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# Growth and Oxidative Stress of Brittlewort (*Nitella pseudoflabellata*) in Response to Cesium Exposure

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**Abstract** The present study evaluated the impact of cesium ($^{133}$Cs) at four concentrations (0, 0.001, 0.01, and 0.1 mg $L^{-1}$) on growth, concentrations of chlorophyll and carotenoid pigments, and oxidative stress responses in the charophyte, *Nitella pseudoflabellata*, over 30 days. Oxidative stress was quantified by measuring anti-oxidant enzyme activities and $H_2O_2$ content. When compared with the control, significantly elevated activity levels of the anti-oxidative enzymes ascorbic peroxidase, catalase and guaiacol peroxidase were observed at 0.1 mg $L^{-1}$ (all $p < 0.05$), even though the $H_2O_2$ level was not significantly elevated. Carotenoid and chlorophyll a and b pigment levels were significantly reduced (all $p < 0.05$) at Cs exposures of 0.01 and 0.1 mg $L^{-1}$. Photosynthetic efficiency (i.e., $F_v/F_m$) was significantly reduced ($p < 0.05$) at Cs concentrations $\geq$0.001 mg $L^{-1}$. Significant reduction ($p < 0.05$) of plant growth (i.e., shoot length) was also observed after 1 week of exposure at Cs concentrations $\geq$0.001 mg $L^{-1}$. Our results suggested that Cs exposure reduced plant growth and affected plant functioning via activating the defense mechanism against oxidative stress in *Nitella*.

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Aquatic systems are challenged by an array of fluctuations in abiotic stress vectors. In particular, anthropogenic inputs, including heavy metals and other toxic substances, have been prominent in the last few decades and have exceeded the tolerance limits for certain species in aquatic systems (Nagajyoti et al. 2010). Stable Cs ($^{133}$Cs) is an alkali metal that originates from an aluminosilicate mineral called pollucite (White and Broadley 2000). The major anthropogenic sources of $^{133}$Cs are mining of pollucite ores and the production and use of Cs compounds in electronic and energy production (especially coal-burning power plants) (ATSDR 2004). The Cs concentrations found in freshwater and marine ecosystems range from $1 \times 10^{-5}$ to $12 \times 10^{-3}$, and $5 \times 10^{-4}$ to $2 \times 10^{-3}$ mg $L^{-1}$, respectively (Komarov and Bennett 1983), whereas soils have been reported to contain 0–26 mg $kg^{-1}$ $^{133}$Cs (Cook et al. 2007). Although the naturally occurring Cs levels are harmless, Cs accumulation over longer time periods can be toxic to plants (Bystrzejewska-Piotrowska et al. 2005; Hampton et al. 2004). Therefore, $^{133}$Cs accumulation may pose a risk to aquatic plants in areas with anthropogenic inputs in the long run.

Aquatic flora provide a wide spectrum of ecological functions and play a crucial role in maintaining the integrity of aquatic ecosystems. Their functions include the provisioning of habitats, refuge and food for fish and other invertebrates, primary production, retention of substances, and contributions to biogeochemical cycles (Bennett et al. 2005; Bornette and Puijalon 2011; Folkard 2011; Nepf 2012). Algae are plant-like aquatic organisms containing chlorophyll for photosynthesis, while their bodies are not

differentiated into true leaves, stems or roots. The macroalgae of the taxon Charophyta, which are commonly known as stoneworts/brittleworts, are a group of non-vascular hydrophytes commonly found in many regions, with potential for phytoremediation (García 1994; Gomes and Asaeda 2009; Siong and Asaeda 2009).

The effects of Cs exposure on growth, metabolism and genetics have been reported for some terrestrial plants (Hampton et al. 2004; Kanter et al. 2010; Zhu and Smolders 2000). However, there is little information regarding the impact of Cs exposure and Cs accumulation on aquatic flora, including charophytes. Furthermore, Cs-induced stress responses in charophytes are largely unknown, and the relationship between plant stress and the Cs accumulation in aquatic plants remains unclear. In this study, we used the charophyte, *Nitella pseudoflabellata*, as a model species to evaluate our hypothesis that Cs exposure would cause oxidative stress and negatively impact growth of the algae.

## Materials and Methods

*Nitella pseudoflabellata* plants were obtained from a laboratory maintained culture tank which was previously collected from nearby paddy fields. A glass beaker (1 L) with a layer (~2 cm) of commercial river (90 % <1 mm) sand purchased from the local market (DOIT, Saitama, Japan) with 1 % Hoagland's nutrient solution (800 mL) was used as an experimental unit. Cs solutions (CsCl, purity was 99 %) and Hoagland's nutrient medium were prepared from pure analytical-grade chemicals (Wako chemicals, Osaka, Japan) and distilled water. The total nitrogen (TN) and total phosphorus (TP) contents of the medium were 2.1 and 0.3 mg $L^{-1}$, respectively. Each treatment, with three replicates (n = 3), was randomly allocated into 12 (4 × 3) glass beakers in a complete randomized design. Six similar size apical tips of *N. pseudoflabellata* [initial length (IL) ~2–3 cm] were planted in each beaker.

The measured initial Cs concentrations of the 0.001, 0.01 and 0.1 mg $L^{-1}$ exposures were 0.005, 0.010 and 0.142 mg $L^{-1}$, respectively. The lowest test concentration approximated the upper limit of concentrations reported for polluted waters in the literature. Light intensity was maintained at ~100 µmol $m^{-2}$ $s^{-1}$ using florescent lamps with a photoperiod of 12 h light and 12 h dark. The average temperature of the glass beakers was maintained at 24 ± 1°C throughout the experimental period (30 days).

The shoot length of *Nitella* was measured once per week. At the end of the experiment, the final shoot length (FL), shoot elongation rate (SER, SER = (FL − IL)/time)), Cs content, pigment concentration (chlorophyll-a, chlorophyll-b, and carotenoids) and the stress responses of plants were compared. Plant stress was assayed by measuring the chlorophyll fluorescence, $H_2O_2$ concentration and antioxidant enzyme activities. To characterize the antioxidant enzyme activities, ascorbic peroxidase (APX), catalase (CAT) and guaicol peroxidase (POD) activities were assayed. The chlorophyll fluorescence was determined using the chlorophyll fluorescence imaging technique (FC 1000-H; Photon Systems Instruments, Drasov, Czech Republic), and the maximum quantum efficiency of photo-system II photochemistry ($F_v/F_m$) was calculated (DeEll and Toivonen 2003). It should be noted that there were some attached algae grown in the microcosm. The algae were carefully removed with the aid of forceps before analysis.

The pigments (chlorophyll and carotenoids) were extracted by keeping fresh *N. pseudoflabellata* (~5 mg) overnight in *N*,*N*-dimethylformamide. After extraction, the absorbance was measured spectrophotometrically (Shimadzu UV mini 1210, Kyoto, Japan) at the wave lengths of 663.8, 646.8 and 480 nm. The pigment contents were calculated according to Wellburn (1994). For the stress assay, plant materials (~100 mg fresh weight (FW)) were ground to extract hormone and antioxidants using an ice-cold phosphate buffer (50 mM, pH = 6.0) which contained polyvinylpyrrolidone (PVP). After extraction, extracts were centrifuged at 3000 rpm for 20 min at 4°C. The supernatant was separated and stored at −80°C until analysis. The $H_2O_2$ content was determined according to Jana and Choudhuri (1982). Briefly, 750 µL of extract was mixed with 2.5 mL of 0.1 % titanium sulfate in 20 % $H_2SO_4$ (v/v). The mixture was centrifuged at 5000×*g* for 15 min at room temperature, and the intensity of the resulting yellow color was measured at 410 nm. The $H_2O_2$ concentration was estimated using a standard curve and the $H_2O_2$ content is presented as µmol $g^{-1}$ FW.

Catalase activity was assayed following Aebi (1984). Briefly, the reaction mixture contained 100 µL of 10 mM $H_2O_2$, 2.00 mL of 100 mM potassium phosphate buffer (pH = 7.0) and 500 µL of extract. The decrease in absorbance at 240 nm was recorded for 0.5 min. The CAT activity was calculated using the extinction coefficient of 40 $mM^{-1}$ $cm^{-1}$. APX activity was determined according to Nakano and Asada (1981). The reaction mixture contained 100 µL of extract, 200 µL of 0.5 mM ascorbic acid in 50 mM potassium phosphate buffer (pH = 7.0) and 2.00 mL of 50 mM potassium phosphate buffer (pH = 7.0). The reaction was started after adding 60 µL of 1 mM $H_2O_2$. The decrease in absorbance at 290 nm was recorded every 15 s. The APX activity was calculated using the extinction coefficient of 2.8 $mM^{-1}$ $cm^{-1}$. Guaiacol peroxidase activity was measured based on guaiacol oxidation according to MacAdam et al. (1992), The reaction mixture contained 3.0 mL of 50 mM potassium phosphate buffer (pH = 6), 40 µL of 30 mM $H_2O_2$ and

50 µL of 0.2 M guaiacol. The reaction was initiated by adding 100 µL of enzyme extract, and the absorbance was measured immediately and then every 15 s for 3 min. The rate of absorbance change was calculated, and the POD activity was determined using the extinction coefficient of 26.6 $mM^{-1}$ $cm^{-1}$. All enzyme activities are presented as nkat $g^{-1}$ FW ('nkat' designates nanokatal, where one katal is the amount of enzyme that converts one mole of substrate per second) (Dybkaer 2001).

For metal analysis (Cs and K), the remaining plants at the end of the experiment were dried at 65°C in an oven (Eyela NDO-700, Tokyo, Japan) until a constant weight was achieved. A 20 mg dry sample was digested with 60 % $HNO_3$ for 1 h at 125°C. After cooling, 30 % $H_2O_2$ was added and the mixture was further digested until it finished bubbling (Plank 1992). The extraction was adjusted to 10 mL using milli-Q water and used to measure K. Cs speciation (i.e., organically bound, inorganically bound and exchangeable) were determined using dry samples following the method described by Siong and Asaeda (2009). Briefly, dried sample was mixed with 10 mL of 1 M $MgCl_2$ for 30 min to obtain the exchangeable fraction. The residue after former extraction was extracted using 10 mL of 1 M NaOAc for 5 h and the carbonate-bound fraction was obtained. The residue in the NaOAc was digested using a mixture of $HNO_3$ and $H_2O_2$ to extract the organic-bound fraction. The solution was evaporated to approximately 5 mL and then diluted using distilled water to a final volume of 25 mL. The content of each Cs species was summed to determine the total Cs content. Air/acetylene flame atomic absorption spectrophotometry (Shimadzu AA-6300, Kyoto, Japan) was used for metal analyses according to standard methods (APHA 1998).

Quality assurance and control (QA/QC) procedures were carried out for Cs estimation from water and plant samples. Method detection limit (MLD) of Cs estimation by atomic absorption spectrophotometry was calculated from seven replicate analyses with 99 % confidence level. The MLD of Cs estimation was 0.0175 mg $L^{-1}$. During Cs analysis by flame atomization, the matrix effects were negligible for both water samples and plant extract. The standard addition technique was used with atomic absorption spectrophotometry. The concentrations obtained for the standard reference material were always within the 95 % CI of certified values. Recalibration of Cs standards was performed after every 10 determinations.

All of the data were analyzed and figures were created by using R (R Development Core Team 2010). Data were presented as the mean ± standard deviation (SD) (n = 3). The homogeneity of variance test and Levine's check for equality of variances were performed on the datasets prior to the statistical analysis to verify the assumptions of normal distribution and homogeneity of variances. 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. Pearson's correlation analyses were conducted to determine the relationships between concentrations of cesium in media and growth, and biochemical parameters.

## Results and Discussion

Plants grew and were alive until the end of the experiment (30 days) in all of the treatments, while the shoot lengths increased with increasing exposure duration irrespective of the treatments (Fig. 1). However, Cs exposure significantly affected the plant growth ($F = 7.80$, $p = 0.01$). The longest shoots were observed in the control, followed by the 0.001, 0.01 and 0.1 mg $L^{-1}$ Cs treatments. The final lengths (length at harvest, Fig. 1) of the control plants were statistically similar to the shoot length of the 0.001 mg $L^{-1}$ treatment. The shoot elongation rate (SER) of *N. pseudoflabellata* varied significantly among the treatments ($F = 13.5$, $p < 0.01$), with SERs in the control, 0.001, 0.01 and 0.1 mg $L^{-1}$ Cs treatments of 2.2 ± 0.1, 2.1 ± 0.1, 1.9 ± 0.0 and 1.7 ± 0.1 mm $day^{-1}$, respectively. The observed trend in SER was in close agreement with the trend observed for the final length (FL). The FL of the control plants was approximately 1.5-fold longer than that of the plants exposed to the highest Cs concentration (0.1 mg $L^{-1}$) (Fig. 1). The trends in growth reduction for charophytes after exposure to chromium (0.8 mg $L^{-1}$), cadmium (0.025–0.15 mg $L^{-1}$) and zinc (0.15–1 mg $L^{-1}$) for 35 days (Gomes and Asaeda 2009; Hawa Bibi et al. 2010; Siong and Asaeda 2009) were similar to the results in this study. Furthermore, the total length inhibition in *Spiroplasma floricola* was reported after exposure to ~1.13 mg $L^{-1}$ Cs (Chang 1986). A similar growth reduction was also reported for *Arabidopsis thaliana* (Hampton et al. 2004). Even though the accumulation of excess metals in plants inhibits growth, some toxic metals appear to promote growth at very low concentrations. For example, Strauss (1980) observed a better growth rate in *Chara fragilis* and *Chara vulgaris* after growing in a medium that contained minute contents of Cs (0.007–0.003 mg $L^{-1}$).

The Cs treated plants of this study (0.001–0.1 mg $L^{-1}$) contained significantly higher concentrations of Cs (Fig. 2a) when compared to the control plants ($F = 6.20$, $p = 0.05$). In addition, there was a positive correlation between Cs accumulation and the exposure concentration ($r = 0.81$, $p = 0.02$). There was a decreasing trend in the K content in plants with increasing Cs concentration (Fig. 2b). The $Cs^+$ ion shows similar properties as $K^+$ and thus both ions

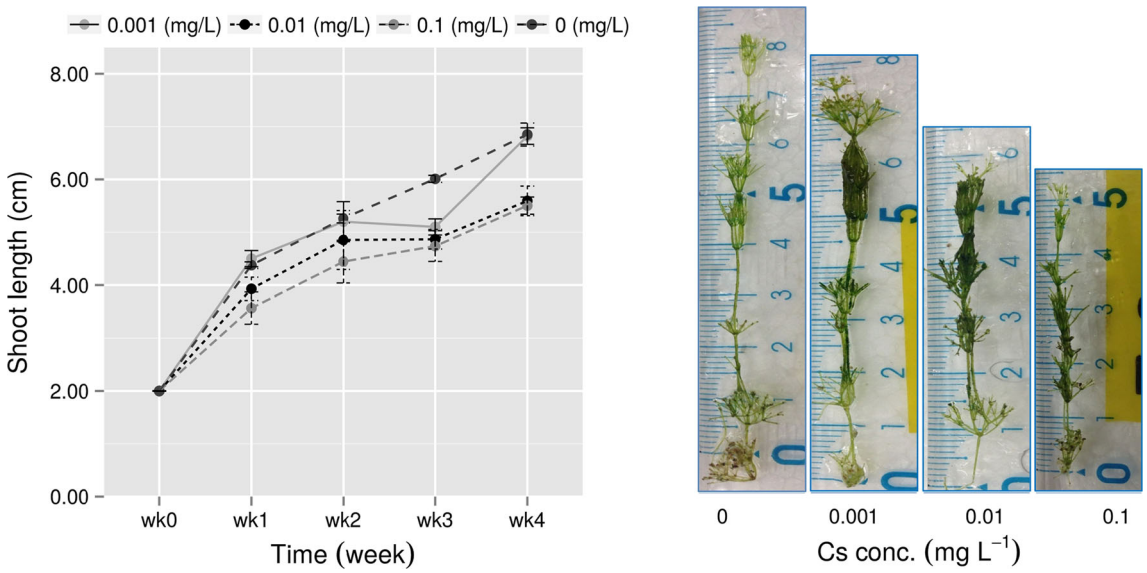


**Fig. 1** Growth of *Nitella* over 30 days (*left* temporal growth pattern, *right* plants at the end of the experiment)

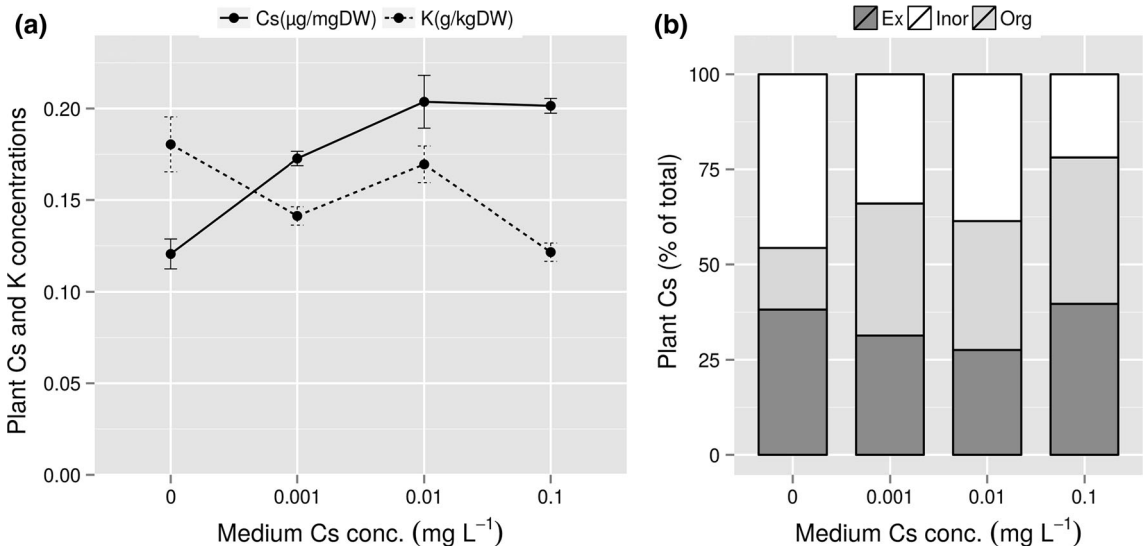


**Fig. 2** Variation in Cs concentrations in *Nitella* **a** total Cs and K content in plant and **b** concentration of exchangeable (Ex), inorganically (Inor), and organically (Org) bound Cs species

compete with $K^+$ binding sites; therefore, K starvation might occur in stressed plants (Hampton et al. 2004; Isaure et al. 2006). We observed a decreasing trend in the K content in plants with increasing Cs concentration (Fig. 2a). But the former trend was not statistically significant. The Cs accumulation might reduce the $K^+$ uptake. As K is one of the major nutrients in plants, this may impact cellular metabolism, leading to reduced growth in *Nitella*.

According to the speciation analysis (Fig. 2b), the organically bound Cs fraction (Cs-ORG/Total Cs) of *Nitella* was significantly different among treatments ($F = 6.68$, $p = 0.04$). Further, this fraction was positively correlated to the total Cs content of the plant ($R = 0.94$, $p < 0.01$). Similar concentrations of exchangeable Cs (EX) were observed in the control, 0.001 and 0.01 mg $L^{-1}$, while the plants in 0.1 mg $L^{-1}$ had significantly higher concentrations of EX Cs ($F = 28.2$, $p < 0.01$). The organically bound Cs (Org) was assumed to gradually accumulate in plants with the Cs exposure (Fig. 2b), and this could be considered as a sign of the bioaccumulation of Cs. This fraction in Cs-treated plants was 60–80 µg $g^{-1}$ DW, nearly three-fold higher than that of the control plants (~20 µg $g^{-1}$ DW). However, the behavior of inorganically bound (IB) accumulation deviated from the former

observations, as we observed an increasing trend followed by a decline at higher Cs exposure.

Photosynthesis plays a prime role in plant functioning, and thus maintenance of appropriate chlorophyll levels in plant cells is essential for plant functioning. Chlorophyll levels decreased in *Nitella* in response to Cs exposure (Fig. 3). Therefore, the reduced plant growth could also be associated with the reduced chlorophyll content in stressed *Nitella*. Cesium exposure significantly affected chlorophyll-a ($F = 5.50$, $p = 0.02$) and chlorophyll-b ($F = 4.76$, $p = 0.04$) concentrations (Fig. 3). The negative impact of Cs exposure on photosynthesis was further explained by the correlations with shoot length observed for chlorophyll-a ($r = 0.62$, $p = 0.046$) and chlorophyll-b ($r = 0.55$, $p = 0.076$). The reduction in chlorophyll and carotenoids in the Cs treated plants may be explained by the degradation of some enzymes, which were essential in pigment biosynthesis (Shalygo et al. 1997). Similar to the present study, concentration-dependent reduction of chlorophyll was observed in barley leaves after exposure to CsCl for 8 h (Shalygo et al. 1997).

Generally, stress-free plants exhibit the optimum value (0.83) of the maximum quantum yield of PSII [i.e., the efficiency of PSII ($F_v/F_m$)] for most plant species, whereas this optimum $F_v/F_m$ ratio decreases when plants are stressed, indicating the phenomenon of photo-inhibition (Atapaththu and Asaeda 2015; Maxwell and Johnson 2000). The observed $F_v/F_m$ ratios were significantly different among the treatments ($F = 12.04$, $p < 0.01$). The highest ratio was observed for the control plants ($0.80 \pm 0.01$), which was close to the optimum value. Similar to the present study, lower $F_v/F_m$ ratios have been reported for charophytes (Gomes and Asaeda 2009), algae (Lu et al. 2000) and aquatic macrophytes (Valderrama et al. 2013) in response to metal toxicity. Therefore, the observed reduction in $F_v/F_m$ ratio suggests that photoinhibition occurred in Cs-treated *Nitella*.

When compared with the control group, the observed activities of CAT, POD and APX indicated the activation of oxidative defense mechanisms responding to Cs exposure (Fig. 3). The stress factors (i.e., response of antioxidant enzymes, efficiency of PS-II) are highly correlated to plant growth and photosynthesis. Plants produce different forms of reactive oxygen species in stress conditions, especially in chloroplasts, mitochondria, peroxisomes etc. Therefore, the metal induced oxidative stress might damage either the chlorophyll structure or chlorophyll membranes (Dinakar et al. 2012). This possibility would appear to be supported by the significant negative correlations in our study between chlorophyll-a concentration and APX and POD activities (Table 1). Further, chlorophyll fluorescence ($F_v/F_m$ ratio) and SER were negatively correlated with CAT, POD and APX (Table 1). Therefore, the Cs-induced stress was considered to have impacted the photosynthetic mechanism of *Nitella*.

The APX activity of plants was significantly different among the treatments ($F = 49.10$, $p = 0.00$), and the activity of this enzyme increased responding to exposure concentration (Fig. 3). Further, the former relationship was clearly explained by the positive correlation between APX activity and the Cs content of plants ($r = 0.79$, $p = 0.02$). The APX activity at the 0.1 mg $L^{-1}$ Cs treatment was

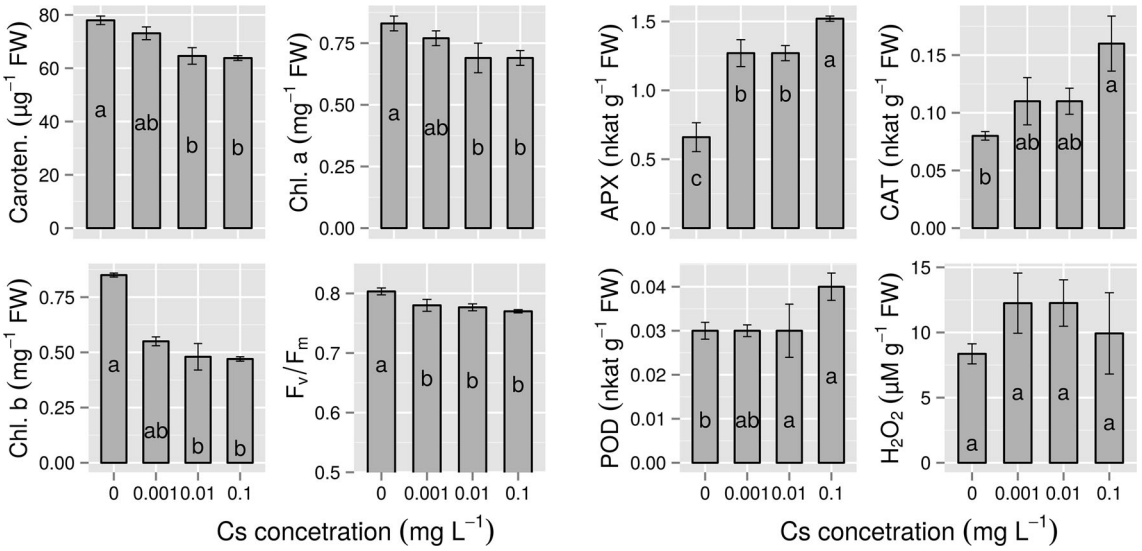


**Fig. 3** The observed changes in pigments (caroten.: Carotenoids, Chl. a: chlorophyll-a, Chl. b: chlorophyll-b), chlorophyll fluorescence ($F_v/F_m$) and stress responses ($H_2O_2$ content, APX and POD activity). *Different letters* in *each bar* indicate treatments were significantly different based on ANOVA followed by Tukey's post hoc test ($p < 0.05$)

**Table 1** Correlation between pigments, stress responses and plant growth

| | Chl- a | Carotenoids | $F_v/F_m$ | APX | CAT | $H_2O_2$ | POD |
|---|---|---|---|---|---|---|---|
| Carotenoids | 0.92*** | | | | | | |
| Fv/Fm | 0.69* | 0.61* | | | | | |
| APX | −0.71* | −0.66* | −0.91*** | | | | |
| CAT | −0.57 | −0.54 | −0.62* | 0.76* | | | |
| $H_2O_2$ | −0.11 | −0.13 | −0.48 | 0.41 | 0.30 | | |
| POD | −0.72* | −0.48 | −0.87*** | 0.81* | 0.59 | 0.34 | |
| SER | 0.59 | 0.58 | 0.76** | −0.83** | −0.66* | −0.44 | −0.60 |

*Chl-a* chlorophyll-a, $F_v/F_m$ efficiency of PSII, *APX* ascorbic peroxidase, *CAT* catalase, *POD* peroxidase, *SER* shoot elongation rate, n = 4

*, ** and *** Significant levels of 0.05, 0.01 and 0.001, respectively

approximately two-fold higher than that of the control group. Similarly, the CAT activity of plants was significantly different ($F = 5.7$, $p = 0.02$) among the treatments (Fig. 3), with the CAT activity of plants exposed to 0.1 mg $L^{-1}$ Cs being approximately two-fold higher than that of the control plants (Fig. 3). The POD activity also varied significantly among the treatments ($F = 6.35$, $p = 0.02$), where elevated levels of POD activity were exhibited in plants exposed to 0.1 and 0.01 mg $L^{-1}$ treatments. However, the POD activity of the plants exposed to the lowest Cs concentration (0.001 mg $L^{-1}$) was not statistically different either from the control or other treatments.

Cs is known to be a potentially toxic mineral element that is released into the environment and taken up by plants (Qi et al. 2008). Due to the large hydrated ion radius of Cs, the free mobile single electron can react with water and oxygen to form reactive oxygen species (Sahr et al. 2005), leading to the activation of the anti-oxidative defense system in plants. The increased activity of antioxidant enzyme activities (POD, CAT and APX) indicated the activation of defense mechanisms against the Cs-induced oxidative stress in *Nitella*. Similarly, the Cs application resulted in the induction of peroxidases, catalases, and an increased amount of metabolites, such as glutathione, in other plants (Ghosh et al. 1993). In the present study, the $H_2O_2$ content was not significantly different among the four treatments ($F = 2.65$, $p = 0.12$).

Cs exposure negatively impacted the chlorophyll content and significantly reduced the growth of *Nitella*. The antioxidant activities (POD, CAT and APX) changed in an exposure dependent manner where elevated levels of activities were detected in plants exposed to the highest Cs (0.1 mg $L^{-1}$) concentration. In summary, the Cs content of the stressed plants was significantly higher than that of the control plants. Cs ($^{133}Cs$) induced oxidative stress and negatively affected photosynthetic function and growth in *Nitella*. However, the present study merely studied the effects of stable Cs on charophytes. Even though the ability of radioactive Cs ($^{137}Cs$) elimination was reported for the charophyte; *Chara braunii* (Fukuda et al. 2014), the impacts of radioactive Cs on function and stress physiology of charophytes remain unclear, and further studies are recommended.

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