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ECOTOXICOLOGICAL EFFECTS OF MINING POLLUTANTS FROM THE ROMANIA SERBIA CROSS-BORDER AREA

1 Effects of copper

The toxic effects of copper on numerous aquatic flora and fauna has been studied intensely over the past 10 years. Biological indicators exhibit a tremendously wide range of sensitivity to copper with toxic effects noted at pCu as low as 10 for some algae, while aquatic macrophytes appear to have a much higher tolerance for copper (pCu < 5.0) (Yahya M. Nor, 1987).

Copper, an element required by most forms of life in minute quantities, is a widely distributed heavy metal. In large quantities, however, copper can become a serious health hazard to both plants and animals (Cvjetko et. al, 2010)..

In general, there is a consensus that free cupric ions are more toxic if compared with other chemical forms such as organically complexed copper. The toxicity of copper, however, is mitigated by the presence of naturally occurring organic compounds in waters through complexation. The regulatory function of dissolved humic matter will continue to be a vital one for as long as copper is discharged into aquatic environments (Yahya M. Nor, 1987).

Chlorella sp. was more sensitive to CuSO₄. Exposure to CuSO₄ reduced the chlorophyll-a, chlorophyll-b, and carotenoid contents of the algal cells. At 96 h, CuSO₄ induced a significant increase in the production of reactive oxygen species (ROS) and degree of lipid peroxidation. Clumping of cells occurred in cultures exposed to CuSO₄. (Wan, Jun-Kit, et al., 2018).

Copper sulphate is a common algaecide applied to ponds to keep phytoplanktonic blooms under control, especially those prone to cyanobacterial development. The copper toxicity was



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evaluated for two phytoplanktonic species with the same size and shape: one chlorophyceae *Chlorella vulgaris* and one cyanobacteria *Microcystis aeruginosa*. The results of analysis showed:

1. that regardless of the cell type, as copper concentrations increased, cell division rate and autofluorescence decreased.
2. a difference in sensitivity according to the length of exposure to copper: 24 or 48 h;
3. that *M. aeruginosa* showed a greater sensitivity than *C. vulgaris* in the 24 h copper exposure assay according to esterase activity data. At the same time, Copper affects the light harvesting complex, phycobilisomes, the photosystem I (PSI) and II (PSII) reaction centres for *M. aeruginosa* and *C. vulgaris* (Hadjoudja, Souad, et al., 2009).

The effect of copper for *Raphidocelis subcapitata* was studied using various physiological and toxic targets. The algae were exposed to seven—five of which are ecologically relevant for European surface waters—copper concentration ranging from 0.5-100 $\mu\text{g Cu l}^{-1}$ during a 3-month period. Significant decreases in algal biomass, pigment diversity and autotrophic index were observed in algal cultures acclimated to 0.5 $\mu\text{g Cu l}^{-1}$ and 100 $\mu\text{g Cu l}^{-1}$. Chlorophyll a content (mean \pm standard deviation) increased from 8.4 ± 3.1 to $28.6 \pm 7.5 \times 10^{-14}$ g per cell and carotenoid content (mean \pm standard deviation) increased from 3.7 ± 0.8 to $7.1 \pm 1.2 \times 10^{-14}$ g per cell for algae exposed to 1 and 100 $\mu\text{g Cu l}^{-1}$, respectively. Intracellular copper increased from 0.099 to 20.6×10^{-15} g Cu per cell and adsorbed copper increased from 0.026 to 1.8×10^{-15} g Cu per cell for algae acclimated for 12 weeks to 0.5 and 100 $\mu\text{g Cu l}^{-1}$, respectively (Bossuyt et al., 2004).

Among the metals, Cu is classified as extremely toxic. Deficiencies in Cu resulted in chlorosis of *L. minor fronds* and low concentrations of Cu interfered with the floral induction in *L. minor* and *L. gibba* (Fflbin and Hough, 1979 & Dirilgen, 1994).

Excess Cu inhibited both frond growth and frond multiplication of *L. paucicostata* and it decreased the content of chlorophyll a and photosynthetic CO_2 uptake in *L. minor* (Fflbin and Hough, 1979 & Dirilgen, 1994)

Exposure to 48 $\mu\text{g L}^{-1}$ Cu was shown to significantly reduce (91%) net Ca^{2+} uptake which is strongly correlated with shell deposition and corresponding snail growth. Snails (*Lymnaea stagnalis*) exposed to 48 $\mu\text{g L}^{-1}$ Cu also exhibited reduced ammonia excretion, a marked hemolymph acidosis, and a compensatory increase in titratable acid excretion. The reduction in net Ca^{2+} uptake was hypothesized to be a secondary effect of Cu-induced inhibition of carbonic anhydrase, but no reduction in carbonic anhydrase activity was detected. Overall, it remains unclear whether inhibition of Ca^{2+} uptake is a direct result of Cu exposure or, along with the other observed physiological effects, is secondary to an unidentified primary mode of toxic action (Brix et al., 2011).

Any addition of total copper will result in lethality for *Daphnia magna*. A possible explanation for this effect could be due to a physiological mechanism based on changes in the reactivity of



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sulfhydryl-containing proteins or enzymes is suggested as a possible explanation for this effect (Andrew,1977).

Aquatic organisms, such as midges, are very sensitive to copper when it is in aqueous forms but not so when present in bound forms. Sediment in aquatic environments, such as lakes and streams, acts as a sink for copper. Copper has been reported to inhibit egg development in an estuarine copepod, reduce the percentage of salmonid egg hatching and adversely affect embryogenesis of sea urchin eggs and coral-reef echinoids. Copper in the water did not affect the embryonic development of the midge. While 0.74 mg/L of copper in water is adequate to produce a 50% mortality in fourth instar larvae of *C. decorus* in 48 hr concentrations of copper up to 5 mg/L did not adversely affect embryonic development of the midge. Therefore, eggs are assumed to possess a protective mechanism in the case of copper. Insect eggs are normally surrounded by a vitelline membrane and chorion, or "shell", and a layer of wax on the interior. The chorion is a complex structure consisting of layers of proteins and lipoproteins. These layers might chemically combine with copper and prevent it from entering the interior of the egg also found defense mechanisms against CuSO_4 in snail eggs (*Oncomelania formosana*) (Kosalwat et al., 1987).

Recent in vitro studies have demonstrated that copper may induce apoptosis triggering the activation of caspase 3, a central effector of apoptotic cell death at fish (*O. niloticus*). The available data, in fish (*O. niloticus*), suggests the existence of equivalent apoptotic pathways to those of mammals, making fish useful models for studying apoptosis. However, fish caspase genes have only recently been started to be sequenced bringing to light the possibility of their use in toxicological studies. Information about the biochemical mechanism of copper-induced apoptosis is relatively scarce and the studies made until now, mainly in vitro, suggest that copper may induce apoptotic cell death through different pathways. Nonetheless, it has previously been demonstrated that copper overload of cells induces the activation of caspase-3 terminal enzyme of both extrinsic and intrinsic apoptotic pathways, thus suggesting its involvement in the copper-induced apoptotic death. To contribute to the understanding of copper action on caspase-3 and in general on apoptotic pathways the cDNA encoding for *O. niloticus* caspase-3 was sequenced and its identification was made with the support of several analyses, as reported in Section 3. Then, the in vivo effects of the continuous exposure to two different copper concentrations were investigated in *O. niloticus* gills. The health of the fish used was previously evaluated, revealing a stress condition, with osmoregulatory disruptions and several histopathological changes in the gill epithelia (Monteiro et al., 2009).

Increasing levels of CuSO_4 and an increase in time of exposure would inhibit growth and cause a yellowing of leaves through damage of chlorophyll (Cvjetko et. al, 2010).

Toxicity of copper for monocots, was evaluated by *Allium cepa* bioassay. The roots of *A. cepa* bulbs exposed to Cu^{2+} (3 mg L⁻¹) individually or in mixtures with Cd^{2+} (0.1 mg L⁻¹) or/and Cr^{6+} (0.1 mg L⁻¹) exhibited the highest growth inhibition, mitotic index depression and nuclear abnormalities. Root



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tip cells exposed to Cr⁶⁺ or Cd²⁺ alone or in mixture displayed significant chromosomal aberrations in comparison to the controls.

The effects of increasing concentrations of soil copper on several leaf structural parameters in *Origanum vulgare* plants were studied to determine the effect of copper toxicity. Copper-stressed leaves were small and chlorotic and underwent a thickening of their lamina, due principally to an increase in the number and volume of mesophyll cells. The number of stomata and glandular and non-glandular hairs increased significantly. Chloroplasts of mesophyll cells declined dramatically in number and volume. Grana and stroma thylakoids of chloroplasts did not undergo any noticeable alterations, but starch grains disappeared, plastoglobuli became larger and the double membrane limiting the chloroplast became dilated. Leaf chlorosis was determined by total chlorophyll analysis and measurement of the leaf Cu, Fe and Mg content. The effects of copper toxicity on oregano leaves comprised significant structural alterations which react reduced metabolic activity (Panou-Filothou, H., 2001).

Copper accumulated in exposed plants *Phaseolus vulgaris* caused severe symptoms such as chlorosis and necrosis as well as a dramatic reduction in dry weight production. The results suggest that while excess copper slightly alters the activity of the antioxidative enzyme system in young expanding leaves of bean plants, it exerts its toxicity primarily through causing a disturbance in the nutrient balance (Bouazizi et al., 2010).

The effect of copper chloride on the level of chlorophyll proline, protein, and abscisic acid in sunflower (*Helianthus annuus L.*) seedlings were investigated. Copper stress caused significant increase of the abscisic acid contents in roots, shoots and leaves of seedlings. The increase was dependent on the copper salt concentration. Enhanced accumulation of proline in the leaves of seedlings exposed to copper was determined, as well as a decrease of chlorophyll and total protein. It was observed that the level of chlorophyll and total protein remarkably decreased as copper concentration increased to 0.6 mM, although the levels of proline and abscisic acid in the leaves of plants were increased - a dose-dependent behavior. The same trends were also observed with the level of abscisic acid of stems and roots. Copper has dose-dependent effects on chlorophyll, proline, protein, and abscisic acid level of sunflower (*Helianthus annuus L.*) seedlings (Zengin et al., 2007).

Animals exposed to 30 ppm copper and higher concentrations may experience vomiting, diarrhea, cramps, nausea, and even liver or kidney damage. (Cvjetko et al., 2010).

Earthworms (*Eisenia fetida*) held in soils containing 53 mg Cu/kg DW show a 50% reduction in cocoon production in 56 days; 32 mg Cu/kg soil had no effect on cocoon production (Spurgeon et al. 1994). The LC50 (56 days) value for earthworms is 555 mg Cu/kg DW soil; no deaths occur at 210 mg/kg soil during this period. Copper is more toxic to *Eisenia fetida* than are salts of cadmium, zinc, or lead (Spurgeon et al. 1994). Copper adversely affects the earthworm *Lumbricus rubellus*. Concentrations of 150 mg Cu/kg surface soil from an accidental spill of copper sulfate in grasslands reduced earthworm



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populations by about 50%; surface soil concentrations of 260 mg Cu/kg kill almost 100% of the *Lumbricus*. Copper is most toxic to *Lumbricus* at low soil pH (4.8-7.1) and at low temperatures Eisler et al.,1998).

In general, birds retain a very small portion of copper and other metals ingested. It is therefore noteworthy that livers of some canvasbacks collected in Louisiana and livers of some mute swans (*Cygnus olor*) from England both contain more than 2,000 mg Cu/kg DW. In the case of mute swans, several thousands of milligrams of copper per kilogram dry weight occur in the blackened livers; blackening is attributed to ingestion of flakes of copper-based antifouling paints (Bryan and Langston 1992).

Tree swallows (*Tachycineta bicolor*) nesting near acidified aquatic ecosystems accumulate sufficient copper from the diet to induce elevated hepatic metallothionein concentrations (St. Louis et al. 1993).

At the mammals copper homeostasis plays an important role in the prevention of copper toxicity. After copper requirements are met, excess copper absorbed into gastrointestinal mucosal cells is bound to metallothionein and excreted when the cell is sloughed. Copper that eludes the intestinal barrier is stored in the liver or incorporated into bile and excreted in feces. The most likely pathway for the entry of toxic amounts of copper would-be long-term inhalation or entry through the skin. Both pathways allow copper to pass unimpeded into the blood. The levels of copper in the mammalian body are held constant by alterations in the rate and amount of copper absorbed, its distribution, and rate and route of excretion. Many factors interfere with copper absorption including competition for binding sites, as with zinc; chelation, as with phytates; and interaction with ascorbic acid, which aggravates copper deficiency by decreasing copper absorption and—with excess copper—reduces the toxic effects (Eisler et al.,1998)

Compared to animals from a normal site, muskrats (*Ondatra zibethicus*) from a site contaminated by copper and other chemicals have higher concentrations of copper in kidneys, and have smaller spleens, larger adrenals, less fat, and lower body weight (Halbrook et al. 1993).

Organisms	Metal	EC ₅₀	References
<i>Chlorella vulgaris</i>	Cu	0,66 μM	(Hadjoudja, Souad, et al., 2009)
	CuSo ₄	150 μM	(Wan, Jun-Kit, et al., 2018)
<i>Raphidocelis subcapitata</i>	Cu ²⁺	0.03-0.28 mg/L ⁻¹	Horvatic, Janja, et al., 2007)
<i>Lemna minor</i>	Cu	0.47 mg/L	(Khella et Nabila, 2009).
	Cu	1·1 mg/L ⁻¹	(Wang et Wuncheng. 1986)



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<i>Daphnia magna</i>	Free Cu ²⁺	129.3 ± 72.7 µg/L	(Christiansen et al., 2011)
	Free Cu ²⁺	102.4 ± 124.5 µg/L	(Christiansen et al., 2011)
<i>Mytilus edulis</i>	Cu	5,8 mg/L-	(MacFarlane et al., 2002)
<i>Mytilus trossolus</i>	Cu	99 µg L ⁻¹ (86-101)	(Roesijadi et al.,1987)
<i>Chironomus decorus</i>	Cu	1.602 mg / kg.	(Kosalwat et al.,1987)
<i>Phaseolus raditus</i>	Cu	335 mg/L	(Lee et al., 2008)
<i>Zea mays</i>	Cu	6,5gM	(Ouzounidou,G. 1995)
<i>T. aestivum</i>	Cu	570 mg/L	(Lee et al., 2008)
<i>Helix aspersa</i>	Cu	1200 µg/g ⁻¹	(Gomot-De Vaufleury, A., 2000)
<i>Caenorhabditis elegans</i>	Cu	0,68 mg / L	(Jiang et al., 2016)
<i>Folsomia candida</i>	CuSO ₄	564 ±50.2 mg Cu/kg	(Neves, J. et al., 2019)
	Cu(OH) ₂	57±50.8 mg Cu/kg	(Neves, J. et al., 2019)
<i>Eisenia foetida</i>	Cu	57(51–62) mg/kg ⁻¹	(Bustos et al., 2015)
<i>Gallus gallus do- mesticus</i>	Cu	2,9 g / L	(Gupta,G.,& Gardner, W. 2005)

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