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AMMONIA TOXICITY TO THE FRESHWATER INVERTEBRATES *Polycelis felina* (PLANARIIDAE, TURBELLARIA) AND *Echinogammarus echinosetosus* (GAMMARIDAE, CRUSTACEA)

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SUMMARY

Ammonia is one of the most widespread pollutants in aquatic ecosystems of industrialized countries. It is found in a freshwater solution as two different chemical species, the unionized form (NH_3) and the ionized form (NH_4^+), this chemical equilibrium is controlled principally by pH and water temperature. Toxicity of ammonia is caused principally by the NH_3. In spite of its high toxicity, there is little toxicological data available on the NH_3 effects on freshwater invertebrate species. The aim of this work was to assess the toxic effects of NH_3 on the survival and movement of two species of benthic freshwater invertebrates: the amphipod *Echinogammarus echinosetosus* and the planarian *Polycelis felina*. The LC_{50}, LC_{10}, $LC_{0.01}$, EC_{50}, EC_{10} and $EC_{0.01}$ values (mg/L NH_3-N) at 24, 48, 72 and 96 hours of exposure were calculated for both species. The $LC_{0.01}$ and $EC_{0.01}$ values were considered as short-term safe concentrations to avoid mortality and inmobility in both species of invertebrates. These concentrations could be used as preliminary safe levels for episodic exposures to unionized ammonia. Additionally the comparison of our results with other species of freshwater amphipods and planarians show the relatively high sensibility of *P. felina* to unionized ammonia. This species may be a good indicator of unionized ammonia pollution.

KEYWORDS: unionized ammonia, amphipod, planarian, toxicity, invertebrates, safe level.

INTRODUCTION

Freshwater ecosystems are affected by different sources of pollution, such as agricultural runoff, industrial effluents, organic waste discharges, atmospheric deposition and nutrient enrichment from aquaculture [1-5]. All these factors increase the concentrations of ammonia, especially the industrial effluents because ammonia is a staple raw material in many industrial processes, and as a consequence a common end-product [5]. Ammonia is also a natural product of organic matter degradation, and therefore is an habitual component of the effluents from sewage treatment plants [5]. As a consequence of all these anthropogenic pollution sources, ammonia is one of the most widespread and serious toxic water contaminants in industrialized countries [5, 6]. This compound can be found in a freshwater solution as two different chemical species, the unionized form (NH_3) and the ionized form (NH_4^+), whose chemical equilibrium is controlled principally by pH and water temperature [2, 7]. High ammonia concentrations may cause toxicity on aquatic animals, especially the unionized ammonia, which is the toxic form of ammonia [2, 3, 5, 8]. Unionized ammonia has a high solubility in lipids and it diffuse easily across cellular membranes, causing several toxic effects on aquatic animals such as hyperexcitability, convulsions, gill hyperventilation and epithelial necrosis [1, 9-11]. In spite of this toxicity, there is less literature available on its effects on aquatic invertebrate species and benthic organisms than on fish [5, 10, 12].

Among freshwater benthic invertebrates, several species of planarians and amphipods have been widely used in aquatic toxicological studies, showing a high sensitivity to several toxics, including ammonia, nitrite, nitrate, cadmium and several insecticides [2, 13-18]. Additionally, these invertebrates are usually an important component of macrobenthic communities in freshwater ecosystems, playing a key trophic role: planarians as predators feeding on living invertebrates, and amphipods as shredders processing allochthonous and autochthonous plant material into more digestible food for other invertebrates [13, 15, 19-22]. Additionally amphipods may be a common food source for freshwater predatory fish and macroinvertebrates [23-24].

The aim of this research is to evaluate the short-term toxicity of unionized ammonia on the survival and movement of two species of freshwater invertebrates; the planarian *Polycelis felina* (Dalyell) (Planariidae, Turbellaria) and the amphipod *Echinogammarus echinosetosus* (Pinkster) (Gammaridae, Crustacea), under laboratory conditions. *P. felina* is relatively abundant in several freshwater European ecosystems, and the genus *Echinogammarus* (Stebbing) is found in a wide geographical area extending from Europe and North Africa to Asia Minor. *E. echinosetosus* is an endemic amphipod of Iberian Peninsula found in the upper and middle reaches of several Spanish rivers [20, 25]. The use of endemic/local species in ecotoxicological studies can contribute to the establishment of regional safe concentrations and to know the local effects of pollution [26].

MATERIALS AND METHODS

Individuals of *P. felina* and *E. echinosetosus* were collected from two upper reaches (site A and B, respectively) of the Henares River (Guadalajara province, Central Spain). Physico-chemical characteristics of both reaches are presented in Table 1. Invertebrates were transported to the laboratory in plastic containers full with river water. Once in the laboratory, amphipods (gravid adults and precopulatory pairs were rejected) and planarians were progressively acclimatized to the test water conditions in independent glass aquaria (1.5 L) for seven days prior to start laboratory bioassays. The test water used in the acclimatization and bioassays was bottled drinking water without chlorine (Table 1). During this acclimatization week, amphipods were fed with stream-conditioned poplar leaves (*Populus* sp.) and planarians with chicken liver and gravid amphipods every two days.

TABLE 1 - Physicochemical parameters for water of both sites in the Henares River (site A for *Polycelis felina* and site B for *Echinogammarus echinosetosus*) and for toxicity test water. Mean values ± standard deviations are presented for each parameter. Water analyses were performed following standardized methods described in ref. [27].

Physicochemical parameter	Toxicity tests	Henares River (site A)	Henares River (site B)
Conductivity (µS)	755.3±25.6	536.4±13.6	852.5±106.4
pH	8.1±0.30	7.7±0.09	8.1±0.12
Alkalinity (mg/L $CaCO_3$)	200.2±0.3	294.9±27.8	267.1±31.5
Calcium (mg/L Ca^{2+})	77.8±3.0	84.4±17.5	103.0±9.4
Chloride (mg/L Cl^-)	56.0±2.7	6.2±0.6	95.9±22.0
Dissolved oxygen (mg/L)	6.6±0.3	8.7±0.7	11.6±2.4
Water temperature (ºC)	15.0±1.0	11.3±1.8	11.7±5.8
NO_3-N (mg/L)	2.7±0.3	1.5±1.4	1.3±1.0
NO_2-N (mg/L)	<0.005	<0.005	<0.005
NH_3-N (mg/L)	<0.002	<0.002	0.0024±0.004

Two independent short-term (4d) bioassays were conducted. Unionized ammonia solutions and controls were renewed daily. Both bioassays were carried out in glass vessels, with 0.1L of control water or unionized ammonia solutions, in triplicate. Vessels were covered with perforated plastic foil in order to reduce water evaporation. No aireation was supplied during the tests to avoid significant oxidation of ammonia. Five exposure concentrations and control were tested for each species. In the case of planarians, nominal concentrations of unionized ammonia were 0.14, 0.27, 0.34, 0.51 and 0.69 mg/L NH_3-N, and ten individuals were used per vessel. Nominal concentrations in the amphipod bioassay were 0.52, 1.04, 1.56, 2.09 and 2.61 mg/ L NH_3-N, and eight individuals were used per vessel. Nominal unionized ammonia solutions were prepared daily from an ammonia stock solution of 100 mg/L NH_4-N+NH_3-N, which were prepared by dissolving the required amount of ammonium chloride (NH_4Cl, PANREAC, Spain, Lot No. 149959380, reported purity 99.5%) in 1000 mL of test water. Ammonium salt was previously dried at 60ºC during 48 hr. The concentrations of unionized ammonia as nitrogen (NH_3-N) were calculated using the aqueous ammonia equilibrium on the basis of mean values of water temperature and pH [7]. Water temperature, pH, dissolved oxygen and total ammonia concentrations were measured daily. Total ammonia concentrations were assessed by spectrophotometry (Spectroquant® Merck, Germany. Detection limit = 0.002 NH_3-N) in accordance with American Public Health Association [27]. Two parameters were monitored at 24 hr intervals in each vessel: the number of dead invertebrates, to calculate LC values, and the proportion of affected individuals, that include dead and inactive organisms, to calculate EC values [28]. A planarian was considered to be dead when the body tissues started to degenerate, and inactive when no displacement was observed after a gentle touch with a soft-brush. The death of an amphipod was admitted when no swimming displacement and no movement of any body part were observed after a touch with a glass stick. An amphipod was considered to be inactive if not swimming displacement was observed but some body part was active (such as pleopods, uropods or antenna movements). Dead invertebrates were removed every day. Glass vessels were renewed daily. $LC_{0.01}$, LC_{10}, LC_{50}, $EC_{0.01}$, EC_{10} and EC_{50} values for 24, 48, 72 and 96 hr, and their respective 95% confidence limits, were calculated using the multifactor probit analysis (MPA) [29]. This methodology solves the concentration-time-response equation via the iterative reweighed least square technique, LC and EC values being calculated by a multiple linear regression. The dependent variable is the probit of the proportion of animals responding to each concentration, and the independent variables are exposure time and unionized ammonia concentrations. Mean actual concentrations of unionized ammonia were used to calculate the LC and EC values for each bioassay. Actual concentrations of unionized ammonia for the *P. felina* bioassay were 0.13, 0.26, 0.33, 0.52 and 0.70 mg/L NH_3-N, and 0.69, 1.13, 1.63, 2.21 and 2.80 mg/L NH_3-N for the *E. echinosetosus* bioassay. In

the present study, $LC_{0.01}$ or $EC_{0.01}$ was considered to be calculated short-term safe concentrations of unionized ammonia for each test species. These parameters refer to the unionized ammonia concentrations that affect 0.01% individuals of the test population for each species [16, 30]. The $LC_{0.01}$ value has been shown to be an appropriate safe level of unionized ammonia to avoid mortality and feeding activity reduction in the freshwater amphipod *Eulimnogammarus toletanus* [16]. Additionally, $LC_{0.01}$ values for the aquatic snail *Potamopyrgus antipodarum* did not caused mortality in a behavioural bioassay with this snail [30, 31].

After the *E. echinosetosus* bioassay, animal body lengths from the antennal base to third uropod were measured using an ocular micrometer. Before the bioassay with *P. felina*, their body lengths were measured with a Delta-T area meter (Cambridge, UK); each planarian was placed in a Petri disc with test water and its image was recorded in a computer through a camera, after its body length was measured. Differences in body length between each unionized ammonia treatment and control were assessed by means of an analysis of variance for each bioassay (ANOVA-Dunnett test) [32]. Significant ($P<0.05$) differences in LC and EC values between both species were considered if 95% confidence limits did not overlap for the same exposure time [29, 33].

RESULTS AND DISCUSSION

All unionized ammonia concentrations used in both bioassays caused mortality which was proportional to exposure time and unionized ammonia concentrations. No dead or inactive animals were found in the control vessels after 96 hr. No significant body length differences between each treatment and the control were found for any of the bioassays ($P>0.05$; Dunnett test). Mean body length ± standard deviations in the *E. echinosetosus* bioassay were 7.4±1.4 mm for the control and 7.3±1.7, 7.4±1.8, 7.3±2.1, 7.4±1.4 and 7.8±1.8 mm for each treatment of increasing unionized ammonia concentrations, respectively. In the *P. felina* bioassay mean body lengths were 7.9±2.0 mm for the control, and 7.0±1.9, 7.3±2.1, 7.3±2.1, 7.5±2.1 and 7.3±1.8 mm for the treatments. LC and EC values for each exposure time are presented in Table 2. *P. felina* was more sensitive to lethal effects of unionized ammonia than *E. echinosetosus*, since its LC_{50}, LC_{10}, EC_{50}, EC_{10} and $EC_{0.01}$ values were significantly lower than those for *E. echinosetosus* ($P<0.05$; 95% confidence limits did not overlap). In contrast, $LC_{0.01}$ values for each exposure time were similar between both species (Table 2).

The comparison of our 96 hr LC_{50} values (mg/L NH_3-N) with other results reported in bibliography for amphipods and planarians showed that *P. felina* has the 2nd highest sensitivity to lethal effects of unionized ammonia (Table 3). In the case of *E. echinosetosus*, its sensitivity was intermediate. The planarian *Polycelis tenuis* showed a higher value of 96 hr LC_{50} than *P. felina* (0.58) [2]. Only the amphipod *Crangonyx pseudogracilis* showed a similar sensitivity to that of planarians, with a 96 hr LC_{50} value of 0.36 (0.17-0.59) mg/L NH_3-N, as reported by Prenter *et al.* [34]. However, Arthur *et al.* [35] reported a high tolerance for the same species to unionized ammonia (LC_{50} 96 hr of 2.57 mg/L NH_3-N). The causes for this discrepancy could be the small size of animals used by Prenter *et al.* [34] and intraspecific differences between the populations studied by both authors. Other amphipod with a high sensitivity to lethal and sublethal effects of unionized ammonia was *Eulimnogammarus toletanus* [16]. The 96 hr LC_{50} values of the amphipod *Hyalella azteca* have been found to depend on the water hardness, hard water (270 mg/L $CaCO_3$) reducing the toxicity of ammonia [36]. The other amphipods (*Gammarus duebeni celticus* and *Gammarus pulex*) showed a higher tolerance to unionized ammonia than *P. felina*, with values of 1.15 and 1.54-1.69 mg/L NH_3-N, respectively [2, 34]. These values were very similar to 96 hr LC_{50} value of *E. echinosetosus* (1.22; Table 3).

As expected, calculated EC values were more sensitive than LC values for both species (Table 2). Furthermore, the proportion of individuals affected by unionized ammonia was clearly higher than the corresponding lethal values in the case of *P. felina*. This difference was especially higher after 96 hr of exposure time between $LC_{0.01}$ (0.12 mg/L NH_3-N) and $EC_{0.01}$ (0.04 mg/L NH_3-N) to *P. felina*, although differences could not be statistically com-

TABLE 2 - 24, 48, 72 and 96 hr $LC_{0.01}$, LC_{10}, LC_{50}, $EC_{0.01}$, EC_{10} and EC_{50} values of NH_3-N (mg/L) for *E. echinosetosus* and *P. felina*. 95% confidence limits are presented in parentheses.

Species	Parameter	24 hr	48 hr	72 hr	96 hr
Echinogammarus echinosetosus	$LC_{0.01}$	0.40 (0.26-0.53)	0.25 (0.16-0.33)	0.21 (0.13-0.29)	0.19 (0.12-0.27)
Polycelis felina	$LC_{0.01}$	0.21 (0.05-0.33)	0.14 (0.03-0.22)	0.13 (0.03-0.19)	0.12 (0.03-0.18)
Echinogammarus echinosetosus	LC_{10}	1.33 (1.10-1.55)	0.82 (0.69-0.93)	0.70 (0.58-0.80)	0.65 (0.53-0.75)
Polycelis felina	LC_{10}	0.45 (0.24-0.62)	0.31 (0.19-0.37)	0.27 (0.16-0.33)	0.26 (0.15-0.32)
Echinogammarus echinosetosus	LC_{50}	2.51 (2.18-2.91)	1.55 (1.44-1.66)	1.32 (1.21-1.43)	1.22 (1.10-1.34)
Polycelis felina	LC_{50}	0.67 (0.47-1.04)	0.47 (0.39-0.58)	0.41 (0.34-0.53)	0.39 (0.31-0.51)
Echinogammarus echinosetosus	$EC_{0.01}$	0.36 (0.24-0.49)	0.23 (0.15-0.31)	0.20 (0.12-0.27)	0.18 (0.11-0.25)
Polycelis felina	$EC_{0.01}$	0.09 (0.05-0.14)	0.06 (0.03-0.08)	0.05 (0.02-0.07)	0.04 (0.02-0.06)
Echinogammarus echinosetosus	EC_{10}	1.18 (0.98-1.38)	0.75 (0.63-0.85)	0.64 (0.53-0.74)	0.60 (0.48-0.69)
Polycelis felina	EC_{10}	0.29 (0.22-0.36)	0.18 (0.14-0.21)	0.15 (0.12-0.18)	0.14 (0.11-0.16)
Echinogammarus echinosetosus	EC_{50}	2.20 (1.92-2.55)	1.39 (1.29-1.50)	1.20 (1.09-1.30)	1.11 (1.00-1.22)
Polycelis felina	EC_{50}	0.54 (0.46-0.65)	0.33 (0.30-0.36)	0.28 (0.25-0.31)	0.26 (0.23-0.29)

TABLE 3 - Comparison between 96 hr LC_{50} values of NH_3-N (mg/L) for several species of freshwater amphipods (A) and planarians (P) reported in bibliography. Water temperature and pH of test water are presented for each bioassay.

Species	Group	LC_{50}	pH	T (ºC)	Reference
Hyalella azteca^{+++}	A	3.76*	6.5-8.5	25	[36]
Crangonyx pseudogracilis	A	2.57*	8.0-8.2	4.0-24.9	[35]
Hyalella azteca^{++}	A	2.37*	6.5-8.5	25	[36]
Gammarus pulex	A	1.69*	7.8-8.0	11.5	[2]
Gammarus pulex	A	1.54	7.8-8.1	12.0-13.0	[34]
Echinogammarus echinosetosus	A	1.22	8.1	15.0	This study
Gammarus duebeni celticus	A	1.15	7.8-8.1	12.0-13.0	[34]
Hyalella azteca^{+}	A	0.86*	6.5-8.5	25	[36]
Eulimnogammarus toletanus	A	0.65	8.0	15.4	[16]
Polycelis tenuis	P	0.58*	7.8-8.0	11.5	[2]
Polycelis felina	P	0.39	8.1	15.0	This study
Crangonyx pseudogracilis	A	0.36	7.8-8.1	12.0-13.0	[34]

*Mean value of several LC_{50} values
$^{+++}$Hard water (270 mg/L $CaCO_3$) $^{++}$Moderately hard water (100 mg/L $CaCO_3$) $^{+}$Soft water (≤42 mg/L $CaCO_3$)

TABLE 4 - Short-term safe concentrations ($LC_{0.01}$ 96 hr) of NH_3-N (mg/L) reported in bibliography for several freshwater invertebrate species. 95% confidence limits are presented in parentheses.

Species	$LC_{0.01}$ 96 hr	Reference
Echinogammarus echinosetosus [a]	0.19 (0.12-0.27)	This study
Potamopyrgus antipodarum [b]	0.16 (0.04-0.30)	[30]
Eulimnogammarus toletanus [a]	0.15 (0.09-0.21)	[16]
Polycelis felina [c]	0.12 (0.03-0.18)	This study

a = amphipod, b = mollusc, c = planarian

pared (95% confidence limits overlap). Our results showed a good dose-response relation between unionized ammonia concentrations and this endpoint for each exposure time. This contrasts with the study of Newton *et al.* [28], who found a high variability and subjectivity in this endpoint for the mollusc *Lampsilis cardium*. Our results showed that the proportion of affected individuals is a sensitivity endpoint, especially in the case of the planarian *P. felina*, whose EC_{50} values to 96, 72 and 48 hr were significantly lower than their corresponding LC_{50} values ($P<0.05$; 95% confidence limits did not overlap) (Table 2). Therefore, the proportion of affected individuals can be a sensitivity and unexpensive endpoint to short-term planarian bioassays.

Ammonia discharges in aquatic ecosystems usually occur as episodes of short duration [3]. Moreover, as a consequence of the daily variation of pH and water temperature in freshwater ecosystems with high alkalinity, the unionized ammonia fraction may reach toxic concentrations to aquatic organisms during short-term periods [31, 37]. Therefore, it is important to determine safe short-term concentrations for unionized ammonia, which may be indicated by $LC_{0.01}$ (or EC) values. These short-term safe concentrations have been shown to be a good level to avoid reduction of feeding activity and survival on the freshwater amphipods *Eulimnogammarus toletanus* [16]. For aquatic invertebrates reported in bibliography, $LC_{0.01}$ values to 96 hr ranged from 0.12 to 0.19 mg/L NH_3-N (Table 4). However, variation in water hardness, velocity and substrate could modify these safe short-term levels [16].

CONCLUSION

Overall, we conclude that *P. felina* showed a relatively high sensitivity to short-term lethal effects of unionized ammonia. The proportion of affected individuals has been shown to be a short-term sensitivity endpoint, especially in the case of the freshwater planarian *P. felina*. $LC_{0.01}$ or $EC_{0.01}$ values can be used as short-term safe level to avoid adverse effects to episodic pollution of unionized ammonia.

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