Fw: Photosynthetica - Manuscript ID 1145-03-2015

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-----Original Message-----
From: photosynthetica@ueb.cas.cz
Sent: Saturday, March 14, 2015 3:48 PM
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Subject: Photosynthetica - Manuscript ID 1145-03-2015

14-Mar-2015

Dear Prof. Nose:

Your manuscript entitled "An improved method for the simultaneous determination of photosynthetic O2 evolution and CO2 consumption of Rhizopora mucronata leaves under aqueous condition" has been successfully submitted online and is presently being given full consideration for publication in Photosynthetica.

Your manuscript ID is 1145-03-2015.

Please mention the above manuscript ID in all future correspondence or when calling the office for questions. If there are any changes in your street address or e-mail address, please log in to ScholarOne Manuscripts at https://mc.manuscriptcentral.com/photos and edit your user information as appropriate.

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Thank you for submitting your manuscript to Photosynthetica.

Sincerely,
Photosynthetica Editorial Office

Dear Editor-in-Chief of Photosynthetica and Reviewers

Thank you very much for reading our manuscript and giving us many kind advices. We would like to show our responses to editor and reviewers here, and provide the revised manuscript.

The blue color and additional of comments and supplementary file in the revised manuscript were in order to respond the suggestions of 1st, 2nd reviewers and associate editor.

Our explanation for the reviewers and associate editor comments is as follows.

Best regards

Akihiro Nose, Prof. Dr.
Corresponding Author

Explanatios of the reviewers' comments:

Reviewer #1: In this manuscript, the authors simultaneously measured rates of photosynthetic O2 evolution and CO2 consumption of Rhizophora mucronata leaves using conventional Clark-type O2 electrode and CO2 sensor under aqueous condition. They examined effects of sample status (slice or chip), pH and NaHCO3 concentration on both rates. They showed that the photosynthetic quotient (PQ, O2 evolution to CO2 consumption) values were always higher than one, and suggested that photorespiration did not occur under this condition. Some data were interested. But firstly they should show the raw data of changes in O2 and CO2 concentrations in the cuvette using the O2 electrode of Hansatech and the CO2 sensor of Presens as the supplementary data.

>> Can both sensors similarly respond to the gas concentration in the cuvette? Was there any time lag between the changes of concentrations?

Explanation: Thank you for the valuable comments and we are also grateful for the time and energy you expended on our behalf. We agree with your suggestion. As our responses, we show the raw data of changes in O_2 and CO_2 concentrations in the cuvette using the O_2 electrode of Hansatech and the CO_2 sensor of Presens especially related with time lag as the supplementary data (see **Page 15, Supplementary 1**).

- Yes, there was the lag period between the changes of O_2 and CO_2 concentrations. We found the maximum lag period for CO2 consumption was less than 2 min, but was about 3 min for O2 evolution (see **Page 15, Supplementary 1**). Generally, lag period of O_2 evolution after light activation was slightly longer than CO2 consumption but not in significance level. Furthermore, The O_2 evolution and CO_2 consumption rates were calculated from the initial slopes of the curves during linear photosynthetic activity after lag period finished (after around 4 min)**.**

>> Also, why did they measure the photosynthetic rate at 25˚C? Is it a bit low for this species?

Explanation: Moore et al (1973) reported that P_{max} of mangrove *Rhizophora* and *Laguncularia* was obtained at leaf temperature near or below 25 ^{0}C. However, some latter reports indicate that the relationship between the maximum photosynthetic rate and leaf temperature indicated a wide peak between 29 and 34 ^{0}C (Okimoto et al. 2007). Therefore, next step we consider and also want to proceed more detail response of mangrove photosynthetic O_2 evolution and CO_2 consumption rate simultaneously at 30˚C.

Reviewer #2: An improved method for simultaneous determination of photosynthetic O2 evolution and CO2 consumption of Rhizophora mucronata leaves Tengku et al. The paper compares measurement of photosynthesis by traditional gas exchange with liquid phase O2 electrodes in combination of CO2 optopodes. The innovative method seems to function properly and provide comparative measurements to those from gas exchange at high light but not at low light. The paper is within the scope of Photosynthetica, and it is an interesting one. The best recommendation will be to test the method further before to publish the results and may be the paper will become in a Regular one and not just a Brief Comunication. In case the Editor y other reviewers agree that paper can be published, which is acceptable as well, I have some suggestions to improve the text.

>> Introduction: Two and half pages are too long for a brief communication. It should be cut substantially.

Explanation: Thank you very much for your kind suggestions about our paper writing. We agree that some statements which are not important substantially should be cut. As our responses, we make some revisions:
- We omit statement "On these occasions, independent biochemical studies and photosynthesis analyses have been simultaneously conducted to estimate the effects of stress on leaf photosynthesis." (see **Page 1, Line 11**)

- We delete "Furthermore, the possibility of directly determining the O_2 evolution and CO_2 consumption rate of leaf samples simultaneously and to monitor how rates change in response to stimuli will add to our understanding of single leaf to complex ecosystem mechanisms (Strovas *et al*. 2010)." (see **Page 1, Line 11**) and also omit Strovas *et al*. (2010) from references list (see **Page 13, Line 277)**

- We delete statements "The PQ value provides fundamental information on metabolic pathways (Taddei *et al.* 2008), balanced growth (Davies *et al.* 2003) and useful to clarify the primary productivity of an ecosystem (Lee and Bong 2006)." (See **Page 1, Line 23**) and also delete it from references list (see **Page 10, Line 221; Page 11 Line 247)**.

- We omit reference "They belong to the C_3 photosynthetic group of plants, but may also be classified as "seaweeds", since they can grow in submerged and highly saline conditions (Kawamitsu *et al*. 2003)." (See **Page 2, Line 31**) and also delete it from references list (see **Page 11, Line 243)**.

>> Materials and Methods: The text should be arranged tidily to easy the understanding of this section.

Explanation: We add some information in the Material and Methods (blue color) to make it understanding easily.

>> Results and discussion: It requires to be shortened as well. I suggest that the final paragraph is arranged in a way that final take home part of the paper is the positive side. It is

good to write that the methods requires further improvements but I am not sure that should be the last message in the paper.

Explanation: We agree with your suggestion. As our responses, we close the final paragraph with the positive side (see **Page 9, Line 203-205)** and does not make the statement "the methods requires further improvements" as the last message in this manuscript (see **Page 8, Line 188-190**).

>> Figure, Table and References are Ok.

Explanation: Thank you very much for your kind words about our manuscript.

Associate Editor: Both reviewers are positive. Please revise the manuscript incorporating their suggestions. The editor also has some comments.

>> Please clarify that the authors used a liquid phase electrode. What was a closed chamber using an aqueous phase of the leaf disc oxygen electrode? In Line 174, they also stated that they used the leaf-disc oxygen electrode.

Explanation: Thank you for the valuable corrections and suggestions. We already changed leaf-disc oxygen electrode with a Clark oxygen electrode type polarographic sensor (see **Page 3, Line 56-57; Page 7, line 167-168; and Abstract, line 29-30**).

>> Please clearly state the oxygen concentration? The buffer was equilibrated with the air (21% O2)?

Explanation: The sensor and buffer was equilibrated with saturation air 21% and also zero oxygen line by using nitrogen bubble. We add this information in Material and method (see **Page 3, Line 68-71**).

>> The respiration rates in the dark (Figure 2) were grater in the liquid phase measurements than that obtained in the air measurement. Also the respiratory quotient (CO2/O2) was lower than 1.0. Was this reproducible? Perhaps, the greater respiratory rates were due to wounding?

Explanation: Although cutting leaves into small pieces result produce high O_2 evolution, we also agree that the wounding also due to O_2 consumption that will increase respiratory rate.

>> Because the photosynthesis quotients obtained were always greater than 1.0, the authors claimed that photorespiration was suppressed. However, the dependence of the photosynthetic rate on the bicarbonate concentrations indicates that the photosynthesis was not saturated by 5 mM or 10 mM bicarbonate ion. Thus, at these concentrations photorespiration would occur. However, the PQ values were also above 1.0? This editor noticed error bars were greater at low bicarbonate. Could the authors explain the discrepancy?

Explanation: Yes, beside low performance in low light condition, the other obstacle of our method is the demand for high carbon dioxide source from bicarbonate (see **Page 7, Line 144-145**). The PQ values above 1.0 were representative of mean values. However, some measurement also gain PQ values bit lower than 1.0.

>> If photorespiration is completely suppressed like the measurement in 5% CO2 level, the rate of photosynthesis will be much greater than the rate

measured in 370 µmol mol–1 CO2 in air. It is necessary to explain the similar maximum values in Figure 2.

Explanation: yes, in comparation with gas exchange method, the maximum photosynthetic rate in photosynthetic O2 evolution and CO2 consumption was achieved under very high carbondiokside condition. We add this information in result and discussion (see **Page 9, Line 199-201**).

>> There are some technical problems as well. For all modifications, follow „Instructions for authors“ and “Detailed instructions for preparation of papers“ carefully.

Explanation: We try to improve our manuscript writing based on “Instructions for authors“ and “Detailed instructions for preparation of papers“.

Leaf O_2 evolution and CO_2 consumption are fundamental mechanisms that support oxygen and carbon ecosystems from the individual plant to the global scale. Based on the photosynthesis chemical formula, which justifies that the ratio of O_2 evolution to CO_2 fixation is 1:1 (Espie 1986), the traditional estimation of photosynthetic gas exchange has been evaluated either by O_2 evolution or CO_2 consumption. However, in an intact leaf, some physiological functions that synthesise and consume O_2 and CO_2 may vary, particularly under stress conditions (Wu *et al*. 2014), photorespiration (Rosenberg *et al*. 1995) and other oxygenative functions (Taddei *et al*. 2008). This means that the ratio of O_2:CO_2 during photosynthesis in intact leaves is not always 1:1.

The simultaneous estimation of O_2 and CO_2 has been done using isotope-Gas Chromatography-Mass Spectrometry (GC-MS) with $^{13}CO_2$ and $^{18}O_2$ (Isobe *et al*. 2011). However, the method is unpopular because the equipment is very expensive (Sipior *et al*. 1996). In this study, we tried to improve the potential for a convenient evaluation of O_2 evolution and CO_2 consumption in photosynthesis by using an O_2 electrode and CO_2 optodes simultaneously. The main advantages of optodes are that they can be used in non-invasive systems, oxygen and carbon dioxide are not consumed by the optodes, measurements are possible over a wide temperature range, and there is no mechanical stress (Warkentin *et al*. 2007). If this simultaneous method is convenient, it becomes a useful mechanism to more easily study the physiological effects of photosynthesis.

A simultaneous measurement of O_2 evolution and CO_2 consumption during photosynthesis is also essential in order to calculate the photosynthetic quotient (PQ), which is described as the molar ratio of the rate of O_2 production to the rate of CO_2 utilization (Williams and Robertson 1991). Some ecosystem productivity studies have been made with the assumption that PQ = 1 (Suzumura *et al.* 2002, Nielsen

Commented [Zia1]: deleted Furthermore, the possibility of directly determining the O_2 evolution and CO_2 consumption rate of leaf samples simultaneously and to monitor how rates change in response to stimuli will add to our understanding of single leaf to complex ecosystem mechanisms (Strovas *et al*. 2010).

Commented [Zia2]: deleted
On these occasions, independent biochemical studies and photosynthesis analyses have been simultaneously conducted to estimate the effects of stress on leaf photosynthesis.

Commented [Zia3]: deleted
The PQ value provides fundamental information on metabolic pathways (Taddei *et al.* 2008), balanced growth (Davies *et al.* 2003) and useful to clarify the primary productivity of an ecosystem (Lee and Bong 2006).

and Nielsen 2006) that could affect data interpretation of tropical productivity (Taddei *et al.* 2008).

Mangroves represent an important coastal ecosystem in tropical areas. During the seedling stage, the red mangrove (*Rhizophora mucronata* L.) lives periodically in submerged conditions like seaweed or macroalgae. Our previous work (Ulqodry *et al*. 2014) explored the photosynthetic performance of *R. mucronata* leaves using the gas exchange method. This method had a high precision and was rapid (Moore *et al*. 1973, Sobrado 2005, Okimoto *et al*. 2007) but was limited under aqueous conditions as the Infra-Red Gas Analyser is sensitive to water immersion (Gevaert *et al*. 2011). The advent of a new type of optical electrodes, the so-called opt(r)odes, facilitated the estimation of the *R. mucronata* photosynthetic rate under aqueous conditions. Previous studies have applied optodes for oxygen and carbon independently in the water column, sediments and plant tissues (Gansert *et al.* 2001, Glud *et al*. 2005, Berggren *et al*. 2012).

In this study, we examined the photosynthetic O_2 evolution and CO_2 consumption rates of *R. mucronata* leaves under aqueous condition. In a simultaneous experiment we used a liquid-phase O_2 electrode and CO_2 optodes to demonstrate their interdependence and differences and compared the results with those of the gas exchange method. The determination of PQ values and light-saturated photosynthetic rate (P_{max}) of *R. mucronata* under aqueous conditions was investigated.

Propagules of *R. mucronata* were obtained from a mangrove area on Galang Island, Batam District, Indonesia (0° 45' N, 104° 15' E). Propagules were initially grown in a heated greenhouse at the Laboratory of Tropical Crop Improvement, Saga University, Japan (33° 14' N, 130° 17' E). The fully expanded leaves from 3–4 mangrove seedlings were used as materials.

Commented [Zia4]: deleted: They belong to the C_3 photosynthetic group of plants, but may also be classified as "seaweeds", since they can grow in submerged and highly saline conditions (Kawamitsu *et al*. 2003).

Leaves were collected early each morning, vacuum-infiltrated with the buffer and stored in the dark until required. One essential consequence of this treatment was the inactivation of rubisco, so that the photosynthetic rates were approximately 10% of those generally observed from leaves taken directly from a plant (Brown 1998). The leaf sample was sliced into squares of approximately 1 mm^2. The leaves were sliced under a 50 mM HEPES buffer containing 0.5 mM $CaSO_4$ and transferred into the electrode chamber that contained the same buffer.

Photosynthetic O_2 evolution and CO_2 consumption were measured simultaneously in a closed chamber using an aqueous phase of a Clark oxygen electrode type polarographic sensor (*Hansatech*, Norfolk, UK) with a 'pCO2 mini' optodes sensor (*PreSens GmbH*, Regensburg, Germany) that was inserted into the chamber. The optodes system guarantees a high temporal resolution and a measurement without drift, oxygen consumption, or gas exchange between the incubation chamber and the environment (Warkentin *et al*. 2007). The chamber was equipped with a water jacket to maintain the temperature at 25°C. Periodic checking ensured that the highest illumination intensity did not result in a rapid increase in temperature. Light was provided by a slide projector lamp and the lens system focussed the light into the electrode compartment. The photosynthetically active radiation (PAR) in the chamber was measured with a quantum sensor (*model QRT1*, *Hansatech*, Norfolk,UK). It is important that the slices do not obstruct the rotation of the magnetic flea and also the sensor of pCO_2 mini. To achieve maximum accuracy, a two point calibration of sensor and buffer was equilibrated with saturation air 21% and also zero oxygen line by using nitrogen bubble. This process also removed any dissolved CO_2 from the medium, such that the added $NaHCO_3$ was the only carbon source available.

Photosynthetic O_2 evolution and CO_2 consumption of *R. mucronata* leaves under aqueous conditions were measured at various pH levels, $NaHCO_3$ concentrations and PAR levels at a temperature of 25°C. The relationship between the pH of the buffer and apparent photosynthetic rate was measured at pH 6.0, 6.5, 7.0, 7.5, 8.0, and 9.0 with $NaHCO_3$ 20 mM as carbon dioxide source under saturated PAR 1,000 µmol m^{-2} s^{-1}. The effect of different $NaHCO_3$ concentrations (0, 5, 10, 20 and 40 mM) was measured at pH 7.5 and a saturated PAR of 1,000 µmol m^{-2} s^{-1}. In relation to light intensity, PAR values in the chamber were maintained in decreasing levels from 1,000 to 50 µmol m^{-2} s^{-1} by placing various distance between projector lamp and the chamber. For a dark respiration measurement, the electrode chamber was wrapped in two layers of aluminium foil.

The O_2 electrode signal was recorded using Oxygraph Plus System software (*Hansatech*, Norfolk, UK) as a real-time chart recorder simulation. Simultaneously, the CO_2 consumption was measured in the same chamber every 5 s using pCO_2 View v1.0.2 software (*PreSens GmbH*, Regensburg, Germany). There was a lag period less than 2 min for CO_2 consumption, and about 3 min for O_2 evolution after light activation (Supplementary 1). Generally, lag period of O_2 evolution was slightly longer than CO_2 consumption but not in significance level. Furthermore, The O_2 evolution and CO_2 consumption rates were calculated from the initial slopes of the curves during linear photosynthetic activity after lag period finished.

As a comparison, the photosynthetic rate based on gas exchange in the air was also performed on leaf pairings similar to those used to measure O_2 evolution and CO_2 consumption under aqueous conditions. Measurements of leaf gas exchange were conducted using a portable open-flow gas exchange system (*LI-6400*, *Li-COR*, Lincoln,

NE, USA). The effect of light intensity on the photosynthetic rate was measured from PAR 1,000 to 0 µmol m^{-2} s^{-1} (1,000; 500, 250, 100, 50, 0 µmol m^{-2} s^{-1}) under leaf temperature, VpdL and CO_2 input were 25^0C, 1.7 $\pm$ 0.3 kPa, and 370 µmol mol^{-1}, respectively. The light responses of the photosynthetic rate was determined using the rectangular hyperbola model (Okimoto *et al.* 2008) to specify the P_{max} of *R. mucronata* leaves (Ulqodry *et al.* 2014) in air and under aqueous conditions.

Analysis of variance (ANOVA) was performed using statistiXL Version 1.x. Significant differences between treatments were further evaluated using the Tukey HSD test (P<0.05).

We began the experiment by comparing the most suitable leaf shape that resulted in highest O_2 evolution and CO_2 consumption, between small slice pieces (1 mm^2) and a larger, chip shape (1 cm^2). Previous results indicated that cutting leaves into small pieces can be negligible during O_2 evolution measurement under aqueous condition (Kawamitsu and Boyer 1999). Our results showed that a small *R. mucronata* leaf sample had significantly had higher O_2 evolution and CO_2 consumption rates compared with the larger, chip shape (Fig. 1-*I*). This suggests that slicing the leaf tissues facilitates the increasing of gas exchange across the boundary layer at the tissue surface (Brown 1998). This eliminates the effect of stomatal resistance for CO_2 diffusion, and free CO_2 molecules or HCO_3^- ions may penetrate more easily into the tissue of the leaf slice, resulting in a higher photosynthetic rate (Ishii *et al*. 1977).

The most important factors for measuring net photosynthetic rate (P_N) in aqueous conditions are the pH and carbonate system of the reaction mixture. Dissolved carbon dioxide in water occurs in three inorganic forms, free aqueous carbon dioxide (free CO_2), bicarbonate (HCO_3^-) and carbonate ions (CO_3^{2-}). If the equilibrium is affected by

121 a change in pH, this could potentially influence P_N (Riebesell *et al*. 2007). The P_N in
122 response to pH exhibited a similar pattern for both O_2 evolution and CO_2 consumption,
123 with higher with associated with intermediate pH values of 7.0–7.5 compared with low
124 and high pH (Fig. 1-*II*). Under a high pH condition of 8.0–9.0, free molecular CO_2
125 decreased and bicarbonate increased (Chen and Durbin 1994). This meant that the free
126 CO_2 in the reaction mixture became limiting, reducing P_N. This result also demonstrated
127 that the main carbon utilised as the substrate for *R. mucronata* leaf photosynthesis was
128 free CO_2 molecules rather than bicarbonate. Almost all terrestrial plants use only free
129 CO_2 for photosynthesis, however, many seaweeds or macroalgae use both free CO_2 and
130 external bicarbonate in water as a source of carbon for photosynthesis (Kawamitsu and
131 Boyer 1999, Pierini and Thomaz 2004). However, the photosynthetic rate that was
132 detected, even at a pH of 9, indicated that *R. mucronata* leaves used bicarbonate as an
133 additional source of carbon under low free CO_2 conditions. The requirement of
134 Photosystem II (PSII) for bicarbonate (carbonate) has been observed for intact leaves,
135 isolated thylakoids and PSII-enriched membrane fragments from oxygenic
136 photosynthesisers (Shevela *et al*. 2012). Bicarbonate is required for the regulation of
137 photosynthetic electron transport on the acceptor side of PSII (Wydrzynski and
138 Govindjee 1975), and is probably also involved in the mechanism of O_2 evolution
139 on the oxidising side of PSII (Stemler 2002).

140 This study was unable to demonstrate that high free CO_2 under low pH
141 condition (< 7.0) resulted in a high P_N. It described that leaf O_2 evolution and CO_2
142 consumption were strongly related to leaf intracellular conditions. Berge *et al*. (2010)
143 pointed out that as the pH dropped the H^+ concentration increased which may affect
144 intracellular pH, which may affect intracellular pH, membrane potential, energy

partitioning, and enzyme activity. For this reason, aqueous acidification may reduce P_N through direct pH effects.

Variation in P_N responses to $NaHCO_3$ concentrations also showed almost similar trends for both O_2 evolution and CO_2 consumption. The P_N increased with higher $NaHCO_3$ concentrations until reaching the saturation point at 20 mM (Fig. 1-*III*). The high bicarbonat saturation point indicated that our method need very high carbondioxide source. Particularly in submerged plants, P_N may be limited by a low availability of dissolved inorganic carbon (Maberly and Spence 1983, Adamec 1997). The interesting finding was that although there was no significant difference between O_2 evolution and CO_2 consumption, O_2 evolution values were always higher than CO_2 consumption values under the different pH and $NaHCO_3$ concentrations. This result is important if we want to explore the PQ of *R. mucronata* leaves under aqueous conditions. To be useful, PQ should be determined using the net rate of O_2 involved per CO_2 fixed simultaneously and can be described as,

$$PQ = \frac{O_2\ evolution}{CO_2\ consumption}$$

Stoichiometrically, a PQ value equal to unity (PQ = 1.00) assumes hexose production with ammonium as the N source (Rosenberg *et al*. 1995). If this simple photosynthesis physiology was replaced by an ecological summation of protoplasm production, including carbohydrates, protein, lipids, and nucleic acids, then the theoretical PQ would be higher (Williams and Robertson 1991). Theoretical PQ values typically range from 1.0–1.3 (Rosenberg *et al*. 1995). The PQ values of *R. mucronata* leaves in different pH and $NaHCO_3$ concentrations ranged from 1.04–1.28 with no significant difference among them (Fig. 1-*IV*). Purely based on stoichiometric and theoretical considerations of PQ values, results similar or higher than unity would be

169 expected. A PQ of 1.0 infers that the sole product of photosynthesis is carbohydrate
170 and a PQ > 1.0 indicates that more reduced compounds are produced, such as fats
171 and proteins (Chisholm 1998). Our results also suggested that the simultaneous
172 measurement of O_2 evolution and CO_2 consumption by using a Clark oxygen electrode
173 type polarographic sensor and 'pCO2 mini' optodes sensor provided a simple, stable
174 and precise measurement of net PQ under aqueous conditions.

175 The net PQ values in all measurements was never less than unity and confirmed
176 that photorespiration did not occur under aqueous conditions. A possible explanation for
177 a PQ less than unity would be photorespiration (glycolate production) as a result of
178 oxygenase activity of ribulose-1,5-bisphosphate (RuBP) carboxylase at high ambient
179 oxygen concentrations (Rosenberg *et al*. 1995). Photorespiration that decreases PQ
180 occurs when rubisco, which principally functions as carboxylase, is substituted by the
181 oxygenase function (Taddei *et al*. 2008). In terrestrial C_3 plants, photorespiratory
182 consumption of O_2 can account for 25% of rubisco activity (Falkowski and Raven 1997).
183 Conversely, photorespiration is assumed to be of minor importance to aquatic plants
184 compared with terrestrial C_3 plants (Laws *et al*. 2000), because submerged
185 environmental conditions, such as fairly constant oxygen and total inorganic carbon
186 concentrations, does not favour photorespiration (Rosenberg *et al*. 1995).

187 In order to characterise the functioning of photosynthetic apparatus of *R.*
188 *mucronata* in air and aqueous conditions, the light curves of P_N for similarly paired
189 leaves were estimated. In Fig. 2, at low light levels (PAR < 500 μmol photons m^{-2} s^{-1}),
190 the photosynthetic rate of O_2 evolution and CO_2 consumption under aqueous conditions
191 was lower than the photosynthetic CO_2 exchange in air. This result is likely to be related
192 to the reduction of low light utilisation while the leaf slices were rotated under aqueous

conditions. Another possible explanation for this was that our method worked well under light saturation compared with light limitation. Therefore, we need to improve the simultaneous measurements of photosynthetic O_2 evolution and CO_2 consumption under aqueous conditions in low light conditions.

The light saturation points for all P_N measurements (CO_2 exchange in air, O_2 evolution under aqueous condition and CO_2 consumption under aqueous condition) were similar at PAR levels around 500–1,000 μmol photons m^{-2} s^{-1}. The P_{max} which demonstrates the potential photosynthetic capacity of *R. mucronata* leaves (Ulqodry *et al*. 2014), was also determined. All experiments produced comparable results with similar P_{max} values of 13.37, 13.11 and 12.31 μmol m^{-2} s^{-1} for CO_2 exchange in air, O_2 evolution under aqueous conditions and CO_2 consumption under aqueous conditions, respectively. In comparation with gas exchange, the maximum photosynthetic rate in photosynthetic O_2 evolution and CO_2 consumption under aqueous condition was achieved under very high carbon dioxide condition. The P_{max} value and daily period of irradiance when plants were in the water and air would be useful as an indicator of primary production (Zimmerman *et al*. 1994). The similar P_{max} values suggested that all treatments resulted in a high capacity to adjust the photosynthetic apparatus under light saturation conditions.

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Thank you very much

Sincerely,
Ivana Štětinová

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BRIEF COMMUNICATION

An improved method for the simultaneous determination of photosynthetic O_2 evolution and CO_2 consumption in *Rhizophora mucronata* leaves

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Abstract

The photosynthetic gas exchange has been assessed traditionally either as O_2 evolution or CO_2 consumption. In this study, we used a liquid-phase O_2 electrode combined with CO_2 optodes to examine simultaneously photosynthesis in intact leaves of mangrove *Rhizophora mucronata*. We verified suitable conditions for leaf photosynthetic rates by assessing pH levels and $NaHCO_3$ concentrations and compared these to the gas exchange method at various PAR levels. The photosynthetic rate in response to pH exhibited a similar pattern both for O_2 evolution and CO_2 consumption, and higher rates were associated with intermediate pH compared with low and high pH values. The net photosynthetic quotient (PQ) of *R. mucronata* leaves ranged from 1.04–1.28. The PQ values, which were never lesser than 1, suggested that photorespiration did not occur in *R. mucronata* leaves under aqueous conditions. The similar maximum photosynthetic rates suggested that all measurements had a high capacity to adjust to not clear the photosynthetic apparatus under a light saturation condition. The simultaneous measurements of O_2 evolution and CO_2 consumption using the Clark oxygen electrode polarographic sensor with the CO_2 optode sensor provided a simple, stable, and precise measurement of PQ under aqueous and saturated light conditions.

Additional key words: carbon dioxide consumption; oxygen evolution; photosynthetic performance.

Leaf O_2 evolution and CO_2 consumption are fundamental mechanisms that support oxygen and carbon ecosystems from the individual plant to the global scale. Based on the photosynthesis chemical formula, which justifies that the ratio of O_2 evolution to CO_2 fixation is 1:1 (Espie 1986), the traditional estimation of photosynthetic gas exchange has been evaluated either by O_2 evolution or CO_2 consumption. However, in an intact leaf, some physiological functions that synthesise and consume O_2 and CO_2 may vary, particularly under stress conditions (Wu *et al*. 2014), photorespiration (Rosenberg *et al*. 1995), and other oxygenative functions (Taddei *et al*. 2008). This means that the ratio of O_2:CO_2 during photosynthesis in intact leaves is not always 1:1.

The simultaneous estimation of O_2 and CO_2 has been done using isotope-gas chromatography-mass spectrometry (GC-MS) with $^{13}CO_2$ and $^{18}O_2$ (Isobe *et al*. 2011). However, the method is unpopular because the equipment is very expensive (Sipior *et al*. 1996). In this study, we tried to improve the potential for a convenient evaluation of O_2 evolution and CO_2 consumption in photosynthesis by using the O_2 electrode and CO_2 optodes simultaneously. The main advantages of optodes are that they can be used in non-invasive systems, oxygen and carbon dioxide are not consumed by the optodes, measurements are possible over a wide temperature range, and there is no mechanical stress (Warkentin *et al*. 2007). If this simultaneous method is convenient, it becomes a useful mechanism to study more easily physiological effects of photosynthesis.

A simultaneous measurement of O_2 evolution and CO_2 consumption during photosynthesis is also essential in order to calculate the photosynthetic quotient (PQ), which is described as the molar ratio of the rate of O_2

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Abbreviations: P_{max} – light-saturated photosynthetic rate; P_N – net photosynthetic rate; PQ – photosynthetic quotient; RuBP – ribulose-1,5-bisphosphate.
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production to the rate of CO_2 utilization (Williams and Robertson 1991). Some ecosystem productivity studies have been made with the assumption that PQ = 1 (Suzumura *et al*. 2002, Nielsen and Nielsen 2006); it could affect data interpretation of tropical productivity (Taddei *et al*. 2008).

Mangroves represent an important coastal ecosystem in tropical areas. During the seedling stage, red mangrove (*Rhizophora mucronata* L.) lives periodically in submerged conditions similar to seaweed or macroalgae. Our previous work (Ulqodry *et al*. 2014) explored the photosynthetic performance of *R. mucronata* leaves using the gas-exchange method. This method showed a high precision and was rapid (Moore *et al*. 1973, Sobrado 2005, Okimoto *et al*. 2007), but it was limited under aqueous conditions as the infra-red gas analyser was sensitive to water immersion (Gevaert *et al*. 2011). The advent of a new type of optical electrodes, the so-called opt(r)odes, facilitated the estimation of the *R. mucronata* photosynthetic rate under aqueous conditions. Previous studies have applied optodes for oxygen and carbon independently in the water column, sediments, and plant tissues (Gansert *et al*. 2001, Glud *et al*. 2005, Berggren *et al*. 2012).

In this study, we examined the photosynthetic O_2 evolution and CO_2 consumption rates of *R. mucronata* leaves under aqueous condition. In a simultaneous experiment, we used a liquid-phase O_2 electrode and CO_2 optodes to demonstrate their interdependence and differences and compared the results with those of the gas-exchange method. The determination of PQ values and light-saturated photosynthetic rate (P_{max}) of *R. mucronata* under aqueous conditions was investigated.

Propagules of *R. mucronata* were obtained from a mangrove area on the Galang Island, Batam District, Indonesia (0°45'N, 104°15'E). Propagules were initially grown in a heated greenhouse at the Laboratory of Tropical Crop Improvement, Saga University, Japan (33°14'N, 130°17'E). The fully expanded leaves from 3–4 mangrove seedlings were used as materials.

The leaves were collected in early morning, vacuum-infiltrated with the buffer, and stored in the dark until required. One essential consequence of this treatment was the inactivation of Rubisco, so that the photosynthetic rates were approximately 10% of those generally observed from leaves taken directly from a plant (Brown 1998). The leaf sample was sliced into squares of approximately 1 mm^2. The leaves were sliced under a 50 mM HEPES buffer containing 0.5 mM $CaSO_4$ and transferred into the electrode chamber that contained the same buffer.

Photosynthetic O_2 evolution and CO_2 consumption were measured simultaneously in a closed chamber using an aqueous phase of a Clark oxygen electrode type polarographic sensor (*Hansatech*, Norfolk, UK) with a '*pCO_2 mini'* optodes sensor (*PreSens GmbH*, Regensburg, Germany) that was inserted into the chamber. The optodes system guarantees a high temporal resolution and a measurement without drift, oxygen consumption, or gas exchange between the incubation chamber and the environment (Warkentin *et al*. 2007). The chamber was equipped with a water jacket to maintain temperature at 25°C. Periodic checking ensured that the highest illumination intensity did not result in a rapid increase in temperature. Light was provided by a slide projector lamp and the lens system focused the light into the electrode compartment. The photosynthetically active radiation in the chamber was measured with a quantum sensor (model *QRT1*, *Hansatech*, Norfolk,UK). It was important that the slices did not obstruct the rotation of the magnetic flea and also the sensor of *pCO_2 mini*. To achieve maximum accuracy, a two point calibration of the sensor and buffer was equilibrated with saturation air 21% and also zero oxygen line by using nitrogen bubble. This process also removed any dissolved CO_2 from the medium, such that the added $NaHCO_3$ was the only carbon source available.

Photosynthetic O_2 evolution and CO_2 consumption of *R. mucronata* leaves under aqueous conditions were measured at various pH levels, $NaHCO_3$ concentrations, and PAR levels at temperature of 25°C. The relationship between the pH of the buffer and apparent photosynthetic rate was measured at pH 6.0, 6.5, 7.0, 7.5, 8.0, and 9.0 with 20 mM $NaHCO_3$ as carbon dioxide source under saturation PAR 1,000 μmol(photon) m^{-2} s^{-1}. The effect of different $NaHCO_3$ concentrations (0, 5, 10, 20, and 40 mM) was measured at pH 7.5 and a saturation PAR of 1,000 μmol(photon) m^{-2} s^{-1}. In relation to light intensity, PAR values in the chamber were maintained in decreasing levels from 1,000 to 50 μmol(photon) m^{-2} s^{-1} by placing various distance between the projector lamp and the chamber. For a dark respiration measurement, the electrode chamber was wrapped in two layers of aluminium foil.

The O_2 electrode signal was recorded using *Oxygraph Plus System* software (*Hansatech*, Norfolk, UK) as a real-time chart recorder simulation. Simultaneously, the CO_2 consumption was measured in the same chamber every 5 s using *pCO_2 View v1.0.2* software (*PreSens GmbH*, Regensburg, Germany). There was a lag period lesser than 2 min for CO_2 consumption, and about 3 min for O_2 evolution after light activation (Fig. 1S, *supplement available online*). Generally, the lag period of O_2 evolution was slightly longer than that of CO_2 consumption, but insignificantly. Furthermore, the O_2 evolution and CO_2 consumption rates were calculated from the initial slopes of the curves during a linear photosynthetic activity rate? after the lag period finished**.**

As a comparison, the photosynthetic rate based on gas exchange in the air was also performed on leaf pairings? similar to those used to measure O_2 evolution and CO_2 consumption under aqueous conditions. Measurements of leaf gas exchange were conducted using a portable open-flow gas exchange system (*LI-6400*, *Li-COR*, Lincoln,

NE, USA). The effect of light intensity on the photosynthetic rate was measured from PAR 1,000 to 0 µmol(photon) m^{-2} s^{-1} (1,000; 500, 250, 100, 50, 0 µmol m^{-2} s^{-1}) with leaf temperature, VpdL? and CO_2 input of 25^0C, 1.7 ± 0.3 kPa, and 370 µmol mol^{-1}, respectively. The light responses of the photosynthetic rate was determined using the rectangular hyperbola model (Okimoto *et al*. 2008) to specify the P_{max} of *R. mucronata* leaves (Ulqodry *et al*. 2014) in air and under aqueous conditions.

Analysis of variance (*ANOVA*) was performed using *StatistiXL Version 1.x*. Significant differences between treatments were further evaluated using the *Tukey*'s HSD test (P<0.05).

We began the experiment by comparing the most suitable leaf shape that would result in the highest O_2 evolution and CO_2 consumption, *i.e*., small slice pieces (1 mm^2) and a larger, chip shape ones (1 cm^2). Previous results indicated that cutting leaves into small pieces can be negligible during O_2 evolution measurement under aqueous condition (Kawamitsu and Boyer 1999). Our results showed that the small *R. mucronata* leaf sample exhibited significantly higher O_2 evolution and CO_2 consumption rates to the larger, chip shape pieces (Fig. 1*A*). This suggests that slicing the leaf tissues facilitated increasing gas exchange across the boundary layer at the tissue surface (Brown 1998). This eliminates the effect of stomatal resistance for CO_2 diffusion, and free CO_2 molecules or HCO_3^- ions may penetrate more easily into the tissue of the leaf slice, resulting in a higher photosynthetic rate (Ishii *et al*. 1977).

The most important factors for measuring the net photosynthetic rate (P_N) in aqueous conditions were pH and a carbonate system of the reaction mixture. Dissolved carbon dioxide in water occurs in three inorganic forms: free aqueous carbon dioxide (free CO_2), bicarbonate (HCO_3^-), and carbonate ions (CO_3^{2-}). If the equilibrium is affected by a change in pH, this could potentially influence P_N (Riebesell *et al*. 2007). The P_N in response to pH exhibited a similar pattern for both O_2 evolution and CO_2 consumption, with higher with associated with intermediate not clear, rephrase pH values of 7.0–7.5 compared to low and high pH (Fig. 1*B*). Under a high pH condition of 8.0–9.0, free molecular CO_2 decreased and bicarbonate increased (Chen and Durbin 1994). This meant that free CO_2 in the reaction mixture became limiting and it reduced P_N. This result also demonstrated that the main carbon form utilised as the substrate for *R. mucronata* leaf photosynthesis was free CO_2 molecules rather than bicarbonate. Almost all terrestrial plants

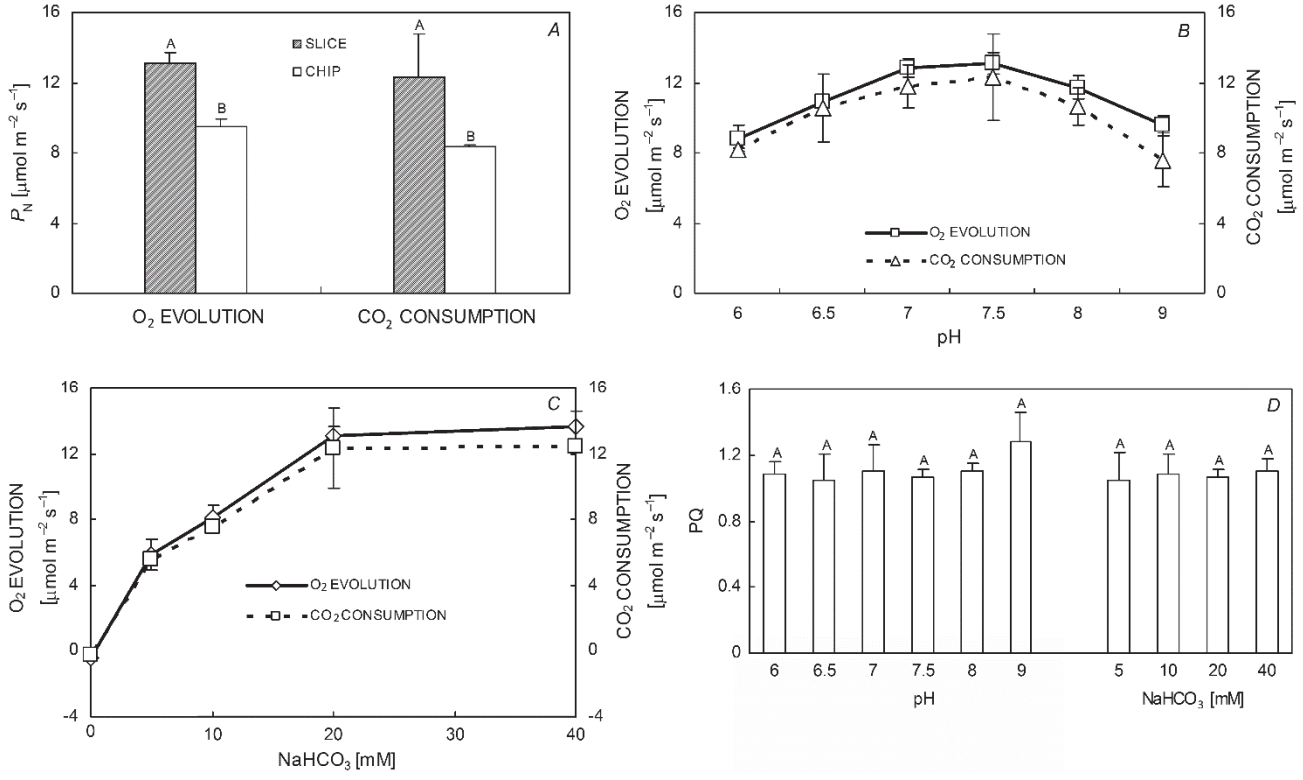

Fig. 1. Photosynthetic O_2 evolution and CO_2 consumption of *R. mucronata* leaves under aqueous conditions measured in various: sample forms (*A*), pH levels (*B*), and $NaHCO_3$ concentrations (*C*); and the photosynthetic quotient (PQ) values as ratio of O_2 evolution and CO_2 consumption (*D*). The conditions of the measurements: temperature of 25°C and PAR of 1,000 µmol(photon) m^{-2} s^{-1}. Values are means ± SD. *Different letters* over bars represent significant differences among all treatments (P<0.05, *Tukey*'s HSD test).

use only free CO_2 for photosynthesis, however, many seaweeds or macroalgae use both free CO_2 and external bicarbonate in water as the source of carbon for photosynthesis (Kawamitsu and Boyer 1999, Pierini and Thomaz 2004). However, the photosynthetic rate, which was detected even at pH 9, indicated that *R. mucronata* leaves used bicarbonate as an additional source of carbon under low free CO_2 conditions. The requirement of PSII for bicarbonate (carbonate) has been observed for intact leaves, isolated thylakoids, and PSII-enriched membrane fragments from oxygenic photosynthesisers (Shevela *et al*. 2012). Bicarbonate is required for the regulation of photosynthetic electron transport on the acceptor side of PSII (Wydrzynski and Govindjee 1975), and is probably also involved in the mechanism of O_2 evolution on the oxidising side of PSII (Stemler 2002).

This study was unable to demonstrate that high free CO_2 under low pH condition (<7.0) resulted in high P_N. It seemed that leaf O_2 evolution and CO_2 consumption were strongly related to leaf intracellular conditions. Berge *et al*. (2010) pointed out that as pH dropped the H^+ concentration increased which may affect intracellular pH, membrane potential, energy partitioning, and enzyme activity. For this reason, aqueous acidification may reduce P_N through direct pH effects.

Variation in P_N responses to $NaHCO_3$ concentrations also showed almost similar trends for both O_2 evolution and CO_2 consumption. P_N increased with higher $NaHCO_3$ concentrations until reaching the saturation point at 20 mM (Fig. 1*C*). The high bicarbonat saturation point indicated that our method needed very rich carbon dioxide source. Particularly, in submerged plants, P_N may be limited by a low availability of dissolved inorganic carbon (Maberly and Spence 1983, Adamec 1997). The interesting finding was that although there was no significant difference between O_2 evolution and CO_2 consumption, the O_2 evolution values were always higher than those of the CO_2 consumption values under the different pH and $NaHCO_3$ concentrations. This result is important if we want to explore the PQ of *R. mucronata* leaves under aqueous conditions. To be useful, PQ should be determined using the net rate of O_2 involved per CO_2 fixed simultaneously and can be described as:

$$\mathrm{PQ} = \frac{\mathrm{O_2\ evolution}}{\mathrm{CO_2\ consumption}}$$

Stoichiometrically, the PQ value equal to 1.00 assumes a hexose production with ammonium as the N source (Rosenberg *et al*. 1995). If this simple photosynthesis physiology was replaced by an ecological summation of protoplasm production, including carbohydrates, proteins, lipids, and nucleic acids, then the theoretical PQ would be higher (Williams and Robertson 1991). Theoretical PQ values typically range from 1.0 to 1.3 (Rosenberg *et al*. 1995). The PQ values of *R. mucronata* leaves under the different pH and $NaHCO_3$ concentrations ranged from 1.04–1.28 with no significant difference among them (Fig. 1*D*). Purely based on stoichiometric and theoretical considerations of the PQ values, results similar or higher than 1.0 would be expected. PQ of 1.0 infers that the sole product of photosynthesis is carbohydrate, while PQ>1.0 indicates that more reduced compounds are produced, such as fats and proteins (Chisholm 1998). Our results also suggested that the simultaneous measurement of O_2 evolution and CO_2 consumption by using the polarographic sensor of Clark oxygen electrode and the '*pCO_2 mini'* optode sensor provided simple, stable, and precise measurements of net PQ under aqueous conditions.

The net PQ values in all measurements was never less than 1.0 and it confirmed that photorespiration did not occur under aqueous conditions. A possible explanation for a PQ lesser than 1.0 would be photorespiration (glycolate production) as a result of oxygenase activity of Rubisco at high ambient oxygen concentrations (Rosenberg *et al*. 1995). Photorespiration that decreases PQ occurs when Rubisco, which principally functions as carboxylase, is substituted by its oxygenase function (Taddei *et al*. 2008). In terrestrial C_3 plants, photorespiratory consumption of O_2 can account for 25% of Rubisco activity (Falkowski and Raven 1997). Conversely, photorespiration is assumed to be of minor importance to aquatic plants compared with terrestrial C_3 plants (Laws *et al*. 2000), because submerged environmental conditions, such as fairly constant oxygen and total inorganic carbon concentrations, does not favour photorespiration (Rosenberg *et al*. 1995).

In order to characterise the functioning of photosynthetic apparatus of *R. mucronata* in air and under aqueous conditions, the light curves of P_N for similarly paired leaves were estimated. In Fig. 2, at low light levels [PAR < 500 μmol(photon) m^{-2} s^{-1}], the photosynthetic rate of O_2 evolution and CO_2 consumption under aqueous conditions was lower than the photosynthetic CO_2 exchange in air. This result is likely to be related to the reduction of low light utilisation while the leaf slices were rotated under aqueous conditions. Another possible explanation was that our method worked well under light saturation compared to light limitation. Therefore, we needed to improve the simultaneous measurements of photosynthetic O_2 evolution and CO_2 consumption under aqueous conditions under low light conditions.

The light saturation points for all P_N measurements (CO_2 exchange in air, O_2 evolution under aqueous condition, and CO_2 consumption under aqueous condition) were similar at PAR levels around 500–1,000 μmol(photon) m^{-2} s^{-1}. The P_{max}, which demonstrates the potential photosynthetic capacity of *R. mucronata* leaves (Ulqodry *et al*. 2014), was also determined. All experiments produced comparable results with similar P_{max} values of 13.37, 13.11, and 12.31 μmol m^{-2} s^{-1} for CO_2 exchange in air, O_2 evolution under aqueous conditions, and CO_2 consumption under aqueous conditions, respectively. In comparation with gas

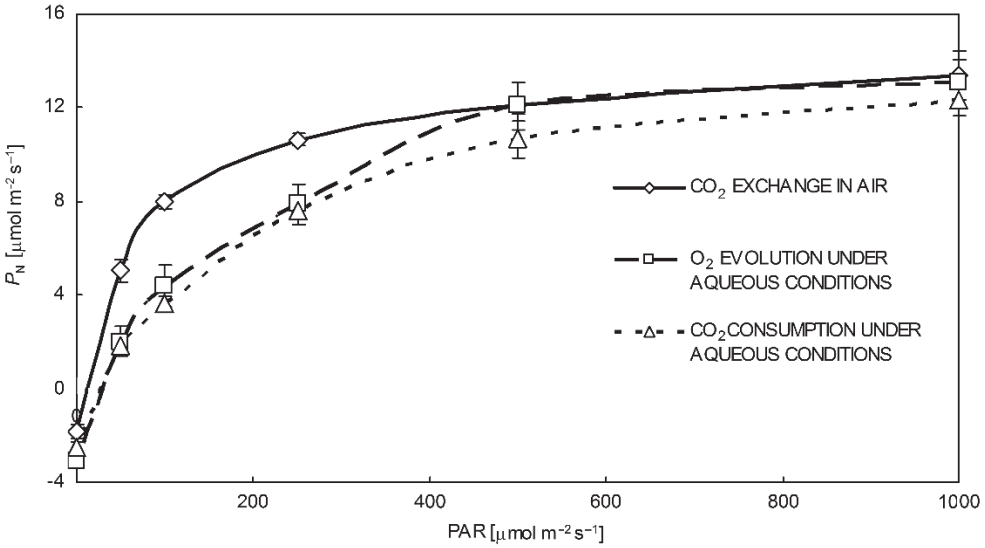

Fig. 2. Response of net photosynthetic rate (P_N) to increasing PAR in the *R. mucronata* leaves. Measurements in air were made with a portable open-flow gas-exchange system, *LI-6400* (CO_2 exchange in air) and measurements under aqueous conditions were made simultaneously with an aqueous-phase O_2 electrode (O_2 evolution) and '*pCO_2 mini*' optode system (CO_2 consumption). Temperature was 25°C for all measurements. Values are means ± SD.

exchange, the maximum photosynthetic rate in photosynthetic O_2 evolution and CO_2 consumption under aqueous condition was achieved under very high carbon dioxide concentration. The P_{max} value and daily period of irradiance, when plants were in the water and air, would be useful as an indicator of primary production (Zimmerman *et al*. 1994). The similar P_{max} values suggested that all treatments resulted in a high capacity to adjust the photosynthetic apparatus under light saturation conditions.

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