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Toxic Effects of Unionized Ammonia on Survival and Feeding Activity of the Freshwater Amphipod *Eulimnogammarus toletanus* (Gammaridae, Crustacea)

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High concentrations of ammonia may be present in freshwater ecosystems as a consequence of organic pollution and nutrient enrichment. The foremost anthropogenic sources of ammonia are urban and agricultural runoff, animal farming, industrial wastes and sewage effluents (Williams et al. 1986; Maltby 1995). In a freshwater solution, ammonia can be found as two different chemical species, the unionized form (NH_3) and the ionized form (NH_4^+), this chemical equilibrium depending on water temperature and pH (Emerson et al. 1975; Williams et al. 1986). The toxicity of ammonia is related to the unionized form (Alabaster and Lloyd 1982; Williams et al. 1986; Maltby 1995; Frías-Espericueta et al. 2000), since the ionized form has little toxicity (Alabaster and Lloyd 1982; Williams et al. 1986).

Unionized ammonia at relatively elevated concentrations may cause mortality and other toxic effects on aquatic animals (Alabaster and Lloyd 1982; Williams et al. 1986; Maltby 1995; Frías-Espericueta et al. 2000). In the case of aquatic invertebrates, the toxic effects of ammonia are mediated by damage to the respiratory surfaces and by changes in the haemolymph pH (Colt and Armstrong 1981). Additionally, as gills are involved in the excretion of ammonia, this process is also hindered by high external ammonia concentrations (Spicer and McMahon 1994; Maltby 1995).

Amphipods have been cited as representative shredders of many freshwater ecosystems that play an important role in detritus processing (Cummins 1979). However, there is a lack of knowledge on the effects of unionized ammonia on the feeding activity of these crustaceans. Therefore, the aim of this study is to assess the toxic effects of unionized ammonia on the survival and feeding activity, under laboratory conditions, of the freshwater amphipod *Eulimnogammarus toletanus* (Pinkster & Stock) (Gammaridae, Crustacea) during short-term exposures. This Palearctic species is relatively abundant in unpolluted calcareous streams of the Iberian Peninsula. The endpoint of feeding activity, measured either as egestion rate or as feeding rate, has been previously used to assess the effects of ammonia and other toxicants (e.g., pyrene, phenanthrene, cadmium, zinc) on several aquatic invertebrates (Lotufo and Fleeger 1996; Leppänen et al. 1998; Frías-Espericueta et al. 2000).

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MATERIALS AND METHODS

Amphipods were collected from an unpolluted upper reach of the Henares river (Guadalajara, Central Spain). Physico-chemical characteristics of river water are presented in Table 1. In the laboratory, organisms were distributed into glass aquaria (gravid adults and precopulatory pairs were rejected) and acclimatized to test water (bottled drinking water without chlorine) conditions for a week prior to laboratory experiments (Table 1). During acclimatization, animals were fed with stream-conditioned poplar (*Populus* sp.) leaves.

A first short-term bioassay (4 d) was conducted in triplicate to assess the effects of unionized ammonia on the survival of *E. toletanus*. Twenty-one glass vessels, each containing 0.1 L of test water and 8 amphipods, were used. They were covered with a perforated plastic foil to prevent water evaporation. No aeration was supplied in order to avoid ammonia oxidation. A control and six nominal concentrations of unionized ammonia were used, in triplicate (0.55, 0.75, 0.96, 1.17, 1.37 and 1.78 ppm NH_3-N). The control had less than 0.01 ppm NH_3-N (Table 1). Nominal concentrations were prepared by dissolving the required amounts of ammonium chloride (NH_4Cl, PANREAC, Spain, Lot No. 149959380, reported purity 99.5%) on pre-established volumes of test water. Mortality observations, water renovation and parameter measurements (pH, water temperature and ammonia concentrations) were carried out at 24 hr intervals. The concentrations of unionized ammonia as nitrogen were determined using aqueous ammonia equilibrium calculations on the basis of mean values of water temperature and pH (Emerson et al. 1975). The 24, 48, 72 and 96 hr $LC_{0.01}$, LC_{10} and LC_{50} values, and their respective 95% confidence limits, were calculated using multifactor probit analysis (MPA) software (US Environmental Protection Agency 1991). The 120 and 144 hr $LC_{0.01}$, LC_{10} and LC_{50} values were also calculated by extrapolation using the MPA software. The MPA methodology solves the concentration-time-response equation simultaneously via the iterative reweighed least squares technique (multiple linear regression). The dependent variable is the probit of the proportion responding to each concentration, and the independent variables are exposure time and toxicant concentration. Mean actual concentrations of unionized ammonia were used for calculating LC values (0.50, 0.73, 0.88, 1.06, 1.39 and 1.85 ppm NH_3-N). These concentrations were measured by spectrophotometry (detection limit = 0.01 ppm NH_3-N) in accordance with American Public Health Association (1995). In this study, 24, 48, 72, 96, 120 and 144 hr $LC_{0.01}$ values are considered as short-term safe concentrations of unionized ammonia for *E. toletanus*, since these calculated values refer to the concentration of unionized ammonia affecting 0.01% individuals of the test population. Amphipods were not fed during toxicity test. Dead animals were removed every day.

A second short-term bioassay (6 d) was conducted to assess the toxic effects of unionized ammonia on the feeding activity of *E. toletanus*. This bioassay was performed comparing a control and three nominal concentrations of unionized ammonia (0.06, 0.22 and 0.33 ppm NH_3-N). Six replicates per treatment and

control were used (glass vessels of 0.1 L). The control had less than 0.01 ppm NH_3-N (Table 1). Test solutions were prepared as previously indicated for the first short-term bioassay. The egestion rate (mg dry wt feces/mg dry wt amphipod/d) was used as a feeding activity endpoint on the basis of the methodology developed by Leppänen et al. (1998). Four 2.2 cm-diameter discs of poplar leaves were added to each vessel, so that the mean leaf dry weight per vessel was 88.5 mg. These discs were previously conditioned for 7 d in glass aquaria with stream water and oxygenation to allow the growth of fungi and bacteria on their surfaces. Water oxygenation was produced with airpumps and airstones. Fecal pellets, daily produced in each glass vessel, were collected with a Pasteur pipette and stored at 5°C in glass tubes. After 2 d, both the amphipods and their fecal pellets from three replicates were dried at 60°C for 72 hr. The remaining three replicates were subsequently processed in the same way after 4 d more. Thus, the egestion rate was estimated after 2 d and 6 d. Water renovation and measurements of pH, water temperature and ammonia concentrations were carried out at 24 hr intervals. The mean actual concentrations of unionized ammonia were 0.04, 0.18 and 0.30 ppm NH_3-N per respective treatment. Significant ($P<0.05$) differences in egestion rate between the control and each treatment were examined by means of an analysis of variance (ANOVA-Dunnett test) (Zar 1984) for each exposure time (2 d and 6 d).

Table 1. Physicochemical characteristics of both Henares river water and toxicity test water.

	Toxicity tests	Henares river
Conductivity (μS)	392±7	536.4±13.6
pH	8.0±0.1	7.7±0.1
Calcium (ppm)	45.4±0.9	87.2±14.6
Dissolved oxygen (ppm)	6.4±0.5	8.7±0.7
Water temperature (°C)	15.5±0.7	11.3±1.8
NO_3-N (ppm)	0.6±0.08	1.6±1.3
NO_2-N (ppm)	<0.005	<0.005
NH_3-N (ppm)	<0.01	<0.01

Mean values ± standard deviations are presented for each parameter. Water analyses were performed following standard methods described by American Public Health Association (1995).

After the bioassays, the body length from antennal base to third uropod of amphipods was measured with an ocular micrometer. Differences in body length between control and NH_3-N treatments were assessed by means of an analysis of variance for the first short-term bioassay (ANOVA-Dunnett test) (Zar 1984), and through a Kruskal-Wallis test for the second short-term bioassay due to the absence of normality of data (Zar 1984).

RESULTS AND DISCUSSION

In the first short-term bioassay, all unionized ammonia concentrations caused mortality on amphipods, this lethal effect being proportional to the unionized

ammonia concentration and the exposure time. No mortality was found in control vessels after 96 hr. Mean values of body length in control and the six NH_3-N treatments were 5.8±1.4, 5.8±1.2, 5.8±1.0, 6.2±1.1, 6.8±1.0, 5.9±0.9 and 5.9±1.1 mm, respectively. The control-treatment differences were not significant (P>0.05; Dunnett test).

The $LC_{0.01}$, LC_{10} and LC_{50} values of NH_3-N, and their 95% confidence limits, for *E. toletanus* are presented in Table 2. From the comparison of LC_{50} values for *E. toletanus* with LC_{50} values for other freshwater amphipods (Table 3), we can see that *E. toletanus* was more sensitive to NH_3-N toxicity than *Crangonyx pseudogracilis*, *Hyalella azteca* and *Gammarus pulex*, but more tolerant than *Paracalliope fluviatilis* (Table 3). The result of this comparison suggests that amphipods with a wide geographical range in freshwater ecosystems, *C. pseudogracilis*, *H. azteca* and *G. pulex*, have a relatively high tolerance to the toxicity of unionized ammonia, while amphipods with a narrow geographical range, the Iberian amphipod *E. toletanus* and the Australian amphipod *P. fluviatilis*, are more sensitive to the NH_3-N toxicity. This might explain the potencial influence of unionized ammonia on the distribution of freshwater amphipods in organic polluted rivers. A similar NH_3-N influence was suggested for an exotic Spanish population of the aquatic snail *Potamopyrgus antipodarum* (Alonso and Camargo 2003).

Table 2. 24, 48, 72, 96, 120 and 144 hr $LC_{0.01}$, LC_{10} and LC_{50} values of NH_3-N (ppm) for *Eulimnogammarus toletanus*.

	$LC_{0.01}$	LC_{10}	LC_{50}
24 hr	0.29 (0.18-0.39)	0.75 (0.61-0.86)	1.23 (1.09-1.40)
48 hr	0.19 (0.12-0.26)	0.49 (0.41-0.55)	0.80 (0.75-0.86)
72 hr	0.17 (0.10-0.22)	0.43 (0.35-0.48)	0.70 (0.64-0.76)
96 hr	0.15 (0.09-0.21)	0.40 (0.32-0.46)	0.65 (0.59-0.72)
120 hr	0.15 (0.09-0.20)	0.38 (0.30-0.44)	0.62 (0.56-0.69)
144 hr	0.14 (0.09-0.20)	0.37 (0.29-0.43)	0.61 (0.54-0.68)

95% confidence limits are presented in parentheses.

The second short-term bioassay showed that unionized ammonia affected the egestion rate of *E. toletanus* (Figure 1). No mortality was found in control vessels after 2 d and 6 d. Mean values of body length were 6.0±1.2, 6.2±1.0, 6.0±0.9 and 6.0±0.9 mm for control and treatments. The control-treatments differences were not significant (P>0.05; Kruskal-Wallis Test). The egestion rates did not differ between control vessels and NH_3-N treatments after 2 d of exposure (P>0.05; Dunnett Test). However, the egestion rate tended to be higher than that in the control at 0.04 and 0.18 ppm NH_3-N (Figure 1). This suggests that the egestion rate in *E. toletanus* may be stimulated at low concentrations of unionized ammonia. Similar results were found by Lawrence and Poulter (1998) for ventilation response of *Gammarus duebeni* to copper, and by Crane et al. (1995) for feeding rate of *Gammarus pulex* exposed to malathion 60 (organophosphorus insecticide). In contrast, the egestion rate of amphipods at 0.30 ppm NH_3-N was significantly lower than that in control vessels after 6 d of exposure (P<0.05;

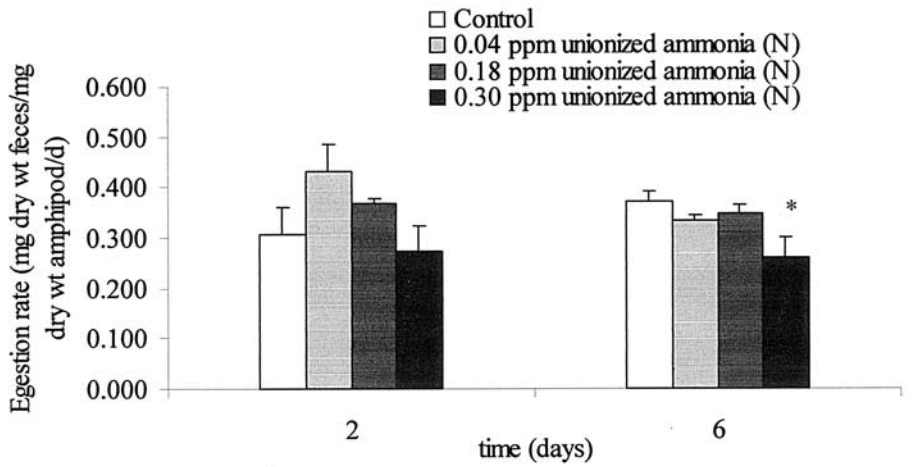

Figure 1. Egestion rate (mg dry wt feces/mg dry wt amphipod/d) of *E. toletanus* exposed to different levels of unionized ammonia during 2 and 6 d. Asterisk shows significant differences between control and treatment for each exposure time (ANOVA; Dunnett test; $P<0.05$).

Dunnett Test). At 0.04 and 0.18 ppm NH_3-N, after 6 d of exposure, the egestion rate was not significantly different from that in control vessels ($P>0.05$; Dunnet Test) (Figure 1). The 144 hr (6 d) $LC_{0.01}$ for *E. toletanus* was 0.14 (0.09-0.20) ppm NH_3-N (Table 2). The no observed effect concentration (NOEC) for the second bioassay was 0.18 ppm NH_3-N after 6 d of exposure, this value being within the 95% confidence limits of the calculated $LC_{0.01}$ value after 6 d. This confirms that the calculated safe concentration of 0.14 ppm NH_3-N is a good limit to avoid mortality and reduction in feeding activity of *E. toletanus* exposed to unionized ammonia stress. However, changes in water hardness, velocity and substrate could either reduce or increase this safe level (Thomas et al. 1991; Ankley et al. 1995).

Table 3. LC_{50} values of NH_3-N (ppm) for several species of freshwater amphipods.

	LC_{50} 24 hr	LC_{50} 48 hr	LC_{50} 96 hr	Reference
Crangonyx pseudogracilis	-	-	1.63-5.63 (1.42-6.91)	Arthur et al. 1987
Hyalella azteca (in hard water)	-	-	2.14-5.38 (1.68-6.04)	Ankley et al. 1995
Gammarus pulex	2.64	2.08	1.69	Williams et al. 1986
Eulimnogammarus toletanus	1.23 (1.09-1.40)	0.80 (0.75-0.86)	0.65 (0.59-0.72)	Present study
Paracalliope fluviatilis	0.31 (0.23-0.45)	0.18 (0.14-0.24)	-	Hickey and Vickers 1994

95% confidence limits are presented in parentheses.

Overall, it is concluded that *E. toletanus* exhibited a relatively high sensitivity to

the short-term toxicity of NH_3-N. In order to protect this species against NH_3-N pollution, a safe level of a maximum NH_3-N concentration of 0.20 ppm for short-term exposures (6 d) is recommended.

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