Ann. Limnol. - Int. J. Lim. 53 (2017) 271–280
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DOI: 10.1051/limn/2017013

Available online at:
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RESEARCH ARTICLE

Physiological and biochemical responses of *Egeria densa* to different sediment redox conditions

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Received: 31 March 2017; Accepted: 28 June 2017

Abstract – Under anaerobic or low oxygen regimes of aquatic environments, the sediment redox condition is one of the major abiotic stresses for plant growth and plays an important role in the distribution of species. In this study, we assessed the effects of reduced conditions, and macro and microelements associated with different redox-potential regimes on a submerged macrophyte, *Egeria densa*, in a microcosm experiment. Five treatments, namely, control (C), reduced (R), highly reduced (HR), transferred water from a reduced tank (RW), and transferred water from a highly reduced tank (HRW) were tested. To assess the effect of H_2S, *E. densa* was exposed to five NaHS (H_2S donor) concentrations (*viz.*, 0, 0.01, 0.05, 0.1, and 0.2 mM NaHS) in a separate microcosm experiment. The concentrations of macro and microelements in the experimental microcosms increased significantly compared to the control in the process of attaining R and HR treatment conditions. Plants exposed to low redox environments showed a significant reduction in the growth rate, photosynthetic pigments, and indole acetic acid; those treatments also showed an excess generation of hydrogen peroxide, peroxidase activities, catalase activities, and ascorbate peroxidase activities compared to plants exposed to the control. Our study suggests that in reduced conditions, low oxygen and high CO_2 concentrations result in a stress that has stronger effects on plants in terms of stress responses compared to soluble macro and microelements. This study will improve our ability to predict the dynamics of wetland aquatic vegetation and thus facilitate the formulation of wetland management and restoration strategies.

Keywords: redox condition / dissolved oxygen / dissolved hydrogen sulfide / oxidative stress / submerged macrophyte

1 Introduction

Macrophytes, especially submerged aquatic macrophytes, are considered important environmental tools for aquatic communities due to their roles in oxygen production, nutrient cycling, control of water quality, and sediment stabilization; likewise, they provide habitats and shelter for aquatic life (Ponnamperuma, 1972; Vardanyan *et al.*, 2008). These plants are crucial for the stabilization of the clear water state in different types of water bodies and can restore damaged aquatic environments by concealing anti-algal allelochemicals, removing nutrients from the water, and improving the water quality of aquatic ecosystems (van Donk and van de Bund, 2002; Collins *et al.*, 2005; Chang *et al.*, 2007); for example, in shallow, mesotrophic and eutrophic lakes (Perrow *et al.*, 1997; Scheffer, 1998). During their life cycle, aquatic macrophytes frequently encounter a combination of biotic and abiotic stress factors. Soil anoxia, waterlogging, submergence, and eutrophication are the major abiotic stresses for plant growth. In aquatic ecosystems, soil flooding and eutrophication change the physical and chemical characteristics of the soil, which lead to higher respiration rates. In turn, these higher respiration rates result in reduced conditions or low dissolved oxygen (DO) in poorly mixed waters (Pezeshki and DeLaune, 2012; Khan *et al.*, 2014). Recently, considerable research has been performed on the effects of reducing conditions on the mobility or availability of metals in soils (Charlatchka and Cambier, 2000; Miao *et al.*, 2006; Yu *et al.*, 2007). However, more studies with different species and at different eco-geographical settings are needed to completely understand the phenomena of low redox conditions on submerged macrophytes.

The redox potential, E_h, in wetland soils varies from −400 mV to +700 mV (Pearsall and Mortimer, 1939; Mortimer, 1941), and it is measured by the oxidation reduction potential (ORP). This condition results in two major problems for plant growth. First, O_2 decreases while other gases such as CO_2 and H_2S increase in the soil and water. Second, several macro and micro elements change to toxic forms under a low redox potential due to the activity of microbes (Atwell *et al.*, 2014). During the change of the soil redox status (from high E_h

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to low E_h), a series of redox reactions occur, which include denitrification (NO_3^- is reduced to N_2), manganese reduction (Mn^{4+} to Mn^{2+}), iron reduction (Fe^{3+} to Fe^{2+}), sulfate reduction (SO_4^{2-} to H_2S, S^{2+} or HS^-), methanogenesis and accumulation of acetic and butyric acids produced by microbial metabolism (Ponnamperuma, 1984; Patrick *et al.*, 1996; Kinsman-Costello *et al.*, 2015). In the laboratory, several methodologies were used to establish reduced conditions in sediments by numerous authors. Among them, glucose/sucrose was widely used as reducing material in the soils (Terrados *et al.*, 1999; Wu *et al.*, 2009; Zaman and Asaeda, 2013), which, is cost effective and can produce highly reduced conditions (∼−250 mV) within a short period of time. Rice straws and chopped plant materials were also used by certain authors as organic matter to create reduced conditions in soils (Marin *et al.*, 1993; Miao *et al.*, 2006). In the present experiment, glucose is used as a reducing agent to decrease the soil E_h by microbial fermentation.For the management of aquatic ecosystems, it is important to understand the interaction between aquatic plants and environmental factors. In reduced aquatic systems, macrophytes, especially submerged macrophytes, are affected by the reduced conditions of the soils. However, under the reduced conditions, it is difficult to separate the effect of low oxygen and high CO_2 concentration from that of phytotoxic by-products on aquatic macrophytes. Such information is necessary to understand the importance of submerged macrophytes for the restoration of the degraded aquatic systems. Especially for eutrophic lake restorations, understanding the anoxia tolerance and metal toxicity levels of aquatic plants is particularly vital. Certain aquatic macrophytes have a high tolerance to anoxia but a low tolerance to metal toxicity and vice versa. The water quality (dissolved oxygen increase) can be improved in these types of anoxic waterbodies by natural (wind and wave action) or artificial (aeration) ways (McGinnis *et al.*, 2004). The authors hypothesized that under reduced conditions, the gaseous compounds (low oxygen and high CO_2) have more effect on submerged macrophytes than the soluble macro and micro elements could do in water. To test the hypothesis, an experiment was conducted using water transferred from reduced (R) and highly reduced (HR) tanks (described in Section 3.1), which thus contains the same amount of macro and micro elements as the R and HR tank. Specifically, there is a lack of knowledge on the physiological and biochemical effects of low oxygen concentrations and phytotoxic stressors on *Egeria densa* Planch. The overall aim of this study was to assess the physiological and biochemical stress responses of *E. densa* under different sediment redox conditions. In this regard, the growth, chlorophyll content, indole acetic acid (IAA), malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and anti-oxidant enzymes (guaiacol peroxidase [POD], catalase [CAT], ascorbate peroxidase [APX]) of *E. densa* are measured after the exposure of different redox conditions.

2 Experimental methodology

E. densa was sampled from Hofu river, Hiroshima, Japan (34°11′390″ N, 131°39′249″ E). After carefully washing with tap water, the plants were cultured in glass aquaria under laboratory conditions. These plants were maintained for subsequent use in the main experiments. Before the beginning of the experiment, approximately 10 cm apical tips of the study plants were planted in the separate experimental microcosms (15.7 cm × 15.7 cm × 24.5 cm) and were left for three weeks for adapting to the laboratory conditions, which later used for the experiment. No algal growth was recognized in all the experimental processes. Commercial river sediments (grain size, 85% <1 mm), which contained 3.12 ± 0.17% ($n = 3$) of organic matter, were used for plant culture and experimental condition preparation. In addition, 5% Hoagland solution (Hoagland and Arnon, 1950) was used as growth media following the methods of Atapaththu and Asaeda (2015), where less than 10% of the Hoagland solution was suggested as suitable experimental culture conditions for *Elodea nuttallii* due to its better growth and lower oxidative stress. All the experiments were conducted in a growth chamber at a controlled temperature of 23 ± 4 °C. The photon flux density was maintained at approximately ∼100 μmol m^{-2} s^{-1} provided by fluorescent lamp tubes with a photoperiod of 12 h light and 12 h dark.

3 Experimental conditions

3.1 Experiments on the reduced condition effects

In the first series of experiments, the effects of reduced conditions and reduced water on *E. densa* were investigated. *E. densa* was incubated in control (C), reduced (R), highly reduced (HR) water, and in water transferred from reduced (RW) and highly reduced (HRW) tanks. Each condition was replicated three times, and a range of redox potential for each condition was maintained; that is, control (C, +300 mV to +350 mV), reduced (R, +5 mV to −30 mV), highly reduced (HR, −230 mV to −180 mV), transferred water from reduced tank (RW, +300 mV to +350 mV), and transferred water from highly reduced tank (HRW, +300 mV to +350 mV). The flow chart of experimental methodology is described in Figure 1. Briefly, a 6 l (15.7 cm × 15.7 cm × 24.5 cm) glass vessel, hermetically sealed with an air-tight lid, was used for each experiment. The microcosms (MC) were filled with 600 g of air-dried commercial soil and deionized water in a 1:5 ratio. The total experimental period was 28 days (21 days for condition preparation and 7 days for plant stress observation), as plants exposed to R and HR conditions showed brown discoloration and increased mortality after 7 days. The reduced and highly reduced conditions were produced in six microcosms by adding 3 and 5 g of glucose, respectively, on the 1st, 3rd, 5th, and 14th day (Yu *et al.*, 2007; Zaman and Asaeda, 2013). After the condition prepared at 21st days, air bubbling was conducted (to raise the dissolved oxygen in water) in three reduced and highly reduced treatments. Water from these six tanks was further transferred into new tanks to establish the RW and HRW treatment microcosms. The water loss was less than 200 ml during the transfer as pore water was also transferred after the centrifugation of the soil. The water loss was adjusted by adding distilled water to maintain 3 l for all the experimental tanks. Approximately 600 g of air-dried commercial soil was used as substrate for the RW and RHW treatments. Before the plantation, the pH was adjusted and maintained between 5.0 and 5.5 by adding 0.1 M KOH or 0.5 M HCl in every microcosm, including the control condition. In the control condition, the water was gently

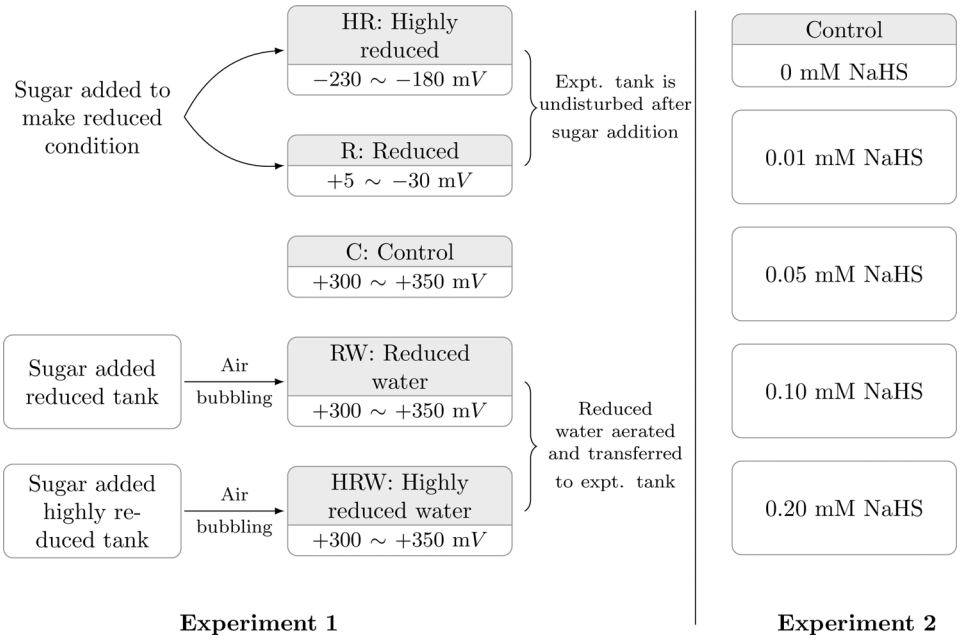

Fig. 1. Flow chart of the experimental conditions. In the 1st series of experiments, R, HR, RW, and HRW denote reduced, highly reduced, transferred water from reduced tank and transferred water from highly reduced tank, respectively. In the 2nd series of experiments, different concentrations of H_2S were applied.

and carefully mixed with the aid of a plastic rod to avoid possible stratifications. On the 21st day, six plants were collected from the experimental tanks and planted in each microcosm after measuring the initial length and weight. The experiments continued for 7 days (until the 28th day). The physiological and biochemical parameters of plants were measured at the end of the experiment. During the experimental period, the redox potential (E_h) and the pH were measured each day by a portable pH/ORP meter (Thermo Scientific Orion Star and Star Plus Meter and POT-101M, SIBATA, Japan).

3.2 Experiments on the H_2S effects

In a second series of experiments, the effects of the H_2S concentration on the growth and stress responses of *E. densa* were investigated. A seven-day hydroponic experiment was conducted to observe the growth and biochemical responses of *E. densa* exposed to five levels of H_2S concentrations (control, 0.01, 0.05, 0.1, and 0.2 mM). The H_2S concentrations were selected based on the first series of experimental results (Tab. 1). Experimental beakers were sealed with nontoxic silicon to prevent H_2S gas from escaping. To achieve the desired H_2S concentrations, sodium hydrogen sulfide (NaHS, Sigma–Aldrich, Japan) was used as a hydrogen sulfide (H_2S) donor (Zhang *et al.*, 2010; Hu *et al.*, 2012; Hou *et al.*, 2013; Ali *et al.*, 2015; Parveen *et al.*, 2017). The targeted H_2S concentrations were achieved by adding 10 mM NaHS via syringe, after deoxygenating the media with inert gas. The culture medium of each treatment was changed every day due to the relatively short half-life of H_2S (12–37 h depending on the conditions) (Napoli *et al.*, 2006). The H_2S concentration of the water was measured colorimetrically every day by extracting 5 ml of the medium (Cline, 1969). The 1st and 2nd series of experiments were conducted in parallel under similar laboratory conditions.

3.3 Sediment and water analyses

Sediment samples were air-dried and sieved with a sieve machine. The particle sizes of the sediments were determined according to the protocol of the American Society for Testing and Materials (ASTM D422-63, 2002). The organic matter content of the sediments was measured with the Walkley–Black method (Walky and Black, 1934). Approximately 100 ml of water was collected from the microcosms on 21st day (total experimental period 21 + 7 days) of the experimental period and passed through a Whatman 42 filter paper. The water samples were kept in a refrigerator at 4 °C for further nutrient analyses. The total phosphorous (TP) was measured with the ascorbic acid method (John, 1970). The concentration of iron (Fe), manganese (Mn), zinc (Zn), lead (Pb), calcium (Ca), magnesium (Mg), copper (Cu), and potassium (K) of the water samples were measured with an atomic absorption spectrophotometer (AAS; Shimadzu AA-660G) using the direct air-acetylene flame method. The precipitated Fe in the water was measured by the filtration of water on 0.42 µm membrane filters, which were further treated with 8 ml nitric acid (2 M) (Immers *et al.*, 2014).

3.4 Measurement of dissolved oxygen (DO), dissolved CO_2 and dissolved hydrogen sulfide (H_2S)

DO and dissolved CO_2 was measured every day by a dissolved oxygen and temperature meter (HI 9146, Japan) and carbon dioxide meter (DKK-TOA Corporation, Japan). Dissolved H_2S, which is the sum of un-ionized hydrogen sulfide (H_2S), bisulfide ions (HS^-), and sulfide ions (S^{2-}), was determined colorimetrically using the methylene blue method (Cline, 1969). For each set of samples, NaHS was used as a calibration standard, and the results were expressed in mM.

Table 1. Dissolved oxygen (DO, mg l^{-1}), dissolved CO_2 (mg l^{-1}), dissolved H_2S (mM), and concentration of elements (mg l^{-1}, mean ± SD) in the water of experimental microcosms under different treatments ($n = 3$).

	C	R	HR	RW	HRW	$F_{(4,10)}$	P
DO (mg l^{-1})	6.0 ± 0.5[a]*	0 ± 0[b]	0 ± 0[b]	5.7 ± 0.3[a]	5.3 ± 0.3[a]	273.5	<0.01
CO_2 (mg l^{-1})	6.0 ± 0.4[a]	14.6 ± 3.6[b]	34.2 ± 3.1[c]	7.7 ± 0.4[a]	7.9 ± 0.7[a]	86.5	<0.01
H_2S (mM)	0 ± 0[a]	0.03 ± 0.0[b]	0.04 ± 0.0[c]	0.00 ± 0.0[a]	0.00 ± 0.0[a]	71.35	<0.01
pH	5.3 ± 0.1	5.1 ± 0.1	5.1 ± 0.1	5.1 ± 0.1	5.1 ± 0.1	2.4	<0.1
Ca	5.2 ± 0.3[c]	22.2 ± 1.2[b]	57.1 ± 1.4[a]	22.3 ± 0.8[b]	51.4 ± 3.8[a]	398.6	<0.01
Mg	8.2 ± 0.8[c]	15.1 ± 0.6[b]	25.7 ± 3.4[a]	14.4 ± 0.6[b]	23.4 ± 2.0[a]	44.6	<0.01
K	1.0 ± 0.2[b]	2.5 ± 0.5[a]	2.6 ± 0.3[a]	2.3 ± 0.3[a]	2.6 ± 0.26[a]	12.1	<0.01
Fe	2.3 ± 0.03[c]	10.4 ± 1.2[b]	16.4 ± 2.5[a]	9.7 ± 1.6[b]	15.8 ± 1.5[a]	53.6	<0.01
Cu	0.1 ± 0.0[c]	0.4 ± 0.1[b]	0.6 ± 0.0[a]	0.4 ± 0.05[b]	0.6 ± 0.03[a]	54.0	<0.01
Zn	0.1 ± 0.0[b]	0.2 ± 0.1[b]	0.8 ± 0.1[a]	0.2 ± 0.03[b]	0.8 ± 0.05[a]	59.4	<0.01
Mn	0.2 ± 0.0[c]	3.7 ± 0.3[b]	4.3 ± 0.0[a]	3.6 ± 0.07[b]	4.6 ± 0.12[a]	141.5	<0.01
Pb (μg l^{-1})	0.04 ± 0.0	0.05 ± 0.0	0.04 ± 0.0	0.05 ± 0.0	0.05 ± 0.0	1.5	<0.2
Cd (μg l^{-1})	0.05 ± 0.01	0.06 ± 0.0	0.07 ± 0.01	0.06 ± 0.02	0.062 ± 0.03	2.9	<0.1
TP	0.5 ± 0.13[c]	2.3 ± 0.3[b]	3.1 ± 0.1[a]	2.4 ± 0.00[b]	2.7 ± 0.00[a]	59.9	<0.01
NH_4-N	0.07 ± 0[c]	0.29 ± 0.08[b]	0.52 ± 0.07[a]	0.30 ± 0.07[b]	0.50 ± 0.06[a]	82.1	<0.01

* Different superscripts in the same row indicate significant differences among treatments; the same superscript letter as the control indicates no significant differences. Different lowercase letters indicate significantly different values analyzed with an ANOVA followed by a Tukey's test; $P < 0.05$.

3.5 Plant sampling and growth measurements

At the end of the experiment, plant samples were collected for physical and chemical analyses. The samples were washed with distilled water to remove the soil and dried by blotting with laboratory tissue. The final shoot length and fresh weight were measured to calculate the plant growth rate. The same plant was used to analyze the chlorophyll and carotenoid contents and for a fluorescence measurement. Other plants were used for hormone, enzyme, and lipid peroxidation analyses.

The relative growth rate (RGR) was calculated with the following equation:

$$\mathrm{RGR} = \frac{\ln W_2 - \ln W_1}{T_2 - T_1}$$

where RGR is the weight increasing rate in mg day^{-1}, and W_1 and W_2 are the plant's initial and final fresh weights at times (days) T_1 and T_2, respectively. T_1 and T_2 are the initial time (1st day) and the final time (7th day). The shoot growth rate (SGR) was calculated with the following equation:

$$\mathrm{SGR} = \frac{L_2 - L_1}{T_2 - T_1}$$

where L_1 and L_2 are the initial and final shoot lengths (mm) at times (days) T_1 and T_2, respectively. As such, SGR was expressed in mm day^{-1}.

3.6 Photosynthetic pigments and chlorophyll fluorescence determination

The chlorophyll and carotenoid contents were extracted by placing fresh leaves and stems in 5 ml of *N*,*N*-dimethylformamide for 24 h in the dark at 4 °C (Rashid *et al.*, 2010). The total chlorophyll and carotenoid contents were determined spectrophotometrically at 665, 649, and 470 nm (UV–vis spectrophotometer, Shimadzu, Japan) using the equations given by Porra *et al.* (1989) and expressed in mg g^{-1} FW.

The chlorophyll fluorescence measurements were performed with a handy fluorocam (FC1000-H, Photon Systems Instruments, Czech Republic) using auto image segmentation. Fresh leaf samples were placed in a petri dish separately with 20 ml of water taken from the same tank and each sample was subjected to a dark adaptation for 15 min before the fluorescence measurement. The maximum photochemical efficiency of PSII (F_v/F_m) was automatically calculated using the following equation:

$$\frac{F_v}{F_m} = \left(\frac{F_m - F_0}{F_m}\right)$$

where F_m and F_0 are the maximum and minimum fluorescence in dark adapted stress (DeEll and Toivonen, 2003).

3.7 Hydrogen peroxide (H_2O_2), guaiacol peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and indole acetic acid (IAA) assays

For the analysis of endogenous H_2O_2 concentrations, samples were extracted with cold acetone following the methods of Cervilla *et al.* (2007). Approximately 100 mg of plant samples were extracted with cold acetone, and 1 ml of aliquot was mixed with 200 μl of 0.1% titanium dioxide in 20% (v/v) H_2SO_4. The mixture was centrifuged at 5000 rpm for 15 min. The intensity of the yellow color was measured spectrophotometrically at 415 nm. H_2O_2 concentrations were estimated using a standard curve prepared from known concentrations of H_2O_2 and are presented as μmol g^{-1} FW.

A phosphate buffer (0.1 mM) at pH 6 with 0.1 g polyvinylpyrrolidone (PVP) was used to make extracts for the analysis of the guaiacol peroxidase (POD, EC 1.11.1.7), catalase (CAT, EC 1.11.1.6), and ascorbate peroxidase (APX, EC 1.11.1.11) activities. POD was assayed according to the method of Goel *et al.* (2003). The reaction mixture containing 1.2 µl of 30% H_2O_2, 1.68 µl of 100% guaiacol, and 2.89 ml of 0.1 M potassium phosphate buffer (pH 6.0) was freshly mixed. The reaction was initiated with the addition of 0.1 ml of enzyme extract. The change in absorbance was recorded at 470 nm at an interval of 15 s for 3 min using an extinction coefficient of 26.6 $mmol^{-1}\,cm^{-1}$. The CAT activity was determined following the methods of Aebi (1984). The CAT activity was calculated using the extinction coefficient of 40 $mmol^{-1}\,cm^{-1}$. The APX activity was assayed following the methods of Nakano and Asada (1981). The decrease in absorbance at 290 nm was recorded every 15 s for 3 min. The APX activity was calculated using the extinction coefficient of 2.8 $mmol^{-1}\,cm^{-1}$. The POD, CAT, and APX activities are presented as µmol/min/g FW.

The concentration of IAA, the most abundant form of auxins in plant tissues, was measured using the Salowski reagent (Gordon and Weber, 1951). Approximately 100 mg of fresh weight (FW) from the apical tip was grounded in 2.5 ml of distilled water and centrifuged at 5000 × *g* at 20 °C for 15 min. After extracting, 1 ml of the supernatant was added to 2 ml of the Salowski reagent, and the color development was measured after 1 h at 530 nm (Ellawala *et al.*, 2011). The results are presented as µg g^{-1} FW.

3.8 Statistical analyses

The homogeneity of variance and the Levine's tests, which assessed the equality of variances, were performed on the datasets prior to the statistical analysis to verify the assumptions of normal distribution and homogeneity of variances. The data were presented as the mean ± standard deviation (SD; $n = 3$). The data recorded at the end of the experiment were subjected to a one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test to evaluate the mean difference at the 0.05 significance level. All the statistical analyses were performed using SPSS version 16.

4 Results

4.1 Water quality and element concentrations in treatment microcosm

All the experimental microcosms were run for 21 days to satisfy the programed conditions. The parameters of the experimental tanks before the *E. densa* planting are shown in Table 1. During the seven days of the experiment, the redox potential (E_h) and pH of the experimental tanks were maintained in the range of the programed conditions. In the R and HR conditions, low DO concentrations (0 mg l^{-1}) and high levels of CO_2 and H_2S were obtained. Instead, in the RW and HRW microcosms, the DO concentration was raised by air bubbling (~6 mg l^{-1}), whereas both CO_2 and H_2S declined. No significant differences ($P < 0.05$) were observed for DO, CO_2 and H_2S, with respect to the control, and these results are presented in Table 1. The concentrations of other elements are also shown in Table 1. The concentrations of elements (except Pb and Cd) were significantly higher in the water of the R, HR, RW, and HRW microcosms than those in the control microcosm. Among the analyzed elements, the Ca and Fe concentrations increased approximately 4 times and 5 times in the R and RW conditions, respectively, and 10 times and 8 times in RW and HRW conditions, respectively, compared to the control. Increments of Cd and Pb in the R, HR, RW, and HRW conditions were not significantly different from the control.

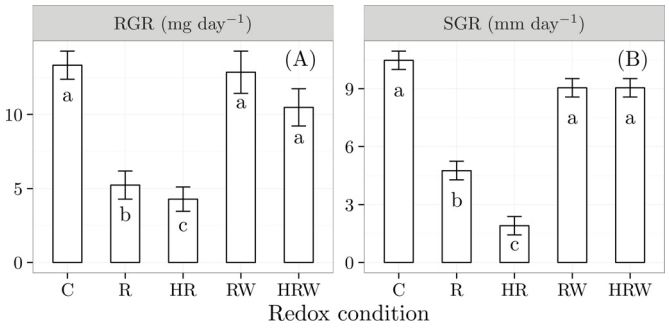

Fig. 2. Relative growth rate (RGR) (A) and shoot growth rate (SGR) (B) of *E. densa* under control (C), reduced (R), highly reduced (HR), transferred water from reduced tank (RW), and transferred water from highly reduced tank (HRW) conditions. Values are the means of three replicates ± SD. Bars with different letters are significantly different at $P < 0.05$, $n = 3$.

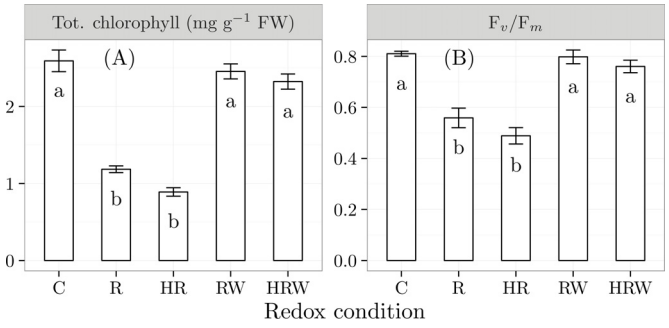

Fig. 3. Total chlorophyll and F_v/F_m values of *E. densa* under control (C), reduced (R), highly reduced (HR), transferred water from reduced tank (RW), and transferred water from highly reduced tank (HRW) conditions. Values are the means of three replicates ± SD. Bars with different letters are significantly different at $P < 0.05$, $n = 3$.

4.2 Physiological and biochemical responses of plant

The growth rate of plants is shown in Figure 2. The growth rate in the R and HR conditions was lower for both RGR ($F = 14.7$, $P < 0.0$) and SGR ($F = 56.7$, $P < 0.0$) than those of the control, RW, and HRW conditions. The RGR and SGR were statistically similar between the control and RW and HRW conditions ($P < 0.97$); however, SGR significantly differed between the R and HR conditions ($P < 0.05$). Figure 3 shows the total chlorophyll concentrations in plants and the F_v/F_m values (the average value of the maximum quantum yield PSII) of plants exposed to different conditions. The chlorophyll concentration decreased the R and HR condition ($P < 0.05$), whereas there was no significant difference

observed between the RW and HRW treatments and the control. The F_v/F_m decreased in both the R and HR conditions; however, it did not significantly decrease in the RW and HRW conditions compared to the control.

Figure 4A shows that the highest H_2O_2 concentration was observed in the HR condition, followed by R, HRW, RW, and the control conditions. The activities of guaiacol peroxidase (POD), ascorbate peroxidase (APX), and catalase (CAT) are also shown in Figure 4B–D, respectively. As with H_2O_2, these enzymes were also the highest in plants subjected to the HR condition, followed by the R condition. Likewise, no statistically significant differences were observed in plants exposed to the RW, HRW, and control conditions ($P < 0.05$) for the above mentioned enzymes. The combined data on shoot growth and IAA concentrations of experimental plants revealed that the shoot growth strongly declined with decreasing IAA concentrations (Fig. 5).

4.3 Response of *E. densa* to H_2S concentration

The SGR, RGR, total chlorophyll, and F_v/F_m values of *E. densa* grown in different levels of H_2S concentrations are shown in Table 2. All the parameters decreased when plants were exposed to more than 0.05 mM H_2S ($P < 0.05$). The total chlorophyll concentration was significantly lower in 0.2 mM H_2S and gradually increased with decreasing H_2S concentrations ($P < 0.05$).

Instead, the H_2O_2 concentration gradually increased with increasing H_2S concentrations. The highest H_2O_2 concentration was found in plants grown under a concentration of 0.2 mM H_2S, and there was a significant difference compared to the plants exposed to a 0.1 mM H_2S concentration ($P < 0.05$). The H_2O_2 concentrations of plants exposed to 0, 0.01, and 0.05 mM H_2S were not significantly different from each other (Tab. 2). Similarly, the POD activity showed similar trends, and the highest POD activity was observed in plants exposed to 0.2 mM H_2S. There were no significant differences observed in plants exposed to control, 0.01, and 0.05 mM H_2S in terms of growth and stress responses (Tab. 2).

Pearson correlation coefficients among SGR (mm/day), RGR (mg/day), total chlorophyll (mg/g FW), F_v/F_m, hydrogen peroxide (H_2O_2, µmol/g FW), guaiacol peroxidase (POD, µmol/min/g FW), catalase (CAT, µmol/min/g FW), ascorbate peroxidase (APX, µmol/min/g FW), and IAA (µmol/g FW) in *E. densa* after exposure to experiment 1 and experiment 2 are listed in Table 3. Significant positive correlations ($P < 0.01$ and 0.05) were observed among RGR, total chlorophyll, F_v/F_m and IAA with SGR, and negative correlations were observed for H_2O_2, POD, CAT, APX with SGR in both redox and H_2S treatments. Figure 5 shows the correlation between IAA and growth rate (SGR, RGR) of plants subjected to different redox conditions and H_2S concentrations. IAA had a strong positive correlation with SGR ($R^2 = 0.92$) and RGR ($R^2 = 0.82$). In addition, the lowest IAA and growth rate were observed when the plants were exposed to the R and HR conditions. There was a significant negative correlation ($R^2 = 0.91$) observed between the presence of H_2O_2 and the total chlorophyll concentration in different redox and H_2S treatments (Tab. 3 and Fig. 6). High H_2O_2 and low chlorophyll concentrations were observed when plants were exposed to R, HR, and 0.1, 0.2 mM H_2S

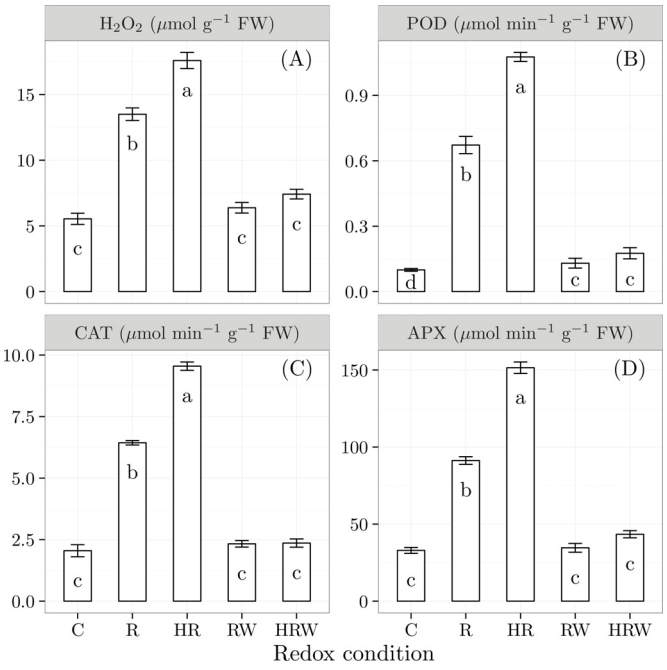

Fig. 4. Hydrogen peroxide (H_2O_2) (A), guaiacol peroxidase activities (POD) (B), catalase activity (CAT) (C), and ascorbate peroxidase activities (APX) (D) of *E. densa* under control (C), reduced (R), highly reduced (HR), transferred water from reduced tank (RW), and transferred water from highly reduced tank (HRW) conditions. Values are the means of three replicates ± SD. Bars with different letters are significantly different at $P < 0.05$, $n = 3$.

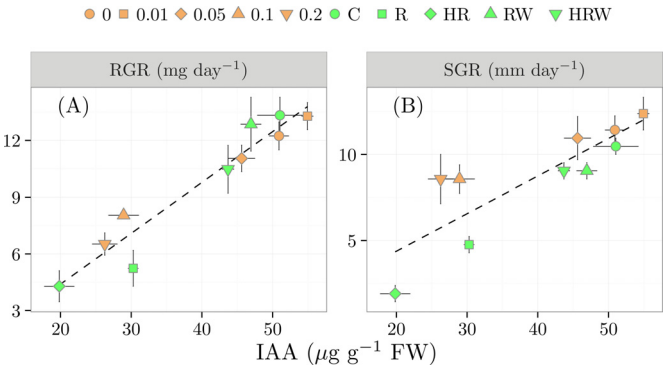

Fig. 5. Relationship between IAA and growth rate of *E. densa* under control (C), reduced (R), highly reduced (HR), transferred water from reduced tank (RW), transferred water from highly reduced tank (HRW) conditions, and 0, 0.01, 0.05, 0.1, 0.2 mM H_2S concentrations. Values are the means of three replicates ± SD, $n = 3$.

concentrations. Figure 6 shows the relationship among POD (A), CAT (B), and APX (C) with H_2O_2 under different redox and H_2S conditions.

5 Discussion

In aquatic ecosystems, the sediment redox conditions are produced by a complex regulatory system, which depends on several factors such as mineral composition, organic content of

Table 2. Growth and stress responses of *E. densa* exposed to different dissolved H_2S concentrations ($n = 3$).

	H_2S concentration (mM)					$F_{(4,10)}$	P
	0	0.01	0.05	0.10	0.20		
SGR (mm/day)	*11.43 ± 1.4[a]	12.38 ± 1.7[a]	11.0 ± 2.1[a]	8.57 ± 1.4[b]	8.57 ± 2.5[b]	2.53	0.01
RGR (mg/day)	12.24 ± 1.3[a]	13.29 ± 1.3[a]	11.05 ± 1.2[a]	8.05 ± 0.3[b]	6.5 ± 1.0[c]	20.88	<0.01
Total Chl. (mg/g) FW	2.4 ± 0.2[a]	2.4 ± 0.2[a]	2.2 ± 0.1[a]	1.7 ± 0.1[b]	0.9 ± 0.1[c]	53.1	<0.01
F_v/F_m	0.81 ± 0.03[a]	0.79 ± 0.01[a]	0.77 ± 0.03[a]	0.69 ± 0.05[b]	0.59 ± 0.11[c]	7.74	<0.01
H_2O_2 content (μmol/g FW)	4.77 ± 0.4[c]	4.97 ± 0.6[c]	5.33 ± 0.4[c]	9.96 ± 0.3[b]	16.50 ± 1.9[a]	90.35	<0.01
POD activity (μmol/min/g FW)	0.23 ± 0.01[c]	0.22 ± 0.04[c]	0.26 ± 0.02[c]	0.46 ± 0.06[b]	0.58 ± 0.04[a]	53.01	<0.01

* Different lowercase letters indicate significantly different values analyzed with an ANOVA followed by a Tukey's test; $P < 0.05$.

Table 3. Pearson correlation coefficients among SGR (mm/day), RGR (mg/day), total chlorophyll (mg/g FW), F_v/F_m, hydrogen peroxide (H_2O_2, μmol/g FW), guaiacol peroxidase (POD, μmol/min/g FW), catalase (CAT, μmol/min/g FW), ascorbate peroxidase (APX, μmol/min/g FW), and IAA (μg/g FW) in *E. densa* after exposure to experiment 1 and experiment 2, $n = 3$.

	SGR	*RGR*	Total Chl.	F_v/F_m	H_2O_2	POD	CAT	APX
Experiment 1								
RGR	0.90**							
Total Chl.	0.96**	0.92**						
F_v/F_m	0.94**	0.83**	0.94**					
H_2O_2	−0.95**	−0.86**	−0.97**	−0.95**				
POD	−0.96**	−0.86**	−0.96**	−0.94**	0.98**			
CAT	−0.97**	−0.87**	−0.98**	−0.94**	0.98**	0.99**		
APX	−0.96**	−0.85**	−0.96**	−0.93**	0.98**	0.99**	0.99**	
IAA	0.96**	0.86**	0.96**	0.9**	−0.96**	−0.96**	−0.97**	−0.95**
Experiment 2								
RGR	0.63*							
Total Chl.	0.68**	0.86**						
F_v/F_m	0.66**	0.83**	0.86**					
H_2O_2	−0.53*	−0.89**	−0.94**	−0.86**				
POD	−0.72**	−0.92**	−0.94**	−0.86**	0.94**			
CAT	−0.21	−0.67*	−0.59*	−0.67**	0.76**	0.63*		
APX	−0.48	−0.75**	−0.79**	−0.83**	0.85**	0.78**	0.77**	
IAA	0.69*	0.94**	0.87**	0.85**	−0.88**	−0.94**	−0.68**	−0.74**

* Correlation is statistically significant at the 0.05 level.
** Correlation is statistically significant at the 0.01 level.

the soil, temperature and gas exchange with the overlying water. This system not only deprives oxygen but also produces highly dissolved CO_2 and accumulates other phytotoxic compounds (H_2S, NH_4N, dissolved Fe, among others) (Pezeshki and DeLaune, 2012). In this study, the concentration of elements in water, especially Ca, Mg, Fe, Mn, increased at R, HR, RW, and HRW treatments, which was also supported by the previous author (Charlatchka and Cambier, 2000; Yu *et al.*, 2007). In spite of the presence of those elements in RW and HRW conditions, plants showed no significant growth and chlorophyll concentration differences after the exposure in these two conditions. The results supported that plants were not affected by the elements present in RW and HRW conditions. In the present experiment, the H_2S produced, even in highly reduced conditions, was 0.04 mM. The effect of the H_2S concentration on *E. densa* was tested separately and showed that plants could survive until they were exposed to 0.05 mM H_2S (Tab. 2). Simultaneously, when plants were subjected to other conditions (RW and HRW), they also showed no significantly negative effects on *E. densa* (Figs. 2–4). This result indicates that the anoxic condition itself might be stressful for the plants rather than the toxic materials produced in the anoxic sediments as long as the production of H_2S remains below 0.05 mM.

The plant growth rate decreased when the plants were exposed to the R and HR conditions. The low growth rate of shoots and roots in low E_h conditions is a common response in several wetland plants (Pezeshki, 2001; Wu *et al.*, 2009; Zaman and Asaeda, 2013). However, there are two possibilities for the reduction of the growth rate of plants under reduced

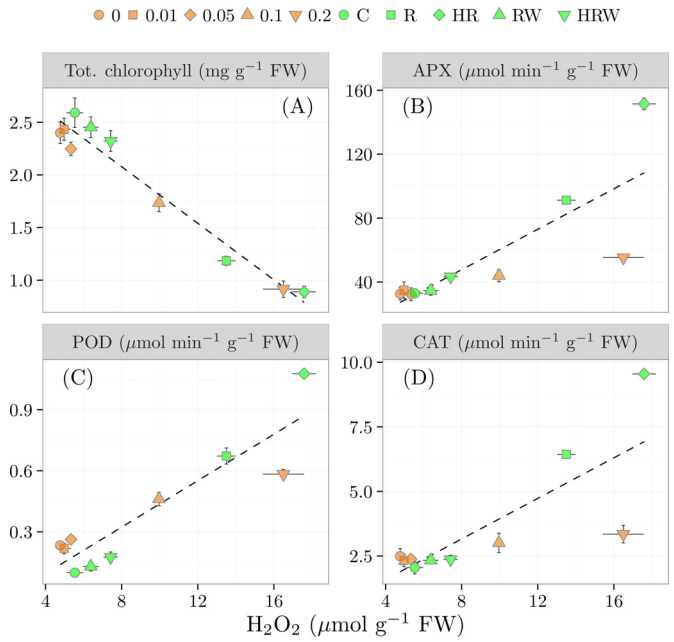

Fig. 6. Relationship with H_2O_2 and chlorophyll a (A), H_2O_2 and POD (B), H_2O_2 and APX (C), and H_2O_2 and CAT (D) of *E. densa* under control (C), reduced (R), highly reduced (HR), transferred water from reduced tank (RW), and transferred water from highly reduced tank (HRW) conditions, and 0, 0.01, 0.05, 0.1, 0.2 mM H_2S concentrations. Values are the means of three replicates ± SD, $n = 3$.

conditions. One possibility is the reduction of auxin (a common growth hormone for all plants) and the other is the production of reactive oxygen species (ROS). Auxin is related to the growth of aquatic macrophytes and may be one of the dominant factors for controlling the shoot elongation (Ellawala *et al.*, 2011; Zaman and Asaeda, 2013). In the present study, the growth rate was completely regulated by auxin, measured as IAA concentration, which was substantially affected by E_h and decreased with increasing depletion levels of O_2. Figure 5 clearly shows that both the RGR and SGR depended on IAA. In the control, TWR, and TWHR conditions, the growth rate increased with increasing levels of IAA. In contrast, the IAA and growth rate decreased in the R and HR conditions. With the level of produced H_2S (0.05 mM), no significant effects were observed, which is also supported by Parveen *et al.* (2017). These results indicate that the reduction in the growth rate of the plants in the reduced conditions was mainly due to the destruction of the IAA caused by the sediment anoxia rather than by the production of phytotoxic elements (Zaman and Asaeda, 2013). However, Barko *et al.* (1991) reported that the low biomass production of *P. pectinatus* on sediments with high organic matter (>10%) concentration is due to high concentrations of inorganic constituents such as soluble reduced iron and manganese and soluble sulfide or organic constituents such as methane, ethylene, phenols and alcohols formed under anaerobic conditions.

The exposure to a low oxygen concentration triggered oxidative stress in aquatic macrophytes (Zaman and Asaeda, 2013; Wu *et al.*, 2015). In the present experiment, the observed high H_2O_2 concentration in plants subjected to the R, HR, and 0.1, 0.2 mM H_2S conditions had a sign of oxidative stress. There was a strong positive correlation between plants exposed to low redox potential (R, HR) and the concentration of H_2O_2, which is also supported by Zaman and Asaeda (2013). Instead, there was no correlation between plants exposed to released phytotoxic elements (RW and HRW) and the concentration of H_2O_2. Therefore, the production of H_2O_2 might be due to a decrease in the chlorophyll concentration. A reduced chlorophyll concentration and F_v/F_m values were also observed in plants exposed to the R and HR conditions. The loss of chlorophyll may have disturbed the photosynthetic machinery; thus, the electron transport rate of PSI and PSII could not work properly, which led to the generation of H_2O_2. In turn, the ROS accumulation can further impede the chlorophyll biosynthesis, either directly or indirectly, by inhibiting the activity of the photosynthetic machinery (Asada, 1994; Dominguez *et al.*, 2008). Apparently, no physiological changes were observed in plants exposed to C, RW, and HRW conditions. In contrast, plants grown under R and HR conditions showed a brown discoloration. We can assume that the observed H_2O_2 accumulation in tissues may promote the loss of leaves because a high H_2O_2 accumulation was identified as an important mechanism of leaf senescence (Wang *et al.*, 2008).

Plants show protective behaviors by scavenging free radicals to prevent oxidative damage (Blokhina *et al.*, 2003). This characteristic is particularly crucial for photosynthetic organisms that constantly generate ROS during normal photosynthesis. In plants, catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (POD) are considered highly important for scavenging H_2O_2 (Noctor and Foyer, 1998; Zhang *et al.*, 2007). POD is an essential component of the plant growth and senescence processes. Therefore, it is considered a stress marker enzyme with a high affinity for H_2O_2 (Andrews *et al.*, 2002; Zaman and Asaeda, 2013). Moreover, it is activated as a short-term stress response (Martínez Domínguez *et al.*, 2010) and also affects lignin and ethylene synthesis and the decomposition of IAA. IAA and H_2O_2 have antagonistic effects on the cell cycle progression (Kovtun *et al.*, 2000; Pasternak *et al.*, 2005). The high concentration of cellular H_2O_2, along with elevated POD, CAT, and APX activities in R and HR conditions, suggest that the ROS scavenging system was activated under low oxygen concentration. Moreover, these three activities can also be used as a low E_h stress marker with a high affinity to H_2O_2.

In the present study, it was observed that despite the growth reduction under low E_h conditions, *E. densa* showed an adaptation behavior through the antioxidative responses. Therefore, it can be suggested that the sediment anoxia itself can influence the distribution and abundance of *E. densa*, which is less tolerant to low E_h in terms of stress responses. Likewise, in the present experiment, low O_2 and high CO_2 concentrations were responsible for the plant senescence, rather than H_2S and other elements, if the produced amounts do not exceed the level determined in this experiment.

It is important to understand the behavior of macrophytes under different abiotic stresses for the management of aquatic ecosystems. The results of the present study indicate that low oxygen along with high CO_2 concentrations acts as one of the important abiotic stresses for the degradation of macrophytes in aquatic systems. Our study also suggests that in reduced conditions, low oxygen and high CO_2 concentrations result in a stress that has stronger effects on plants in terms of stress

responses compared to other factors such as soluble macro and microelements. This study will improve our ability to predict the dynamics of wetland aquatic vegetation and thus facilitate the formulation of wetland management and restoration strategies. However, field or *in situ* investigations should be required for further studies to gain insight into the interactions between sediment conditions and submerged macrophytes.

Acknowledgements. This research was financially supported by a Research Grant-in-Aid (15H04045) from the Japan Society for the Promotion of Science, the River Fund by the River Foundation in Japan, and the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT). The authors also wish to acknowledge the laboratory members of the Ecological Engineering Laboratory of the Saitama University, Japan, for their immense help during the experimental work.

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Cite this article as: Parveen M, Asaeda T, Rashid MH. 2017. Physiological and biochemical responses of *Egeria densa* to different sediment redox conditions. *Ann. Limnol. - Int. J. Lim.* 53: 271–280