Physiological responses of *Ulva pertusa* and *U. armoricana* to copper exposure

Taejun Han [a,*], Sung-Ho Kang [b], Ji-Sook Park [a],
Han-Kyun Lee [a], Murray T. Brown [c]

[a] *Division of Biology and Chemistry, University of Incheon, Incheon 402-749, Republic of Korea*
[b] *Korea Polar Research Institute, Incheon 406-840, Republic of Korea*
[c] *School of Biological Sciences, University of Plymouth, Plymouth, Devon, PL4 8AA, United Kingdom*

Abstract

A comparative study of copper (Cu) toxicity and tolerance in two species of *Ulva* from Korea, the native *Ulva pertusa* and alien *Ulva armoricana*, was conducted by examining the effects on growth, pigmentation, chlorophyll fluorescence, antioxidant capacity and nitrate reductase activity. Toxic effects of Cu were less expressed in *U. armoricana* than in *U. pertusa*. At lower concentrations (25–$50\,\mu g\,L^{-1}$), exposure to Cu did not affect thallus growth of *U. armoricana*, whilst growth was significantly reduced in *U. pertusa*. An increase in chlorophyll concentrations was observed in *U. armoricana* exposed up to $100\,\mu g\,L^{-1}$, whereas Cu caused a significant chlorophyll reduction in *U. pertusa*. Chlorophyll *b* was reduced to a lesser extent than chlorophyll *a* by higher Cu concentrations. In *U. armoricana*, the maximum efficiency of photosystem II, minimum fluorescence, maximum electron transport rate and non-photochemical quenching were unaffected by Cu except at the highest concentration tested. *U. pertusa* showed a significant decrease in those parameters at much lower Cu concentrations. It was notable that in this alga the maximum efficiency of photosystem II was reduced at higher Cu concentrations than relative electron transport rate. Elevated concentrations of Cu induced a strong activation of antioxidant activity in *U. armoricana*, whereas the generation of high levels of reactive oxygen species probably decreased the non-enzymatic antioxidant defense system in *U. pertusa*. An increase in the nitrate reductase activity of *U. armoricana* at 50–$100\,\mu g\,L^{-1}$ Cu coincided with the increase in chlorophyll contents, whereas *U. pertusa* showed a significant decrease at the higher Cu concentration. Differences in the sensitivity of the two species of *Ulva* to Cu may influence their competitive interactions in Korean coastal waters experiencing temporal increases in the loading of heavy metals.
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1. Introduction

Species of the green seaweed genus *Ulva* are generally considered to be tolerant of a wide range of environmental stressors, including metals (Correa et al., 1996; Fletcher, 1996) and because of their high accumulation capacity for metals they are used as bioindicators of metal pollution (Haritonidis and Malea, 1999; Lee and Wang, 2001). However, the results obtained from recent studies on *U. pertusa* suggest that this species might be more sensitive to toxic chemicals, including metals, than other *Ulva* species and some freshwater microalgae and aquatic plants (Han and Choi, 2005; Han et al., 2007). This conclusion is derived from comparisons with published data (e.g. EC_{50}s) and does not take account of differences in the culture conditions used in the various studies, which can influence the toxicity of metals (Brown and Newman, 2003). Therefore, to establish whether related species have evolved differential tolerance to metals, comparative studies using material collected from the same location need to be undertaken. Despite much published data on the impacts of metals on macroalgae very few studies have investigated directly inter-specific differences in response to metal exposure.

U. pertusa Kjellman is native to Korea and forms extensive mats in shallow coastal waters due to its fast rate of growth and high reproductive capacity (Han et al., 2003). This seaweed plays an important role as a primary producer, contributing directly and indirectly to higher trophic levels, and provides habitat and nursery grounds for fish and invertebrates. Recently, extensive populations of the related alien species, *Ulva armoricana* Dion et al. have been recorded from the eastern coasts of Korea, and found growing in the same communities as *U. pertusa* (Han et al., 2006). The introduction of any new seaweed raises concerns about the potential risks to the indigenous biota by, for example, pre-empting space and resources for related species, reducing food and shelter for consumers, threatening biodiversity and, more generally, disturbing the dynamics of near-shore ecosystems (Carlton, 2000; Occhipinti-Ambrogi and Savini, 2003). In the case of *U. armoricana* there is evidence for environmental problems associated with its introduction into French coastal waters including damage to shellfish cultivation through fouling, production of large-scale green tides and catastrophic decomposition of the algal mats resulting in dystrophic conditions (Dion and Le Bozec, 1996). Compared with other *Ulva* species, *U. armoricana* displays a high photosynthetic potential, nutrient uptake performance and wide temperature optimum (Dion and Le Bozec, 1996; Dion et al., 1998), but its response to metal pollution has not previously been investigated. As both species co-occur in Korean waters impacted to varying degrees by anthropogenically derived chemical pollutants, there is an opportunity to investigate their responses to metal-pollution and to compare their levels of resistance.

Although copper (Cu) is essential for the metabolism of eukaryotic organisms it is generally considered to be one of the most toxic metals (Lobban and Harrison, 1994; Gledhill et al., 1997). At concentrations beyond a certain threshold, Cu induces phytotoxic symptoms in algae such as: inhibition of growth, photosynthesis and fertility, disruption of development, chlorosis and damage to membranes (Gledhill et al., 1997; Brown and Newman, 2003; Nielsen et al., 2003a,b; Eklund and Kautsky, 2003). In off-shore seawater, reported values for total Cu concentration vary from 0.002 to $6 \mu g L^{-1}$, but the concentration may increase several fold in coastal waters affected by waste derived from e.g., industrial activities, sewage, antifouling paints and pesticides (Kim and Han, 1999; Brown and Newman, 2003). In Korean coastal waters and estuaries dissolved Cu concentrations range between 4 and $14 \mu g L^{-1}$ (Korea Maritime Ministry), and these levels are likely to increase with time as a result of industrial growth and population increase (Lee et al., 1998).

To assess the relative sensitivities of the two species of *Ulva* to Cu, several end-points at different levels of biological organization were used. Such an approach allows for a more detailed evaluation of the response to an environmental stressor than using a single physiological parameter, and can provide information on the mechanism(s) of resistance to that stressor (Brown and Newman, 2003). Five parameters were selected: growth, pigmentation, chlorophyll fluorescence, antioxidant and nitrate reductase activities, all of which are known to be affected, to varying degrees, by Cu in other plants and algae (e.g. Cid et al., 1995; Ouzounidou et al., 1997; Brown and Newman, 2003).

2. Materials and methods

2.1. Culture of plants

U. pertusa and *U. armoricana* were collected from sites near Ahnin on the well-conserved eastern coast of Korea (37.4°N, 129.1°E). Unialgal stock cultures were maintained in artificial seawater medium, incubated at 15 °C and 10–15 μmol photons $m^{-2} s^{-1}$ of white fluorescent light (FL400, Kum-Ho, Seoul, South Korea) on a 12-h photoperiod. The medium was prepared by dissolving commercial sea salts (Coralife, Energy Savers, California, USA) in deionized water to a concentration of 35‰, to which was added 1 mM KNO_3 and 0.1 mM K_2HPO_4 as nutrients.

Algal disks (Ø 6 mm) were cut from the middle region of healthy thalli and placed in flasks (15 ml volume per well) with medium containing different concentrations of Cu. The medium was gently bubbled with air. A stock solution of copper chloride ($CuCl_2$) was prepared from a concentrated standard in deionized water acidified with 1N hydrochloric acid (HCl) (Junsei, Tokyo, Japan). Appropriate volumes of the stock solution were added so that the final concentrations were in the range 25–250 $\mu g L^{-1}$ Cu. Cultures were established under optimal conditions for growth i.e.:15 °C, 80–100 μmol photons $m^{-2} s^{-1}$ of white fluorescent light, 12:12-h LD photoperiod and salinity of 35‰. Controls consisted of the artificial seawater medium without added Cu. There were four replicates per treatment.

2.2. Growth

Following exposure to experimental treatments for 3 days, the algal samples were harvested to determine changes to disk area, measured using a computer-assisted image analyzer (MV200, Samsung, Seoul, Korea). Relative growth rates (RGR) were calculated using the equation:

$$RGR\,(\%/day) = \frac{\ln A_t - \ln A_0}{t} \times 100\%$$

where A_0 and A_t are the areas of disks at time zero and after t days, respectively.

2.3. Chlorophyll concentrations

Chlorophyll concentrations were determined using a Specord spectrophotometer (S10, Zeiss) after extraction of disks in 100% methanol for at least 24 h in the dark at 4 °C. For chlorophyll *a* and *b* contents the methanolic extracts were measured at 666 nm (chlorophyll *a*; chl *a*) and at 653 nm (chlorophyll *b*; chl *b*) using the equations outlined in Hipkins and Baker (1986). The chl *a/b* ratio was also calculated for all disks.

2.4. Chlorophyll fluorescence

Chlorophyll fluorescence was measured using a pulse amplitude modulated (PAM) fluorometer (Diving-PAM, Walz,
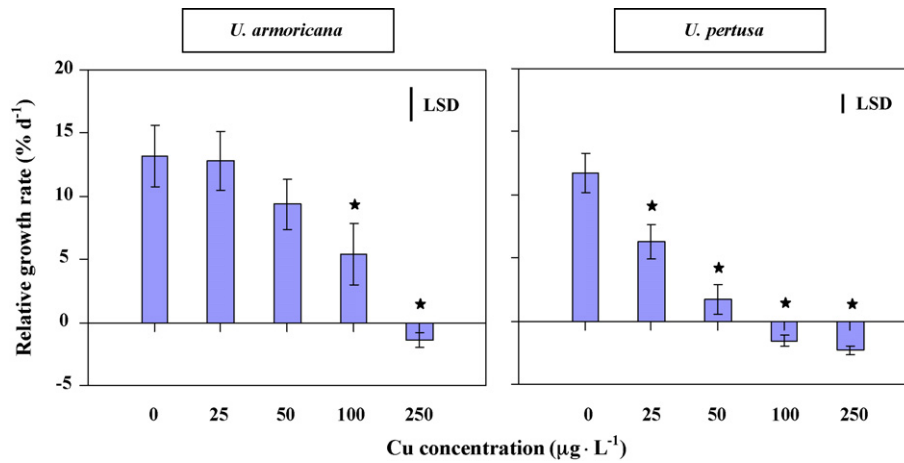
Fig. 1. Daily relative growth rate of *Ulva armoricana* and *U. pertusa* after a 3-day exposure to a range of Cu concentrations. Mean $\pm$ 95% confidence interval ($n = 11$) are shown. Values with an asterisk are significantly different at $P < 0.05$ from those of the controls.

Effeltrich, Germany). Samples were initially dark adapted for 15 min before chl fluorescence readings were taken. F_m, the maximum fluorescence yield of dark-adapted samples and F_0, the initial fluorescence yield, were recorded. The maximum quantum yield of PSII in the dark-adapted state is expressed as the ratio of variable to maximal chl fluorescence (F_v/F_m), derived from $(F_m - F_0)/F_m$.

Rapid light curves were measured using 10 s pulses of actinic light increased stepwise from 0 to 1517 μmol photons $m^{-2}\,s^{-1}$ (Schreiber, 2004). Relative electron transport rates (rel.ETR) were calculated by multiplying the effective quantum yield ($\Phi_{PSII} = F'_m - F/F'_m$, where F'_m is maximum light-acclimated fluorescence yield and F is light-acclimated fluorescence yield) by photosynthetic photon flux density (PPFD) and plotted against PPFD. The ETR is relative because the absorptance of the thallus was not measured. Maximum electron transport rate (ETR_{max}) was derived from the hyperbolic tangent formulation, rel.ETR $= ETR_{max} \tanh(\alpha I/ETR_{max})$, where α indicates electron transport rate under light-limited conditions, adapted from Jassby and Platt (1976). Alterations in chl fluorescence due to changes in non-photochemical quenching (NPQ) were calculated from $(F_m - F'_m)/F'_m$ (Maxwell and Johnson, 2000).

2.5. Antioxidant activity

Non-enzymatic antioxidant activity of disks was measured by DPPH (α,α-diphenyl-β-picrylhydrazyl) scavenging activity. Thallus disks were homogenized in 2 ml of absolute ethanol with a mortar and pestle and the extracts centrifuged at 10,000 rpm for 15 min. A 0.5-ml aliquot of the supernatant was mixed with a 0.5-mM DPPH ethanol solution (0.25 ml) and 100 mM of acetate buffer (pH 5.5, 0.5 ml). After incubating in the dark for 30 min, the absorbance of the mixture was measured at 517 nm to determine DPPH scavenging activity (Abe et al., 1998). Activity is expressed as $(A_{control} - A_{treated})/A_{control} \times 100\%$, where $A_{control}$ and $A_{treated}$ are the absorbances at 517 nm of control and copper-treated samples.

2.6. Nitrate reductase (NR) activity

NR activity was assayed as described by Jun and Kim (1989) with some modifications. After a 72-h culture period under different Cu treatment conditions (50 and 100 μg L^{-1}), disks were harvested and weighed before each was transferred to a flask containing 20 ml of fresh medium with 20 mM sodium nitrate, 4% n-propanol and chloramphenicol (2 μg L^{-1}) for 3-h incubation in the dark. NR activity was determined by incubating 1 ml supernatant with 3N HCl and 0.02% *N*-1-naphthyl-ethylenediamine dihydrochloride, at room temperature for 20 min. The amount of nitrite produced was determined by measuring the absorbance at 540 nm. One unit of NR activity is defined as that which produced 1 mol $NO_2^{-1}\,h^{-1}$.

2.7. Statistical analysis

One-way analysis of variance (ANOVA) was performed to confirm significant differences in response. Multiple comparison test by the least significance difference (LSD) was then carried out to find out significant differences at $P = 0.05$ in response from controls.

3. Results

3.1. Effects of copper on growth

Toxic effects of Cu on growth were strongly dependent on species. The mean RGR of *U. armoricana* was not significantly affected by 25–50 μg L^{-1} Cu but at 100 μg L^{-1} Cu the mean RGR was reduced to 41% of the control and at 250 μg L^{-1} the RGR was negative (-1.4%/day), indicating a shrinkage of the disks (Fig. 1). *U. pertusa* was less tolerant to Cu with a 43% reduction of growth even at the lowest test concentration (25 μg L^{-1}) and negative mean RGRs at 100 and 250 μg L^{-1} Cu (Fig. 1).
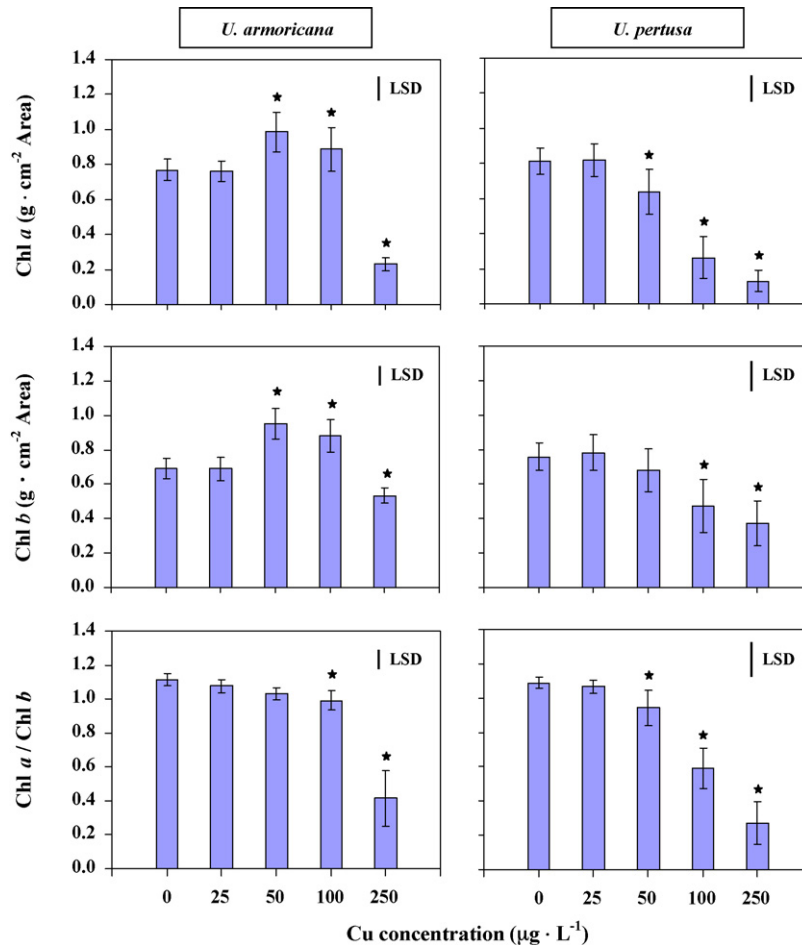
Fig. 2. Chlorophyll concentrations of *Ulva armoricana* and *U. pertusa* after a 3-day exposure to a range of Cu concentrations. Mean ± 95% confidence interval ($n = 11$) are shown. Values with an asterisk are significantly different at $P < 0.05$ from those of the controls.

3.2. Effects of copper on chlorophyll concentrations

Fig. 2a and b show the changes in the concentrations of chl *a* and *b* with increasing Cu for both species. When exposed to 50 μg L^{-1} Cu the concentrations of both pigments, relative to the controls, increased by 29–36.2% in *U. armoricana* but decreased by 14–23% in *U. pertusa*. At 100 μg L^{-1} Cu, *U. armoricana* still exhibited 5–26% higher concentrations of chl *a* and *b* than the controls, whereas the same treatment reduced the chlorophyll contents of *U. pertusa* by 42–70%. At the highest tested concentration of Cu, the two pigments were significantly reduced in both species, although to lesser extent in *U. armoricana*. For both species, reductions in chl *b* concentrations at 50–250 μg L^{-1} Cu were less than for chl *a*. The ratio of chl *a*/*b* (Fig. 2c) remained stable in the Cu concentration range between 25 and 50 μg L^{-1} but significantly decreased at 100 (11%) and 250 μg L^{-1} (63%) for *U. armoricana* whereas in *U. pertusa* there was a significant decrease in the ratio at concentrations greater than 25 μg L^{-1}.

3.3. Effects of copper on photosynthetic activity

A significant decline in the maximum quantum yield of PS II, as measured by F_v/F_m, was apparent at 250 μg L^{-1} Cu for *U. armoricana* and 100 μg L^{-1} for *U. pertusa* (Fig. 3). *U.*

armoricana exhibited a relatively stable F_0 up to 100 μg L^{-1} Cu but at 250 μg L^{-1} values declined significantly. In *U. pertusa*, F_0 was unaffected by Cu concentrations 25–50 μg L^{-1}, but values were significantly lower at 100 and 250 μg L^{-1} Cu.

A significant reduction of ETR$_{max}$ occurred at 250 μg L^{-1} Cu for *U. armoricana* while even the lowest tested Cu concentration caused 19% reduction in *U. pertusa*, followed by further significant reductions at higher concentrations.

In *U. armoricana*, values of NPQ remained unaltered in disks exposed to concentrations less than 250 μg L^{-1} Cu, whereas in *U. pertusa* NPQ decreased significantly from control values at all external concentrations of Cu. In both species NPQ values fell to just above zero at 250 μg L^{-1}.

3.4. Effects of copper on non-enzymatic antioxidant activity

In *U. armoricana*, exposure to concentrations of 25 to 100 μg L^{-1} Cu resulted in a stimulation of antioxidant activity, reaching its maximum at 100 μg L^{-1} (Fig. 4). In contrast, the activities of antioxidants showed a decreasing tendency in *U. pertusa* exposed to elevated Cu. At 250 μg L^{-1} Cu antioxidant activities of both species were significantly reduced to 20–30% of control values.
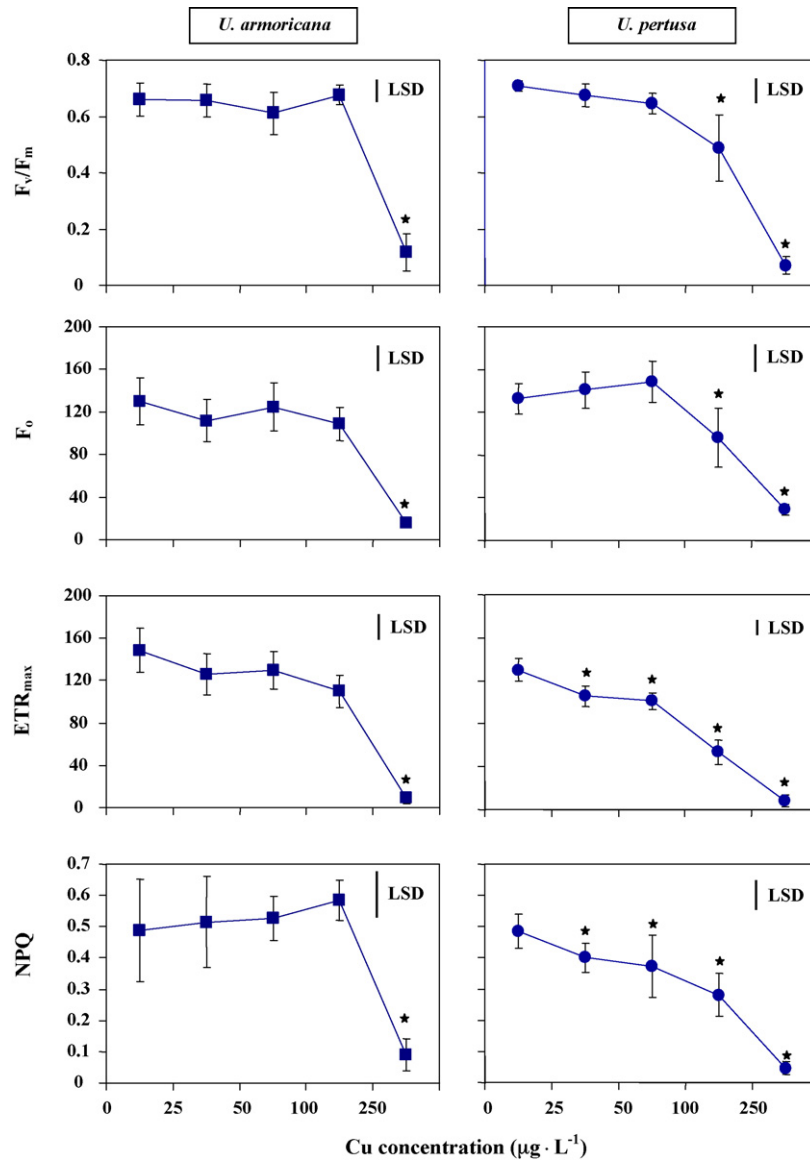
Fig. 3. Chlorophyll fluorescence parameters, F_v/F_m, F_0, ETR_{max}, and NPQ, measured on *Ulva armoricana* and *U. pertusa* after a 3-day exposure to a range of Cu concentrations. Mean ± 95% confidence interval ($n = 11$) are shown. Values with an asterisk are significantly different at $P < 0.05$ from those of the controls.

Fig. 4. Non-enzymatic antioxidant activity of *Ulva armoricana* and *U. pertusa* after a 3-day exposure to a range of Cu concentrations. Mean ± 95% confidence interval ($n = 11$) are shown. Values with an asterisk are significantly different at $P < 0.05$ from those of the controls.

Fig. 5. NR activity of *Ulva armoricana* and *U. pertusa* after a 3-day exposure to 50–100 μg L^{-1} Cu. Mean ± 95% confidence interval ($n = 11$) are shown. Values with an asterisk are significantly different at $P < 0.05$ from those of the controls.
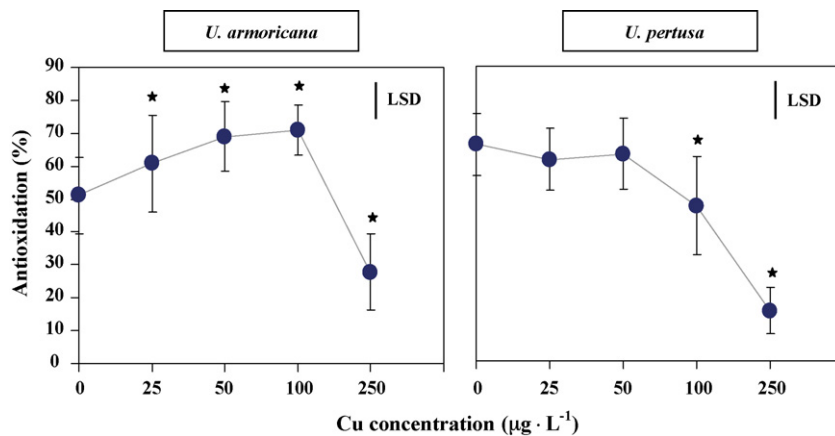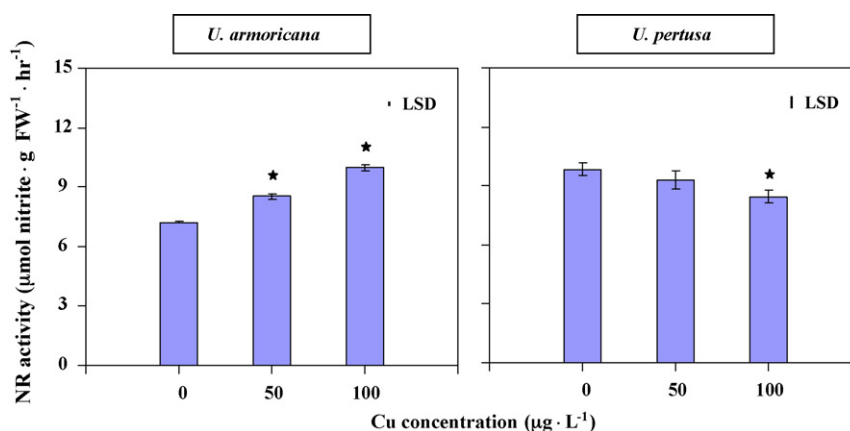
3.5. Effects of copper on nitrate reductase activity

Copper induced differential effects on NR activity of the *Ulva* species. *U. armoricana* showed 18 and 38% increase in NR activity relative to the controls after exposure to 50 and 100 μg L^{-1} Cu, respectively (Fig. 5). In contrast, a significant decrease in NR activity (14%) was observed in *U. pertusa* exposed to 100 μg L^{-1} Cu.

4. Discussion

Differences in the response of the two species of *Ulva* to Cu exposure have been identified. The results obtained from measurements at different levels of biological organization show that *U. armoricana* is more tolerant to copper than *U. pertusa* within the concentration range used in the current study. Of the various endpoints measured, growth was found to be the most sensitive. The threshold concentration that resulted in a significant reduction in relative growth rate (RGR) was 25 μg L^{-1} for *U. pertusa* compared with 100 μg L^{-1} for *U. armoricana*. Studies on other *Ulva* species have also reported that significant reductions in growth occur at concentrations greater than 100 μg L^{-1}; for example, in *U. compressa* (L.) Nees von Esenbeck growth inhibition occurred in the range 114–6354 μg L^{-1} Cu (Reed and Moffat, 1983; Correa et al., 1996) and in *U. fasciata* Delile 50% growth inhibition was recorded at 850 μg L^{-1} Cu (Lee et al., 2005). On exposure to 250 μg L^{-1} shrinkage of thallus disks was observed in both species, a finding that has been reported previously in the red seaweed *Gracilariopsis longissima* (S.G. Gmelin) Steentoft, LM Irvine and Farnham and the brown seaweed *Fucus vesiculosus* L. exposed to similar concentrations of Cu (Bryan and Gibbs, 1983; Brown and Newman, 2003). Copper-induced interference with cell division and/or expansion has been proposed as a possible reason for the observed reduction in growth (e.g. Stauber and Florence, 1987). This may be linked to a decrease turgor and/or a change in cell wall elasticity due to copper toxicity (Brown and Newman, 2003).

Uncoupling of growth from photosynthetic electron transport at elevated concentrations of Cu was evident in both species of *Ulva*. An increase in Cu from 25 to 50 μg L^{-1} caused a significant reduction in growth in *U. pertusa* but had no apparent effect on photosynthetic electron transport, while for *U. armoricana* a similar response was observed only at higher concentrations (50–100 μg L^{-1}). These results imply that the initial site of toxic action does not occur in pathways associated with O$_2$ evolution. Similar conclusions have been reached for several microalgae and seaweeds (e.g. Lumsden and Florence, 1983; Nalewajko and Olaveson, 1995; Brown and Newman, 2003). For example, Brown and Newman (2003) reported that while growth rates of the red seaweed *G. longissima* were reduced significantly at 12.5 μg L^{-1}, no observable changes in photosynthesis were evident until 250 μg L^{-1} Cu. They concluded that this uncoupling of growth from photosynthesis at low concentrations of Cu was most likely due to the energy captured by the light reactions being utilized to combat the toxic effects of Cu through induction of protective mechanisms. Further support for this view is provided by the results obtained in the current study.

Photosynthetic activity of *U. pertusa* and *U. armoricana*, as determined from the chlorophyll fluorescence parameters, was not significantly affected until the seaweeds were exposed to 100 and 250 μg L^{-1} Cu, respectively. The decrease in activity was related to changes in the chlorophyll content of disks. A reduction in chlorophyll, due to either a decrease in biosynthesis or increase in the rate of degradation, is a commonly observed symptom of toxicity in algae (Gledhill et al., 1997). Copper is known to regulate the pigment content and composition of the pigment-protein complexes of PS II (Prasad and Strzalka, 1999). For *U. pertusa*, exposure to 50 μg L^{-1} was found to significantly lower the concentration of chl *a*, and the chl *a/b* ratio, with further progressive reductions at the higher concentrations, whereas the concentration of chl *b* was only affected at higher concentrations of Cu. The greater susceptibility of chlorophyll *a* to Cu exposure has also been observed in a freshwater macrophyte, *Ceratophyllum demersum* L. (Coontail) and some lichens (Chettri et al., 1998; Rama Devi and Prasad, 1998). Of note was the lack of change in F_v/F_m and F_0 in *U. pertusa* exposed to Cu concentrations less than 100 μg L^{-1} although chl *a* declined significantly. This disparity might imply that the chl *a* affected by exposure to 50 μg L^{-1} Cu was mostly a component of the

PS I since PS I consists almost entirely of chl *a* while PAM fluorescence probes only PS II.

In contrast, there was an increase in chlorophyll concentrations of *U. armoricana* exposed to 50–100 μg L^{-1} of Cu. Moreover, there was no decrease in either F_v/F_m or F_0 at 100 μg L^{-1}, although the RGR was reduced, albeit not to the same extent as in *U. pertusa*. These findings provide further support for a trade-off between energetic resources being used for pigment biosynthesis at the expense of growth. Such a stimulatory effect of excess of Cu on chlorophyll accumulation has previously been observed in copper-tolerant higher plants (Baszyński et al., 1982; Maksymiec and Baszyński, 1996). More generally, significant increases in chlorophyll have been found to occur in response to a range of environmental stresses and are associated with stress resistance (Zhang et al., 2005). For example, in *U. rigida* C. Agardh increases in chl *a* and *b* were considered to be an efficient protective mechanism against ultraviolet-B radiation (UV-B) (Altamirano et al., 2000). It has been noticed in the aquatic plant, *Lemna gibba* that copper and UV radiation elicit similar stress responses (Babu et al., 2003). In both species, the significant decline in F_0 and F_v/F_m at concentrations greater than 100 μg L^{-1} Cu coincided with negative RGRs and are a result of marked reductions in light absorption and PS II efficiency, respectively due to the destruction of chlorophyll molecules (Capsi et al., 1999).

Relative ETR provides an approximation of the rate of electrons pumped via PS II into the photosynthetic chain (Schreiber, 2004). Copper has been shown to inhibit photosynthetic electron transport through damage to both the donor and acceptor sides of PS II (Pätsikkä et al., 1998). In principle, blockage of electron transport by Cu could lead to a decrease in F_v/F_m (Karukstis, 1991). In *U. armoricana*, changes in ETR$_{max}$ paralleled those of F_v/F_m, whereas in *U. pertusa,* ETR$_{max}$ showed a significant decrease at lower Cu concentrations than did F_v/F_m. The disparity between the responses of F_v/F_m and ETR$_{max}$ reflects inhibition downstream of PS II, suggesting that the down-regulation of PS II electron transport is a result of inhibition of Calvin cycle enzymes and Rubisco (Yu et al., 2002).

Transition metals such as Cu catalyze the formation of hydroxyl radicals ($^{\bullet}$OH) from the non-enzymatic chemical reaction (Haber-Weiss reaction) between superoxide (O$_2^{\bullet-}$) and H$_2$O$_2$ (Winterbourn, 1982). Therefore, the presence of excess Cu can promote oxidative damage by increasing the cellular concentrations of reactive oxygen species (ROS) (Pinto et al., 2003). In chloroplasts, ROS cause inactivation and degradation of Rubisco and other components of the Calvin cycle (Pätsikkä et al., 1998), and lipid peroxidation, resulting in the disruption to photosynthetic pigments (Pätsikkä et al., 2002). The tolerance of photosynthetic organisms to such stress factors is associated with the antioxidant capacity, as the increased levels of antioxidant constituents can prevent stress damage. Copper is known to induce the activity of antioxidants such as ascorbate and gluthathione via the ascorbate-gluthathione cycle, which plays an important role in detoxification of certain ROS (Panda et al., 2003). However, the responses of antioxidant and enzyme activities in photosynthetic organisms exposed to Cu have not been fully resolved, with their stimulation and inhibition both being

reported (Mazhoudi et al., 1997). In the present study, increase in non-enzymatic antioxidant activity of *U. armoricana* after exposure to sublethal levels of Cu may alleviate the deleterious effects of copper while decreased antioxidant capacity in *U. pertusa* may have resulted from Cu-induced decline of antioxidant activity (Gallego et al., 1996). It is hypothesized, therefore, that the antioxidant capacity of *U. pertusa* was overloaded by the high levels of ROS produced in copper-treated thallus of this species, although further study is required to confirm this.

While NPQ was unaltered in *U. armoricana* by copper treatments up to 100 μg L^{-1}, it declined at 25 μg L^{-1} in *U. pertusa*. The copper-induced decrease in NPQ might be explained by a decrease in the rate of electron transport in the pathway P680 → pheophytin → Q$_A$ → Q$_B$ or to an increase in the rate of the back reaction, Q$_A{}^-$ → P680$^+$ (Horton and Bowyer, 1990).

Nitrate reductase is one of the most important enzymes of any biosynthetic pathway in the algae and is involved in the assimilation of nitrate, the primary nitrogen source (Berges, 1997). In macroalgae, NR activity is known to be regulated by external factors such as light and nitrogen availability, and by an internal biological clock (Davison and Stewart, 1984; Lopes et al., 1997; Chow et al., 2004). It is interesting to note that in *U. armoricana* the activity of NR increased after exposure to 50–100 μg L^{-1} Cu, compared with the controls, and was coincident with increases in the concentration of chlorophyll and the reduction in RGR. As far as we are aware, Cu-induced stimulation of NR activity has never been reported before. In higher plants, it has been observed that some heavy metals cause *de novo* synthesis of NR isoenzymes (Panda and Patra, 1998). By comparison the Cu-induced reduction in NR activity in *U. pertusa* concurs with other reports of the toxic effects of elevated levels of Cu on NR activity in microalgae (Rai et al., 1994; Tripathi et al., 2004). There are three possible mechanisms by which Cu could have inhibited NR activity: (1) direct modification of thiol groups, (2) oxidative stress occurring through interference with the electron flux from PS II to PS I, and (3) reduced photosynthesis (González-Moreno et al., 1997). Given that ETR$_{max}$ was reduced at 50 μg L^{-1} Cu while NR activity remained constant excludes option (3) as an explanation for the reduced NR activity in *U. pertusa*. The reduction in both DPPH and NR activity at 100 μg L^{-1} Cu would suggest that oxidative damage, as a result of Cu toxicity, is the most likely explanation for these observations.

5. Conclusion

Ulva species are generally considered to be tolerant of the effects of heavy metals compared with many other macroalgae (Haritonidis and Malea, 1999; Lee and Wang, 2001). For the first time we have identified two species of *Ulva*, growing together on the east coasts of Korea, that differ in their sensitivity to Cu. The alien species *U. armoricana* appears to be more tolerant to Cu exposure than the native *U. pertusa* in terms of growth, pigmentation, photosynthesis, antioxidant capacity and NR activity. The observed uncoupling of growth from photosynthetic activity at higher concentrations of Cu provided sufficient energy for the induction of protective measurements, including the synthe-

sis of chlorophyll and antioxidants, to combat Cu-toxicity in *U. armoricana*. This greater level of tolerance to Cu, and potentially other metals, displayed by *U. armoricana*, together with its ability to rapidly colonize disturbed habitats, could result in the native *U. pertusa* being out-competed and displaced and, as a consequence, the biodiversity and functioning of near-shore marine ecosystems in Korea being modified.

Acknowledgements

References

Abe, N., Murata, T., Hirota, A., 1998. Novel 1,1-diphenyl-2-picryhydrazyl-radical scavengers, bisorbicillin and demethyltrichodimerol, from a fungus. Biosci. Biotechnol. Biochem. 62, 661–662.

Altamirano, M., Flores-Moya, A., Figueroa, F.L., 2000. Long-term effects of natural sunlight under various ultraviolet radiation conditions on growth and photosynthesis of intertidal *Ulva rigida* (Chlorophyceae) cultivated *in situ*. Bot. Mar. 43, 119–226.

Babu, T.S., Akhtar, T.A., Lampi, M.A., Tripuranthakam, S., Dixon, D.G., Greenberg, B.M., 2003. Similar responses are elicited by copper and ultraviolet radiation in the aquatic plant *Lemna gibba*: implication of reactive oxygen species as common signals. Plant Cell Physiol. 44, 1320–1329.

Baszyński, T., Król, M., Krupa, Z., Ruszkowska, M., Wojcieska, U., Wolińska, D., 1982. Photosynthetic apparatus of spinach exposed to excess copper. Z. Pflanzenphysiol. 108, 385–392.

Berges, D., 1997. Algal nitrate reductase. Eur. J. Phycol. 32, 3–8.

Brown, M.T., Newman, J.E., 2003. Physiological responses of *Gracilariopsis longissima* (S.G. Gmelin) Steentoft, L.M. Irvine and Farnham (Rhodophyceae) to sublethal copper concentrations. Aquat. Toxicol. 64, 201–213.

Bryan, G.W., Gibbs, P.E., 1983. Heavy metals in the Fal Estuary, Cornwall: a study of long-term contamination by mining waste and its effects on estuarine organisms. Mar. Biol. Assoc. UK Occas. Publ. 2, 1–109.

Capsi, V., Droppa, M., Horváth, G., Malkin, S., Marder, J.B., Raskin, V.I., 1999. The effect of copper on chlorophyll organization during greening of barley leaves. Photosynth. Res. 62, 165–174.

Carlton, J.T., 2000. Global change and biological invasions in the oceans. In: Mooney, H.A., Hobbs, R.J. (Eds.), Invasive Species in a Changing World. Island Press, Washington, DC, pp. 31–53.

Chettri, M.K., Cook, C.M., Vardaka, E., Sawidis, T., Lanaras, T., 1998. The effect of Cu, Zn and Pb on the chlorophyll content of the lichens *Cladonia convoluta* and *Cladonia rangiformis*. Environ. Exp. Bot. 39, 1–10.

Chow, F., de Oliveira, M.C., Pedersén, M., 2004. In vitro assay and light regulation of nitrate reductase in red alga *Gracilaria chilensis*. J. Plant Physiol. 161, 769–776.

Cid, A., Herrero, C., Torres, E., Abalde, J., 1995. Copper toxicity on the marine microalgae *Phaeodactylum tricornutum*: effects on photosynthesis and related parameters. Aquat. Toxicol. 31, 165–174.

Correa, J.A., González, P., Sánchez, P., 1996. Copper-algal interaction: inheritance or adaptation. Environ. Monit. Assess. 40, 41–54.

Davison, I.R., Stewart, W.D.P., 1984. Studies on nitrate reductase activity in *Laminaria digitata* (Huds.) Lamour. II. The role of nitrate availability in the regulation of enzyme activity. J. Exp. Mar. Biol. Ecol. 79, 65–78.

Dion, P., Le Bozec, S., 1996. The French Atlantic coasts. In: Schramm, W., Nienhuis, P.H. (Eds.), Ecological Studies: Marine Benthic Vegetation. Recent Changes and the Effects of Eutrophication, vol. 12. Springer, Berlin, pp. 251–264.

Dion, P., de Reviers, B., Coat, G., 1998. *Ulva armoricana* sp. Nov. (Ulvales, Chlorophyta) from the coasts of Brittany (France). I. Morphological identification. Eur. J. Phycol. 33, 73–80.

Eklund, B., Kautsky, L., 2003. Review on toxicity testing with marine macroalgae and the need for method standardization-exemplified with copper and phenol. Mar. Poll. Bull. 46, 171–181.

Fletcher, R.L., 1996. The occurrence of "Green Tides"—a review. In: Schramm, W., Nienhuis, P.H. (Eds.), Ecological Studies: Marine Benthic Vegetation. Recent Changes and the Effects of Eutrophication, vol. 12. Springer, Berlin, pp. 7–44.

Gallego, S.M., Benavídes, M.P., Tomaro, M.L., 1996. Effect of heavy metal ion excess on sunflower leaves: evidence for involvement of oxidative stress. Plant Sci. 121, 151–159.

Gledhill, M., Nimmo, M., Hill, S.J., Brown, M.T., 1997. The toxicity of copper (II) species to marine algae, with particular reference to macroalgae. J. Phycol. 33, 2–11.

González-Moreno, S., Gómez-Barrera, J., Perales, H., Moreno-Sánchez, R., 1997. Multiple effects of salinity on photosynthesis of the protist *Eugelna gracilis*. Physiol. Plant. 101, 777–786.

Han, T., Choi, G.-W., 2005. A novel marine algal toxicity bioassay based on sporulation inhibition in the green macroalga *Ulva pertusa* (Chlorophyta). Aquat. Toxicol. 75, 202–212.

Han, T., Han, Y.-S., Kain, J.M., Häder, D.-P., 2003. Thallus differentiation of photosynthesis, growth, reproduction, and UV-B sensitivity in the green alga *Ulva pertusa* (Chlorophyta). J. Phycol. 39, 712–721.

Han, Y.-S., Kong, J.-A., Kim, H.-S., Han, T., 2006. Morphological characteristics of *Ulva* species in Ahnin on the eastern coast of Korea. In: Proceedings of the Korean Society of Phycology Meeting, vol. 20, p. 58.

Han, Y.-S., Brown, M.T., Park, G.S., Han, T., 2007. Evaluating aquatic toxicity by visual inspection of thallus color in the green macroalga *Ulva*: testing a novel bioassay. Environ. Sci. Technol. 41, 3667–3671.

Haritonidis, S., Malea, P., 1999. Bioaccumulation of metals by the green alga *Ulva rigida* from Thermaikos Gulf, Greece. Environ. Poll. 104, 365–372.

Hipkins, M.F., Baker, N.R., 1986. Photosynthesis: Energy Transduction: A Practical Approach. IRL Press, Oxford, 199 pp.

Horton, P., Bowyer, T.R., 1990. Chlorophyll fluorescence transients. In: Harwood, J.L., Bowyer, T.R. (Eds.), Methods in Plant Biochemistry. Lipids, Membranes and Aspects of Photobiology. Academic Press, Sandiego, CA, pp. 259–295.

Jassby, A.D., Platt, T., 1976. Mathematical formulation of the relationship between photosynthesis and light for phytoplankton. Limnol. Oceanogr. 21, 540–547.

Jun, B.-O., Kim, D.H., 1989. Studies on nitrate reductase in *Ulva pertusa*. 1. *In vivo* nitrate reductase activity. Kor. J. Phycol. 4, 63–67.

Karukstis, K.K., 1991. Chlorophyll fluorescence as a physiological probe of the photosynthetic apparatus. In: Scheer, H. (Ed.), Chlorophylls. CRC Press, London, pp. 770–797.

Kim, J.-K., Han, T., 1999. Effects of inorganic nutrients and heavy metals on growth and pigmentation of the green alga, *Ulva pertusa* Kjellman. Kor. J. Environ. Biol. 17, 427–438.

Lee, W.-Y., Wang, W.-X., 2001. Metal accumulation in the green macroalga *Ulva fasciata*: effects of nitrate, ammonia and phosphate. Sci. Total Environ. 278, 11–22.

Lee, K.W., Kang, H.S., Lee, S.H., 1998. Trace metals in the Korean coastal environment. Sci. Total Environ. 214, 11–19.

Lee, T.-M., Huang, Y.-L., Chen, M.-H., 2005. Copper induction of phosphorus deficiency in *Ulva fasciata* (Ulvales, Chlorophyta). Phycologia 44, 620–628.

Lobban, C.S., Harrison, P.J., 1994. Seaweed Ecology and Physiology. Cambridge University Press, Cambridge, p. 366.

Lopes, P.F., Oliveira, M.C., Colepicolo, P., 1997. Diurnal fluctuation of nitrate reductase activity in the marine red alga *Gracilaria tenuistipitata* (Rhodophyta). J. Phycol. 33, 225–231.

Lumsden, B.R., Florence, T.M., 1983. A new algal assay procedure for the determination of the toxicity of copper species in seawater. Environ. Technol. Lett. 4, 271–276.

Maksymiec, W., Baszyński, T., 1996. Different susceptibility of runner bean plants to excess copper as a function of the growth stages of primary leaves. J. Plant Physiol. 149, 217–221.

Maxwell, K., Johnson, G.N., 2000. Chlorophyll fluorescence—a practical guide. J. Exp. Bot. 345, 659–668.

Mazhoudi, S., Chaoui, A., Ghorbal, M.H., Ferjani, E.E., 1997. Response of antioxidant enzymes to excess copper in tomato (*Lycopersicon esculentum*, Mill.). Plant Sci. 127, 129–137.

Nalewajko, C., Olaveson, M.M., 1995. Differential responses of growth, photosynthesis, respiration, and phosphate intake to copper in copper-tolerant and copper-intolerant strains of *Scenedesmus acutus* (Chlorophyceae). Can. J. Bot. 73, 1295–1303.

Nielsen, H.D., Brownlee, C., Coelho, S.M., Brown, M.T., 2003a. Interpopulation differences in inherited copper tolerance involve photosynthetic adaptation and exclusion mechanisms in *Fucus serratus*. New Phytol. 160, 157–165.

Nielsen, H.D., Brown, M.T., Brownlee, C., 2003b. Cellular responses of developing *Fucus serratus* embryos exposed to elevated concentrations of Cu^{2+}. Plant Cell Environ. 26, 1737–1744.

Occhipinti-Ambrogi, A., Savini, D., 2003. Biological invasion as a component of global change in stressed marine ecosystems. Mar. Poll. Bull. 46, 542–551.

Ouzounidou, G., Moustakas, M., Strasser, R.J., 1997. Site of action of copper in the photosynthetic apparatus of maize leaves: kinetic analysis of chlorophyll fluorescence, oxygen evolution, absorbance changes and thermal dissipation as monitored by photoacoustic signals. Aust. J. Plant Physiol. 24, 81–90.

Panda, S.K., Patra, H.K., 1998. Role of nitrate and ammonium ions on chromium toxicity in developing wheat seedlings. Proc. Natl. Acad. Sci. India 70, 75–80.

Panda, S.K., Chaudhury, I., Khan, M.H., 2003. Heavy metals induce lipid peroxidation and affect antioxidants in wheat leaves. Biol. Plantarum. 46, 289–294.

Pätsikkä, E., Aro, E.-M., Tyystjärvi, E., 1998. Increase in the quantum yield of photoinhibition contributes to copper toxicity in vivo. Plant Physiol. 117, 619–627.

Pätsikkä, E., Kairavuo, M., Sersen, F., Aro, E.-M., Tyystjärvi, E., 2002. Excess copper predisposes photosystem II to photoinhibition in vivo by outcompeting iron and causing decrease in leaf chlorophyll. Plant Physiol. 129, 1359–1367.

Pinto, E., Sigaud-Kutner, T.C.S., Leitáo, M.A.S., Okamoto, O.K., Morse, D., Colepicolo, P., 2003. Heavy metal-induced oxidative stress in algae. J. Phycol. 39, 1008–1018.

Prasad, M.N.V., Strzalka, K., 1999. Impact of heavy metals on photosynthesis. In: Prasad, M.N.V., Hagemeyer, J. (Eds.), Heavy Metal Stress in Plants. Springer, Berlin, pp. 117–138.

Rai, P.K., Mallick, N., Rai, L.C., 1994. Effect of Cu and Ni on growth, mineral uptake, photosynthesis and enzyme activities of *Chlorella vulgaris*. Biomed. Environ. Sci. 7, 56–67.

Rama Devi, S., Prasad, M.N.V., 1998. Copper toxicity in *Ceratophyllum demersum* L. (Coontail), a free floating macrophyte: response of antioxidant enzymes and antioxidants. Plant Sci. 138, 157–165.

Reed, R.H., Moffat, L., 1983. Copper toxicity and copper tolerance in *Enteromorpha compressa* (L.) Grev. J. Exp. Mar. Biol. Ecol. 69, 85–103.

Schreiber, U., 2004. Pulse-amplitude-modulation (PAM) fluorometry and saturation pulse method: an overview. In: Papageorgiou, G.C., Govindjee (Eds.), Chlorophyll *a* Fluorescence: A Signature of Photosynthesis. Springer, Amsterdam, pp. 279–319.

Stauber, J.L., Florence, T.M., 1987. Mechanism of toxicity of ionic copper and copper complexes to algae. Mar. Biol. 94, 511–519.

Tripathi, B.N., Mehta, S.K., Gaur, J.P., 2004. Recovery of uptake and assimilation of nitrate in *Scenedesmus* sp. previously exposed to elevated levels of Cu^{2+} and Zn^{2+}. J. Plant Physiol. 161, 543–549.

Winterbourn, C.C., 1982. Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts is a feasible source of hydroxyl radicals *in vivo*. Biochem. J. Lett. 205, 461–463.

Yu, J.-Q., Zhou, Y.-H., Huang, L.-F., Allen, D.J., 2002. Chill-induced inhibition of photosynthesis: genotypic variation within *Cucumis sativus*. Plant Cell Physiol. 43, 1182–1188.

Zhang, X., Ervin, E.H., Schmidt, R.E., 2005. The role of leaf pigment and antioxidant levels in UV-B resistance of dark- and light-green Kentucky bluegrass cultivars. Hortscience 130, 836–841.