Acute Toxicity of Atrazine, Endosulfan Sulphate and Chlorpyrifos to *Vibrio fischeri*, *Thamnocephalus platyurus* and *Daphnia magna*, Relative to Their Concentrations in Surface Waters from the Alentejo Region of Portugal

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Abstract Ecotoxicological effects of the herbicide atrazine and the insecticides endosulfan sulphate and chlorpyrifos were evaluated using a test battery comprising aquatic organisms from different trophic levels. According to the categories established in the EU legislation, atrazine can be considered non-harmful for the species tested, while the insecticides can be considered very toxic for the crustaceans. The results of acute toxicity tests showed that the sensitivity of organisms were as follows: *Thamnocephalus platyurus* > *Daphnia magna* > *Vibrio fischeri*. Chlorpyrifos may act as a toxic compound in the aquatic environment of Guadiana River, as it may be detected in water at levels that promote toxic effects.

Keywords Chlorpyrifos · Endosulfan sulphate · Atrazine · Aquatic ecotoxicity bioassays

The use of pesticides in agriculture may lead to contamination of surface and ground waters by drift, runoff, drainage and leaching (Cerejeira et al. 2003). Alentejo region (South of Portugal) is an important Portuguese agricultural area. The insecticides endosulfan sulphate and chlorpyrifos and the herbicide atrazine are three of the pesticides most frequently used in Alentejo region crops. These compounds were chosen taking in account their concentration in the surface water of Alentejo region, mainly in Guadiana River, and their environmental significance. The herbicide atrazine may reach values above maximum admissible concentration (MAC) allowed by Portuguese Legislation for surface waters (Decreto-Lei n°236/98 1998). Despite that, atrazine did not pose a significant threat to the aquatic environment. However, Solomon et al. (1996) cautioned that, when atrazine is retained in small, standing-water reservoirs or has repeated inputs to a reservoir, damage can occur in the aquatic ecosystem. The organophosphorous (chlorpyrifos) and organochlorine (endosulfan sulphate) compounds were insecticides which promoted high acute and chronic toxicity to aquatic species (Wan et al. 2005; Zalizniak and Nuugeoda 2006). Furthermore, some reports have indicated that endosulfan and atrazine may act as endocrine disruptors, and therefore can promote changes in aquatic population reproduction (Oehlmann and Schulte-Oehlmann 2003; McKinlay et al. 2008). Additionally, these pesticides are on the list of hazardous substances or priority pollutants reported by 2000/60/EC European Water Framework Directive. The pollutants on this list have been selected due to their environmental relevance and toxic effects to aquatic organisms.

The bioassays used were luminescent inhibition of *Vibrio fischeri* (bacterial species), *Daphnia magna* immobilization and *Thamnocephalus platyurus* mortality (crustacean species). The pesticides’ acute toxicity was classified according to EU-Directive, which establishes toxicity categories for aquatic organisms, based on the values of effective concentration (EC₅₀) (EC 1996).

The purposes of this study were to evaluate the acute toxicity of atrazine, endosulfan sulphate and chlorpyrifos using three species of non-target aquatic organisms, in
3.0 per animals water (ASTM 1998) and a 16 h light:8 h dark photoperiod/C176 maintained in the lab at 20°C ± 1°C. The marine bacteria luminescence was determined.

Two replicates, per treatment, were used. The inhibition of marine bacteria was calculated.

Materials and Methods

Atrazine PESTANAL® (97.4% purity); endosulfan sulphate PESTANAL® (97.7% purity) and chlorpyrifos PESTANAL® (99.2% purity) were obtained from Riedel-de-Häen Labormedikalien GmbH. Stock solutions were prepared with dimethyl sulfoxide (DMSO) obtained from Merk® (>99.0% purity) as a carrier solvent (Hernando et al. 2007). Working stock solutions were prepared using MilliQ® water (resistivity > 18 MΩ cm) and made immediately prior to the test. The maximum amount of DMSO added, in all experiments, was lower than 0.01% (v/v). Prior to the development of toxicity tests, the solution of DMSO in MilliQ® water was analysed to check the absence of toxic effects for the organisms used. The solution of DMSO prepared within the percentage of 0.01% (v/v) did not show toxic effects. A DMSO negative control was included in all experiments. The experiments were performed with nominal concentrations of pesticides, which were not measured during the acute bioassays.

Luminotox® was used to evaluate the inhibition of the marine bacteria V. fischeri luminescence (NRRL B-11177), according to the protocol “DR LANGE luminescent bacteria test” following ISO 11348-2 (1998). The bacteria V. fischeri was supplied, as liquid-dried solution, by Dr. Lange GmbH & Co KG, Düsseldorf. The solution was stored at –20°C and re-hydrated prior to the test. Tests were carried out on 50.0%, 25.0%, 12.5%, 6.25% and 3.125% of the stock pesticides nominal concentrations. Two replicates, per treatment, were used. The inhibition of the bacteria natural light emission was measured against a non-toxic control (2% NaCl solution). The samples were maintained at a temperature of 15°C ± 0.5°C. The pH of the pesticides samples was in the range of 6.5–7.0. For each sample, bioluminescence was measured before and after the desired incubation period (15 and 30 min). The concentration of each pesticide that reduced 50% of the bacteria luminescence was determined.

D. magna were obtained from continuous cultures maintained in the lab at 20°C ± 1°C with ASTM hard water (ASTM 1998) and a 16 h light:8 h dark photoperiod at a light intensity of 100–1,000 Lx. Animal density was 15 animals per 800 mL. Daphnids were fed with algae (Pseudokirchneriella subcapitata) with a density of 3.0 × 10⁵ cells mL⁻¹ (an equivalent carbon content of 2.65 mg C mL⁻¹) three times per week. To evaluate the effects of the three pesticides on daphnids immobility/mortality, tests were conducted in accordance with the standard protocol ISO 6341 (1996) of the International Organization for Standardisation. The nominal concentrations tested were the following: chlorpyrifos (0.50 × 10⁻³, 0.65 × 10⁻³, 0.75 × 10⁻³, 0.80 × 10⁻³, 0.90 × 10⁻³, 1.00 × 10⁻³ mg L⁻¹), endosulfan sulphate (0.20, 0.40, 0.60, 0.75, 0.80, 0.90, 1.00 mg L⁻¹) and atrazine (30.0, 35.0, 40.0, 60.0, 70.0, 80.0, 100 mg L⁻¹). Four replicates, per treatment group, were used with five juveniles (<24 h) per glass jar, filled with 25 mL of test concentration. DMSO with a nominal concentration of 0.01% (v/v) was used in the control group. Animals were not fed during tests. ASTM hard water had a total hardness of 160–180 mg L⁻¹ CaCO₃, pH range of 7.5–8.0 and a conductivity of 580 μS cm⁻¹. During the experiment the pH, temperature, dissolved oxygen, conductivity and ammonium were monitored. After 48 h daphnids were observed for their mobility/death and the EC₅₀ (%) was determined.

To assess the effects of the three pesticides on the mortality of T. platyurus, tests were carried out in accordance to the standard operational procedure provided in THAMNOTOXKIT F™ (Persoone 1999). The concentrations tested were the following: chlorpyrifos (0.40 × 10⁻³, 0.60 × 10⁻³, 0.80 × 10⁻³, 1.00 × 10⁻³, 1.20 × 10⁻³ mg L⁻¹), endosulfan sulphate (0.20, 0.40, 0.60, 0.80, 1.00, 1.20 mg L⁻¹) and atrazine (20.0, 40.0, 60.0, 80.0, 100 mg L⁻¹). The nominal concentrations of pesticides were obtained in synthetic freshwater (water included in the test kit, also used as a control). Larvae of shrimp T. platyurus (<24 h), obtained by the hatching of cysts, were incubated in a 24-well plate test system, 10 crustaceans per well (1.00 mL of test solution), three replicates per treatment, at 25°C for 24 h in the dark. The pH varied within 7.0–7.5. Animals were not fed during tests. The number of dead shrimp, for each treatment, was used as endpoint and the EC₅₀ (%) was calculated.

For all bioassays, a reference test with potassium dichromate (K₂Cr₂O₇) from Merk® was performed as a positive control. The sensitivity of the organisms was in accordance with the followed protocols. The control groups had a survival rate above 90%.

Values of EC₅₀ (%) were calculated using the Probit analysis (MINITAB STATISTICAL Software™ 2000). For the Luminotox bioassays the EC₅₀ (%) values were determined using LUMISsoft 4 Software™.

Results and Discussion

The tested pesticides covered different classes of compounds with different mechanisms of action. Thus, the various toxicity assays could be assessed for their abilities
to respond to a range of possible interactions between the toxic chemicals and the organisms. Table 1 showed that chlorpyrifos was the pesticide with the highest acute toxic effect to the bacteria *V. fischeri* (30 min EC$_{50}$ = 2.84 mg L$^{-1}$). The short-term effect promoted by this insecticide was above that reported by Somasundaram et al. (1990). Endosulfan sulphate promoted a slight toxic effect on *V. fischeri* (30 min EC$_{50}$ = 11.2 mg L$^{-1}$) as indicated in Table 1. Atrazine was the least toxic pesticide for this species (30 min EC$_{50}$ = 69.4 mg L$^{-1}$) (Table 1). According to the toxicity categories based on EC$_{50}$ values, established in European legislation: “very toxic” to aquatic organisms (EC$_{50}$ ≤ 1 mg L$^{-1}$), “toxic” (EC$_{50}$ in the range of 1–10 mg L$^{-1}$), and “harmful” (EC$_{50}$ in the range of 10–100 mg L$^{-1}$); chlorpyrifos can be classified as a “toxic” compound to this aquatic organism, while atrazine and endosulfan sulphate can be classified as “harmful”. *V. fischeri* was the most tolerant organism to these three pesticides, in accordance with Strachan et al. (2001), who indicated that bacteria are not as sensitive to insecticides and herbicides as other aquatic organisms.

The toxic effects promoted in *D. magna* by the pesticides showed a similar trend with the other freshwater organisms tested. Chlorpyrifos promoted the highest toxic effect with a 48 h EC$_{50}$ = 0.74 × 10$^{-3}$ mg L$^{-1}$ (Table 1). Similar values of 48 h EC$_{50}$ for this pesticide, were obtained by other authors (Tomlin 1994; Moore et al. 1998). Zalizniak and Nugegoda (2006) reported a 48 h EC$_{50}$ = 0.50 × 10$^{-3}$ mg L$^{-1}$ for *D. carinata*, which suggests a similar sensitivity of both daphnid species. All these results showed the high sensitivity of the crustaceans to organophosphorus insecticides, presenting very low EC$_{50}$ values for the most part of these compounds. This may be due to the mechanism of action of this class of chemicals, which inhibit cholinesterase enzymes (Fulton and Key 2001). Endosulfan sulphate also showed a high acute toxic effect to *D. magna* (48 h EC$_{50}$ = 0.92 mg L$^{-1}$). A similar result was obtained by Lemke (1980). Barry et al. (1995) reported a value of 48 h EC$_{50}$ = 0.756 mg L$^{-1}$ for *D. carinata*. These data indicate that 48 h EC$_{50}$ values for endosulfan sulphate for both daphnid species were in the same range. Nevertheless, Wan et al. (2005) obtained an acute toxic effect (48 h EC$_{50}$ = 2.12 mg L$^{-1}$) for *D. magna*, a value that is higher than that reported above. According to the toxicity categories, chlorpyrifos and endosulfan sulphate can be classified as “very toxic”, for this aquatic species. Concerning atrazine, the results indicated that, in short exposures, this herbicide was not toxic.

### Table 1: Acute toxicity (EC$_{50}$) of selected pesticides to bacterial species (*Vibrio fischeri*) and freshwater species (*Thamnocephalus platyurus*; *Daphnia magna*). EC$_{50}$ values are expressed as means (95% confidence intervals)

<table>
<thead>
<tr>
<th></th>
<th><em>Vibrio fischeri</em></th>
<th><em>Thamnocephalus platyurus</em></th>
<th><em>Daphnia magna</em></th>
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<td></td>
<td>30 min EC$_{50}$</td>
<td>24 h EC$_{50}$</td>
<td>48 h EC$_{50}$</td>
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<tr>
<td>Atrazine</td>
<td>69.4 (68.8-70.0)$^a$ (n=2)</td>
<td>36.7 (23.2-50.3)$^a$ (n=3)</td>
<td>35.5 (26.3-44.7)$^a$ (n=4)</td>
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<td></td>
<td>39.9 (35.4-44.9)$^b$ $^a$ &gt; 39.9$^c$</td>
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<td>Endosulfan sulphate</td>
<td>11.2 (8.69-13.6)$^a$ (n=2)</td>
<td>0.58 (0.50-0.658)$^a$ (n=3)</td>
<td>0.92 (0.87-0.97)$^a$ (n=4) 2.12 (1.45-3.99)$^c$ 0.74 $^c$</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>2.84 (2.52-3.16)$^a$ (n=2) 46.0x10$^{-3}$ $^d$$^e$</td>
<td>0.53x10$^{-3}$ (0.26x10$^{-3}$,0.79x10$^{-3}$)$^a$ (n=3)</td>
<td>0.74x10$^{-3}$(0.69x10$^{-3}$,0.79x10$^{-3}$)$^a$ (n=4) 0.60x10$^{-3}$ $^e$ 0.17x10$^{-3}$ $^h$</td>
</tr>
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</table>

$^a$ Data reported in this work  
$^b$ Tchounwoul et al. (2000)  
$^c$ Hernando et al. (2007)  
$^d$ Somasundaram et al. (1990)  
$^e$ Wan et al. (2005)  
$^f$ Lemke (1980)  
$^g$ Moore et al. (1998)  
$^h$ Tomlin (1994)  
$^*$ 95% confidence intervals not reported
for *D. magna*. Latt Phyu et al. (2004) measured a 48 h EC$_{50}$ = 24.6 mg L$^{-1}$ for *D. carinata*, and reported that this species may be more sensitive than *D. magna*. However, for both biosensors, this herbicide can be considered as “harmful”, according to the EU-Directive (EC 1996).

To our knowledge, this was the first study about the toxic effects of these three pesticides on *T. platyurus*. The pesticide which exhibited the greatest toxic effect in *T. platyurus* was chlorpyrifos (24 h EC$_{50}$ = 0.53 × 10$^{-3}$ mg L$^{-1}$), followed by endosulfan sulphate (24 h EC$_{50}$ = 0.58 mg L$^{-1}$) and atrazine (24 h EC$_{50}$ = 36.7 mg L$^{-1}$) (Table 1). The highly toxic effect induced by chlorpyrifos in the crustaceans was in line with the observations made by Barron and Woodburn (1995), who indicated acute toxicity to the majority of aquatic invertebrates, between 1.0 × 10$^{-2}$ and 1.0 × 10$^{-3}$ mg L$^{-1}$. Both insecticides were classified as “very toxic” to this organism. Atrazine was classified as “harmful” for this aquatic species. The result was not surprising since its mechanism of action was to inhibit the photosynthetic electron transport and cell division in plants, and crustaceans do not have this biochemical pathway (Bowyer et al. 1991). *T. platyurus* showed a great sensitivity for the two classes of insecticides tested than *D. magna*. The high sensitivity of this crustacean was also reported by other authors for different classes of contaminants, such as pyrethroids (Sánchez-Fortún and Barahona 2005) and trace metals (nanosize and bulk particles of zinc, copper and titanium dioxide) (Heinlaan et al. 2008). However, for other compounds such as prednisone and dexamethasone, *T. platyurus* was less sensitive than *D. magna* (DellaGreca et al. 2004).

For evaluating the potential ecologic risk impact of these pesticides in aquatic ecosystems of the Alentejo Region, we examined the relationship between the acute toxic values obtained from the laboratory studies with the most sensitive species, and the quantified values of these pesticides in surface waters of Guadiana River. The values of these pesticides in surface waters were already reported by other authors (Cerejeira et al. 2003), and in a study by our group at Alqueva reservoir (integrated catchments of Guadiana) (Palma et al. 2007, submitted). This study was performed during 2006–2007 at nine sampling points in the Alqueva reservoir. Water samples were collected bimonthly (at 50 cm of depth, and stored in an amber bottle under cool conditions in the dark). The pesticides analyses were performed using gas chromatography according to DIN EN ISO 6468 (1996), with a limit of quantification of 0.01 μg L$^{-1}$.

The results suggested that endosulfan sulphate is “very toxic” for invertebrates; however, its concentrations in surface water of Alentejo (<0.01–1.80 μg L$^{-1}$) were approximately 2,000 times lower than the concentrations which promoted acute toxic effects. Therefore, it is not considered to be a hazardous compound for acute exposures in Alentejo waters. Atrazine is “toxic” to aquatic animal species when it reached values 600 times higher than those measured in surface water (0.01–5.50 μg L$^{-1}$), so it can be considered a non-toxic compound without any acute risk impact to animal life. However, some plant species may be affected at concentrations near the upper end of this range (Solomon et al. 1996). Chlorpyrifos is the only one of the three pesticides analysed which may constitute a potential risk to aquatic animal species from acute exposures, since its concentrations in surface water of the Alentejo Region (<0.01–0.36 μg L$^{-1}$) were in the same range as the EC$_{50}$ values obtained in acute toxicity studies.

Results from this study confirmed that the toxicity test response, to the same pesticide, is strongly dependent on the sensitivity of the species used. The insecticides were more toxic than herbicides to the aquatic species tested. Furthermore, the freshwater crustacean species were more sensitive to all the pesticides than the bacteria species. Concerning the crustacean species, the results indicated that the most sensitive organism for the insecticides was *T. platyurus*. These results suggest that this species may be a good alternative for standard acute toxicity assays using *Daphnia* sp., and may justify its use in programs of aquatic risk assessment. This investigation provides evidence that the most toxic pesticide was chlorpyrifos, and that its toxicity for crustacean species was in line with the actual concentrations found in surface waters of the Alentejo Region. The results suggest that chlorpyrifos may have a real ecologic impact in this aquatic ecosystem, and may influence the density of some crustacean populations. This indicates a need for performing chronic studies with concentrations measured in surface waters of the Alentejo Region, and possibly with the surface waters themselves.

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**References**


