# Long-Term Effects of Ammonia on the Behavioral Activity of the Aquatic Snail *Potamopyrgus antipodarum* (Hydrobiidae, Mollusca)

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**Abstract** An appropriate approach to assess the effect of toxicants on aquatic animals is to monitor behavioral endpoints, as they are a link between physiological and ecological processes. A group that can be exposed long-term to low toxic concentrations is benthic macroinvertebrates, as their mobility in aquatic ecosystems is relatively limited. Therefore, the study of behavioral long-term effects in this group is suitable from an ecological point of view, as behavioral effects can appear before mortality. During the last decades there has been an increase in ammonia concentrations in freshwater ecosystems, threatening aquatic animals. The present study focuses on the long-term effects (40 days) of nonionized ammonia on the behavioral activity of the aquatic snail *Potamopyrgus antipodarum*. One control and three ammonia concentrations (0.02, 0.07, and 0.13 mg N–NH$_3$/L) were used in triplicate, and the activity of snails (as mean time to start normal movement) and immobility were recorded for each treatment after 0, 10, 20, 30, and 40 days of continuous exposure to nonionized ammonia. The results show that *P. antipodarum* presented a high tolerance to lethal long-term effects of nonionized ammonia, as no animal died during the bioassay. However, the behavioral activity of snails was a very sensitivity endpoint, as a mean nonionized ammonia concentration of 0.07 mg N–NH$_3$/L affected *P.*

*antipodarum*. The results are discussed and compared with the available literature on long-term effects of ammonia on freshwater macroinvertebrates. Additionally, the ammonia water quality criteria, NOECs, LOECs, and long-term LCs are discussed on the basis of the current available data for freshwater macroinvertebrates.

Conventional short-term bioassays apply high concentrations of toxicants, causing death of the animals after a few days of exposure (2–4 days). However, natural ecosystems normally exhibit very low concentrations of chemical compounds, but over a long time (Calevro et al. 1999; Gerhardt et al. 2002). Therefore, conventional short-term bioassays do not properly assess the real effects of actual toxicant concentrations in natural ecosystems, as they are usually nonlethal to aquatic animals at short-term exposures (Rand 1985; Cheung et al. 2002; Scott and Sloman 2004). A more appropriate approach to assess the effect of toxicants on aquatic animals is to monitor behavioral endpoints, as behavioral alterations can appear at lower chemical concentrations, and they are a good link between physiological and ecological processes (Rand 1985; Cheung et al. 2002; De Lange et al. 2006a, b; Scott and Sloman 2004). These endpoints take into account the effects of toxicants in an ecological context (Scott and Sloman 2004) and can be used as nondestructive measures for repeated toxicological monitoring (Gerhardt et al. 2002). Behavioral responses have been studied using different techniques and methodologies, showing a high sensitivity through the different taxonomic groups and toxics, including insects, crustaceans, molluscs, oligochaeta, fish, and amphibians (Kramer et al. 1989; Gerhardt et al. 1994; Richardson et al. 2001; Alonso and Camargo 2004a; Craig and Laming

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2004; Scott and Sloman 2004; Schriks et al. 2006; Gerhardt 2007a, b).

Among the aquatic animals, benthic macroinvertebrates are particularly susceptible to long-term exposure to pollution, as their mobility in aquatic ecosystems is relatively limited (Rosenberg and Resh 1993). In this group different behavioral endpoints have been studied, such as alterations in swimming activity, ventilation, foraging and predator avoidance, pollution avoidance, and reproductive behavior (Watts et al. 2001; Wallace and Estephan 2004; Riddell et al. 2005; De Lange et al. 2006a, b; Gerhardt 2007b). Although most of them have shown higher sensitivity to different toxicants than traditional acute bioassays, behavioral endpoints are not used for regulatory uses or for a probabilistic risk assessment of toxicants, as they are considered less standardized than traditional bioassays (with mortality, growth, and reproduction as endpoints) (Scott and Sloman 2004).

Among behavioral endpoints, the movement abilities of invertebrates (e.g., swimming, sliding, time to start normal activity, feeding activity) are important endpoints, as they permit animals to feed, to avoid predators and pollution, to reproduce, etc. (Golding et al. 1997; Alonso and Camargo 2004b; Cold and Forbes 2004). Therefore, any effect on the behavior caused by a toxicant may potentially alter the structure and function of natural ecosystems, as those alterations can reduce the fitness of the invertebrates (Jones et al. 1991; Cold and Forbes 2004). The different movements of invertebrates can be assessed in different ways through different techniques, either automatically or manually (Kramer et al. 1989; Alonso and Camargo 2004a; Gerhardt 2007a). Therefore, the use of behavioral endpoints can promote a significant improvement in the ecological risk assessment of toxicants, as they can appear at lower concentrations than mortality.

One compound that can alter the behavior of macroinvertebrates is ammonia (Alonso and Camargo 2004a, b, 2006; Craig and Laming 2004; Kirkpatrick et al. 2006). Ammonia is a component of the nitrogen cycle, and under natural conditions it derives from decomposition of organic matter and animal excretion. Additionally, ammonia concentrations can be increased as a consequence of different anthropogenic activities, such as animal and farming runoff, atmospheric deposition, industrial wastes, and urban effluents (Vitousek et al. 1997; Camargo and Alonso 2006; Passel et al. 2007). As a consequence, ammonia is one of the most widespread pollutants in developed countries (Abel 2000). In aquatic ecosystems, ammonia can exist in two chemical species, nonionized ($NH_3$) and ionized ($NH_4^+$), whose equilibrium is controlled by both pH and water temperature (Emerson et al. 1975). Ionic strength also plays a role in ammonia equilibrium, but it is considered small, so it is normally ignored for freshwater

ecosystems (U.S. Environmental Protection Agency 1999; Passel et al. 2007). The nonionized form is toxic to aquatic animals, whereas the ionized form is only toxic at high concentrations and low pH values (Abel 2000; Camargo and Alonso 2006; Passel et al. 2007). Nonionized ammonia has a high solubility in lipids, diffusing easily across cellular membranes, causing an increase in gill ventilation, hyperexcitability, convulsions, epithelial necrosis, and collapse of gill lamellae (Alabaster and Lloyd 1982; Russo 1985; Rebelo et al. 2000; Camargo and Alonso 2006). All these alterations can modify the normal movements of aquatic animals. Despite ammonia's being a common toxin, its potential effect on the behavior of aquatic macroinvertebrates has scarcely been studied at long-term exposures. Previous studies have demonstrated that nonionized ammonia can alter the movements of several freshwater macroinvertebrates, at least for short-term exposures (Alonso and Camargo 2004a, b, 2006).

The main goal of this study is to assess the long-term toxicity of nonionized ammonia on two behavioral activity endpoints of the aquatic snail *Potamopyrgus antipodarum* (Hydrobiidae, Mollusca): time to start the normal movement and immobility. The effect of ammonia toxicity on the behavioral activity of *P. antipodarum* has been previously recorded in short-term bioassays (Alonso and Camargo 2004a). The high tolerance of this snail to human perturbations (including tolerance to ammonia) has been suggested to be a relevant trait to become an invasive species throughout the world (Alonso and Castro-Diez 2008). However, to get a full picture of its invading success, it is necessary to study its long-term tolerance to ammonia. Additionally, other long-term studies on the toxicity of ammonia to benthic macroinvertebrates and safe levels to aquatic ecosystems are reviewed and compared with our results. The present study can contribute to clarify the importance of behavioral endpoints in the assessment of long-term ammonia toxicity in aquatic invertebrates and, also, the assessmment of the potential risk of ammonia to freshwater ecosystems.

## Materials and Methods

Animals were collected from benthos and macrophytes of a nonpolluted reach of the Henares River (central Spain). Snails were transferred to the laboratory in a plastic box filled with river water. In the laboratory, snails were kept in glass aquaria (1.5 L) and acclimated to test water (bottled drinking water without chlorine) through a sequential diluting process for a week prior to the bioassay (i.e., 15% of the river water was replaced by test water during the first day, 30% during the second day, and so on, until reaching 100% test water). The mean physicochemical parameters

of the test water were as follows: conductivity, 784 μS/cm; pH 8.1; water temperature, 15.3°C; dissolved oxygen, 6.8 mg $O_2$/L; calcium, 90.8 mg $Ca^{2+}$/L; nitrate, 1.15 mg N–$NO_3$/L; and nonionized ammonia, <0.002 mg N–$NH_3$/L. These parameters were very similar to those for Henares River (Alonso and Camargo 2004a, b). During acclimation, animals were fed with aquatic macrophytes collected in the Henares River, dry spinach, and fish food (gold fish food, Specipez; Spain). Macrophytes were used as substratum and food, and they were provided at the beginning of the acclimation. Every 2 days, dry spinach and fish food (1:1) were provided ad libitum.

The monitored behavioral endpoints were based on the methodologies of Cheung and Wong (1999) and Alonso and Camargo (2004a). Briefly, the first behavioral endpoint was the time (seconds) spent by each snail to start the sliding movement after manipulation (=time to start normal movement). When an individual was picked up with forceps and placed at the bottom of the test glass vessel (height, 55 mm; diameter, 77 mm; test water volumen, 0.1 L), snails retracted their body into the shell; then the time spent to start the sliding movement was recorded with a chronometer. Normal movement was considered when the snail soft body was out of the shell and the animal slid on the bottom vessel using its foot. When snails did not move after 360 s they were considered to be immobile, which was recorded as the second behavioral endpoint. Both behavioral endpoints were monitored using a stereomicroscope equipped with an optic fiber beam.

A long-term (40-day) bioassay was conducted. A control (<0.002 mg/L N–$NH_3$) and three nominal concentrations of nonionized ammonia were used in triplicate (0.02, 0.07, and 0.14 mg/L N–$NH_3$). Glass vessels with a volume of 0.1 L were used, placing 10 P. antipodarum individuals (mean shell length, 3.3 ± 0.5 mm) in each vessel. The time to start normal movement of each individual from each vessel and the number of immobile snails per vessel were recorded after 0, 10, 20, and 40 days of continuous exposure to nonionized ammonia. When a snail was immobile its operculum was touched with forceps: if it retracted its soft body, the animal was considered to be alive; if not, it was considered to be dead. Nominal nonionized ammonia solutions, controls, and vessels were renewed every 2 days. Snails were fed ad libitum every 4 days; the same amount of food (50% dry spinach and 50% Specipez) was supplied to each vessel during 1 h before solution/vessel renewal.

Nominal ammonia concentrations were prepared from a stock solution of 100 mg/L total ammonia (N–$NH_4$ + N–$NH_3$). Stock solution was made from ammonium chloride ($NH_4Cl$; PANREAC, Spain; Lot No. 149959380, with a reported purity of 99.5%) and was prepared by weighing and dissolving the required amount of salt in 1000 mL of test water. Ammonia stock solution was renewed for every

solution. A small climatic chamber with a controlled temperature of 15°C was used for the bioassay. Vessels were placed in the chamber and covered with a perforated plastic foil to reduce water evaporation. No air pump was used, to avoid ammonia oxidation. Every 2 days the water temperature, pH, dissolved oxygen, and actual concentrations of total ammonia were measured using the standard methods developed by the APHA (1995). The actual concentrations of nonionized ammonia were calculated using the mean values of pH and water temperature that were monitored during the bioassay (Alleman 1998). After bioassay, shell lengths of all animals were measured using a stereomicroscope equipped with a micrometer.

The effect of nonionized ammonia on the first behavioral endpoint at each time point was assessed using a one-way analysis of variance (ANOVA). The dependent variable was the mean time (seconds) spent to start the normal movement of active snails per vessel. Differences in activity between treatments and controls for each exposure time (0, 10, 20, and 40 days) were assessed via a Dunnett test. The heterogeneity of variance across exposure times was tested using Levene's test. When variances were heterogeneous, data were log-transformed and retested to ensure the homogeneity of variance. The normality of data was not tested, as the ANOVA is robust with respect to the assumption of the underlying populations' normality (Zar 1984). The nonionized ammonia effect on the proportion of immobile snails was assessed for each exposure time using the nonparametric test of Kruskal-Wallis. Differences between control and each treatment for each exposure time were assessed using the Mann Whitney U-test. A p value <0.05 was considered statistically significant. For both behavioral endpoints the highest concentration with no significant differences from the control was considered the NOEC (no observed effect concentration), and the lowest one with a significant difference as the LOEC (lowest observed effect concentration). Statistical analyses were performed using SPSS 12.0 software.

## Results and Discussion

The mean actual (n = 40) concentrations of nonionized ammonia (mean actual total ammonia) for each treatment were 0.02 ± 0.003 (0.50 mg N–$NH_4$ + N–$NH_3$/L), 0.07 ± 0.015 (2.10 mg N–$NH_4$ + N–$NH_3$/L), and 0.13 ± 0.017 (3.88 N–$NH_4$ + N–$NH_3$/L) mg N–$NH_3$/L; the mean (±SD) pH was 8.1 ± 0.3 (n = 60); and the water temperature, 15.3 ± 0.5°C (n = 120). All concentrations cited in the results are mean actual concentrations of N–$NH_3$ (mg/L). No significant differences in mean shell length between control and treatments were found in the bioassay (p > 0.05; Tukey test). Mean (±SD) shell lengths for control and increasing

ammonia treatments were $3.3 \pm 0.50$, $3.4 \pm 0.47$, $3.2 \pm 0.51$, and $3.3 \pm 0.52$ mm, respectively.

For the first monitored endpoint (time to start normal movement), all treatments—except that with the lowest concentration (0.02)—delayed the start of the snail movement with respect to the control. For the mean and high concentrations (0.07 and 0.13), the significant effect appeared after 10 days of exposure ($p < 0.05$; Dunnett test) (Fig. 1). This effect was significant for the higher-concentration treatments during the entire bioassay, especially at the last exposure time (40 days). The second monitored endpoint (percentage immobile snails) was significantly increased by the highest concentration with respect to the control after 10 days ($p < 0.05$; Mann Whitney $U$-test) (Fig. 2). The same trend was observed after 20, 30, and 40 days of exposure. The NOEC and LOEC values for both behavioral endpoints are presented in Table 1. The most sensitive endpoint was time to start normal movement as, after 40 days of exposure to
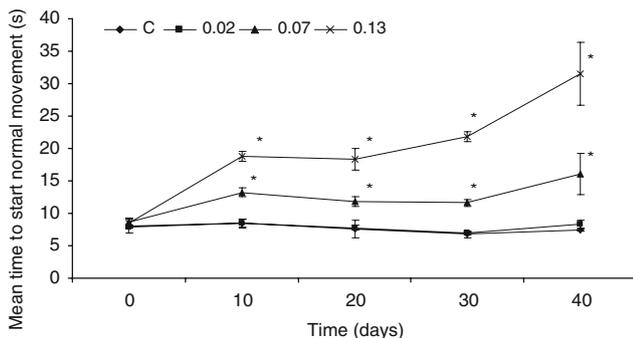


**Fig. 1** Mean time (s) $\pm$ SD to start normal movement after exposure to nonionized ammonia (0, 10, 20, 20, and 40 days). Asterisks indicate significant differences between control (C) and treatments (0.02, 0.07, and 0.13 mg N–NH₃/L) for each exposure time (ANOVA; Dunnett test; $p < 0.05$)
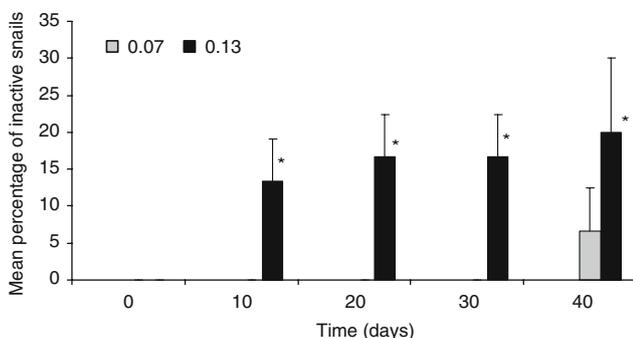


**Fig. 2** The mean percentage of inactive snails in treatments affected by nonionized ammonia. The number of inactive snails was zero in the control and the lowest nonionized ammonia treatment (0.02 mg N–NH₃/L). Asterisks indicate significant differences between treatment (0.07 and 0.13 mg N–NH₃/L) and control for each exposure time (Mann Whitney $U$-test; $p < 0.05$)

nonionized ammonia, the NOEC value was the lowest (0.02 mg/L N–NH₃). Proportion of inactive snails was a less sensitive endpoint (NOEC, 0.07 mg/L N–NH₃), and no mortality was recorded during the bioassay (NOEC, 0.13 mg/L N–NH₃).

Our results show a relatively high tolerance of this invasive snail to lethal long-term exposure to nonionized ammonia, as no animal died during the bioassay. This fact agrees with the high tolerance shown by this snail to short-term exposures to nonionized ammonia in a natural population (LC₅₀ 4 days, 2.02 mg/L N–NH₃ [Alonso and Camargo 2003]). This high tolerance to lethal effects of nonionized ammonia is corroborated when our results are compared with the short-term effects of nonionized ammonia on other aquatic invertebrates. A concentration of 0.09 (0.06–0.18) mg/L N–NH₃ caused the death of 50% of the population of the mollusk *Lampsilis cardium* after 10 days (Newton et al. 2003). Two days of exposure to the same concentration caused mortality to the crustacean *Paracalliope fluviatilis* (Hickey and Vickers 1994). Nonionized ammonia concentrations ranging from 0.10 to 0.23 mg/L N–NH₃ caused mortality after 4 days to three species of mollusks (*Lampsilis fasciola*, *L. cardium*, and *Villosa iris*) (Mummert et al. 2003; Newton et al. 2003). Ammonia also affected the activity of the planarian *Polycelis felina*, with an EC₁₀ 4-day value of 0.14 (0.11–0.16) mg/L N–NH₃ (Alonso and Camargo 2006). All these concentrations were lethal to several species of invertebrates with short-term exposure. However, similar concentrations did not cause mortality to *P. antipodarum* with long-term exposure.

The long-term effects of nonionized ammonia to several species of benthic macroinvertebrates are presented in Table 1. To our knowledge, the international bibliography reports laboratory studies for only four species (the crustacean *Hyalella azteca* and the mollusks *Sphaerium novaezelandiae*, *Villosa iris*, and *Lampsilis siliquoidea*) (Borgmann 1994; Hickey and Martin 1999; Wang et al. 2007) and one chronic mesocosm study (Hickey et al. 1999). All these laboratory studies showed a high sensitivity to nonionized ammonia, as the studied species were killed by concentrations within the range used in the present study. However, all these studies focused on mortality and growth effects, rather than on behavioral responses, except that by Hickey and Martin (1999), who studied morbidity in *Sphaerium novaezelandiae* after chronic exposure to ammonia (60 days). Those authors found similar sensitivities between mortality and number of moribund (e.g., inability to rebury). Only Alonso and Camargo (2004a) reported a high tolerance of *P. antipodarum* to short-term exposures recording the same endpoint, as they found a reduction of activity after 10 days of exposure to a nonionized ammonia concentration of

**Table 1** Long-term toxic effects of nonionized ammonia on *Potamopyrgus antipodarum* (NOEC and LOEC for activity, immobility, and mortality); also, long-term ammonia data for other benthic macroinvertebrates

| Species | Group | Endpoint | Value (mg N–NH$_3$/L) | Time (days) | Water temperature (°C) | pH | Reference |
|---------|-------|----------|-----------------------|-------------|------------------------|-----|-----------|
| *Sphaerium novaezelandiae* | Mollusk | LC$_{50}$ | 0.04 (0.03–0.05) | 60 | 20 | 7.5 | Hickey and Martin (1999) |
| *Sphaerium novaezelandiae* | Mollusk | LOEC$_{mortality}$ | 0.05 | 60 | 20 | 7.5 | Hickey and Martin (1999) |
| *Sphaerium novaezelandiae* | Mollusk | NOEC$_{mortality}$ | 0.01 | 60 | 20 | 7.5 | Hickey and Martin (1999) |
| *Potamopyrgus antipodarum* | Mollusk | LOEC$_{activity}$ | 0.07 | 40 | 15.3 | 8.1 | This study |
| *Potamopyrgus antipodarum* | Mollusk | NOEC$_{activity}$ | 0.02 | 40 | 15.3 | 8.1 | This study |
| *Potamopyrgus antipodarum* | Mollusk | LOEC$_{immobility}$ | 0.13 | 40 | 15.3 | 8.1 | This study |
| *Potamopyrgus antipodarum* | Mollusk | NOEC$_{immobility}$ | 0.07 | 40 | 15.3 | 8.1 | This study |
| *Potamopyrgus antipodarum* | Mollusk | NOEC$_{mortality}$ | 0.13 | 40 | 15.3 | 8.1 | This study |
| *Sphaerium novaezelandiae* | Mollusk | LC$_{50}$ | 0.17 (0.12–0.27) | 30 | 20 | 7.5 | Hickey and Martin (1999) |
| *Hyalella azteca* | Crustacean | LC$_{50}$ | 0.08–0.11 | 28 | 25 | 8.0–8.4 | Borgmann (1994) |
| *Villosa iris* | Mollusk | NOEC$_{survival}$ | 0.06 | 28 | 20 | 8.2–8.4 | Wang et al. (2007) |
| *Villosa iris* | Mollusk | LOEC$_{survival}$ | 0.12 | 28 | 20 | 8.2–8.4 | Wang et al. (2007) |
| *Villosa iris* | Mollusk | NOEC$_{growth}$ | 0.006 | 28 | 20 | 8.2–8.4 | Wang et al. (2007) |
| *Villosa iris* | Mollusk | LOEC$_{growth}$ | 0.03 | 28 | 20 | 8.2–8.4 | Wang et al. (2007) |
| *Lampsilis siliquoidea* | Mollusk | NOEC$_{growth}$ | 0.02 | 28 | 20 | 8.2–8.4 | Wang et al. (2007) |
| *Lampsilis siliquoidea* | Mollusk | LOEC$_{growth}$ | 0.04 | 28 | 20 | 8.2–8.4 | Wang et al. (2007) |

*Note*: Data are arranged by experiment duration (days). Water temperature and pH for each study are included. When only the total ammonia was reported in the original data (Wang et al. 2007), the nonionized ammonia was calculated using the water temperature and mean range of pH levels provided by the authors

0.05 mg/L N–NH$_3$. The NOEC activity value reported in that study coincided with the value found in our study (0.02 mg/L N–NH$_3$). Regarding community effects, the mesocosm study by Hickey et al. (1999) showed that several parameters assessing community structure (number of EPT taxa, taxon richness, and quantitative macroinvertebrate community index) were not affected by ammonia. However, ammonia caused a significant reduction in the abundance of particular taxa (*Deleatidium* sp.; Ephemeroptera: Leptophebiidae and *Coloburiscus humeralis*; Ephemeroptera: Oligoneuriidae). This fact shows that monospecies studies can be more sensitive to deleterious ammonia effects than community parameters.

Although *P. antipodarum* presents a high tolerance to nonionized ammonia, with both short- and long-term exposures, activity (as time to start normal movement) was a very sensitive endpoint, as it was affected by low nonionized ammonia concentrations (0.05–0.07 mg/L N–NH$_3$) (Alonso and Camargo 2004a; see Table 1). For this species, a concentration of 0.02 mg/L N–NH$_3$ can be considered the NOEC to avoid behavioral effects at pH ~ 8.1 (Alonso and Camargo 2004a; Alonso 2005). The water quality criteria for nonionized ammonia reported for several aquatic fauna ranged from 0.01 to 0.10 mg N–NH$_3$/L (U.S. Environmental Protection Agency 1986, 1999; Environment Canada 2001; Alonso 2005; Camargo and Alonso 2006; Passel et al. 2007). Although these criteria have been developed on the basis of different

situations (e.g., pH and temperature, ecosystems) and animal groups (e.g., fish and invertebrates), the highest values may not be protective enough for some freshwater macroinvertebrates, as ammonia is highly toxic to this group of animals, especially for mollusks in the Unionidae family (Newton et al. 2003). For *P. antipodarum*, long-term and subchronic exposures to nonionized ammonia concentrations from 0.05 to 0.13 mg N–NH$_3$/L affected behavioral activity (Alonso and Camargo 2004a; present study), showing that the NOEC of freshwater macroinvertebrates must be lower than 0.05 mg N–NH$_3$/L to avoid behavioral effects. However, for ecosystems with pH values <7.5 the toxicity of ammonia must consider the ionized ammonia fraction (NH$_4^+$), as it can be very high (Passel et al. 2007). Additionally, our study highlights that a species that survives long-term exposures to high ammonia concentrations may be behavioral-sensitive to relatively low concentrations.

The estimation of NOECs to avoid behavioral effects is relevant from an ecological viewpoint, as toxicants can alter the behavior of snails, for example, the feeding activity, time to start normal movement, and ability to avoid polluted conditions, retract their foot, and close their operculum (Burris et al. 1990; this study). All these factors affect ecological interactions of animals (e.g., ability to avoid predation) and individual fitness-related traits (e.g., growth rate and reproduction). However, the behavioral endpoints have been criticized because they include an

important component of subjectivity and variability between and within individuals, especially when qualitative behavioral endpoints are used (Gerhardt et al. 1994). For these reasons they are scarcely used for regulatory uses or for probabilistic risk assessment of toxicants (Scott and Sloman 2004). However, the studied behavior in *P. antipodarum* can be easily observed and objectively quantified, avoiding qualitative subjectivity.

On the basis of our study, we conclude that the inclusion of quantitatively assessed behavioral endpoints of activity in the assessment of ecological risk of ammonia and water quality guideline derivations can be a suitable improvement, as they have been demonstrated to be more sensitive than classical toxicological endpoints (e.g., mortality). Regulatory guidelines for aquatic pollutants have traditionally been based on acute tests (e.g., mortality) and, also, on growth and reproduction. However, acute tests do not consider alterations in behavior, which can make animals unviable under environmental conditions (Scott and Sloman 2004). Our study shows that *P. antipodarum* has a behavioral property that can be objectively quantified in laboratory bioassays and, therefore, applied in ecotoxicology. However, further studies are needed to assess other factors that can also modify animal behavior (e.g., age, light intensity), before including this endpoint in the ecological risk assessment of ammonia.

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