

RESEARCH ARTICLE

Effects of short-term exposure to different salinity levels on *Myriophyllum spicatum* and *Ceratophyllum demersum* and suitability of biomarkers to evaluate macrophyte responses to salinity stress

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Received: 13 April 2020; Accepted: 24 August 2020

Abstract – Sea-level rise caused by global warming is leading to increased freshwater salinization, which causes significant stress on aquatic ecosystems and species, including macrophytes. To form a better understanding of the responses of macrophytes to salinity stress, we assessed biochemical, pigmentation and growth responses of *Myriophyllum spicatum* L. and *Ceratophyllum demersum* L. exposed to different salinity levels (0, 1.5, 2.5, 5.0, and 10 ppt). For both species, elongation rates decreased, and levels of photosynthetic pigments (chlorophyll *a* and chlorophyll *b*) increased at higher salinities (5 ppt and 10 ppt). Anthocyanin and H₂O₂ concentrations increased in *M. spicatum* but decreased in *C. demersum* with the increase in salinity. The activities of antioxidant enzymes (guaiacol peroxidase, catalase, and ascorbate peroxidase) were different between two species and fluctuated along the salinity gradient. *M. spicatum* and *C. demersum* exhibit species-specific salinity sensitivities, reaching different physiological statuses at each salinity level. Elongation rates were significantly correlated with several biochemical parameters in a species-specific manner. These correlations can be used in evaluating the expected responses of these two species to salinity changes. The species-specific responses of most parameters measured in the present study suggests the inapplicability of common biochemical responses across species.

Keywords: Critical salinity / Macrophytes / River salinization / Salinity stress / Sea-level rise

1 Introduction

The salinization of freshwater ecosystems is an increasing environmental problem worldwide, which is directly or indirectly associated with human activities. Intensive agricultural practices, modification of waterways, and the use of rock salt for road construction are examples of human activities that directly affect freshwater salinity (Kaushal *et al.*, 2005; Lind *et al.*, 2018; Nielsen *et al.*, 2003). Moreover, sea-level rise (SLR) caused by climate change is increasingly leading to salinization of aquifers, estuaries, coastal lakes, wetlands, and rivers (Bhuiyan and Dutta, 2012; Mulamba *et al.*, 2019; Schallenberg *et al.*, 2003). The degree of SLR is expected to increase, and sea level is estimated to rise to 2 m in the worst-case scenarios (Bamber *et al.*, 2019). The impact of SLR on

freshwater salinization is especially relevant in lowland rivers, which will face more salt contamination than usual, sometimes up to many kilometers upstream (Bhuiyan and Dutta, 2012; Mulamba *et al.*, 2019; Rice *et al.*, 2014; Vu *et al.*, 2018). Furthermore, reduced river flow and discharge during dry spells and droughts (climate change increases drought duration and severity) facilitate the intrusion of seawater (Chiogna *et al.*, 2018).

Macrophytes play a crucial role in determining the characteristics and functions of aquatic ecosystems (Franklin *et al.*, 2008). The abundance and distribution of macrophytes are closely associated with water body hydrology, morphology, nutrient availability, sediment characteristics, and physical and chemical parameters of water (color, turbidity, temperature, pH, and dissolved oxygen; Feldmann and Nöges, 2007; Hrivnák *et al.*, 2009; Rameshkumar *et al.*, 2019). Macrophytes occupy and colonize water bodies with favorable growth conditions, which vary according to the species (Cao *et al.*, 2019).

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They respond to tolerate changes in environmental conditions, which may lead to alterations in macrophyte community composition (Barko *et al.*, 1986; Søndergaard *et al.*, 2010). When ambient conditions exceed tolerable levels, macrophyte growth is suppressed, and the plants eventually perish.

High salinity in freshwater bodies has adverse effects on freshwater ecosystems (Cañedo-Argüelles *et al.*, 2019). This is especially true for aquatic plants, which, in contrast to many animals, are unable to migrate to unaffected sites. Plants respond to salinity changes by adjusting their morphology and physiology according to their salinity tolerance (Twilley and Barko, 1990) and salt concentration, which at extreme levels causes plant death. Therefore, the excessive and prolonged intrusion of saltwater into freshwater bodies is expected to cause a further increase in salinity, leading to the elimination of freshwater macrophytes; however, studies have shown that some levels of salinity are tolerable (Borgnis and Boyer, 2016; Thouvenot and Thiébaud, 2018). Future aquatic vegetation management strategies, especially in coastal areas, will have to consider the effects of altered salinity levels. Understanding the tolerance and responses of macrophytes to changes in salinity levels could assist with the monitoring and management of such ecosystems.

Myriophyllum spicatum L. and *Ceratophyllum demersum* L. are common submerged macrophytes species that are widely distributed in low-flow rivers, lakes, and ponds (Germ *et al.*, 2006; Keskinan *et al.*, 2007). These two species have common characteristics, such as rapid growth, low light preference, and fragment distribution. However, *M. spicatum* is a rooted species, while *C. demersum* does not form roots and is found free-floating or anchored to the substrate. These two species also present distinct responses to environmental stresses, including different heavy metals absorption (Keskinan *et al.*, 2007), sensitivity to ultraviolet B radiation (Germ *et al.*, 2006), and selenium toxicity (Mechora *et al.*, 2011). Because of the characteristics referred to above, several studies have investigated the responses of *M. spicatum* and *C. demersum* to changes in environmental conditions, including salinity changes (Li *et al.*, 2011; Thouvenot *et al.*, 2012). However, little is known about the biochemical responses of these two species to salt stress, since most studies focused on morphological adjustments rather than biochemical changes under different salinity conditions (Borgnis and Boyer, 2016; Thouvenot *et al.*, 2012; Thouvenot and Thiébaud, 2018).

Because little work has been done on the biochemical responses of *M. spicatum* and *C. demersum* to salt stress, we investigated the effects of exposing these two species to different salinity levels for a short time period as an initial study. We focused on changes in biochemical composition (hydrogen peroxide and antioxidant enzymes levels), pigmentation (chlorophyll *a*, chlorophyll *b*, carotenoids, and anthocyanins levels), and growth rate parameters of macrophytes maintained under controlled temperature, light, and nutrient conditions, but under different salinity conditions, for 5 days. Further, we investigated the relationship between parameters under salinity influence to evaluate the suitability of biochemical and pigmentation responses as biomarkers to manage these two macrophytes.



Fig. 1. Representative images of experimental tanks containing the two species, *Myriophyllum spicatum* and *Ceratophyllum demersum*, after five days of continuous exposure to different salinity levels (0, 1.5, 2.5, 5, and 10 ppt). *C. demersum* plants exposed to 10 ppt salinity kept their leaves closed even when exposed to light; at 5 ppt leaves remained partially closed.

2 Materials and methods

2.1 Macrophyte stock culture

Macrophytes were collected in large quantities from river colonies of the Kanto region, Japan, and healthy plants were cultured in 20–30 L glass aquariums as the stock culture. Each stock tank was containing 4–5 cm layer of nutrient washed river sand and selected healthy plants were fixed to the sand with the help of a thumb forceps. Light was maintained at $80\text{--}85\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ photosynthetically active radiation (PAR) intensity at plant depth provided by LED lamps with a color rendering index of 80 (Model LT-NLD85L-HN, Ohm Electric, Toshima, Japan). The light period was set at 12 h light, 12 h dark. Tanks were kept in a temperature-controlled experiment room at $25 \pm 2\ ^\circ\text{C}$ continuously. A commercial aquaponics solution (Hyponex concentrated nutrient solution, Hyponex, Osaka, Japan) at 5 ppm was used as a nutrient source.

2.2 Experimental plants and salinity treatments

Plant cuttings (~8 cm) from healthy stock plants were obtained and washed with 5% Hoagland solution; any attached algae were removed. Cuttings were immediately planted in separate tanks filled with 5% Hoagland solution containing nutrients washed river sand layer (~3 cm). Light conditions were the same as described for the stock tanks. Plant cuttings were kept in tanks for one week for acclimatization and then transferred to experimental tanks (25 × 15 × 15, height × width × length) containing nutrients washed river sand (~3 cm) and 4 L of 5% Hoagland solution. In each experimental tank, 3 cuttings of *C. demersum* or 4 cuttings of *M. spicatum* were planted. Planting was done by inserting ~2.5 cm of cutting into the sand with the help of a thumb forceps. Light conditions were maintained at $80\text{--}85\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ PAR intensity at plant depth and 12:12 light cycle. Water temperature was maintained at $25 \pm 1.0\ ^\circ\text{C}$. Each tank had a general-purpose aquarium air pump to maintain the CO₂ supply (Fig. 1). Plant cuttings were kept under these conditions for 3 days

prior to the treatments. Any water loss due to evaporation was replenished with distilled water daily.

After 3 days, plants were exposed to different salinities using a mixture of salts at a fixed ratio to achieve the desired salinity conditions. The salt mixture contained: 2.08% NaCl, 0.94% MgCl₂·6H₂O, 0.132% CaCl₂·2H₂O, and 0.06% KCl (Rout and Shaw, 2001). The salts were dissolved in a beaker using nutrient solutions taken from each tank. Then, the solution was added back slowly into the respective tanks and slowly mixed with a plastic rod without disturbing the plants. The different salinity levels were: 0 ppt, 1.5 ± 0.1 ppt, 2.5 ± 0.1 ppt, 5.0 ± 0.2 ppt, and 10 ± 0.5 ppt, which were confirmed using a portable salinity tester (Eutech Expert CTS Pocket Tester, Thermo Scientific, Waltham, MA, USA). All salinity exposure experiments were performed in triplicate. After 5 days of salinity exposure, plants had their length recorded and were put in resealable bags after blotting and stored in ice. Then samples were stored at -80 °C until further analysis.

2.3 Elongation measurement

The length of each plant cutting was measured at the beginning of the experiment (when the salt mixture was added to the tank) and at the end of the experiment (on the 5th day of exposure). The length was measured using a millimeter-scale in relation to the sand layer in the experiment tank while plants were still inside the tank. The elongation of plants is expressed as percent change over initial length after 5 days exposure.

2.4 Pigments quantification

Photosynthetic pigment content was estimated after extracting pigments from samples using N, N-dimethylformamide. Pigments were extracted by incubating 150 mg of samples collected from the upper part of the plants with 5 mL of N, N-dimethylformamide for 24 h in the dark at room temperature (25–27 °C). Then, the optical absorptions were spectrophotometrically measured at wavelengths of 664, 647, and 480 nm (UV-1280, Shimadzu, Kyoto, Japan). The concentrations of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and total carotenoids (Car) were calculated using the equation provided by Wellburn (1994) and are expressed as milligrams per gram of dry weight (mg/g DW) for each pigment.

Anthocyanin content was measured spectrophotometrically, as described in Nakata and Ohme-Takagi (2014) but with modifications. Approximately 50 mg of samples collected from the upper portion of the plants were pulverized in liquid nitrogen and mixed with 2 mL of extraction buffer containing 45% (v/v) methanol and 5% (v/v) acetic acid in distilled water. After centrifugation at 3,500 rpm for 15 min at 20 °C, the supernatant was collected, and optical absorption at 637 and 530 nm was measured (UV-1280, Shimadzu, Kyoto, Japan). Anthocyanin content was calculated, taking one anthocyanin unit as equivalent to one absorbance unit in 1 mL of extraction solution (Teng *et al.*, 2005).

2.5 Hydrogen peroxide and antioxidant enzymes

The activities of antioxidant enzymes, guaiacol peroxidase (GPX), catalase (CAT), and ascorbate peroxidase (APX), as

well as H₂O₂ levels, were measured spectrophotometrically (UV-1280, Shimadzu, Kyoto, Japan). For these measurements, approximately 200 mg of each sample were crushed in liquid nitrogen in the presence of polyvinylpyrrolidone. Extraction was done by adding 5 mL of 0.05 M phosphate buffer (pH 6.0) to the crushed samples. Then the extraction mixture was centrifuged at 3,500 rpm for 10 min at 4 °C, and the supernatant was collected and stored at -80 °C until further analysis.

Hydrogen peroxide content was measured by mixing 750 µL of the supernatant with 2.5 mL of 0.1% (w/v) titanium sulfate in 20% (v/v) H₂SO₄ (Satterfield and Bonnell, 1955) and incubating the assay mixture at room temperature (25–27 °C) for 30 min. Then, the assay mixture was read at 410 nm. Hydrogen peroxide concentration in samples was estimated by comparing absorbance values with those of an H₂O₂ standard curve and was expressed as µmol g⁻¹ FW.

Guaiacol peroxidase activity was measured by mixing 100 µL of extract supernatant with 40 µL of 30 mM H₂O₂ and 50 µL of 25 mM guaiacol (MacAdam *et al.*, 1992). The reaction was initiated with the addition of supernatant into the reaction medium. The absorbance at 420 nm was recorded every 10 s for 3 min. Based on the rate of absorbance increase, GPX activity was expressed as µmol min⁻¹ g⁻¹ FW using the extinction coefficient of 26.6 mmol⁻¹ cm⁻¹.

Catalase activity was measured by reacting 100 µL of 10 mM H₂O₂, 2 mL of 100 mM potassium phosphate buffer (pH 7.0), and 500 µL of extract supernatant (Aebi, 1984). The reaction was initiated with the addition of supernatant into the reaction medium. The absorbance at 240 nm was recorded every 10 s for 3 min. Based on the rate of absorbance decrease, CAT activity was expressed as µmol min⁻¹ g⁻¹ FW using the extinction coefficient of 40 mmol⁻¹ cm⁻¹.

Ascorbate peroxidase was measured according to Nakano and Asada (1981). The reaction mixture contained 100 µL of extract supernatant, 200 µL of 0.5 mM ascorbic acid in 50 mM potassium phosphate buffer (pH 7.0), and 2 mL of 50 mM potassium phosphate buffer (pH 7.0). The assay was started with the addition of 60 µL of 1 mM H₂O₂. The absorbance at 290 nm was recorded every 10 s for 3 min. Based on the rate of absorbance decrease, APX activity was expressed in µmol min⁻¹ g⁻¹ FW using the extinction coefficient of 2.8 mmol⁻¹ cm⁻¹.

2.6 Data analysis

The levels of antioxidant enzymes, H₂O₂, and anthocyanin in plants were normalized relative to those in the control group (0 ppt salinity). This was done by dividing the results of each salinity exposure group by those of the corresponding 0 ppt exposure group for each replicate. Data were analyzed using ANOVA followed by the Duncan post hoc test. Significant differences between experimental groups were evaluated using Student's *t*-test assuming equality of variance. Regression relationship analysis were used to evaluate relationships between parameters (H₂O₂, antioxidative enzymes, photosynthetic pigments, anthocyanin, and elongation). For all analyses, *P* values below 0.05 were considered to indicate statistically significant differences. Statistical comparisons were performed using IBM SPSS Statistics, version 25 (IBM, Armonk, NY, USA). Regression relationships and descriptive statistical analyses, as well as data visualization, were

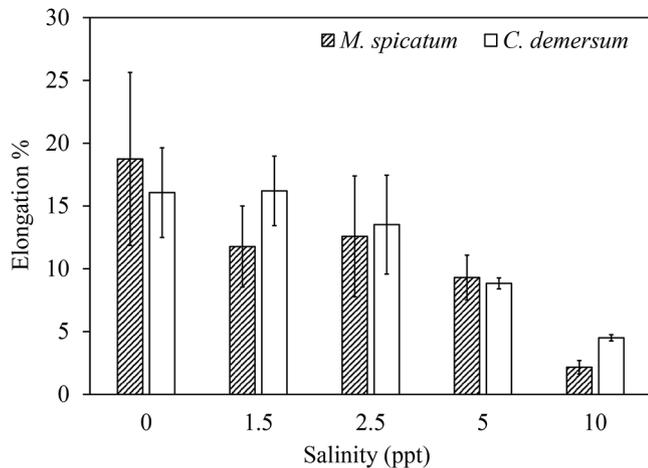


Fig. 2. Changes in plant length of *Myriophyllum spicatum* and *Ceratophyllum demersum* expressed as elongation (%) after five days of exposure to different salinity levels (0, 1.5, 2.5, 5, and 10 ppt). Error bars represent the standard deviation.

calculated and presented using Microsoft Excel 2016 (Microsoft, Redmond, WA, USA).

3 Results

Elongation rates of *M. spicatum* were significantly reduced in plants exposed to any salinity level compared with that of control plants maintained at 0 ppt (Fig. 1, ANOVA $P < 0.01$ for 5 and 10 ppt; ANOVA, $P < 0.05$ for 1.5 and 2.5 ppt; Fig. 2). There was no significant difference between *M. spicatum* groups exposed to 1.5 ppt, 2.5 ppt, and 5 ppt salinities (ANOVA, $P > 0.05$). The elongation of *M. spicatum* plants exposed to 10 ppt salinity was lower than that of all other groups (ANOVA, $P < 0.01$). Compared with the control group, the elongation of *M. spicatum* in the 10 ppt group decreased by 8.6-fold. The elongation rate of *C. demersum* was not significantly affected by the exposure to 1.5 or 2.5 ppt salinities (ANOVA, $P > 0.05$; Fig. 2). Elongation was significantly reduced in plants exposed to 5 ppt salinity compared with that of plants in low salinity (0, 1.5, and 2.5 ppt) groups, (ANOVA, $P < 0.05$), but it remained significantly higher than the elongation of plants in the 10 ppt group (ANOVA, $P < 0.01$). The elongation rate of *C. demersum* exposed to 10 ppt salinity was approximately 3.5-fold lower than that of plants in the 0 ppt group (Fig. 2). Further, the usual leaf-closing mechanism (closed in the dark and open under light) of *C. demersum* was altered at 5 ppt and 10 ppt salinities: leaves remained fully closed under 10 ppt salinity exposure and partially closed under 5 ppt salinity exposure, regardless of light or dark phase. Under 1.5 ppt and 2.5 ppt salinities exposure, the leaf-closing mechanism of *C. demersum* was unaffected (Fig. 1).

Chl *a* level in *M. spicatum* differed between 2.5 ppt and 5 ppt salinity levels (*t*-test, $P < 0.05$). The rest of the salinity levels were statistically not different (ANOVA, $P > 0.05$; Fig. 3a). The Chl *b* levels of 5 ppt and 10 ppt groups were increased significantly than other groups (ANOVA, $P < 0.05$; Fig. 3b). In *C. demersum*, Chl *a* and Chl *b* levels of 1.5 ppt and

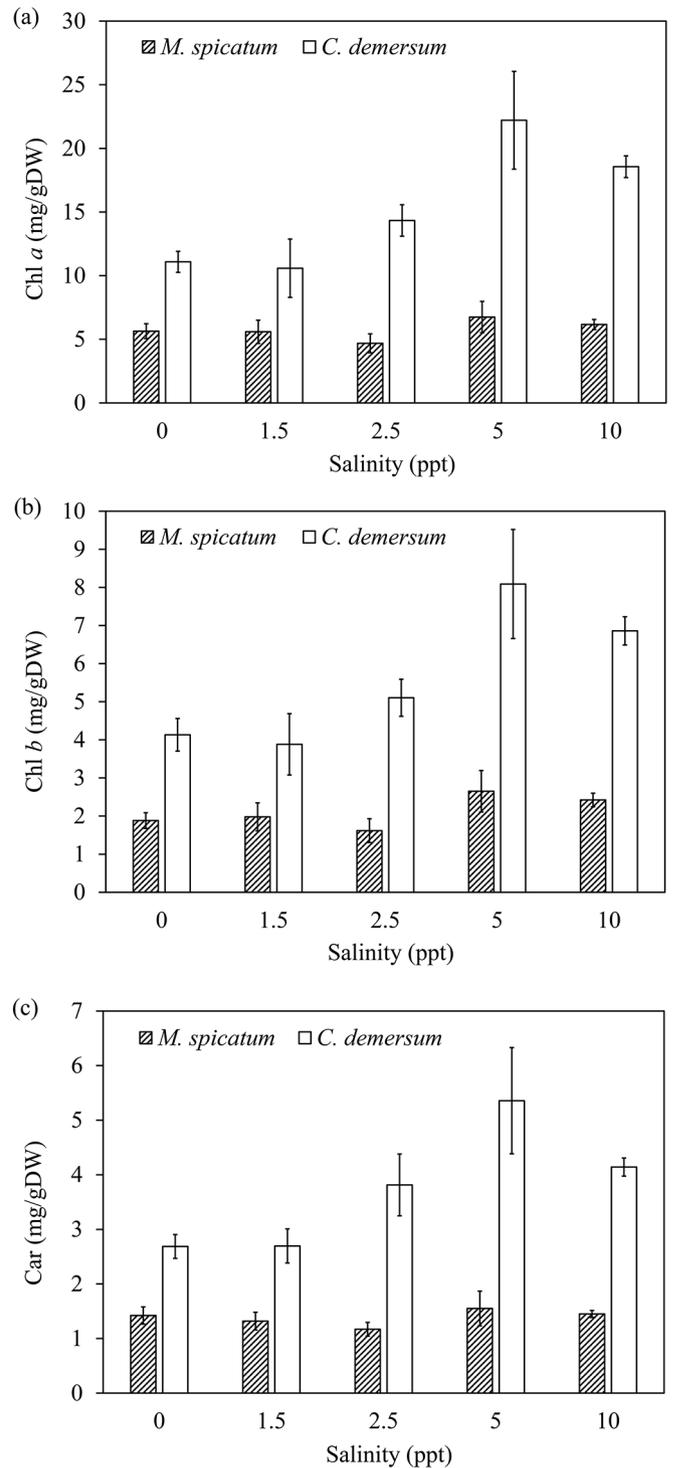


Fig. 3. Levels of photosynthetic pigments, (a) chlorophyll *a* (Chl *a*), (b) chlorophyll *b* (Chl *b*), and (c) carotenoids (Car), in *Myriophyllum spicatum* and *Ceratophyllum demersum* after exposure to different salinity levels (0, 1.5, 2.5, 5, and 10 ppt) for five days. Results are expressed as micrograms per gram of fresh weight ($\mu\text{g/g FW}$). Error bars represent the standard deviation.

2.5 ppt salinities were not different from 0 ppt (ANOVA, $P > 0.05$; Fig. 3a and b). However, Chl *a* and Chl *b* levels significantly increased in *C. demersum* exposed to 5 ppt and

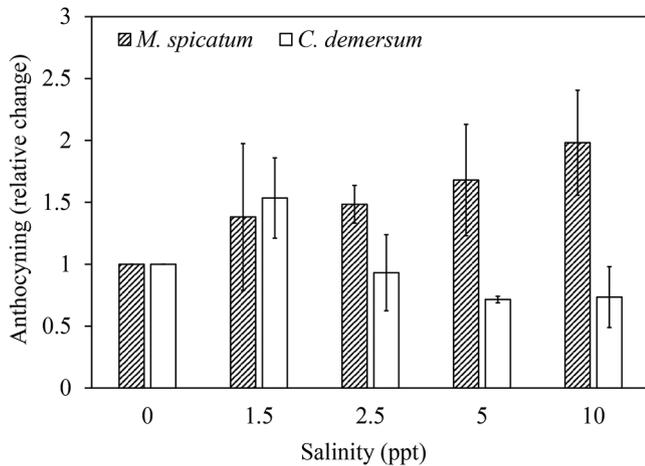


Fig. 4. Relative change of anthocyanin content in *Myriophyllum spicatum* and *Ceratophyllum demersum* after exposure to different salinity levels (0, 1.5, 2.5, 5, and 10 ppt) for five days. Anthocyanin levels were normalized to those of the respective control group. Error bars represent the standard deviation.

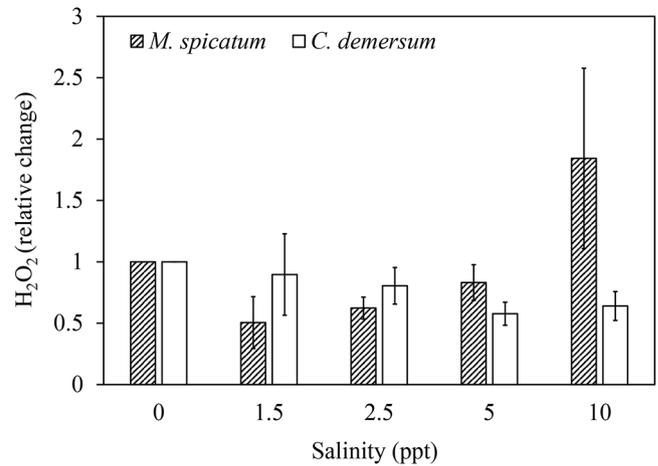


Fig. 5. Relative change of hydrogen peroxide levels in *Myriophyllum spicatum* and *Ceratophyllum demersum* after exposure to different salinity levels (0, 1.5, 2.5, 5, and 10 ppt) for five days. Hydrogen peroxide levels were normalized to those of the respective control group. Error bars represent the standard deviation.

10 ppt salinities compared with other experimental groups (ANOVA, $P < 0.01$). There was no significant difference in Chl *a* and Chl *b* levels of *C. demersum* exposed to 5 ppt and 10 ppt salinities (t -test, $P > 0.05$). The levels of Car in *M. spicatum* were not significantly affected by any of the salinity groups compared with the 0 ppt group (ANOVA, $P > 0.05$; Fig. 3c). In the case of *C. demersum*, Car content did not differ between 0 ppt and 1.5 ppt groups (t -test $P > 0.05$; Fig. 3c). Plants exposed to 2.5 ppt and 10 ppt salinity had higher Car levels than those in 0 ppt and 1.5 ppt groups (ANOVA, $P < 0.01$). The *C. demersum* exposed to 5 ppt salinity had the highest Car content, which is higher than the Car contents of other salinity groups (ANOVA, $P < 0.01$; Fig. 3c).

Anthocyanin content in *M. spicatum* showed an increasing trend as salinity increased (Fig. 4). In plants exposed to 2.5 ppt, 5 ppt, and 10 ppt salinities, anthocyanin levels increased significantly compared with the 0 ppt group (ANOVA, $P < 0.01$), but there was no significant difference between these three groups (ANOVA, $P > 0.05$). No significant difference was observed between *M. spicatum* exposed to 1.5 ppt salinity and other groups due to high variability (t -test, $P > 0.05$). The concentration of anthocyanins in *M. spicatum* exposed to 10 ppt salinity increased significantly compared with that in the 0 ppt and 2.5 ppt groups (ANOVA, $P < 0.05$), whereas anthocyanin content in the 1.5 ppt and 5 ppt groups did not differ significantly (ANOVA, $P > 0.05$). A different pattern was observed for *C. demersum*, for which the anthocyanin content reached the highest value in plants exposed to 1.5 ppt salinity, which was significantly higher than all other groups (ANOVA, $P < 0.05$). Anthocyanin levels tended to decrease in *C. demersum* exposed to 5 ppt and 10 ppt salinities compared with the 0 ppt group, but statistically significant changes were detected only for 10 ppt exposure (ANOVA, $P < 0.05$; Fig. 4).

Hydrogen peroxide levels tended to decrease in *M. spicatum* exposed to 1.5 ppt, 2.5 ppt, and 5 ppt salinities compared with those in the control group (Fig. 5). In 1.5 ppt and 2.5 ppt groups, H₂O₂ content was significantly lower than

that in plants maintained at 0 ppt (ANOVA, $P < 0.05$). There was no significant difference in H₂O₂ content between the 5 ppt group and the control group ($P > 0.05$). *Myriophyllum spicatum* exposed to 10 ppt salinity had the highest H₂O₂ content, which was significantly higher than that in all other groups (ANOVA, $P < 0.01$). In the case of *C. demersum*, H₂O₂ concentration showed a decreasing trend as salinity increased (Fig. 5). Hydrogen peroxide levels significantly decreased in *C. demersum* exposed to 5 ppt and 10 ppt salinities compared with those in the 0 ppt group (ANOVA, $P < 0.01$). Plants in the 5 ppt and 10 ppt groups did not differ significantly from each other (t -test, $P > 0.05$). *Ceratophyllum demersum* plants exposed to either 1.5 ppt or 2.5 ppt salinity were not significantly different from the control group in terms of H₂O₂ content (ANOVA, $P > 0.05$).

In *M. spicatum*, GPX activity showed an increasing trend as salinity increased up to 2.5 ppt when it reached the highest level (Fig. 6a); GPX activity was significantly higher in plants exposed to 2.5 ppt salinity than it was in all other groups (ANOVA, $P < 0.05$). In 1.5 ppt and 2.5 ppt groups, GPX activity was significantly higher than that in the control group (ANOVA, $P < 0.01$). As salinity increased to 5 ppt and 10 ppt, GPX activity tended to decrease and stabilize at levels similar to those observed in plants exposed to 1.5 ppt salinity. Nevertheless, GPX activity was still higher in *M. spicatum* exposed to 5 ppt and 10 ppt salinities than in control plants. ($P < 0.05$). With the exception of the 2.5 ppt group (t -test, $P > 0.05$), GPX activity increased in *C. demersum* exposed to saltwater (1.5 ppt, 5 ppt, and 10 ppt) compared to that in the control group (ANOVA, $P < 0.01$). Guaiacol peroxidase activity increased by 6.9-fold and 2.4-fold in *C. demersum* exposed to 10 ppt and 5 ppt salinities, respectively, compared with the control group (ANOVA, $P < 0.01$; Fig. 6a).

The exposure to saltwater at all levels significantly increased CAT activity in *M. spicatum* compared with the 0 ppt group (ANOVA, $P < 0.01$; Fig. 6b). There were no significant differences between 1.5 ppt and 2.5 ppt groups (t -test, $P > 0.05$) and between 5 ppt and the 10 ppt groups

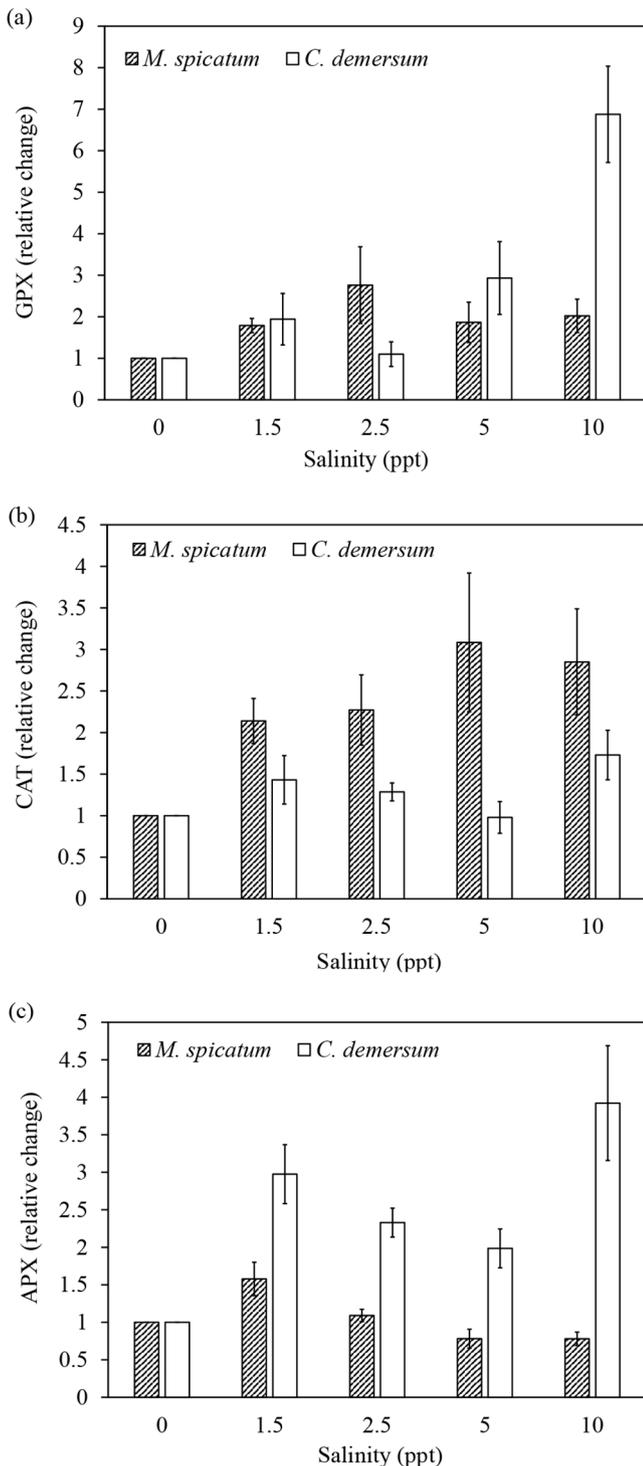


Fig. 6. Relative change of antioxidant enzymes activities, (a) guaiacol peroxidase (GPX), (b) catalase (CAT), and (c) ascorbate peroxidase (APX), in *Myriophyllum spicatum* and *Ceratophyllum demersum* after exposure to different salinity levels (0, 1.5, 2.5, 5, and 10 ppt) for five days. Enzymatic activities were normalized to those of the respective control group. Error bars represent the standard deviation.

(*t*-test, $P > 0.05$) in terms of CAT activity. The highest CAT activity levels were observed in *M. spicatum* exposed to 5 ppt salinity. The 5 ppt and 10 ppt groups were significantly higher

than that in 1.5 ppt and 2.5 ppt groups (ANOVA, $P < 0.05$). For *C. demersum*, CAT activity significantly increased in plants exposed to 1.5 ppt, 2.5 ppt, and 10 ppt salinities compared with that in the 0 ppt group (ANOVA, $P > 0.05$; Fig. 6b). Catalase activity in the 5 ppt group was not significantly different from that in the 0 ppt, 1.5 ppt, and 2.5 ppt groups (ANOVA, $P > 0.05$). The highest CAT activity for *C. demersum* was recorded in the 10 ppt group but was not significantly different from that in 1.5 ppt and 2.5 ppt groups (ANOVA, $P > 0.05$; Fig. 6b).

In *M. spicatum*, APX activity was the highest in plants exposed to 1.5 ppt salinity, whose APX levels were significantly higher than all other groups (ANOVA, $P < 0.01$; Fig. 6c). From 1.5 ppt to 10 ppt, APX activity tended to decrease and return to control levels. Ascorbate peroxidase activity in the 2.5 ppt group was not different from that in the 0 ppt group (*t*-test, $P > 0.05$), but it was significantly higher than that in plants exposed to 5 ppt and 10 ppt salinities. There was no significant difference between *M. spicatum* exposed to 5 ppt and 10 ppt salinities (*t*-test, $P > 0.05$), but these two groups had APX activity levels significantly lower than those in the other salinity groups (ANOVA, $P < 0.05$). The exposure to saltwater at all levels significantly increased APX activity in *C. demersum* compared with the control group ($P < 0.01$; Fig. 6c). From 1.5 ppt to 5 ppt, APX activity tended to reduce in *C. demersum*. There was no significant difference between APX activity in plants exposed to 1.5 ppt and 2.5 ppt salinities (*t*-test, $P > 0.05$), while APX activity in *C. demersum* exposed to 5 ppt salinity was significantly lower than that in the 1.5 ppt group (*t*-test, $P < 0.05$). There was no significant difference between *C. demersum* exposed to 2.5 ppt and 5 ppt salinities (*t*-test, $P > 0.05$). *Ceratophyllum demersum* plants exposed to 10 ppt salinity had higher APX activity than that in plants exposed to 2.5 ppt and 5 ppt; (ANOVA, $P < 0.01$); APX activity in the 10 ppt group was not significantly different from that of the 1.5 ppt group due to high variability (*t*-test, $P > 0.05$; Fig. 6c).

3.1 Relationships between parameters

Hydrogen peroxide levels showed a strong regression linear relationship with elongation rates in *M. spicatum* ($R^2 = 0.965$), whereas this relationship followed a second order polynomial distribution ($R^2 = 0.863$) in *C. demersum* (Tab. 1). The relationships between H_2O_2 content and levels of photosynthetic pigments (Chl *a*, Chl *b*, and Car) were weak for *M. spicatum* ($R^2 = 0.168$, 0.227, and 0.209 respectively), whereas these relationships were linear and strong for *C. demersum* ($R^2 = 0.942$, 0.944, and 0.858 respectively). As in the H_2O_2 levels, the relationships between elongation rates and photosynthetic pigments (Chl *a*, Chl *b*, and Car) were weak for *M. spicatum* ($R^2 = 0.190$, 0.394, and 0.077 respectively), whereas these relationships followed second-order polynomial distributions and strong at for *C. demersum* ($R^2 = 0.945$, 0.930, and 0.968 respectively). Further, elongation rates had a strong linear relationship with anthocyanin content in *M. spicatum* ($R^2 = 0.807$) and a second-order polynomial relationship with anthocyanin content in *C. demersum* ($R^2 = 0.701$). In *M. spicatum*, GPX activity had a weak second-order polynomial relationship with elongation ($R^2 = 0.4824$), whereas this relationship was linear and strong

Table 1. Regression relationships between different parameters of *Myriophyllum spicatum* and *Ceratophyllum demersum* under salinity exposure.

Parameters	Equation	R ²
H ₂ O ₂ vs. Elongation		
<i>M. spicatum</i>	Elongation = 0.1078 (H ₂ O ₂ ²) - 2.7379 (H ₂ O ₂) + 23.557	0.965
<i>C. demersum</i>	Elongation = 0.2127 (H ₂ O ₂) + 4.4377	0.863
H ₂ O ₂ vs. Photopigments		
<i>M. spicatum</i>		
	Chl <i>a</i> = 0.5943 (H ₂ O ₂) + 5.194	0.168
	Chl <i>b</i> = 0.3778 (H ₂ O ₂) + 1.747	0.227
	Car = 0.1248 (H ₂ O ₂) + 1.262	0.209
<i>C. demersum</i>		
	Chl <i>a</i> = -4.169 (H ₂ O ₂) + 44.343	0.942
	Chl <i>b</i> = -1.518 (H ₂ O ₂) + 16.171	0.944
	Car = -0.892 (H ₂ O ₂) + 9.939	0.858
Photopigments vs. Elongation		
<i>M. spicatum</i>		
	Chl <i>a</i> = -3.4177 (Elongation) + 30.615	0.190
	Chl <i>b</i> = -9.0176 (Elongation) + 29.943	0.394
	Car = -11.503 (Elongation) + 26.81	0.077
<i>C. demersum</i>		
	Chl <i>a</i> = -0.1609 (Elongation ²) + 2.5185 (Elongation) + 10.628	0.944
	Chl <i>b</i> = -0.0542 (Elongation ²) + 0.8421 (Elongation) + 4.340	0.930
	Car = -0.0479 (Elongation ²) + 0.8519 (Elongation) + 1.348	0.968
Anthocyanin vs. Elongation		
<i>M. spicatum</i>	Antho = -0.5321 (Elongation) + 20.707	0.807
<i>C. demersum</i>	Antho = 0.2324 (Elongation ²) + 3.5127 (Elongation) + 32.494	0.701
GPX vs. Elongation		
<i>M. spicatum</i>	GPX = -0.1352 (Elongation ²) + 2.0481 (Elongation) + 26.426	0.482
<i>C. demersum</i>	GPX = -115.46 (Elongation) - 2095.5	0.824
CAT vs. Elongation		
<i>M. spicatum</i>	CAT = -0.185 (Elongation) + 5.74	0.423
<i>C. demersum</i>	CAT = -0.1353 (Elongation ²) + 3.0291 (Elongation) + 28.597	0.719
APX vs. Elongation		
<i>M. spicatum</i>	APX = 0.1966 (Elongation) + 13.658	0.400
<i>C. demersum</i>	APX = 0.3927 (Elongation ²) + 9.6447 (Elongation) + 78.637	0.485

Chl *a*, chlorophyll *a* content; Chl *b*, chlorophyll *b* content; Car, carotenoids content; Antho, anthocyanin content; GPX, guaiacol peroxidase activity; CAT, catalase activity; APX, ascorbate peroxidase.

in *C. demersum* ($R^2=0.824$). A weak and linear relationship was found between CAT activity and elongation in *M. spicatum* ($R^2=0.423$), whereas this relationship was strong and followed a second-order polynomial distribution in and *C. demersum* ($R^2=0.719$). In the case of APX activity and elongation rate, a scattered distribution was observed for both *M. spicatum* and *C. demersum* ($R^2=0.399$ and 0.485 , respectively; [Tab. 1](#)).

4 Discussion

We assessed the effects of short-term exposure to different salinities on the growth rate and biochemical adjustments of two key macrophyte species, *M. spicatum* and *C. demersum*. The results indicated both shared and species-specific responses. The two species shared the same trend in elongation rates. On the other hand, the H₂O₂ and anthocyanin levels in response to salinity followed opposite trends. The antioxidant and photosynthetic pigments (Chl *a*, Chl *b*, and Car) responses were showed deviated responses between two species. Therefore, *M. spicatum* and *C. demersum* will exhibit different

levels of physiological responses for salt stress. In terms of elongation, *C. demersum* had a higher tolerance to salinity compared with *M. spicatum*. Its elongation was not significantly affected at 1.5 ppt salinity, while that of *M. spicatum* was significantly reduced at this level. Nevertheless, both species maintained elongation above 10% after 5 days exposure to 2.5 ppt or lower salinities. Therefore, it is expected that *M. spicatum* and *C. demersum* can maintain adequate elongation rates when exposed to salt water up to 2.5 ppt for a short period of time. However, according to extended duration studies on macrophytes responses to salinity ([Thouvenot and Thiébaud, 2018](#); [Warwick and Bailey, 1997](#)), deviations in responses of these two species expected from short duration exposure, which is to be tested in further experiments.

The levels of photosynthetic pigments (Chl *a*, Chl *b*, and Car) in both species increased at high salinity levels (though the pigments levels of *M. spicatum* were statistically not significant). Photosynthetic pigments concentrations in macrophytes have been reported to either increase or decrease in response to salinity exposure depending on the species and light intensity ([Twilley and Barko, 1990](#)). For example, under

low light conditions (light intensity not mentioned), Chl *a* content increases in *M. spicatum* as salinity increases (Twilley and Barko, 1990). In the present study, the 80–85 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic active radiation density in which plants were maintained is appropriate for the normal functioning of plants but can be considered as low light compared with natural conditions. Alternatively, the increase in photosynthetic pigment levels can be related to the excess Mg^{2+} in the artificial seawater used in the present study, which contains 0.94% MgCl_2 and, thus, increases Mg^{2+} supply as salinity increases. Magnesium is the most important ion for the synthesis of the central structure of chlorophyll molecules, and its increased availability stimulates the formation of chlorophyll (Candan and Tarhan, 2003; Terpstra and Lambers, 1983). Therefore, we speculate that the rise in pigment content in response to high salinity might also be related to the increased Mg^{2+} supply. However, further experiments are needed to evaluate the effect of artificial seawater containing Mg^{2+} on plant Mg^{2+} content.

In macrophytes under stress exposure, H_2O_2 content usually increases with the severity of the stress until a tolerance threshold level is reached (Asaeda *et al.*, 2018). However, H_2O_2 content tended to decrease as salinity increased in *C. demersum*. The anthocyanin content also followed similar trends to those of the H_2O_2 levels for both species. The anthocyanin content of plants usually increases in response to high salinity stress (Eryilmaz, 2006), as well as to nutrient deficiency, cold stress, and excessive light (Ahmed *et al.*, 2015; Liang and He, 2018; Pietrini *et al.*, 2002). Increased anthocyanin content is also considered to be a strategy to enhance antioxidant defenses in plants (Liu *et al.*, 2018) in response to reactive oxygen species (ROS) accumulation in plants under stress (Xu and Rothstein, 2018).

Antioxidant enzyme activities were not correlated with higher salinity or H_2O_2 content. Moreover, changes in the activity of antioxidant enzymes in response to high salinity differed between species. For example, CAT activity increased under all salt exposure conditions in *M. spicatum*, whereas it was rather stable in *C. demersum*. The patterns of GPX and APX activities changes were distinct between the two species, indicating that the antioxidant systems of *M. spicatum* and *C. demersum* respond differently to the salinity stress. Changes in antioxidant enzyme activities reflect distinct physiological states under different salinity levels. The salinity exposure of some terrestrial plant species also exhibited increased antioxidant activities without relating to a salinity gradient (Hishida *et al.*, 2014; Srivastava *et al.*, 2015). Therefore, exposure to high salinity stress is expected to elicit different antioxidant responses depending on the salinity level and species, which may not follow a consistent trend.

4.1 Biochemical parameters and elongation relationships

Temperature, light intensity, light duration, growth stage, and nutrient supply are the main factors that determine plant responses to stresses. In the present study, plants were maintained under controlled conditions within the optimal

ranges for both species. However, in the field, where environmental conditions frequently change, these macrophytes might respond differently to changes in salinity compared with the responses recorded here. Despite changing environmental conditions, the correlations between physiological and morphological parameters are expected to predict the condition of the species under stress exposure. In that regard, we found strong correlations between some parameters, although their levels fluctuated along the salinity gradient. The regression relationships between H_2O_2 levels and elongation rates, and anthocyanin contents and elongation rates are significant (Tab. 1), and this warrants further investigation into their suitability for evaluation of expected growth responses under field conditions.

The correlations between elongation rates and pigment levels in *M. spicatum* and *C. demersum* were described mostly by linear regression distribution and sometimes by second-order polynomial regression distributions. These relationships are candidate approaches to estimate the expected growth response of macrophytes to salinity influences based on biochemical parameters or vice versa. However, as our findings indicate, such correlations are species-specific. Therefore, any application of these correlations for macrophyte monitoring should be carefully selected in relation to the species. Moreover, the responses of other macrophytes to long-term salinity exposure are expected to differ from those reported here and, thus, warrant further research.

5 Conclusion

Findings of the present research indicate the occurrence of species-specific responses, even under lower salinity levels. These distinct responses are not always correlated with salinity, indicating that macrophytes reach specific physiological statuses at each salinity level. The species-specific responses of most parameters measured in the present study suggest the inapplicability of common biochemical responses across species. On the other hand, the correlations between H_2O_2 levels and elongation rates, and anthocyanin content and elongation rates for both species have potential applications in the monitoring and management of *M. spicatum* and *C. demersum*. These two macrophyte species should be further studied for long-duration salinity exposure to distinguish short duration and long duration exposure responses. There can be a desiccating effect of on plant physiology and morphology, especially at higher salinity exposure, which was not measured in the present experiment. Considering the desiccating effect in further studies is recommended for understanding the plant response mechanism under salinity stress.

Acknowledgments. This work was supported by the annual budget allocations of the Saitama University, Japan.

Supplementary Material

Supplementary Figure S1.

The Supplementary Material is available at <https://doi.org/10.1051/limn/2020021>.

References

- Aebi H. 1984. Catalase in Vitro. *Methods Enzymol* 105: 121–126.
- Ahmed NU, Park JI, Jung HJ, Hur Y, Nou IS. 2015. Anthocyanin biosynthesis for cold and freezing stress tolerance and desirable color in *Brassica rapa*. *Funct Integr Genomics* 15: 383–394.
- Asaeda T, Senavirathna MDHJ, Xia LP, Barnuevo A. 2018. Application of hydrogen peroxide as an environmental stress indicator for vegetation management. *Engineering* 4: 610–616.
- Bamber JL, Oppenheimer M, Kopp RE, Aspinall WP, Cooke RM. 2019. Ice sheet contributions to future sea-level rise from structured expert judgment. *Proc Natl Acad Sci* 116: 11195–11200.
- Barko J, Adams M, Clesceri N, 1986. Environmental factors and their consideration in the management of submersed aquatic vegetation: a review. *J Aquat Plant Manag* 24: 1–10.
- Bhuiyan MJAN, Dutta D. 2012. Assessing impacts of sea level rise on river salinity in the Gorai river network, Bangladesh. *Estuar Coast Shelf Sci* 96: 219–227.
- Borgnis E, Boyer KE. 2016. Salinity tolerance and competition drive distributions of native and invasive submersed aquatic vegetation in the upper San Francisco estuary. *Estuar Coasts* 39: 707–717.
- Candan N, Tarhan L. 2003. Relationship among chlorophyll-carotenoid content, antioxidant enzyme activities and lipid peroxidation levels by Mg²⁺ deficiency in the *Mentha pulegium* leaves. *Plant Physiol Biochem* 41: 35–40.
- Cañedo-Argüelles M, Kefford B, Schäfer R. 2019. Salt in freshwaters: causes, effects and prospects – Introduction to the theme issue. *Philos Trans R Soc B Biol Sci* 374: 20180002.
- Cao Y, Zhi Y, Jeppesen E, Li W. 2019. Species-specific responses of submersed macrophytes to simulated extreme precipitation: a mesocosm study. *Water* 11: 1160.
- Chiogna G, Skrobanek P, Narany TS, Ludwig R, Stumpp C. 2018. Effects of the 2017 drought on isotopic and geochemical gradients in the Adige catchment, Italy. *Sci Total Environ* 645: 924–936.
- Eryılmaz F. 2006. The relationships between salt stress and anthocyanin content in higher plants. *Biotechnol Equip* 20: 47–52.
- Feldmann T, Nöges P. 2007. Factors controlling macrophyte distribution in large shallow Lake Vörtsjärv. *Aquat Bot* 87: 15–21.
- Franklin P, Dunbar M, Whitehead P. 2008. Flow controls on lowland river macrophytes: a review. *Sci Total Environ* 400: 1–3.
- Germ M, Mazej Z, Gaberšček A, Sedej TT. 2006. The response of *Ceratophyllum demersum* L. and *Myriophyllum spicatum* L. to reduced, ambient, and enhanced ultraviolet-B radiation. *Hydrobiologia* 570: 47–51.
- Hishida M, Ascencio-Valle F, Fujiyama H, Orduño-Cruz A, Endo T, Larrinaga-Mayoral JA. 2014. Antioxidant enzyme responses to salinity stress of *Jatropha curcas* and *J. cinerea* at seedling stage. *Russ J Plant Physiol* 61: 53–62.
- Hrivnák R, Otahel'ová H, Valachovic M. 2009. Macrophyte distribution and ecological status of the Turiec River (Slovakia): changes after seven years. *Arch Biol Sci* 61: 297–306.
- Kaushal SS, Groffman PM, Likens GE, Belt KT, Stack WP, Kelly VR, Band LE, Fisher GT. 2005. Increased salinization of fresh water in the northeastern United States. *Proc Natl Acad Sci* 102: 13517–13520.
- Keskinkan O, Goksu MZL, Yuceer A, Basibuyuk M. 2007. Comparison of the adsorption capabilities of *Myriophyllum spicatum* and *Ceratophyllum demersum* for zinc, copper and lead. *Eng Life Sci* 7: 192–196.
- Li F, Xie Y, Chen X, Pan Y, Deng Z, Li X. 2011. Plant distribution can be reflected by physiological responses to salinity of three submersed macrophytes from the Modern Yellow River Delta. *Fundam Appl Limnol* 179: 159–167.
- Liang J, He J. 2018. Protective role of anthocyanins in plants under low nitrogen stress. *Biochem Biophys Res Commun* 498: 946–953.
- Lind L, Schuler MS, Hintz WD, Stoler AB, Jones DK, Mattes BM, Relyea RA. 2018. Salty fertile lakes: how salinization and eutrophication alter the structure of freshwater communities. *Ecosphere* 9: e02383.
- Liu Y, Tikunov Y, Schouten RE, Marcelis LFM, Visser RGF, Bovy A. 2018. Anthocyanin biosynthesis and degradation mechanisms in Solanaceous vegetables: a review. *Front Chem* 6: 52.
- MacAdam JW, Nelson CJ, Sharp RE. 1992. Peroxidase activity in the leaf elongation zone of tall fescue: I. Spatial distribution of ionically bound peroxidase activity in genotypes differing in length of the elongation zone. *Plant Physiol* 99: 872–878.
- Mechora Š, Cuderman P, Stibilj V, Germ M. 2011. Distribution of Se and its species in *Myriophyllum spicatum* and *Ceratophyllum demersum* growing in water containing se (VI). *Chemosphere* 84: 1636–1641.
- Mulamba T, Bacopoulos P, Kubatko EJ, Pinto GF. 2019. Sea-level rise impacts on longitudinal salinity for a low-gradient estuarine system. *Clim Change* 152: 533–550.
- Nakano Y, Asada K. 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiol* 22: 867–880.
- Nakata M, Ohme-Takagi M. 2014. Quantification of Anthocyanin Content. *Bio-Protocol* 4: e1098.
- Nielsen DL, Brock MA, Rees GN, Baldwin DS. 2003. Effects of increasing salinity on freshwater ecosystems in Australia. *Aust J Bot* 51: 655–665.
- Pietrini F, Iannelli MA, Massacci A. 2002. Anthocyanin accumulation in the illuminated surface of maize leaves enhances protection from photo-inhibitory risks at low temperature, without further limitation to photosynthesis. *Plant Cell Environ* 25: 1254–1259.
- Rameshkumar S, Radhakrishnan K, Aanand S, Rajaram R. 2019. Influence of physicochemical water quality on aquatic macrophyte diversity in seasonal wetlands. *Appl Water Sci* 9: 12.
- Rice KC, Hong B, Shen J. 2014. Assessment of salinity intrusion in the James and Chickahominy Rivers as a result of simulated sea-level rise in Chesapeake Bay, East Coast, USA. *J Environ Manag* 111: 61–69.
- Rout NP, Shaw BP. 2001. Salt tolerance in aquatic macrophytes: Ionic relation and interaction. *Biol Plant* 44: 95–99.
- Satterfield CN, Bonnell AH. 1955. Interferences in the titanium sulfate method for hydrogen peroxide. *Anal Chem* 27: 1174–1175.
- Schallenberg M, Hall CJ, Burns CW. 2003. Consequences of climate-induced salinity increases on zooplankton abundance and diversity in coastal lakes. *Mar Ecol Prog Ser* 251: 181–189.
- Søndergaard M, Johansson LS, Lauridsen TL, Jørgensen TB, Liboriussen L, Jeppesen E. 2010. Submersed macrophytes as indicators of the ecological quality of lakes. *Freshw Biol* 55: 893–908.
- Srivastava AK, Srivastava S, Lokhande VH, D'Souza SF, Suprasanna P. 2015. Salt stress reveals differential antioxidant and energetics responses in glycophyte (*Brassica juncea* L.) and halophyte (*Sesuvium portulacastrum* L.). *Front Environ Sci* 3: 19.

- Terpstra W, Lambers JWJ. 1983. Interactions between chlorophyllase, chlorophyll a, plant lipids and Mg²⁺. *Biochim Biophys Acta (BBA)/Protein Struct Mol* 746: 23–31.
- Thouvenot L, Haury J, Thiébaud G. 2012. Responses of two invasive macrophyte species to salt. *Hydrobiologia* 686: 213–223.
- Thouvenot L, Thiébaud G. 2018. Regeneration and colonization abilities of the invasive species *Elodea canadensis* and *Elodea nuttallii* under a salt gradient: implications for freshwater invasibility. *Hydrobiologia* 817: 193–203.
- Twilley RR, Barko JW. 1990. The growth of submersed macrophytes under experimental salinity and light conditions. *Estuaries* 13: 311–321.
- Vu DT, Yamada T, Ishidaira H. 2018. Assessing the impact of sea level rise due to climate change on seawater intrusion in Mekong Delta, Vietnam. *Water Sci Technol* 77: 1632–1639.
- Warwick NWM, Bailey PCE. 1997. The effect of increasing salinity on the growth and ion content of three non-halophytic wetland macrophytes. *Aquat Bot* 58: 73–88.
- Wellburn AR. 1994. The spectral determination of Chlorophylls a and b, as well as total Carotenoids, using various solvents with spectrophotometers of different resolution. *J Plant Physiol* 144: 307–313.
- Xu Z, Rothstein SJ. 2018. ROS-Induced anthocyanin production provides feedback protection by scavenging ROS and maintaining photosynthetic capacity in Arabidopsis. *Plant Signal Behav* 13: e1451708.

Cite this article as: Senavirathna MDHJ, Wijesinghe NA, Liu Z, Fujino T. 2020. Effects of short-term exposure to different salinity levels on *Myriophyllum spicatum* and *Ceratophyllum demersum* and suitability of biomarkers to evaluate macrophyte responses to salinity stress. *Ann. Limnol. - Int. J. Lim.* 56: 23