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Bioaccumulation and Elimination of Waterborne Mercury in the Midge Larvae, *Chironomus riparius* Meigen (Diptera: Chironomidae)

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Abstract Here, mercury kinetics and behavioural effects in the midge larvae under a water-only exposure were assessed. Uptake and elimination of waterborne mercury were described by using a one-compartment kinetic model. Results show that midges were able to readily accumulate the heavy metal (BCF = 450), presenting a fast uptake, up to 13.1 μg Hg g of animal^{-1} at the end of the exposure period. Elimination was slow, with c.a. 39 % of the mercury in larvae being depurated after 48 h in clean medium. Behaviour did not present differences upon exposure or elimination, but a trend to increase ventilation was noticed during the exposure period.

Keywords Toxicokinetics · Online biomonitoring · Mercuric chloride · Water only exposure

The Minamata (Japan) incident in the 1950's triggered the global concern regarding the mercury contamination hazard. Mercury is a very common contaminant in the environment, having natural sources—such as volcanism—and anthropogenic origins such as coal burning, mine tailings or industrial effluents (Morel et al. 1998; Wolfe et al. 1998). Due to its high volatility, it can also be dispersed via atmospheric transportation and deposited in other regions, thus being available to biota even in regions distant from any point source, especially in the form of Hg(II) or MeHg, after being methylated (Morel et al. 1998). Despite the imposed legislation having reduced/minimized mercury discharges to the environment in the last decades, mercury legacy in sediments and soils continues to be a matter of great concern at a global scale, since it is a persistent contaminant that bioaccumulates even at low concentrations and exhibits high toxicity towards some aquatic organisms (Azevedo-Pereira and Soares 2010). In fact, mercury can present an increased ecological risk because it can be biomagnified throughout the trophic chain (Morel et al. 1998). In aquatic environments, benthic fauna plays an important role in the food web, representing a link with higher trophic levels (Armitage et al. 1995). This way, contamination affecting these ecological communities can affect the distribution and abundance of benthic fauna, and any deleterious effects of pollutants in these organisms can be consequently reflected in the whole ecosystem (Fleeger et al. 2003). One of the most ubiquitous and cosmopolitan benthic group that can be found in both lotic and lentic environments, playing an important role as prey for fish, is the aquatic diptera *Chironomus* spp. (Armitage et al. 1995). The midge *Chironomus riparius* (Meigen) is commonly used to assess sediment toxicity both in situ and in the laboratory (OECD 2004), and can also be used in water only toxicity tests (Lydy et al. 2000) which is a route that should not be neglected as an exposure pathway (although benthonic, they also live in close contact with the water body). Mercury ecotoxicity on chironomids have focused mainly on bioaccumulation (e.g. Rossaro et al. 1986),

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morpho-physiological, and behavioural parameters (e.g. Azevedo-Pereira and Soares 2010). Because accumulation of mercury by aquatic organisms is rapid and its depuration is slow (especially in the case of organomercury compounds, like methylmercury), it has an increased potential for biomagnification (Eisler 1987). Thus assessing bioaccumulation and relate it with other parameters is important to understand how potentially toxic agents and their concentration in the media and tissues can affect the organism physiological responses. Any contaminant affecting an organism implies an uptake of the toxicant and is closely related to its ability to accumulate and eliminate the substance. According to Nuutinen et al. (2003; after McCarty and Mackay 1993), toxicological processes may comprise three general steps: the first one includes the period of time upon which an organism is exposed to a certain chemical and the relative bioavailability of that chemical during the exposure (exposure phase); the second includes the uptake, distribution, metabolism, and elimination of the bioavailable portion of the toxicant (toxicokinetics phase); and the third involves the biological response resulting from the chemical arriving at the site(s) of toxic action in the organism and acting to produce its toxic effect(s) in a time dependant manner (toxicodynamics phase). Integrating the complexity of the organisms' physiological processes and mechanisms with the environmental stimuli that cause them (e.g. metal accumulation), organism behavioural responses will contribute to better perceive what happens in contaminated ecosystems (Dell'Omo 2002). These behavioural alterations can be measured using biomonitors, giving us a fast, measurable and discriminate sub-lethal response, which have been used in multiple ecotoxicological tests, including tests with chironomids (Azevedo-Pereira and Soares 2010). The present study aimed to simulate what could happen if a possible chlor-alkali discharge occured, by evaluating the kinetic performance (bioaccumulation and elimination) of waterborne mercuric choride in the benthic invertebrate *C. riparius* Meigen and establishing a relationship between its toxicokinetics and ecotoxicity using behaviour as a relevant endpoint.

Materials and Methods

Chironomus riparius Meigen (Diptera, Chironomidae) larvae were obtained from a laboratory culture established for more than 4 years in conditions described in Azevedo-Pereira and Soares (2010). Prior to the test, several egg ropes were removed from the culture and transferred into a crystallizing dish with hard water ASTM medium (ASTM 1980) and placed at 20°C. When the larvae ecloded they were transferred again into a beaker with acid-washed inorganic fine sediment as substrate and ASTM hard water until they reached the size needed for the tests. Mercuric (II) Chloride (HgCl2; Merck KGaA, Darmstadt, Germany) was used to prepare the appropriate stock solutions of mercury with ultra-pure water, and the concentration determined by a Mercury Analyser from LECO—AMA254 (St. Joseph, Michigan, USA)—see details below. Stock solution concentration was 9.113 mg Hg L^{-1} and it was stored at room temperature, protected from light and periodically analysed. Tests solutions were prepared by diluting the stock solution in ASTM hard water, in order to reach the pre-established concentration of 38 μg Hg L^{-1}. This bioassay was performed in static conditions as a water only toxicity test, at 20 ± 1°C with a 16 h:8 h light:dark photoperiod. No substrate was used in the uptake experiments to avoid possible sorption to sediment. Eighty 350 mL plastic beakers containing 150 mL of test solution were prepared. One control and one concentration (38 μg Hg L^{-1}) were used, separated in 40 beakers per medium. The concentration was chosen based on the EC_{20} for growth, obtained in previous tests with the same species (Azevedo-Pereira and Soares 2010): EC_{20} (95 % CI) = 31.77 (7.50–50.30) μg L^{-1}. The medium in plastic beakers was allowed to stabilize for 4 h, upon which five 3rd instar larvae per each test vessel were carefully transferred—larval instars were verified according to its length. All larvae were previously unfed for 12 h in order to clear their guts of any organic particles. They remained unfed until the end of the test. All beakers were covered with perforated Parafilm™ in order to slow evaporation but assuring proper ventilation. Five sampling time periods were chosen from preliminary tests (1.30, 3, 6, 12 and 24 h after starting the exposure period). Three replicates per concentration were taken during these sampling period and water and animals analysed. Animals were removed and placed in a crystallizing dish with clean ASTM water for 5 min, in order to remove any possible Hg particles that might have been adsorbed to their surface. Afterwards, the animals were quickly passed through filter paper to remove any superficial water and promptly weighed (fresh weight) before being analysed for mercury content as a composite sample of five animals per replicate. Forty mL of each test solution were acidified (pH < 2.00, HNO3 65 % Fluka, Switzerland) and preserved for a maximum period of 96 h, upon which 40 μL of each test solution were analyzed as a measure of waterborne Hg. Simultaneously, a behavioural study using the Multispecies Freshwater Biomonitor (MFB) was conducted. This equipment records online the behaviour of aquatic species quantitatively, by measuring changes in impedance caused by organisms that move freely inside specific test chambers that have electrodes which generate an alternating current and detect the changes in this electrical field (description available in Azevedo-Pereira and Soares 2010). As a result of these changes in the electrical field, different chironomid behaviours can be assigned to different

frequencies, such as locomotion (low frequency movements like crawling, on the range 0.5–2.5 Hz) or ventilation (high frequency movements which involve undulation of the body in a regular pattern, on the range 3.0–8 Hz). These frequencies are obtained by previous tests comprising visual observation and simultaneous recording with the equipment and were also described in other studies (Azevedo-Pereira and Soares 2010). The data generated is presented as the percentage of time that the organism spends on each activity. In this parallel behavioural study, the test chambers were placed in 3L plastic beakers with the respective concentration (one beaker for control—ASTM hard water—and another for the 38 μg L^{-1} Hg concentration). Seven animals for control and ten animals for the 38 μg L^{-1} Hg concentration were exposed individually in the chambers. For each beaker, three additional empty chambers were added as blank replicates. The MFB recorded automatically for 4 min in every 10 min (equivalent to 6 recordings per hour) during the 24 h exposure period. After this period, 3 larvae were removed and analysed for mercury, following the procedures stated above. Water samples from the beakers containing the chambers were also acidified for posterior analysis. Bioconcentration factor (BCF) was estimated, at 24 h, using the following formula:

$$BCF = \frac{C_a}{C_w} \quad (1)$$

where Ca = concentration of mercuric chloride in the animal (μg g^{-1}) and Cw = concentration of mercuric chloride in the water (μg g^{-1}). After the exposure period, larvae were gently removed from each test beaker, placed in a crystallizing dish with clean ASTM water and transferred to glass vials with clean hard water ASTM, following the procedure adopted in the previous contaminated replicates. The larvae remained unfed. A time frame was established to analyse elimination of Hg in these specimens: three replicates per treatment were withdrawn and water and biological samples analysed after 3, 6, 12, 24, 48 and 72 h in clean medium. Biota and water sample processing for the determination of the mercury content was as described above. Regarding the behavioural patterns, the animals that were inside the MFB test chambers during the exposure experiments were removed, rinsed through ASTM hard water and then transferred individually into new test chambers that were placed inside 3L plastic beakers containing clean ASTM hard water, following the same procedure adopted in the previous contaminated test chambers. The biomonitor maintained the same recording patterns as described above, until the end of the 72 h post-exposure period. Physical–chemical parameters were measured at the beginning and every 48 h until the end of test.

The test solutions used to contaminate the medium were analysed before being added to the test vessels. All samples, including biota and preserved acidified water samples, were analysed directly by atomic absorption spectrometry (AAS), using an Advanced Mercury Analyser (AMA254—Mercury Analyser from LECO, St. Joseph, Michigan, USA). In this procedure the sample goes under thermal decomposition and the mercury vapour is collected by a gold amalgamator, as described by Hall and Pelchat (1997). For this work purpose, procedure was adapted—drying time 60 s, decomposition time of 150 s and waiting time of 45 s—in accordance with the volume and weight of the material used here. The accuracy of the data was assessed using the reference DORM-3 (fish protein certified reference material for trace metals) from the National Research Council Canada. In these experiments, the uptake and elimination kinetics of mercuric chloride in the organisms was described using a one-compartment model, allowing a simultaneous estimation of the assimilation (a) and elimination (k) rates. We used the constant exposure model (Sousa et al. 2000), applying the following equations:

For $t \leq t_c$

$$Q_t = \frac{a}{k}\left(1 - e^{-kt}\right) \quad (2)$$

And for $t > t_c$

$$Q_t = \frac{a}{k}\left(1 - e^{-kt_c}\right)e^{-k(t-t_c)} \quad (3)$$

where Q_t = concentration in the organisms at time t (μg Hg g of $animal^{-1}$); a = assimilation rate (μg Hg g of $animal^{-1}$ $day{-}1^{-1}$); k = elimination rate constant (d^{-1}); t = time (d); and t_c = time at which the animals were transferred to uncontaminated medium (d). The parameters used in the toxicokinetics model were estimated using a nonlinear estimation with the Quasi-Newton method for calculating least squares. Data from behavioural experiments were arcsin square root transformed to stabilise variances across treatments and two-way ANOVA's were performed, with mercuric chloride concentrations and hours as treatments. Where applicable, results are presented as mean ± SE. For all statistical tests the significance level was set at $p \leq 0.05$.

Results and Discussion

Preliminary tests (results not shown) indicated that a 24 h exposure period was sufficiently long to ensure adequate accumulation of mercury in the midges and to reach the beginning of the accumulation plateau. Since the test design implied the animals to stay unfed for 120 h, the

experimental procedure complied only a 24 h exposure period. This short term bioassay was intended to understand the toxicokinetics of waterborne mercury, without the integration of other factors such as metal adsorption by sediment, inorganic contaminated particles ingestion or food intake by the organisms, thus presenting only the influence of waterborne mercury on the larvae, which can not be disregarded in a real context. Other short term kinetic bioassays with chironomids, with no sediment or food addition, have been successful in determining toxicokinetic parameters and developing kinetic models that describe uptake and elimination of other chemicals such as DDE and 2-chlorobiphenyl (Lydy et al. 2000). In the uptake and elimination experiments the physical parameters pH, dissolved oxygen, and conductivity averaged 7.86 ± 0.11, 6.67 ± 0.3 mg L^{-1}, 500 ± 2.9 µS cm^{-1}, respectively, throughout the test duration. Upon stabilization, concentrations in the contaminated beakers were maintained relatively constant during the uptake experimental period (31 ± 3 µg L^{-1}). Mortality was measured as immobilization and was observed after 48 h of exposure, with 13 % mortality for controls at the end of the test. Cannibalism among chironomids was registered at 96 h, hence observations made at that time were not considered because results could be flawed. The use of a one compartment model allowed the simultaneous estimation of the uptake and elimination rates in ASTM hard water. The rate of Hg uptake in *C. riparius* larvae was quite rapid, being detected at the first sampling period just after initial exposure (Fig. 1), reaching an average value of 13.1 µg of Hg g of animal^{-1} at the end of the exposure period. The estimated kinetic parameter for assimilation rate (a) was 0.663 µg of Hg g of animal^{-1} day^{-1}. This quick process is comparable with the cadmium (Cd; Group B metal, like Hg) bioaccumulation in the same species (Timmermans et al. 1992) and Hg bioaccumulation rate in other invertebrates, like marine macrobenthonic species [*Scrobicularia plana* and *Hediste diversicolor* (Cardoso et al. 2009)]. Within the 48 h time frame upon which the elimination period occurred, the mercury concentration in the organisms started to decrease mildly just after they were transferred into clean ASTM hard water, reaching an average value of 8.03 µg of Hg g of animal^{-1} at the end of this period—only c.a. 39 % of the total mercury was eliminated in the time frame considered. The elimination rate ($k = 0.012$ day^{-1}) was however substantially lower than the one found for the same species in Cd toxicokinetic studies [0.20 day^{-1} for larvae from non-adapted populations (Postma et al. 1996)], probably due to the fact that in the present study the animals were not fed, thus excretion rates are expected to be much lower. Tsui and Wang (2007) also referred that the elimination process of Hg would be slow for *Daphnia magna*. The BCF found for mercury in our experiment was 450, much lower than the BCF found for Cd (6850) by Timmermans et al. (1992) and the BCF found for Hg in other studies done with the same species (1657; data not presented in this paper). Both experiments comprised feeding and the presence of a substrate (paper fibers or sediment). The low BCF in this experiment might be due to the established short elimination period, but also because this metal was relatively less bioavailable, since the midges were not fed nor had sediment in the beakers, as stated above. Any possible adsorption of Hg to the exoskeleton that could influence the results of the AAS analysis (and consequently have an impact on the elimination phase results) was not assessed, but other previous studies reported that metal adsorption to the exoskeleton was low compared to the uptake in internal tissues, like Cd uptake in the same species, where only an insignificant portion was adsorbed to the exoskeleton (Timmermans et al. 1992) or where 90 % of the metal was found in the guts (Postma et al. 1996). Even rinsing larvae in ASTM hard water in spite of acidified water did not prove to have significant differences among concentrations of metal obtained from exposed larvae (Timmermans et al. 1992, for Cd).

Faster elimination rates of metals in studies with feeding might be related with desorption and repartioning of the metal between the gut and clean sediment particles passing through it or even with higher excretion rates (since food intake can lead to higher metabolic rates). In fact, Tsui and Wang (2007) refer that for *Daphnia magna* assimilation efficiency of Hg is somewhat dependent on food density. In

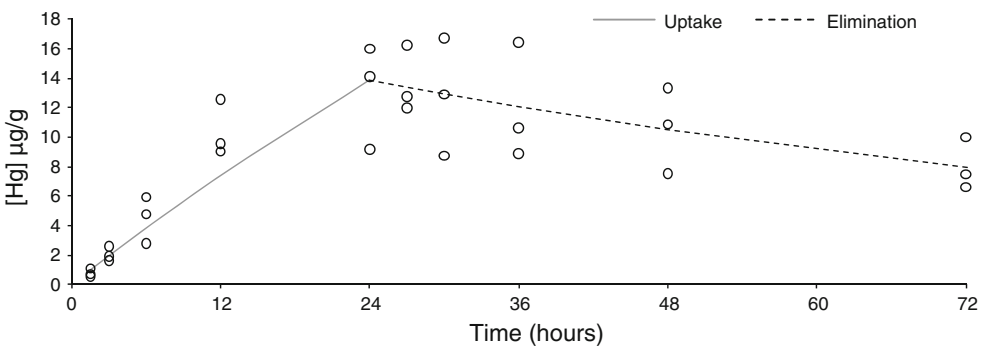

Fig. 1 Kinetic behaviour of mercuric chloride in *Chironomus riparius* during uptake and elimination phases. Organisms were exposed to contaminated ASTM hard water. Data was fitted by non-linear regression (see text for further explanation)

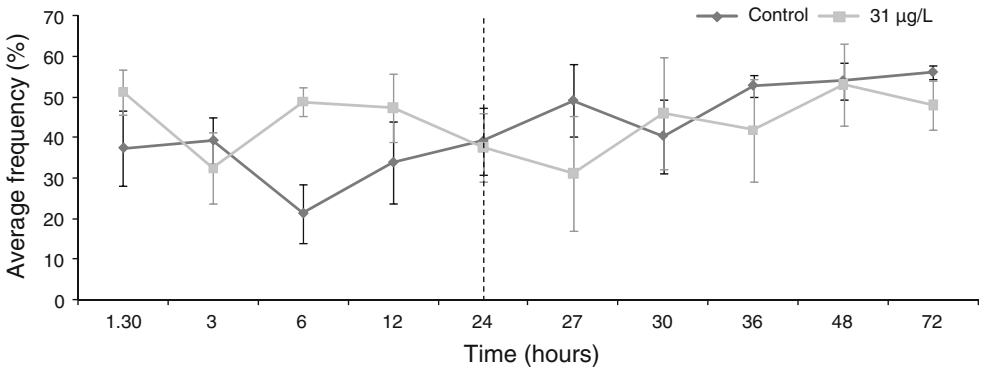

Fig. 2 Average activity frequencies of locomotion of *Chironomus riparius* when exposed to a concentration of 31 µg Hg L^{-1} for a period of 24 h. *Dashed line* represents the end of the uptake phase and the beginning of the elimination phase

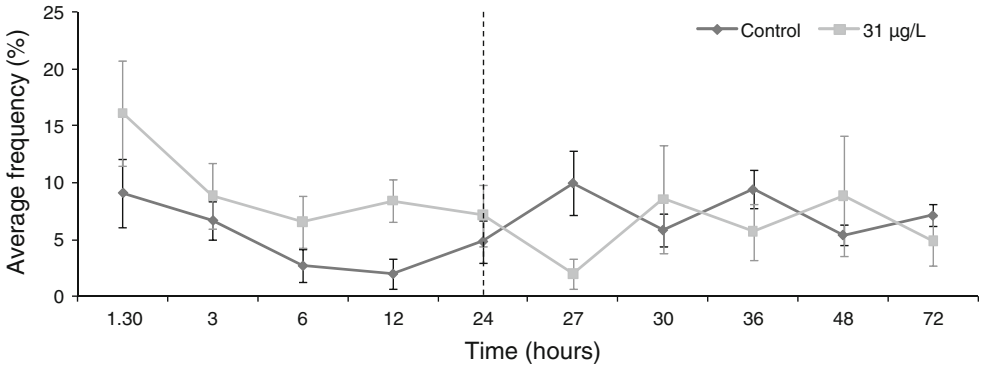

Fig. 3 Average activity frequencies of ventilation of *Chironomus riparius* when exposed to a concentration of 31 µg Hg L^{-1} for a period of 24 h. *Dashed line* represents the end of the uptake phase and the beginning of the elimination phase

this work any puzzling factors that could affect waterborne mercury bioaccumulation were avoided (as stated above), e.g. since fine sediments particles could adsorb Hg, therefore affecting the water concentration. In this research, no statistically significant differences were found for behavioural patterns, neither during the uptake and elimination phases (two-way ANOVA, $p > 0.05$), nor between exposed and control organisms (two-way ANOVA, $p > 0.05$). This is probably due to the fact that 24 h toxicant exposure was not enough to produce significant effects on either locomotion or ventilation of the midges. In fact, a very similar pattern was found between the control and exposed midges in locomotion activities, in both uptake and elimination phases (Fig. 2). In previous work (Azevedo-Pereira and Soares 2010), statistical significant differences for *C. riparius* locomotion at a similar concentration (40.88 µg Hg L^{-1}) after 4 days exposure to the same toxicant was not found—effects were only reported after 10 days of exposure. Regarding ventilation, the organisms exposed to the stress agent presented a tendency to increase their activity frequencies, albeit not showing any significant differences when compared with the control (Fig. 3), which is usually linked with attempts to escape from contaminated areas (Janssens de Bisthoven et al. 2004). After being transferred to clean medium, pre-exposed larvae rapidly normalised their behavioural activities when compared with non-exposed larvae (Fig. 3), thus suggesting that a quick contamination period is not enough to produce different behavioural effects on exposed midges. Although this type of experiments does not always represent ecologically realistic scenarios, they are important to know the evolutionary pattern of a chemical in the environment and in the organisms with time, evidencing the importance of the exposure route. Moreover, the model developed for this study underestimates the feeding exposure route, since in water environments the conjugation with particles dominates the movement and fate of mercury (Schoellhamer 1996), but gives us an understanding of how *C. riparius* slowly eliminates Hg and highlights the toxic effects of waterborne Hg, since this rapid bioaccumulation and slow elimination can promote serious consequences at higher trophic levels. In further studies, one should consider reducing the number of sampling periods during the experiment, as well as integrate other compartments: sediment and food (abundancy or shortage as in the present case), separately and combined, in order to fully understand the mechanisms underlying Hg bioaccumulation by this key freshwater sentinel species.

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