Short Communication

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 LC50, LC10, and LC0.01 values (mg/L NO2−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 LC50 values of 2.09 and 2.59 mg/L NO2−N, respectively. In contrast, the planarian P. felina showed a higher tolerance to nitrite, with a 96 h LC50 value of 60.0 mg/L NO2−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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Keywords: nitrite; amphipods; planarians; short-term toxicity; freshwater; invertebrates

Nitrite (NO2−) 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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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 metahemoglobin. 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 Palearctic 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 (Gualdalajara, 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 NO2−N for E. echinosetosus and 0.75, 1.5, 2.25, 3, and 6 mg/L NO2−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 NO2−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 NO2−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 (NaNO2) (SIGMA, Steinheim, Germany, Lot No. 91H1563, 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 LC50, LC10 and LC0.01 values, with their respective 95% confidence limits, were calculated using the multifactor probit analysis (MPA) software.

### TABLE I. Physicochemical characteristics of both Henares river water (sites A and B) and toxicity test water

<table>
<thead>
<tr>
<th>Physicochemical Parameter</th>
<th>Tests</th>
<th>Site A</th>
<th>Site B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conductivity (μS)</td>
<td>733.5 ± 6.9</td>
<td>536.4 ± 13.6</td>
<td>852.5 ± 106.4</td>
</tr>
<tr>
<td>pH</td>
<td>8.1 ± 0.30</td>
<td>7.7 ± 0.09</td>
<td>8.1 ± 0.12</td>
</tr>
<tr>
<td>Calcium (mg/L)</td>
<td>77.8 ± 3.0</td>
<td>84.4 ± 17.5</td>
<td>103.0 ± 9.4</td>
</tr>
<tr>
<td>Chloride (mg/L)</td>
<td>56.0 ± 2.7</td>
<td>6.2 ± 0.6</td>
<td>95.9 ± 22.0</td>
</tr>
<tr>
<td>Dissolved oxygen (mg/L)</td>
<td>6.9 ± 0.3</td>
<td>8.7 ± 0.7</td>
<td>11.6 ± 2.4</td>
</tr>
<tr>
<td>Water temperature (°C)</td>
<td>15.5 ± 0.6</td>
<td>11.3 ± 1.8</td>
<td>11.7 ± 5.8</td>
</tr>
<tr>
<td>NO3—N (mg/L)</td>
<td>2.7 ± 0.3</td>
<td>1.5 ± 1.4</td>
<td>1.3 ± 1.0</td>
</tr>
<tr>
<td>NO2—N (mg/L)</td>
<td>&lt;0.005</td>
<td>&lt;0.005</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>NH3—N (mg/L)</td>
<td>0.004 ± 0.003</td>
<td>&lt;0.002</td>
<td>0.0024 ± 0.004</td>
</tr>
</tbody>
</table>

*a Mean values ± standard deviations are presented for each parameter.

*b Water analyses were performed following standardized methods described by APHA (1995).
between control and nitrite treatments for each bioassay significant differences were found in mean body length \( E. \) toletanus after finishing the bioassays. Mean body length in the and exposure time. No mortality was found in control ves-
ware (Norusis, 2004).

statistical analyses were performed using SPSS 12.0 soft-
ance of variance (ANOVA-Dunnett test; Zar, 1984). All

centrations of nitrite for each bioassay were used to calcu-
posure time and toxicant concentrations. Mean actual con-
dependent variable is the probit of the proportion responding
sented in Table II. \( P. \) felina was the least sensitive inverte-rate to nitrite short-term toxicity for all LC values (LC50,
trate—time–response equation via the iterative reweighed
dependent variable is the probit of the proportion responding

to nitrite short-term toxicity in comparison with \( P. \) felina. After 24 h of exposure, the most sensitive inver-
vertebrate was \( E. \) echinosetosus \( (P < 0.05; \) 95% confidence limits did not overlap). Both
pecies of amphipod \( (E. \) echinosetosus and \( E. \) toletanus) showed high sensitivity to nitrite toxicity in comparison

95% Confidence limits in parentheses.

PF—Polycelis felina; EE—Echinogammarus echinosetosus; ET—Eulimnogammarus toletanus.

Time (hours).

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

\( ^{a} \)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 concentra-
tion–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 ex-
posure time and toxicant concentrations. Mean actual con-
centrations of nitrite for each bioassay were used to calcu-
late the LC values: 28.4, 50.5, 99.0, 152.5, 190.0, and 295.0
mg/L NO2—N for \( P. \) felina; 0.99, 2.00, 3.06, 4.11, and 5.16
mg/L NO2—N for \( E. \) echinosetosus; and 0.65, 1.40, 2.10,
2.85, and 6.08 mg/L NO2—N for \( E. \) toletanus. These con-
centrations were measured by spectrophotometry (detection
limit \( = 0.005 \) mg/L NO2—N) in accordance with APHA
(1995). We considered the 24, 48, 72, and 96 h \( \text{LC}_{0.01} \) val-
ues 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
NO2—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 soft-
ware (Norusis, 2004).

All nitrite concentrations caused mortality in all the bio-
assays, this mortality increasing with nitrite concentrations
and exposure time. No mortality was found in control ves-
s after finishing the bioassays. Mean body length in the
\( E. \) toletanus, \( E. \) echinosetosus, and \( P. \) felina bioassays was
\( 5.6 \pm 1.0, 7.5 \pm 1.3, \) and \( 7.3 \pm 1.8 \) 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 bio-
assay, and their respective 95% confidence limits, are pre-

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Environmental Toxicology DOI 10.1002/tox
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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