

Response to Copper and Sodium Chloride Excess in *Spirulina* sp. (Cyanobacteria)

F. Deniz · S. D. Saygideger · S. Karaman

Received: 18 October 2010 / Accepted: 29 April 2011 / Published online: 11 May 2011
© Springer Science+Business Media, LLC 2011

Abstract Physiological responses of the cyanobacterium, *Spirulina* sp., were evaluated following exposure to copper (0.1 and 1.0 mg/L) and sodium chloride (0.2 and 0.4 mol/L) for 7 days. Growth and chlorophyll *a* content exhibited decreases at most exposure levels, while increases occurred for malondialdehyde at all exposure levels. Proline content was increased at the higher exposure levels. Carotenoid levels of *Spirulina* sp. were not significantly changed. Increased amounts of malondialdehyde were indicative of free radical formation in *Spirulina* sp. under the stress, while increasing levels of proline pointed to the occurrence of a scavenging mechanism. Concentrations of copper in *Spirulina* sp. decreased with increasing concentrations of NaCl.

Keywords Copper · NaCl · Malondialdehyde · Proline · *Spirulina* sp.

Heavy metals are important environmental pollutants and many of them are toxic even at very low concentrations (Tanyolac et al. 2007). Copper (Cu) is a trace element that is an essential component of the biochemical functions of photosynthetic organisms (Kovacik et al. 2008). However, in excessive concentrations this metal becomes powerfully toxic for phytoplankton communities, where it can affect electron transport through photosystem II and modify or

inhibit fundamental enzymatic activities (Pinto et al. 2003). Cu contamination is widespread as a result of mining, smelting, use of Cu in fungicides and other activities. Salinity is also one of the major abiotic stresses affecting plant growth, development and productivity (Rahnama and Ebrahimzadeh 2004). Plants exposed to salt stress undergo changes in their metabolism in order to prevent the deleterious effects of the stress. They have to cope with the lower water potential in their environment, the presence of toxic ions, mainly Na⁺, and the difficulties that salt exerts on the uptake of essential nutrients.

Spirulina sp. is planktonic photosynthetic filamentous cyanobacterium (Vonshak et al. 1982), identified by the main morphological feature of the genus, i.e. the arrangement of multicellular cylindrical trichomes in a helix along the entire length of the filaments. Native people have harvested the biomass of *Spirulina* from Chad Lake (Africa) and Texcoco Lake (Mexico) as a source of food for centuries (Vonshak 1997). *Spirulina* is rich in protein, polyunsaturated fatty acids, pigments, vitamins and phenolics (Richmond 1992). Moreover, the biomass of *Spirulina* has been used to remove unwanted materials such as heavy metals from waste waters (Chojnacka et al. 2004, 2005). *Spirulina* sp. is a suitable model for toxicity evaluations because of its small size, rapid growth, and ease of culture. The main objective of the study was to evaluate the physiological responses of *Spirulina* sp. to copper and sodium chloride individually and in combination.

Materials and Methods

Spirulina sp. was provided from Cukurova University, Faculty of Fisheries (Adana, Turkey) and was cultured in *Spirulina* medium (Schlösser 1982) under aseptic

F. Deniz (✉) · S. Karaman
Department of Biology, Faculty of Arts and Science,
Kahramanmaraş Sutcu Imam University,
46100 Kahramanmaraş, Turkey
e-mail: f_deniz@windowslive.com

S. D. Saygideger
Department of Biology, Faculty of Arts and Sciences,
Gaziantep University, 27310 Gaziantep, Turkey

conditions. The stock and test cultures were maintained at $28 \pm 2^\circ\text{C}$ in a sterile chamber illuminated with 21-W daylight tubes providing a light intensity of $2,000 \pm 200$ lux around the culture vessels following a 16:8-h light/dark regime. A pH of 9.0 was maintained for the appropriate growth of *Spirulina* sp..

To examine the effects of copper and salt on *Spirulina* sp., Cu (II) (as CuCl_2 , 0.1 and 1.0 mg/L), and NaCl (0.2 and 0.4 mol/L) were added separately to the growth medium. The effects of Cu (II) and NaCl combinations (0.1 + 0.2, 0.1 + 0.4, 1.0 + 0.2 and 1.0 + 0.4 mg/L + mol/L) were also examined. The stock solutions were prepared in distilled water. For the control experiment, *Spirulina* sp. was grown under identical culture conditions without adding any Cu (II) and NaCl. The 7-day-old cultures were harvested by filtration through a Millipore membrane filter (0.45 μm) and washed twice with distilled water.

The progressive growth of *Spirulina* sp. was determined over a period of 7 days by measuring absorbance at 560 nm, and by weighing dry mass. The procedures followed for the quantification of chlorophyll *a* (Chl-*a*) and carotenoid were previously described by Lichtenthaler and Wellburn (1985). The malondialdehyde (MDA) content of *Spirulina* sp. was determined by the thiobarbituric acid reaction following the method of Hodges et al. (1999) and it was calculated from the absorbance at 532 nm by an UV–Vis spectrophotometer (GBC, Cintra 202). The measurements were corrected for non-specific turbidity by subtracting the absorbance at 600 nm and the results were expressed as mmol/mL. Proline was determined according to Bates et al. (1973). The cultures suspended in 5 mL of 3% sulfosalicylic acid were centrifuged at 4,000 rpm for 10 min, 2 mL of supernatant and 2 mL of ninhydrin were added in 2 mL glacial acetic acid, and they were incubated at boiling temperature for 1 h. The mixture was extracted with toluene, and proline was quantified spectrophotometrically at 520 nm from the organic phase. The total concentrations of Cu (II) in the extracts of *Spirulina* sp. were analyzed with an atomic absorption spectrometer (Perkin Elmer, AA 400). The instrument was zeroed with a 2% HNO_3 blank. A certified stock solution of $1,005 \pm 2 \mu\text{g}$ Cu (II)/mL (Inorganic Ventures, Lot No Z-CU02080) was used for the calibration of the instrument.

In order to evaluate the results, the all data were analyzed using one-way ANOVA. *Post hoc* comparisons were made with the least significant difference (LSD) test. A *p* value <0.05 was considered significant.

Results and Discussion

In this study, growth of *Spirulina* sp. was significantly affected by Cu (II) and NaCl treatments. The effects of Cu

(II) and NaCl on growth of *Spirulina* sp. are depicted in Fig. 1. Growth decreased gradually with increased metal and salt concentrations, compared with the control. The combined treatment of 1.0 mg/L Cu (II) + 0.4 mol/L NaCl caused maximum reduction in growth. This was visually apparent, as well, by yellowing and fragmentation of filaments. In an earlier study, *Synechocystis aquatilis* showed a decrease in growth with increasing metal ion concentration at initial exposures of 0.01–0.05 mg/L Cu (II) (Shavryina et al. 2001). Reduction in growth of a cyanobacterium, *Scytonema javanicum*, with increasing NaCl concentration (50–200 mM) was determined (Tang et al. 2007). The reduction in growth could be due to inhibition of normal cell division by the metal and salt, as it has been reported for *Padina boeagesenni* exposed to Cu (Mamboya et al. 1999), *Anabaena flos-aquae* exposed to Cu and Cd (Surosz and Palinska 2004) and *Scenedesmus obliquus*

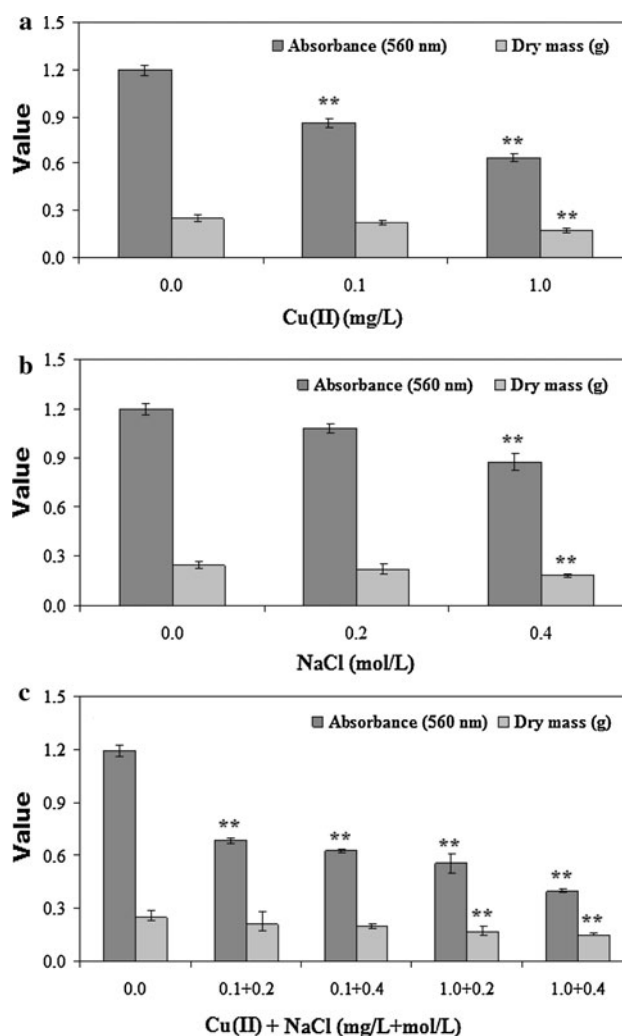


Fig. 1 Effect of Cu (II) (a), NaCl (b) and combinations of Cu (II) and NaCl solution (c) on the growth of *Spirulina* sp. Each data point is the mean $\pm$ SE of three replicates (**p* < 0.05; ***p* < 0.01)

exposed to NaCl (Demetriou et al. 2007). The decrease in the rate of cell division caused by metals is primarily attributed to their binding to sulfhydryl groups which are important for regulating the plant cell division (Visviki and Rachlin 1991). The deleterious effects of excessive salinity on plant growth are associated with nutritional imbalance, effect on specific ions, and a combination of these two factors (Ashraf 1994; Marschner 1995).

The highest concentration of Chl-*a* was found in *Spirulina* sp. treated with the control, and Chl-*a* in *Spirulina* sp. was measured in the control group, with decreased levels occurring at increased concentrations of Cu(II) and NaCl (Table 1). No significant differences were found in the levels of carotenoid. The decrease of the Chl-*a* content is usual in plants that have been exposed to Cu (II) or other metals and salt, and it has previously been observed in cyanobacteria (0.5–6 mg/L Cu (II)) (Surosz and Palinska 2004; Tang et al. 2007). It has been suggested that the content of Chl-*a* is a useful parameter to determine the physiological conditions of plants subjected to heavy metals and salts (Kebede 1997; Chettri et al. 1998). The degradation of pigment is usually the result of oxidation induced by contaminants. In the present study, Cu (II) concentrations over 1 mg/L and NaCl concentrations over 0.4 mol/L were particularly toxic, as the values of the Chl-*a* were significantly different from all the other treatments. Cu (II) and NaCl proved to have a damaging effect on the pigment content of *Spirulina* sp..

The levels of MDA were significantly modified by the Cu (II) and NaCl treatment (Table 1). MDA content increased with increasing metal and salt concentrations in the culture medium. MDA production was highest at the 1.0 mg/L Cu (II) + 0.4 mol/L NaCl treatment, followed by 1.0 mg/L Cu (II) + 0.2 mol/L NaCl, and then 1.0 mg/L Cu (II) alone. The level of MDA is considered as a measure of the degree of lipid peroxidation. Lipid peroxidation is linked to the production of O_2^- . The presence of high

amounts of transitional metals such as Cu (II) favors the enhanced generation of HO from O_2^- through the Fenton reaction (Luna et al. 1994). Thus, the increased levels of MDA suggest that Cu (II) ions stimulated free radical formation in *Spirulina* sp.. Increased MDA levels have also been reported in several higher plants (Luna et al. 1994; Chaoui et al. 1997). The increased production of free radicals on exposure of microorganisms to salt stress has also been reported previously (Price and Hendry 1991; Alia et al. 1993).

Increases in proline content were directly proportional to concentration of Cu (II) and NaCl. The percentage increases of proline, relative to the control value of 27.2 nmol/g, were 2.6 and 18.7% at the 0.1 and 1.0 mg/L Cu (II) treatments, respectively; and 4.4 and 16.9% at the 0.2 and 0.4 mol/L NaCl treatments, respectively. In the combination treatments, proline content increased 8.0, 15.3, 19.3 and 26.3% at the Cu (II) (mg/L) and NaCl (mol/L) combinations of 0.1 + 0.2, 0.1 + 0.4, 1.0 + 0.2, and 1.0 + 0.4, respectively. Increases in proline concentration under heavy metal and salt stresses have been reported in some higher plants and in the cyanobacteria *Spirulina*, *Anabaena* and *Cylindrospermum* (Alia and Pardha Saradhi 1991; Bassi and Sharma 1993; Sultan and Fatma 1999; Jetley et al. 2004; Kumar et al. 2004; Rahn-ama and Ebrahimzadeh 2004; Chris et al. 2006; Choudhary et al. 2007). Increases in proline might be an adaptive mechanism for reducing the level of accumulated NADH and the acidity ($2NADH + 2H^+$). Binding with metal ions due to the chelating ability of proline can also be a defense mechanism for survival. An increase in proline content is indicative of an effort by plant cells to reduce stress caused by metal ions and NaCl.

The concentration of Cu (II) within *Spirulina* sp. increased with increased Cu (II) exposure, and decreased with increased NaCl exposure ($R^2 = 1$) (Fig. 2). The maximum Cu (II) concentration was determined in

Table 1 Values of the physiological parameters (mean $\pm$ SE) measured in *Spirulina* sp. in different concentrations of Cu (II), NaCl and combination of Cu (II) and NaCl solutions

Treatment	Concentration	Chl- <i>a</i> (mg/g)	Carotenoids (mg/g)	MDA (mmol/mL)	Proline (nmol/g)
Control	0.0	10.0 $\pm$ 0.23	4.0 $\pm$ 1.96	29.9 $\pm$ 0.18	27.2 $\pm$ 0.06
Cu (II) (mg/L)	0.1	9.1 $\pm$ 0.06*	3.8 $\pm$ 0.06 ^{ns}	31.8 $\pm$ 0.22**	27.9 $\pm$ 0.64 ^{ns}
	1.0	7.3 $\pm$ 0.33**	3.2 $\pm$ 0.15 ^{ns}	34.4 $\pm$ 0.43**	32.3 $\pm$ 0.55**
NaCl (mol/L)	0.2	9.5 $\pm$ 0.12 ^{ns}	3.7 $\pm$ 0.06 ^{ns}	31.4 $\pm$ 0.34*	28.4 $\pm$ 0.91 ^{ns}
	0.4	8.0 $\pm$ 0.09**	3.4 $\pm$ 0.09 ^{ns}	33.2 $\pm$ 0.61**	31.8 $\pm$ 0.36**
Cu (II) + NaCl (mg/L + mol/L)	0.1 + 0.2	8.2 $\pm$ 0.22**	3.3 $\pm$ 0.09 ^{ns}	32.3 $\pm$ 1.10 ^{ns}	30.1 $\pm$ 0.91 ^{ns}
	0.1 + 0.4	7.6 $\pm$ 0.07**	3.0 $\pm$ 0.03 ^{ns}	33.3 $\pm$ 0.67*	32.1 $\pm$ 0.49*
	1.0 + 0.2	6.9 $\pm$ 0.03**	2.9 $\pm$ 0.09 ^{ns}	35.8 $\pm$ 1.18**	33.3 $\pm$ 2.07**
	1.0 + 0.4	6.5 $\pm$ 0.15**	2.6 $\pm$ 0.09 ^{ns}	36.7 $\pm$ 1.28**	35.2 $\pm$ 0.53**

* $p < 0.05$; ** $p < 0.01$; ^{ns} not significant at significance level 0.05

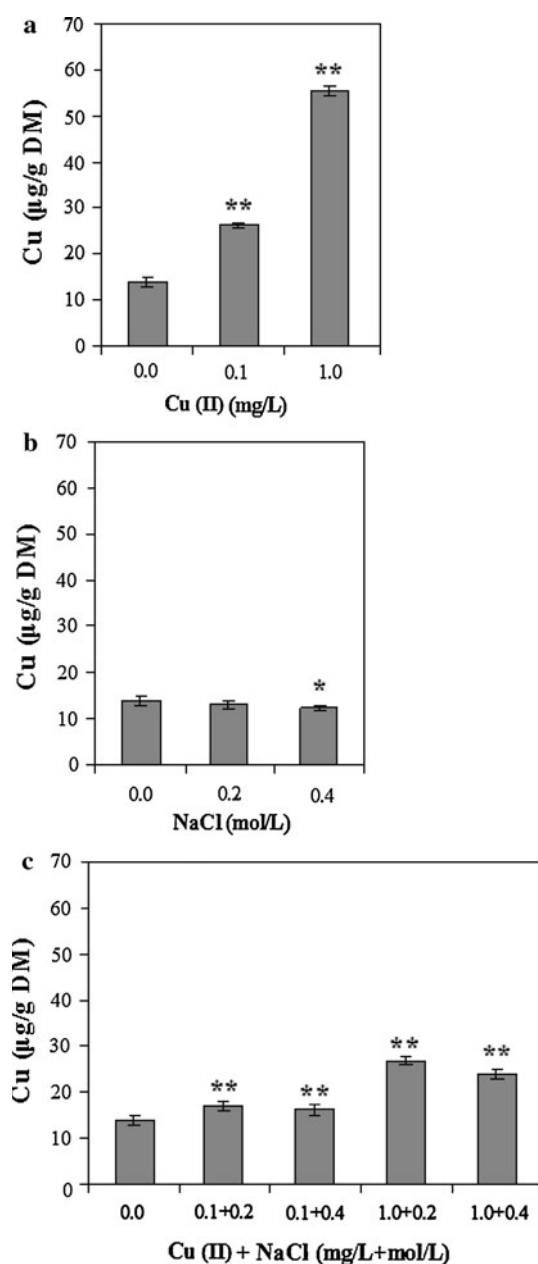


Fig. 2 Effect of Cu (II) (a), NaCl (b) and combinations of Cu (II) and NaCl solution (c) on the copper concentration of *Spirulina* sp.. Each data point is the mean $\pm$ SE of three replicates (* $p < 0.05$; ** $p < 0.01$)

Spirulina sp. treated with 1.0 mg/L Cu (II) solution. Relative to the control, the percentages for Cu (II) uptake were 90.5 and 303.0% following exposure to 0.1 and 1.0 mg/L Cu (II), respectively; and 5.3 and 11.2% following exposure to 0.2 and 0.4 mol/L NaCl, respectively. The uptake percentages for Cu (II) uptake in the combination treatments were 23.3, 16.9, 93.4, and 74.2% following exposure to the combination treatments of 0.1 + 0.2, 0.1 + 0.4, 1.0 + 0.2, and 1.0 + 0.4 mg/L Cu (II) + mol/L NaCl, respectively. Under salt stress, reduced Cu (II) uptake has

been observed in higher plants such as *Triticum aestivum* (El-Enany 2002), but the mechanism for this decreased uptake is still unknown. It may be due to changes in the composition and type of cell wall binding sites, leading to differential absorption and/or cation exchange (Carreras and Pignata 2007). Another reason may be the competitive interaction of metal and salt ions for binding sites on the cell wall.

In conclusion, this study has shown the following: (1) *Spirulina* sp. showed a reduction in growth caused by Cu (II) and NaCl, (2) Chl-*a* content decreased significantly with increasing concentrations of Cu (II) and NaCl, (3) carotenoid levels were not affected by Cu (II) or NaCl, (4) MDA contents of *Spirulina* sp. increased with increasing concentrations of Cu (II) and NaCl, (5) proline, a universal protectant from various stressors, increased in concentration with increasing Cu(II) and NaCl concentrations, and (6) Cu (II) uptake in *Spirulina* sp. was decreased with increased NaCl concentration.

References

- Alia, Pardha Saradhi P (1991) Proline accumulation under heavy metal stress. *J Plant Physiol* 138:554–558
- Alia, Pardha Saradhi P, Mohanty P (1993) Proline in relation to free radical production in seedlings of *Brassica juncea* raised under sodium chloride stress. *Plant Soil* 155(156):497–500
- Ashraf M (1994) Breeding for salinity tolerance in plants. *Crit Rev Plant Sci* 13:17–42
- Bassi R, Sharma SS (1993) Proline accumulation in wheat seedling exposed to zinc and copper. *Phytochemistry* 33:1339–1342
- Bates LS, Waldren RP, Teare D (1973) Rapid determination of free proline for water stress studies. *Plant Soil* 39:205–297
- Carreras HA, Pignata ML (2007) Effects of the heavy metals Cu^{2+} , Ni^{2+} , Pb^{2+} , and Zn^{2+} on some physiological parameters of the lichen *Usnea amblyoclada*. *Ecotox Environ Safe* 67:59–66
- Chaoui A, Mazhoudi S, Ghorbal MH, Ferlani EE (1997) Cadmium and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities in bean (*Phaseolus vulgaris* L.). *Plant Sci* 127:139–147
- Chettri MK, Cook CM, 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
- Chojnacka K, Chojnacka A, Gorecka H (2004) Trace element removal by *Spirulina* sp. from copper smelter and refinery effluents. *Hydrometallurgy* 73:147–153
- Chojnacka K, Chojnacka A, Gorecka H (2005) Biosorption of Cr^{3+} , Cd^{2+} , and Cu^{2+} ions by blue-green algae *Spirulina* sp.: kinetics, equilibrium and the mechanism of the process. *Chemosphere* 59:75–84
- Choudhary M, Jetley UK, Khan MA, Zutshi S, Fatma T (2007) Effect of heavy metal stress on proline, malondialdehyde, and superoxide dismutase activity in the cyanobacterium *Spirulina platensis*-S5. *Ecotox Environ Safe* 66:204–209
- Chris A, Zeeshan M, Abrahama G, Prasad SM (2006) Proline accumulation in *Cylindrospermum* sp. *Environ Exp Bot* 57:154–159

- Demetriou G, Neonaki C, Navakoudis E, Kotzabasis K (2007) Salt stress impact on the molecular structure and function of the photosynthetic apparatus. The protective role of polyamines. *BBA-Bioenergetics* 1767:272–280
- El-Enany AE (2002) Wheat seedlings response to interactive effects of Cu and salinity. *Developments in plant and soil sciences. Plant nutrition*. Springer, Netherlands, pp 424–425
- Hodges DM, Delong JM, Forney CF, Frange RK (1999) Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. *Planta* 207:604–611
- Jetley UK, Choudhary M, Fatma T (2004) Evaluation of biochemical productivity cyanobacterium *Spirulina platensis*-S5. under heavy metal stress. *Asian J Chem* 16:1524–1528
- Kebede E (1997) Response of *Spirulina platensis* (= *Arthrospira fusiformis*) from Lake Chitu, Ethiopia, to salinity stress from sodium salts. *J Appl Phycol* 9:551–558
- Kovacik J, Backor M, Kadukova J (2008) Physiological responses of *Matricaria chamomilla* to cadmium and copper excess. *Environ Toxicol* 23:123–130
- Kumar S, Jetley UK, Fatma T (2004) Tolerance of *Spirulina platensis*-S5 and *Anabaena* sp. to endosulfan an organochlorine pesticide. *Ann Plant Physiol* 18:103–107
- Lichtenthaler HK, Wellburn AR (1985) Determination of total carotenoids and chlorophylls-a and b of leaf in different solvents. *Biochem Soc Trans* 603:591–592
- Luna CM, Gonzalez VS, Trippi VS (1994) Oxidative damage caused by excess copper in oat leaves. *Plant Cell Physiol* 35:11–15
- Mamboya FA, Pratap HB, Mtolera M, Bjork M (1999) The effect of copper on the daily growth rate and photosynthetic efficiency of the brown macroalgae *Padina boergesenii*. In: Richmond MD, Francis J (eds) *Proceedings of the conference on advances on marine sciences, Tanzania*, pp 185–192
- Marschner H (1995) *Mineral nutrition of higher plants*. Academic Press, London
- Pinto E, Sigaud-Kutner TCS, Leitao MAS, Okamoto OK, Morse D, Colepicolo P (2003) Heavy metal-induced oxidative stress in algae. *J Phycol* 39:1008–1018
- Price AH, Hendry GA (1991) Iron-catalyzed oxygen formation and its possible contribution to drought damage in nine native grasses and three cereals. *Plant Cell Environ* 14:477–484
- Rahnama H, Ebrahimzadeh H (2004) The effect of NaCl on proline accumulation in potato seedlings and cali. *Acta Physiol Plant* 26:263–270
- Richmond A (1992) *Spirulina*. In: Borowitzka MA, Borowitzka LJ (eds) *Microalgal biotechnology*. Cambridge University Press, London
- Schlösser UG (1982) Sammlung von Algenkulturen. *Ber Deutsch Bot Ges* 95:181–276
- Shavyrina OB, Gapochka LD, Azovskii AI (2001) Development of tolerance for copper in cyanobacteria repeatedly exposed to its toxic effect. *Biol Bull* 28:183–187
- Sultan S, Fatma T (1999) Phytotoxicity of heavy metals on *Spirulina platensis*. *Phykos* 38:87–92
- Surosz W, Palinska KA (2004) Effects of heavy metal stress on cyanobacterium *Anabaena flos-aquae*. *Arch Environ Contam Toxicol* 48:40–48
- Tang D, Shi S, Li D, Hu C, Liu Y (2007) Physiological and biochemical responses of *Scytonema javanicum* (cyanobacterium) to salt stress. *J Arid Environ* 71:312–320
- Tanyolac D, Ekmekci Y, Unalan S (2007) Changes in photochemical and antioxidant enzyme activities in maize (*Zea mays* L.) leaves exposed to excess copper. *Chemosphere* 67:89–98
- Visviki I, Rachlin JW (1991) The toxic action and interactions of copper and cadmium to the marine alga *Dunaliella minuta* in both acute the chronic exposure. *Arch Environ Contam Toxicol* 2:271–275
- Vonshak A (1997) *Spirulina platensis* (Arthrospira): physiology, cell-biology and biotechnology. Taylor and Francis, London
- Vonshak A, Abeliovich A, Boussiba S, Arad S, Richmond A (1982) Production of *Spirulina* biomass: effects of environmental factors and population density. *Biomass* 25:341–349