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Sensitivity of three unionid glochidia to elevated levels of copper, zinc and lead

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ABSTRACT

Key-words: *glochidia, ecotoxicity, copper, zinc, lead, unionid species*

In this study glochidia as most sensitive life-stage of bivalves were used to evaluate the toxicity of copper, zinc and lead. Sensitivity of three species, *Anodonta anatina*, *Pseudanodonta complanata* and *Unio tumidus* were compared to copper and zinc, based on 24 and 48 h LC_{50}s. The copper 24 h LC_{50} showed the lowest value (26.8 $\mu g \cdot L^{-1}$) for *Unio tumidus* glochidia. After 48 h exposure, close LC_{50} values, 18.9 and 19.0 $\mu g \cdot L^{-1}$, were recorded for *A. anatina* and *U. tumidus* glochidia, respectively. *U. tumidus* glochidia exhibited the highest sensitivity to zinc (48 h LC_{50} = 134.2 $\mu g \cdot L^{-1}$) followed by *P. complanata* (48 h LC_{50} = 201.6 $\mu g \cdot L^{-1}$) and *A. anatina* (48 h LC_{50} = 233.5 $\mu g \cdot L^{-1}$). Toxicity of lead to *P. complanata* glochidia was estimated, 24 and 48 h LC_{50}s of lead to 374.6 and 260.8 $\mu g \cdot L^{-1}$, respectively. No observed effect concentrations (NOECs) for the tested metals exhibited species differences. All NOECs exceeded environmental quality standards (EQS), therefore these species are at risk only in such European freshwaters which have extreme concentration of these metals. Synergistic effect was reported for the combinations of Cu + Zn and Cu + Pb, additive effect was reported for Zn + Pb.

RÉSUMÉ

Sensitivité de trois glochidies d'unionidés à des niveaux élevés de cuivre, zinc et plomb

Mots-clés : *glochidie, écotoxicité, cuivre, zinc, plomb, espèces d'unionidé*

Dans cette étude, les glochidies comme stade de vie le plus sensible de bivalves ont été utilisées pour évaluer la toxicité du cuivre, du zinc et du plomb. La sensitivité de trois espèces, *Anodonta anatina*, *Pseudanodonta complanata* et *Unio tumidus*, aux cuivre et zinc a été comparée, à partir des LC_{50} 24 et 48 h. La LC_{50} 24 h du cuivre présente la valeur la plus basse (26,8 $\mu g \cdot L^{-1}$) pour les glochidies de *Unio tumidus*. Après 48 h d'exposition, des valeurs LC_{50} proches (18,9 et 19,0 $\mu g \cdot L^{-1}$) ont été enregistrées pour les glochidies de *A. anatina* et *U. tumidus* respectivement. Les glochidies de *U. tumidus* montrent la sensibilité la plus forte au zinc (48 h LC_{50} = 134,2 $\mu g \cdot L^{-1}$) suivie de *P. complanata* (48 h LC_{50} = 201,6 $\mu g \cdot L^{-1}$) et *A. anatina* (48 h LC_{50} = 233,5 $\mu g \cdot L^{-1}$). La toxicité du plomb vis à vis des glochidies de *P. complanata* a été estimée : LC_{50} 24 et 48 h respectivement 374,6 et 260,8 $\mu g \cdot L^{-1}$. Les concentrations sans effet observé (NOECs) pour les métaux

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testés montrent des différences selon les espèces. Toutes les NOEC sont au-dessus du standard de qualité environnementale (EQS), par conséquent ces espèces ne seront à risque que dans les eaux douces européennes où les concentrations de ces métaux seraient extrêmes. Un effet synergique est constaté pour les combinaisons Cu + Zn et Cu + Pb, un effet additif pour Zn + Pb.

INTRODUCTION

Although some metals are essential for life (Engel and Sunda, 1979), all metals are toxic at high concentrations and for some there is a narrow range of concentrations between what is essential and what is toxic. Elevated metal concentrations can cause severe reduction or elimination of intolerant species, thereby having a significant effect on the diversity and trophic structure of the biological community (Peterson, 1986). In this study three metals were selected for assessing their toxic effect, all of them might pose potential hazard in Lake Balaton and in freshwater ecosystems in general. In aquatic habitats, copper is a predominant pollutant since the copper based compounds are used as herbicides, algicides, and molluscicides (WHO, 1993). Its levels range from 0.04–294 $\mu g \cdot L^{-1}$ (An and Kampbell, 2003) reaching 20 000 $\mu g \cdot L^{-1}$ in critical situations (Goodyear and McNeill, 1999). Organo-zinc compounds are ingredients in several fungicides, antibiotics and lubricants (WHO, 2001). Exposure of aquatic organisms to elevated levels of zinc has been found to induce many hazardous biophysiological alterations as antioxidant stress to *Perna perna* and *Unio tumidus* (Cossu *et al.*, 2000; Franco *et al.*, 2006). The necessity to study the ecotoxicological impact of mixture of pollutants on aquatic ecosystems is increasing worldwide (Shaw *et al.*, 2006). The simultaneous exposure to several metals might lead to synergistic or antagonistic effects (Enserink *et al.*, 1991). For example, Hanstčn *et al.* (1996) found that Mn (400 $\mu g \cdot L^{-1}$) had no effect on Cu and Cd, but increased the toxicity of Zn.

Most freshwater mussels have a complex life cycle including a larval stage, called glochidia, which is an obligatory parasite on fish or other vertebrates (Pennak, 1989). In case of many species they are released from the brooding chambers in the gills (marsupia) of the females into the surrounding water where they will encyst upon a host. They are able to survive in the water from 1 to 14 days searching for the host (Mackie, 1984; ASTM, 2006). Freshwater mussels (family Unionidae) are in serious global decline and in urgent need of protection and conservation (Newton *et al.*, 2008). In line with, this research presents the ecotoxicological testing of copper, lead and zinc alone or in combination on the glochidia as the most sensitive life stage of three freshwater mussels (family Unionidae). Although the toxicity of Cu and Zn to the glochidia of different unionids has been examined earlier (Pynnönen, 1995; Hanstčn, 1996; Conners and Black, 2004; Milam *et al.*, 2005; Gillis *et al.*, 2008), the toxicity of Pb has not been extensively studied. Furthermore, copper, zinc and lead interactions are not yet explained. Three species were selected as test organisms: *Anodonta anatina*, *Pseudoanodonta complanata* and *Unio tumidus*, as they co-exist in Hungarian ponds and lakes. At present there are no data available on the comparison of toxicity exerted by Cu, Zn and Pb on these three species. Moreover, *P. complanata* is a protected species in Hungary, therefore its sensitivity to environmental pollutants is of special concern.

MATERIALS AND METHODS

> *COLLECTION AND LABORATORY CARE OF THE MUSSELS*

A. anatina, *P. complanata* and *U. tumidus* gravid females were collected from Marcali reservoir (South of Lake Balaton) in mid-November, 2009. They were kept in glass aquaria for one week to allow them to get acclimatized to laboratory conditions. The water of the aquaria was changed weekly using water collected from Csigere stream (47°7.081'N, 17°25.244'E) (near

Devecser village west Veszprém, Hungary) which contained natural food for the mussels. The aquaria were well aerated, basic water quality parameters were as follows: temperature 16–18 °C, dissolved oxygen 7.08–9.07 $mg{\cdot}L^{-1}$, pH 7.98–8.72, conductivity 825–883 $\mu s{\cdot}cm^{-1}$. These parameters were measured using multi HQ 40d with electrode for each measured item.

> GLOCHIDIA COLLECTION AND TOXICITY TESTING

Collection of mature glochidia was carried out as prescribed by Johnson *et al.* (1993). First, the marsupia were flushed with water using 35 mL syringe. The collected glochidia were washed several times with partially reconstituted hard water, 170 $mg{\cdot}L^{-1}$ $CaCO_3$ and pH 8.38 (ASTM, 2006) in order to remove the mucus and dead ones. The viability of each larva was checked prior to use by testing its ability to close the valves while exposed to concentrated NaCl solution, 240 $g{\cdot}L^{-1}$. This was done by counting the number of larvae with opened valve before and after to the exposure to salt solution. Glochidia collected from three gravid females were pooled, except those of *A. anatina* as only one gravid female could be attained. Only, glochidia with viability $\geqslant$ 90% were used for the bioassay.

One hundred glochidia for each replicate were exposed to 100 mL of five serial concentrations of copper sulphate (in triplicate order) as a source of Cu (5, 10, 20, 40, 80 and 120 $\mu g{\cdot}L^{-1}$) and zinc sulphate as a source of Zn (50, 100, 200, 400, 800, and 1600 $\mu g{\cdot}L^{-1}$). Due to paucity of collected glochidia, only those of *P. complanata* were exposed to serial dilutions of $PbCl_2$ as a source of Pb (50, 100, 200, 400, 800, and 1600 $\mu g{\cdot}L^{-1}$). Also, they were exposed to a mixture of tested metals, Cu 40 + Zn 200; Cu 40 + Pb 400 and Pb 400 + Zn 200 $\mu g{\cdot}L^{-1}$. All tests were done in 250 mL flasks in a triplicate order at room temperature (20–22 °C) for 24 and 48 h. Flasks were covered to keep out extraneous contaminants and bacteria and also, to minimize evaporation. The distinction between dead and alive glochidia was done by counting the number of larvae with opened valve (N) and then placing some drops of concentrated sodium chloride. The alive glochidia normally close the valve; the glochidia with opened valve were than counted again (M). The percentage of survival was calculated as follows: (M – N/M)100. Accordingly, the percentage mortality was recorded. For each test chamber in each treatment, other than the controls, the mortality rate was corrected using Abott's formula (Abott, 1925): $E = 100(A - M)/(100 - M)$ where E is the mortality rate, adjusted for the controls, A is the mortality in the test and M is the average mortality in the control treatments.

> STATISTICAL ANALYSIS

One way analysis of variance (ANOVA) was done using Origin software (Origin 7.5) to get the significant difference ($P \leqslant 0.05$) between the groups (Sanders *et al.*, 1985). The estimation of toxicant concentration resulting 50% larval mortality of the exposed organisms (LC_{50}s) and its confidence limit were calculated using US EPA probit analysis program version 1.5. No-observed effect concentrations (NOECs), as the uppermost concentration being not significantly different from controls ($P \leqslant 0.05$), were also calculated, using Origin 7.5 software.

To test the interactive effect of both metals, the theoretically expected effect (E) of the binary mixtures on the test organisms was evaluated using the formula proposed by Kungolos *et al.* (1999) and Hadjispyrou *et al.* (2001). It was calculated using the following formula: $E = P_1 + P_2 - P_1P_2/100$, where E is the theoretical expected effect, P_1 is the inhibition caused by chemical A and P_2 is the inhibition caused by chemical B. This model is based on the hypothesis that the toxicants act concurrently and not successively on the affected organism (Kungolos *et al.*, 2009). The toxicity of binary mixtures was assessed in triplicate order. The observed toxicity of the chemical mixture (O), which was assessed experimentally, and the expected mixture toxicity (E), which was estimated based on the previous equation were compared. Toxic effect can be considered antagonistic or more than additive (synergistic) only if the observed effect (O) is significantly lower or higher, respectively, than the theoretically expected (E) value at $P \leqslant 0.05$.

Table I
Acute copper, zinc and lead as LC_{50}s and no-observed effect concentrations (NOEC) ($\mu g \cdot L^{-1}$) at 24 and 48 h for glochidia of some unionid species.

Tableau I
Toxicité du cuivre, zinc et plomb mesurée par la LC_{50} et concentrations sans effet observé (NOEC) ($\mu g \cdot L^{-1}$) à 24 h et 48 h pour les glochidies de quelques espèces d'unionidés.

Metals	Species	24 h			48 h		
		Control survival (%)	LC_{50}	NOEC	Control survival (%)	LC_{50}	NOEC
Copper	*A. anatina*	91.5 ± 0.8	31.2 (21.6–47.3)[a]	9	91.4 ± 0.5	18.9 (10.0–31.1)[a]	8
	P. complanata	91.5 ± 0.7	34.3 (29.9–39.4)[a]	12	90.8 ± 0.5	29.3 (25.4–34.0)[a]	10
	U. tumidus	93.6 ± 0.7	26.8 (23.2–30.9)[b]	12	90.5 ± 0.2	19.0 (16.2–22.0)[b]	8
Zinc	*A. anatina*	91.6 ± 0.8	361.1 (314.9–415.5)[a]	90	90.3 ± 0.5	233.5 (199.8–271.9)[a]	60
	P. complanata	91.5 ± 0.7	262.5 (230.5–298.9)[b]	70	90.8 ± 0.5	201.6 (175.5–230.5)[a]	55
	U. tumidus	93.6 ± 0.8	180.6 (55.4–208.3)[c]	60	90.5 ± 0.2	134.2 (16.1–153.5)[b]	50
Lead	*P. complanata*	91.5 ± 0.8	374.6 (321.0–440.1)	32	90.0 ± 0.5	260.8 (226.0–300.5)	29

Confidence limit indicated between brackets. Control data expressed as Mean ± SE. Data based on corrected mortality, where corrected mortality = 100(A – M)/(100 – M) where A mortality of test and M average mortality of control. Means of different superscript articles in same column are significantly different at $P \leqslant 0.05$.
Les limites de confiance sont entre parenthèses. Les données témoins sont des moyennes ± SE. La mortalité observée est corrigée selon la formule mortalité corrigée = 100(A – M)/(100 – M) où A est la mortalité du test et M la mortalité moyenne du témoin. Les lettres en exposant indiquent des différences significatives dans la colonne ($P \leqslant 0,05$).

RESULTS

> *SINGLE EFFECT OF THE METALS*

Viability of the glochidia in the control were 91.5 ± 0.8 and 91.4 ± 0.5% for *A. anatina*, 91.5 ± 0.7 and 90.8 ± 0.5% for *P. complanata* and 93.6 ± 0.7 and 90.5 ± 0.2% for *U. tumidus*, after 24 and 48 h respectively (Table I). The Cu 24 h LC_{50}s for glochidia of the tested mussels ranged from 26.8 (23.2–30.9 $\mu g \cdot L^{-1}$) to 34.3 (29.9–39.4 $\mu g \cdot L^{-1}$) being *U. tumidus* as the most and *P. complanata* as the least sensitive (Table I). 48 h LC_{50}s showed similar tendency. *A. anatina* glochidia showed medium sensitivity, with 24 h and 48 h LC_{50}s of 31.2 (21.6–47.3) and 18.9 (10.0–31.1) $\mu g \cdot L^{-1}$, respectively. 24 h and 48 h NOECs were also calculated from the mortality data, these values fell very close to each other: 9 and 8 $\mu g \cdot L^{-1}$ for *A. anatina*, 12 and 10 $\mu g \cdot L^{-1}$ for *P. complanata* and 12 and 8 $\mu g \cdot L^{-1}$ for *U. tumidus*.
U. tumidus glochidia proved to be the most sensitive to Zn, too, with the lowest 24 and 48 h LC_{50}s of 180.6 (55.4–208.3) and 134.2 (16.1–153.5) $\mu g \cdot L^{-1}$, respectively (Table I). The lowest Zn sensitivity was reported for the glochidia of *A. anatina*, giving 24 and 48 h LC_{50}s 361.1 (314.9–415.5) and 233.5 (199.8–271.9) $\mu g \cdot L^{-1}$, respectively. For Zn, *P. complanata* showed medium sensitivity, with 24 and 48 h LC_{50}s of 262.5 (230.5–298.9) and 201.6 (175.5–230.5) $\mu g \cdot L^{-1}$, respectively. Acute Zn NOECs at 24 and 48 h were: 90 and 60 $\mu g \cdot L^{-1}$ for *A. anatina*, 70 and 55 $\mu g \cdot L^{-1}$ for *P. complanata* and 60 and 50 $\mu g \cdot L^{-1}$ for *U. tumidus*, respectively.
The data in Table I show the Pb 24 and 48 h LC_{50}s to the glochidia of *P. complanata*, being 374.6 (321.0–440.1) and 260.8 (226.0–300.5) $\mu g \cdot L^{-1}$. Comparing the toxicity of the tested metals, it can be concluded that the order of toxicity towards *P. complanata* glochidia was Cu > Zn > Pb. The Pb NOECs calculated for *P. complanata* glochidia were 32 and 29 $\mu g \cdot L^{-1}$ after 24 and 48 h, respectively.

> *BINARY EFFECT OF THE METALS*

The data presented in Table II show the binary interactive effect of Cu, Zn and Pb on the glochidia of *P. complanata.* The binary interactive effect of Cu and Zn resulted in significantly

Table II
Comparison between theoretically expected and observed interactive effects of the binary mixtures of copper, zinc and lead on corrected mortality of glochidia of Pseudanodonta glochonata*.*

Tableau II
Comparaison entre effets interactifs attendus et observés de mélanges binaires de cuivre, zinc et plomb sur la mortalité corrigée des glochidies de *Pseudanodonta glochonata.*

	24 h		48 h	
Metal interactions ($\mu g \cdot L^{-1}$)	Observed corrected mortality (O)	Expected corrected mortality (E)	Observed corrected mortality (O)	Expected corrected mortality (E)
Cu 40 + Zn 200	85.3 ± 0.93↑	64.11 ± 3.29	90.18 ± 2.13↑	74.12 ± 2.14
Cu 40 + Pb 400	89.62 ± 1.88⁺	82.74 ± 4.12	92.25 ± 0.95↑	85.35 ± 1.22
Pb 400 + Zn 200	79.73 ± 1.51⁺	69.36 ± 4.55	87.67 ± 1.53⁺	86.21 ± 1.42

Where corrected mortality = 100(Mc – Mt)/Mc. Number of replicates are 3. ↑ is significantly higher than E, more than additive. ⁺ Non-significantly higher value of O than E means additive effect. Significantly lower O value than E means antagonistic effect.
Mortalité corrigée = 100(Mc – Mt)/Mc. Le nombre de réplicats est de 3. ↑ est significativement plus grand qu'un simple effet additif. ⁺ Valeur de O égale à E, simple effet additif. Valeur O plus petite que E : effet antagoniste.

higher observed effect in comparison to the expected one, so they most probably exerted synergistic effect. The combination of Cu and Pb after 24 h exposure showed additive effect, whereas after 48 h their interaction was found to be synergistic. The lowest toxic effect was reported for the combination of Pb and Zn (additive effect at both exposure periods).

DISCUSSION

Sensitivity of the three tested species to Cu fell in the same range, with minor differences. For *A. anatina* glochidia, 24 h LC_{50} was close to the 40 $\mu g \cdot L^{-1}$ (24 h LC_{50}) reported earlier by Hanstčn *et al.* (1996). Although no comparative data are available for *P. complanata*, studies involving different species might indicate that the sensitivity of *P. complanata* and *U. tumidus* also fell in the range generally determined for freshwater bivalves (Jacobson *et al.*, 1997; Wang *et al.*, 2007; Gillis *et al.*, 2008).

Regarding Zn sensitivity, the tested species exhibited considerably higher differences. *U. tumidus* was the most sensitive species, followed by *P. complanata and A. anatina*. 24 h LC_{50} of *U. tumidus* (180.6 $\mu g \cdot L^{-1}$) was close to the value reported (173.5 $\mu g \cdot L^{-1}$) for *Ceriodaphnia dubia* (Cooper *et al.*, 2009). For *A. anatina* glochidia, the 24 h LC_{50} was close to 380 $\mu g \cdot L^{-1}$ (24 h LC_{50}) reported earlier by Hanstčn *et al.* (1996). The susceptibilities of the glochidia of *A. anatina* and *P. complanata* to Zn were more or less similar to those reported formerly for *A. cygnea* (Pynnönen, 1995).

Considering the toxicity of lead, there are no comparative data for *P. complanata*. However, 48 h LC_{50} was close to 208.8 $\mu g \cdot L^{-1}$ (48 h LC_{50}) reported by Cooper *et al.* (2009) for *C. dubia*.

It is important to analyse if response of the tested glochidia actually indicate environmental risk. Neal *et al.* (1996) give environmental quality standard (EQS) limits for copper, zinc and lead as follows: 6–10 $\mu g \cdot L^{-1}$ for copper, 50–75 $\mu g \cdot L^{-1}$ for zinc and 8–18 $\mu g \cdot L^{-1}$ for lead.

Acute copper no-observed effect concentrations (NOECs, $\mu g \cdot L^{-1}$) at 24 and 48 h for glochidia of the three species were: *A. anatina* 9 and 8 $\mu g \cdot L^{-1}$, *P. complanata* 12 and 10 $\mu g \cdot L^{-1}$, and *U. tumidus* 12 and 8 $\mu g \cdot L^{-1}$. These values are well above copper concentrations in Lake Balaton which were measured by Nguyen *et al.* (2005) and they found that the concentration range varied between 0.22 to 0.59 $\mu g \cdot L^{-1}$. Although no environmental risk posed by copper is present in Lake Balaton, sensitivity of all three species falls in the environmentally relevant range of 0.5 to 100 μg $Cu \cdot L^{-1}$ as defined by Bossuyt and Janssen (2003), showing that they can be at risk in some freshwater habitats.

The situation is very similar for zinc. Acute Zn NOECs ($\mu g \cdot L^{-1}$) at 24 and 48 h for glochidia of the three species were: 90 and 60 for *A. anatina*, 70 and 55 for *P. complanata* and 60 and 50 for *U. tumidus*. As reported by Nguyen *et al.* (2005) Zn concentration in Lake Balaton fell in the range of 0.22–1.9 $\mu g \cdot L^{-1}$ which are within EQS limits. Although unionid species seem not to be at risk in Lake Balaton, Zn levels measured in other freshwater habitats exceed NOEC values recorded in our experiment, meaning that in Europe the ecological risk cannot be neglected. For example, in River Rhine as high as 330 μg $Zn \cdot L^{-1}$ concentration was reported (Neal *et al.*, 1996) which is considerably higher than these NOEC values.

Ecological risk of Pb follows a very similar pattern. 24 and 48 h acute NOECs for *P. complanata* were 32 and 29 $\mu g \cdot L^{-1}$, respectively. According to Neal *et al.* (1996), characteristic level in Lake Balaton is between 0.11–3.54 $\mu g \cdot L^{-1}$ which fells within EQS. As the reported Pb NOECs were higher than these EQS and environmental realistic levels in Lake Balaton, Pb does not pose environmental risk for *P. complanata* in this ecosystem but in some European freshwater habitats environmental concentrations might well exceed these NOEC values (*e.g.* Neal *et al.*, 1996; Nedjai, 2007).

Regarding the toxicity order, the glochidia of the tested mussels exhibited higher sensitivity for Cu than Zn. Such toxicity order is reported earlier for *A. cygnea* (Pynnönen, 1995). Also it is reported by Loayza-Muro and Elias-Letts (2007) for *Anodontites trapesialis* using filtration rate as another end point. The glochidia of *P. complanata* biotest exhibited the following decreasing sensitivity order: Cu, Zn, Pb. There are no comparative data for *P. complanata*, but Cooper *et al.* (2009) reported the same toxicity order for *Daphnia magna* and *Ceriodaphnia dubia*.

The data reported herein showed that the interactive effect of Cu + Zn were synergistic as indicated by the mortality of *P. complanata* larvae. Synergistic actions of the mixture of Cu with either Zn or Pb were reported by Cooper *et al.* (2009) using *C. dubia* and *D. magna* bioassays. However, in our study, the interaction of Cu + Pb was found additive. Interaction of metals may be influenced by the tested species, the combination of metals and water quality (Otitoloju, 2002; Kungolos *et al.*, 2009). Spehar and Fiandt (1986) indicated that the same combination of metals (arsenic, cadmium, chromium, copper, mercury and lead) showed different interactive effects depending on both the species exposed and the endpoint tested. Utgikar *et al.* (2004) reported that the toxic effects of binary mixtures were substantially higher than simple addition of toxicity of the individual metals.

Our study was especially targeted to examine the sensitivity of *P. complanata*. This species is not only protected in Hungary but is listed as 'Near Threatened' on the IUCN red list (IUCN, 2006). Gillis *et al.* (2008) when testing glochidia of nine unionids found that the endangered species are less tolerant to the contaminants than non-endangered species. However, in our case, *P. complanata* exhibited lower sensitivity than either *U. tumidus* or *A. anatina*.

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