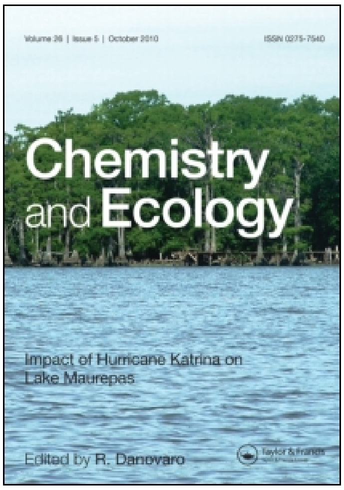

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Effects of animal starvation on the sensitivity of the freshwater amphipod *Gammarus pulex* to cadmium

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Populations of amphipods experience different food availabilities during the year. This may alter their sensitivities to toxicants. However, there is scarce information about the effects of starvation on the tolerance to pollutants, and no data are available for the species *Gammarus pulex*. Our aim was to evaluate the effects of different levels of starvation on the short-term mortality of *G. pulex* on exposure to cadmium. Four levels of starvation (0, 3, 5 and 7 days without food) were assessed using two exposure modes: semi-static (4 days exposure to 0.10, 0.20 and 0.35 mg Cd·L^{-1}) and two pulses (2 and 6 h) of 2 mg Cd·L^{-1}. LT_{50} and $peLT_{50}$ values (post exposure) were calculated for each concentration and pulse, respectively. Our results show that starvation modifies the sensitivity of *G. pulex*. In general, at the lowest cadmium concentration (0.10 mg Cd·L^{-1}) less-starved animals in semi-static exposure showed higher sensitivity to cadmium than more-starved animals. This trend was reversed for the highest cadmium exposure. Non-starved animals were more sensitive to cadmium applied in a short pulse than starved animals. Because natural populations are exposed to different food availability, starvation status has to be taken into account to assess the risk of toxicants.

Keywords: invertebrates; toxicity; sensitivity; freshwater; starvation; natural pollution

1. Introduction

Amphipods play a key role in freshwater ecosystems. These shredders are involved in the breakdown of coarse particulate organic matter and are also an important source of food for predators [1,2]. Amphipods are very often used in ecotoxicology and risk assessment because they are sensitive to a wide range of toxicants [3–6]. *Gammarus pulex* (Gammaridae, Crustacea) is one of the most important invertebrates in northern European streams because it can occur in high densities in the field [7]. This species has been amply used in ecotoxicological and ecological studies during recent decades [7–11].

Densities of detritivorous invertebrates tend to be linked with spatiotemporal variations in allochthonous organic matter [12,13]. Shredder-detritivores can become growth-limited by a

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shortage of leaf litter in late spring and summer [12,14,15], affecting their nutritional level and thus their fitness [16–19]. The nutritional state of animals may alter the sensitivity of the populations to pollution events [20]. For example, the tolerance of aquatic animals to metals is affected by food quantity or starvation periods prior to toxicant exposure [20,21]. The sensitivity to cadmium of the laboratory-reared estuarine amphipod *Leptocheirus plumulosus* was very similar between fed and starved animals [22]. By contrast, starved marine amphipods, with fewer whole-body lipids, were less tolerant to the toxicity of cadmium and tributyltin [23]. However, information about the effects of previous starvation on the tolerance of freshwater macroinvertebrates to toxicants is scarce [24], especially for the commonly-used *G. pulex*.

Several human activities, such as smelting plants, combustion of fossil fuel, battery manufacture, paints and fertilisers, can increase the cadmium concentration in freshwater ecosystems [6,25]. High concentrations of cadmium have been found in polluted ecosystems. For example, concentrations up to $0.07\,\mathrm{mg\,Cd{\cdot}L^{-1}}$ have been found in polluted bodies of water in Belgium [26], and values as high as $0.29\,\mathrm{mg\,Cd{\cdot}L^{-1}}$ have been found in polluted water from some Canadian lakes [27]. In a river in Argentina the maximum cadmium concentration was $1.7\,\mathrm{mg{\cdot}L^{-1}}$ [28]. Cadmium is a nonessential heavy metal which can be accumulated in the gills and hepatopancreas of crustaceans, causing damage to cells and disrupting enzymatic reactions [6,20]. Several species of crustaceans, including *G. pulex*, have shown high sensitivity to cadmium toxicity [6,8,11,25]. In addition, natural populations of lotic ecosystems can be exposed to short pollution events after surface run-offs or accidental spillages [7,29]. Given that cadmium tends to accumulate in sediments, and is present in the water column for short periods only [25], short exposures (from a few hours to a few days) are more likely than chronic exposure for animals that dwell in water column. For these reasons, cadmium was chosen as the toxicant model in our study.

The aim of this study was to evaluate the effects of different levels of starvation on the sensitivity of *G. pulex* to cadmium through two different exposure modes: semi-static and pulse. Our hypothesis is that a longer starvation period will increase the sensitivity of the exposed animals, independent of the mode of exposure. We tested our hypothesis by performing different laboratory experiments in order to elucidate the isolated effect of starvation status.

2. Materials and methods

2.1. *Amphipod collection and acclimatisation*

Amphipods were collected using three sieves (1, 2 and 4 mm mesh size) from an unpolluted reach of the Heelsum Stream (51°58′ N, 5°45′ E) near Wageningen (The Netherlands). Invertebrates collected using 1 and 2 mm sieves (juveniles) were transferred to the laboratory using plastic containers (5 L) and kept in an aerated aquarium in a climatic chamber with controlled temperature ($15 \pm 1\,^{\circ}\mathrm{C}$). Animals were fed with stream-conditioned poplar (*Populus* sp.) leaves and were progressively acclimatised to the test water (Dutch Standard Water; DSW) [30] for one week prior to the bioassays. DSW has a nominal calcium concentration of $54.4\,\mathrm{mg\,Ca^{2+}{\cdot}L^{-1}}$ and a magnesium concentration of $17.7\,\mathrm{mg\,Mg^{2+}{\cdot}L^{-1}}$. Given than juveniles of *G. pulex* have been reported to be more sensitive to short-term cadmium toxicity [8,31], they were selected for this study.

2.2. *Starvation treatments*

Four levels of starvation were used. These starvation treatments consisted of groups of amphipods that were kept without food for 0, 3, 5 and 7 days prior to the start of the bioassays (see below).

McNulty et al. [24] showed that periods up to 7 days without food were more critical for the survival of older than younger amphipods of *Hyalella azteca*, but based on this study, we chose 7 days of starvation for juveniles of *G. pulex*. For each starvation level and exposure, four or five 300 mL glass beakers filled with DSW were used; 35–40 individuals were added to each beaker. Starvation treatments started 7 days before the beginning of the bioassays. In the 7-day starvation beakers, six 30 mm diameter plastic discs were added per beaker to provide refuge, but there was no food supply. In the other starvation treatments (0, 3 and 5 days), three conditioned 30 mm diameter leaf discs (which provided food and refuge) and three plastic discs of the same size (which provided refuge only) were added per beaker. After 2 and 4 days, all leaf discs were replaced by plastic discs in the 5- and 3-day starvation treatments, respectively. Amphipods from the 0-day starvation treatment were allowed to feed until the start of the bioassays. Faeces were removed daily from the beakers with a plastic pipette in order to avoid the use of faeces as food. Additional conditioned leaf discs had to be provided to low starved animals (0–3 days) during starvation treatments, showing that these animals were feeding properly during the starvation period prior to the bioassays.

2.3. *Design of the semi-static and pulse bioassays*

Two types of bioassays were performed: semi-static and pulse. In the semi-static exposure bioassay, amphipods were exposed to cadmium for 96 h with renewal of toxic solutions and control every 48 h. Three nominal cadmium concentrations (0.10, 0.20 and 0.35 mg $Cd{\cdot}L^{-1}$) and a control were used for each starvation level (0, 3, 5 and 7 days) in triplicate, with 10 animals in each replicate. After 7, 24, 31, 48, 55, 72, 79 and 96 h the number of dead amphipods was monitored in each treatment.

In the pulse exposure bioassay, one nominal short-term lethal cadmium concentration (2 mg $Cd{\cdot}L^{-1}$) was chosen. Two pulses (2 and 6 h) of continuous cadmium exposure were applied to amphipods from each starvation level (0, 3, 5 and 7 days). The selected Cd concentration was higher than in semi-static bioassay to cause a lethal effect on amphipods after the exposure. For each starvation level, including the control and cadmium pulse, five replicates were used, with 10 animals in each replicate. After 2 or 6 h of exposure, animals from each starvation level were transferred to control water (DSW). The same handling was applied to controls after 6 h (from DSW to new DSW). After 6, 24, 31, 48, 55, 72, 79 and 96 h the number of dead amphipods was monitored in all treatments.

Cadmium concentrations for both bioassays were selected to obtain a mortality response at short-term exposure on the basis of previous short-term bioassays conducted in our laboratory [31]. Cadmium solutions were prepared from a stock solution of 82.8 mg $Cd{\cdot}L^{-1}$, which was prepared dissolving the required amount of cadmium chloride in DSW ($CdCl_2$, Aldrich, lot no. 188165). Because our main objective was to compare the effects of contrasting starvation levels on amphipod sensitivity to short-term cadmium exposure, the nominal concentrations were not confirmed, assuming that these were similar in our different starvation treatments. The same protocol was used in a previous study to assess contrasting sensitivities between *Gammarus* species to ivermectin and cadmium [31]. In addition, because the experimental design was very similar between treatments (same vessels, number of animals, etc.), the cadmium dynamic in water column (e.g. dissipation, adsorption) was assumed to be very similar. Animals were not fed during the bioassays. An amphipod was considered to be dead when neither swimming nor movement were observed after touching the animal with a small plastic pipette. Dead amphipods were removed in each observation for all bioassays. Observations were conducted using a stereoscopic microscope. Bioassays were conducted in a controlled chamber at $15 \pm 1\,^{\circ}C$, 60% humidity and 12 h light photoperiod. Physical–chemical properties were monitored every 2 days during the bioassays. Mean ($\pm$SD) values were: conductivity $623.4 \pm 13\,\mu S{\cdot}cm^{-1}$, dissolved oxygen concentration $7.9 \pm 0.3\,\mathrm{mg\,O_2 \cdot L^{-1}}$ and pH 7.9 ± 0.12. At the end of bioassays the length of amphipods, from antennal base to the third uropod, was measured using a micrometer situated in a stereoscopic microscope. Mean size ($\pm$SD) for the animals of semi-static bioassays was 4.69 ± 0.79 mm, and 4.91 ± 0.91 mm for the pulse bioassays.

2.4. *Statistical analysis*

Lethal time values (LT_{50}) for each concentration (0.10, 0.20 and 0.35 mg $\mathrm{Cd \cdot L^{-1}}$) of the semi-static bioassay, and post-exposure lethal time values ($peLT_{50}$) for each pulse (2 and 6 h of 2 mg $\mathrm{Cd \cdot L^{-1}}$)

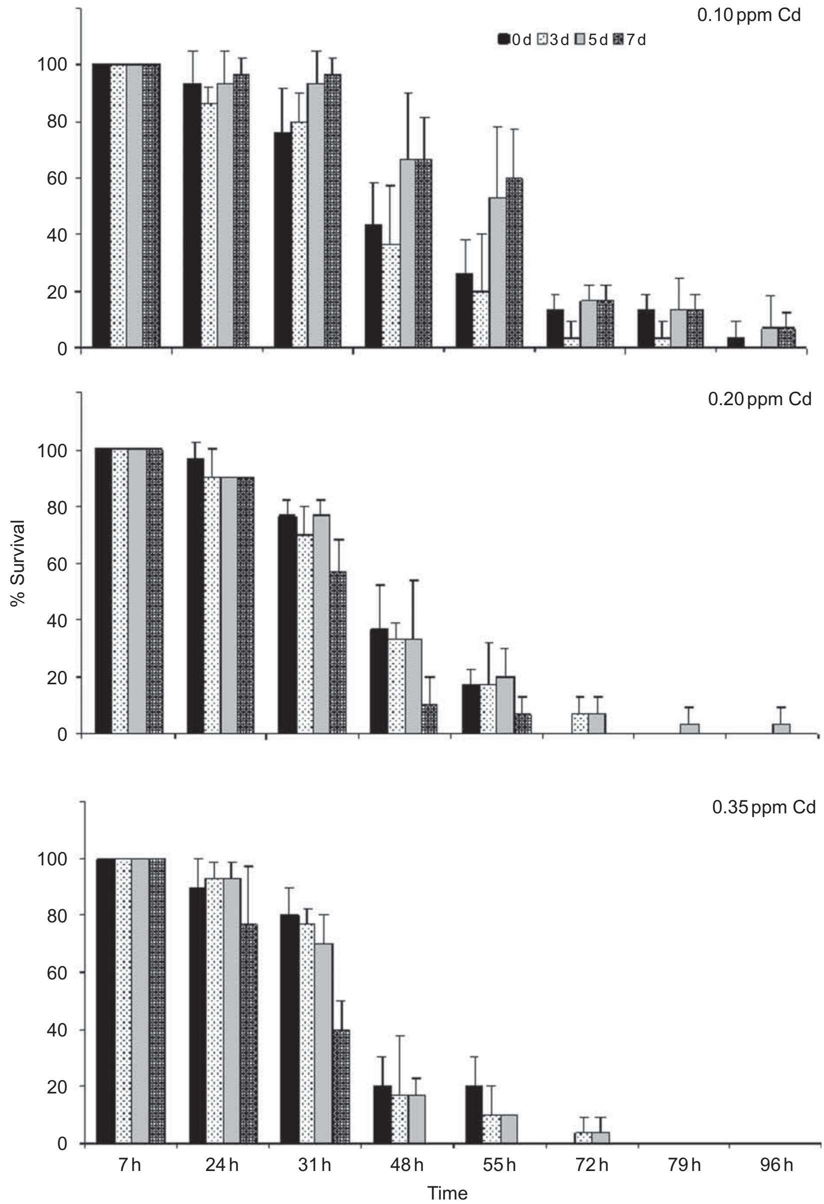

Figure 1. Mean percentage of survival (+SD) for each semi-static cadmium exposure (0.10, 0.20 and 0.30 mg $\mathrm{Cd \cdot L^{-1}}$) and for each starvation period.

and their 95% confidence limits were calculated using probit regression analysis [32–34]. The dependent variable was the probit of the proportion of animals responding at each time point, and the independent variable was the logarithm of exposure time. Regression analyses were performed using SPSS v. 15 software [35]. For each cadmium concentration (0.10, 0.20 and 0.35 mg·L^{-1}) or pulse (2 and 6 h) a regression model was conducted, using starvation as a factor with four levels (0, 3, 5 and 7 days). This model requires that the regression of all factor levels be parallel. Therefore, we conducted a parallelism test to compare slopes between starvation levels for each concentration or pulse. If the regression models showed parallelism ($p > 0.05$; parallelism test) a common slope model was used for all levels of the factor [34]. Otherwise, an independent model has to be conducted for each level of the factor. Statistical differences for LT_{50} and $peLT_{50}$ values between starvation treatments for each cadmium concentration (semi-static exposure) or between starvation treatments for each cadmium pulse (2 and 6 h) were conducted by means of a Z-test [36]. This analysis compares LT or LC values with overlapped confidence limits. For the Z-test a Bonferroni correction was applied to reduce the probability of rejecting the null hypothesis being true, therefore differences between LT_{50} or $peLT_{50}$ were considered if $p < 0.0084$.

3. Results

The mean maximum mortality recorded in controls for the bioassays was <9%. The mean mortality values in controls of semi-static bioassays for 0, 3, 5 and 7 days were 3.3, 2.2, 1.1 and 7.8%, respectively (no significant differences between mortality of starvation treatments were found, $p > 0.05$; ANOVA). In the case of pulse bioassays, mortalities were 8, 8, 4 and 2%, respectively with no significant differences between mortality of starvation treatment ($p > 0.05$; ANOVA). In the semi-static bioassays all cadmium concentrations caused mortality in *G. pulex* (Figure 1). In the pulse bioassays, no animal died after 2 h of exposure to 2 mg Cd·L^{-1}. The effects of the longest pulse (6 h) were more pronounced than that of the shortest (Figure 2). The LT_{50} values for the semi-static bioassays are shown in Table 1. In the lowest cadmium concentration (0.10 mg·L^{-1}), higher levels of starvation (5 and 7 days) showed a higher tolerance to cadmium than the lowest starvation levels (0 and 3 days) ($p < 0.0084$; Z-test). In the case of the intermediate cadmium concentration (0.20 mg·L^{-1}), all starvation tratments showed similar LT_{50} values ($p > 0.0084$; Z-test). In the highest cadmium concentration (0.35 mg·L^{-1}), LT_{50} was lower for the 7-day starvation treatment than for the other starvation levels ($p < 0.0084$; Z-test).

$peLT_{50}$ values for the longest pulse (6 h) (Table 1) were not affected by starvation treatments ($p > 0.0084$; Z-test). In the case of the shortest pulse (2 h), the three highest starvation treatments showed a higher tolerance to cadmium than the lowest starvation period ($p < 0.0084$; Z-test).

4. Discussion

The results of this study showed that different periods of starvation prior to exposure to cadmium can modify the sensitivity of *G. pulex*, either in semi-static or pulse exposures. An interaction was found between starvation and cadmium concentration in the semi-static exposure, because less-starved animals tended to be more sensitive to the lowest cadmium concentrations, whereas the reverse was true for the highest concentration. In the case of pulse exposure, sensitivity to cadmium was affected by starvation at the shortest pulse. The longest pulse caused a rapid death in all amphipods, independent of the starvation level. Therefore, the hypothesis that higher starvation increased the sensitivity of *G. pulex* to cadmium should be rejected for semi-static exposure to the lowest concentration and for the shortest pulse exposure. A likely cause of this result is that

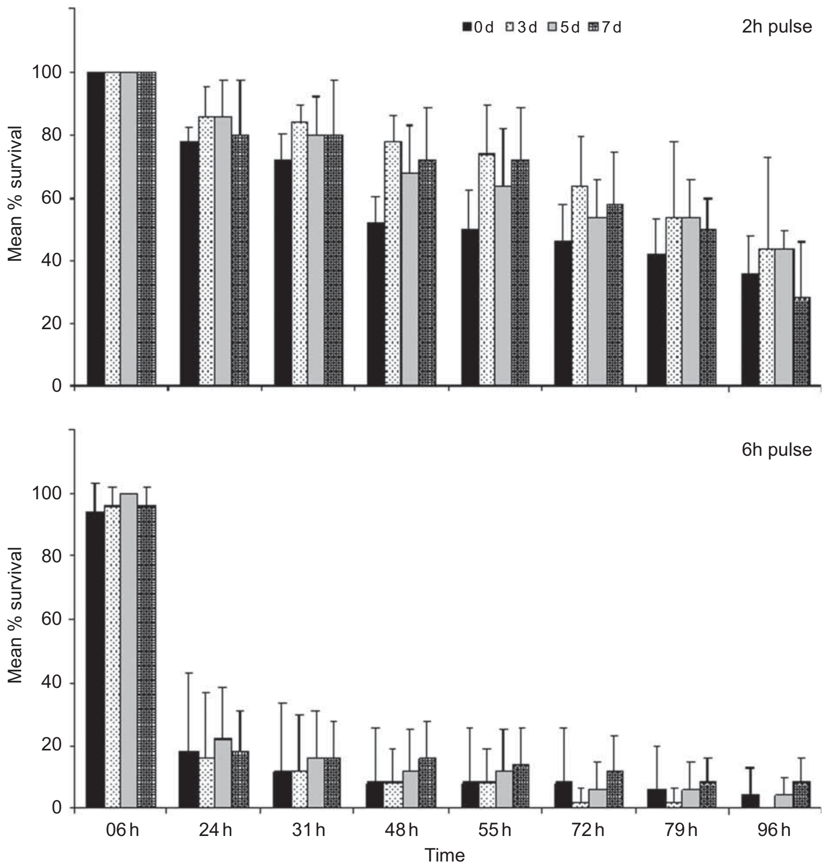

Figure 2. Mean percentage of survival (+SD) for each cadmium pulse (2 and 6 h to 2.0 mg Cd·L^{-1}) and starvation period for each post-exposure time.

Table 1. LT_{50} or $peLT_{50}$ values and their 95% confidence limits of *Gammarus pulex* for each starvation period (0, 3, 5 and 7 days) and each semi-static (0.10, 0.20 and 0.35 mg Cd·L^{-1}) and pulse (2 and 6 h) treatment.

Cd treatment	0 days	3 days	5 days	7 days
LT_{50} (h)				
Semi-static (0.10 mg Cd·L^{-1})	43.1 (38.5–47.9)^a	42.4 (37.7–47.9)^a	52.8 (47.1–58.9)^b	53.3 (47.7–59.2)^b
Semi-static (0.20 mg Cd·L^{-1})	40.4 (36.1–45.0)^a	39.8 (35.8–44.1)^a	40.6 (36.8–44.8)^a	34.4 (30.6–38.6)^a
Semi-static (0.35 mg Cd·L^{-1})	38.5 (34.9–42.5)^b	39.1 (35.2–43.5)^b	36.3 (32.8–40.0)^b	28.0 (24.6–31.9)^a
$peLT_{50}$ (h)				
Pulse 2 h (2.0 mg Cd·L^{-1})	59.4 (50.4–70.5)^a	99.2 (82.3–124.6)^b	83.2 (69.9–102.0)^b	84.0 (70.4–103.7)^b
Pulse 6 h (2.0 mg Cd·L^{-1})	21.3 (14.2–30.7)^a	14.1 (8.9–20.5)^a	10.7 (6.3–15.6)^a	12.6 (8.1–17.5)^a

Notes: Different letters show significant differences between starvation periods for each cadmium concentration or pulse ($p < 0.0084$; Z-test). For each concentration or pulse, models for starvation periods had parallel slopes ($p > 0.05$; parallelism test), therefore a common slope model was used for each concentration or pulse. The Pearson goodness of the probit model for each concentration or pulse was not significant ($p > 0.05$), showing that the model fit was adequate.

starvation reduces the amphipods' energy supply, depressing their locomotion and ventilation activity, and therefore reducing cadmium uptake, as reported for several metals and species [37]. Hervant et al. [38] found a reduction in ventilation activity during starvation for several species of hypogean crustacean, although this trend was not so clear for epigean amphipods. A reduction in locomotion activity in *G. pulex* individuals after 7 days without food was recorded by Peeters et al. [39] using an automatic Multispecies Freshwater Biomonitor (MFB) device. The ventilation activity of this amphipod was also depressed in a starvation experiment (2 days) conducted in our laboratory using MFB (unpublished data). Therefore, there is experimental evidence that relatively short periods of starvation cause a reduction in the activity (both swimming and ventilation) of *G. pulex*. However, cadmium concentrations above a threshold probably cannot be compensated for the reduction of ventilation activity, exceeding the toxicity threshold for cadmium. Because gills are an important pathway for metal uptake from water [20], a reduction in the ventilation rate (caused by starvation or/and by low concentrations of cadmium) could be a good short-term physiological/behavioural mechanism by which to reduce cadmium uptake [37]. Another potential explanation for the interaction between starvation levels and cadmium concentration may be attributed to damage to the gill lamellae. A shortening in secondary lamellae after long starvation periods has been reported in fish [40], thus both a long starvation period and low cadmium exposure may contribute to damaged gill tissues, reducing metal uptake. Therefore, a low gill ventilation rate or/and a shortening of the respiratory surface caused by starvation may reduce the lethality of low cadmium concentrations. An additional explanation is that authophagy is increased by starvation, protecting against pollutant toxicity by removing oxidatively damaged organelles and proteins. Therefore, it may also contribute to reduce sensitivity to cadmium in starved juveniles.

In our experiment, we used juveniles of *G. pulex*. However, when adult amphipods are used in cadmium bioassays, other factors (e.g. gender or reproduction stage of females) have to be taken into account, because sexually mature males have shown higher tolerance to cadmium than sexually mature females [41]. Within mature females, those carrying older embryonic stages were more tolerant than females carrying earlier embryonic stages [41]. Toxic effects of cadmium on non-starved amphipods may be higher in adult females because the energy demand for reproduction is higher than in adult males [41]. Therefore, the likely ameliorating effects of starvation on cadmium toxicity can differ between adult females and males, because animals with less energy can show a higher tolerance to low short-term cadmium exposures.

The results of pulse exposure to cadmium showed that at the end of short pulses (2 or 6 h) no or very low mortality was observed, but a few hours later most of the animals died, especially in the longest pulse. A similar trend has been found for other species of freshwater amphipod (*Eulimnogammarus toletanus* and *Hyalella azteca*) on exposure to nitrite and copper, respectively [42,43]. Therefore, the post monitoring of delayed mortality after short-term exposures to toxicants should be taken into account, in order to obtain a more realistic assessment of pulse exposure [7,32,43–45]. For this, we suggest the use of $peLT_{50}$ (or $peLC_{50}$) values to improve accuracy of pulse ecotoxicology bioassays, especially during very short pulses, where delayed mortality is relatively more important than in longer exposures [43]. These values may contribute to a more realistic assessment of the adverse effects of toxicants after exposure, including the recovery capacity of animals and their detoxification mechanisms. In addition, several toxicokinetic and toxicodynamic models have been developed in recent years, to simulate the dynamics of toxicants in the whole organism or to predict effects [46–49]. Because animal concentration is surrogated to the environmental concentration, these models can contribute to descriptions of the dynamics of delayed mortality after toxicant exposure when no pulse data are available.

The sensitivity of *G. pulex* to the short-term effects of cadmium has been reported by several authors [6,31,50,51]. The LC_{50} values at 96 h ranged from 0.02 to 0.60 mg $Cd \cdot L^{-1}$ in different exposures (continuous, semi-static, etc.) and/or concentrations (nominal or actual). These values are within the range of our results, because ~50% of amphipods died in the semi-static bioassay at 0.10–0.20 mg $Cd \cdot L^{-1}$ and after 48 h of exposure. However, we recommend the use of juveniles for bioassays with cadmium, as they have been previously shown to be the most sensitive stage [8,31].

In the ecological risk assessment (ERA) procedure, data from laboratory bioassays (e.g. LC_{50}, EC_{50}) are used to estimate safe concentrations for the whole ecosystem [42,52]. Most of the data derive from standardised tests, performed with animals in optimal conditions (e.g. non-starved animals). However, natural populations present different feeding conditions as a consequence of competition, seasonality, habitat, life stages, etc. Therefore, for a more realistic ecological risk assessment the different feeding conditions of animals should be tested, especially in the case of the amphipods. If only non-starved animals are used in laboratory studies, the obtained safe concentrations may overestimate the toxicity of cadmium.

5. Conclusions

We conclude that the short-term toxicity of cadmium to juveniles of *G. pulex* was different depending on the previous starvation periods. A semi-static exposure to low cadmium concentration and high starvation level resulted in a higher tolerance to the short-term lethal effects of cadmium. This trend was similar for short-term pulses (2 h). Therefore, to improve the assessment of cadmium toxicity in freshwater amphipods, the previous state of starvation has to be taken into account. In addition, for a more realistic ecological risk assessment procedure, the different feeding conditions of animals should be tested, especially in the case of the amphipods.

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