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TOXIC EFFECT OF COPPER ON GROWTH, PIGMENTS, PROTEINS AND SOME ENZYMES OF NITROGEN ASSIMILATION IN THE DIAZOTROPHIC CYANOBACTERIUM *ANABAENA VARIABILIS*

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Abstract

The influence of Cu^{2+} toxicity on dry mass, chl.a, carotenoids, protein content, Cu^{2+} uptake, activities of some enzymes of nitrogen assimilation and alkaline phosphatase in the diazotrophic cyanobacterium *Anabaena variabilis* was evaluated. Lower Cu^{2+}concentrations stimulated the dry weight, chl.a, and protein content, whereas, higher concentrations were inhibitory. In contrast, a prominent increase in the carotenoids content was detected in response to all Cu^{2+} treatments. *A. variabilis* showed high uptake capacity of Cu^{2+}. The accumulation capacity is directly proportional to the external Cu^{2+} concentrations. A stimulation in the activities of glutamine synthetase (GS), NADH-glutamate synthetase (GOGAT), nitrate reductase (NR), nitrogenase and alkaline phosphatase (AP) in response to low Cu^{2+} concentrations was noted. However, higher ones inhibited the enzymes activities with different degrees. The enzymes could be arranged according to their tolerance to Cu^{2+} toxicity in the following order: AP > NR > GOGAT > GS > nitrogenase.

Keywords**:** *Anabaena variabilis*, Copper, Glutamine synthetase, Glutamate synthetase, Nitrate reductase, nitrogenase, Alkaline phosphatase.

Introduction

Metals are important pollutants in the aquatic environment. Metal contamination occurs as a result of human activities and affects organisms at the biochemical, cellular, community and population level (Bajguz, 2000). Microalgae are the first organisms affected by heavy metals discharged in aquatic environments (Cid *et al*., (1996) because they are directly in contact with the medium separated only by the cytoplasmic membrane and the cell wall. High concentrations of metals are known to disrupt algal metabolism either by inactivating the photosynthetic machinery, enzymatic pathways or by altering the nutrient transport and availability (Rai *et al*., 1998).

Cyanobacteria are phylogenetically the oldest group of oxygen evolving photosynthetic prokaryotes occupying an important place in both aquatic and terrestrial ecosystems (Dohler, 1986) because of their ability to utilize atmospheric nitrogen. Therefore, any threat to their existence will bring about an imbalance in the nitrogen status of entire ecosystems.

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Copper is an essential micronutrient for growth, metabolism and enzyme activities of various algae and cyanobacteria, it is a component of thylakoidal plastocyanin (Cavet *et al*., 2003). However, it also a proven inhibitor of algal growth at high concentrations (Abdel-Monem *et al*., 1998; Bajguz, 2000; Franklin *et al*., 2000; Einicker-Lamas *et al*., 2002 and Bossuyt and Janssen , 2004).

In this contribution, we report the inhibitory effect of copper in terms of growth, pigments, protein, metal uptake, and some enzymes of nitrogen assimilatiom as nitrate reductase (NR), glutamine synthetase (GS), NADH-glutamate synthetase (GOGAT), nitrogenase as well as alkaline phosphatase (AP) in the diazotrophic cyanobacterium *Anabaena variabilis*.

Materials and Methods

Anabaena variabilis (Kütz.) (obtained from Prof. Kleiner, Mikrobiologie Institut, Universität Bayreuth, Germany) was grown axenically in batch cultures in the nutrient medium recommended by Allen and Stanier (1968) at 26$\pm$1°C, pH 7.5, under 75 µ mol m^{-2} s^{-1} PAR photon flux . The cultures were aerated with a mixture of 97% air and 3% CO_2. Stock solution of $CuSO_4.5H_2O$ was prepared in glass-distilled water and sterilized by passing through Millipore membrane filter (0.22µm).

The dry weight was determined according to the method described by Ahmed and Osman (1973). The contents of chlorophyll a and carotenoids were estimated by the methods adopted by Jeffrey and Humphrey (1975) and Jensen and Liaaen (1959), respectively. Protein content was estimated by the method described by Lowry *et al*. (1951). Copper uptake was estimated using a Perkin Elmer 2380 atomic absorption spectrophotometer. The residual Cu^{2+} was determined in the cell free medium (after centrifugation at 5000 rpm for 5 min) after acidification to pH $\leq$ 2, according to Ting *et al*. (1989).

The exponentially growing cultures were centrifuged, the cells washed two times with cold imidazol buffer (20mM, pH 7.6), resuspended in 0.8 ml imidazol buffer, sonnicated in ice path four times (20 sec. Each), centrifuged for 2 min. and the supernatant was used as raw extract for estimation of enzymatic activities. Glutamine synthetase activity (EC.6.3.1.2) was assayed by the method described by Kohlhaw *et al*. (1965). NADH- glutamate synthetase (E.C.1.4.1.13) activity was estimated using the method of Meers *et al*. (1970). Nitrate reductase (E.C.1.6.6.1) activity was determined according to Camm and Stein (1974).

In vivo nitrogenase activity of *A. variabilis* was measured by estimating the reduction of acetylene to ethylene using gas chromatograph (Dani 1000) (Hardy *et al*., 1973). Alkaline phosphatase activity in cells exposed to different Cu^{2+} concentrations was monitored by the method of Tabatabai and Bremner (1969).

Results and Discussion

Some metals play indispensable roles in cell growth and maintenance of metabolic functions, but when their concentrations in the environment increase above a threshold, many cellular changes can be detected as a response to the stress provoked. Results obtained indicated that low concentrations of Cu^{2+} (25 and 50 µM) stimulated the dry biomass of *Anabaena variabilis* by 15 and 5%, respectively. The increase in metal concentrations was accompanied by a gradual decline in the dry yield (Table 1). In this connection, Bossuyt and Janssen (2004) reported that the optimal range of Cu^{2+} was 1 - 35 µg l^{-1} for *Pseudokirchneriella subcapitata* biomass, growth rate and pigment diversity. Yan *et al*. (2001) found that safety concentration of Cu^{2+}, for *Chlorella pyrenoidosa*'s growth was 31.8 µg /L, and 96 h-EC50 was 67.3 µg /L. Whereas, Cu^{2+} concentrations required to inhibit growth rate of *Chlorella sp*. by 50% (72-h EC_{50}) was 1.5 µg l^{-1} (Franklin *et al*., 2000), denoting that the tolerance of algae to copper toxicity was species dependent. Inhibition of algal growth by copper was reported in *Anabaena doliolum* (Rai *et al*. 1991), *Nostoc calcicola* (Verma *et al*., 1993), *Nostoc muscorum* (Pandy and Chatterjee, 1999), *Chlorella vulgaris* (Bajguz, 2000), *Scenedesmus acutus* (Abdel-Monem *et al*., 1998), *Euglena gracilis* (Einicker-Lamas *et al*., 2002) *Phaeodactylum tricornutum* (Cid *et al*., 1996), diatoms (Pistocchi, 2000). Visviki and Rachlin (1991) attributed the depressed growth in *Dunaliella minuta* by copper to its binding to the sulfhydryl groups which are important in regulating cell division since Cu^{2+} possessed higher affinity for sulfur complexation than other metals. On the other hand, growth induction by lower heavy metals concentrations may be attributed to the ability of heavy metals to prevent the export of metabolites into the bathing medium (De Filippis *et al*., 1981) or to their ability to substitute Zn in some metalloenzymes in vitro and in vivo (Price and Morel 1990).

A concentration of 25 µM Cu^{2+} stimulated the chl.a content by 7% above the control value. Thereafter, the increase in metal concentration was accompanied by successive reduction in chl. a content. The drop was highly remarkable at the higher doses. Stimulation in chlorophyll biosynthesis at lower Cu^{2+} doses was reported by Fisher and Jones (1981) in *Asterionella japonica*. On the other hand, reduction in chl.a biosynthesis by Cu^{2+} was reported in *Anabaena doliolum* (Tripathi *et al*., 2003), *Nostoc muscorum* (Pandy and Chatterjee, 1999) in *Oocystis nephrocytioides* (Soldo *et al*., 2005) and in *Chlorella vulgaris* (Lam *et al*., 1999). The reduction in chlorophyll biosynthesis was attributed to an inhibition in the reductive steps in chlorophyll biosynthetic pathway by heavy metals (De- Filippis *et al*., 1981). Consequently, Cu^{2+} inhibited the gross photosynthesis rate in *Enteromorpha flexuosa* (Andrade *et al*., 2004). Also, Cu^{2+} inhibited photosynthetic carbon fixation, O_2 evolution, chlorophyll fluorescence in *Chlorella vulgaris* (Singh *et al*., 2004) and in cyanobacteria (Osman *et al*., 1996).

Table 1: Effect of different Cu^{2+} concentrations on dry biomass, chl.a , carotenoids, protein content and percentage of Cu^{2+} uptake by *Anabaena variabilis* after eight days of growth. (Mean of 3 replicates ± S.E.)

Cu^{2+} Conc. (µM)	Dry biomass (mg/50ml)	Chl. a (µg/ml)	Carotenoids (µg/ml)	Protein content % control	% of Cu^{2+} uptake
Cont.	65 ± 1.3	0.68 ± 0.01	0.88 ± 0.03	100	-
25	74 ± 2.2	0.73 ± 0.02	0.97 ± 0.04	113 ± 3.4	33 ± 1.9
50	68 ± 1.7	0.65 ± 0.01	0.99 ± 0.05	120 ± 4.8	45 ±1.4
100	46 ± 0.9	0.44 ± 0.01	1.01 ± 0.03	108 ± 4.3	53 ± 2.7
125	31 ± 0.9	0.31 ± 0.01	1.03 ± 0.02	82 ± 4.1	65 ± 2
150	17 ± 0.4	0.17 ± 0.01	1.06 ± 0.03	62 ± 1.9	78 ± 3.1

All values are significant at $p > 0.001$ for dry biomass, carotenoids, $p > 0.01$ for chl. a and $p > 0.0001$ for protein and % of Cu uptake.

On the other hand, a prominent rise in carotenoids content was noticed at all Cu^{2+} concentrations used, specially at higher ones (Table 1). In the plant system, carotenoids play a significant role in scavenging $^1O_2$. The significant rise in carotenoid content under copper stress might offer protection to the chlorophyll and photosynthetic membrane from photooxidative damage (Mallick and Rai 1999). This result can be interpreted in the sense that, *A. variabilis* responded to Cu^{2+} toxicity by inductions of several antioxidants, including diverse enzymes and the synthesis of low molecular weight compounds such as carotenoids and glutathione (Pinto *et al*., 2003). In this regard, Rai *et al*. (1991) reported that Cu^{2+} increased carotenoids biosynthesis compared to Chl.a in *Anabaena doliolum*. Also, Chlorophyll *a* and carotenoid contents increased in *Pseudokirchneriella subcapitata* exposed to 1 and 100 µg Cu l^{-1} (Bossuyt and Janssen 2004). A concentration dependent increase in lipid peroxidation, carotenoid and activity of superoxide dismutase was observed in the green microalga *Chlorella vulgaris* following copper exposure (Mallick, 2004). However, Elevated concentrations of Cu^{2+} and Zn^{2+} inhibited growth, protein, chlorophyll a, and carotenoid contents in *Anabaena doliolum* (Tripathi *et al*. 2003).

As indicated in table 1, low Cu^{2+} concentrations (25, 50, 100 µg Cu l^{-1}) stimulated significantly the protein content of *A. variabilis* by 13, 20 and 8%, respectively. Similar increment in protein was reported in *Nostoc muscorum* and *Calothrix fusca* by El-Naggar *et al*. (1999) in response to cobalt and lead. On the other hand, higher Cu^{2+} concentrations (125 and 150 µg) inhibited the protein content by 18 and 38%, respectively. The reduction in the total soluble proteins by Cu^{2+} was observed earlier in *Anabaena doliolum* (Tripathi *et al*., 2003). It could be suggested that accumulation of protein at low heavy metal concentrations may be one of the ways through which the algae can abolish their

toxic effects through the accumulation of metallothionine (high sulfhydryl containing proteins of low molecular weights). The cyanobacterial metallothionine was found to be induced in the presence of heavy metals salts with regulation at the level of transcription (Olafson, 1986) and it has been indicated in *Synechococcus*, it was found to be similar to eukaryotic metallothioneins (Olafson *et al*., 1988). A second interpretation for protein increment is the increase in respiration leading to the utilization of carbohydrate in favor of protein accumulation. Such interpretation has been supported by the results obtained by El-Naggar (1993), who observed that low concentrations of Cu^{2+} stimulated the respiration of *Chlorella vulgaris* and *Scenedesmus bijuga*. On the other hand, inhibition of protein accumulation induced by higher concentrations of heavy metals may be attributed to the toxic action of these heavy metals on the enzymatic reactions responsible for protein biosynthesis (Kobbia *et al.*, 1985; Rai *et al.*, 1991; El-Naggar, 1993; Nassar, 2000).

Results show that the percentage of Cu^{2+} uptake increased as its concentration increased in the culture media (Table, 1). Positive linear relationship between heavy metals uptake and its concentration in the media was detected for Cu^{2+} in *Phormidium* (Wang *et al.*, 1998), *Oscillatoria anguistissima* (Ahuja *et al.*, 1997) and *Oocystis nephrocytioides* (Soldo *et al.*, 2005); for Ni^{2+} in *Scenedesmus acutus* (Xiaoleijin *et al.*, 1996); for Co^{2+} and Zn^{2+} in *Chlorella salina* (Garnham *et al.*, 1992).

The high uptake capacity of *A. variabilis* (78 % of the added Cu^{2+}) which was detected in culture treated with 150µM suggest an increase in cell permeability and also supports the damage of biomembanes caused by Cu^{2+} (Rai *et al.*, 1998). The accumulation of Cu^{2+} by *A. variabilis* may be due to the binding of Cu^{2+} to active binding sites on the cell wall (Cho *et al.*, 1994; Kretschmer *et al.*, 2004), binding to intracellular large molecules as polyphosphate bodies (Hashemi *et al.*, 1994; Kretschmer *et al.*, 2004), proteinaceous metallothioneins (Skowronski *et al.*, 1998), extracellular ligands (Soldo *et al.*, 2005) or sequestered within vacuoles (Einicker-Lamas *et al.*, 2002; Andrade *et al.*, 2004).

Copper is one of the most powerful phytochelatin synthesis activators (Rauser, 1995). These metal binding peptides have been found to be formed by Glu, Cys and Gly in a 3.2 : 2.7 : 1 ratio (Howe and Merchant 1992), denoting the importance of these amino acids in the tolerance to metals. In *A. variabilis*, the effect of Cu^{2+} on some enzyme activities of Glu biosynthesis (NR, GS and NADH-GOGAT) has been studied.

The reduction of nitrate to ammonium is catalysed by the successive action of nitrate reductase and nitrite reductase (Devriese *et al.*, 2001). Nitrate reductase was hardly affected by the sublethal Cu^{2+} concentrations used, the activity was stimulated by 12 and 5% in cultures treated with 25 and 50µM Cu^{2+}, respectively. High Cu^{2+} concentrations (125 and 150µM) inhibited the enzyme activity by 10 and 25 %, respectively (Table 2). The observed reduction in NR

activity at high Cu^{2+} concentrations may be referred to that Cu^{2+} inhibited the nitrate uptake at higher doses as reported in *Chlamydomonas* by Devriese *et al.* (2001). Furthermore, metals were found to inactivate nitrate reductase through altering its structure (non- competitive inhibition) (Rai *et al.*, 1998). In this regard, Cu^{2+} inhibited NR and NO3- uptake in a concentration-dependent manner, with the latter process being inhibited more strongly than the former in *Scenedesmus* species (Tripathi *et al.*, 2004).

Table 2: Effect of different Cu^{2+} concentrations on the activities of nitrate reductase, glutamate synthetase, glutamine synthetase, nitrogenase and alkaline phosphatase of *Anabaena variabilis* after eight days of growth. (Mean of 3 replicates ± S.D)

Cu^{2+} Conc. (µM)	Nitrate reductase (NR) µmol mg^{-1} protein h^{-1}	Glutamate synthetase (GOGAT) µg mg^{-1} protein h^{-1}	Glutamine synthetase (GS) µg mg^{-1} protein h^{-1}	Nitrogenase µmolC_2H_4 mg^{-1} dwt h^{-1}	Alkaline phosphatase (AP) µmol mg^{-1} protein h^{-1}
Cont.	**1.8 ± 0.05**	**6.8 ± 0.07**	**3.4 ± 0.07**	**7.8 ± 0.16**	**7.2 ± 0.07**
25	**2 ± 0.1**	**8.2 ± 0.2**	**3.9 ± 0.1**	**8.2 ± 0.08**	**7.8 ± 0.16**
50	**1.9 ± 0.1**	**7.7 ± 0.2**	**3.5 ± 0.1**	**7.9 ± 0.24**	**8.1 ± 0.08**
100	**1.7 ± 0.09**	**7.1 ± 0.1**	**2.9 ± 0.06**	**7.0 ± 0.3**	**9.1 ± 0.09**
125	**1.6 ± 0.05**	**6.1 ± 0.12**	**2.04± 0.02**	**5.1 ± 0.05**	**6.8 ± 0.2**
150	**1.4 ± 0.6**	**4.8 ± 0.14**	**1.2 ± 0.02**	**2.0 ± 0.04**	**6.5 ± 0.13**

All values are significant at p> 0.001 for NR, GOGAT, nitrogenase, p > 0.01 for GS and AP

The incorporation of ammonium into carbon skeletons, as α-amino group of L-glutamate is catalysed by the glutamine synthetase (GS)- glutamate synthetase (GOGAT) cycle (Vega *et al.*, 1991). Cu^{2+}concentrations up to 100µM caused significant rise in NADH-glutamate synthetase activity, while higher doses inhibited the enzyme activity by 10 and 30%, respectively. Devriese *et al.* (2001) reported also that NADH-glutamate synthetase activity was not affected by Cd^{2+} up to 100µM in *Chlamydomonas reinhardtii.*

Adverse environmental conditions like heavy metals stress affect N assimilation in plants and algae and lead to either an increase or decrease in glutamine synthetase (GS) activity depending on the species (Kumar and Dubey, 1999). GS was more sensitive to Cu^{2+} toxicity showing higher and significant inhibition (40 and 65%) in response to higher Cu^{2+} doses (125 and 150µM), respectively. However, 25 µM Cu^{2+}stimulated the enzyme activity by 14% above the control value. LD_{50} of Cu^{2+} caused 37% inhibition in GS activity in *Anabaena doliolum* (Rai *et al.*, 1998) denoting that the sensitivity of GS to Cu^{2+} toxicity is

species dependent. These results could be compared with those previously described for *Chlamydomonas reindardtii*, where nitrite reductase was scarcely inhibited, NADH-glutamate synthetase was not affected, whereas, glutamine synthetase decreased in response to heavy metals stress (Devriese *et al.*, 2001).

Nitrogenase, the key enzyme for atmospheric nitrogen fixation in diazotrophic cyanobacteria, showed higher sensitivity to copper toxicity. Although, 25µM Cu^{2+} slightly stimulated the enzyme activity by 6%, further increase in metal concentrations was accompanied by successive reductions in nitrogenase activity which reached 35 and 74% in cultures treated with 125 and 150µM, respectively. Inhibition of nitrogenase activity by Cu^{2+} was reported earlier in *Anabaena doliolum* (Rai *et al.*, 1998), *Nostoc muscorum* (Pandy and Chatterjee, 1999) and in *Nostoc calcicola* (Singh *et al.*, 1987). The reduced nitrogenase activity may be due to that Cu^{2+} like other metals reduced heterocyst frequency or disturbed its plasma membrane permeability (Rai *et al.*, 1990; Trehan and Maneesha, 1991, 1994), and limited supply of photosynthetically generated ATP (Rai and Tiwari, 1999).

Generally, the inhibition in the activities of many enzymes with copper and other heavy metals has been attributed to the inactivation of enzymic proteins by the interactions of heavy metals with functional-SH groups present on enzymes (Kumar and Dubey, 1999).

Alkaline phosphatase activity has been considered an ideal marker to assess the phosphorus availability and morphological reaction in cyanobacteria (Verma *et al.*, 1993). Results show that the activity of intracellular alkaline phosphatase (AP) stimulated significantly by 8, 12 and 27% in cultures treated with 25, 50, 100 µM Cu^{2+}, respectively, whereas, higher concentrations (125 and 150µM) inhibited the enzyme activity by 6 and 10%, respectively (Table 2).

Algae store Pi intracellurarly as polyphosphate, the synthesis of polyphosphate bodies increased in response to Cu^{2+} toxicity as mechanism of tolerance for sequestering metals (Hashemi *et al.*, 1994; Kretschmer *et al.*, 2004). At the same time, Cu^{2+} caused phosphate depletion as reported in *Anabaena doliolum* (Tripathi *et al.*, 2003). So, the marked stimulation in AP activity in response to Cu^{2+} could be interpreted in the sense that the enzyme was activated for the hydrolysis of polyphosphate bodies to meet Pi requirements (Verma *et al.*, 1993 and Lee, 2000). Such interpretation could be supported by the findings of Macro and Orus (1988) who reported that several intracellular phosphatases have a role in the hydrolysis of intracellular polyphosphates. Furthermore, P-deficiency was found to increase the activity of intracellular phosphatases in microalgae (Lee, 2000). In accordance with our results, Alvarez and Jerez (2004) reported that *A. ferrooxidans* cells exposed to high copper concentrations showed a rapid decrease in polyphosphate levels with a concomitant increase in exopolyphosphatase activity and a stimulation of phosphate efflux. Their results revealed that heavy metals stimulate polyphosphate hydrolysis and the metal-

phosphate complexes formed are transported out of the cell as part of a possibly functional heavy metal tolerance mechanism.

On the other hand, the inhibition of AP activity in response to higher concentrations of Cu^{2+} points towards an indirect involvement of Cu^{2+} through cellular depletion of energy essential to drive enzyme activity rather than direct competition between metal and enzyme as suggested by Verma *et al.*, (1993). In this connection, Rai *et al.*, (1998) reported that LD_{50} Cu^{2+} inhibited the AP activity in *Anabaena doliolum.*

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التأثير السام للنحاس على النمو، الأصباغ، البروتين وبعض إنزيمات تمثيل النيتروجين في السيانوبكتيريا المثبتة للنيتروجين أنابينا فاريابيليس

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أجرى هذا البحث لدراسة التأثيرالسام للنحاس على الوزن الجاف وكلوروفيل أ والكاروتينات ومحتوى البروتين وكذلك تراكم النحاس ونشاطات بعض الإنزيمات المسئولة عن تثبيت النيتروجين وإنزيم الفوسفاتيز القاعدي في السيانوبكتيريا المثبتة للنيتروجين أنابينا فاريابيلي .

أدت المعاملة بالتركيزات المنخفضة إلى زيادة كل من الوزن الجاف وكلوروفيل أ ومحتوى البروتين بينما كانت التركيزات العالية مثبطة . على العكس من ذلك لوحظت زيادة واضحة في محتوى الكاروتينات استجابة لكل تركيزات النحاس المستخدمة . أظهرت أنابينا فاريابيليس قدرة عالية لتراكم النحاس وتناسب هذا التراكم طرديا مع تركيزات النحاس المضافة .

وقد أظهرت الدراسة زيادة في نشاطات إنزيمات الجلوتامين سينثيتيز، الجلوتامات سيينثيتيز، النيترات ريداكتيز، النيتروجينيز والفوسفاتيز القاعدي استجابة للتركيزات المنخفضة من النحاس بينما أدت التركيزات العالية إلى تثبيط نشاطات الإنزيمات بدرجات متفاوتة ويمكن ترتيب الإنزيمات تبعا لقدرتها على تحمل سمية النحاس بالترتيب الآتي :-

الفوسفاتيز القاعدي > نيترات ريداكتيز > جلوتامات سينثيتيز > جلوتامين سينثيتيز > النيتروجينيز.