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Effects of Various Copper Forms on the Freshwater Alga, *Scenedesmus quadricauda* (TURP.) BRÉB. Strain Greifswald 15

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Algae are internationally accepted organisms that are used to evaluate the potential hazard of chemicals on aquatic ecosystems (Klaine and Lewis 1995; OECD 2002). Anthropogenic influxes of copper into lake waters are common in both industrial and rural regions. It can move through the hydrosphere, through rivers, groundwater, dry dust fall, and by precipitation (Drever 1997). The prevalence of Cu in industrial activities, coupled with its mobility and toxicity, contributes to the problem of Cu contamination. Cu is an essential nutrient to nearly all forms of life, from fish to nanoplankton. However, seemingly minute Cu concentrations can be fatal to some species of aquatic life (Bruland et al. 1991). There is a small range of Cu concentrations that are neither nutrient limiting nor toxic. Under Cu stress, some biota produce Cu-binding proteins and ligands, and in doing so, reduce its toxicity (Moffett and Brand 1996). With regards to water quality, species diversity and abundance, it is important to study the sources and sinks of Cu and the processes that determine Cu availability and toxicity in different concentrations. A number of studies have been conducted and show clearly that the toxicity of the copper ion in water media is dependent on its activity (or „free" concentration) rather than on the total level of copper present in the solution (Lüderitz et al. 1989).

Copper occurs in natural waters primarily as Cu(II) predominantly in complexed form. Free Cu may be present, but is generally a minor species (Stumm and Morgan 1981). Copper reacts with both inorganic and organic chemicals in solution and in suspension, resulting in a multitude of chemical forms. Because the cupric ion is highly reactive, it forms moderate to strongly complexed solutes and precipitates with many inorganic and organic constituents of natural waters and is readily sorbed onto surfaces of suspended solids. Even thought it is present in water in many forms, the toxicity of copper to aquatic life has been shown to be related primarily to activity of the cupric ion, and possibly to some of the hydroxyl complexes (Allen and Hansen 1996).

Imidazole is a heterocyclic aromatic organic compound present in important biological building blocks such as histidine (an essential amino acid), histamine (the decarboxylated compound from histamine) and other biologically important

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compounds. Imidazole has two nitrogen atoms. The one is slightly acidic, while the other is basic. Many drugs contain an imidazole ring, for example antifungal drugs and nitroimidazoles. Imidazole alkaloids contain one or more imidazole moieties as a part of its structure (Langer et al. 2004). Some imidazole compounds inhibit the biosynthesis of ergosterol, required in cell membrane of fungy. They have antibacterial, antifungal, antiprotozoal, and anthelmintic activity. Several distinct metyl-, phenyl-, benzylimidazoles are therapeutically useful agents against either superficial or systematic infection. In complexes with copper they are often used as antirheumatics. Benzimidazole is a dicyclic compound having imidazole ring fused to benzene. Benzimidazole structure is a part of the nucleotide portion of vitamin B_{12} and the nucleus in some drugs such as proton pump inhibitors and anthelmintic agents. Imidazole and its derivatives are widely used as intermediates in synthesis of organic and inorganic target compounds including pharmaceuticals, agrochemicals, dyes, photographic chemicals, corrosion inhibitors, epoxy curing agents, adhesives and plastic modifiers (Kráľová et al. 1998). Their possible input to the environment is from various anthropogenic activities. No ecotoxicological data are available for any of these compound types and their environmental affects have not been documented.

This study focuses on Cu(II) cyanato-complexes with biologically active heterocyclic imidazole ligands and their ecotoxicological effects determined via changes of the algal growth, photosynthetic pigments production, and Cu accumulation in algal cells.

MATERIALS AND METHODS

Scenedesmus quadricauda (TURP.) BRÉB. strain Greifswald 15 growth inhibition, chlorophyll contents reduction and Cu accumulation in the algae were determined. Stock cultures of algae were maintained in nutrient medium containing, in distilled water, the following chemicals (mg/L): NH_4Cl (1.5), $MgCl_2.6H_2O$ (1.2), $CaCl_2.2H_2O$ (1.8), $MgSO_4.7H_2O$ (1.5), KH_2PO_4 (0.16), $NaHCO_3$ (0.5); (μg/L): $FeCl_3.6H_2O$ (0.8), $Na_2EDTA.2H_2O$ (1.0), H_3BO_3 (1.85), $MnCl_2.4H_2O$ (4.15), $ZnCl_2$ (0.03), $CoCl_2.6H_2O$ (0.015), $CuCl_2.2H_2O$ (0.0001), $Na_2MoO_4.2H_2O$ (0.07) (ISO 1989). The pH of control and with tested compounds supplemented media was set to 7.2 and cultures were incubated at 22 ± 2 °C, under continuous white light with the intensity level of 70 $\mu E/m^2/s$. Algal cultures were maintained in 250 mL Erlenmeyer flasks and suspensions were twice a day agitated on shaker. The algal inoculum for the tests was taken from the exponentially growing pre-culture and added aseptically to all vessels with 50 mL of sterilized medium in concentration 25,000 coenobia/mL (coenobia - four cells connected in one unit). Diluted stock solutions (0.1 mL/50 mL) of tested compounds were spiked to individual cultures directly after inoculation to obtained corresponding range of test concentrations. In control vessels the same volume of distilled water was added. The test duration was 15 days. The growth rate was determined every two days by using spectrophotometer (λ=750 nm) (Bolier and Donze 1989). The

chlorophyll *a* (Chl*a*) and chlorophyll *b* (Chl*b*) content was determined by spectrophotometer (Fargašová et al. 1999) from the same samples used to determine the growth rate. Chlorophyll was determined in 95% (v/v) ethanol extract measuring absorbance at 665 and 649 nm and its amount was calculated under the following equations:

$$Chla = 13.70(A_{665}) - 5.76(A_{649})$$
$$Chlb = 25.80(A_{649}) - 7.96(A_{665})$$

in μg/mL. The spectrophotometer used was capable of measuring accurately cell density as low as 10^4 coenobia/mL.

The type of statistical method to be used for processing the data was a computerized regression analysis of the concentration – response relationship (Toxicity – STATISTICA Data Cz). The analysis aims at quantitatively describing the concentration response curve in the form of a mathematical regression function Y = f (C). Used inversely C = f (Y), IC_x values, including IC_{50}, and their 95% confidence limits can be calculated. The advantage of such an analysis over a graphic estimation is that it is objective, and allows the possibility of estimating confidence limits around the reported figures (OECD 2002).

Accumulation of Cu by the algae from complexes as well as from $Cu(NO_3)_2$ compound was determined using an energy disperse multichannel X-ray fluorescent analyzer (model Canberra 8100, U.S.A. – detection limits reliably below 1 μg/L) equipped with a digital data recorder (Fargašová et al. 1999). As reference material P-ACHK Essential and Toxic Elements in Green Algae from Institute of Radioecology and Applied Nuclear Techniques, Košice, Slovak Republic were used. Prior to Cu accumulation measurement, the algal cells were washed by distilled water to remove any copper possibly remaining at the cell surface. For Cu accumulation analysis tested compounds were added into the cultivation medium in concentration equal to 10 μg Cu/L. Accumulation was expressed relative to dry weight (DW).

During the study $Cu(NO_3)_2 \cdot 4H_2O$ (p.a.) (MERC Darmstadt, FRG) and Cu(II) cyanato-complexes with heterocyclic N-donor imidazole ligands - $Cu(NCO)_2(iz)_2$, $Cu(NCO)_2(Meiz)_2$, $Cu(NCO)_2(Me_2iz)_2$ and $Cu(NCO)_2(Mebz)_2$ were the test compounds (iz = imidazole; Meiz = 2-methylimidazole; Me_2iz = 1,2-dimethylimidazole; Mebz = 2-methylbenzimidazole). Complexes $Cu(NCO)_2L_2$ (L is iz, Meiz, Me_2iz or Mebz) have tetragonal stereochemistry. They contain coordinate molecules of imidazole or its derivatives and anionic NCO^- ligands. All imidazole ligands are coordinated to Cu(II) through the nitrogen atom and the cyanate anions are N-bounded to Cu(II) (Mašlejová et al. 1980). In aqueous solutions $Cu(NO_3)_2$ is present as aquacomplex $[Cu(H_2O)_6]^{2+}$, NO_3^- anion is not coordinated. Cu(II) cyanato-complexes were prepared at the Department of Inorganic Chemistry, Faculty of Chemical and Food Technology, Slovak Technical University in Bratislava (Slovak Republic). The modes of coordination of ligands in the complexes had been inspected by means of infrared absorption spectra (Mašlejová et al. 1980). Stock solutions were prepared in DMSO. The

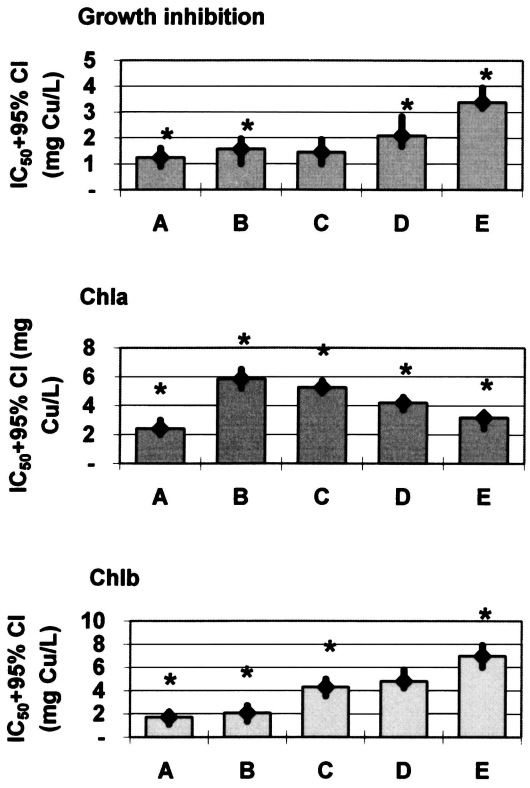
final concentration of DMSO in all tested and control vessels with 50 mL of cultivation media was 0.4%. The measured concentrations of all tested substances followed a geometric progression from 0.1 to 10 μg Cu/L. For each compound, 10 various concentrations in the level of effect from 10 to 90 % algal growth inhibition were chosen.

Copper in stock solutions was determined chelatometrically after decomposition of the compounds by H_2SO_4 and $K_2S_2O_8$ before each test. The elemental analyses were carried out using a Flash EA 112 Analyzer. Three replicates of each test substance concentration and six identical controls were prepared for all measurements.

RESULTS AND DISCUSSION

The Cu(II) complexes inhibited growth and chlorophyll content in alga *S. quadricauda* less than $Cu(NO_3)_2$. Inhibitory activity of the complexes tested expressed by IC_{50} values varied for alga growth, Chl*a* and Chl*b* content in the range of 1.6 $[Cu(NCO)_2(iz)_2]$ to 3.4 μg Cu/L $[Cu(NCO)_2(Mebz)_2]$, 3.2 $[Cu(NCO)_2(Mebz)_2]$ to 5.8 μg Cu/L $[Cu(NCO)_2(iz)_2]$ and 2.1 $[Cu(NCO)_2(iz)_2]$ to 7.0 μg Cu/L $[Cu(NCO)_2(Mebz)_2]$, respectively (Fig. 1.). The decreasing inhibitory orders were: for alga growth and Chl*b* content: $Cu(NO_3)_2$>$Cu(NCO)_2(iz)_2$> $Cu(NCO)_2(Meiz)_2$>$Cu(NCO)_2(Me_2iz)_2$>$Cu(NCO)_2(Mebz)_2$; for Chl*a* content: $Cu(NO_3)_2$ > $Cu(NCO)_2(Mebz)_2$ > $Cu(NCO)_2(Me_2iz)_2$ > $Cu(NCO)_2(Meiz)_2$ > $Cu(NCO)_2(iz)_2$. The variances between the inhibitory activities of the studied Cu(II) complexes were, in most cases, significant and the ligands effect was pronounced. This statement contradicts that of Kráľová et al. (1998) conclusions for two types of Cu(II) complexes, $CuX_2.H_2O$ and CuX_2L_y (where X = flufenamate, mefenamate, niflumate, naproxenate; L = nicotinamide, N,N-diethyl-nicotinamide, ronicol, caffeine, methyl-3-pyridylcarbamate; y = 1 or 2) who assumed that the Cu(II) ions are mainly responsible for *Chlorella vulgaris* alga growth and chlorophyll content. We presumed that decrease of algal growth and chlorophyll content in algae by the studied Cu(II) complexes could be in connection with changes in the biosynthesis of chlorophyll. This would be caused by the replacement of Mg^{2+} ions by Cu^{2+} (Kowalewska et al. 1987), with changes in the structure of the lipid membranes due to their peroxidation (Sandmann and Böger 1980) and with retardation of the synthesis of the D_1 protein in PS2 (Vavilin et al. 1995). $Cu(NO_3)_2$ had in this study the strongest inhibitory effect on algal growth and chlorophylls content.

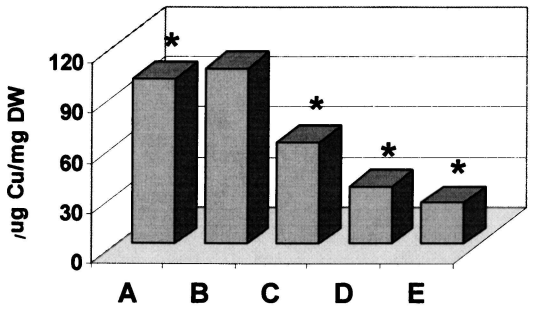
When the inhibitory effects for algal growth and Chl*b* content were compared with their molecular weight and lipophilicity, adverse effects decreased with increasing of these parameters. Explanation for this fact could be found in the results obtained from the determination of Cu amount accumulated in the algal cells after 15 days cultivation (Fig. 2.). These results confirmed that the highest is molecular weight and lipophilicity, and the lowest is the penetration of Cu(II)



Chla – chlorophyll *a*; Chlb – chlorophyll *b*; * - significant differences between all compounds; **A** – $Cu(NO_3)_2.4H_2O$; **B** – $Cu(NCO)_2(iz)_2$; **C** – $Cu(NCO)_2(Meiz)_2$; **D** – $Cu(NCO)_2(Me_2iz)_2$; **E** – $Cu(NCO)_2(Mebz)_2$

Figure 1. IC_{50} values and their 95% CI (μg Cu/L) for algal growth inhibition and chlorophyll content

complexes and subsequent Cu(II) accumulation in *S. quadricauda* cells. The accumulation order was $Cu(NCO)_2(iz)_2$=$Cu(NCO)_2(NO_3)_2$> $Cu(NCO)_2(Meiz)_2$> $Cu(Me_2iz)_2$>$Cu(Mebz)_2$ and Cu(II) accumulated amount in algal cells varied in the range of 24.6 [$Cu(Mebz)_2$] to 105.5 μg Cu/mg DW [$Cu(NCO)_2(iz)_2$] (Fig. 2.). Differences between molecular weight of $Cu(NCO)_2(iz)_2$ and $Cu(NO_3)_2$ are very low. This indicated nearly the same accumulated amount of Cu from both these compounds. The similar dependency between molecular weight and toxicity, penetration and accumulation in algae was also confirmed for some other Cu(II), Fe(III) and Cd(II) complexes (Kráľová et al. 1998; Ondrejkovičová et al. 2000; Fargašová and Ondrejkovičová 2002). Copper accumulation from tested



* - significant differences between all compounds; standard deviation 6% or less
A – $Cu(NO_3)_2.4H_2O$; **B** - $Cu(NCO)_2(iz)_2$; **C** - $Cu(NCO)_2(Meiz)_2$; **D** - $Cu(NCO)_2(Me_2iz)_2$; **E** - $Cu(NCO)_2(Mebz)_2$;

Figure 2. Cu accumulation from Cu(II) complexes and $Cu(NO_3)_2$ in alga cells (µg Cu/mg DW)

Cu(II) complexes is influenced by spherical structure of the complex and by variations in the active absorption centers situated on the cell wall surface. Cu(II) ion is known to influence algal growth and algal cell wall structure (Fargašová 2001). However, on the basis of results presented, it may also be concluded that the nature of the ligands affects Cu(II) complexes, inhibitory effects on algal growth and photosynthetic pigments production, as well as cellular accumulation of copper. The statement that copper binding with biological active ligands decreased its bioavailability and would therefore tend to be associated with increased copper IC_{50} or LC_{50} values (U.S. EPA 2003) was fully confirmed during our tests. Metal bioavalability may also be modified by competitive interactions at the biotic ligand.

Studied complexes exhibit tetragonal structures formed by $Cu(NCO)_2L_2$ units, in which the Cu(II) atom exhibits a square planar coordination by four N-atoms from two imidazole ligands and two NCO-groups. Complexes tested have small axial interaction upon the steric properties of the given imidazole ligands. In the investigated complexes, imidazole ligands are bonded to copper atoms similarly as in the cases of biologically active (N-salicylidene-glutamato)(R-imidazole) copper(II) complexes (R = 1-methyl or 2-methyl) (Langer et al. 2004). It appears that the size of the ligand in the immediate vicinity of the copper ion is the dominating factor in determining the properties of the complexes. Bulkiness and steric effect of imidazole ligands was increased in the order iz < Meiz < Me_2iz < Mebz. This fact is reflected in decreased toxicity for particular complexes, as well as for Cu accumulation in algal cells. Weaker axial interaction was noticed for more bulky benzimidazole ligand (Mebz) which penetrates into the algal cells in the lowest amount and indicated the lowest toxicity for growth inhibition and Chl*b* production from all complexes tested. Completely opposite dependency for Chl*a*

could be explained through different structure of both pigments that can interact with the study complexes in various ways.

Over the past decade considerable attention has been given to the affects of the toxicity of potentially toxic trace metals in the dissolved phase through a change in the chemical form, or species, of the trace metal. Trace metals such as Cd, Cu, Zn and others can form complexes with a number of inorganic and organic ligands or complexing agents that are present in natural water (Allen 2002). Many metal complexes are also input into the environment from various antropogenic activities. To such complexes belong also presented Cu(II) complexes with imidazole ligands for which no ecotoxicological data are available and their environmental impact has not been documented. How biotic ligands influence metal toxicity in natural waters depends on water quality criteria and the number of inorganic and organic ligands or complexing agents interacted with the metals. The chemical speciation of trace metals greatly influence their biological effects. Nonetheless, no clear consensus currently exists as to when metal complexes are bioavailable, especially for field conditions. Recently, the U.S. EPA has incorporated the Biotic Ligand Model (BLM) into their regulatory framework. The BLM is an effective tool to estimate toxicity of dissolved metals through the use of mathematically integrated traces of metal interaction with solution phase ligands to predict its speciation and its subsequent interaction with receptor sites on the organism (Allen 2002; U.S. EPA 2003; De Schamphelaere and Janssen 2004).

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