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

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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 to measure changes in expression levels of key genes involved in C₄ metabolism. We found that very low levels of oxygen (i) altered the photophysiology of *Z. muelleri*, a characteristic of C₃ mechanism of carbon assimilation, and (ii) decreased the expression levels of phosphoenolpyruvate carboxylase and carbonic anhydrase. These molecular-physiological results suggest that regulation of the photophysiology of *Z. muelleri* might involve a close integration between the C₃ and C₄, or other CO₂ 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.

Keywords Diffusive boundary layer · Photosynthesis · Respiration · RT-qPCR · Seagrass

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 year⁻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 nighttime 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; Staehrl 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 O2 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 C3 plants on the biochemical criteria of short-term inorganic 14C incorporation products and the ratio of ribulose-1,5-bisphosphate carboxylase–oxygenase (RuBisCO) to phosphoenolpyruvate carboxylase (PEPC) activities, although Thalassia testudinum has C4 metabolism (Benedict and Scott 1976) and Halophila stipulacea has C$_{5}$–C$_{4}$ intermediate metabolism by these criteria (Beer et al. 1980, 2002; Koch et al. 2013). The C3 CO2 fixation process begins with RuBisCO which catalyses the carboxylation using CO$_2$ (and H$_2$O) 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 O2 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 C4 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. C4 photosynthesis involves a higher steady-state CO$_2$ concentration than that available to RuBisCO in biochemically and physiologically defined C4 photosynthesis with diffusive entry of CO$_2$ from the bulk external medium to RuBisCO, i.e. C4 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 C4 mechanism has an advantage over the C3 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$_4$ plants, to a much smaller extent, the 2-phosphoglycolate from the oxygenase reaction is metabolized 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$_4$ plants relative to C$_3$ 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. beccarii), showed short-term inorganic 14C 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 14C labelling is the most conclusive method 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)
The occurrence of PEPC and aspartate aminotransferase in *H. becarii* does not signify a C₄ pathway, since these enzymes are ubiquitous in plants (Aubry et al. 2011). HCO₃⁻ is the predominant inorganic C species in seawater (at pH 8.16: 85.8% HCO₃⁻, 0.4% CO₂ and 13.7% CO₂²⁻, Pierrot et al. 2006) with CO₂ at about the same concentration (mol m⁻³ of fluid medium) as in air. However, the diffusion coefficient for CO₂ in water is about 10⁻⁴ than 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), CO₂ diffusion to the leaf surface, and O₂ 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 HCO₃⁻ is lower than that of CO₂ (Raven 1984), the quantitative predominance of HCO₃⁻ in seawater means that it can support a larger flux to the leaf surface in response to a given potential for CO₂ assimilation in the leaf, provided the leaf can use HCO₃⁻. Some seagrasses can utilize HCO₃⁻ either directly via active transport into epidermal cells (Beer and Rehnberg 1997), or more commonly, indirectly by dehydrating HCO₃⁻ to CO₂ via the enzyme carbonic anhydrase (CA) in the epidermal cell wall (Beer et al. 1980) usually interacting with co-localized 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 C₄ 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 (Beer et al. 1980) has subsequently been shown to be invalid. Some freshwater submerged aquatic plants and some terrestrial C₄ members of the Chenopodiaceae utilize C₄ photosynthesis via the fixation of external inorganic C by PEPC, and the fixation of CO₂ (regenerated from C₄ 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 C₄ carbon fixation pathway in seagrass transcriptome could provide evidence relevant to the argument that seagrasses are not strictly C₃ plants.

For instance, several genes coding for PEPC, a cytosolic enzyme essential for the C₄ 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 HCO₃⁻ to produce oxaloacetate (as described previously), a key intermediate in the C₄ 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, 2014; Raven and Farquhar 1990; Aubry et al. 2011). 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 C₄ photosynthesis and also to other aspects of metabolism in the 4 completely sequenced tracheophytes (2 with C₃ photosynthesis, 2 with C₄ photosynthesis) and 1 completely sequenced C photosynthesis bryophyte. For PEPC, the 2 C₄ plants had 3 or 4 copies of the gene, while the 2 C₃ 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 C₃ plant.

Furthermore, genes encoding CA were also detected in the Z. muelleri transcriptome (unpublished data). CA catalyses the reversible interconversion of HCO₃⁻ to CO₂ (HCO₃⁻ + H⁺ = CO₂ + H₂O). CAs are also involved in several non-photosynthetic reactions in plants (Raven 2014 and references therein), possibly including provision of respiratory CO₂ to HCO₃⁻ 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 C₄-based and other CCMs, as well as in C₃ photosynthesis (Aubry et al. 2011; Raven 2014; Raven and Beardall 2016).

However, expression of C₄ photosynthetic metabolism in some submerged freshwater relatives of seagrasses is a function of environmental conditions (low CO₂, high O₂) of terrestrial C₄ 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 O₂ concentration and hence the potential for RuBisCO oxygenase activity, noting that the other light-dependent O₂ 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).

**Materials 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′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 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 h, 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 containers resulting in a total of 8 containers (4 replicate containers ×2 sumps/O$_2$ treatments = 8 containers in total). Each container contained 2 individual shoots of *Z. muelleri* (8 tanks ×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 Fig. S2). 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 Fig. S3). The control tank setup 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 AS, Aarhus, Denmark; Revsbech 1989) with a fast response time (<0.5 s) mounted on a motorized micromanipulator (Unisense AS, Aarhus, Denmark). The microsensor was connected to a multimeter (Unisense Microsensor Multimeter AS, Aarhus, Denmark) and interfaced with a PC running dedicated data acquisition and positioning software (SensorTrace PRO; Unisense AS, 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 O₂ concentrations for micro-profiles were measured at 100 µm increments using the motorized micromanipulator (Unisense Motorised Micromanipulator A/S, Aarhus, Denmark) controlled by dedicated positioning software (SensorTrace PRO). Linear calibration of the O₂ microsensor was obtained from signal readings in 100% air-saturated seawater and anoxic seawater (seawater amended via N₂ bubbling and the O₂ scavenger sodium dithionite) at air-saturated seawater and pH and temperature.

Seagrass maximum quantum efficiency of photosystem II (Fₘ/Φₘ; 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 (F₀) was recorded using a weak measuring light, which was then followed by a saturating pulse (irradiance of 3500 µmol photons m⁻² s⁻¹ for 0.8 s) to measure maximal fluorescence (Fₘ). Under these conditions, Fₘ/Fₘ⁰ ratios provide a measure of maximal PSII photochemical efficiency (Fᵥ = Fₘ – F₀) 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 O₂ 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 O₂ concentration gradient until it intersects with the constant O₂ concentration in the overlaying water. These O₂ micro-profiles were ran-].

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 O₂). 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 O₂ 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

where $D_0$ is the diffusion coefficient of O₂ in seawater at the experimental salinity and temperature ($2.2088 \times 10^{-5}$ cm⁻² s⁻¹; tabulated values taken from http://www.unisense.com) and $\frac{\Delta C}{\Delta z}$ is the gradient of the linear O₂ concentration within the DBL.

The measured O₂ 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 O₂ consumption (Spilling et al. 2010):

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

where $\alpha$ is the initial slope of the P–I curve in the light-limited 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 O₂ produced via photosynthesis equals the respiratory demands, using the following equations (e.g. Lichtenberg and Kühl 2015):

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

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

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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 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.

**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-well plates in a Step One Plus™ 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 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):

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

All the RT-qPCR efficiencies obtained with the different primers were between 96 and 104%, with a calibration coefficients > 0.969 (Table 1, see Supplementary Fig. S4). 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.

| Name     | Accession number | Forward primer | Reverse primer | Length (bp) | T<sub>m</sub> | C<sub>T</sub> | Efficiency (%) |
|----------|------------------|----------------|----------------|-------------|-------------|-------------|----------------|
| GAPDH    | Zoma_C_c6252     | CGGTTACCTGAGCCACCTCGT | CAAGGCTGGGATTTGTTA | 79          | 59.9        | 25          | 88             |
| EloF     | Zoma_C_c59090    | AAGCAAAAGGCTACCTTGAT | TCTGCTGCTTTTCTTCTTCT  | 82          | 59.9        | 24          | 104            |
| Calmodulin | Zoma_B_i07192    | ATCCATCTTGCTTCTTTGCG | CACTGTGAATCCACTCGTGTTG | 197         | 60.1        | 23          | 114            |
| TubB     | Zoma_Concontig120 | GGCAAAATCTCTCCGTCAGA | TCCAGATCCAGTTCCACCTC  | 195         | 60.0        | 24          | 88             |
| Actin    | Zoma_ZMF02257    | TAAGGTGCTGCTCTCCTCTG | ACTCGGCTGTTGCAATCCACCA | 104         | 60.4        | 26          | 110            |
| PolyA    | Zoma_C_c36619    | GCTGTCGTTCAACTTCTCTC | ATGACCGCATTATAATCTGC | 112         | 59.9        | 29          | 93             |
| S4       | Zoma_Concontig219 | GCTGTGCTACAGAAGCGCA | GTGTTATCAAAAGCATCTCG | 108         | 59.7        | 29          | 114            |
| PEPC-1   | KMZ56135         | AGGCAAAATCTCGAAGCTTCA  | GAGGAGCAGTGTTGACAGAA  | 84          | 60.1        | 29          | 99             |
| PEPC-2   | KMZ58048         | TGCCGGCCTAGACCTCAGAG | TCTGCTCTGCTGTTGGCAAG  | 91          | 60.0        | 28          | 97             |
| γ-CA     | KMZ56166         | AGGTCAATGCTGCTCTCTC | CAGCAACCAATCCGGTTCTT | 110         | 60.1        | 28          | 104            |

Table 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/) primer sequences, amplicon length, melting temperature, geometric mean of cycle threshold (CT) and RT-qPCR efficiency are indicated. GAPDH: 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
Data from RT-qPCR was analysed using the Step One Plus™ 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 Fig. S5) 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 \( \alpha, P_{max}, R, E_{k} \) 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 \( \gamma \) 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 container locations 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 \( \sim 0.02 \)-cm-thick DBL at the leaf surface of \( Z. \) muelleri at all irradiances tested in both the control and low \( O_2 \) conditions (Fig. 1). The average \( O_2 \) concentration at the leaf tissue surface of the control plants increased from 203 to 352 \( \mu \)mol \( O_2 \) L\(^{-1}\) as a response to an increasing incident irradiance from 0 to 100 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\), as compared to an increase from 3.6 to 187 \( \mu \)mol \( O_2 \) 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 for incident photon irradiances of 0, 25 and 50 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\) (permutational \( t \) test, \( t4 = 4.7575, P = 0.0026 \); \( t4 = 12.526, P = 0.0002 \); \( t4 = 5.4299, P = 0.0032 \), respectively, Fig. 2). Comparison between the dark respiration rates (\( R \)) and the initial slope of the P–I curve (\( \alpha \)), 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 \( \alpha \); \( t4 = 6.8879, P = 0.0018 \); \( t4 = 5.2964, P = 0.0064 \), respectively, Table 2); with a decrease in \( R \) but an increase in \( \alpha \).

In contrast, between incident irradiances of 200–700 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\), there was only a slight increase of \( \sim 2 \) \( \mu \)mol \( O_2 \) L\(^{-1}\) in the control plants, from 386 to 388 \( \mu \)mol \( O_2 \) L\(^{-1}\), and an increase of \( \sim 50 \) \( \mu \)mol \( O_2 \) L\(^{-1}\) in the low \( O_2 \)-treated plants from 194 to 246 \( \mu \)mol \( O_2 \) L\(^{-1}\). This translated to net oxygen flux values that were not statistically different among the 200, 500 and 700 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\) irradiance treatments (permutational \( t \) test, \( t4 = 2.2527, P = 0.088 \); \( t4 = 0.65804, P = 0.5314 \); \( t4 = 1.147, P = 0.306 \), respectively, Fig. 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, \( t4 = 0.2956, P = 0.7974 \), Table 2).

Calculations of the \( E_{k} \) of \( Z. \) muelleri plants, in the control and low \( O_2 \) conditions, yielded an average saturation irradiance of \( \sim 60 \) and 51 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\), respectively (Table 2), values that are not statistically different (permutational \( t \) test, \( t4 = 1.2571, P = 0.2892 \); Table 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, \( t4 = 6.5624, P = 0.0012 \), Table 2) which were calculated to be at incident irradiances of \( \sim 5.19 \) and 0.71 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\), respectively (Table 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 Fig. S5). These two
reference genes were then used to evaluate target gene expression profile in *Z. muelleri* under low O₂. The relative quantification demonstrated a significant down-regulation of the PEPC-1 and γ-CA genes for seagrass incubated in low O₂ when compared to control (Fig. 3). Specifically, there was a ~2.2-fold decrease in PEPC-1 gene expression in low O₂-treated samples relative to control (permutational t test, $t_6 = 2.9916, P = 0.0188$, Fig. 3). Similarly, a ~2.8-fold decrease was observed in γ-CA (permutational t test, $t_6 = 3.3414, P = 0.0072$: Fig. 3). No statistical difference was observed in the expression level of the PEPC-2 gene (permutational t test, $t_6, P = 0.0664$, Fig. 3), although a decreasing trend was observed in low O₂-treated samples when compared to the controls.

**Discussion**

**Photosynthetic parameters**

We observed that by lowering the O₂ concentration in the water-column, the net photosynthetic rates of *Zostera muelleri* were enhanced in the light-limited region of the P–I curve (Fig. 2). Further, we confirmed that these results were not due to impacts on the health of the *Z. muelleri* photosystems as shown by the $F_v/F_m$ which remained ≥ 0.7 for the duration of the experimental period (as measured via PAM-fluorometry, see Supplementary Fig. S6). This shows that *Z. muelleri* has a higher photosynthetic activity with an increased CO₂:O₂ 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
Table 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_s) 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).

|                | α       | P_max (nmol O_2 cm^{-2} h^{-1}) | R (nmol O_2 cm^{-2} h^{-1}) | E_c (µmol photons m^{-2} s^{-1}) | E_s (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*                          |

Values are given as a mean ± SEM (n = 3); with their corresponding P values (permutation t test), where * indicates significant difference between treatments on a 5% level.

Fig. 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.

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 (Fig. 2; Table 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 regard 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 O₂ produced via photosynthesis results in similar O₂ microclimates in and around leaves within both treatments, owing to an internal and external build-up of O₂ 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 O₂ and control treatment at photon irradiances ≥ 100 µmol photons m⁻² s⁻¹ (Figs. 1, 2; Table 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 C₄ 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 O₂ conditions, we found a significant 2.2-fold decrease in PEPC-1 gene expression (Fig. 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 (Fig. 3), there was a decline in response to low O₂ conditions. As mentioned in the introduction, although PEPC activity has been widely invoked as evidence of C₄ 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 O₂ 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 O₂, it is important that the implications of these results are not overly extrapolated. Overall, our results indicate that, when exposed to low water-column O₂, Z. muelleri plants (i) increase their photosynthetic activity, a characteristic of C₃ plant photosynthesis and (ii) down-regulate genes coding for PEPC, suggesting that these low O₂ conditions yield lower energy costs of photosynthesis.

HCO₃⁻ can be utilized through extracellular dehydration via CA (Millhouse and Strother 1986a; Beer and Rehberg 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 HCO₃⁻ (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 respiration, probably as a HCO₃⁻ 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: Fig. 3) observed in Z. muelleri plants exposed to low O₂ suggests that this enzyme might be more critical for photosynthesis under ambient O₂ levels (i.e. lower DIC:O₂) than under low O₂ levels.

In the freshwater aquatic monocot Hydrilla verticillata, C₄-type photosynthesis is induced in C₃-type photosynthesizing leaves under warmer temperatures, limited CO₂, increased O₂ 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 a 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 O₂) had one sump (100 L 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 O₂ 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, 2012, 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 Fig. S2).

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 Fig. S3). 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 $\pm0.01$ pH). Since this process occurs continuously, the pH deviates between 0.01 and 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 10 s (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 setup are clearly needed to give more informative results.

Conclusion

Ambiguous metabolic properties such as the ability to use HCO$_3^-$ and a C$\text{}_4$-type photosynthetic quantum efficiency have led to some seagrass species being classified as C$\text{}_3$–C$\text{}_4$ 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$\text{}_3$ plants, and (ii) low O$_2$ levels induce the down-regulation of PEPC and $\gamma$-CA genes. While regulation of these genes might not be strictly associated with a C$\text{}_4$ biochemistry, our data suggest that when the conditions are favourable for the carboxylation reaction of RuBisCO, Z. muelleri down-regulates its CCM(s), thus altering its photosynthesis. 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, $\gamma$-CA and RuBisCO and measurements of short-term (2–5 s) 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.

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Compliance with ethical standards

Conflict 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.

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