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Ameliorating Effect of Chloride on Nitrite Toxicity to Freshwater Invertebrates with Different Physiology: a Comparative Study Between Amphipods and Planarians

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Abstract High nitrite concentrations in freshwater ecosystems may cause toxicity to aquatic animals. These living organisms can take nitrite up from water through their chloride cells, subsequently suffering oxidation of their respiratory pigments (hemoglobin, hemocyanin). Because NO_2^- and Cl^- ions compete for the same active transport site, elevated chloride concentrations in the aquatic environment have the potential of reducing nitrite toxicity. Although this ameliorating effect is well documented in fish, it has been largely ignored in wild freshwater invertebrates. The aim of this study was to compare the ameliorating effect of chloride on nitrite toxicity to two species of freshwater invertebrates differing in physiology: *Eulimnogammarus toletanus* (amphipods) and *Polycelis felina* (planarians). The former species presents gills (with chloride cells) and respiratory pigments, whereas in the latter species these are absent. Test animals were exposed in triplicate for 168 h to a single nitrite concentration (5 ppm NO_2-N for *E. toletanus* and 100 ppm NO_2-N for *P. felina*) at four different environmental chloride concentrations (27.8, 58.3, 85.3, and 108.0 ppm Cl^-). The number of dead animals and the number of affected individuals (i.e., number of dead plus inactive invertebrates) were monitored every day. LT_{50} (lethal time) and ET_{50} (effective time) were estimated for each species and each chloride concentration. LT_{50} and ET_{50} values increased with increases in the environmental chloride concentration, mainly in amphipods. Results clearly show that the ameliorating effect of chloride on nitrite toxicity was more significant in amphipods than in planarians, likely because of the absence of gills (with chloride cells) and respiratory pigments in *P. felina*. Additionally, this comparative study indicates that the ecological risk assessment of nitrite in freshwater ecosystems should take into account not only the most sensitive and key species in the communities, but also chloride levels in the aquatic environment.

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Introduction

Nitrite (NO_2^-) is a natural component of the nitrogen cycle in aquatic systems (Wetzel 2001; Philips *et al*. 2002). This ion is an intermediate oxidation form between ammonia (NH_3+NH_4^+) and nitrate (NO_3^-), being able to be found in unpolluted freshwater ecosystems at concentrations between 0.001 and 0.005 ppm NO_2-N (Kelso *et al*. 1997; Wiesche and Wetzel 1998; Wetzel 2001). Nevertheless, point and nonpoint pollution sources (*e.g.*, industrial wastewater effluents, municipal sewage effluents, runoff from agriculture, emissions to the atmosphere and the subsequent atmospheric depositions) have significantly increased nitrite concentrations (as well as the concentrations of ammonia and nitrate) in freshwater ecosystems (Lewis and Morris 1986; Meybeck *et al.* 1989; Gleick 1993; Smil 2001; Philips *et al*. 2002; Camargo and Alonso 2006).

Elevated concentrations of inorganic nitrogen compounds (*i.e.*, ammonia, nitrite, and nitrate) in freshwater

ecosystems can induce ecotoxicological processes because they are toxic to aquatic animals (Lewis and Morris 1986; Alcaraz and Espina 1994; Jensen 1995; Philips *et al*. 2002; Beketov 2004; Camargo *et al.* 2005; Camargo and Alonso 2006). In the case of nitrite, its toxicity is better documented to fish than to other aquatic animals (Camargo and Alonso 2006), although freshwater invertebrates can have higher toxicological relevance because they usually show a much wider tolerance range to pollutants (Buikema and Voshell 1993; Camargo and Alonso 2006). Among freshwater invertebrates, planarians and amphipods exhibit high sensitivity to various toxicants (Best *et al*. 1981; Pantani *et al*. 1997; Alonso and Camargo 2004, 2006b). In addition, these two animal groups are important components of the benthic macroinvertebrate communities because they play key trophic roles as predators (planarians) and shredders (amphipods) (Lock and Reynoldson 1976; Cummins and Klug 1979; Thorp and Covich 2001; Alonso and Camargo 2004, 2006a).

Aquatic animals can take nitrite up from water through their chloride cells, located in the gills (fish and crustaceans) and in the anal papillae (*Chironomus* larvae, Diptera) (Lewis and Morris 1986; Alcaraz and Espina 1994; Jensen 1995; Neumann *et al*. 2001). The rate of chloride active uptake is hindered by high external nitrite concentrations, because NO_2^- exhibits high affinity for the Cl^- uptake mechanism (Jensen 1995, 2003). Once inside the animal, nitrite can cause dysfunctions of the oxygen-carrying pigments, leading to hypoxia and finally death (Alcaraz and Espina 1994; Jensen 1995; Philips *et al*. 2002; Camargo and Alonso 2006). The oxidation of respiratory pigments seems to be especially active in hemoglobin (fish pigment), being less important in hemocyanin (crustacean pigment) (Jensen 1995, 2003). Other physiological alterations, such as modifications in cardiac activity, reductions in extracellular chloride concentration and muscle K^+ content, hyperventilation, extracellular alkalosis, and enzymatic alterations, have also been observed in aquatic animals exposed to nitrite ions (Jensen 1995, 2003; Das *et al*. 2004; Camargo and Alonso 2006).

Because both nitrite ions and chloride ions compete for the same site of active transport, nitrite toxicity to freshwater animals may be mitigated by elevated chloride concentrations in the aquatic environment (Alcaraz and Espina 1994; Cheng and Chen 1998; Camargo and Alonso 2006). This ameliorating effect of chloride is well documented in some freshwater species, especially in fish (Lewis and Morris 1986; Tomasso 1986; Alcaraz and Espina 1994; Bartlett and Neumann 1998; Tomasso *et al*. 2003; Camargo and Alonso 2006). Similarly, it has been found that sodium and potassium can reduce ammonia toxicity to some aquatic invertebrates (Borgmann and Borgmann 1997; Beketov 2002). However, there is scarce knowledge on the toxic effects of nitrite to wild freshwater invertebrates and their interaction with chloride ions (Glass 1996; Kelso *et al*. 1999; Neumann *et al*. 2001; Alonso and Camargo 2006a). Furthermore, the ameliorating effect of chloride on nitrite toxicity to freshwater invertebrates, lacking specific respiratory structures and pigments (*e.g.*, planarians), has been amply ignored.

The aim of this study was to compare the ameliorating effect of chloride on nitrite toxicity to two species of freshwater invertebrates differing in physiology: the amphipod *Eulimnogammarus toletanus* (Pinkster & Stock) (Gammaridae, Crustacea), and the planarian *Polycelis felina* (Dalyell) (Planariidae, Turbellaria). Amphipods bear gills and hemocyanin for oxygen transport, whereas planarians neither have specific respiratory structures nor pigments (Thorp and Covich 2001). A higher protective effect of chloride must hence be expected in amphipods than in planarians. Additionally, the Iberian amphipod *E. toletanus* has been found to be very sensitive to nitrite toxicity, whereas *P. felina* has shown a high tolerance to this inorganic nitrogen compound at relatively low chloride levels (56 ppm Cl^-; Alonso and Camargo 2006a). Nevertheless, as far as we know, the protective effect of chloride has not yet been examined in these species.

Materials and Methods

Test Organisms and Acclimatization

Invertebrates were collected from an unpolluted upper reach of the Henares River (Guadalajara province, Central Spain). Planarians were collected from the underside of stones using a soft paintbrush, and amphipods were collected with a hand-net (0.250 mm). Animals were transported to the laboratory in plastic containers filled with water from the sampling reach. Once in the laboratory, individuals of each test species were introduced into glass aquaria (1.0 L) and progressively acclimatized to test water (bottle drinking water without chlorine). In general, the Henares River water and test water showed similar physicochemical characteristics (Table 1). Additionally, four different concentrations of chloride were established in test water: the first one was a baseline mean chloride concentration of 27.8 ppm Cl^- (Table 1), and the rest were 58.3, 85.3, and 108.0 ppm Cl^- (see Experimental Design below). The acclimatization period lasted a week prior to the start of bioassays. Precopulatory pairs and gravid amphipods were rejected for the experiment. During acclimatization, amphipods were fed with stream-conditioned poplar leaves (*Populus* sp.), and planarians with gravid amphipods. No food was supplied during bioassays.

Table 1 Mean values (± SD) of physicochemical characteristics of both Henares River water in the sampling site and test water[a]

	Test water	Henares water
Conductivity (μS)	429.7 ± 17.8	536.4 ± 13.6
pH	8.0 ± 0.3	7.7 ± 0.09
Water temperature (°C)	14.3 ± 0.9	11.3 ± 1.8
Dissolved oxygen (ppm)	7.4 ± 0.2	8.7 ± 0.7
Chloride (ppm)	27.8 ± 1.2	6.2 ± 0.6
Calcium (ppm)	39.1 ± 1.6	84.4 ± 17.5
NH_3-N (ppm)	<0.002	<0.002
NO_2-N (ppm)	<0.005	<0.005
NO_3-N (ppm)	2.3 ± 0.8	1.5 ± 1.4

[a] Water analyses were performed following the standard methods of American Public Health Association (1995)

Experimental Design

An independent bioassay (168 h) was conducted in triplicate for each species using small glass vessels (capacity of 0.1 L). Test solutions and controls were renewed daily. All vessels were covered with a perforated plastic foil in order to reduce water evaporation. Eight individuals were randomly assigned to each vessel. In both bioassays, animals were exposed to one lethal concentration of nitrite at four mean concentrations of chloride (27.8, 58.3, 85.3, and 108.0 ppm Cl^-) and to two controls, with 27.8 ppm Cl^- and 108.0 ppm Cl^-. These ranges of chloride concentrations have been found in relatively unpolluted reaches of the Henares River (Alonso and Camargo 2006a). Nominal nitrite concentrations were 5.0 and 100.0 ppm NO_2-N for the amphipod and planarian bioassays, respectively, being around twice of the 96-h LC_{50} values obtained at 56 ppm Cl^- for both species (Alonso and Camargo 2006a). These test nitrite concentrations were chosen to ensure lethal effects on both species, and to test the possible ameliorating effect of different chloride concentrations on a short-term exposure to a lethal nitrite concentration in each species. Nitrite solutions for each bioassay were prepared daily from nitrite stock solutions of 100 ppm NO_2-N plus 27.8, 58.3, 85.3, or 108.0 ppm Cl^-, by dissolving the required amount of sodium nitrite (NO_2Na, Sigma, Steinheim, Germany, lot no. 97H1563, reported purity of 99.5%) in 1000 mL of chloride test water. Chloride solutions were prepared at the beginning of the bioassays by dissolving the required amount of sodium chloride (NaCl, Panreac, Spain, lot no. 142060830, reported purity of 99.0%) in 5000 mL of test water, which had a baseline concentration of 27.8 ppm Cl^- (Table 1). Both salts were weighted after drying at 60°C during 48 h. Water temperature, pH, dissolved oxygen, and chloride and nitrite concentrations were monitored daily. Actual chloride and nitrite concentrations were measured using the argentometric method and the spectrophotometric method, respectively (Spectroquant-Merk®, Germany. Detection limit = 0.005 ppm NO_2-N) (APHA 1995).

Bioassay Monitoring and Statistical Analysis

In both bioassays, two parameters were monitored every 24 h over a period of 7 days: the proportion of dead invertebrates and the proportion of affected individuals (*i.e.*, dead plus inactive invertebrates) (Newton *et al.* 2003; Alonso and Camargo 2006b). An amphipod was considered dead when neither swimming displacement nor movement of any body part were observed after touching the animal with a glass stick, and inactive when no swimming displacement was observed but some body part was active (such as pleopods, uropods, antenna, or gills). The death of a planarian was established when body tissues started to decompose. A planarian was considered inactive when body contraction and no displacement were observed after touching the animal with a soft paintbrush, but body tissues were intact. The endpoint of affected animals was chosen because it has been previously observed and considered as a useful sensitive endpoint for freshwater invertebrates (Alonso and Camargo 2006b). Dead invertebrates were removed every day.

Lethal time (LT_{50}) and effective time (ET_{50}) and their respective 95% confidence limits were calculated for each chloride concentration in each species. The multifactor probit analysis software (MPA; a software developed by U.S. Environmental Protection Agency 1991) was used to calculate LT_{50} and ET_{50} values. The time (hours) was considered as the independent variable (log transform), and the probit of the proportion of invertebrate responding to each time was the dependent variable. One of the advantages of using probit analyses is that 100% responses really are not needed to calculate LC_{50}, LT_{50}, EC_{50}, or ET_{50} values (U.S. Environmental Protection Agency 1991).

Before starting the planarian bioassay, *P. felina* body lengths were measured using a Delta-T leaf area meter (Cambridge, UK). After finishing the *E. toletanus* bioassay, the body length from antennal base to third uropod was measured for each amphipod with an ocular micrometer. Differences in body lengths between treatments were assessed through a one-way analysis of variance followed by a post-hoc Tukey test for each bioassay. LT_{50} and ET_{50} values were considered to be significantly different ($p < 0.05$) between chloride concentrations within a species when 95% confidence limits did not overlap (U.S. Environmental Protection Agency 1991; Mummert *et al.* 2003). Ratio test was conducted to assess significant differences ($p < 0.05$) between chloride concentrations within each

Table 2 LT_{50} and ET_{50} values (expressed in hours) for *Eulimnogammarus toletanus* and *Polycelis felina* after exposures to a mean actual concentration of 5.1 and 101.3 ppm NO_2-N, respectively, at four different mean actual concentrations of chloride*

	Eulimnogammarus toletanus		*Polycelis felina*	
Chloride (ppm)	LT_{50} (h)	ET_{50} (h)	LT_{50} (h)	ET_{50} (h)
27.8	45.1[a] (36.8–53.0)	<24[¶]	36.5[a] (29.7–44.4)	<24[¶]
58.3	88.1[b] (73.4–104.2)	51.1[a] (42.1–43.2)	47.9[b] (37.3–57.8)	<24[¶]
85.3	135.1[c] (114.0–179.6)	97.9[b] (83.0–115.6)	71.1[c] (61.9–79.6)	<24[¶]
108.0	148.0[c] (129.0–187.4)	127.1[c] (113.6–144.9)	72.1[c] (60.9–83.4)	<24[¶]

[¶] ET values could not be calculated

* LT_{50} and ET_{50} values were calculated using the multifactor probit analysis software (MPA) software (US Environmental Protection Agency 1991). The time (hours) was considered as the independent variable (log transform), and the probit of the proportion of individuals responding to each time was the dependent variable. Letters (a, b, c) indicate significant differences between chloride concentrations for each LT_{50} or ET_{50} value. 95% confidence limits are presented in parentheses

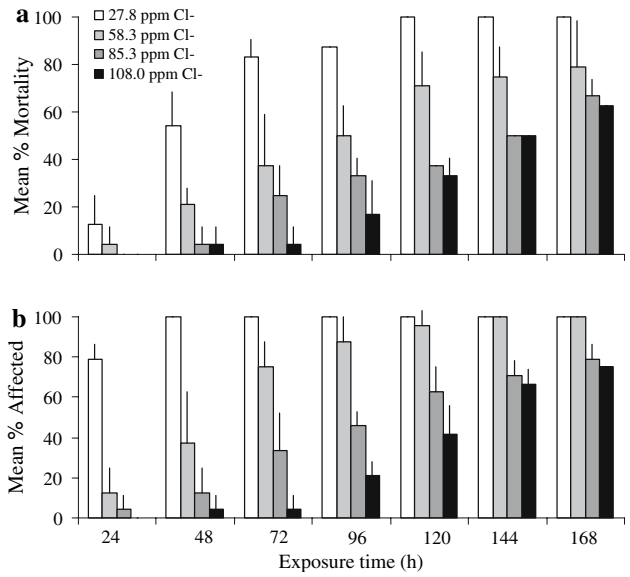

Fig. 1 Mean percentages (+SD) of mortality **(a)** and affected individuals **(b)** for *Eulimnogammarus toletanus* exposed to 5.1 ppm NO_2-N through seven different exposure times (hours) and at four different chloride concentrations (ppm Cl^-)

species when LT_{50} and ET_{50} confidence limits overlapped (Wheeler *et al*. 2006). A regression analysis between chloride concentrations (independent variable) and LT_{50} values (dependent variable) was conducted for each species to elucidate the possible ameliorating effect of chloride.

Results and Discussion

All animals in control vessels (27.8 and 108.0 ppm Cl^-, and <0.005 ppm NO_2-N) survived after 7 days. Inactive animals in these controls were lower than 10% for amphipods and 0% for planarians. Mean body lengths were 6.4 ± 1.0 mm for planarians and 5.6 ± 0.8 mm for amphipods, with no significant difference being found among treatments (including controls) ($p > 0.05$; Tukey test). Mean actual concentrations of nitrite (ppm NO_2-N) in amphipod and planarian bioassays were 5.1 ± 0.1 (n = 14) and 101.3 ± 1.9 (n = 14), respectively. The mean mortality and the proportion of affected animals for *E. toletanus*, at each environmental chloride concentration and exposure time, are shown in Figure 1, and the mean mortality for *P. felina* is shown in Figure 2. LT_{50} and ET_{50} values, and their respective 95% confidence limits, are presented in Table 2 for each test species and each chloride concentration. In both test species, LT_{50} values increased in parallel to the environmental chloride concentration (Figure 3), being significantly different between chloride concentrations ($p < 0.05$; 95% confidence limits did not overlap), except for the two highest concentrations in which they did not significantly differ ($p > 0.05$; Ratio test). ET_{50} values for *E. toletanus* at 27.8 ppm Cl^- could not be calculated, because more than 50% of individuals were affected after 24 h of exposure to 5.1 ppm NO_2-N (Figure 1). The rest of ET_{50} values paralleled the trend of LT_{50} values (Table 2), except for the two highest concentrations in which they differed significantly ($p < 0.05$; Ratio test). ET_{50} values for *P. felina* could not be calculated, because all individuals were inactive after 24 h of exposure to 101.3 ppm NO_2-N at all chloride concentrations (data not shown). The regression analysis between the environmental chloride concentrations and LT_{50} values was positive and significant for both test species, with R^2 values being higher than 0.90 (Figure 3).

The present investigation has clearly shown that chloride ions may protect freshwater invertebrates against nitrite toxicity. This ameliorating effect of chloride has been previously found in the amphipod *Gammarus* sp., in which an artificial supplement of chloride (70 ppm Cl^-) reduced its mortality, and in the dipteran *Chironomus* sp., in which chloride reduced the adverse effects of nitrite on

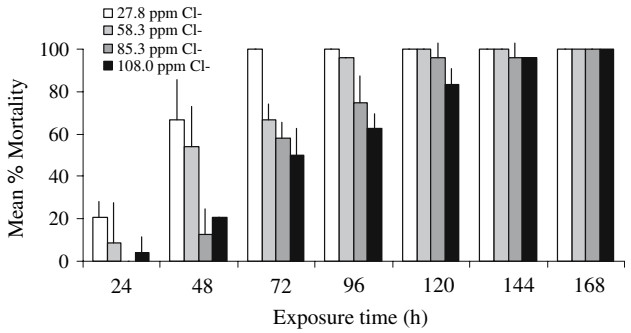

Fig. 2 Mean percentages (+SD) of mortality for *Polycelis felina* exposed to 101.3 ppm NO_2-N through seven different exposure times (hours) and at four different chloride concentrations (ppm Cl^-)

the development of its anal papillae (Glass 1996; Kelso *et al*. 1999; Neumann *et al*. 2001). The Cl^-/NO_2^- competition system is believed to be operational in *Gammarus* sp., and in the case of *Chironomus* sp. it seems to be associated with the anal papillae. Furthermore, Camargo (2004) found that chloride ions increased the tolerance of aquatic larvae of the net-spinning caddisfly *Hydropsyche tibialis* to fluoride toxicity, suggesting that this ameliorating effect was the result of competition between $F^-$and Cl^- ions for the same active transport site. In this connection, our regression analysis (Figure 3) shows that chloride ions reduced significantly the short-term toxicity of nitrite ions in two contrasting species of freshwater

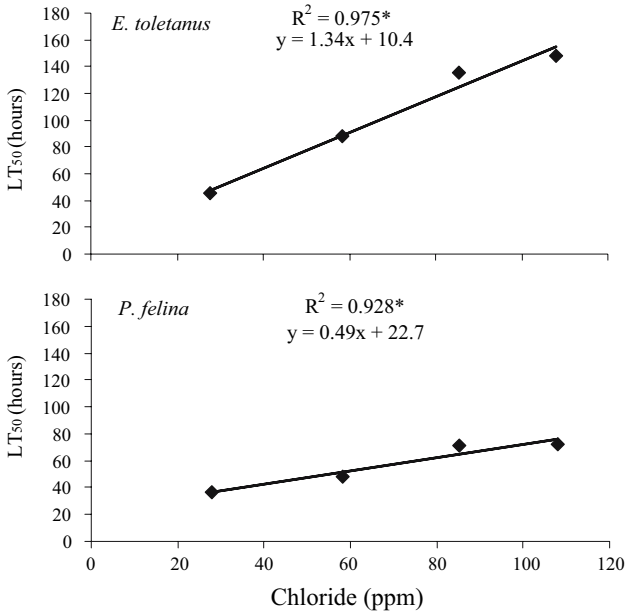

Fig. 3 Regression analysis between LT_{50} values (hours) and chloride concentrations (ppm Cl^-) for *Eulimnogammarus toletanus* (exposed to 5.1 ppm NO_2-N) and *Polycelis felina* (exposed to 101.3 ppm NO_2-N). Asterisk indicates a significant linear relationship between both variables ($p < 0.05$)

invertebrates, and consequently the results might be used to extrapolate chloride effects to other related freshwater invertebrates.

There is strong evidence that the gills are the organs of active ion transport in several freshwater groups, including crayfish and amphipods (Barnes *et al*. 1993; Thorp and Covich 2001). In addition, the respiratory singular pigment in these animal groups is hemocyanin (Ruppert and Barnes 1994). By contrast, planarians have no specific respiratory structures and pigments, the gas exchange being performed by simple diffusion through their body walls (Barnes *et al*. 1993; Ruppert and Barnes 1994; Kolasa 2001). In the absence of specific respiratory structures and pigments, the uptake of Cl^- and NO_2^- across the body walls appears as a likely possibility in planarians (Kolasa 2001). The uptake of NO_2^- might be both via active and passive transport. Indeed, with the highest environmental nitrite concentration used in the present study, passive transport could be an important way for uptake, even if its permeability is low. The latter issue could also explain the high tolerance of planarians to the nitrite toxicity.

Our results highlight that nitrite safe levels in the aquatic environment may be higher the higher the chloride concentrations. This fact seems to be especially relevant for assessing the environmental impact of industrial and sewage effluents, because they often introduce high amounts of both chloride ions and inorganic nitrogen compounds into the recipient freshwater ecosystems (Meybeck *et al.* 1989; Gleick 1993; Smil 2001; Orozco *et al*. 2003). Overall, we conclude that the presence of high chloride concentrations in the aquatic environment may cause an ameliorating effect on nitrite toxicity to the two test freshwater invertebrate species, particularly regarding the sensitive amphipod *E. toletanus*. Therefore, the influence of chloride ions on the toxicity of nitrite ions must be taken into account when the ecotoxicological risk assessment of nitrite is performed in freshwater ecosystems. A similar recommendation was pointed out by Camargo (2004) with regard to the real risk of fluoride ions for freshwater invertebrates. However, it would be necessary to get further information on the ameliorating effects of chloride ions on long-term nitrite toxicity to freshwater invertebrates and other animals in order to determine more realistic nitrite safe levels with different environmental chloride concentrations.

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