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RESEARCH PAPER

Mouthpart Deformities of *Chironomus plumosus* Larvae Caused by Increased Concentrations of Copper in Sediment from Carp Fish Pond

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Abstract

Elevated concentrations of heavy metals in water can cause deformities of the mouthparts in chironomid larvae reared on artificial sediment. This investigation is an attempt to study effects of increased copper concentrations on mouthparts deformities of Chironomus plumosus larvae reared on natural sediment obtained from carp pond. Since the bioavailability of heavy metals is greater in artificial substrates than in natural ones usage of carp pond sediment, especially due to its low heavy metals content according to sediment quality guidelines, should provide more realistic assessment of biomarker potential of chironomid mouthparts deformities in biomonitoring of copper sediment pollution.

It is demonstrated in the experiment that an increase of copper concentration in sediment leads to progressive increase in the frequency and severity of deformities of the mentum in C. plumosus larvae. Thus, shortening of median teeth in 2.0±0.2% individuals was the only deformity recorded in control tanks (at 15 µgg-1 Cu), at 30 µgg-1 Cu its frequency increased to 16±2%, and shortening of median-lateral teeth appeared at the same frequency, while at 60 µgg-1 Cu frequency of both deformities increased to 24±2% and 28±4%, respectively, in addition to the appearance of tooth loss in 16±3% of individuals.

Introduction

Under conditions of global and local pollution of surface water in the presence of industrial and agricultural waste, constantly increasing amounts of toxic substances are accumulating in the sediment of inland waters. It is known that the sediment and processes transpiring in it are of great significance for quality of the water in a given aquatic ecosystem. Many substances (including heavy metals) that in water form weakly soluble carbonates, sulphates and sulphides are deposited in the sediment, where they accumulate (Jovanović, 2012). Introduced into an organism, they can be found in virtually all tissues and organs. ~~Only by monitoring the content of metals and living communities in ecosystems is it possible to fully understand the extent to which an aquatic ecosystem is polluted (Gremyatchikh, Tomilina & Grebenyuk, 2009).~~

Use of living organisms in biomonitoring of aquatic ecosystems is preferable to traditional chemical analyses of water quality because freshwater organisms, by virtue of their continual presence in the water, respond to all ecological stressors. Changes on the organism level can be more useful than changes on the community level in biomonitoring of aquatic ecosystems (Al-Shami, Rawi, Nor, Ahmad & Ali, 2010). Information about changes occurring on the level of organisms in response to the presence of heavy metals can be more important because they respond before the community does. Such individual responses can indicate the danger of heavy metal contamination in the earliest phases of pollution (Gremyatchikh, Tomilina & Grebenjuk, 2009~~Gremyatchikh *et al.*, 2009~~).

One of the ways of following the status of aquatic ecosystems is by studying their benthic macroinvertebrates, among which chironomids occupy a special position (Beneberu & Mengistou, 2014). In view of the fact that chironomid larvae inhabit almost all types of aquatic habitats and, in contrast to aquatic insects that disappear under the influence of pollution (Plecoptera, Ephemeroptera, Trichoptera), are found in places burdened with organic and inorganic substances (Beneberu & Mengistou, 2014) as well as heavy metals (Clements, Cherry & Cairns, 1988; Janssens de Bisthoven, Thimmermans & Ollevier, 1992), it is clear why they represent a key group in the benthic community (Armitage, Cranston & Pinder, 1995; Péry, Mons & Garric, 2004; Čerba, Mihaljević & Vidaković, 2010; Čerba, Mihaljević & Vidaković, 2011; Milošević, Simić, Stojković & Živić, 2012; Milošević, Simić, Stojković, Čerba, Mančev, Petrović & Paunović, 2013; Milošević, Stojković, Čerba, Petrović, Paunović & Simić, 2014) and why they are used as test organisms in studies of sediment toxicity (Péry et al., 2004; Di Veroli, Goretti, Paumen, Kraak & Admiraal, 2012; Reynolds & Ferrington, 2002; Grebenjuk & Tomilina, 2014; Ebau, Rawi, Din & Al-Shami, 2012; Janssens de Bisthoven et al., 1998; Martinez et al., 2003). Additionally, ~~Chironomids~~ chironomids are successfully used as bioindicators of the degree of toxicity in aquatic ecosystems because they complete the greater part of their life cycle in situ~~. Chironomid larvae inhabit nearly all bottom types~~ and play an important role in the circulation of matter and flow of energy in an aquatic ecosystem (~~Grebenyuk~~ Grebenjuk & Tomilina, 2014). In the course of their feeding, chironomid larvae can metabolize various contaminants present in the sediment and water. Such compounds can be responsible for the occurrence of abnormalities in certain parts of the body, especially the ~~oral apparatus~~mouthparts. The occurrence of deformities can be regarded as a warning sign indicating degradation of an aquatic environment by chemical pollutants (Beneberu & Mengistou, 2014). To be specific, pathomorphological deviations in the mouth structures of chironomids have been registered since the early 1970s. Hamilton and Saether (Hamilton & Saether, 1971) were the first to indicate that deformities in chironomids can be used to measure the extent to which aquatic ecosystems are polluted. It was shown latter on that elevated concentrations of zinc, lead, copper and arsenic in water can cause deformities of the ~~oral apparatus~~mouthparts in chironomid larvae reared on an artificial sediment unburdened by heavy metals (Janssens de Bisthoven, Vermeulen & Ollevier, 1998; Martinez, Moore, Schaumloffel & Dasgupta, 2003; Martinez, Moore, Schaumloffel & Dasgupta, 2006; Beneberu & Mengistou, 2014).

Copper sulphate is still frequently used to reduce the possibility of algal blooms in water of fish farms, as well as for treatment of fish illnesses caused by bacteria and parasites (FAO, 2017; Marković, 2010). Therefore, unlike other toxic metals, copper concentrations are frequently elevated in carp pond sediment as a consequence of its regular maintaining. The presence of copper in the body of chironomid larvae indicates the possible presence of heavy metals in carp meat, since these larvae are natural food of carp. Moreover, because chironomid larvae feed predominantly by scraping and filtering (Moog, 2002), eventual changes in the mentum that occur due to the presence of copper in the sediment make feeding difficult, with the result that the larvae stop eating and die, thereby leaving the carp without a source of natural food on the fish farm. Therefore, establishing ~~the~~ a fast, inexpensive and reliable method for detection of elevated copper concentrations in carp pond sediment is of great importance.

The aim of this study was to test the effects of artificially elevated copper concentration in carp pond sediment, as a natural environment of *Chironomus plumosus* (Linnaeus, 1758) on its mouthparts morphology in order to test if it can be used as an indicator of sediment copper pollution. Therefore, except for addition of appropriate concentrations of copper sulphate, carp pond sediment was not altered in any other way, in order to keep conditions for ~~*Ch. plumosus*~~*C. plumosus* growth and development in experimental tanks as close as possible to the natural ones. Analysis of deformities in the mentum of chironomids is a simple method of ~~establishing~~ monitoring ~~the presence of~~ copper contamination in the sediment of carp farms, as well as its potential presence in meat of the farmed carp.

Materials and Methods

Samples and Design of Experiments

Chironomus plumosus larvae were collected in the NL2 experimental fish pond (with a bottom base area of 300 m^2) of the Little Danube Center of Fisheries and Applied Hydrobiology, Faculty of Agriculture, University of Belgrade (Serbia). Material was sampled with a Van Veen dredge having a surface area of 260 cm^2, and chironomids were then separated on site from the sediment with the aid of a sieve for counting purposes. Total of 350 third ~~or fourth~~ instar larvae were collected out of which 300 were used for rearing in experimental tanks and 50 for chemical analysis of heavy metal concentrations. For chemical analysis of heavy metal

Table 1. Concentrations of heavy metals (in μg g^{-1}) in the sediment and in ~~*Ch. plumosus*~~*C. plumosus* larvae from carp pond NL2

	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	Zn
Sediment	0,481	20,550	100,134	15,000	31942,953	696,644	50,705	36,107	60,067
~~*Ch. plumosus*~~*C. plumosus*	0,021	0,322	2,216	11,972	652,597	20,447	1,767	14,102	13,366

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presence in sediment 50 g of composite sediment sample was collected from pond NL2. Analysis of chironomids and sediment for the presence of heavy metals was performed at the Institute of Chemistry, Technology and Metallurgy of Belgrade University's Center of Chemistry and presented in Table 1. Copper concentration in fish pond sediment was 15 µg g^{-1} of dry weight (Table 1).

Sediment for the experiment was taken from NL2 experimental fish pond, dried in a Sterimatic ST-11 sterilizer for four hours at 100°C and homogenized by mixing. Homogenized sediment was divided at four equal parts, one control with no additional copper added (i.e. coper concentration of 15 µg g^{-1} of dry weight), and three treated parts each mixed with copper (II) sulphate-pentahydrate (Alkaloid, Skoplje, proanalysis) in concentrations of 30, 60 and 120 µg g^{-1} of dry weight, respectively. The concentrations of copper were selected on the basis of their being between the target concentration (36 µg g^{-1}) and the maximum permissible concentration (110 µg g^{-1}) of copper in sediment according to Serbian regulations (Official Gazette, No. 50/2012), as well as between the threshold effect concentration (TEC, 31.6 µg g^{-1}) and the probable effect concentration (PEC, 140 µg g^{-1}) according to MacDonald, Ingersoll and Berger (2000). Each part of sediment was used as bottom substratum in three experimental tanks (9 cm x 9 cm x 14 cm) forming a layer with average height of 2 cm. A measured volume (400 ml) of distilled water was added to each aquarium. Water and sediment were left for two days to achieve equilibration (OECD, 2004) of suspended firm substances. Each experimental tank was used for rearing of 25 larvae of *Ch. plumosus*. As a result control and each treatment consisted of three independent groups of 25 individuals.

The aquariums were under a light regime consisting of 16h of light followed by 8 h of darkness (Martinez *et al*., 2006). They were equipped with aerators and had an average temperature of 17±1°C and pH value of 5-7. Larvae were fed with the finely ground extruded feed used to nourish carp (38% protein), every larva receiving 0.4 mg per day (Kuvangkadilok, 1994). Experiment lasted for 20 days, since the life cycle of ~~*Ch. plumosus*~~*C. plumosus* is no longer than 39 days (Vedamanikam, 2009) and sometimes shorter depending on water temperature (Pinder, 1986). At the end of experiment larvae were separated from the sediment (by straining through a sieve). Live specimens were then separated from the dead ones. Only surviving larvae were used for further analysis. They were fixed in 96% alcohol. Microscope slides of mouthparts were made according to Namiotko, Danielopol and Baltanás (2011). These slides were examined under a Carl Zeiss Axioskop 40 microscope and then photographed with a Canon PowerShot A80 camera.

Analysis of Heavy Metals in the Sediment and Chironomid Larvae

Microwave Digestion

Sediment and Chironomidae larvae samples were first homogenized, and the samples were then dried at 105° C to constant weight. The dried samples were homogenized and powdered. The total dissolution of the about 0.1 g solid samples for ICP-OES studies was performed on an advanced microwave digestion system (ETHOS 1, Milestone, Italy) using HPR-1000/10S high pressure segmented rotor. In the digestion, samples were mixed in each clean vessel with 15 ml HCl (37%, analytical grade, Sigma Aldrich), 3 mL HNO_3 (67%, analytical grade, Sigma Aldrich) and 2 mL HF (48%, analytical grade, Merck, Germany) and then heated with microwave energy for 30 min. The temperature was controlled with a predetermined power program. Digestion of the samples was carried out for 20 min at a constant temperature of 220°C, with a prior warm up linearly over 10 min to 220°C. After cooling and without filtration, the solution was diluted to a fixed volume of 25 mL into volumetric flask with ultrapure water. Ultrapure water with a resistivity of 18.2 MΩ·cm (equal to 0.05 µS/cm) was prepared using a Barnstead™ GenPure™ Pro (Thermo Scientific, Germany).

Instrumental Analysis

The content of elements, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn in prepared solution samples performed by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), using a iCAP 6500 Duo ICP (Thermo Fisher Scientific, Cambridge, United Kingdom) spectrometer with iTEVA operational software. The spectrometer was equipped with a RACID86 Charge Injector Device (CID) detector, concentric nebulizer, quartz torch, and alumina injector. The optical system purged with argon and the Echelle polychromator thermostated at 38°C. The instrumental conditions were optimized to obtain sufficient sensitivity and precision. Standards for the instrument calibration were prepared on the basis of multielement plasma standard solutions: SS-Low Level Elements ICV Stock (10 mg/L) and ILM 05.2 ICS Stock 1 (500 mg/L). For each digested sample, the ICP-OES measurement was carried out in triplicate (n=3). The reliability of measurements was approved by relative standard deviation of 0.5 - 3%.

Assessment of Sediment Quality

The enrichment factor (EF) and geoaccumulation index (I_{geo}) were calculated to assess anthropogenic impact on the sediment. The following equation (Loska,

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Cebula, Pelczar, Wiechula & Kwapulinski, 1997) was used to calculate EF

$$EF = (M_x * Fe_b) / (M_b * Fe_x)$$

where M_x, M_b, Fe_x and Fe_b are the concentrations of metal (M) and Fe in the sample and in the background reference, respectively. As ~~the~~ background reference, we used the metal concentrations in average continental shale (Díaz-de Alba, Galindo-Riaño, Casanueva-Marenco, García-Vargas, & Kosore, 2011).

The geoaccumulation index determines pollution levels by comparing current metal content with preindustrial levels (Müller, 1981). It was calculated using the following equation:

$$I_{geo} = \log(M_x / 1.5 * M_b)$$

Biota-Sediment Accumulation Factor

In order to quantify the ability of ~~*Ch. plumosus*~~*C. plumosus* larvae to accumulate selected heavy metals from the sediment, the biota-sediment accumulation factor (BSAF) was used. It was obtained as the ratio of concentration of a metal in ~~*Ch. plumosus*~~*C. plumosus* larvae (MCh) and its concentration in the sediment (Ms): BSAF = MCh / Ms.

Analysis of Data

Data were expressed as means ± standard error. Samples were compared statistically using unpaired t-tests at a 5% level of significance (P<0.05) with the aid of Sigma Plot 11 software (Systat Software Inc., USA).

Results and Discussion

~~It is known that the sediment and processes transpiring in it are of great significance for quality of the water in a given aquatic ecosystem. Many substances (including heavy metals) that in water form weakly soluble carbonates, sulphates and sulphides are deposited in the sediment, where they accumulate (Jovanović, 2012). Introduced into an organism, they can be found in virtually all tissues and organs. In view of the fact that chironomid larvae inhabit almost all types of aquatic habitats and, in contrast to aquatic insects that disappear under the influence of pollution (Plecoptera, Ephemeroptera, Trichoptera), are found in places burdened with organic and inorganic substances (Beneberu & Mengstou, 2014) as well as heavy metals (Clements, Cherry & Cairns, 1988; Janssens de Bisthoven, Thimmermans & Ollevier, 1992), it is clear why they represent a key group in the benthic community (Armitage, Cranston & Pinder, 1995; Péry, Mons & Garric, 2004; Čerba, Mihaljević & Vidaković, 2010; Čerba, Mihaljević & Vidaković, 2011; Milošević, Simić, Stojković & Živić, 2012; Milošević, Simić, Stojković, Čerba, Mančev, Petrović & Paunović, 2013; Milošević, Stojković, Čerba, Petrović, Paunović & Simić, 2014) and why they are used as test organisms in studies of sediment toxicity (Péry et al., 2004; Di Veroli, Goretti, Paumen, Kraak & Admiraal, 2012; Reynolds & Ferrington, 2002; Grebenjuk & Tomilina, 2014; Ebau, Rawi, Din & Al-Shami, 2012; Janssens de Bisthoven et al., 1998; Martinez et al., 2003).~~ It is known that the feeding of chironomid larvae by scraping and filtering causes their ~~oral apparatus~~mouthparts to be directly exposed to harmful substances. Other than in feeding, larvae of the family Chironomidae use materials from the sediment to construct the tube around their body which serves to generate respiratory currents and protects them from predators (Dudgeon, 1994), whereby they are directly exposed to copper and other metals present in the sediment (Reynolds & Ferrington, 2002). Certain species, like those of the genus

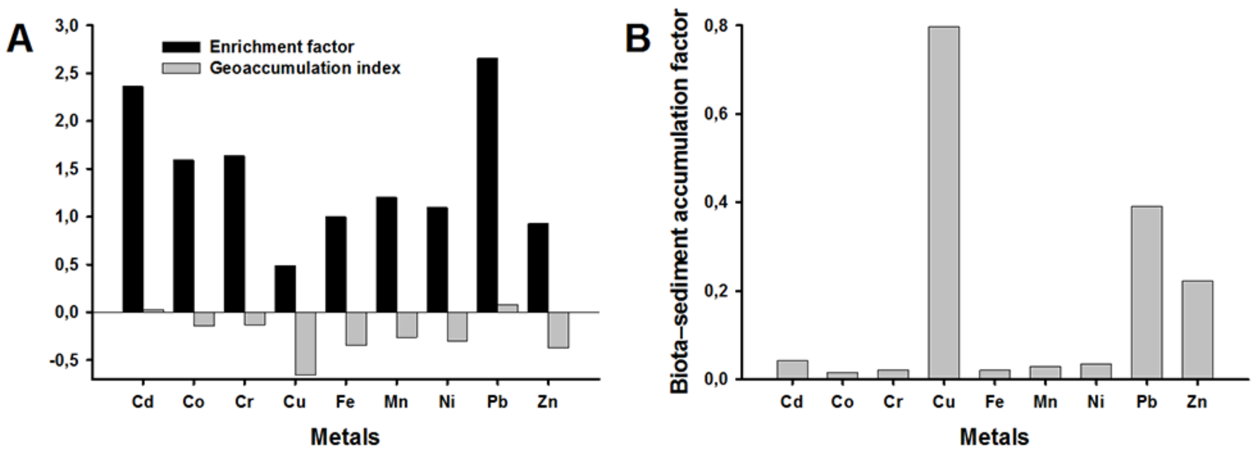

Figure 1. A) Values of the Enrichment factor and Geoaccumulation index for heavy metals determined in the sediment of the carp pond NL2. B) Biota-sediment accumulation factor for ~~*Ch. plumosus*~~*C. plumosus* larvae collected in carp pond NL2.

Chironomus, are capable of increasing their tolerance to metals and stress caused by the presence of heavy metals (Postma, Kyed & Admiraal, 1995; Klerks & Weis, 1987; Posthuma & Van Straalen, 1993).

Since sediment from the fish farm which the larvae were sampled was used as the substrate in experiments testing the influence of copper on morphology of the ~~oral apparatus~~mouthparts of chironomid larvae, the composition of heavy metals was determined both in the substrate and larvae (Table 1). When the obtained values are analyzed in relation to published sediment quality guidelines for freshwater ecosystems (MacDonald *et al.*, 2000), only the concentration of chromium is significantly higher than the threshold effect concentration (TEC, 43.4 µg g^{-1}), at which toxic effects are not very probable, but it is still far lower than the probable effect concentration (PEC, 111 µg g^{-1}), at which toxic effects are probable. The concentration of lead is on the very boundary of TEC (35.8 µg g^{-1}), while the concentrations of other metals are significantly lower than TEC values. A similar conclusion can be reached when the determined concentrations of metals are compared with the maximum values permissible for sediment (Official Gazette, No. 50/2012), since all concentrations are significantly lower than those levels.

For a more detailed description of the degree of anthropogenic influence on the sediment, two more indices customarily used to quantify it were calculated, namely the enrichment factor (EF) and the geoaccumulation index (I_{geo}) (Figure 1A). It can be seen from Fig. 1A that values of EF for the majority of metals are less than 2, which indicates their deficit or minimal enrichment (Cheng, Mana, Nie & Wong, 2013). Only the values for lead and cadmium are somewhat greater than 2, indicating moderate enrichment of the sediment with these metals in relation to the reference state.~~, i.e., the presumed composition of heavy metals in the pre-industrial era, which was determined on the basis of metal concentrations in average continental shale (Díaz-de Alba *et al.*, 2011)~~. For the majority of determined metals (Co, Cr, Cu, Fe, Mn, Ni, Zn), the values of I_{geo} are less than zero, which means that we are dealing with unpolluted sediment. Again, only for lead and cadmium are the values of I_{geo} insignificantly greater than zero, which means that the sediment can be considered unpolluted to moderately polluted with these two metals (Cheng *et al.*, 2013). It is important to stress that by far the lowest values of EF and I_{geo} are obtained precisely for copper and that its concentration in the tested sediment is more than two times less than TEC (31.6 µg g^{-1}), which, together with low values for the other metals, makes the sediment from fish farm pond NL2 very suitable for use as a substrate in further experiments. In the present investigation, preference is given to the natural over the artificial type of substrate even though the latter is characterized by the absence of contaminants and greater reproducibility of composition, since it has been demonstrated that the bioavailability of heavy metals is greater in artificial substrates than in natural ones, which makes it difficult to extrapolate the results obtained in an experiment to the state of affairs in natural populations (Harrahy & Clements, 1997).

Influence of the sediment on the composition of heavy metals in ~~*Ch. plumosus*~~*C. plumosus* larvae (Table 1) was quantified with the aid of the biota-sediment accumulation factor (BSAF, Fig. 1B). The values of BSAF indicate that the level of accumulation of Cd, Co, Cr, Fe, Mn and Ni in relation to the sediment is very low. A significant exception is observed for copper, whose ~~BASF~~ BSAF value is close to one, but also for lead and zinc, which are characterized by 2.5 and 4.5 times lower concentrations in larvae than in the sediment (Fig. 1B). In absolute terms, iron is characterized by the greatest concentration in the body of larvae, which was to be expected owing to the high content of hemoglobin in specimens of the genus *Chironomus*. Such a high concentration is nevertheless 49 times lower than in the sediment, which can be explained by the fact that the bulk of iron in the sediment is found in a form that is difficultly available to living organisms. The high values of BSAF for Cu and Zn can be attributed to the fact that they are essential elements for which strictly controlled mechanisms of assimilation must exist, while their concentrations in the sediment are relatively low, as is indicated by the lowest values of EF and I_{geo} (Figure 1A). Moreover, assimilation of the required amounts of these metals by larvae caused their concentrations in larvae to approach in greater measure the relatively low values in the sediment. Such reasoning cannot be applied to lead, both because it is not essential, but rather only a toxic element, and because it is characterized by the highest values of EF and I_{geo} (Figure 1A). Precisely when we analyse BSAF values for the tested heavy metals and chironomid larvae are we able to discern that they are very heterogeneous, both with respect to the absolute values obtained for individual elements in different studies, and in regard to the ratio of values between different elements. Thus, in the case of lead, BSAF was only 0.03 in the study of Reinhold, Hendriks, Slager and Ohm (1999) and was similar in the study of Hamidian, Zareh, Poorbagher, Vaziri and Ashrafi (2016), whereas in the study of Rezaei, Kamali and ~~-~~Shapoori (2011) the values of ~~BASF~~BSAF for lead were significantly higher and ranged from 0.26 to 3.9. The situation is similar in the case of other elements. The cause of such heterogeneity lies in the fact that the availability of heavy metals to larvae is dictated by many factors, the most significant of which are the representation of magnesium and iron oxides and presence of organic substances in the sediment (Shea, 1988; Young & Harvey, 1991). Moreover, these factors can exert completely different action on availability of the same element, depending on the dominant chemical form in which it is found (Bervoets, Blust, De Wit & Verheyen, 1997). Apart from that, it has been demonstrated that the extent of bioaccumulation declines with increase in the concentration of a certain element in the environment (DeForest, Brix & Adams, 2007). It is obvious that bioaccumulation of heavy metals from the sediment by chironomid larvae is a very complex process, that its usefulness as a bioindicator of heavy metal pollution varies greatly depending on composition of the sediment and that additional studies are needed for an adequate understanding of the mechanisms governing this dependence.

In order to establish whether morphological changes in the ~~oral apparatus~~mouthparts can be regarded as an indicator of increased copper concentration in the sediment, we tested the effects of adding copper in a series of concentrations ranging from that found in the fish farm's sediment (15 µg g^{-1} all the way up to 120 µg g^{-1}), which is somewhat higher than the maximum permissible concentration of copper in sediment (Official Gazette, No. 50/2012). At a concentration of 15 µg g^{-1} Cu, the only type of deformity is shortening of the median teeth, which occurs in 2.0±0.2% of individuals (Figure 2). This is the "mildest" form of deformity of the mentum (Martinez *et al.,* 2006; Al-Shami *et al.,* 2010), which along with its low representation indicates that the given concentration of copper in combination with other heavy metals in the sediment of pond NL2 exerts negligible influence on the occurrence of such deformities, thereby justifying further use of the fish farm's sediment in the control group.

At a concentration of 30 µg g^{-1} Cu, the representation of shorter median teeth increases with statistical significance to 16 ± 2% (*P* = 0.004) and a new deformity appears, namely shorter median-lateral teeth, also in 16 ± 2% of individuals. Total deformity incidence also increase to 31 ± 3% (*P* = <0.001). At a concentration of 60 µg g^{-1} Cu, the representation of shorter median-lateral teeth, ~~and lateral~~ median teeth and total deformities increases with statistical ~~-~~significance to 24 ± 2% (*P* = 0.036), ~~and~~ 28±4% (*P* = 0.048), and 69 ± 7% (*P* = 0.007) respectively, in addition to which a new deformity appears, namely tooth loss in 16 ± 3% of examined individuals (Figure 2). At the highest concentration (120 µg g^{-1}), the representation of shorter median teeth declines with statistical significance to 12 ± 4% (*P* = 0.047), that of shorter median-lateral teeth remains unchanged (24 ± 2%), and the share of individuals with tooth loss increases to 20 ± 3%~~)~~, but the increase is not statistically significant (Figure 2). Total deformity incidence slightly decreases to 55 ± 7% but with no statistical significance.

It is evident that both the representation and the severity of deformities detected in the mentum of chironomid larvae increase with increase in the concentration of copper in the medium. The greatest concentration of copper is an exception. This departure can be attributed to a statistically significant increase of larval mortality at the given concentration in relation to the control and the other two tested concentrations (Figure ~~2~~3). To be specific, increase in the concentration of copper in the substrate leads to a gradual increase of larval mortality, but it is not statistically significant

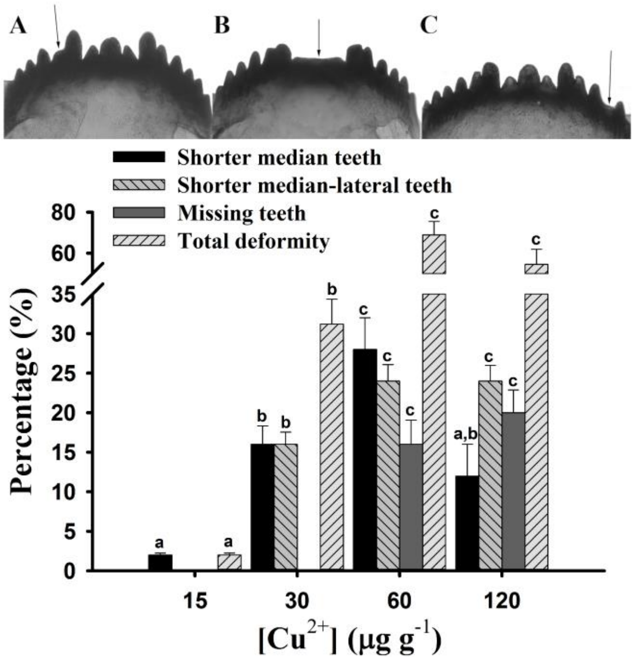

Figure 2. Percentage representation of shorter~~-~~median teeth ~~median-lateral teeth~~, shorter ~~median teeth~~median-lateral teeth, tooth loss and ~~number of dead specimens~~total mouthparts deformities of ~~*Ch. plumosus*~~*C. plumosus* larvae as a function of copper concentration in the substrate. Upper images: A) shorter median-lateral tooth; B) shorter median tooth; C) tooth loss. Values in the frame of each type of response that are designated by ~~the same~~ different letters (a,b,c) shows statistically significant differences ~~are different with statistical significance~~ (P<0.05).

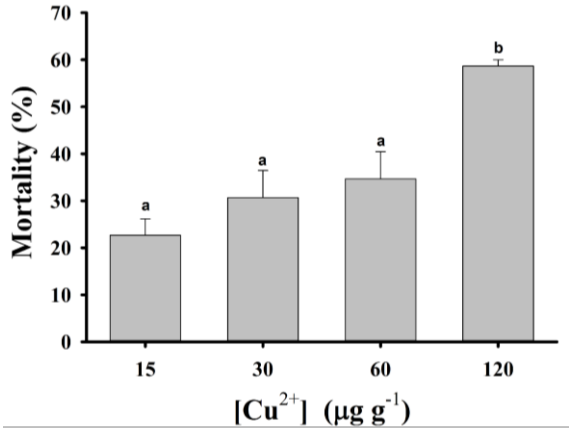

Figure 3. Dependence of *C. plumosus* larvae mortality on copper concentration in the substrate. Bars showing statistically significant differences (P<0.05) are marked with different letters (a, b).

initially and ranges from 23 ± 4% (15 µg g^{-1}) to 35 ± 6% (60 µg g^{-1}), a significant increase in the level of larval mortality to 59 ± 1% occurring only at a concentration of 120 µg g^{-1} (Figure 2). Such high mortality reduced size of the experimental group to 10 to 11 individuals from the starting 25 specimens, which casts doubt on reliability of the obtained results only at a concentration of 120 µg g^{-1}. The great mortality at a concentration of 120 µg g^{-1} can be attributed to the fact that the highest concentration of copper in our experiment is 10 µg g^{-1} greater than the maximum permissible concentration of copper in sediment (Official Gazette, No. 50/2012).

The types of deformities of larvae at the lowest concentration in the study of Martinez *et al*. (2003), where the monitored copper concentrations were similar to those in our experiment, were tooth loss, fused teeth and median tooth division. In our experiment, the most frequent type of deformity at the lowest concentration was occurrence of shorter median teeth, but tooth loss and fused teeth were absent. In contrast to the indicated study, where the most frequent deformities at medium copper concentrations were tooth loss and fused teeth, in our experiment the most frequent deformities at these concentrations were on the median and median-lateral teeth, which were shorter. At the greatest copper concentration, the most frequent deformities were tooth loss, divided median teeth and fused teeth in the cited study of Martinez *et al*. (2003), whereas in our experiment it is impossible to say what type of deformity is the most frequent due to the elevated mortality of chironomid larvae under conditions of the highest concentrations. A high rate of mortality at high copper concentrations was also recorded elsewhere (Martinez *et al*., 2006) and can be attributed to inability of the larvae to use their ~~oral apparatus~~mouthparts owing to the high degree of its deformity, thereby creating a situation in which some specimens are incapable of neutralizing metal stress through metabolism or excretion (Krantzberg & Stokes, 1989).

On the basis of the results obtained in this investigation and those of previous studies, it is entirely clear that increase in the concentration of copper leads to progressive increase in the frequency and severity of deformities appearing in the ~~oral apparatus~~mouthparts of chironomids. However, the type of deformities occurring as a function of copper concentration is fairly variable. All in all, it can be stated that deformities of the ~~oral apparatus~~mouthparts in chironomids have great potential as indicators of pollution with copper and can be used for rapid detection of the presence of this metal in aquatic ecosystems.

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