Copper contaminated water mediated biochemical changes on charophyte species *Chara braunii*

V P Ranawakage¹, T Fujino¹ and A Herbst²

1 Department of Environmental Science and Technology, Saitama University, Saitama 338-8570, Japan
2 University of Rostock, Biosciences, Albert-Einstein-Straße 3, 18059 Rostock, Germany
E mail: vranawaka@gmail.com

**Abstract.** Over the past few decades water bodies have been heavily contaminated by the heavy metals thus charophytes communities tended to disappear from their own habitat niches. In this experiment we hypothesized that elevating Cu concentrations and increasing exposure time deviates the biochemical responses of *Chara braunii*. For evaluating this hypothesis we considered changes in plant reactive oxygen species (ROS) mainly as hydrogen peroxide. Thereafter, scavenging antioxidant activities were measured (POD, CAT) while pigment content assessed means of Chl *a*, Chl *b* and Carotenoids. Each treatment contains 3 replicates and subjected to four levels of Cu concentrations (0ppm, 1ppm, 5ppm, and 10ppm) for over four days respectively. Compared to the control, all dosages of Cu concentrations and exposure times were caused to trigger *H₂O₂* generation. Observed results revealed that dramatic increases of *H₂O₂* level on the 1<sup>st</sup> day of copper contamination by the 10ppm concentration. Conversely, after 1<sup>st</sup> day *H₂O₂* content continuously tended to decreases and in 4<sup>th</sup> day it shown the lowest value. Moreover, *C. braunii* exhibited significant increase in the catalase activity and peroxidase activity for detoxifying Cu toxicity for short duration whereas inhibited antioxidant activity on prolonged exposure. Consequently, chlorophyll pigments content impaired significantly and deteriorated plant color suggests subjected plant were under extreme stress. This study results indicated that exposure to Cu contaminated water is lethal for charophyte growth and the physiological process by enhancing oxidative damages to the cells.

1. Introduction
Charaophytes are early pioneering macroscopic algae species that taxonomically close relatives of the terrestrial plants [1]. Besides, characean species consider as rapid colonizers [2] and play key roles to stabilize ecosystem function as providing habitats, foods, refuges for aquatic fauna [3]. Their habitats distribution took place in streams, rivers and wetlands across the world [4,5]. However, last few decades as a result of heavy water pollution in many countries stonewort’s population and distribution declined [6] dramatically. Moreover, this habitat loss triggered by the agriculture and urban development activities done by the human enrolment [7].
In aquatic environment heavy metals can be considered as most devastating contaminant [8] while contamination of water bodies cause serious damage to the aquatic biodiversity [9]. In surface water bodies heavy metal can be forms as natural activity or by anthropogenic activities [10]. Among the major heavy metals Cu contaminations plays major role in aquatic pollution. For an example application of cu for kill mollusks, fungi, algae can be consider as extreme toxicity of particular heavy metal [11].The copper requirement for plant growth and development vary with the species [12] and it
is species-specific. Whereas, if the copper concentration exceed the threshold level, it caused a toxic effect for the aquatic species [13]. In coastal seawater, natural Cu concentration remained between 0.008 and 0.050 µM [14] whereas river water it recorded as 10µg/L [15]. Furthermore, copper concentration in surface waters ranged from 0.0005 to 1mg/L. The higher concentration of heavy metals caused the most toxic effect to the aquatic organism. Nevertheless, recent research findings were suggest that in future toxic Cu concentration may furthermore elevated with the acidification of the oceans [16]. However, microalgae species are considered as good source of bio monitors and bio indicators considering the accumulation of toxic metals [17]. Furthermore, research findings discussed the importance of microalgae species as absorbent of various metal ions because of their higher growing capacity [18]. However, little attention has been paid to the charophyte species biochemical resistive capacity against the Cu toxicity, which might be more insightful for manage the quality of the aquatic ecosystem.

In this study, we used plant species *Chara braunii* is well observed Charaophytes species belongs to the non-corticated characean group. It is one of the most abundant Charaophytes species available within the Japanese peninsula [19]. Besides, stonewort’s occurred in a wide variety of aquatic habitats [20] while the majority of characeae lived in fresh water bodies that pH range between 8 or higher [21]. This particular charralean species not limited to the deep waters but also grows in shallow water habitats as rice fields. Considering the above mentioned habitat locations, we conducted this experiment to evaluate possible biochemical changes taken place under copper contamination.

2. Materials and methods
After one month culture, healthily grown *C. braunii* plants were randomly selected and put in 1L glass bottles for Cu treatment. The plant density per one bottle maintained as 6 plants while nutrient substituted with 10% Hoagland solutions without Cu used as growth medium. Each treatment contained three replicates while all 4 treatments contained 72 seedlings respectively. The four corresponded Cu concentrations were arranged as 0ppm, 1ppm, 5ppm and 10ppm as complete randomized block design thus Cu concentrations were chosen according to the antecedent literature. The growth chamber light intensity maintained 100 µmolm⁻²s⁻¹ with 12h/8hr dark cycle at 25 °C. During the 4 day experimental period plant enzymatic activities measured daily basis by harvesting 3 plants from each treatment.

2.1 Sampling and extracts preparation.
Harvested *C. braunii* plants were thoroughly cleaned blotted and approximately 100 mg of fresh plant samples were extracted in chilled (50mM pH 6.0) potassium phosphate buffer with polyvinylpyrrolidone (PVP). Homogenate was centrifuged at 3000 g for 10 minutes at 4°C. The obtained suspension was stored in -80°C for analysis of H₂O₂ and scavenging antioxidative assays. The chlorophyll *a*, chlorophyll *b* and carotenoids content were measured calorimetrically [22] extractions in N, N-Dimethyl-formamide after 24 hours. Hydrogen peroxide concentrations were estimated using a standard curve plotted from the serial concentrations of H₂O₂ followed by method of [23]. CAT activity (EC 1.11.1.6) was determined following the protocol of [24]. The Guaiacol peroxidase activity (EC 1.11.1.7) was obtained using an extinction coefficient of 26.6 mM⁻¹ cm⁻¹. Each enzyme activity above is expressed as µmol min⁻¹ g⁻¹ FW fresh weight basis. The experiments were replicated thrice (n=3) for each growth condition.
3. Results

Figure 1. Effect of exposure time on Chlorophyll a (A) Chlorophyll b (B), Carotenoids (C) in Chara braunii. Values represent mean and standard deviation (n=3) at P< 0.05.

Copper toxicity had significant effect on pigment concentration on the Chara braunii. After 1st day, compared to the control treatment Cu exposure caused 72%, 75.74 and 87.28% reduction of Chl a content in 1ppm, 5ppm and 10ppm treatments respectively, at 2nd day the relative value significantly decreased by 76.56, 88.13 and 91.26 respectively, and at 3rd day reduction percentage stood at 77.84, 90.33 and 91.88 respectively (Figure 1A). Observed decreasing trend pattern for chlorophyll a continued until the last day of experiment and reached the lowest value at 4th day. Copper toxicity influenced the Chl b concentration with the increasing toxicity (Figure 1B) and time duration (p<0.05) while interaction effect of two factors was not significant (Table 1) .The plants grown in control had the highest Chl b level, followed by plants grown 1ppm and 5ppm treatments, in contrast to the lowest content available in 10ppm subjected plants over the period. Most Significant degradation in carotenoids content was observed for 4th day with 10ppm concentration compared to the control (Figure 1C). However, between two higher cu dosages (10 ppm and 5ppm) carotenoids degradation was not significantly different (p>0.05).
Figure 2. Effect of exposure time on H$_2$O$_2$ (A), Guaiicol peroxidase (POD) (B), Catalase activity (CAT) (C) in Chara braunii. Values represent mean and standard deviation (n=3) at P<0.05.

Reactive oxygen species was measured as H$_2$O$_2$ content. H$_2$O$_2$ content was significantly increased in plant propagules exposed to the higher copper treatments (Figure 2A). Till the 4th day H$_2$O$_2$ concentration was increased up to the 21.46 µmol g$^{-1}$ FW with compared to the control besides the consecutive days indicated the reduction of available H$_2$O$_2$ content. The H$_2$O$_2$ variation was significantly influenced by the concentration, Time and interactions of two factors (Table 1).

Copper treated plants exhibited accelerated Catalase (CAT) and guaiacol peroxidase activity (POD) at all concentrations and limited to shorter durations. However, prolonged exposure tended to inhibit both antioxidants scavenging process. Considering the POD activity, increase in copper level in water was significantly related to an increase in scavenging activity for over time (Figure 2B). Moreover, interactions of different copper concentrations and exposure time also significantly caused for the changes in POD activity during the experiment. The mean POD concentration in C. braunii plants subjected to the 10ppm Cu level was 58.22% and 68.16 higher than to the 5ppm and 1ppm dosages whereas 215.08% greater than to the control after 1st day. However, this upregulated guaiacol peroxidase activity significantly reduced to lower level by the end of experimental period. A gradual increase was observed in CAT activity under 10 ppm concentrations in contrast to the control at 1st day approximately 2.69 fold and this peaked concentration reached the lowest level at 4th day (Figure 2C). Furthermore, the activity of catalase was significantly related to copper concentrations as well as to both stress factors and their interactions (Table 1).
Table 1. Results of the two-way ANOVA of the effect of copper concentration and exposure time

| Response          | Factor          | F     | P           |
|-------------------|-----------------|-------|-------------|
| Chlorophyll a     | Concentration   | 889.3 | <0.0001     |
|                   | Time            | 6.17  | <0.0001     |
|                   | concentration × time | 1.09  | 0.391       |
| Chlorophyll b     | Concentration   | 700.33| <0.0001     |
|                   | Time            | 5.52  | <0.0001     |
|                   | concentration × time | 1.86  | 0.095       |
| Carotenoids       | Concentration   | 1124.76 | <0.0001  |
|                   | Time            | 5.14  | <0.0001     |
|                   | concentration × time | 1.17  | 0.345       |
| Total chlorophyll | Concentration   | 865.10| <0.0001     |
|                   | Time            | 5.83  | 0.003       |
|                   | concentration × time | 1.34  | 0.255       |
| H2O2              | Concentration   | 99.31 | <0.0001     |
|                   | Time            | 158.51| <0.0001     |
|                   | concentration × time | 61.42 | <0.0001     |
| POD               | Concentration   | 31.137| <0.0001     |
|                   | Time            | 49.44 | <0.0001     |
|                   | concentration × time | 12.81 | <0.0001     |
| CAT               | Concentration   | 46.91 | <0.0001     |
|                   | Time            | 36.04 | <0.0001     |
|                   | concentration × time | 5.67  | <0.0001     |

Figure 3. The appearance of Chara braunii leaves under different treatments of Cu under a light microscope. A: control, B: 1ppm, C: 5ppm, D: 10ppm.

4. Discussion
Exposure to the different heavy metals caused to the disruption of cellular and photosynthesis process of the plants. According to our results copper contamination clearly related to the photosynthesis pigment destruction and aligned with the previous research findings [25]. As a result of chlorophyll damage, we observed chlorotic leafs from the Chara braunii plants from the second day of the copper
exposure over all three copper gradients. Marschner [26] explained chlorosis as one of the visual symptoms of the excess copper by plants (Figure 3). In the present study chlorophyll content reduction took place in three main pigments mainly as Chlorophyll a, chlorophyll b and carotenoids. As three different pigment varieties Chlorophyll a and chlorophyll b plays important role in function of photosystem [27] while carotenoid fight back against the harmful reactive oxygen species [28]. This downturn of the chlorophyll may be associated with the inhibition of the enzymes [29] and effect of lipid peroxidation thus it is make drastically challenges to maintain survival of the effected species. Furthermore, previous research findings are reveals that this chlorophyll damage is not limited to the Cu whereas different other heavy metals also caused the chlorophyll content deterioration [30].

The elevation of H$_2$O$_2$ content was observed in all Cu treatments whereas it triggered in short durations more drastically. It is clear that heavy metal stress can leads to the excessive generation of reactive oxygen species and noted by several authors [31,32]. Among the major ROS, H$_2$O$_2$ consider as one of the stable molecules that lower concentrations also strongly inhibit the photosynthesis [33] In plant cellular level reactive oxygen species are produced as partial reduction of oxygen. When this ROS are exceeding the cellular antioxidant capacity oxidative stress taken place and create unfavorable condition for plant growth thereafter scavenging activity of the antioxidants falls resulting in oxidative damage. Reactive oxygen species are more often toxic to the organelles thus if this cell environment continuously persists cell death taken place.

Increase in cellular ROS signals the increased synthesis of antioxidants activities like CAT and POD. In this present study CAT and POD activities inhibited for Chara braunii when plants exposed to the longer durations of copper concentrations. This suggests these two antioxidants were not resistive for exceedingly accumulated ROS generated by Cu toxicity. Catalase is more commonly available antioxidant within all plants and animals that having lower affinity for hydrogen peroxide. It converts H$_2$O$_2$ in to the water and oxygen [34] while reduce the toxic concentration of available ROS. In this experiment increase response of CAT activity with increasing copper concentrations which implies that more available substrate for reduces toxicity within Cu treated C. braunii tissues. In contrast, at the end of the experiment period activity reduced to the lower level attributed to the inactivation of antioxidant mechanism over extreme oxidative stress. Similarly, at the 4th day decreased responses of the POD activity against Copper treatments indicated that prolonged exposure is not viable for Charophytes.

5. Conclusion

Copper induced oxidative damage caused detrimental unrecoverable effect for the Chara braunii which shows it is good bio indicator for the water quality assessment related with Cu toxicity. If water bodies are contaminated with the heavy metals the colonization of the Charophytes could be reduced. Consequently, under prolonged contamination all Charophytes may disappear from the particular aquatic ecosystems. Moreover, exhibited defensive antioxidative system activities at early stages showed it can fight back with the free radicles for scavenge ROS for short duration only. These results increase our understanding of biochemical stress responses of Charophytes while to how there coping strategies are vary with the time duration.

6. References

[1] McCourt RM, Delwiche CF, Karol KG. Charophyte algae and land plant origins. *Trends Ecol. Evol.* 2004;19(12):661–6.

[2] Simons J, Ohm M, Daalder R, Boers P, Rip W. Restoration of Botshol (The Netherlands) by reduction of external nutrient load: recovery of a characean community, dominated by Chara connivens. *Hydrobiologia.* 1994;275(1):243–53.

[3] Rey-Boissezon A, Joyce DA. Habitat requirements of charophytes—Evidence of species discrimination through distribution analysis. *Aquat. Bot.* 2015;120:84–91.

[4] Casanova MT, Porter JL. Two new species of Nitella (Characeae, Charophyceae) from arid-zone claypan wetlands in Australia. *Muelleria.* 2013;31:53–9.

[5] Schubert H, Blindow I. Charophytes of the Baltic sea. Gantner; 2004.
[6] Simons J, Nat E. Past and present distribution of stoneworts (Characeae) in The Netherlands. In: Management and Ecology of Freshwater Plants. Springer; 1996. p. 127–35.

[7] Romanov RE. Charophytes (Charales: Streptophyta) of the South of the West Siberian Plain. *Rastit. mir Aziat. Ross.* 2009;1:19–30.

[8] Rai PK. Heavy metal phytoremediation from aquatic ecosystems with special reference to macrophytes. *Crit. Rev. Environ. Sci. Technol.* 2009;39(9):697–753.

[9] Xing W, Wu H, Hao B, Huang W, Liu G. Bioaccumulation of heavy metals by submerged macrophytes: looking for hyperaccumulators in eutrophic lakes. *Environ. Sci. Technol.* 2013;47(9):4695–703.

[10] Vardanyan LG, Ingole BS. Studies on heavy metal accumulation in aquatic macrophytes from Sevan (Armenia) and Carambolim (India) lake systems. *Environ. Int.* 2006;32(2):208–18.

[11] Okocha RO, Adeeji OB. Overview of copper toxicity to aquatic life. *Rep. Opin.* 2012;4(8).

[12] Mantovì P, Bonazzi G, Maestri E, Marmioli N. Accumulation of copper and zinc from liquid manure in agricultural soils and crop plants. *Plant Soil.* 2003;250(2):249–57.

[13] Mal TK, Adorjan P, Corbett AL. Effect of copper on growth of an aquatic macrophyte, Elodea canadensis. *Environ. Pollut.* 2002;120(2):307–11.

[14] Lewis AG. Copper in water and aquatic environments. *ICA Rep.* 1995;71.

[15] Brown BT, Rattigan BM. Toxicity of soluble copper and other metal ions to Elodea canadensis. *Environ. Pollut.* 1979;20(4):303–14.

[16] Pascal P-Y, Fleeger JW, Galvez F, Carman KR. The toxicological interaction between ocean acidity and metals in coastal meiobenthic copepods. *Mar. Pollut. Bull.* 2010;60(12):2201–8.

[17] Murphy V, Hughes H, McLoughlin P. Comparative study of chromium biosorption by red, green and brown seaweed biomass. *Chemosphere.* 2008;70(6):1128–34.

[18] Mohan D, Singh KP. Single-and multi-component adsorption of cadmium and zinc using activated carbon derived from bagasse—an agricultural waste. *Water Res.* 2002;36(9):2304–18.

[19] Imahori K. Ecology, phytogeography and taxonomy of the Japanese Charophyta. Otto Koeltz; 1954.

[20] Moore JA. Charophytes of Great Britain and Ireland. In Botanical Society of the British Isles; 1986.

[21] Beilby MJ. *Chara braunii* genome: a new resource for plant electrophysiology. *Biophys. Rev.* 2019;11(2):235–9.

[22] Wellburn AR. The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. *J. Plant Physiol.* 1994;144(3):307–13.

[23] Jana S, Choudhuri MA. Glycolate metabolism of three submersed aquatic angiosperms during ageing. *Aquat. Bot.* 1982;12:345–54.

[24] Aebi H. [13] Catalase in vitro. In: Methods in enzymology. Elsevier; 1984. p. 121–6.

[25] Monferrà M V, Agudo JAS, Pignata ML, Wunderlin DA. Copper-induced response of physiological parameters and antioxidant enzymes in the aquatic macrophyte Potamogeton pusillus. *Environ. Pollut.* 2009;157(8–9):2570–6.

[26] Marschner H. Mineral Nutrition of Higher Plants Academic Press London 889. 1995.

[27] Kume A, Akitsu T, Nasahara KN. Why is chlorophyll b only used in light-harvesting systems? *J. Plant Res.* 2018;131(6):961–72.

[28] Stahl W, Sies H. Bioactivity and protective effects of natural carotenoids. *Biochim. Biophys. Acta (BBA)-Molecular Basis Dis.* 2005;1740(2):101–7.

[29] Böddi B, Oravec AR, Lehoczki E. Effect of cadmium on organization and photoreduction of protochlorophyllide in dark-grown leaves and etioplast inner membrane preparations of wheat. *Photosynth. (Czech Republic).* 1995.

[30] Malec P, Maleva M, Prasad MN V, Strzálka K. Copper toxicity in leaves of Elodea canadensis Michx. *Bull. Environ. Contam. Toxicol.* 2009;82(5):627–32.

[31] Mithöfer A, Schulze B, Boland W. Biotic and heavy metal stress response in plants: evidence for common signals. *FEBS Lett.* 2004;566(1–3):1–5.
[32] Bhaduri AM, Fulekar MH. Antioxidant enzyme responses of plants to heavy metal stress. Rev. Environ. Sci. Bio/Technology. 2012;11(1):55–69.

[33] Mittler R, Zilinskas BA. Purification and characterization of pea cytosolic ascorbate peroxidase. Plant Physiol. 1991;97(3):962–8.

[34] Jones P, Dunford HB. On the mechanism of compound I formation from peroxidases and catalases. J. Theor. Biol. 1977;69(3):457–70.

Acknowledgments
This research was financially supported by a Research Grant in Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.