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Toxicity of Nitrite to Three Species of Freshwater Invertebrates

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ABSTRACT: Nitrite is a compound with a high toxicity to aquatic animals. Several anthropogenic pollution sources are increasing the concentrations of this component of the nitrogen cycle. Despite this toxicity, there is little available literature on its effects on freshwater invertebrates. Laboratory bioassays were performed to obtain data on the lethal effects of nitrite to three species of freshwater invertebrates: the planarian *Polycelis felina* and the amphipods *Echinogammarus echinosetosus* and *Eulimnogammarus toletanus*. The LC_{50}, LC_{10}, and $LC_{0.01}$ values (mg/L NO_2—N) at 24, 48, 72, and 96 h were calculated for each species. *E. toletanus* and *E. echinosetosus* were the most sensitive species, with 96 h LC_{50} values of 2.09 and 2.59 mg/L NO_2—N, respectively. In contrast, the planarian *P. felina* showed a higher tolerance to nitrite, with a 96 h LC_{50} value of 60.0 mg/L NO_2—N. The obtained results were compared with the reported nitrite data for other freshwater invertebrates. This study may contribute to a more appropriate assessment of the ecological risk of this compound in freshwater ecosystems.
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Nitrite (NO_2^-) is present as a natural component of the nitrogen cycle in freshwater ecosystems (Lewis and Morris, 1986; Philips et al., 2002; Jensen, 2003). This anion derives from the degradation of organic matter, being an intermediate oxidation form between ammonia and nitrate (Lewis and Morris, 1986; Stumm and Morgan, 1996). Two principal genera of bacteria are involved in the oxidation of inorganic N forms through the process of nitrification: ammonia to nitrite by *Nitrosomonas*, and nitrite to nitrate by *Nitrobacter* (Lewis and Morris, 1986; Stumm and Morgan, 1996).

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Nitrite concentrations are increasing in freshwater ecosystems as a consequence of several anthropogenic sources, such as effluents from industries producing metals, dyes and celluloids, urban sewage effluents, and aquaculture (Lewis and Morris, 1986; Jensen, 2003). In addition, loss of nitrification flora, resulting from the toxic action of some chemical compounds (antibiotics, unionized ammonia, etc.), can induce the accumulation of large amounts of nitrite in natural waters (Russo, 1985). Nitrite concentrations higher than 73 mg/L NO_2—N have been found in polluted freshwater environments (Russo, 1985). Nitrite in elevated concentrations can cause environmental problems because of its high toxicity to aquatic animals (Lewis and Morris, 1986; Neumann et al., 2001; Philips et al., 2002; Jensen, 2003). Nitrite can be taken up across gill epithelium and accumulated in body fluids, oxidizing the iron of the fish hemoglobin molecule to methemoglobin, and causing a

TABLE I. Physicochemical characteristics of both Henares river water (sites A and B) and toxicity test water[a,b]

Physicochemical Parameter	Tests	Site A	Site B
Conductivity (μS)	733.5 ± 6.9	536.4 ± 13.6	852.5 ± 106.4
pH	8.1 ± 0.30	7.7 ± 0.09	8.1 ± 0.12
Calcium (mg/L)	77.8 ± 3.0	84.4 ± 17.5	103.0 ± 9.4
Chloride (mg/L)	56.0 ± 2.7	6.2 ± 0.6	95.9 ± 22.0
Dissolved oxygen (mg/L)	6.9 ± 0.3	8.7 ± 0.7	11.6 ± 2.4
Water temperature (°C)	15.5 ± 0.6	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)	0.004 ± 0.003	<0.002	0.0024 ± 0.004

[a] Mean values ± standard deviations are presented for each parameter.
[b] Water analyses were performed following standardized methods described by APHA (1995).

similar reaction with the copper of hemocyanin in crustaceans (Gutzmer and Tomasso, 1985; Rouse et al., 1995; Jensen, 2003), as these oxidized forms are unable to transport oxygen, especially the methemoglobin molecule, causing anoxia and death (Russo, 1985; Meade and Watts, 1995; Rouse et al., 1995; Jensen, 2003). However, nitrite has also been found to cause high mortality in the salmon that exhibited low levels of metahemoglobina. This suggests that other mechanisms also might be involved in the toxicity of nitrite, such as hyperventilation and alterations in cardiovascular function (Russo, 1985; Jensen, 2003).

Although there is good knowledge on the toxic effects of nitrite to several freshwater fish species (Lewis and Morris, 1986; Tomasso, 1986; Jensen, 2003), studies with freshwater invertebrate species are comparatively scarce (Gutzmer and Tomasso, 1985; Ewell et al., 1986; Kelso et al., 1999; Neumann et al., 2001). The aim of this study was to assess the lethal effects of nitrite on three species of freshwater macroinvertebrates under laboratory conditions: the planarian *Polycelis felina* (Dalyell; Planariidae, Turbellaria) and the amphipods *Echinogammarus echinosetosus* (Pinkster; Gammaridae, Crustacea) and *Eulimnogammarus toletanus* (Pinkster & Stock; Gammaridae, Crustacea). These Paleartic species have been selected because these groups have been cited as important predators (planarians) and shredders (amphipods) in many freshwater ecosystems (Boddington and Mettrick, 1977; Cummins and Klug, 1979; Grebe and Schaeffer, 1991; Alonso and Camargo, 2004). Additionally, *E. echinosetosus* and *E. toletanus* have been found to be sensitive species to ammonia and nitrate toxicity (Alonso and Camargo, 2001; Alonso and Camargo, 2004; Camargo et al., 2005). In fact, safe levels of nitrite have not yet been established for fish and aquatic invertebrates.

Amphipods and planarians were collected from two relatively unpolluted upper reaches of the Henares river (Guadalajara, Central Spain). Physicochemical characteristics of water from both reaches (site A for *P. felina* and *E. toletanus* and site B for *E. echinosetosus*) are presented in Table I. Once in the laboratory, each species was distributed into a glass aquarium (1.0 L) and progressively acclimated to test water (bottled drinking water without chlorine, see Table I) for 7 days prior to nitrite bioassays. This test water was selected because its physicochemical properties were similar to the natural conditions of sites where these species dwell (Table I). In the case of amphipods, gravid adults and precopulatory pairs were rejected. During acclimation, amphipods were fed with stream-conditioned poplar (*Populus* sp.) leaves, and planarians were fed every 2 days with chicken liver and gravid adults of the amphipods species.

A short-term bioassay (4 days with daily water renovation) replicated 3 times was conducted for each species, using glass vessels (0.1 L) as experimental units. Vessels were covered with perforated plastic foil in order to reduce water evaporation. No aeration was supplied in order to avoid nitrite oxidation. For amphipod bioassays, a control and five nominal concentrations were used in triplicate for each species (1, 2, 3, 4, and 5 mg/L NO_2—N for *E. echinosetosus* and 0.75, 1.5, 2.25, 3, and 6 mg/L NO_2—N for *E. toletanus*). Eight randomly selected individuals were used per vessel. For the planarian bioassay, a control and six nominal concentrations of nitrite were used in triplicate (30, 50, 100, 150, 200, and 300 mg/L NO_2—N), and 10 randomly selected planarians were used per vessel. Nominal nitrite concentrations were prepared by adding the required volume of a 100 mg/L stock solution of NO_2—N to get the desired nominal final concentration in a final volume of 100 mL of test water. The stock solution was prepared daily by dissolving the required amount of sodium nitrite ($NaNO_2$) (SIGMA, Steinheim, Germany, Lot No. 97H1563, reported purity of 99.5%) in 1000 mL of test water. Planarians and amphipods were not fed during the bioassays. Mortality, pH, water temperature, dissolved oxygen, and nitrite concentrations were monitored daily. Dead animals were removed at 24 h intervals.

The 24, 48, 72, and 96 h LC_{50}, LC_{10} and $LC_{0.01}$ values, with their respective 95% confidence limits, were calculated using the multifactor probit analysis (MPA) software

TABLE II. LC values of NO_2—N (mg/L) for invertebrate test species[a]

Sp[b]	T[c]	$LC_{0.01}$	LC_{10}	LC_{50}
PF	24	45.2 (19.8–80.4)	294 (184–455)	787 (507–1316)
EE	24	0.34 (0.16–0.56)	2.68 (2.02–3.42)	7.89 (6.05–11.1)
ET	24	0.35 (0.14–0.66)	4.72 (2.97–7.58)	18.5 (11.2–36.2)
PF	48	8.13 (3.52–13.8)	52.8 (39.0–65.1)	141 (122–166)
EE	48	0.16 (0.07–0.28)	1.28 (0.96–1.54)	3.76 (3.36–4.30)
ET	48	0.08 (0.03–0.16)	1.10 (0.80–1.38)	4.33 (3.62–5.48)
PF	72	4.59 (1.89–8.04)	29.8 (21.0–37.8)	79.8 (68.3–92.9)
EE	72	0.13 (0.05–0.23)	1.00 (0.71–1.25)	2.93 (2.59–3.34)
ET	72	0.05 (0.02–0.10)	0.68 (0.45–0.89)	2.67 (2.27–3.19)
PF	96	3.45 (1.37–6.18)	22.4 (15.1–29.3)	60.0 (49.2–72.1)
EE	96	0.11 (0.04–0.21)	0.88 (0.61–1.13)	2.59 (2.23–3.00)
ET	96	0.04 (0.01–0.08)	0.53 (0.34–0.72)	2.09 (1.72–2.54)

[a] 95% Confidence limits in parentheses.
[b] PF—*Polycelis felina;* EE—*Echinogammarus echinosetosus;* ET—*Eulimnogammarus toletanus.*
[c] Time (hours).

(U.S. EPA, 1991). This methodology solves the concentration–time–response equation 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 concentrations. Mean actual concentrations of nitrite for each bioassay were used to calculate the LC values: 28.4, 50.5, 99.0, 152.5, 190.0, and 295.0 mg/L NO_2—N for *P. felina*; 0.99, 2.00, 3.06, 4.11, and 5.16 mg/L NO_2—N for *E. echinosetosus*; and 0.65, 1.40, 2.10, 2.85, and 6.08 mg/L NO_2—N for *E. toletanus*. These concentrations were measured by spectrophotometry (detection limit = 0.005 mg/L NO_2—N) in accordance with APHA (1995). We considered the 24, 48, 72, and 96 h $LC_{0.01}$ values to be short-term safe nitrite concentrations for each test species because they affected 0.01% of the individuals of each test population (Alonso and Camargo, 2003).

After the *E. echinosetosus* and *E. toletanus* bioassays, the body length from antennal base to the third uropod of the amphipods was measured with an ocular micrometer. Before the planarian bioassay, the body length of *P. felina* was measured with a Delta-T leaf area meter (Cambridge, UK). Differences in body length between control and NO_2—N treatments for each bioassay were assessed by analysis of variance (ANOVA-Dunnett test; Zar, 1984). All statistical analyses were performed using SPSS 12.0 software (Norusis, 2004).

All nitrite concentrations caused mortality in all the bioassays, this mortality increasing with nitrite concentrations and exposure time. No mortality was found in control vessels after finishing the bioassays. Mean body length in the *E. toletanus*, *E. echinosetosus,* and *P. felina* bioassays was 5.6 ± 1.0, 7.5 ± 1.3, and 7.3 ± 1.8 mm, respectively. No significant differences were found in mean body length between control and nitrite treatments for each bioassay ($P > 0.05$; Dunnett test). Values of LC for each nitrite bioassay, and their respective 95% confidence limits, are presented in Table II. *P. felina* was the least sensitive invertebrate to nitrite short-term toxicity for all LC values (LC_{50}, LC_{10}, and $LC_{0.01}$) and exposure times (24, 48, 72, and 96 h) ($P < 0.05$; 95% confidence limits did not overlap). Both species of amphipod (*E. echinosetosus* and *E. toletanus*) showed high sensitivity to nitrite toxicity in comparison with *P. felina*. After 24 h of exposure, the most sensitive invertebrate was *E. echinosetosus* ($P < 0.05$; 95% confidence limits for LC_{50} values did not overlap), although for 48, 72, and 96 h of exposure, both amphipods showed a similar tolerance.

The comparison of our 96 h LC_{50} values (mg/L NO_2—N) with literature data showed a high tolerance of the planarian *P. felina* to lethal effects of nitrite (60.0 mg/L; Table III). Kelso et al. (1999) reported a 96 h LC_{50} value of 61.6 mg/L for *Polycelis* sp., which is very similar to *P. felina*. Only the invasive snail *Potamopyrgus antipodarum* exhibited a higher 96 h LC_{50} value (535 mg/L; Alonso and Camargo, 2003), being also very tolerant to lethal effects of ammonia and nitrate (Alonso and Camargo, 2003). Both amphipod species showed low tolerance to nitrite toxicity (96 h LC_{50} = 2.09 and 2.59 mg/L for *E. toletanus* and *E. echinosetosus*, respectively). Only the mayfly *Hexagenia* sp. (1.4 mg/L; Kelso et al., 1999) and the Australian crayfish *Cherax quadricarinatus* (1.03 mg/L; Rouse et al., 1995) exhibited lower LC_{50} values. However, in another study, this crayfish species showed a high tolerance to nitrite (25.9 mg/L; Meade and Watts, 1995). Such a discrepancy may be due to the high chloride concentration of the test water used by Meade and Watts (1995) (450 mg/L Cl^-), as this anion has been shown to be an important factor modifying the toxicity of nitrite: an increase of its concentration reduces the nitrite toxicity to several aquatic animals (Gutzmer and Tomasso, 1985; Russo, 1985; Lewis and Morris, 1986; Tomasso, 1986; Jensen, 2003). The likely

TABLE III. 96 h LC_{50} values (mg/L NO_2—N) for several species of freshwater invertebrates[a]

Species	LC_{50}	Cl^-	Reference
Potamopyrgus antipodarum[b]	535	–	Alonso and Camargo (2003)
Polycelis sp.[c]	61.6	30	Kelso et al. (1999)
Polycelis felina[c]	60.0	56.0	Present study
Cherax quadricarinatus[e]	25.9	450	Meade and Watts (1995)
Gammarus sp.[d]	12.3	30	Kelso et al. (1999)
Helisoma trivolvis[b]	10.9	26	Ewell et al. (1986)
Procambarus clarkii[e]	8.91	22	Gutzmer and Tomasso (1985)
Gammarus factiatus[d]	5.89	26	Ewell et al. (1986)
Echinogammarus echinosetosus[d]	2.59	56.0	Present study
Ephemerella sp.[f]	2.5	30	Kelso et al. (1999)
Eulimnogammarus toletanus[d]	2.09	56.0	Present study
Hexagenia sp.[f]	1.4	30	Kelso et al. (1999)
Cherax quadricarinatus[e]	1.03	–	Rouse et al. (1995)

[a] Chloride concentrations (mg/L Cl^-) of test water used in each bioassay are presented.
[b] Snail.
[c] Planarian.
[d] Amphipod.
[e] Crayfish.
[f] Mayfly.

cause of this protective effect is that Cl^- competes with NO_2^- for active transport across the gill cells and into the body cavity (Kelso et al., 1999; Jensen, 2003). In addition, other environmental factors, such as extreme pH values, dissolved oxygen concentration and water temperature, can modified the nitrite toxicity to freshwater fish (Bowser et al., 1983; Watenpaugh et al., 1985; Lewis and Morris, 1986). The mayfly *Ephemerella* sp. showed a tolerance to nitrite similar to *E. echinosetosus* (LC_{50} = 2.5 versus 2.59 mg/L; Kelso et al., 1999), while other freshwater invertebrates showed higher tolerance than both species of amphipods (Table III). In addition, in previous studies these species have also shown high sensitivity to other nitrogen compounds (nitrate and unionized ammonia) with nitrate LC_{50} values (mg/L NO_3—N) to 96 h of 85.0 and 62.5 for *E. toletanus* and *E. echinosetosus*, respectively (Alonso and Camargo, 2001; Camargo et al., 2005), and an unionized ammonia LC_{50} value to 96 h of 0.65 mg/L NH_3—N for *E. toletanus* (Alonso and Camargo, 2004). All these results show the high sensitivity of both amphipod species to inorganic nitrogen compounds. Amphipods could hence be used as biotic sensors of nitrogen enrichment in freshwater ecosystems.

In amphipods, the oxygen is taken up by the gills and transported by hemocyanin, the respiratory pigment of their blood (Ruppert and Barnes, 1994). As nitrite oxidizes the copper of this pigment, it can cause anoxia and death (Meade and Watts, 1995; Rouse et al., 1995). The flatworms have neither respiratory structures nor pigments (Barnes et al., 1993; Ruppert and Barnes, 1994), and gas exchange is conducted across the body wall by simple diffusion (Ruppert and Barnes, 1994). These factors may explain the high tolerance of *P. felina* to nitrite in comparison with the amphipods (*E. echinosetosus* and *E. toletanus*). The high nitrite tolerance of *P. antipodarum*, a mollusk with gills and hemocyanin, may be the consequence of detoxication processes such as a low branchial Cl^-/NO_2^- uptake rate and/or a low nitrite affinity for the uptake mechanism, which have been cited as protective mechanisms for aquatic animals (Jensen, 2003).

Overall, we conclude that the freshwater amphipods (*E. echinosetosus* and *E. toletanus*) showed high sensitivity to nitrite toxicity in comparison with the freshwater planarian *P. felina*. However, to understand the mechanisms of nitrite toxicity to freshwater macroinvertebrates, it is necessary obtain more information on the effects of other environmental factors, such as chloride concentration, dissolved oxygen concentration, extreme pH values, and water temperature to nitrite toxicity. Data from this study may contribute to a more appropriate assessment of the ecological risk posed by this compound.

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