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Effect of Ionic Copper Toxicity on the Growth of Green Alga, *Selenastrum capricornutum*

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Abstract The growth of *Selenastrum capricornutum* in culture was investigated as a function of ionic copper concentrations to verify the toxicity and physiological variations. In a Cu^{++} excess culture (E: Cu^{++} of 130 μg/l), the growth rate (*K*) (0.32) was lower than that of the control culture (C: Cu^{++} of 0.065 μg/l) (0.61) after 8 days and the exponential growth rate (R_E) was also found to be lower in culture E (1.1) than culture C (2.9). On the contrary, the *K* of *S. capricornutum* in trace Cu^{++} culture (T_1 and T_2, 0.72) after 6 days tended to be more increased than culture C (0.68). From 8 to 14 days of culture, the amounts of chlorophylls *a* and *b* were increased in culture C (chlorophyll *a*, 106→126 μg/g dry wt; chlorophyll *b*, 158→208 μg/g dry wt), while the amounts of chlorophylls *a* and *b* were decreased in culture E (chlorophyll *a*, 309→235 μg/g dry wt; chlorophyll *b*, 405→352 μg/g dry wt). The amounts of chlorophylls in ionic copper trace culture (T_1 and T_2) [(chl *a/b*) of T_1: 384/620 μg/g dry wt; (chl *a/b*) of T_2: 320/467 μg/g dry wt] were increased more than the culture C (260/387 μg/g dry wt). However, when photosynthetic rates were normalized to the dry weight of algae, the control culture continued to show higher values than the treated culture (T_1). An appropriate amount of ionic copper (T_1: 26 μg/l) stimulated the growth of *S. capricornutum* than the ionic copper content of 13 μg/l (T_2), while the excess amount of ionic copper (130 μg/l) resulted in the highest toxicity to the growth of *S. capricornutum*.

Key words: Ionic copper toxicity, *Selenastrum capricornutum*, chlorophylls *a* & *b*, photosynthetic rate

Recently, the microalgae have been studied for their physiology [20, 32, 37], water pollution effects [13, 40], and medicinal applications [14]. Most studies involving *S. capricornutum* have been focused on growth responses which are due to interest for using the alga as a bioassay tool to detect and quantify environmental and toxicological influences.

S. capricornutum has been studied for toxicity testing by using flow cytometry [8], toxicity to chemical substances [26, 31, 33], nutrient effects [3, 10], photosynthesis [7, 30], the dependency of the trace elements, and their interactions with Cu, Cd, Zn [2, 38] with copper transport [9].

In particular, copper induces the variation of cell size, duration of the lag phase and the motility of phytoplankton, the modification of the thecal plates, starch grains, and lipid droplets in cell, as well as being an essential micronutrient for favorable growth, pigment synthesis, and photosynthesis [15, 39]. The excess amount of copper in culture medium induces deleterious effects on the biochemical compositions [17]. Copper stress affects algal population, their adaptation phenomena, and the photosynthetic electron transport of thylakoids by chlorophyll fluoroscence [4, 21, 36]. Metaxas and Lewis [22] reported that increasing copper concentration stimulated the exponential growth rate of some diatoms, while the physiological conditions of desmid species and *Chlamydomonas reinhardtii* were very sensitive to copper toxicity and harmful to them [12, 43].

Some studies involve fluorescence-based indicators of photosynthesis as well [21, 29]. Although informative, fluorescence measurements by themselves cannot give a full, clear picture of the actual photosynthetic carbon assimilation of organisms. There is also an interest in fast-growing algae such as *S. capricornutum* as potential producers of useful biomolecules such as proteins and lipids [41], but *S. capricornutum* has received little attention in this regard. The potential of *S. capricornutum* in both toxicological and biotechnological usefulness can be better appreciated if we have a fuller understanding of its physiology. In fact, we earlier carried out the first assessments of its photosynthetic performance and major biosynthetic patterns as a function of irradiance, and found that both photosynthetic performance and biosynthetic patterns can be influenced by culture growth conditions such as nutrient supply [6].

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In the present study, we investigated the growth of *S. capricornutum* in culture by changing the Cu^{++} concentrations in order to verify the toxicity and physiological variations. In addition, we also present here some preliminary studies on the effects of copper, an essential micronutrient that is potentially toxic at relatively low concentrations [17, 19].

MATERIALS AND METHODS

Algal Strain and Culture Conditions

S. capricornutum, a bacteria-free culture (UTCC 37) of fresh water, was obtained from the University of Toronto Culture Center and cultivated on the medium of Fraquil [23]. The photoperiod was for 16/8 h (L/D) at 24°C and cultivation took place in a cooling growth chamber with a light intensity of 102 μmol $m^{-1}s^{-1}$. Cu^{++} was in the form of $CuSO_4 \cdot 5H_2O$. Cu^{++} content of the control culture (C) was regarded as the standard quantity (0.065 μg/l: 9.97×10^{-10} M), according to the recipe for Fraquil medium. The copper excess culture (E) was 130 μg/l of Cu^{++} (0.52×10^{-7} M), while the copper trace media were of 26 μg/l (T_1: 0.1×10^{-7} M) and 13 μg/l (T_2: 0.05×10^{-7} M).

Growth Measurement and Dry Weight

The number of cells was counted with a hemocytometer, simultaneously verified with a Coulter Counter (Beckman Coulter, Miami, U.S.A.). The growth rates and exponential rates were calculated by Guillards equations [11]. For dry weight, samples of 50 ml were homogenized, kept in an oven at 70°C for 3 days, and dryed in a desiccator for one day.

Absorption Spectra and Pigment Analysis

The absorption spectra of samples, measured by spectrophotometery (Varian, Victoria, Australia), ranged from 350 to 700 nm. The concentrations of Chlorophylls *a* and *b* were calculated using the Arnons formula [1].

Photosynthesis and Photosynthate Allocation

Photosynthesis *vs* irradiance (P-I) functions were measured by using a light-gradient incubator and ^{14}C [25]. The total activity was approximately 0.5 μCi ml^{-1} of ^{14}C-sodium bicarbonate, the aliquot volume was 5 ml, and the incubation period was 3 h. The light source was a tungsten-halogen lamp. Incubations were terminated by a filtration process through glass fiber filters (nominal pore size: 0.8 μm). Total activities were verified by liquid scintillation counting, and time-zero activities were determined and subtracted from the final activities to make corrections for background activity and adsorption.

Each filter was desiccated and then subjected to the sequential solvent scheme of Smith *et al.* [34] to determine the allocation of photosynthate to low molecular weight compounds (LMW), lipids, polysaccharides, and proteins. The total photosynthetic carbon incorporation was calculated as the sum of the photosynthate classes. In brief, the sequential extraction involved extraction of the desiccated filters in 2:1 chloroform: methanol for approximately 18 h at -20°C and then it was filtered again to retain the particulate material. The filtrate was mixed with 0.88% KCl, and the chloroform phase was (lipids) separated from the methanol-water phase (LMW) by centrifugation. The remaining particulate material was then extracted in 5% trichloroacetic acid for 1 h at 80°C and filtered again. The filtrate contained primarily polysaccharides with smaller amounts of nucleic acids, while the residual particulate material was mainly protein. All fractions were assayed by liquid scintillation counting.

The P-I responses were fitted to the 2-parameter model of Platt *et al.* [29]:

$$P=P_{max}\ A\ (1-e^{-\forall AI/Pmax}) \quad (1)$$

Where P_{max} is the light-saturated maximum rate of photosynthesis, $\forall$ is the initial slope of the P-I curve (the photosynthetic efficiency), and I is the irradiance received during the incubation period. Systat 5.0 [42] was used to fit the functions by iterative nonlinear regression.

RESULTS

Variations of Growth Rates and Dry Weights

In general, the growth rates (K) and exponential growth rates (R_E) of *S. capricornutum* in each culture C and E were decreased as the culture passage increased (Table 1). In the culture E, the K (0.32) was lower than that of the culture C (0.61) after 8 days of culture (Fig. 1). R_E was lower in culture E (1.1) than in C (2.9). On the contrary, the K of *S. capricornutum* in culture T (0.72) after 6 days

Table 1. Growth rates (K) and exponential growth rates (R_E) of *Selenastrum capricornutum* in cultures, according to excess and trace contents of Cu^{++}.

Culture/Time	K_8	K_{14}	K_{16}	R_E
	(K_n=days of culture)			
C^{++} Excess				
C	0.61	0.44	0.40	2.9
E	0.32	0.30	0.24	1.1
C^{++} Trace		K_6		
C_2		0.68		2.9
T_1		0.72		3.3
T_2		0.72		3.6

C & C_1: control cultures (0.065 μg/l of C^{++}, 9.97×10^{-10} M); E: culture of C^{++} excess (130 μg/l of C^{++}, 0.52×10^{-7} M); T_1: culture of C^{++} trace (26 μg/l of C^{++}, 0.1×10^{-7} M); T_2: culture of C^{++} trace (13 μg/l of C^{++}, 0.05×10^{-7} M); growth rate (K), exponential growth rate (R_E): Guillard 1973.

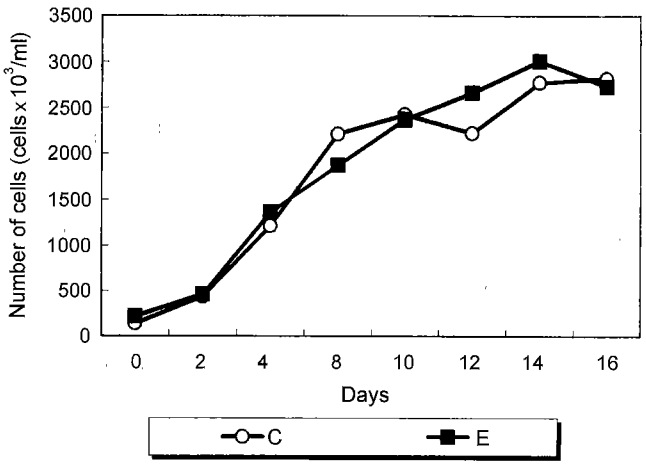

Fig. 1. Evolution of cell growth of *Selenastrum capricornutum* cultured on the control (C: Cu^{++} content of 0.065 μg/l) and excess (E: Cu^{++} content of 130 μg/l) copper in media.

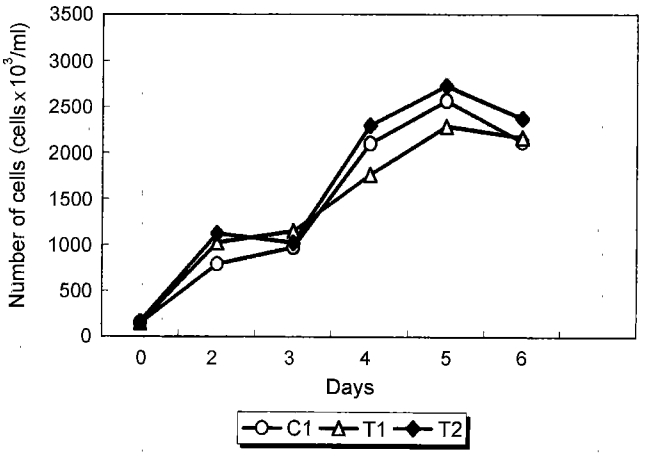

Fig. 2. Evolution of cell growth of *Selenastrum capricornutum* cultured on control culture (C_1) and trace culture (T) of copper: T_1, Cu^{++} content of 26 μg/l; T_2, Cu^{++} content of 13 μg/l).

of culture tended to increase more than culture C_1 (0.68), and the exponential growth rate of *S. capricornutum* seemed to be higher in culture T_2 (3.6) than in culture C_1 (3.0) (Fig. 2). The dry weights of S. *capricornutum* were increased in both cultures C and E (Table 2). On the 8th day of culture, the dry weight of *S. capricornutum* in culture E (23 μg/g fresh wt) was somewhat lower than in culture C (58 μg/g fresh wt), and the dry weight in culture E was lower (88 μg/g fresh wt) than culture C (102 μg/g fresh wt) on 14 days of culture.

Evolution of Pigments

From 8 to 14 days of culture, the amounts of chlorophylls *a* and *b* were increased in culture C (chlorophyll *a*, 106→126 μg/g dry wt; chlorophyll *b*, 158→208 μg/g dry wt), while the amounts of chlorophylls *a* and *b* were decreased in culture E (chlorophyll *a*, 309→235 μg/g dry wt; chlorophyll *b*, 405→352 μg/g dry wt). On the contrary, the amounts of chlorophylls in trace copper cultures (T_1 and T_2) [(chl *a*/*b*) of T_1: 384/620 μg/g dry wt; (chl *a*/ *b*) of T_2: 320/467 μg/g dry wt] were higher than the control culture (C_1) (260/387 μg/g dry wt) (Table 2). It was remarkable that the ratios between chlorophylls *a* and *b* were similar (0.6- 0.8) in all cultures.

The peaks of absorption spectra of *S. capricornutum* were routinely observed at 437, 473, and 674 nm in all cultures, however, the optical densities were highly varied depending on the time and medium of culture.

Photosynthesis and Photosynthate Allocation

Photosynthetic carbon assimilation rates increased with irradiance, reaching a plateau at approximately 500 μmol photons $m^{-2}s^{-1}$ of PAR (Fig. 3). Since there was no evidence of photoinhibition at the higher irradiances, the photosynthetic-light response was fitted into the 2-parameter model (Equation 1) and yielded an R^2 (percent explained variation) >85% for both cultures. The control culture had a P_{max} value of 0.266 mgC l^{-1} h^{-1} (s.e.=0.0059) and an ∀ value of 0.00455 [mgC l^{-1} h^{-1}] [μmol photons m^{-2} s^{-1}]$^{-1}$ (s.e.=0.00013). The treated culture had a P_{max} value of 0.114 mgC l^{-1} h^{-1} (s.e.=0.015) and an ∀ value of 0.000844 [mgC l^{-1} h^{-1}] [μmol photons m^{-2} s^{-1}]$^{-1}$ (s.e.= 0.00001). The ratio of P_{max}: ∀, the light adaptation parameter, was 103.9 μmol photons m^{-2} s^{-1} for the control and 1351.1 μmol photons m^{-2} s^{-1} for the treated culture.

When photosynthetic rates were normalized to the dry weight of algae, the control culture continued to display higher values than the treated culture (T_1). P_{max} was 0.00502 mgC mg dry wt^{-1} h^{-1} in the control and 0.00317 in the treated,

Table 2. Chlorophylls and dry weights of *Selenastrum capricornutum* in cultures, according to excess and trace contents of Cu^{++}.

Cultures (K_n=days of culture)		[Chl *a*/Chl *b*] (μg/g dry wt)		Dry weights (μg/g fresh wt)		
C^{++} Excess	C	E		C	E	
K_8	106/158 (0.7)	309/405 (0.8)		58	23	
K_{14}	126/208 (0.6)	235/352 (0.7)		102	88	
C^{++} Trace	C_1	T_1	T_2	C	T_1	T_2
K_6	260/387 (0.7)	384/620 (0.6)	320/467 (0.7)	52	36	60

Numbers in the parentheses ( ) indicate the ratios between the amounts of chlorophyll *a* and chlorophyll *b*.

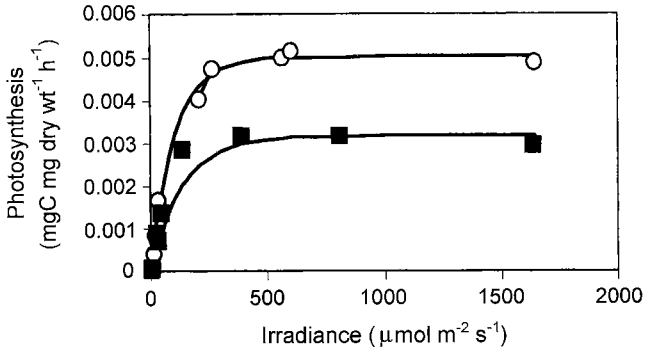

Fig. 3. Biomass-specific photosynthesis *vs* irradiance for the control cultures (○) and the trace cultures (■), with the lines fitted by nonlinear regression.

while ∀ was 0.0000489 [mgC l^{-1} h^{-1}] [μmol photons m^{-2} s^{-1}]$^{-1}$ in the control and 0.0000234 in the treated. The difference in the photosynthetic rates between control and treated cultures was significant at the p=0.05 level according to a t-test, whether or not it was normalized to the dry weight or to the culture volume.

The proportion of labeled photosynthate that was directed to protein was high and independent on irradiance for both cultures, averaging 54.8% for the control and 59.6% for the T_1 culture. The proportion allocated to LMW was low and independent on irradiance in both cultures, averaging 2.4% in the control and 1.4% in the treatment culture. Allocations to lipid and polysaccharide varied with irradiance. Thus, the proportion directed to lipid increased with irradiance, from approximately 10% at the lowest irradiance to more than 20% at the highest irradiance level in both cultures. On the other hand, the proportion allocated to polysaccharide followed the opposite pattern, decreasing from over 30% at the lowest irradiance to about 20% at the highest irradiance level in both cultures (Figs. 4, 5).

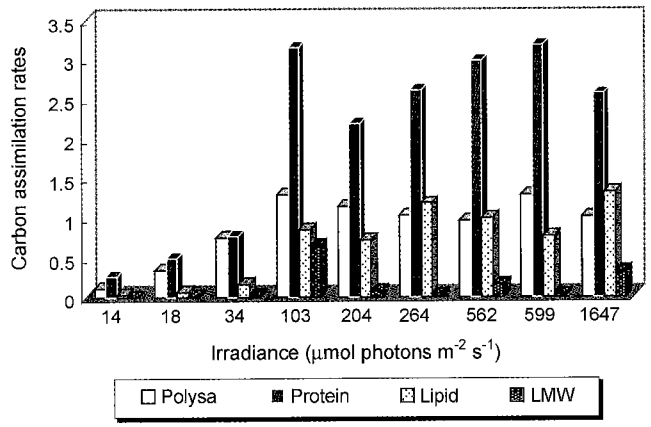

Fig. 4. Allocations of carbon assimilation (^{14}C), using a light-gradient incubator, in *Selenastrum capricornutum* from control culture (C_1).

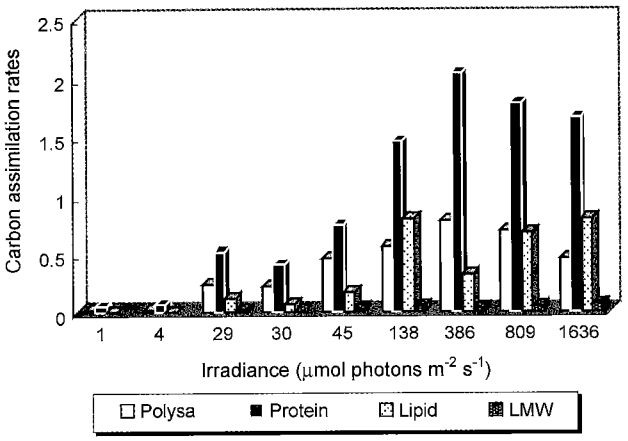

Fig. 5. Allocation of carbon assimilation (^{14}C), using a light-gradient incubation, in *Selenastrum capricornutum* from Cu^{++} trace culture (T_1).

DISCUSSION

The result presented in the present study that the growth rates of *S. capricornutum* in copper-excess medium decreased is in complete agreement with the previous ones for *Asterionella glacialis* and *Chlorella pyrenoidosa* [35]. In these studies, the concentration (130 μg/l) of Cu^{++} excess culture decreased the cell division, as reported by Nielsen and Nielson [24], where Cu^{++} concentration higher than 30 μg/l had a negative influence in that the growth was completely stopped at a concentration of 300 μg/l of Cu^{++}.

Even the concentration of Cu^{++} in the control culture (0.065 μg/l; R_E=2.9) was less sufficient to develop cell division than the trace culture (T_1: R_E=3.3; T_2: R_E=3.6). However, the limiting copper content of culture C may be alleviated and compensated by other metals including trace nutrients (Mn, Zn, Fe, Co, etc.) in the control culture [18].

In culture E, the dry weight of *S. capricornutum* was lower than that of culture C. It is certain that the cell division and other biochemical compositions of *S. capricornutum* might have been decreased due to the toxicity of ionic copper. This result was in accordance with those of Pistocchi *et al.* [28], who showed that the toxicity from excess copper actually inhibited the growth and photosynthesis of phytoplanktons.

In fact, the amount of chlorophylls of this algae was decreased in culture E from 8 to 14 days of culture, while the chlorophyll content of culture T_1 was the highest even in 6 days of culture. What this means is that the photosynthetic activity of *S. capricornutum* in culture T_1 was most favored at the concentration of ionic copper used.

In this study, the most favorable content of copper was 26 μg/l (0.1×10^{-7} M) in T_1, but not 13 μg/l (0.05×10^{-7} M) in T_2, while the highest toxicity occurred at the ionic copper content of 130 μg/l (0.52×10^{-7} M). Then, we could estimate an appropriate amount of ionic copper (26 μg/l)

which stimulated the growth of *S. capricornutum* more than the ionic copper content of 13 μg/l, which was found to be not toxic in the previous result [22].

Therefore, the photosynthesis and photosynthate allocation of *S. capricornutum* between the favorable culture (T_1) with ionic copper amount and the control culture were investigated. Neither of the cultures suffered photoinhibition over the range of irradiances used in this study, which extended up to about 10 times higher than the growth irradiance. This is typical of algae adapting to a moderate irradiance and not suffering from any major impairment of their photosynthetic apparatus. Strongly shade-adapted algae, or those damages suffering from stressors such as UV radiation or excess copper, would be expected to suffer from photoinhibition, as the irradiances were used here [17, 25, 27]. However, the treated culture did display a lower photosynthetic rate per unit volume of culture than the control under both limiting ($\forall$) and saturating (P_{max}) irradiances. The difference could be partly due to the lower biomass of the treated culture, but the difference was still observed when photosynthetic rates were normalized to the algal dry weight. The results thus indicated both a lower photosynthetic efficiency and a lower maximum photosynthetic rate in the cells of the trace Cu^{++} culture. Such an effect would be expected with a suboptimal supply of copper for the photosynthetic apparatus, thereby restricting electron capture in PSII and subsequent electron transfer to PSI and the Calvin cycle [19]. However, additional experiments would be needed to verify this information.

The result on the allocation of photosynthate among macromolecular classes indicated that the algae synthesized primarily protein at all incubation irradiances, and that allocation was independent of the different amounts of copper added in the two cultures. The percentage that was devoted to protein was near the upper limit of the range known for microalgae [6, 34], even at the highest irradiance level. Together with the very low allocation to the LMW class, the high allocation to protein would normally be interpreted to indicate a good physiological condition with respect to the major nutrients, nitrogen and phosphorus [5].

Surprisingly, the algae allocated more photosynthate to lipid, but less to polysaccharide as the incubation irradiance increased. The common pattern among microalgae is to allocate to polysaccharide (specifically, carbohydrates) as the irradiance increases, while allocation to lipid is mostly less variable. The maximum allocation to lipid achieved at the highest irradiance level was well within the commonly-expected range for microalgae, while the allocation at the lowest irradiance level was quite low. *Selenastrum*, grown here at relatively high culture irradiance, most likely had difficulty in synthesizing lipids during the relatively brief assimilation experiments at the lower irradiance level and accumulated polysaccharides by default. At a higher irradiance level, it was able to synthesize lipids more effectively, thereby acheiving a more balanced synthesis of cell constituents [41].

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