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SUB-LETHAL RESPONSES OF THE AQUATIC SNAIL *Potamopyrgus antipodarum* (HYDROBIIDAE, MOLLUSCA) TO UNIONIZED AMMONIA: A TOLERANT INVADING SPECIES

Alvaro Alonso - Julio A. Camargo

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SUMMARY

The behavioural endpoint of activity, as mean time to start the normal movements, was used to assess the toxic effect of unionized ammonia (NH_3-N) on the aquatic invasive snail, *Potamopyrgus antipodarum*. Three bioassays were performed: 1) assessment of organism's tolerance to different toxic concentrations of NH_3-N, 2) determination of the time of recovery after toxic exposure to NH_3-N, and 3) evaluation of the snail size on the tolerance to NH_3-N. In the first bioassay, four concentrations and a control were used (0.02, 0.05, 0.10, 0.18 mg/L NH_3-N) in triplicate, recording the activity for 10 days. In the second, snails were exposed for 24 hours to 0.47, 0.82, and 1.15 mg/L NH_3-N in triplicate, and their activity was recorded during 4 days of post-exposure to NH_3-N. In the third, snails of two shell sizes (3-4 mm and 1.5-2.7 mm) were exposed to 0.28 mg/L NH_3-N and a control, in quadruplicate, the activity was recorded daily for 4 days of exposure and for 3 days of post-exposure. Results showed that NH_3-N affected both the activity and recovery of this snail, although the recovery used to be relatively quick, and the shell size did not affect the tolerance to NH_3-N. It is concluded that *P. antipodarum* is an invasive species with a high tolerance and recovery ability to the toxic effects of NH_3-N, which could partly explain its ecological success to dwell in aquatic ecosystems polluted with organic matter.

KEYWORDS: activity, behaviour, invasive species, *Potamopyrgus antipodarum*, recovery, unionized ammonia.

INTRODUCTION

Ammonia is a natural constituent of aquatic ecosystems that mainly derives from the degradation of organic matter. Nowadays, high concentrations of ammonia are generated from different anthropogenic sources, such as industrial wastes, sewage effluents, animal farming and agricultural runoff [1-3]. As a consequence, ammonia concentrations are increasing in surface waters over normal levels, causing toxic effects on aquatic organisms [4, 5].

Normally, ammonia can appear in a freshwater solution in two chemical species, unionized (NH_3) and ionized (NH_4^+), whose equilibrium is affected both by water temperature and pH [6]. Toxicity to aquatic animals has been related to the unionized form [4, 5], the ionized form having little or no toxicity [1, 4]. Unionized ammonia has a high solubility in lipids and it can diffuse across cellular membranes [7]. In fishes, it causes an increase of gill ventilation, hyperexcitability, convulsions and, finally, death [4, 5]. Disruption of pilaster cells, ephitelial necrosis and hyperplasia of gill cells, and subsequent collapse of gill lamellae, have been found in crabs exposed to ammonia [8]. In freshwater amphipods, unionized ammonia caused a reduction in the energy balance and respiration [9].

The evaluation of the toxic effects on aquatic organisms is usually performed through short-term bioassays, which use supraenvironmental toxic concentrations, and end up with the death of the organisms. Therefore, this approach is not very suited to predict the effects of pollution on natural ecosystems [10]. The best method to meet this aim is the monitoring of natural communities, but it requires years of investigation and a wide knowledge of the community dynamics to distinguish the effects of toxics from the natural fluctuations of populations [11]. The monitoring of behavioural endpoints, instead of mortality, is an alternative sublethal method to assess toxic effects, which allows the use of environmentally realistic toxic concentrations [12, 13]. The behavioural response has been studied in several aquatic invertebrates [14-20]. Most of these studies have dealt with mean-sized individuals of natural populations, despite the evidence of the size effects on the tolerance to unionized ammonia, reported for several aquatic invertebrates [2, 21-23]. This factor might be very important to predict the toxic effects of pollutants in natural populations.

An additional factor affecting freshwater invertebrate populations is the quick spread of a few aloctonous taxa [24], whose invasive ability may rely both on a fast reproduction and on a high tolerance to pollution. This is the case of the aquatic snail *Potamopyrgus antipodarum* Gray (=*P. jenkinsi* Smith) (Hydrobiidae, Mollusca). This snail is an ubiquitous and persistent species native of New Zealand, but it is nowadays established in Australia, Europe and North America [24, 25]. This snail has a fast parthenogenetic reproduction [26], shows a high tolerance to organic pollution [27], and is relatively tolerant to lethal short-term toxicity of ammonia, nitrite and nitrate [3].

This work aims to assess the effect of sublethal NH_3-N concentrations on: 1) the behavioural activity (as mean time to start the movement) of *P. antipodarum*; 2) the time to recover activity after toxic exposure; and 3) the effect of body size on the response to NH_3-N. This study would contribute to quantify the importance of ammonia tolerance in the success of this species, as well as to assess if small individuals are more sensitive to pernicious effects of unionized ammonia. It is expected to find a high resistance to sublethal effects of NH_3-N, a short time of recovery, and a higher tolerance in large snails.

MATERIALS AND METHODS

Snails were collected in relatively unpolluted upper reach of the Henares river (Guadalajara, Central Spain) (Table 1). A part of the snails were sieved in two different shell sizes at the riverside (length of small shell: from 1.5 to 2.7 mm and length of big shell: from 3 to 4 mm). Organisms were transported to the laboratory in plastic containers filled with river water. In the laboratory, snails were distributed into 1.5L-glass aquaria, and acclimated to test water (bottled drinking water without chlorine, Table 1) for a week prior to the bioassays. During acclimatization, snails were fed with aquatic macrophytes collected in the same river reach.

The behavioural endpoint was the time (seconds) spent by each snail to start normal movement after manipulation. Each individual was taken up with a forceps and placed in the centre of the test glass vessel (0.1L), which made them to retract their body within the shell; then, the time spent to start the normal movement was recorded with a chronometer, based on the methodology of Cheung and Wong [16]. All observations were made under a binocular. Snails that did not move after 360 seconds were considered to be inactive. Inactive snails were recorded daily in all bioassays.

Three independent bioassays were conducted. The first bioassay was a static one. A control and four nominal concentrations of unionized ammonia were used in triplicate (0.02, 0.04, 0.09 and 0.17 mg/L NH_3-N), the activity of snails being recorded every 24 hours during 10 days. The second bioassay assessed the recovery time after one day of exposure to a control and three nominal concentrations of unionized ammonia, all in triplicate (0.51, 0.85 and 1.19 mg/L NH_3-N). After 24 hours of exposure snails were transferred to control water and their activity recorded after 24, 48, 72 and 96 hours. The third bioassay aimed to assess the effect of snail size both on NH_3-N tolerance and recovery of activity. The two size classes were exposed to a control and a treatment with a nominal concentration of 0.34 mg/L NH_3-N, both in quadruplicate, during 4 days. Activity was recorded after 24, 48, 72 and 96 hours of the toxic addition. After 4 days of exposure, organisms were transferred to control water and their activities recorded after 24, 48 and 72 hours.

The controls of all the bioassays contained less than 0.002 mg/L NH_3-N. Nominal concentrations were prepared by adding a required volume of a 100 mg/L stock solution of total ammonia (NH_4-N+NH_3-N) to give the desired nominal final concentration. Stock solution was prepared daily by dissolving the required amount of ammonium chloride (NH_4Cl, PANREAC, Spain, Lot No. 149959380 with a reported purity of 99.5%) in 1000 mL of test water. Eight randomly selected snails were assigned to each 0.1L-test glass vessels. Then, vessels were placed in a refrigerator with a regulated temperature of 15±1 ºC and covered with a perforated plastic foil to reduce water evaporation. No ventilation was supplied during the bioassays in order to avoid significant reductions of ammonia concentrations by oxidation. Snails were not fed during the toxicity tests to prevent potential changes in the ammonia concentrations. Test solutions and controls were renewed daily in all the bioassays. Temperature, dissolved oxygen, pH and actual concentrations of total ammonia were measured daily, using the standard methods of the American Public Health Association [28]. Unionized ammonia concentrations were calculated using mean values of temperature, pH and nominal and actual total ammonia concentrations [6]. After the bioassays, the shell length of all snails was measured with an ocular micrometer. No significant differences between mean shell length for control and treatments were found for the first two bioassays (P>0.05; Tukey test). In the third bioassay, significant difference between the shell length of small snails and that of big ones was found (P<0.05; Tukey test) (Table 2).

The effects of unionized ammonia on *P. antipodarum* activity at each time were assessed in the first two bioassays through analysis of variance (ANOVA). In the third bioassay the effect of unionized ammonia (exposure and post-exposure to toxic and control) and size (small and big snails) on the activity of *P. antipodarum* at each time of exposure and post-exposure were assessed through a two-way ANOVA. In all statistical analyses the mean time (seconds) spent to start the normal movement of active snails per vessel was the dependent variable. Differences of activity between treatments and controls were assessed through a Dunnett test for the first two bioassays [29]. Differences in mean shell length between control and treat-

TABLE 1
Mean values (± SD) of physico-chemical parameters in control test water and Henares river water. Water analyses were performed following standardised methods described in ref. [28].

	Control-test water	Henares-river water
Conductivity (µS)	784±7	987±275
pH	8.1±0.1	8.1±0.1
Water temperature (ºC)	15.3±0.7	8.0±4.2
Dissolved oxygen (mg/L)	6.8±0.4	12.7±1.2
Calcium (mg/L)	90.8±0.9	120±24
NO_3-N (mg/L)	1.15±0.08	1.60±0.7
NO_2-N (mg/L)	<0.005	0.009±0.007
NH_3-N (mg/L)	<0.002	<0.002

TABLE 2
Mean concentrations (± SD) of actual total (mg/L NH_4-N+NH_3-N) and unionized (mg/L NH_3-N) ammonia, and mean values of shell length (mm) for each bioassay. Nominal total and unionized ammonia concentrations are presented for each bioassay.

	Nominal total ammonia	Nominal unionized ammonia	Actual total ammonia	Actual unionized ammonia	Shell length	
First Bioassay						
Control				<0.002	3.6±0.4	
Treatment 1 (n=10)	0.63	0.02	0.69±0.1	0.02±0.003	3.5±0.4	
Treatment 2 (n=10)	1.25	0.04	1.51±0.2	0.05±0.007	3.3±0.4	
Treatment 3 (n=10)	2.5	0.09	2.9±0.2	0.10±0.007	3.3±0.5	
Treatment 4 (n=10)	5	0.17	5.16±0.9	0.18±0.031	3.5±0.3	
Second Bioassay						
Control				<0.002	3.0±0.5	
Treatment 1 (n=3)	15	0.51	13.9±0.3	0.47±0.01	3.0±0.5	
Treatment 2 (n=3)	25	0.85	24.1±0.1	0.82±0.003	2.7±0.5	
Treatment 3 (n=3)	35	1.19	33.9±1.9	1.15±0.065	2.7±0.4	
Third Bioassay					Big	Small
Control				<0.002	3.5±0.3	2.4±0.2
Treatment (n=4)	10	0.34	8.1±1.3	0.28±0.044	3.6±0.3	2.4±0.4

ments were examined using Tukey test for each bioassay [29]. Data were tested for heterogeneity of variance using Levene's test [30]. When necessary, data were log-transformed to ensure homogeneity of variance. The effect of unionized ammonia on the percentage of inactive snails for each time was assessed through a Kruskal-Wallis test. Significance was accepted at P<0.05 and all statistical analyses were performed using SPSS 11.5 software.

RESULTS

All concentrations cited in the results are mean actual concentrations of NH_3-N (mg/L, Table 2). In the first bioassay, all treatments, except that with the lowest concentration (0.02 mg/L N-NH_3), delayed the start of snail movement with respect to the control. In the second treatment (0.05 mg/L N-NH_3), the significant effect appeared after 144 hours of exposure; in the third (0.10 mg/L N-NH_3) after 96 hours and in the last (0.18 mg/L N-NH_3), after 24 hours (P<0.05; Dunnett Test) (Table 3).

In the second bioassay, the time to recover activity after 24 hours of post-exposure to unionized ammonia, significantly differed between each treatment and the control (P<0.05; Dunnett test) (Figure 1). After 48 and 72 hours of post-exposure significant difference between treatments and control was only found for the highest concentration (P<0.05; Dunnett Test). After 96 hours of post-exposure to unionized ammonia snails in all treatments performed like the control (P>0.05; Dunnett Test).

In the case of the third bioassay, the effect of exposure to unionized ammonia was significant at all the times (P<0.05; two-way ANOVA, Table 4). The effect of the toxic in the post-exposure time was significant only after 24 hours (Table 4). The effect of size on the time to recover movement after exposure and post-exposure to the toxic compound was only significant 24 and 48 hours after exposure (Table 4). The interaction of size and toxic was significant in the 24 hours-exposure treatment, as the toxic effect was more pronounced in the small than in the big snails (Table 4, Figure 2).

TABLE 3

Mean time (seconds) ± standard error for the time spent to start the movement after toxic exposure in the first bioassay. Asterisks show significant differences between control and treatment for each exposure time (ANOVA; Dunnett test; *P*<0.05).

Time (hr)	Control (<0.002 mg N-NH_3/L)	0.02 mg N-NH_3/L	0.05 mg N-NH_3/L	0.10 mg N-NH_3/L	0.18 mg N-NH_3/L
24	9.9±1.2	13.0±1.5	12.5±3.3	14.8±1.1	25.4±5.9*
48	11.1±0.9	13.2±1.2	12.9±2.0	15.2±1.3	26.2±2.6*
72	11.5±0.9	18.2±3.3	24.0±5.9	24.9±4.1	28.0±4.4*
96	11.8±1.2	15.8±0.6	16.5±0.9	25.6±1.0*	32.8±2.6*
120	10.9±1.3	12.9±1.2	17.1±3.3	25.0±4.7*	37.0±0.2*
144	9.3±0.5	18.8±2.5	23.2±2.9*	29.0±4.5*	43.4±4.7*
168	14.9±2.0	15.9±2.5	29.5±2.4*	34.3±3.4*	41.3±3.7*
192	14.0±1.0	27.3±4.4	36.7±5.0*	39.7±9.3*	54.1±5.9*
216	15.6±3.2	21.6±1.6	29.9±2.2*	33.4±2.5*	60.0±6.9*
240	13.5±1.6	22.3±2.7	30.3±2.9*	30.0±0.9*	39.5±7.0*

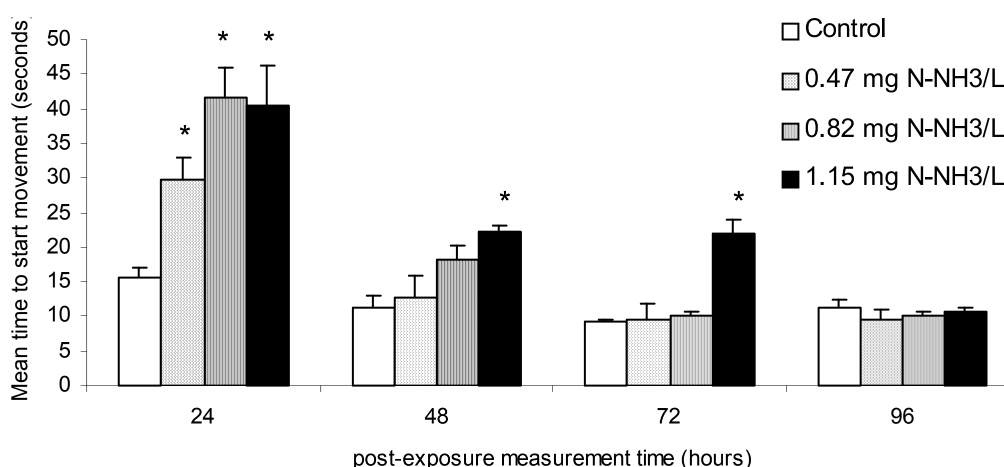


FIGURE 1

Mean time + standard error required by the snails of the second bioassay to start movement after 24, 48, 72 and 96 hours of finishing a 24 hours exposure to 0.47, 0.82 and 1.15 mg/L N-NH_3, and transference back to control water. Asterisks show significant difference between control and treatment for each time (ANOVA; Dunnett test; *P*<0.05).

TABLE 4

Summary of the two-way analysis of variance of the effect of exposure to 0.28 mg N-NH_3/L and size (small 1.5-2.7 mm versus big 3-4 mm) on the activity of *P. antipodarum* for each exposure and post-exposure time.

	Source of variation					
	Exposure to 0.28 mg N-NH_3/L		Size		Size × Exposure to 0.28 mg N-NH_3/L	
	F	*P*	F	*P*	F	*P*
24 hr exposure	303.5	**0.000**	5.99	**0.031**	22.4	**0.000**
48 hr exposure	114.6	**0.000**	6.34	**0.027**	0.14	0.717
72 hr exposure	75.4	**0.000**	1.47	0.248	3.25	0.097
96 hr exposure	151.3	**0.000**	0.39	0.540	0.82	0.384
24 hr post-exposure	16.6	**0.020**	0.15	0.705	0.58	0.461
48 hr post-exposure	4.36	0.059	3.00	0.109	1.68	0.219
72 hr post-exposure	0.01	0.916	0.91	0.359	0.15	0.702

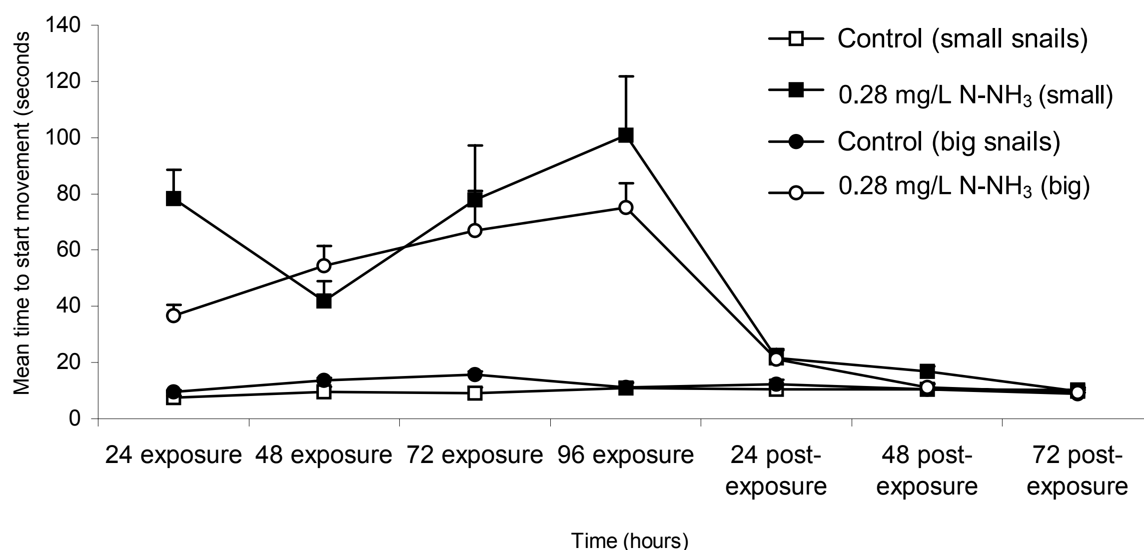


FIGURE 2
Mean time + standard error to start movement for control and treatment of small and big snails, found in the third bioassay after each exposure and post-exposure time (hours).

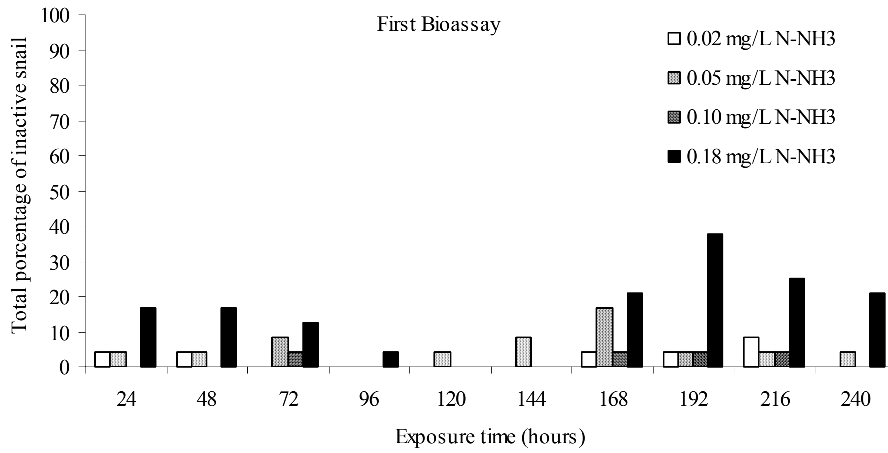
The analysis of the percentage of inactive snails showed a result similar to that of the activity for each bioassay (Figure 3). In the first bioassay, treatment 4 (0.18 mg/l $N\text{-}NH_3$) showed a higher percentage of inactive snails for exposure times over 168 hours. In the second bioassay, all snails exposed to 0.82 and 1.15 mg/l $N\text{-}NH_3$ after 24 hours of exposure were inactive (Figure 3). However, after 24 hours of post-exposure, all snails recovered their activity (Figure 3). In the third bioassay, the percentage of inactive snails increased with the time of exposure, differing from the control after 72 and 96 hours of exposure to unionized ammonia. After 24 hours of post-exposure to unionized ammonia all the snails recovered their normal activity (Figure 3).

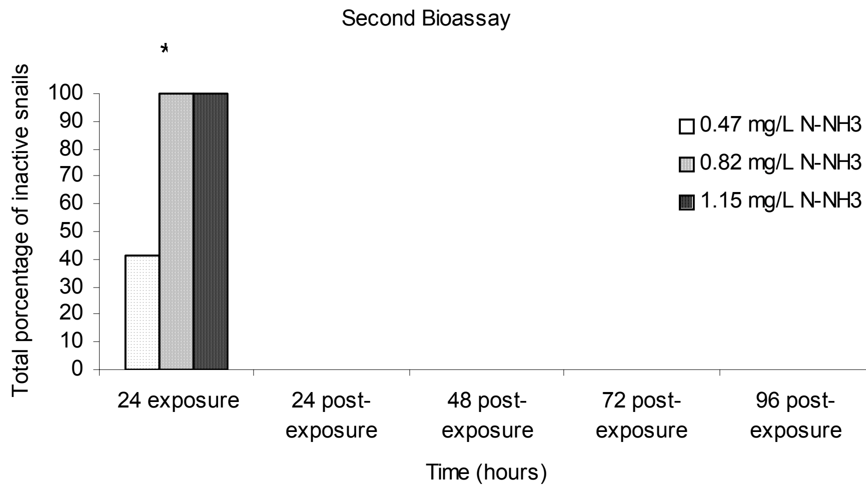
DISCUSSION

This study has demonstrated that sublethal concentrations of unionized ammonia (0.05-1.15 mg/L $N\text{-}NH_3$) caused alterations in the activity of *P. antipodarum*, increasing the time to start the normal movement after exposure, and suppressing movement in part of the test population.

A previous study with the same Spanish exotic population of *P. antipodarum* has demonstrated a great resistance to the lethal effects of unionized ammonia (4-day LC_{50} = 2.02 mg/L NH_3-N, Table 5) [3]. The present concentrations and exposure times (from 1 to 10 days) were, as reported in the literature, enough to kill other aquatic invertebrates (Table 5). The unionized ammonia concentration used in the first and third bioassays (0.18 and 0.28 mg/L $N\text{-}NH_3$, with a exposure time of 10 and 4 days, respectively) killed *Lumbriculus variegatus*, whose 4-day LC_{50} 95% confidence limits ranged from 0.18 to 0.48 mg/L NH_3-N [31]. The unionized ammonia concentration corresponding to 2-day LC_{50} of *Paracalliope fluviatilis* was applied for 10 days and no snail died. Richardson [32] reported for the crustacean *Paratya curvirostris* 1-day LC_{10} 95% confidence limits ranging from 0.99 to 1.90 mg/l NH_3-N. Although, in the second bioassay the snails were exposed to 1.15 mg/L NH_3-N for 1 day, no mortality was found in *P. antipodarum*. This comparison shows the relatively high tolerance of this snail to toxic effects of unionized ammonia.

In addition, we compared our results with others reported in the literature for *P. antipodarum*; the 95% confidence limits for the 4 days LC_{50} values for a New Zealand native population reported by Hickey and Vickers [33] ranged from 0.26 to 0.52 mg/l NH_3-N. In a population from an experimental stream of Great Britain, Watton and Hawkes [21] reported a 4-days LC_{50} values between 0.31 and 0.85 mg/l NH_3-N (Table 5). Alonso and Camargo [3] and the results of the present work show a higher tolerance to ammonia, compared to the above reports. The likely cause for these differences would be the distinct origin of population; in the case of ref. [33], snails come from a native population, and in the case of ref. [21], the organisms were collected from an experimental stream. Other cause would be the different methods used to calculate the LC_{50} values, especially the method in ref. [34] used by the authors of ref. [21]. The results of this method are dependent on the individual who is fitting the data "by eye" [35]. The high tolerance showed by this invasive snail could partly explain the success of this snail to dwell in ammonia polluted european freshwater ecosystems [27].





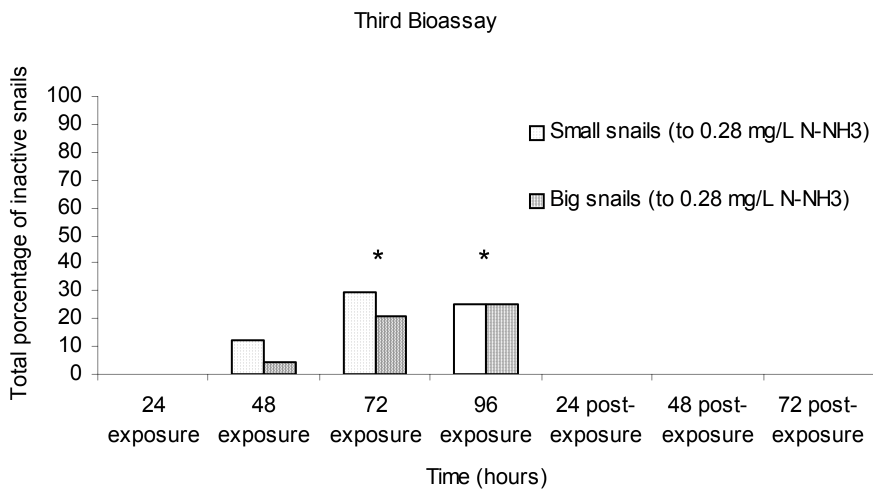


FIGURE 3

Total percentage of inactive snails in each bioassay. The number of inactive snails was zero in every control. Asterisks show significant difference between treatments for each time (Kruskal-Wallis test; *P*<0.05).

TABLE 5

Values of LC_{50} or LC_{10} (mg/L N-NH_3) for some aquatic invertebrates reported in the literature. Unionized ammonia conentrations and/or exposure times used in these studies were similar to those used in the present study. Water temperature (ºC), pH and 95% confidence limits for the LC_{50} or LC_{10} values are presented. Species are ranked in the order of decreasing exposure time.

Species	Group	LC_{50}	LC_{10}	Time (days)	95% Confidence limits	Water temperature (ºC)	pH	Reference
Potamopyrgus antipodarum	Snail	2.02	-	4	1.56-2.45	20.4	8.3	[3]
Potamopyrgus antipodarum	Snail	0.31-0.85	-	4	-	15	7.2-8.0	[21]
Potamopyrgus antipodarum	Snail	0.31-0.44	-	4	0.26-0.52	15-20	7.6-8.2	[33]
Lumbriculus variegatus	Worm	0.29	-	4	0.18-0.48	23	6.5-6.9	[31]
Paracalliope fluviatilis	Crustacean	0.18	-	2	0.14-0.24	15	7.6	[33]
Paratya curvirostris	Crustacean	-	1.23-1.71	1	0.99-1.90	15	7.5-8.1	[32]

The results of the second and third bioassays showed that *P. antipodarum* rapidly recover its activity after an exposure to unionized ammonia, not apparently causing serious damage on the locomotory physiology of *P. antipodarum*. In natural aquatic ecosystems, ammonia discharges usually occur as episodes of short duration [2, 36]. Moreover, when the water has a high alkalinity, the fraction of unionized ammonia may reach toxic levels to aquatic organisms during a short period. In this conditions, the quick recovery to sublethal concentrations together with the low sensitivity to lethal effect of unionized ammonia [3] could explain the success of *P. antipodarum* to dwell in organic polluted and nutrient enriched streams of Europe [27].

The effect of snail size in the tolerance to ammonia revealed by our third bioassay was not very clear, although small snails tended to be less active than big ones through the test period (no significant trend), this trend was the reverse after 48 hours of exposure. Our results partly resembled that of ref. [21], which reported that the juvenile snails (1-2 mm) were less tolerant than the adults (≥4mm) to lethal effects of ammonia. Other studies on other species revealed a different effect of size on the sensitivity to ammonia; ref. [2] concluded that the juveniles of two freshwater crustaceans (*Gammarus pulex* and *Asellus aquaticus*) were less susceptible than the adults to lethal effect of unionized ammonia and hypoxia; ref. [22] reported that the adults of the fingernail clam *Musculium transversum* were more sensitive to ammonia than the younger clams. Hickey and Martin [23] found that adults and juveniles of the freshwater bivalve *Sphaerium novaezelandiae* had a similar sensitivity in a chronic exposure (60 days) to ammonia.

It is concluded that *P. antipodarum* was relatively resistant to the adverse effects of unionized ammonia. This species was able to recover quickly from sublethal toxic stress. The comparison with lethal effect studies of aquatic invertebrates showed a great tolerance of this snail to unionized ammonia in short-term exposures. This high tolerance to this substance could partly explain its ecological success in organic and nutrient enriched aquatic ecosystems. Nevertheless, there is a lack of studies on the toxic effects of unionized ammonia on the behaviour of the aquatic invertebrates, and, especially, on the size-dependent tolerance. Further investigations are required to know which life-history stages are more sensitive to this toxic compound.

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