhydrases. Bioorganic & Medicinal Chemistry Letters 24 (18):4389-4396

Downton W, Bishop D, Larkum AWD, Osmond C (1976) Oxygen inhibition of photosynthetic oxygen evolution in marine plants. Functional Plant Biology 3 (1):73-79

Drechsler Z, Beer S (1991) Utilization of inorganic carbon by *Ulva lactuca*. Plant Physiol 97 (4):1439-1444

Figueroa FL, Jerez CG, Korbee N (2013) Use of in vivo chlorophyll fluorescence to estimate photosynthetic activity and biomass productivity in microalgae grown in different culture systems. Latin American Journal of Aquatic Research 41 (5):801-819. doi:103856/vol41-issue5-fulltext-1

Fourqurean JW, Duarte CM, Kennedy H, Marbà N, Holmer M, Mateo MA, Apostolaki ET, Kendrick GA, Krause-Jensen D, McGlathery KJ (2012) Seagrass ecosystems as a globally significant carbon stock. Nature Geoscience 5 (7):505-509

Greiner JT, McGlathery KJ, Gunnell J, McKee BA (2013) Seagrass restoration enhances "blue carbon" sequestration in coastal waters. PloS one 8 (8):e72469

Greve TM, Borum J, Pedersen O (2003) Meristematic oxygen variability in eelgrass (*Zostera marina*). Limnology and Oceanography 48 (1):210-216. doi:10.4319/lo.2003.48.1.0210

Harlin MM (1995) Changes in major plant groups following nutrient enrichment. Eutrophic Shallow Estuaries and Lagoons CRC Press, Inc, Boca Raton, Florida:173-187

Hellblom F, Axelsson L (2003) External HCO_3^- dehydration maintained by acid zones in the plasma membrane is an important component of the photosynthetic carbon uptake in *Ruppia cirrhosa*. Photosynthesis Research 77 (2-3):173-181

Hellblom F, Beer S, Björk M, Axelsson L (2001) A buffer sensitive inorganic carbon utilisation system in *Zostera marina*. Aquatic Botany 69 (1):55-62

Hurlbert SH (1984) Pseudoreplication and the design of ecological field experiments. Ecological Monographs 54 (2):187-211

Invers O, Zimmerman RC, Alberte RS, Perez M, Romero J (2001) Inorganic carbon sources for seagrass photosynthesis: an experimental evaluation of bicarbonate use in species inhabiting temperate waters. Journal of Experimental Marine Biology and Ecology 265 (2):203-217

Jiménez C, Niell FX, Algarra P (1987) Photosynthetic adaptation of *Zostera noltii* Hornem. Aquatic botany 29 (3):217-226

Jørgensen BB, Revsbech NP (1985) Diffusive boundary layers and the oxygen uptake of sediments and detritus. Limnol Oceanogr 30 (1):111-122. doi:10.4319/lo.1985.30.1.0111

Koch M, Bowes G, Ross C, Zhang XH (2013) Climate change and ocean acidification effects on seagrasses and marine macroalgae. Global Change Biology 19 (1):103-132

Koressaar T, Remm M (2007) Enhancements and modifications of primer design program Primer3. Bioinformatics 23 (10):1289-1291

Kühl M, Cohen Y, Dalsgaard T, Jørgensen B, Revsbech NP (1995) The microenvironment and photosynthesis of zooxanthellae in scleractinian corals studied with microsensors for O_2, pH and light. Mar Ecol Prog Ser 117:159-172

Larkum A, den Hartog C (1989) Evolution and biogeography of seagrasses. In: Larkum AWD, McComb AJ, Shepherd SA (eds) Biology of Seagrasses. 1 edn. Elservier Pub Co, Amsterdam, The Netherlands, pp 112-156

Larkum AWD, Davey PA, Kuo J, Ralph PJ, Raven JA (2017) Carbon-concentrating mechanisms in seagrasses. Journal of Experimental Botany 68 (14):3773-3784. doi:10.1093/jxb/erx206

Larsson C, Lennart A, Ryberg H, Beer S (1997) Photosynthetic carbon utilization by *Enteromorpha intestinalis* (Chlorophyta) from a Swedish rockpool. European Journal of Phycology 32 (1):49-54. doi:10.1080/09541449710001719365

Lee H, Golicz AA, Bayer P, Jiao Y, Tang H, Paterson AH, Sablok G, Krishnaraj RR, Chan C-KK, Batley J (2016) The genome of a southern hemisphere seagrass species (*Zostera muelleri*). Plant Physiol:00868.02016

Les DH, Cleland MA, Waycott M (1997) Phylogenetic studies in Alismatidae, II: evolution of marine angiosperms (seagrasses) and hydrophily. Syst Bot:443-463

Lichtenberg M, Kühl M (2015) Pronounced gradients of light, photosynthesis and O_2 consumption in the tissue of the brown alga *Fucus serratus*. New Phytologist 207 (3):559-569. doi:10.1111/nph.13396

Long MH, McGlathery KJ, Zieman JC, Berg P (2008) The role of organic acid exudates in liberating phosphorus from seagrass-vegetated carbonate sediments. Limnology and Oceanography 53 (6):2616-2626. doi:10.4319/lo.2008.53.6.2616

Martin V, Villarreal F, Miras I, Navaza A, Haouz A, González-Lebrero RM, Kaufman SB, Zabaleta E (2009) Recombinant plant gamma carbonic anhydrase homotrimers bind inorganic carbon. FEBS Letters 583 (21):3425-3430. doi:10.1016/j.febslet.2009.09.055

Mass T, Genin A, Shavit U, Grinstein M, Tchernov D (2010) Flow enhances photosynthesis in marine benthic autotrophs by increasing the efflux of oxygen from the organism to the water. Proceedings of the National Academy of Sciences 107 (6):2527-2531

McLeod E, Chmura GL, Bouillon S, Salm R, Björk M, Duarte CM, Lovelock CE, Schlesinger WH, Silliman BR (2011) A blueprint for blue carbon: toward an improved understanding of the role of vegetated coastal habitats in sequestering CO_2. Frontiers in Ecology and the Environment 9 (10):552-560. doi:10.1890/110004

Millhouse J, Strother S (1986a) The effect of pH on the inorganic carbon source for photosynthesis in the seagrass *Zostera muelleri* Irmisch ex Aschers. Aquatic botany 24 (2):199-209

Millhouse J, Strother S (1986b) Salt-stimulated bicarbonate-dependent photosynthesis in the marine angiosperm *Zostera muelleri*. Journal of Experimental Botany 37 (7):965-976

Olsen JL, Rouzé P, Verhelst B, Lin Y-C, Bayer T, Collen J, Dattolo E, De Paoli E, Dittami S, Maumus F (2016) The genome of the seagrass *Zostera marina* reveals angiosperm adaptation to the sea. Nature 530 (7590):331-335

Papenbrock J (2012) Highlights in seagrasses’ phylogeny, physiology, and metabolism: what makes them special? ISRN Botany 2012. doi:10.5402/2012/103892

Pedersen O, Colmer TD, Borum J, Zavala-Perez A, Kendrick GA (2016) Heat stress of two tropical seagrass species during low tides – impact on underwater net photosynthesis, dark respiration and diel in situ internal aeration. New Phytologist 210 (4):1207-1218. doi:10.1111/nph.13900

Penhale PA, Wetzel RG (1983) Structural and functional adaptations of eelgrass (*Zostera marina* L.) to the anaerobic sediment environment. Canadian Journal of Botany 61 (5):1421-1428

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Research 29 (9):e45-e45

Pierrot D, Lewis E, Wallace D (2006) MS Excel program developed for CO_2 system calculations: ORNL/CDIAC-105a. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, US Department of Energy, Oak Ridge, Tennessee

Pregnall A, Smith R, Kursar TA, Alberte R (1984) Metabolic adaptation of *Zostera marina* (eelgrass) to diurnal periods of root anoxia. Marine Biology 83 (2):141-147

Procaccini G, Olsen JL, Reusch TB (2007) Contribution of genetics and genomics to seagrass biology and conservation. Journal of Experimental Marine Biology and Ecology 350 (1):234-259

Radonić A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A (2004) Guideline to reference gene selection for quantitative real-time PCR. Biochemical and biophysical research communications 313 (4):856-862

Ralph P, Durako MJ, Enriquez S, Collier C, Doblin M (2007) Impact of light limitation on seagrasses. Journal of Experimental Marine Biology and Ecology 350 (1):176-193

Raven JA (1984) Energetics and transport in aquatic plants. A.R. Liss, New York, USA

Raven JA (2014) Speedy small stomata? Journal of Experimental Botany 65 (6):1415-1424. doi:10.1093/jxb/eru032

Raven JA, Beardall J (2016) The ins and outs of CO_2. Journal of Experimental Botany 67 (1):1-13. doi:10.1093/jxb/erv451

Raven JA, Farquhar GD (1990) The influence of N metabolism and organic acid synthesis on the natural abundance of isotopes of carbon in plants. New Phytologist 116 (3):505-529

Roberts DG, Moriarty DJW (1987) Lacunal gas discharge as a measure of productivity in the seagrasses *Zostera capricorni*, *Cymodocea serrulata* and *Syringodium isoetifolium*. Aquatic Botany 28 (2):143-160. doi:http://dx.doi.org/10.1016/0304-3770(87)90036-2

Sánchez R, Cejudo FJ (2003) Identification and expression analysis of a gene encoding a bacterial-type phosphoenolpyruvate carboxylase from *Arabidopsis* and rice. Plant physiology 132 (2):949-957

Schliep M, Pernice M, Sinutok S, Bryant C, York P, Rasheed M, Ralph P (2015) Evaluation of reference genes for RT-qPCR studies in the seagrass *Zostera muelleri* exposed to light limitation. Scientific Reports 5:17051. doi:10.1038/srep17051

Schwarz AM (2004) Contribution of photosynthetic gains during tidal emersion to production of *Zostera capricorni* in a North Island, New Zealand estuary. New Zealand Journal of Marine and Freshwater Research 38 (5):809-818

Short FT, Polidoro B, Livingstone SR, Carpenter KE, Bandeira S, Bujang JS, Calumpong HP, Carruthers TJB, Coles RG, Dennison WC, Erftemeijer PLA, Fortes MD, Freeman AS, Jagtap TG, Kamal AHM, Kendrick GA, Judson Kenworthy W, La Nafie YA, Nasution IM, Orth RJ, Prathep A, Sanciangco JC, Tussenbroek Bv, Vergara SG, Waycott M, Zieman JC (2011) Extinction risk assessment of the world's seagrass species. Biological Conservation 144 (7):1961-1971. doi:http://dx.doi.org/10.1016/j.biocon.2011.04.010

Sinutok S, Hill R, Doblin MA, Kühl M, Ralph PJ (2012) Microenvironmental changes support evidence of photosynthesis and calcification inhibition in *Halimeda* under ocean acidification and warming. Coral Reefs 31 (4):1201-1213. doi:10.1007/s00338-012-0952-6

Sinutok S, Hill R, Doblin MA, Wuhrer R, Ralph PJ (2011) Warmer more acidic conditions cause decreased productivity and calcification in subtropical coral reef sediment-dwelling calcifiers. Limnology and Oceanography 56 (4):1200-1212. doi:10.4319/lo.2011.56.4.1200

Sinutok S, Hill R, Kühl M, Doblin MA, Ralph PJ (2014) Ocean acidification and warming alter photosynthesis and calcification of the symbiont-bearing foraminifera *Marginopora vertebralis*. Marine Biology 161 (9):2143-2154. doi:10.1007/s00227-014-2494-7

Smith RD, Dennison WC, Alberte RS (1984) Role of seagrass photosynthesis in root aerobic processes. Plant Physiology 74 (4):1055-1058

Spilling K, Titelman J, Greve TM, Kühl M (2010) Microsensor measurements of the external and internal microenvironment of *Fucus vsiculosus* (Phaeophyceae). Journal of Phycology 46 (6):1350-1355. doi:10.1111/j.1529-8817.2010.00894.x

Staehr PA, Borum J (2011) Seasonal acclimation in metabolism reduces light requirements of eelgrass (*Zostera marina*). Journal of Experimental Marine Biology and Ecology 407 (2):139-146

Tripp BC, Smith K, Ferry JG (2001) Carbonic anhydrase: new insights for an ancient enzyme. Journal of Biological Chemistry 276 (52):48615-48618. doi:10.1074/jbc.R100045200

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG (2012) Primer3—new capabilities and interfaces. Nucleic Acids Research 40 (15):e115-e115

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome biology 3 (7):1

Vogel S (1994) Life in moving fluids: the physical biology of flow. Princeton University Press, New Jersey, USA

Voznesenskaya EV, Franceschi VR, Kiirats O, Freitag H, Edwards GE (2001) Kranz anatomy is not essential for terrestrial C_4 plant photosynthesis. Nature 414 (6863):543-546

Waghmode A, Joshi G (1983) Significance of phosphoglycollate phosphatase and 3-phosphoglycerate phosphatase in photosynthetic carbon assimilation in some marine plants (*Ceriops*, *Lumnitzera*, *Aegiceras*, *Aeluropus*, *Halophila*). Photosynthetica 17:193-197

Wang F, Liu R, Wu G, Lang C, Chen J, Shi C (2012) Specific downregulation of the bacterial-type PEPC gene by artificial microRNA improves salt tolerance in *Arabidopsis*. Plant Molecular Biology Reporter 30 (5):1080-1087

Webb WL, Newton M, Starr D (1974) Carbon dioxide exchange of *Alnus rubra*. Oecologia 17 (4):281-291

Zeebe RE, Wolf-Gladrow DA (2001) CO_2 in seawater: equilibrium, kinetics, isotopes. vol 65. Gulf Professional Publishing,

Zimmerman RC, Kohrs DG, Steller DL, Alberte RS (1997) Impacts of CO_2 enrichment on productivity and light requirements of eelgrass. Plant Physiology 115 (2):599-607

Chapter 3: Effect of reduced irradiance on ^{13}C uptake, gene expression and protein activity of the seagrass *Zostera muelleri*

Mikael Kim[1], Mathieu Pernice[1], Alexander Watson-Lazowski[2,3], Paul Guagliardo[4], Matt R. Kilburn[4], Anthony W. D. Larkum[1], John A. Raven[1,5] and Peter J. Ralph[1].

AFFILIATIONS

[1]University of Technology Sydney, Climate Change Cluster, Ultimo NSW 2007, Australia

[2]Hawkesbury Institute for the Environment, University of Western Sydney, Penrith, Australia

[3]ARC Centre of Excellence for Translational Photosynthesis, Australian National University, Canberra, Australia

[4]Centre for Microscopy, Characterisation and Analysis, University of Western Australia, Perth, WA, Australia

[5]Division of Plant Science, University of Dundee at the James Hutton Institute, Invergowrie, Dundee DD2 5DA, UK

CORRESPONDING AUTHOR

Mathieu Pernice

[1]Climate Change Cluster, University of Technology Sydney, New South Wales 2007, Australia.

Email: mathieu.pernice@uts.edu.au

Phone: +61295144162

Fax: +61295144079

This article was published on the 4th of October, 2017 in the journal ‘Photosynthesis Research’, volume: 136, issue 2, page: 147-160

Contributions

MK, MP, AWDL, JAR and PJR designed the experiment; MK and MP carried out the experiment; MK and MP performed the nanoSTRING analysis; MK and AWL performed the enzyme assays; PG and MRK performed the nanoSIMS analysis; MK wrote the manuscript with contributions from all authors.

Abstract

Photosynthesis in the seagrass *Zostera muelleri* remains poorly understood. We investigated the effect of reduced irradiance on the incorporation of ^{13}C, gene expression of photosynthetic, photorespiratory and intermediates recycling genes as well as the enzymatic content and activity of Rubisco and PEPC within *Z. muelleri*. Following 48 h of reduced irradiance, we found that i) there was a ~7 fold reduction in 13C incorporation in above ground tissue, ii) a significant down regulation of photosynthetic, photorespiratory and intermediates recycling genes and iii) no significant difference in enzyme activity and content. We propose that *Z. muelleri* is able to alter its physiology in order to reduce the amount of C lost through photorespiration to compensate for the reduced carbon assimilation as a result of reduced irradiance. In addition, the first estimated rate constant (K_{cat}) and maximum rates of carboxylation (V_{cmax}) of Rubisco is reported for the first time for *Z. muelleri*.

Keywords: Seagrass, Stable isotope, Genetics, photosynthesis, photorespiration, nanostring, Australia, *Zostera muelleri*

Kim M, Pernice M, Watson-Lazowski A, Guagliardo P, Kilburn MR, Larkum AWD, Raven JA, Ralph PJ (2019) Effect of reduced irradiance on ^{13}C uptake, gene expression and protein activity of the seagrass *Zostera muelleri*. Marine Environmental Research 149:80-89.

Chapter 4: Effect of carbonic anhydrase inhibitors on photosynthesis, ^{13}C enrichment and gene expression in the seagrass *Zostera muelleri*

Mikael Kim, Mathieu Pernice, Anthony W.D. Larkum, John A. Raven & Peter J. Ralph

Contributions:

MK, MP, AWDL, JAR and PJR designed the experiment; MK and MP carried out the experiment; MK performed the microsensor and ^{13}C uptake analysis; MK and MP performed the nanoSIMS analysis; MK wrote the manuscript with contributions from all authors.

This chapter has been formatted for submission to the journal: Marine Environmental Research.

Abstract

Seagrasses are a group of flowering plants that live in coastal marine environments. The predominant form of dissolved inorganic carbon in seawater is HCO_3^- and seagrasses have been shown to use this as a source of inorganic C for photosynthesis in addition to CO_2. The main methods of HCO_3^- uptake by seagrasses are through either an external or membrane-bound carbonic anhydrase (CA), which catalyses the dehydration of HCO_3^- to CO_2, and/or a localised increase in pH within the diffusive boundary layer, thereby promoting the dissociation of HCO_3^- to CO_2, and active transport of HCO_3^- into the cell. In this study, we investigated the effect that cell-impermeable and cell permeable CA inhibitors, acetazolamide (AZ) and ethoxyzolamide (EZ) respectively, have on the photosynthetic rate, ^{13}C uptake and gene expression in the seagrass *Zostera muelleri*. Following 15 mins of separate AZ or EZ treatments, we observed a significant reduction in the photosynthetic rate in response to both CA inhibitors. Following a 12 h AZ or EZ treatment, we did not observe any significant change in ^{13}C uptake; however, we did observe a significant up-regulation in photosynthetic and photorespiration genes in response to AZ treatment, while with EZ we observed a significant down regulation of genes for photosynthesis, photorespiration and reactive oxygen species detoxification. Although the effect of AZ on gene expression was expected, the opposite effect EZ had on gene expression was surprising. Our results bring a new understanding of photosynthetic carbon fixation and metabolism in *Z. muelleri*, suggesting that this seagrass species is able to compensate for extracellular and cellular CA inhibition by altering its physiology.

Chapter 5: Synthesis, limitations, perspective research and concluding remarks

Investigations into the molecular biology of seagrasses is an emerging field of research (Procaccini et al. 2007; Davey et al. 2016), which has rapidly advanced our knowledge about how seagrasses respond to environmental stimuli at the transcriptional level (Procaccini et al. 2012; Dattolo et al. 2013; Procaccini et al. 2017; Davey et al. 2018; Kim et al. 2018; Kim et al. 2019). Correlating molecular biology with physiological changes has been a valuable tool in revealing the underlying metabolic processes that enable seagrasses to adjust to their dynamic environment, providing further insight into seagrass novel metabolic pathways. As discussed in Chapter 1 of this thesis, seagrass photosynthesis has been a key topic of research due to their ecological significance and their recent global decline primarily as a result of reduced irradiance (Waycott et al. 2009).

The results contained in this thesis add further knowledge to the metabolic pathways associated with *Z. muelleri* carbon metabolism, including the method of HCO_3^- utilisation. By using molecular analytical approaches in each chapter, the effects of different environmental changes on *Z. muelleri* photosynthesis were explored with greater detail than what is possible with traditional physiological analyses alone. Integration of the conclusions drawn in each chapter provide a broader understanding of the molecular mechanisms driving photosynthesis, photorespiration and HCO_3^- utilisation in *Z. muelleri*. In the following synthesis of the results, new conclusions will be drawn followed by a discussion of the limitations identified in each chapter. Finally, to address these limitations, prospective work to improve upon the data presented in this thesis will be suggested.

Synthesis

Photosynthesis in *Z. muelleri*

As discussed in the introduction, the epidermal cell layer contain the vast majority of chloroplasts in *Z. muelleri* leaves and as a result, this layer is thought to be the primary site of photosynthesis (Larkum et al. 1989; Kuo et al. 1990). This is clearly evident in the NanoSIMS image (Figure 4. 1) as the chloroplasts in the epidermal cells are highly enriched in ^{13}C when *Z. muelleri* is exposed to irradiances of ~200 µmol photons m^{-2} s^{-1}. The photosynthetic rate was analysed using two different methods throughout this thesis. In Chapter 2 and 4, O_2 microsensors were used to construct P-I curves and measure rates of photosynthesis respectively while ^{13}C uptake analysis via an isotope ratio mass spectrometer was used in Chapter 3 and 4 to estimate how much carbon was photosynthetically fixed. Interestingly, although the *Z. muelleri* shoots were collected from the same location and acclimated to the same irradiance regime and environmental conditions in Chapter 2, 3 and 4, the photosynthetic rate in Chapter 2 was measured at roughly ~500 nmol O_2 cm^{-2} h^{-1} higher than that in Chapter 3 when the leaf segments were exposed to ~200 µmol photons m^{-2} s^{-1}. Conversely, the ^{13}C uptake levels observed in Chapter 3 and 4 were consistently ~1700 d^{-1} (Figure 3. 1 and Figure 4.2).

Although there was no visible ^{13}C signatures on the surface of the epidermal cells, the presence of epiphytes on the surface of the leaves could contribute to the differences observed between chapters. Kuo et al. (1990) observed the presence of epiphytes on the surface of *Z. muelleri* leaves through a scanning electron microscope despite cleaning of the leaves before processing for microscopy. This epiphyte coverage has been shown to significantly reduce net photosynthesis of *Z. muelleri* leaves (Brodersen et al. 2015); however, as up to 25% coverage of epiphytes would be needed to obtain the differences in photosynthesis observed between Chapter 2 and 4, presence of epiphytes alone would not explain the difference observed.

Photorespiration in *Z. muelleri*

Rates of photorespiration in seagrasses have been suggested as being lower than that of terrestrial plants (Frost-Christensen and Sand-Jensen 1992), potentially due to the presence of a CO_2 concentrating mechanism (Larkum et al. 2017). In each chapter, regulation of photorespiration genes was observed in response to changes in environmental conditions; O_2 concentration in Chapter 2 and irradiance in Chapter 3, along with inhibition of CA in Chapter 4. This suggests that although photorespiration rates may be low in *Z. muelleri*, photorespiration impacts C metabolism in *Z. muelleri* to some extent. We observed that in low O_2 conditions, a gene involved in photorespiration was down-regulated, where as in conditions where CO_2 availability was reduced due to CA inhibition, genes involved in photorespiration were upregulated. Furthermore, in low irradiance conditions where C retention is advantageous, photorespiration gene expression is down-regulated, thereby reducing the loss of C through this process. Although this was reflected in the physiological analyses in Chapter 2 and 3, an opposite effect was observed in Chapter 4 whereby despite the up-regulation of photorespiratory genes, there was an inferred increase in photosynthesis in order to compensate for the CA inhibitor-induced decrease in CO_2 availability. These results illustrate the complexity of photorespiration regulation at the gene level, particularly when considering the impact of multiple environmental factors. Nevertheless, these results support the view of Buapet et al. (2013) who argued that photorespiration rates need to be considered when estimating the carbon budgets of seagrass meadows.

CO_2 concentrating mechanism in *Z. muelleri*

The presence of a form of CCM in seagrasses have been investigated in previous studies (Figure 1. 3). In the context of C_4 photosynthesis, CO_2 must first enter a compartment containing PEPC, whereby CA catalyses the initial hydration of CO_2 to HCO_3^-. The HCO_3^- is then needed for the carboxylation of phosphoenolpyruvate (PEP) via PEPC to form oxaloacetate. In the context of C_3 photosynthesis however, PEPC and CA take on an anaplerotic and photorespiration role respectively. The expression of PEPC and γCA have been analysed throughout this thesis however differential regulation of these genes was only observed in *Z. muelleri* in response to reduced O_2 concentrations and reduced irradiance in Chapter 2 and 3 (Figure 2. 3 and Figure 3. 3 respectively). Interestingly, there were no changes in the expression pattern of these genes in response to CA inhibition as demonstrated in Chapter 3 and, coupled with the apparent regulation of photorespiration as discussed above, supports the assertion that *Z. muelleri* is a C_3 plant.

However, it is clear that CA enhances photosynthesis in *Z. muelleri*. Addition of AZ inhibited the extracellular dehydration of HCO_3^- to CO_2, reducing the amount of HCO_3^--derived CO_2 diffusing into the cell (Figure 5. 1 System A) and thereby reducing the photosynthetic rate by ~25% (Figure 4. 1). Similarly, EZ also inhibited the photosynthetic rate by ~35% in *Z. muelleri* leaves (Figure 4. 1); however, EZ would affect both System A and B (Figure 5. 1). Although the 10% difference in the mean photosynthesis between AZ and EZ could be attributed to the internal dehydration of H^+/HCO_3^- co-transport derived HCO_3^- to CO_2 alone (Figure 5. 1 System B), there was no statistically significant difference between the AZ and EZ treatment. This suggests System B contributes minimally to the total Ci uptake in *Z. muelleri*.

In the presence of EZ, the remaining photosynthetic output of ~65% should therefore be due to CO_2 diffusion into the cell. This could be achieved by the diffusion of CO_2 already present in the seawater along with the enhanced dissociation of HCO_3^- to CO_2 in the diffusive boundary layer (DBL) as a result of a localised decrease in pH (Figure 5. 1 System C). Although System C was not targeted in Chapter 4, a buffer that has been suggested to dissipate the localised decreased pH zone, tris(hydroxymethyl)aminomethane (TRIS) has been used in numerous seagrass species in the past (see references in Table 1. 1). Notably, the effect of TRIS on the photosynthetic output of *Z. muelleri* has not yet been investigated, although in the genetically similar *Z. marina*, photosynthesis was shown to be significantly reduced as a result of TRIS (Invers et al. 2001; Beer et al. 2002).

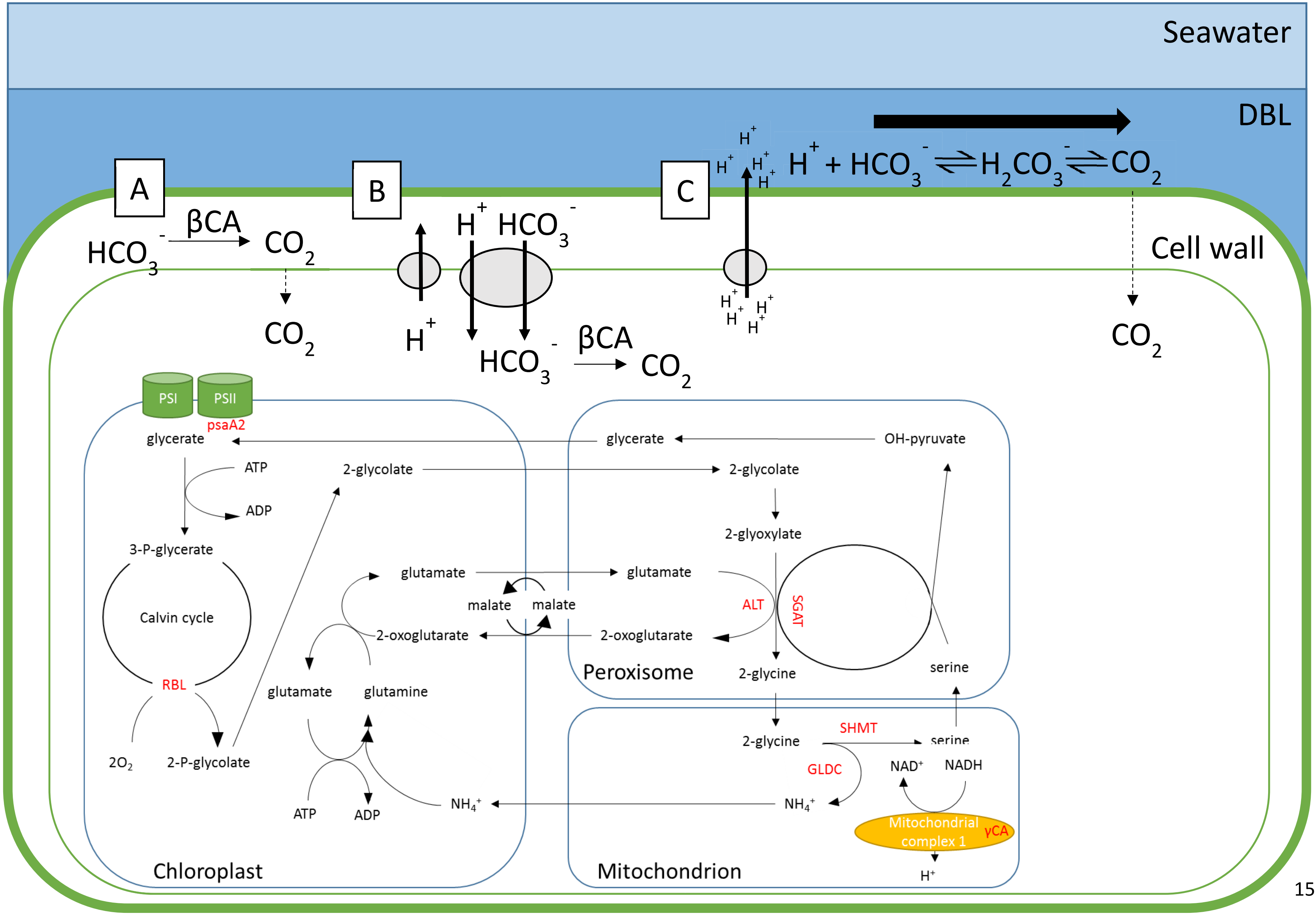

Seawater
DBL
A
B
C
$H^+ + HCO_3^- \rightleftharpoons H_2CO_3 \rightleftharpoons CO_2$
H^+
H^+
H^+
H^+
Cell wall
HCO_3^-
βCA
CO_2
CO_2
H^+
HCO_3^-
H^+
HCO_3^-
βCA
CO_2
H^+
H^+
H^+
H^+
CO_2
PSI
PSII
psaA2
glycerate
ATP
ADP
3-P-glycerate
Calvin cycle
RBL
$2O_2$
2-P-glycolate
2-glycolate
glutamate
malate
malate
2-oxoglutarate
glutamate
glutamine
ATP
ADP
NH_4^+
Chloroplast
glycerate
OH-pyruvate
2-glycolate
2-glyoxylate
glutamate
ALT
SGAT
2-oxoglutarate
2-glycine
serine
Peroxisome
2-glycine
SHMT
serine
GLDC
NAD^+
NADH
NH_4^+
Mitochondrial complex 1
γCA
H^+
Mitochondrion

Figure 5. 1. Conceptual diagram of an epidermal cell illustrating the potential mechanisms for HCO_3^- use in *Z. muelleri*. System A represents the extracellular dehydration of HCO_3^- by a membrane bound CA; System B represents a H^+/HCO_3^- co-transporter that transports HCO_3^- into the cell where it is then dehydrated to CO_2 via an intracellular CA and System C represents the localised reduction in pH within the DBL, enhancing the dissociation of HCO_3^- to CO_2. Main metabolic pathways investigated in this thesis with specific genes targeted are indicated in red.

Limitations

Z. muelleri gene sequence

The genome of the seagrass species *Zostera marina* has been available online since 2016 (Olsen et al. 2016), while only a draft genome for *Z. muelleri* was available before the beginning of this thesis (Lee et al. 2016). Unfortunately, *Z. marina* is not present in Australia, with the closest relative with high genetic similarity being *Z. muelleri* (Davey et al. 2018); however, there are differences in the transcripts of the same genes from *Z. marina* and *Z. muelleri*. These differences had an impact during the primer design step in Chapter 2 of this thesis as several of the primers tested produced multiple PCR products (Figure 5. 2). Therefore, the annotation of the *Z. muelleri* genome by Davey et al. (2018) and subsequent database creation by Sablok et al. (2018) was critical for subsequent primer designs. As a result, this new database; AquaticPlantsDB (http://115.146.91.129/version3/functional.php) was used to design the Capture and Reporter probes (NanoString, USA) for NanoString based gene expression analyses that are reported in Chapters 3 and 4.

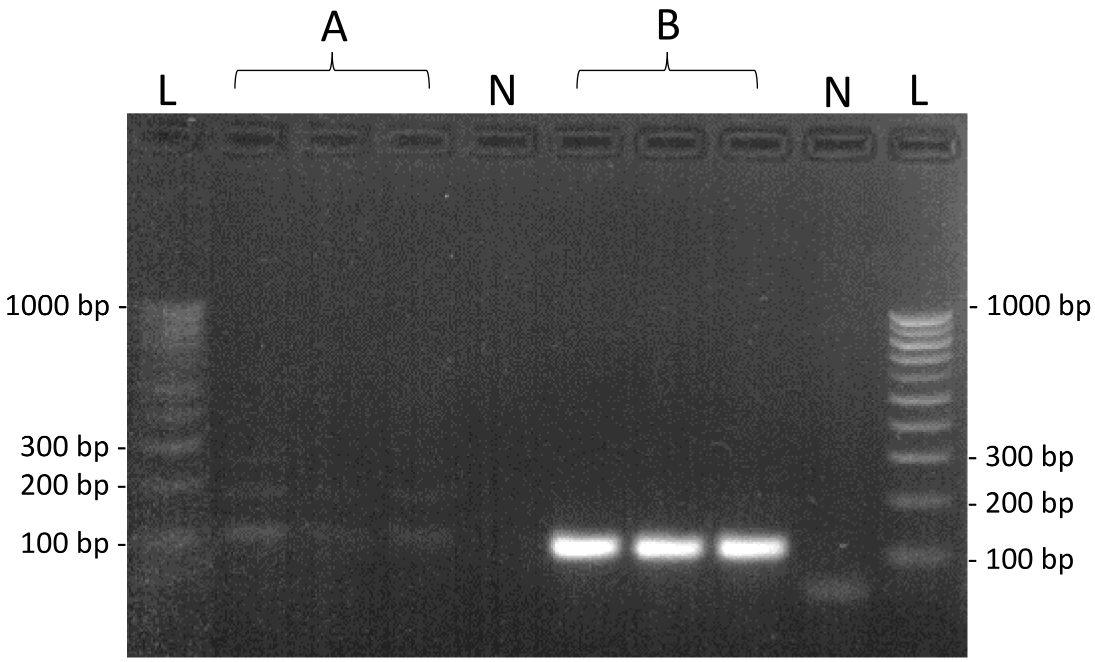


Figure 5. 2. 2.0 % agarose gel depicting multiple PCR products from beta-carbonic anhydrase (βCA) primers (A) and single PCR products from gamma-carbonic anhydrase (γCA) primers (B). Both primer products were expected to be 121 bp and 110 bp for βCA and γCA respectively. DNA ladders (L) and negative controls (N) were added to the remaining lanes.

Despite having access to the *Z. muelleri* genome database, the NanoString analysis results in Chapter 3 and 4 illustrate the need for further work in identifying functional genes or accuracy of annotation. Of the 42 target genes included in the analysis in both chapters (Supplementary table S4.1), only the normalised average counts for 24 of these genes could be considered for the final analysis. This was due to the average raw counts for the remaining 18 genes being too low (Kim et al. 2019), with raw counts below 60 considered to be below the limit of detection (LOD) of the analysis as recommended by the manufacturer. Although the low counts could suggest these genes normally have low expression in *Z. muelleri* under normal conditions, there is the possibility these genes are inactive or incorrectly annotated within the database. In support of this, the genes below the LOD were consistent between the control samples of Chapter 3 and 4.

Alternatively, the LOD can be determined based on the counts of the negative controls built into the analysis. Veldman-Jones et al. (2015) calculated the LOD by taking the average raw counts of the 8 built-in negative controls plus 2 standard deviations however, it was found that between biological replicates, the counts of genes increased in variability as they approached this LOD. Had this method of determining the LOD been adopted in Chapter 3 and 4 of this thesis, the LOD for both analyses would have been different; 63 counts for Chapter 3, and 14 counts for Chapter 4. Although using these different LOD values would not have altered the results and subsequent data interpretation, the varying value of LOD highlights the potential for LOD determination to affect future experiments by excluding or including genes for down-stream analysis.

Gene translation

Throughout Chapter 2, 3 and 4, interpretation of the gene expression analysis was primarily used to identify the metabolic processes that were affected by the treatment, and the trends observed in the expression of those genes were correlated to physiological changes. However, changes in gene expression do not necessarily equate to changes at the translational level as control of gene expression can also occur at this level (Mauch and Schoenwolf 2001). Some of the main ways in which gene expression can be controlled at the level of translation include: i) differential mRNA longevity whereby the half-life of a gene influences the amount of protein that can be translated (Shaw and Kamen 1986), ii) selective inhibition of mRNA translation whereby stores of mRNA are in a dormant state, in some cases by the binding of an inhibitory protein (Smibert et al. 1996) and iii) cytoplasmic localisation whereby the selective transport of some mRNAs to specific locations of the cell can have further down-stream effects (Rebagliati et al. 1985).

Although protein content and activity analysis was explored in Chapter 3 via enzyme assays, this was not repeated in Chapter 2 and 4. This was due to limitations such as biomass and funding availability, a lack of space for aquaria needed for adequate replication and most importantly, the lack of effective methods to extract and analyse proteomic data from *Z. muelleri*. Extraction of proteins from seagrasses is difficult as they have a recalcitrant cell wall and contain compounds such as phenolic compounds, organic acids and proteolytic and oxidative enzymes that interfere with traditional plant protein extraction methods (Papenbrock 2012), therefore the development of an efficient method for *Z. muelleri* protein extraction by Jiang et al. (2017) was a critical first step.

In an attempt to demonstrate relative changes in protein content that would correspond to changes in gene expression, we extracted protein from *Z. muelleri*, then analysed the relative quantity of specific proteins between control and treatment groups via Western blot. Although alternative methods for protein content analysis such as one-dimensional, two-dimensional gel electrophoresis (1-DE and 2-DE respectively) and two-dimensional isoelectric focusing (2-DIEF) have been applied in seagrasses proteomic studies in the past (Spadafora et al. 2008; Mazzuca et al. 2009; Dattolo et al.

2013; Piro et al. 2015; Kumar et al. 2017; Procaccini et al. 2017), we chose Western blots as this allowed for a targeted approach to protein content analysis and allowed for additional down-stream analyses such as immunolocalisation. Through localising these proteins via conjugating proteins with primary, then fluorescent secondary antibodies followed by fluorescence microscopy, functional roles of these proteins can be inferred. To my knowledge, only the localisation of aquaporins and zeatin have been successfully demonstrated in the leaves and apical meristems of *Posidonia oceanica* (Bruno et al. 2009; Serra et al. 2013).

Following successful protein extraction, subsequent Western blot results varied. Antibodies for phoshoenolpyruvate carboxylase (PEPC), Rubisco large subunit (RbcL) and γCA derived from *Arabidopsis thaliana* (Agrisera, Sweden) were tested and while we demonstrated the specificity of PEPC (Jiang et al. 2017) and RbcL (Figure 5. 3), anitbodies of γCA were not specific to the 3 expected isoforms of γCA within *Z. muelleri* (Figure 5. 4).

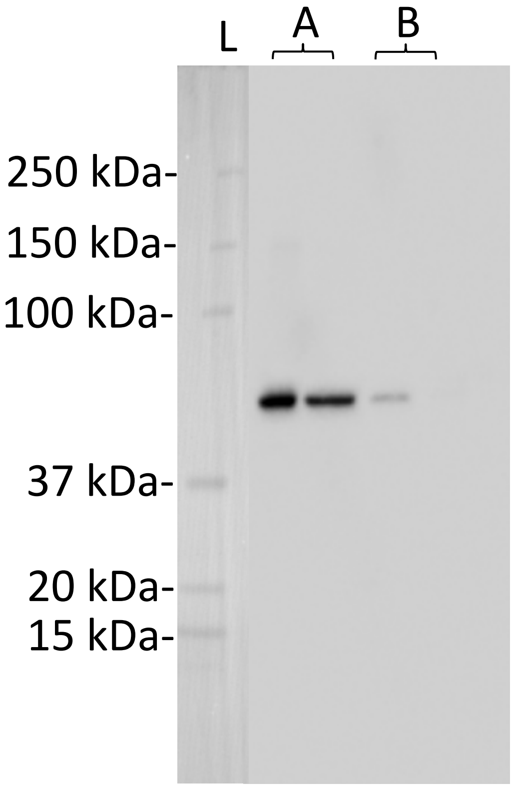


Figure 5. 3. Western blot detection of RbcL. Expected molecular weight was 52.7 kDa. Lanes contained: A) 1/1 dilution, B) 1/5 dilution control group and L) molecular weight ladder (kDa).

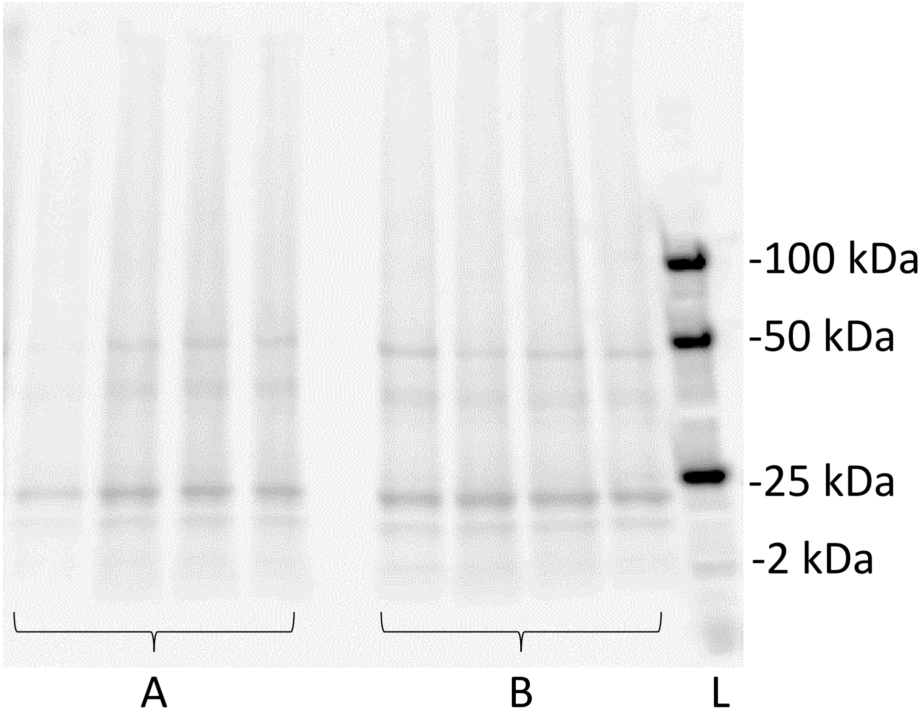


Figure 5. 4. Western blot detection of γCA. Expected molecular weights of 3 isoforms of γCA were: 9.97 kDa, 30 kDa and 27.83 kDa. Lanes contained: A) control group, B) treatment group and L) molecular weight ladder (kDa).

Based on these results, we attempted to localise PEPC and RbcL in *Z. muelleri* leaves using immunolocalisation techniques adapted from Pasternak et al. (2015) and following manufacturer recommended dilutions of primary and secondary antibodies for immunolocalisation analyses. Initially, red and green autofluorescence was detected from *Z. muelleri* leaves (Figure 5. 5A and B, 6A and B), therefore a fluorophore with an excitation/emission of 400/420 nm (Invitrogen, USA) was selected for subsequent analyses. The resulting fluorescence images for PEPC and RbcL (Figure 5. 5C, D and 6C, D respectively) were obtained, however these images do not definitively indicate where these proteins are located. In addition, fluorescence images of RbcL localisation suggest there is a layer of extracellular RbcL adjacent to epidermal cells (Figure 5. 6D). This persisted despite careful cleaning of the leaf surface to remove any epiphytes and bacteria and as a result, we believe it could be some form of artefact.

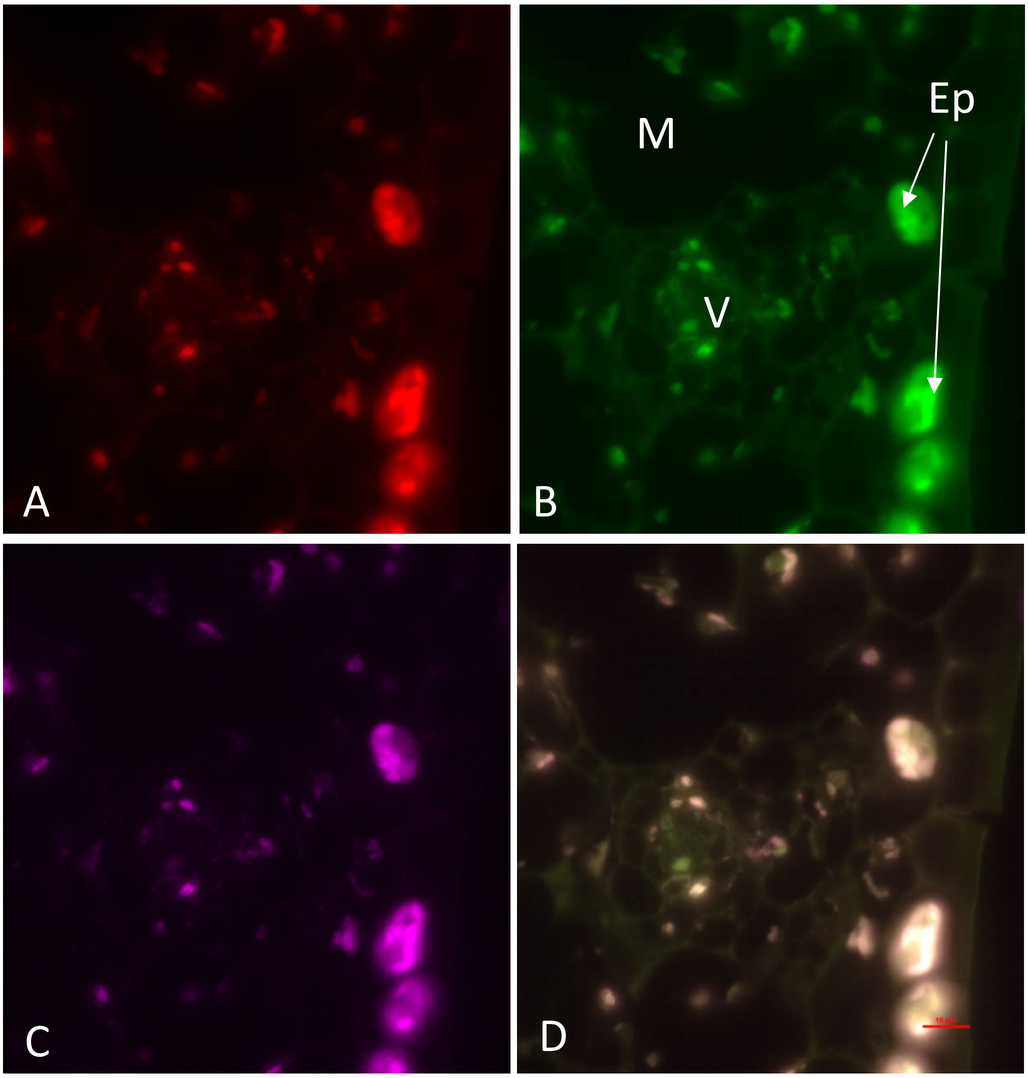


Figure 5. 5. Fluorescence image of a *Z. muelleri* leaf containing: epidermal cells (Ep), mesophyll cells (M) and vascular bundle (V). Red autofluorescence (A), green autofluorescence (B), purple fluorescent secondary antibody specific to PEPC (C), and combined fluorescence image with amber fluorescence indicating combined autofluorescence and PEPC (D). Red bar indicates 10 µm.

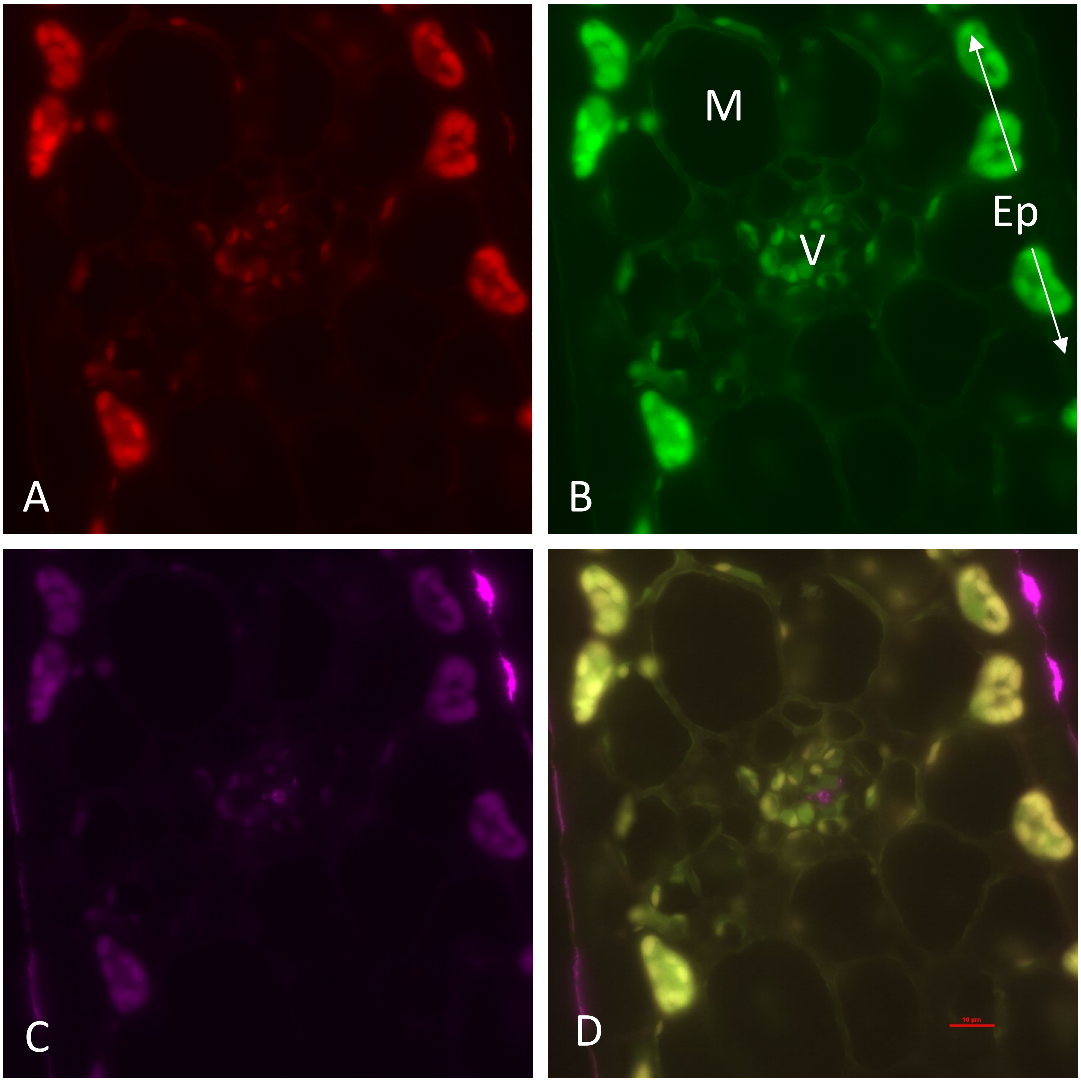


Figure 5. 6. Fluorescence image of a *Z. muelleri* leaf containing: epidermal cells (Ep), mesophyll cells (M) and vascular bundle (V). Red autofluorescence (A), green autofluorescence (B), purple fluorescent secondary antibody specific to RbcL (C), and combined fluorescence image with purple fluorescence indicating location of RbcL (D). Red bar indicates 10 µm.

Prospective research and concluding remarks

Building on the issues identified in the *Z. muelleri* gene sequences, gene translation and immunolocalisation, future research opportunities exist in improving these aspects of molecular research. Presently, I have described the expression patterns of a relatively small number of photosynthetic and photorespiration genes along with a few genes involved in TCA cycle intermediates recycling and reactive oxygen species in response to reduced O_2 environments, reduced irradiance and inhibition of carbonic anhydrase. Considering the complexity of metabolic pathways and the capability of NanoString to analyse up to 800 genes at a time, greater detail can be obtained in prospective studies by increasing the number of genes incorporated into each analysis. Furthermore, additional metabolic pathways can be investigated in this way, providing a holistic view of the interactions between mechanisms that are being affected.

Examining changes in protein content and activity should then be the next step in order to corroborate the changes in gene expression. For this purpose, 1-DE, 2-DE and 2-DIEF can be useful in achieving this goal with subsequent immunolocalisation providing additional information relating to the location of these proteins within the cell. Of particular interest is the localisation of: i) CA for the purpose of understanding its role in HCO_3^- dehydration in *Z. muelleri*, ii) Rubisco as the results of our immunolocalisation indicated an extracellular presence despite our best efforts to remove any contaminants or foreign material on the leaf surface and iii) PEPC as the cellular location of this enzyme could clarify its role in *Z. muelleri* carbon metabolism. Voznesenskaya et al. (2001) showed that spatial compartmentation of PEPC and Rubisco along with separation of different types of chloroplasts and organelles allowed for concentration of CO_2 and subsequent C_4 photosynthesis in a single cell of the terrestrial plant *Borszczowia aralocaspica*. Although C_4 photosynthesis in *Z. muelleri* is unlikely due to the results presented in this thesis, a form of CCM could be demonstrated and characterised through this method.

Interestingly, Digital Spatial Profiling technology developed by NanoString has the capacity to quantify RNA or proteins within regions of interest (ROI) of a tissue sample, allowing for protein or RNA quantification analysis to be considered from a morphological context. This technique involves the incubation of a tissue section with a cocktail of oligo-conjugated antibodies specific to target proteins or RNA probes to target RNA, identification and selection of ROI via light based imaging, exposing ROI to UV light to release oligos or tags followed by collection and analysis of these oligos or tags to quantify proteins or RNA present in the ROI (NanoString Technologies, USA).

Application of these methods could then be applied to populations of *Z. muelleri* that are exposed to different irradiance regimes or concentrations of CO_2/HCO_3^-. Procaccini et al. (2017) identified fluctuations in gene expression and protein abundance in populations of *P. oceanica* that inhabit different depths, highlighting the ability of *P. oceanica* to adapt to these changes. In a similar way, any differences observed in the gene expression or protein abundance in different populations of *Z. muelleri* could provide further insight into the adaptability of *Z. muelleri.* In this way, the importance of regulating specific metabolic pathways in response to different conditions can be explored and ultimately be used to inform seagrass management and conservation efforts.

The work presented in this thesis adds further knowledge to the molecular mechanisms driving carbon metabolism in *Z. muelleri* and presents new techniques in molecular physiology that can be applied to different areas of seagrass research. We identify some of the limitations associated with the application of these techniques and present some of the preliminary work that demonstrate the potential of these methods.

References

Beer S, Bjork M, Hellblom F, Axelsson L (2002) Inorganic carbon utilization in marine angiosperms (seagrasses). Functional Plant Biology 29 (3):349-354

Brodersen KE, Lichtenberg M, Paz L-C, Kühl M (2015) Epiphyte-cover on seagrass (*Zostera marina* L.) leaves impedes plant performance and radial O_2 loss from the below-ground tissue. Frontiers in Marine Science 2:58

Bruno A, Petrarulo M, Salimonti A, Chiappetta A, Bitonti MB (2009) Distinct pattern of zeatin distribution in the shoot apical meristem marks stress conditions in *Posidonia oceanica* (L.) Delile plants. Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology 143 (1):25-33. doi:10.1080/11263500802633220

Buapet P, Rasmusson LM, Gullström M, Björk M (2013) Photorespiration and carbon limitation determine productivity in temperate seagrasses. PloS one 8 (12):e83804

Dattolo E, Gu J, Bayer PE, Mazzuca S, Serra IA, Spadafora A, Bernardo L, Natali L, Cavallini A, Procaccini G (2013) Acclimation to different depths by the marine angiosperm *Posidonia oceanica*: transcriptomic and proteomic profiles. Frontiers in Plant Science 4:195

Davey PA, Pernice M, Ashworth J, Kuzhiumparambil U, Szabó M, Dolferus R, Ralph PJ (2018) A new mechanistic understanding of light-limitation in the seagrass *Zostera muelleri*. Marine Environmental Research 134:55-67. doi:https://doi.org/10.1016/j.marenvres.2017.12.012

Davey PA, Pernice M, Sablok G, Larkum A, Lee HT, Golicz A, Edwards D, Dolferus R, Ralph P (2016) The emergence of molecular profiling and omics techniques in seagrass biology; furthering our understanding of seagrasses. Functional & Integrative Genomics:1-16. doi:10.1007/s10142-016-0501-4

Frost-Christensen H, Sand-Jensen K (1992) The quantum efficiency of photosynthesis in macroalgae and submerged angiosperms. Oecologia 91 (3):377-384

Invers O, Zimmerman RC, Alberte RS, Perez M, Romero J (2001) Inorganic carbon sources for seagrass photosynthesis: an experimental evaluation of bicarbonate use in species inhabiting temperate waters. Journal of Experimental Marine Biology and Ecology 265 (2):203-217

Jiang Z, Kumar M, Padula MP, Pernice M, Kahlke T, Kim M, Ralph PJ (2017) Development of an efficient protein extraction method compatible with LC-MS/MS for proteome mapping in two Australian seagrasses *Zostera muelleri* and *Posidonia australis*. Front Plant Sci 8:1416. doi:10.3389/fpls.2017.01416

Kim M, Brodersen KE, Szabo M, Larkum AWD, Raven JA, Ralph PJ, Pernice M (2018) Low oxygen affects photophysiology and the level of expression of two-carbon metabolism genes in the seagrass *Zostera muelleri*. Photosynth Res 136 (2):147-160. doi:10.1007/s11120-017-0452-1

Kim M, Pernice M, Watson-Lazowski A, Guagliardo P, Kilburn MR, Larkum AWD, Raven JA, Ralph PJ (2019) Effect of reduced irradiance on ^{13}C uptake, gene expression and protein activity of the seagrass *Zostera muelleri*. Marine Environmental Research 149:80-89. doi:https://doi.org/10.1016/j.marenvres.2019.06.004

Kumar M, Padula MP, Davey P, Pernice M, Jiang Z, Sablok G, Contreras-Porcia L, Ralph PJ (2017) Proteome analysis reveals extensive light stress-response reprogramming in the seagrass *Zostera muelleri* (Alismatales, Zosteraceae) metabolism. Frontiers in Plant Science 7 (2023). doi:10.3389/fpls.2016.02023

Kuo J, Ridge RW, Lewis SV (1990) The leaf internal morphology and ultrastructure of *Zostera muelleri* Irmisch ex Aschers.(Zosteraceae): a comparative study of the intertidal and subtidal forms. Aquatic botany 36 (3):217-236

Larkum AWD, Comb AJM, Shepherd SA (1989) Biology of seagrasses: a treatise on the biology of seagrasses with special reference to the Australian region. Elsevier Science Limited, Amsterdam, The Netherlands

Larkum AWD, Davey PA, Kuo J, Ralph PJ, Raven JA (2017) Carbon-concentrating mechanisms in seagrasses. Journal of Experimental Botany 68 (14):3773-3784. doi:10.1093/jxb/erx206

Lee H, Golicz AA, Bayer P, Jiao Y, Tang H, Paterson AH, Sablok G, Krishnaraj RR, Chan C-KK, Batley J (2016) The genome of a southern hemisphere seagrass species (*Zostera muelleri*). Plant Physiol:00868.02016

Mauch TJ, Schoenwolf GC (2001) Developmental Biology. Sixth Edition. By Scott F. Gilbert. American Journal of Medical Genetics 99 (2):170-171. doi:10.1002/1096-8628(2000)9999:999<00::AID-AJMG1133>3.0.CO;2-G

Mazzuca S, Spadafora A, Filadoro D, Vannini C, Marsoni M, Cozza R, Bracale M, Pangaro T, Innocenti AM (2009) Seagrass light acclimation: 2-DE protein analysis in *Posidonia* leaves grown in chronic low light conditions. Journal of Experimental Marine Biology and Ecology 374 (2):113-122

Olsen JL, Rouzé P, Verhelst B, Lin Y-C, Bayer T, Collen J, Dattolo E, De Paoli E, Dittami S, Maumus F (2016) The genome of the seagrass *Zostera marina* reveals angiosperm adaptation to the sea. Nature 530 (7590):331-335

Papenbrock J (2012) Highlights in seagrasses' phylogeny, physiology, and metabolism: what makes them special? ISRN Botany 2012. doi:10.5402/2012/103892

Pasternak T, Tietz O, Rapp K, Begheldo M, Nitschke R, Ruperti B, Palme K (2015) Protocol: an improved and universal procedure for whole-mount immunolocalization in plants. Plant Methods 11 (1):50. doi:10.1186/s13007-015-0094-2

Piro A, Serra IA, Spadafora A, Cardilio M, Bianco L, Perrotta G, Santos R, Mazzuca S (2015) Purification of intact chloroplasts from marine plant *Posidonia oceanica* suitable for organelle proteomics. Proteomics 15 (23-24):4159-4174. doi:10.1002/pmic.201500246

Procaccini G, Beer S, Björk M, Olsen J, Mazzuca S, Santos R (2012) Seagrass ecophysiology meets ecological genomics: are we ready? Marine Ecology 33 (4):522-527

Procaccini G, Olsen JL, Reusch TBH (2007) Contribution of genetics and genomics to seagrass biology and conservation. Journal of Experimental Marine Biology and Ecology 350 (1–2):234-259

Procaccini G, Ruocco M, Marin-Guirao L, Dattolo E, Brunet C, D'Esposito D, Lauritano C, Mazzuca S, Serra IA, Bernardo L, Piro A, Beer S, Bjork M, Gullstrom M, Buapet P, Rasmusson LM, Felisberto P, Gobert S, Runcie JW, Silva J, Olive I, Costa MM, Barrote I, Santos R (2017) Depth-specific fluctuations of gene expression and protein abundance modulate the photophysiology in the seagrass *Posidonia oceanica*. Sci Rep 7:42890. doi:10.1038/srep42890

Rebagliati MR, Weeks DL, Harvey RP, Melton DA (1985) Identification and cloning of localized maternal RNAs from *Xenopus* eggs. Cell 42 (3):769-777. doi:10.1016/0092-8674(85)90273-9

Sablok G, Hayward RJ, Davey PA, Santos RP, Schliep M, Larkum A, Pernice M, Dolferus R, Ralph PJ (2018) SeagrassDB: An open-source transcriptomics landscape for phylogenetically profiled seagrasses and aquatic plants. Scientific Reports 8 (1):2749. doi:10.1038/s41598-017-18782-0

Serra IA, Nicastro S, Mazzuca S, Natali L, Cavallini A, Innocenti AM (2013) Response to salt stress in seagrasses: PIP1;1 aquaporin antibody localization in *Posidonia oceanica* leaves. Aquatic Botany 104:213-219. doi:https://doi.org/10.1016/j.aquabot.2011.05.008

Shaw G, Kamen R (1986) A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 46 (5):659-667. doi:10.1016/0092-8674(86)90341-7

Smibert CA, Wilson JE, Kerr K, Macdonald PM (1996) smaug protein represses translation of unlocalized nanos mRNA in the *Drosophila embryo*. Genes & development 10 (20):2600-2609. doi:10.1101/gad.10.20.2600

Spadafora A, Filadoro D, Mazzuca S, Bracale M, Marsoni M, Cardilio M, Innocenti AM (2008) 2-DE polypeptide mapping of *Posidonia oceanica* leaves, a molecular tool for marine environment studies. Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology 142 (2):213-218. doi:10.1080/11263500802150316

Veldman-Jones MH, Brant R, Rooney C, Geh C, Emery H, Harbron CG, Wappett M, Sharpe A, Dymond M, Barrett JC, Harrington EA, Marshall G (2015) Evaluating Robustness and Sensitivity of the NanoString Technologies nCounter Platform to Enable Multiplexed Gene Expression Analysis of Clinical Samples. Cancer Research 75 (13):2587. doi:10.1158/0008-5472.CAN-15-0262

Voznesenskaya EV, Franceschi VR, Kiirats O, Freitag H, Edwards GE (2001) Kranz anatomy is not essential for terrestrial C_4 plant photosynthesis. Nature 414 (6863):543-546

Waycott M, Duarte CM, Carruthers TJB, Orth RJ, Dennison WC, Olyarnik S, Calladine A, Fourqurean JW, Heck KL, Hughes AR, Kendrick GA, Kenworthy WJ, Short FT, Williams SL (2009) Accelerating loss of seagrasses across the globe threatens coastal ecosystems. Proceedings of the National Academy of Sciences of the United States of America 106 (30):12377-12381

Appendix 1: Low oxygen affects photosynthesis and the level of expression of two carbon metabolism genes in the seagrass *Zostera muelleri*.

Mikael Kim[1], Kasper Elgetti Brodersen[1], Milan Szabo[1], Anthony W. D. Larkum[1], John A. Raven[1,2], Peter J. Ralph[1] and Mathieu Pernice[1].

AFFILIATIONS

[1]Climate Change Cluster, University of Technology Sydney, New South Wales 2007, Australia.

[2]Division of Plant Science, University of Dundee at the James Hutton Institute, Invergowrie, Dundee DD2 5DA, UK

CORRESPONDING AUTHOR:

Mathieu Pernice

[1]Climate Change Cluster, University of Technology Sydney, New South Wales 2007, Australia.

Email: mathieu.pernice@uts.edu.au

Phone: +61295144162

Fax: +61295144079

RUNNING TITLE: LOW O_2 EFFECTS ON *ZOSTERA MUELLERI* PHOTOBIOLOGY

KEYWORDS: Diffusive boundary layer; Photosynthesis; Respiration; RT-qPCR; Seagrass

ACKOWLEDGMENTS

The authors would like to thank UTS and C3 for strategic research support as well as Dr Audrey Commault, Dr Sutinee Sinutok and Paul Brooks for technical assistance. The University of Dundee is a registered Scottish charity, No. 015096.

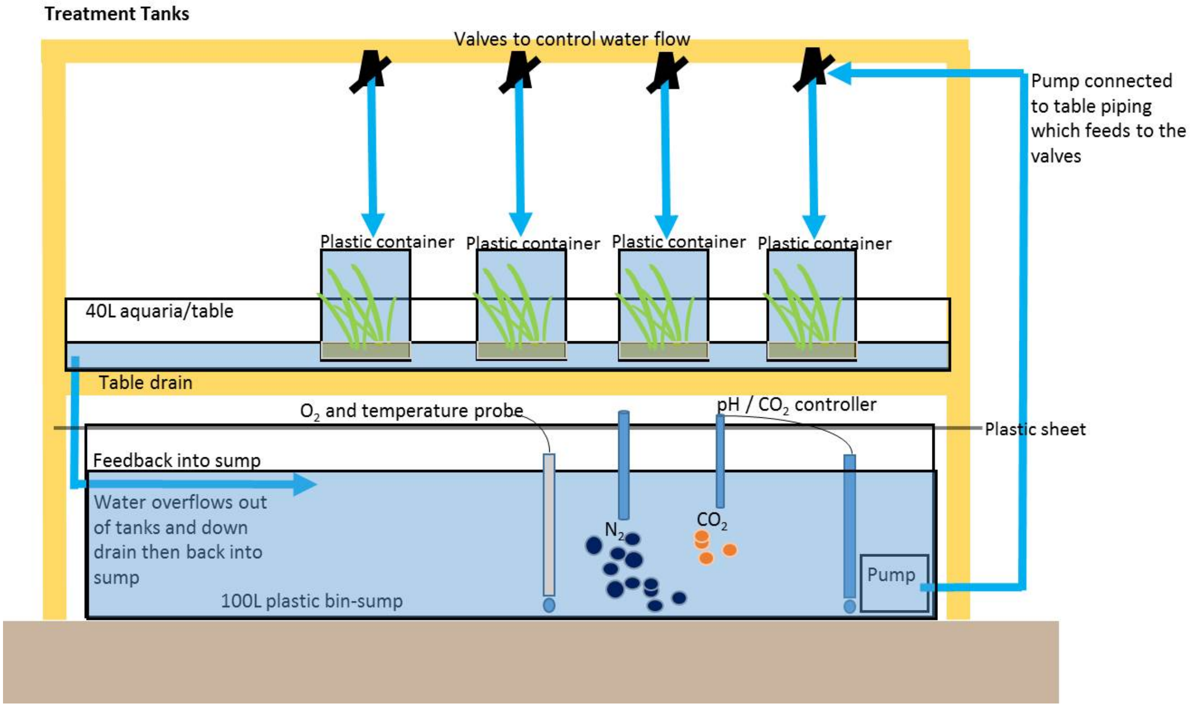
Compliance with Ethical Standards:

Funding: This study was funded by an Australian Research Council Linkage Grant (LP11020045), Climate Change Cluster Honours Scholarship, University of Technology Sydney (MK) and the Augustinus Foundation (KEB).

Conflicts of Interest: The authors declare that they have no conflict of interest.

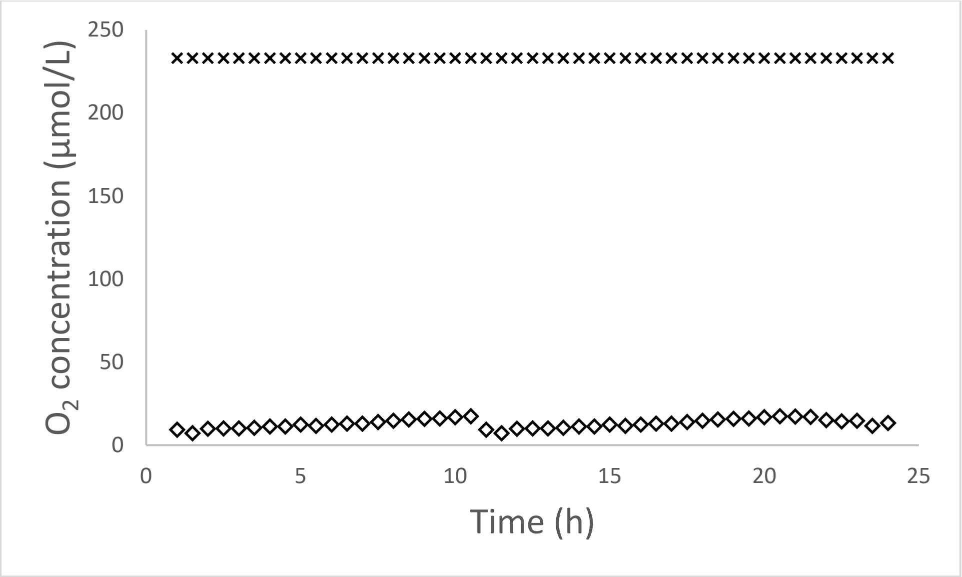
Ethical approval: All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

Supplementary Figures and Tables 1

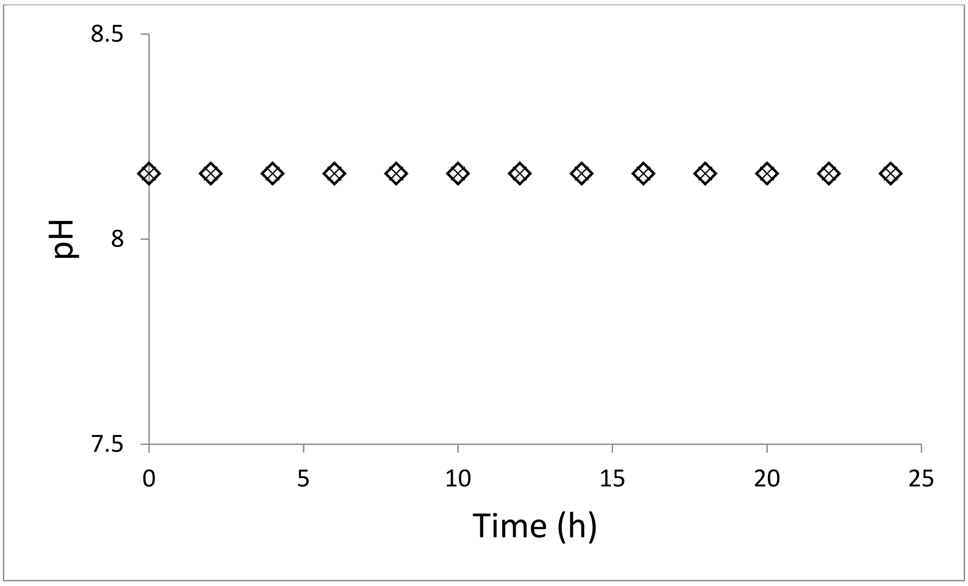


Supplementary Figure S1. 1. Experimental set up and tank replication used for low O_2 treatment.

Control tank set up was the same (just bubbling air instead of N_2 and CO_2).

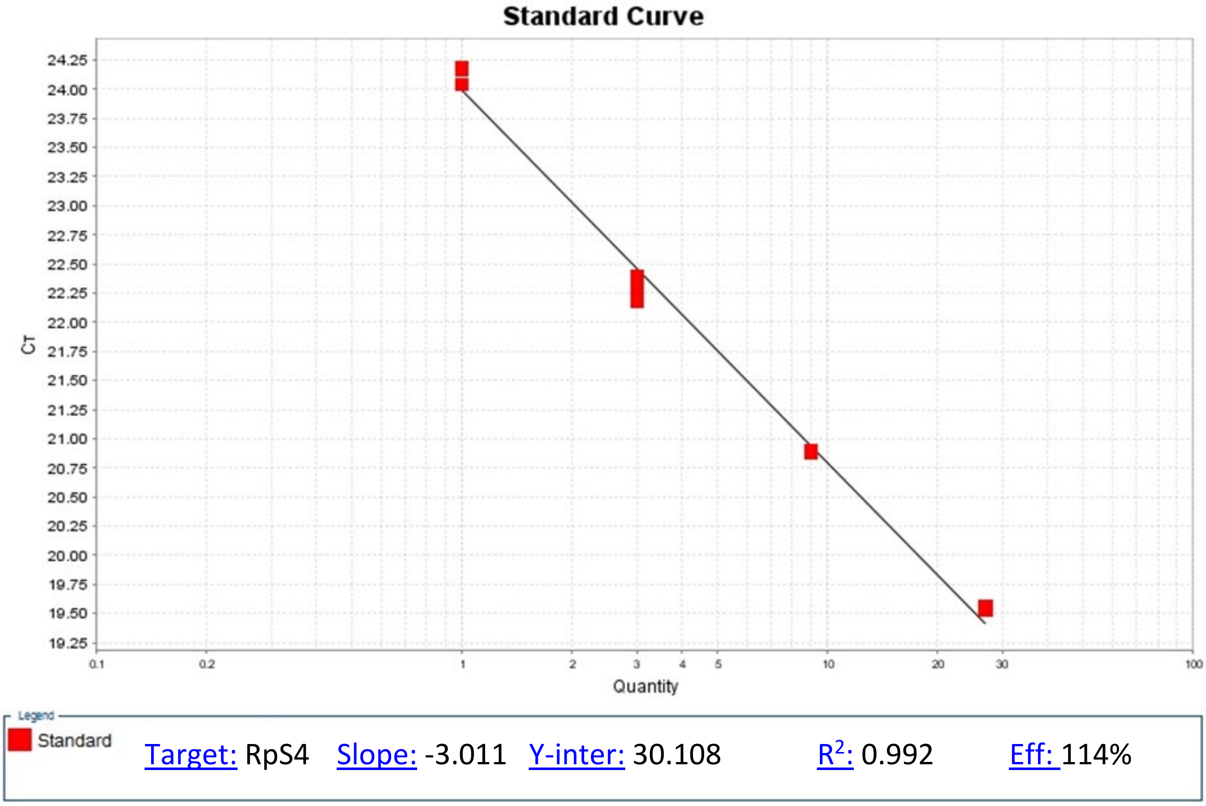


Supplementary Figure S1. 2. Concentration of O_2 in µmol/L over the experimental period in control (crosses) and treatment (open diamonds) tanks.



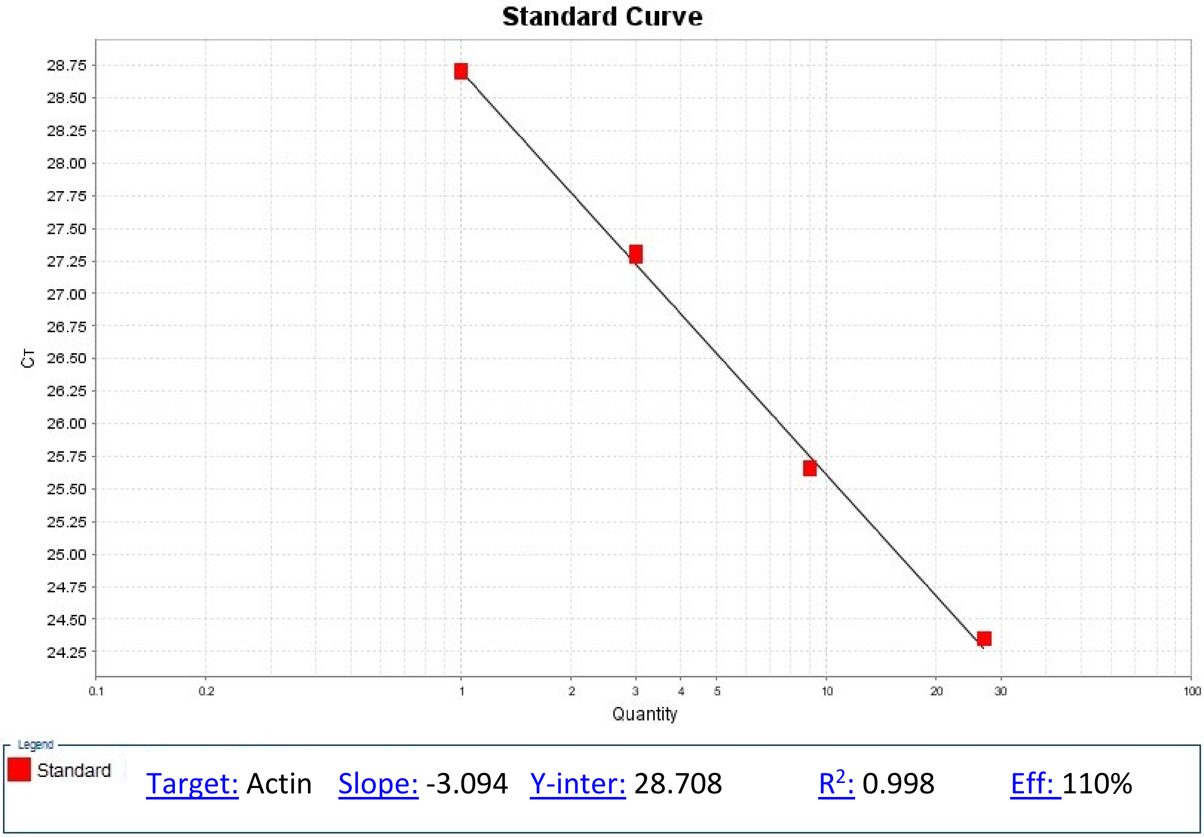
Supplementary Figure S1. 3. pH measurements over the experimental period in control (crosses) and treatment (open diamonds) tanks.

A



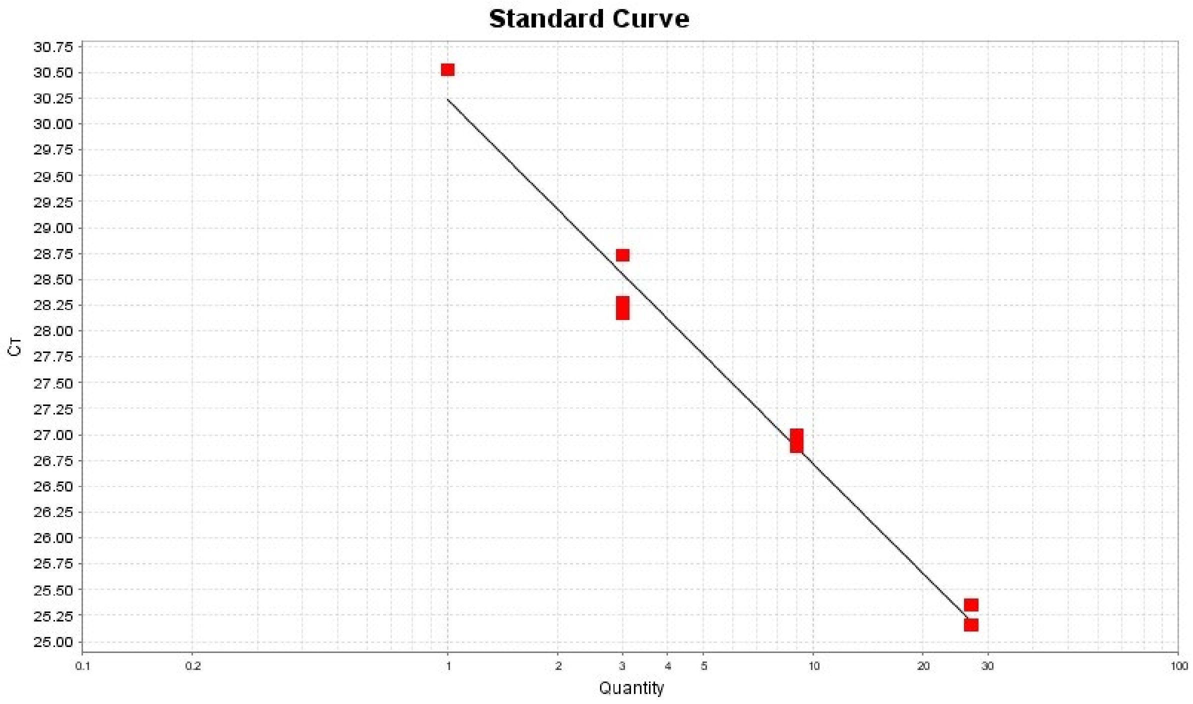
Target: RpS4 Slope: -3.011 Y-inter: 30.108 R^2: 0.992 Eff: 114%

B



Target: Actin Slope: -3.094 Y-inter: 28.708 R^2: 0.998 Eff: 110%

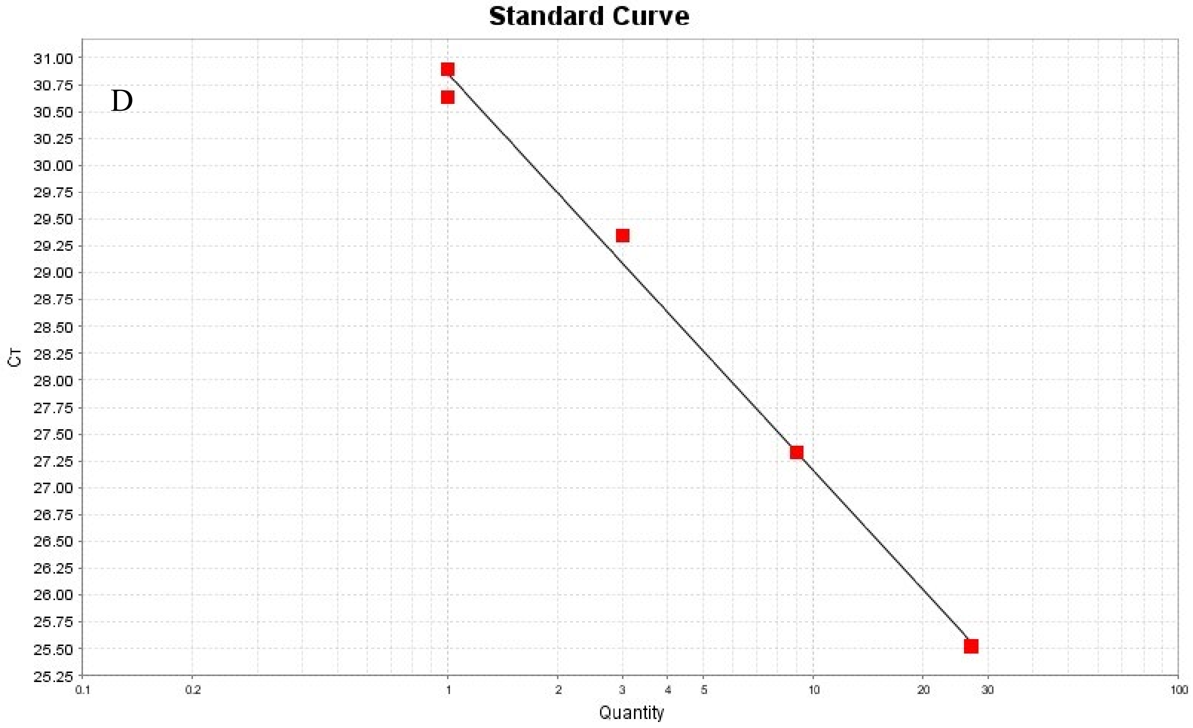
C



Legend

🟥 Standard

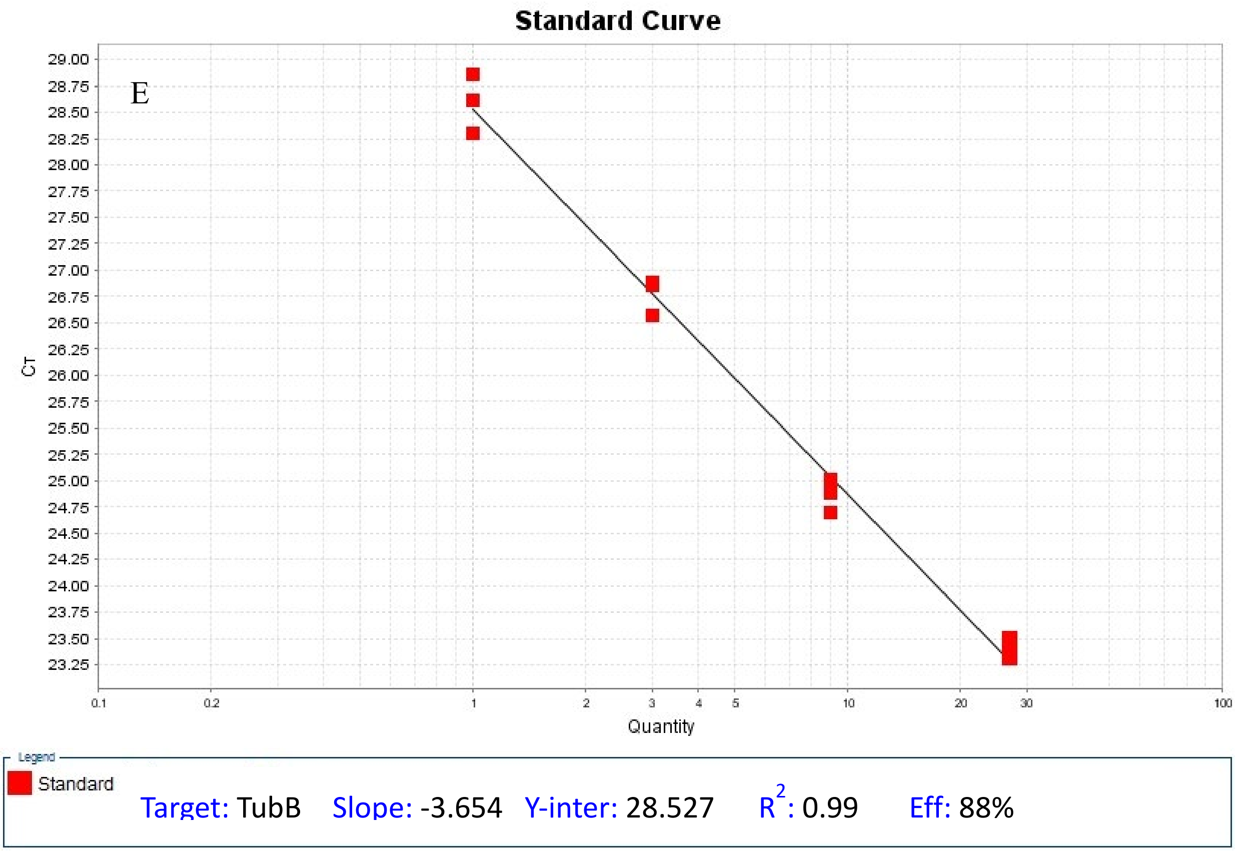
Target: PolyA Slope: -3.509 Y-inter: 30.229 R^2: 0.983 Eff: 93%



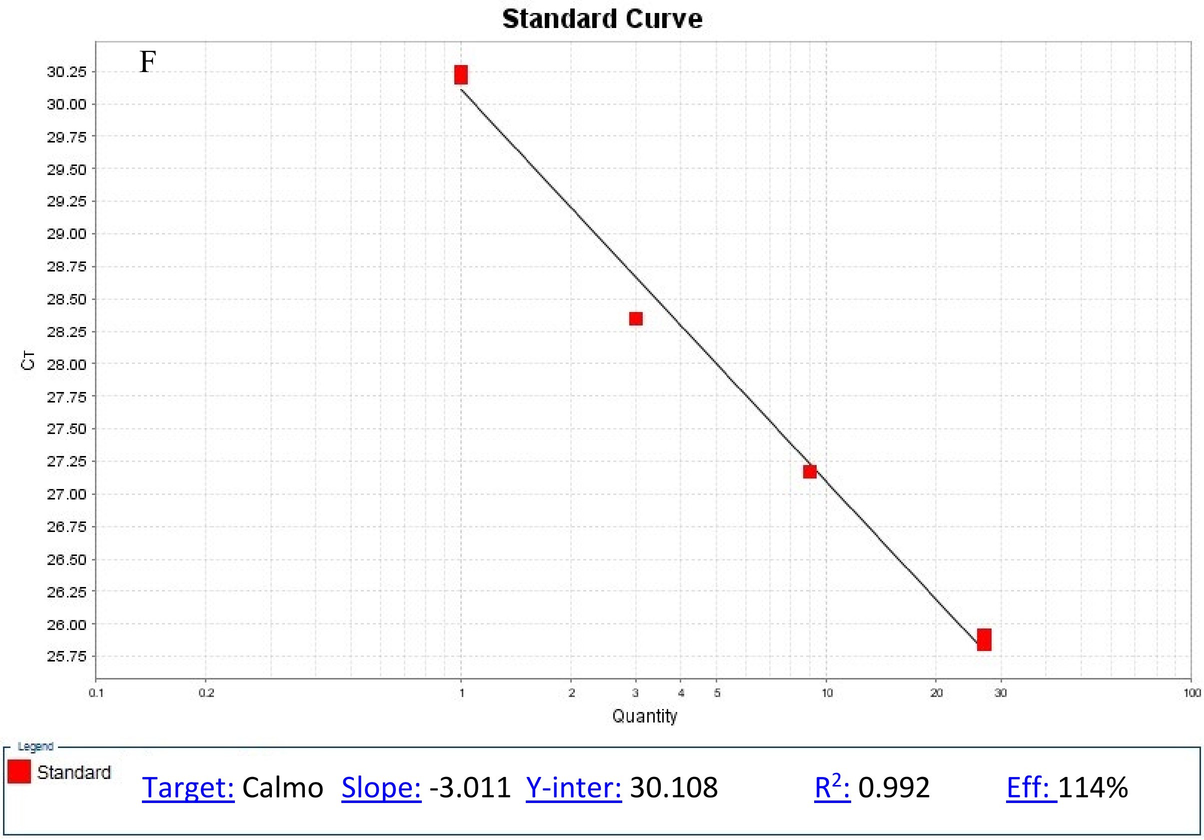
Legend

🟥 Standard

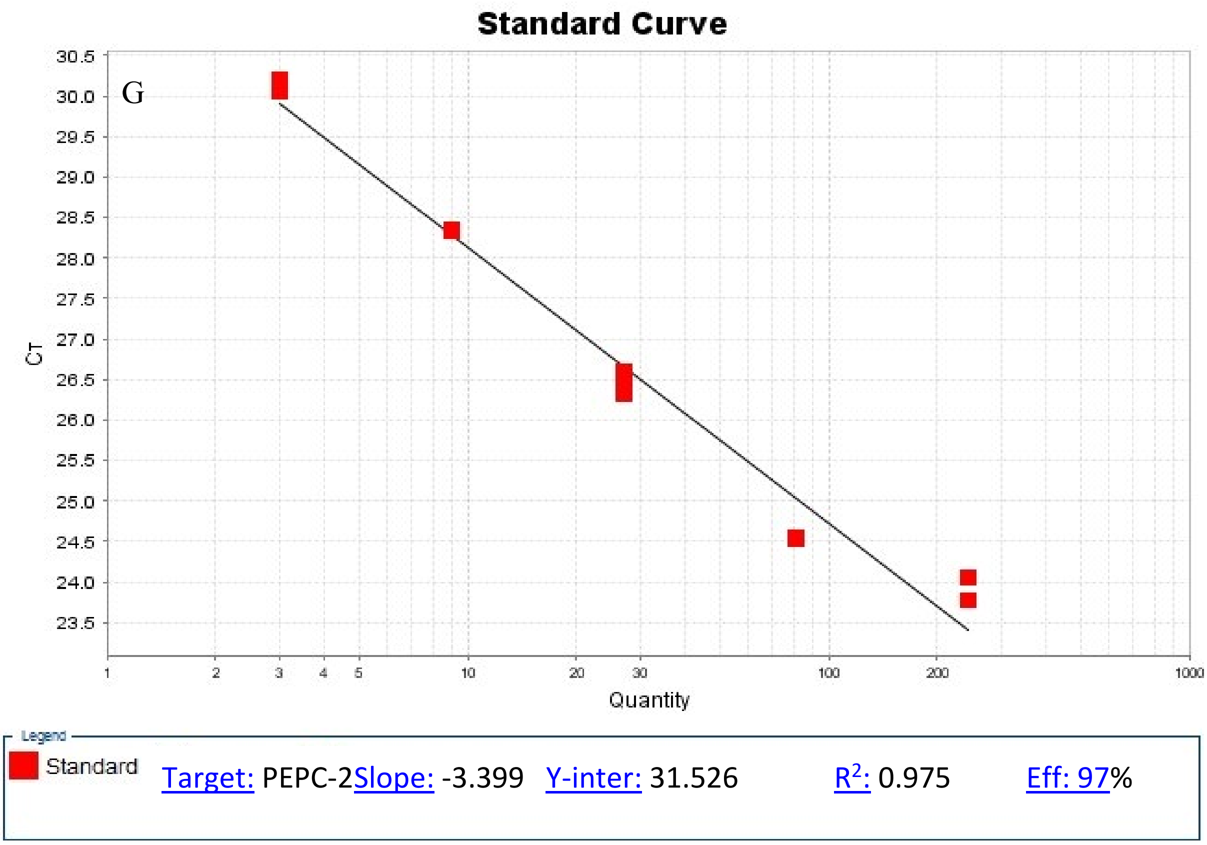
Target: GADPH Slope: -3.662 Y-inter: 30.816 R^2: 0.994 Eff: 88%



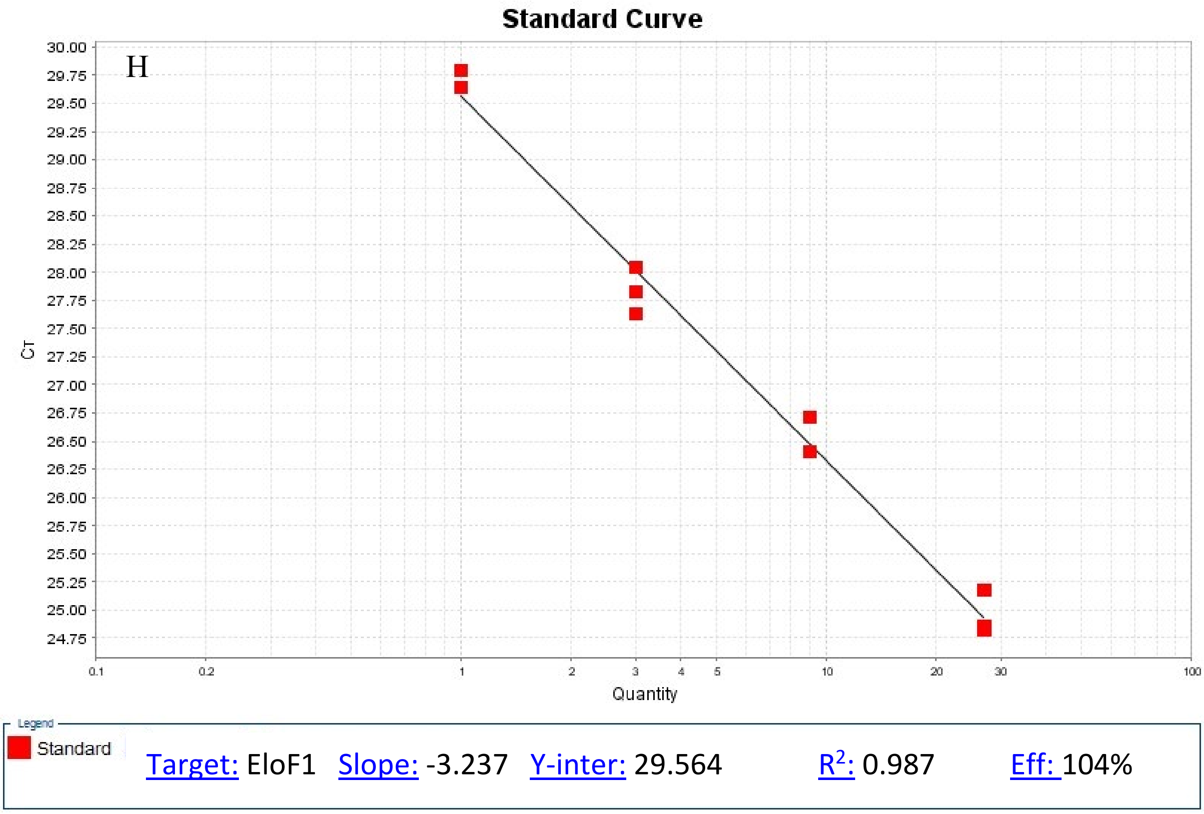
Target: TubB Slope: -3.654 Y-inter: 28.527 R^2: 0.99 Eff: 88%



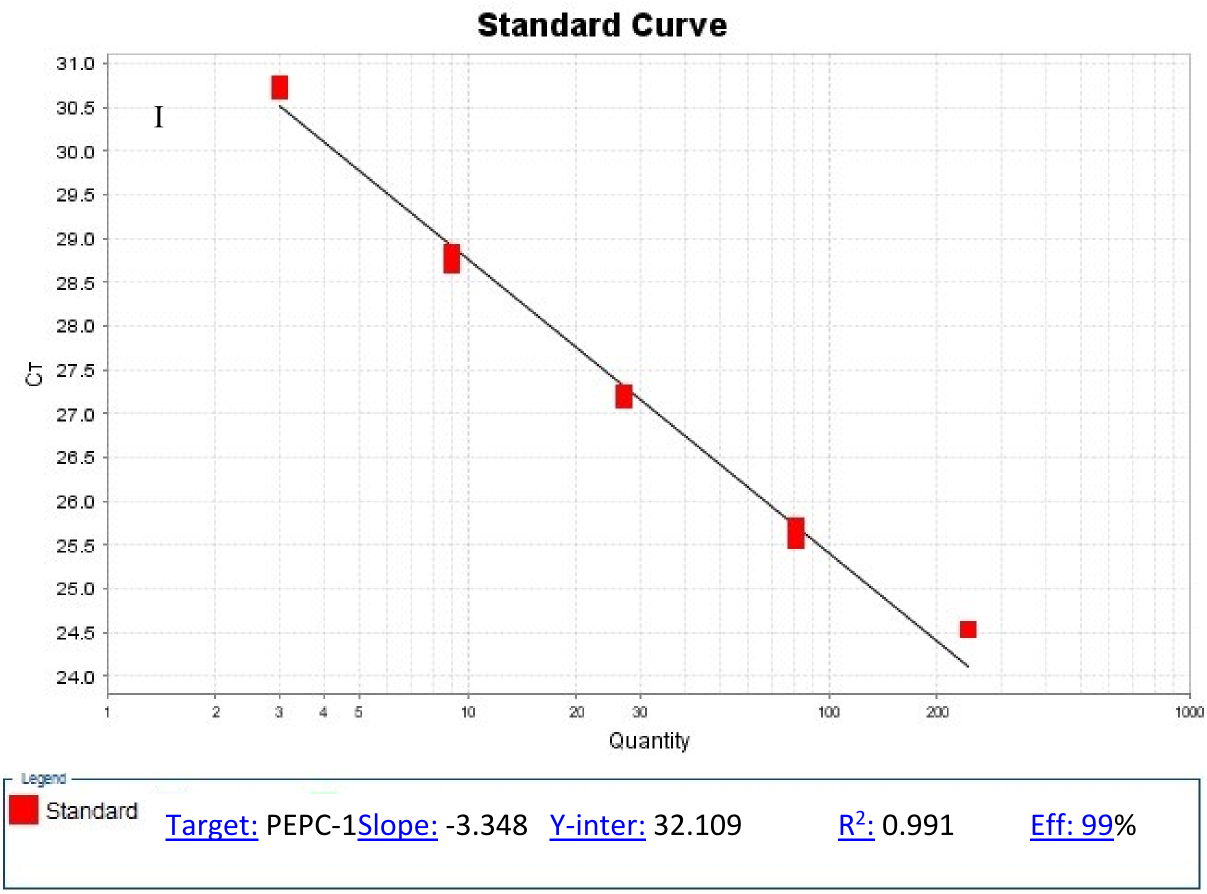
Target: Calmo Slope: -3.011 Y-inter: 30.108 R^2: 0.992 Eff: 114%



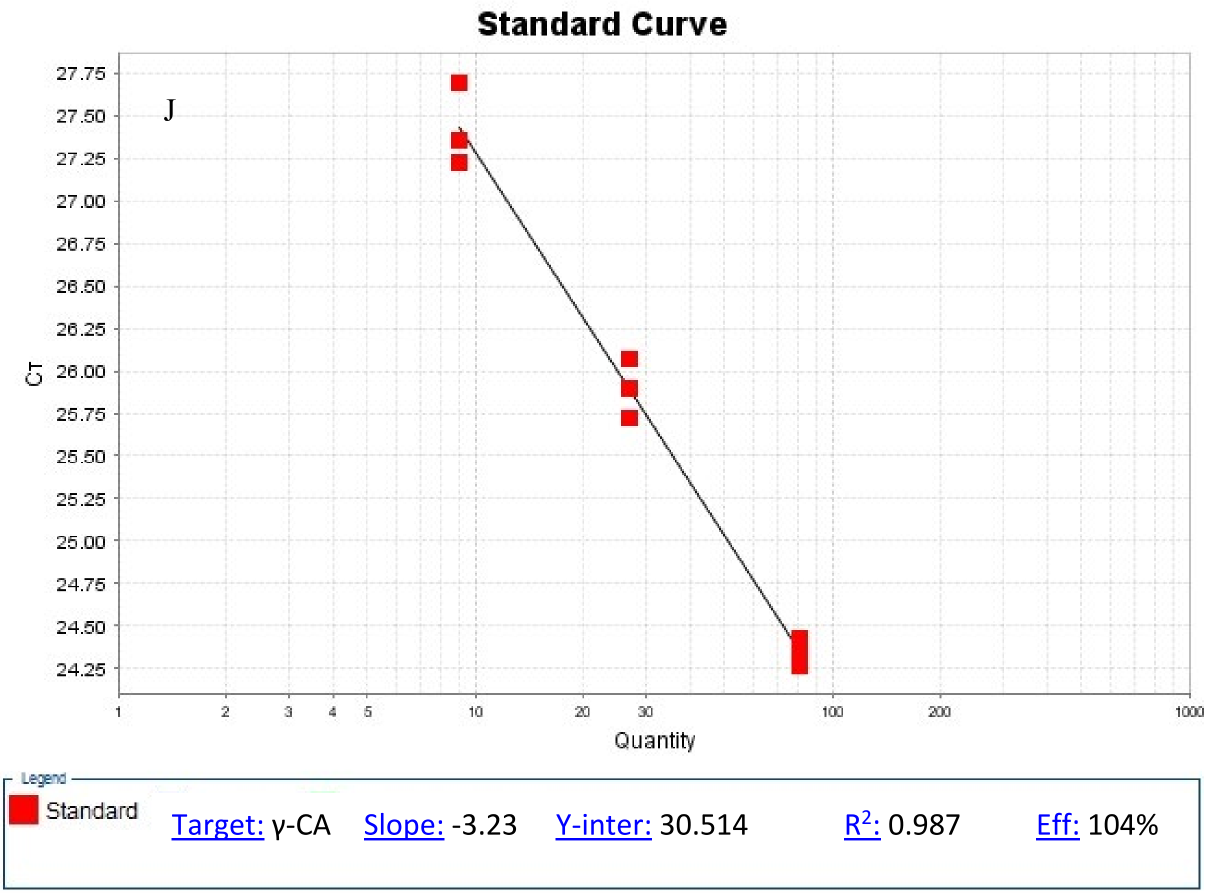
Target: PEPC-2Slope: -3.399 Y-inter: 31.526 R^2: 0.975 Eff: 97%



Target: EloF1 Slope: -3.237 Y-inter: 29.564 R^2: 0.987 Eff: 104%

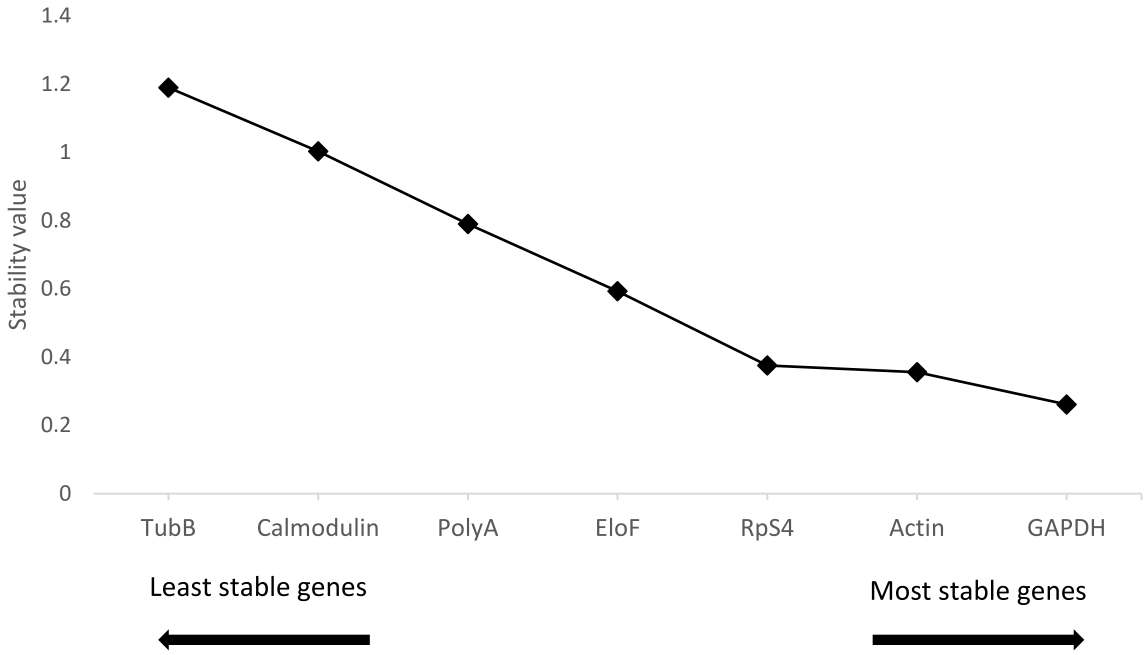


Target: PEPC-1Slope: -3.348 Y-inter: 32.109 R^2: 0.991 Eff: 99%

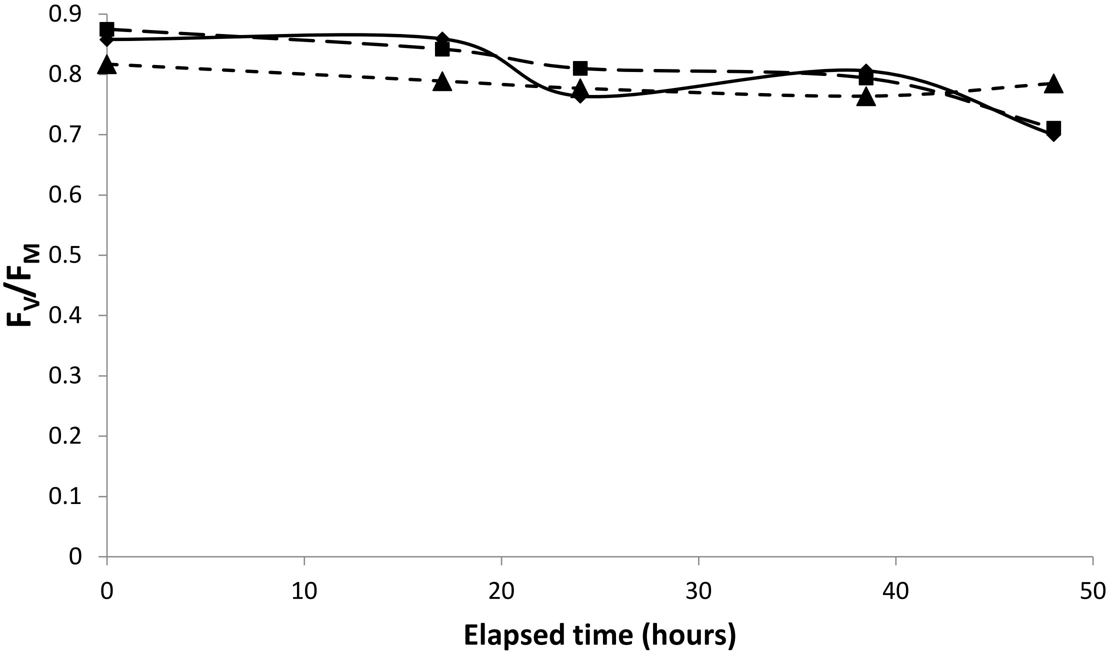


Target: γ-CA Slope: -3.23 Y-inter: 30.514 R^2: 0.987 Eff: 104%

Supplementary Figure S1. 4. Standard curves, efficiency and R^2 for all the reference and target genes investigated in this study: (A) SRp4: 30S ribosomal protein S4; (B) Actin; (C) PolyA: Poly(A) RNA polymerase; (D) GADPH: Glyceraldehyde 3-phosphate dehydrogenase; (E) TubB: Tubulin beta-1 chain; (F) Calmo: Calmodulin; (G) EloF1: Translation initiation factor 1 subunit beta (H) PEPC-2: Phospho*enol*pyruvate carboxylase – isoform 2; (I) PEPC-1: Phospho*enol*pyruvate carboxylase – isoform 1; (J) γ-CA: γ-Carbonic anhydrase



Supplementary Figure S1. 5. Analysis of expression stability of 7 potential reference genes using Normfinder software in Zostera muelleri exposed to low O_2 level. Average expression stability value (lower M values corresponding to more stable gene expression, as defined by Normfinder) for each candidate gene. TubB: Tubulin beta-1 chain; Calmodulin; PolyA: Poly(A) RNA polymerase; EloF: Translation initiation factor 1 subunit beta; RpS4: 30S ribosomal protein S4; Actin; GADPH: Glyceraldehyde 3-phosphate dehydrogenase



Supplementary Figure S1. 6. Maximum quantum yield of photosystem II (F_V/F_M) of leaves: 1 (diamonds), 2 (squares) and 3 (triangles) over the microsensor experimental period of 48 hours as measured by PAM-fluorometry.

Appendix 2: Effect of reduced irradiance on ^{13}C uptake, gene expression and protein activity of the seagrass *Zostera muelleri*

Mikael Kim[1], Mathieu Pernice[1], Alexander Watson-Lazowski[2,3], Paul Guagliardo[4], Matt R. Kilburn[4], Anthony W. D. Larkum[1], John A. Raven[1,5] and Peter J. Ralph[1].

AFFILIATIONS

[1]University of Technology Sydney, Climate Change Cluster, Ultimo NSW 2007, Australia

[2]Hawkesbury Institute for the Environment, University of Western Sydney, Penrith, Australia

[3]ARC Centre of Excellence for Translational Photosynthesis, Australian National University, Canberra, Australia

[4]Centre for Microscopy, Characterisation and Analysis, University of Western Australia, Perth, WA, Australia

[5]Division of Plant Science, University of Dundee at the James Hutton Institute, Invergowrie, Dundee DD2 5DA, UK

CORRESPONDING AUTHOR

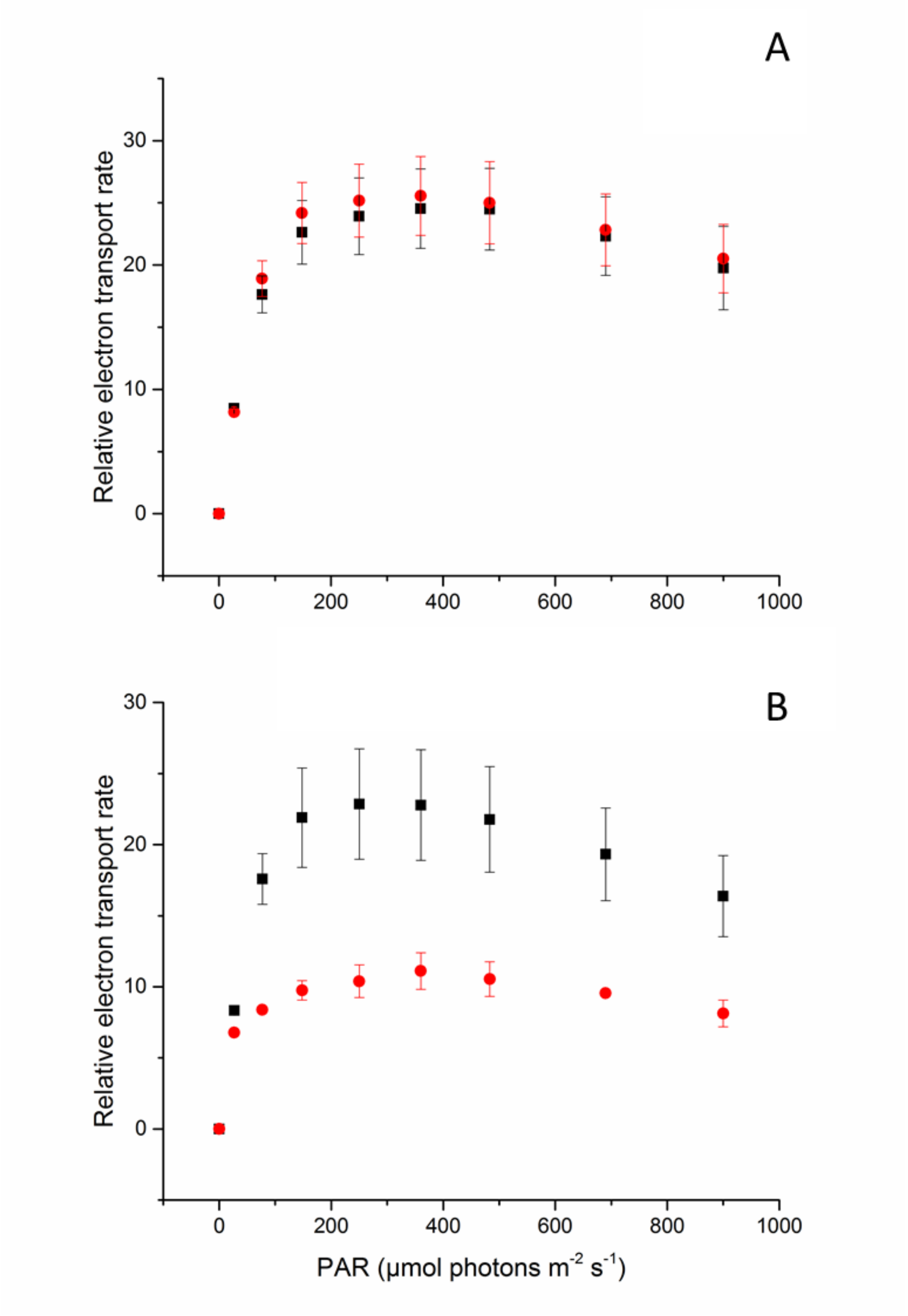
Mathieu Pernice

[1]Climate Change Cluster, University of Technology Sydney, New South Wales 2007, Australia.

Email: mathieu.pernice@uts.edu.au

Phone: +61295144162

Fax: +61295144079


A
Relative electron transport rate
0
10
20
30
0
200
400
600
800
1000
B
Relative electron transport rate
0
10
20
30
0
200
400
600
800
1000
PAR (µmol photons m^{-2} s^{-1})

Supplementary Figure S2. 1. Rapid light curves. Relative electron transport rate measured immediately prior to low light treatment (A) and after 48 h of low light treatment (B). Black squares represent the control group and red circles represent low light treatment group. Error bars are SE, n=4.

Supplementary Table S2. 1. Details of target genes. NanoSTRING Reporter and Capture probes were labelled using their Gene identifiers and Accession number, and designed to target a specific sequence.

Identifier	**Accession**	**Position**	**Target Sequence**
Putative phosphate, pyruvate dikinase regulatory protein (PDRP)	ZM217855.1	1160-1259	TCTGTCGACAAGACAATGCTCGAACTGACCCAACGAGGCATTCACAGCATG CTCCAGTGTCCATCCGGTACCATCTGATACCATGTAGATCACCTTCCCA
Gamma carbonic anhydrase (γCA)	ZM217312.1	684-783	TGGCGCTCTTGTTACTGAGAATACAAGGATTCCTACTGGCGAGGTATGGGC CGGCACACCAGCAAAGTTCCTTAGGAAACTCAAGGATGGGGAAATTGAA
Alanine aminotransferase (ALT)	ZM219590.1	755-854	TGGGTGGTTCACTCATACCCTACTATCTTGAGGAAACTTCTAATTGGGGTCT TGACATTGATGGCCTCCACCAATCAGTAGTTCAGGCTAAAATGAACGG
Serine glyoxylate aminotransferase (SGAT)	ZM212667.1	435-534	CGTGGGAAAGCGCACTCACCAACACTTTATCTCCCGGTGATCGGATCGTTTC GTTTAGCATCGGCCAATTCAGCCTCCTCTGGATCGATCAACAGAAGCG
Glycine dehydrogenase decarboxylating (GLDC)	ZM189396.1	3096-3195	GGTCATTCAAGAATACTGCCGGAATCGAGCCTGAAGATGTTGCCAAACGGC TTATAGACTACGGATTCCATGGCCCGACCATGTCATGGCCTGTTCCTGG
Serine hydroxymethyltransferase (SHMT)	ZM212667.1	435-534	CGTGGGAAAGCGCACTCACCAACACTTTATCTCCCGGTGATCGGATCGTTTC GTTTAGCATCGGCCAATTCAGCCTCCTCTGGATCGATCAACAGAAGCG
Photosystem 1 P700 apoprotein A2 (psaA2)	ZM006917.1	1781-1880	CTTGGCGTGGATATTGGCAGGAATTGATTGAAACTTTAGCATGGGCTCATG AACGCACACCTTTAGCTAATTTAATTCGATGGAGAGATAAGCCGGTGGC
Phosphoenol pyruvate carboxylase (PEPC)	ZM219556.1	1143-1242	GGCTGTCGTGGCCACAGATGAATACCGATCTATTGTTTTCAAAGAACCTCGC

			TTTGTCGAGTATTTCCGGCTTGCGACACCCGAGACAGAGTACGGACGC
Rubisco large subunit (RBL)	ZM194765.1	831-930	TGCCGAAGCTATTTATAAAGCACAATCTGAAACTGGTGAAATTAAAGGGCA TTACTTGAATGCTACTGCGGGTACGTGTGAAGAAATGATAAAAAGAGCC

Appendix 3: Effect of carbonic anhydrase inhibitors on photosynthesis, ^{13}C enrichment and gene expression in the seagrass *Zostera muelleri*

Supplementary Figures and Tables 3

Supplementary Table S3. 1. Details of reference genes and target genes. NanoSTRING Reporter and Capture probes were labelled using their Gene identifiers and Accession number and designed to target a specific sequence.

Identifier	Accession	Position	Target Sequence
putative ubiquitin conjugating enzyme	ZM171867	582-681	AGAACTCGGATTTACCATTGTAACGTGGATCCAAATGGGAACCTTAGCCTTGGGATTTTGAAAGCCAATGAAGGATGGAGTCCTGCATTGACAATCTCCG
UTP:RNA uridylyltransferase 1	ZM243959	2151-2250	CTTTTTACAGCAATGTAGACCTCCCATTCTTCCATGCTTACAGGAAATGAGAGCTACTTATGTTGTGACTGTTGAAGGCAAGAATTGTGCTTTCTTTGAC
Ubiquitin fusion degradation protein	ZM219941	603-702	CTATAAGGAGCCAGAGAGGCCTGCAGCTCCAATCCCTACAAGCAAGGTTTCTATGCAAGCTGAATCTGAAGCAGAACCTGAGGTGCCTAAATTCAACCCC

Glycine hydroxymethyltransferase	ZM227042	1012-1111	AGGAAAGGGTTAAAGGGGACAAACAAACAAGGAAAAGAGATTTTCTATGATTTCGAAGATAAGATCAATGCTGCTGTCTTTCCTGGACTTCAAGGAGGCC
Alanine aminotransferase (ALT)	ZM219590	755-854	TGGGTGGTTCACTCATACCCTACTATCTTGAGGAAACTTCTAATTGGGGTCTTGACATTGATGGCCTCCACCAATCAGTAGTTCAGGCTAAAATGAACGG
Glycine dehydrogenase decarboxylating (GLDC)	ZM189396	3096-3195	GGTCATTCAAGAATACTGCCGGAATCGAGCCTGAAGATGTTGCCAAACGGCTTATAGACTACGGATTCCATGGCCCGACCATGTCATGGCCTGTTCCTGG
photosystem I P700 apoprotein (psaA2)	ZM006917	1781-1880	CTTGGCGTGGATATTGGCAGGAATTGATTGAAACTTTAGCATGGGCTCATGAACGCACACCTTTAGCTAATTTAATTCGATGGAGAGATAAGCCGGTGGC
Serine glyoxylate aminotransferase (SGAT)	ZM212667	435-534	CGTGGGAAAGCGCACTCACCAACACTTTATCTCCCGGTGATCGGATCGTTTCGTTTAGCATCGGCCAATTCAGCCTCCTCTGGATCGATCAACAGAAGCG
Light-harvesting complex II chlorophyll a/b binding protein (LHCB)	ZM222974	701-800	ACCCAGAAGCCTTCGCCGAGTTGAAGGTGAAGGAGTTGAAGAACGGAAGACTGGCCATGTTCTCCATGTTCGGATTCTTCGTTCAGGCCATCGTCACCGG
Glutathione disulfide reductase (GSR)	ZM255687	208-307	TCTACAAATACGGATAAAGTTGTGGGTGTTCATATGTGCGGAGAGGATGCACCAGAAATAATGCAGGGAATTGCTATTGCTGTGAAAGCTGGACTTTCCA

putative pyruvate, phosphate dikinase regulatory protein (PDRP)	ZM217855	1160-1259	TCTGTCGACAAGACAATGCTCGAACTGACCCAACGAGGCATTCACAGCATGCTCCAGTGTCCATCCGGTACCATCTGATACCATGTAGATCACCTTCCCA

Appendix 4: Synthesis, limitations, perspective research and concluding remarks

Supplementary Figures and Tables 4

Supplementary Table S4. 1. Details of all 42 genes included in all NanoString analyses. Reporter and Capture probes were labelled using their Gene identifier and Accession number and designed to target the specified sequence at the specified position. Reference genes are indicated by *.

Gene Identifier	**Accession**	**Target Sequence**	**Position**
2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase-like protein	ZM009775	TGCTTTCCAAAAAAACTTCATCCATGAACTCCTGACTGATGCCATGGTTGATTATATAGAAAAATCCAGAATCTAGGCACGCCTGTTTGAGAAGACGGAC	6973-7072
Abscisic acid 8'-hydroxylase 3	ZM191538	AAGACCATGGATTGGGAGAACAAAGGGACCGAACTGAATCTCATTACTTTTCCTTGATCCCGACAAAGACCATCTGTATTTTGTGGTGAGGTGGTGCAGG	338-437
Alanine aminotransferase (ALT)	ZM219590	TGGGTGGTTCACTCATACCCTACTATCTTGAGGAAACTTCTAATTGGGGTCTTGACATTGATGGCCTCCACCAATCAGTAGTTCAGGCTAAAATGAACGG	755-854
Armadillo-like helical *	ZM249151	CACAGTAAGGCAGTATTCTCTCTTCTAATGCCCTACACAAATCACCAACCACACCAACAGTGATTGTGCAAATCTGGTACTCCGAAAAATTCTGGAGACC	605-704
Aspartate aminotransferase	ZM229092	CCTGATTTCGACACCCCTGCTCCCATCGTCGAGGCTGGGATGAATGCTATACGCGATGGGCATACAAGGTATACTCCAAATGCGGGAACTTTGGAGATTC	449-548
Carbonic anhydrase chloroplastic	ZM226270	CCCATACTTGCTGCCACCCTGCATGTTTTGTCTGTAAACAAGGCTCCACTTCTCAAATGTACAGTCGATGAAATTGTAGTAGCCACCATGAATGGAGAGC	318-417

Chlorophyll a/b binding protein	ZM188022	GTCGAGCATCGGTTGGATGTTTTGCTAGACCGAGTGGGTTGAAAAGCGGACCTCCTGGGTAGCCAGGCTCGAAACCTTCCAAGGAGTCTTCAATACCAAA	345-444
D glucerate 3 kinase chloroplastic	ZM243189	AAGAACAGGCCACAATGAGGAAACAAAATCCTGGAAATGCTTTATTAGAGTTTATTCCCTGCCATTTCAGTTTCGAGGGAATGCAGGGAGTCATGATCTT	197-296
Early light-induced protein	ZM206470	CAAGTGGACCACCCTTCAAGTACTCAGTGAAAACCAATGCAACAAGCCCAAGCATGGCGAGCCTTCCGTTCCACATCTCGGCTTTCGAGCTCATCACCTG	890-989
Eukaryotic translation initiation factor 2 subunit beta *	ZM209462	CTGCCTTGATAGGTGCCACTGATCGAGAAGATCCACACTGCTCGCATCGAAGAAAGAAGAGACGGTTCTCCTTGATAAGAATGGTGTCTGGACTCTTGCA	313-412
Gamma carbonic anhydrase (γCA)	ZM217312	TGGCGCTCTTGTTACTGAGAATACAAGGATTCCTACTGGCGAGGTATGGGCCGGCACACCAGCAAAGTTCCTTAGGAAACTCAAGGATGGGGAAATTGAA	684-783
Glutamate ammonia ligase	ZM187860	CTGGAATTGGCTTAGGGTCAAATGAGAGCACAACACCAGAAATCTCAGTGATCCTCTCAAGAAGGTAGCGTGCAATCCATACTTCGTCACCAGCAGCAAT	555-654
Glutathione disulfide reductase (GSR)	ZM255687	TCTACAAATACGGATAAAGTTGTGGGTGTTCATATGTGCGGAGAGGATGCACCAGAAATAATGCAGGGAATTGCTATTGCTGTGAAAGCTGGACTTTCCA	208-307

Glutathione Peroxidase	ZM200094	AAGATTGGGTATTCAGCCTTGAAGCGTGTGCAAGCAAATTCCACAATCTGTTCGTTGTTTCCGGGCTCCTGCCCTCCAAACTGATTACAAGGAAAGGCCA	424-523
Glutathione synthase	ZM204808	TAGAGCTCAACACGATTGCTAGCTCATTTTCAGGTCTCAGTTGTATTGTCGGTGAACTTCACAGGAACTTGATCAATCATTATGGGAAGAATCTTGGATT	710-809
Glycine dehydrogenase decarboxylating (GLDC)	ZM189396	GGTCATTCAAGAATACTGCCGGAATCGAGCCTGAAGATGTTGCCAAACGGCTTATAGACTACGGATTCCATGGCCCGACCATGTCATGGCCTGTTCCTGG	3096-3195
Glycine hydroxymethyltransferase	ZM227042	AGGAAAGGGTTAAAGGGGACAAACAAACAAGGAAAAGAGATTTTCTATGATTTCGAAGATAAGATCAATGCTGCTGTCTTTCCTGGACTTCAAGGAGGCC	1012-1111
Glyoxylateghydroxypyruvate reductase B	ZM253771	GAACTCGTCAGCCTCAACAATTCTTCTTGCCGCCGACACAGAAAGAGAAGCTGCTAGCTCAGCAGTTGTCTCAGTTAGAACTCCAGGAGTGTTGCCAACA	1027-1126
GRAM domain containing protein gABA responsive	ZM239651	TTGGTTCATGGGGTTTGTTAACTACAGGAGGTCCCTGAAGCACTTGCAGATGGCAATGATGCAGGACACACCATTGAATTTTCGCCAGAGAAGCAAGAGA	715-814
Light-harvesting complex II chlorophyll a/b binding protein (LHCB)	ZM222974	ACCCAGAAGCCTTCGCCGAGTTGAAGGTGAAGGAGTTGAAGAACGGAAGACTGGCCATGTTCTCCATGTTCGGATTCTTCGTTCAGGCCATCGTCACCGG	701-800

Malate dehydrogenase	ZM220964	TCAGCTGATATAATGGCCTCTACACCTTTAACCCCAAGCTTGAGCCTAGAAGCAAAGAAAGGCAGATCTGTCAGTTGAGACTGCACAAATGAACACTCAT	439-538
Manganese superoxide dismutase	ZM212939	ATGTGGAGCTTCACCACCTCCGTCTTTTGTGGGAGCAAGATTCTTCCAGAAAATGGAGTGATTGACATGTCCTCCGCCATTGAACTTGATAGCTCCCTGG	546-645
MngFe superoxide dismutase family	ZM223388	ACACTGGAATATCATTGGGGCAGACATCACAAGAATTATGTGGAAGGCTTGAATAGACAGATTGTTGGAACTGAACTGGATGCGGTTTCATTGGAGGACA	303-402
NAD dependent ME	ZM230971	TCCAATTGAAATTCCAATACCTTGGATCCCAAGATCACCAAGTCCCAATATTCTACTTCCATCTGTGACAACTATCATATCTACCTGATCAGCTGGCCAA	1398-1497
NADH dependent Glutamate synthase 1 cytosolic	ZM256572	TCACATCCGCAAGCACCACGATGCGCCATCCTCTTCAACATCTCGATTCCATCCACCACAATCTTCCTTTTGTACTCTCCAGACAACTCGGCAACAAACC	6300-6399
Phage shock protein A, PspA	ZM007466	CATATATCTTGCTTTCCAAATGCCGAGAATTGGAGACAAGATTGTCAACTACAGTTTTCTGCTGGTCAAGCTGAGATTTCAAAGAAGTTGCATTATCCTG	632-731
Phosphoenol pyruvate carboxylase (PEPC)	ZM219556	GGCTGTCGTGGCCACAGATGAATACCGATCTATTGTTTTCAAAGAACCTCGCTTTGTCGAGTATTTCCGGCTTGCGACACCCGAGACAGAGTACGGACGC	1143-1242

Phosphoglycolate phosphatase	ZM216514	GCCAAATAGTCCATCATAAAAGTTGAAGGCTTTCCTACAACTAGAGGCTCTCTTTGTGTTGAGCCTATAAGGGCTCCAACCATTGATCCGCCACCTGCCC	539-638
Phosphoribulokinase	ZM218838	ACTTCATTGCTGATGTCTAGGTAAATACTGAAGTCCAACAGATCCCGGACACGAGAATCATACATTGGGTGCAGTCCTTCAATGACCAGGATCTTGGGAG	1175-1274
Photosystem I P700 apoprotein (psaA2)	ZM006917	CTTGGCGTGGATATTGGCAGGAATTGATTGAAACTTTAGCATGGGCTCATGAACGCACACCTTTAGCTAATTTAATTCGATGGAGAGATAAGCCGGTGGC	1781-1880
Phototropin 2	ZM253255	CCGGTCCCTGAAGAAAACGACAGTTTCTTCCAATGACTTCATCTGGAGCATACCCAGTCATACTAAAAAAACCAGCACTTGCATAAGTAATAGGGAAGTC	2457-2556
Phytochrome	ZM254591	TCGGGATTCACCTATCAGTCTTGTCAACCAAAGTCCCAGCATAATGGACCTGGTGAAATGTGACGGCACGGCGTTGTACCACCAGGGTAAGTACTTCTCA	1534-1633
Proline dehydrogenase	ZM232377	GCGAGGACGCAAAACTAATCGTATACGGCACCGTGCAAGCGTACTTGAAGGATTCCATCGAAAGACTCGCTCTTGCTTCTGATGCGGCGAGGAAACACGG	1004-1103
Protein transport protein SEC31 *	ZM251805	TGGTACACAAAGATACCTGGGATAATATCTGCATCATCCTATGATGGGAAAATTGGAATTTACAACATGGAGGCTTGTAGCAGACAGACAGTCAATGATG	1208-1307

Putative pyruvate, phosphate dikinase regulatory protein (PDRP)	ZM217855	TCTGTCGACAAGACAATGCTCGAACTGACCCAACGAGGCATTCACAGCATGCTCCAGTGTCCATCCGGTACCATCTGATACCATGTAGATCACCTTCCCA	1160-1259
Putative ubiquitin conjugating enzyme	ZM171867	AGAACTCGGATTTACCATTGTAACGTGGATCCAAATGGGAACCTTAGCCTTGGGATTTTGAAAGCCAATGAAGGATGGAGTCCTGCATTGACAATCTCCG	582-681
Pyruvate phosphate dikinase	ZM204646	CAGACGAATGGTGACCGGAAAGCCATCCATTGCACGGAAAATTCCTTCAAAATCAGCTTTTTGGTAGGGCAAGAGGAGGTTCAATGCCTTCTTTCGCTGC	1142-1241
Rubisco large subunit (RBL)	ZM194765	TGCCGAAGCTATTTATAAAGCACAATCTGAAACTGGTGAAATTAAAGGGCATTACTTGAATGCTACTGCGGGTACGTGTGAAGAAATGATAAAAAGAGCC	831-930
Serine glyoxylate aminotransferase (SGAT)	ZM212667	CGTGGGAAAGCGCACTCACCAACACTTTATCTCCCGGTGATCGGATCGTTTCGTTTAGCATCGGCCAATTCAGCCTCCTCTGGATCGATCAACAGAAGCG	435-534
Serine hydroxymethyltransferase (SHMT)	ZM212667	CGTGGGAAAGCGCACTCACCAACACTTTATCTCCCGGTGATCGGATCGTTTCGTTTAGCATCGGCCAATTCAGCCTCCTCTGGATCGATCAACAGAAGCG	435-534
Ubiquitin fusion degradation protein *	ZM219941	CTATAAGGAGCCAGAGAGGCCTGCAGCTCCAATCCCTACAAGCAAGGTTTCTATGCAAGCTGAATCTGAAGCAGAACCTGAGGTGCCTAAATTCAACCCC	603-702

UTP:RNA uridylyltransferase 1 *	ZM243959	CTTTTTACAGCAATGTAGACCTCCCATTCTTCCATGCTTACAGGAAATGAGAGCTACTTATGTTGTGACTGTTGAAGGCAAGAATTGTGCTTTCTTTGAC	2151-2250
Zeaxanthin epoxidase chloroplastic	ZM232355	TTGACAGACATGAACAGTAAACATGGTACCTGGCTAACCGATAACGAAGGAAGAAGATGCCGCGTTCCACCCAACTTCCCAGTTCGATTTCGTCCATCGG	2145-2244

Appendix 5: Further contributions

Development of an efficient protein extraction method compatible with LC-MS/MS for proteome mapping in two Australian seagrasses *Zostera muelleri* and *Posidonia australis*

frontiers
in Plant Science

ORIGINAL RESEARCH
published: 15 August 2017
doi: 10.3389/fpls.2017.01416

Development of an Efficient Protein Extraction Method Compatible with LC-MS/MS for Proteome Mapping in Two Australian Seagrasses *Zostera muelleri* and *Posidonia australis*

Zhijian Jiang[1,2†], *Manoj Kumar*[2*†], *Matthew P. Padula*[3], *Mathieu Pernice*[2], *Tim Kahlke*[2], *Mikael Kim*[2] *and Peter J. Ralph*[2]

[1] Key Laboratory of Tropical Marine Bio-resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, China, [2] Climate Change Cluster (C3), Faculty of Science, University of Technology Sydney (UTS), Sydney, NSW, Australia, [3] Proteomics Core Facility, University of Technology Sydney (UTS), Sydney, NSW, Australia

The availability of the first complete genome sequence of the marine flowering plant *Zostera marina* (commonly known as seagrass) in early 2016, is expected to significantly raise the impact of seagrass proteomics. Seagrasses are marine ecosystem engineers that are currently declining worldwide at an alarming rate due to both natural and anthropogenic disturbances. Seagrasses (especially species of the genus *Zostera*) are compromised for proteomic studies primarily due to the lack of efficient protein extraction methods because of their recalcitrant cell wall which is rich in complex polysaccharides and a high abundance of secondary metabolites in their cells. In the present study, three protein extraction methods that are commonly used in plant proteomics i.e., phenol (P); trichloroacetic acid/acetone/SDS/phenol (TASP); and borax/polyvinyl-polypyrrolidone/phenol (BPP) extraction, were evaluated quantitatively and qualitatively based on two dimensional isoelectric focusing (2D-IEF) maps and LC-MS/MS analysis using the two most abundant Australian seagrass species, namely *Zostera muelleri* and *Posidonia australis*. All three tested methods produced high quality protein extracts with excellent 2D-IEF maps in *P. australis*. However, the BPP method produces better results in *Z. muelleri* compared to TASP and P. Therefore, we further modified the BPP method (M-BPP) by homogenizing the tissue in a modified protein extraction buffer containing both ionic and non-ionic detergents (0.5% SDS; 1.5% Triton X-100), 2% PVPP and protease inhibitors. Further, the extracted proteins were solubilized in 0.5% of zwitterionic detergent (C7BzO) instead of 4% CHAPS. This slight modification to the BPP method resulted in a higher protein yield, and good quality 2-DE maps with a higher number of protein spots in both the tested seagrasses. Further, the M-BPP method was successfully utilized in western-blot analysis of phosphoenolpyruvate carboxylase (PEPC—a key enzyme for carbon metabolism). This optimized protein extraction method will be a significant stride toward seagrass proteome mining and identifying the protein biomarkers to stress response of seagrasses under the scenario of global climate change and anthropogenic perturbations.

Keywords: seagrass, proteomics, 2D-IEF, *Zostera muelleri*, *Posidonia australis*, LC-MS/MS

Edited by:
Karl-Josef Dietz,
Bielefeld University, Germany

Reviewed by:
Sudisha Jogaiah,
Karnatak University, India
Silvia Mazzuca,
University of Calabria, Italy

***Correspondence:**
Manoj Kumar
manoj.kumar@uts.edu.au

† These authors have contributed equally to this work.

Specialty section:
This article was submitted to Plant Abiotic Stress, a section of the journal Frontiers in Plant Science

Received: *12 April 2017*
Accepted: *31 July 2017*
Published: *15 August 2017*

Citation:
Jiang Z, Kumar M, Padula MP, Pernice M, Kahlke T, Kim M and Ralph PJ (2017) Development of an Efficient Protein Extraction Method Compatible with LC-MS/MS for Proteome Mapping in Two Australian Seagrasses Zostera muelleri and Posidonia australis. Front. Plant Sci. 8:1416. doi: 10.3389/fpls.2017.01416