The use of *Chironomus riparius* larvae to assess effects of pesticides from rice fields in adjacent freshwater ecosystems

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Abstract

A bioassay with *Chironomus riparius* larvae, using larval development and growth as endpoints, was carried out inside a rice field and in the adjacent wetland channel in Portugal, during pesticide treatments (molinate, endosulfan and propanil) to determine impact caused by pesticide contamination in freshwater ecosystems. The bioassay was also performed under laboratory conditions, to assess whether in situ and laboratory bioassays demonstrated comparable results. Growth was inhibited by concentrations of endosulfan (2.3 and 1.9 \( \mu \text{g} L^{-1} \) averages) in water from rice field in both the field and laboratory, and by concentrations of endosulfan (0.55 and 0.76 \( \mu \text{g} L^{-1} \) averages) in water from the wetland channel in the laboratory bioassay, while development was not affected. *C. riparius* larvae were not affected by molinate and propanil concentrations. The results indicate that endosulfan treatments in rice fields may cause an ecological impairment in adjacent freshwater ecosystems. The results also indicate that laboratory testing can be used to assess in situ toxicity caused by pesticide contamination.

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Keywords: *Chironomus riparius*; Bioassays; Endosulfan; Molinate; Propanil; Rice field; Ecological impact; Wetlands

1. Introduction

Several pesticides are globally used in agriculture and may enter aquatic environments through spray drift or runoff events, drainage or leaching, resulting in contamination of surface and ground waters (Leonard et al., 1999; Schulz et al., 2001a, b; Cerejeira et al., 2003). Pesticides used in Portuguese agricultural areas have been found in both surface and ground waters. Insecticides (e.g., endosulfan, lindane, chlorfenvinphos) and herbicides (e.g., propanil, molinate, atrazine) have been detected in the surface waters of three river basins (Tejo, Sado and Guadiana) from 1983 to 1999, and in ground water samples, collected from wells in seven different agricultural areas in the Tejo and Sado basins between 1991 and 1998 (Cerejeira et al., 2003). Waters contaminated with pesticides can cause toxic effects to aquatic flora and fauna (Forney and Davis, 1981; Mulla and Mian, 1981), and may affect human health (Buxton and Stedfast, 1994; Sumner and McLaughlin, 1996).

Rice, along with wheat and corn, is one of the three crops on which many human subsists. Almost two billion people depend on rice for their staple diet (Ronald, 1997). It is also an important crop in terms of pesticide use, particularly herbicides. The use of pesticides during cereal growth may affect the quality of the surrounding aquatic environment. In Portugal, rice is an irrigated crop and water is frequently discharged into surrounding water bodies (Pereira et al., 2000). Studies designed to clarify the potential toxic effects of rice-associated pesticides on aquatic ecosystems are therefore an important tool for biological understanding and environmental management. However, the impact of pesticides on aquatic ecosystems is often difficult to assess because they degrade very quickly (Barry and Logan, 1998), can be absorbed onto the sediments (Peterson and Batley, 1993; Hamer et al., 1999) and peak concentrations that coincide with storm runoff events may be difficult to measure (Cooper, 1996).
The aim of this study was to determine the effect of pesticides used in rice treatments on a selected aquatic macroinvertebrate and assess the potential risk of these chemicals to aquatic ecosystems. A bioassay using *Chironomus riparius* larvae and employing biological responses (development, growth) as endpoints was deployed in a rice field and in the adjacent wetland channel during the period of pesticide spraying. Third instar larvae were deployed in rice fields 48 h after each pesticide treatment (endosulfan, molinate, propanil). Bioassays using contaminated water from the rice fields and conditioned sediments were also conducted under laboratory conditions, at the same time as the in situ bioassay, to assess whether in situ and laboratory tests demonstrated comparable results.

Chironomidae larvae have been recorded as pests of rice growing in many temperate countries (Surakarn and Yano, 1995). The larvae either attack the seed itself, or feed on the roots or shoots of young seedlings (Helliwell and Stevens, 2000; Stevens et al., 2000). Damage can arise through their tunnelling activity in the sediment which can destabilise the root systems of young plants and increase turbidity, reducing photosynthesis and slowing the growth of submerged seedlings (Helliwell and Stevens, 2000). But Chironomids are also important macroinvertebrates in the ecology of the aquatic ecosystems. They are the most widespread and often abundant group of insects in freshwater environments (Pinder, 1995). They provide an important link between different trophic levels, and play a key role in recycling organic matter (Prat and Rieradevall, 1995; Garcia-Berthou, 1999).

2. Material and methods

2.1. Study sites and pesticide treatment

In situ bioassays with *C. riparius* were carried out in rice fields within the Natural Reserve of Azilda Marsh, a protected wetland located near the Mondego river in Central Portugal (Fig. 1). The site designated as highly contaminated was located inside a rice field, representing one of the several rice fields that were treated with pesticides (Fig. 1). The control and low contamination sites were located in a channel bordering the rice fields (Fig. 1). The control site was located upstream and the low contamination site was located downstream from the point where the channel received water from the (highly contaminated) rice fields. This herbicide is soluble in water but adsorbs weakly to soil particles (Wauchope et al., 1992; Weed Science Society of America, 1994). It rapidly breaks down in water due to microbial activity and has a low persistence in soil, with a field half-life of 1–3 days (Wauchope et al., 1992; Weed Science Society of America, 1994).

2.2. Test organisms: culture conditions

*C. riparius* larvae used in the bioassays were obtained from laboratory cultures established at the Biology Department, University of Aveiro. The culture unit was an enclosed transparent acrylic box (120 cm X 120 cm X 60 cm), containing all the conditions necessary to complete the whole life cycle of the chironomids and large enough to allow swarming and copulation of emerged adults (OECD, 2000). Cultures were maintained at 20 ± 2 °C, with a 14 h light: 10 h dark photoperiod. At the start of a new culture ∼120 newborn larvae were introduced into plastic beakers (27 cm X 13 cm X 11.5 cm) containing a 2 cm layer of organic matter free natural sediment (ignited in a muffle furnace for 6 h at 450 °C) from the Bestança river (north Portugal) and ASTM reconstituted hard water (ASTM, 2000). This sediment was considered unpolluted because it came from a river segment known to be free from pollution (Faria et al., 2006). A suspension of ground TetraMin® (Tetrawerke, Germany) of 1 mg larvae−1 day−1 was added as the food source (Naylor and Rodrigues, 1995) and each beaker was gently aerated. Seven days later, larvae were transferred to new culture beakers with fresh media, sediment and food (60 larvae per beaker) until emergence.

2.3. In situ bioassays

Twenty four hours after each pesticide treatment in rice fields, five chambers, with a layer of 5 cm natural sediment previously collected from the Bestança river, were placed in each site, to condition the sediment for in situ and laboratory bioassays. Two additional chambers were deployed, to determine pesticide concentrations in the sediment on day 0 and on day 6 of the in situ bioassay. Six additional chambers were placed in each site to condition sediment during 24 h for laboratory tests. All chambers where placed in sites using a holding structure, constructed with 2 baskets (10 X 21 x 25 cm) made of plastic coated metal wire. In situ chambers were modified from a design by Soares et al. (2005) consisting of PVC tube (20 cm high and 4.5 cm inner diameter) with four openings, two laterals (∼10 cm X 7 cm), one at the top and one at the bottom. The openings were covered with 200-µm nylon mesh to allow contact with the sediment and water flow through the chambers.

![Fig. 1. Study sites where the in situ bioassay were performed.](image-url)
On the first day of the bioassays (day 0), 2 days after pesticide treatment, five third instar larvae of *C. riparius* were introduced into each assay chamber using a plastic pipette via a plastic tube (tube size depended on water depth) glued to the 200-µm nylon mesh that covered the assay chamber (glued with white thermal glue, Elis-Taiwan, TN122/WS, Taiwan, which has been shown to be nontoxic to cladocerans; Pereira et al., 1999), to avoid sediment disturbance. TetraMin<sup>®</sup> (~30 mg in suspension) was added to each of these chambers. The body length and head capsule width of 30 additional larvae were measured to determine initial body length and development stage of larvae. The conditioned sediment of the additional five chambers was collected for use in the laboratory tests and the sediment from the other chambers, previously added were collected for pesticide analysis. Water was also collected at each site to use in laboratory tests and for pesticide analysis.

At the end of the experiment (day 6) in each site, the surviving larvae were collected from the five chambers, killed and preserved in Von Törne conservant (Gama, 1964) for later measurement. Sediment from the sixth chamber was collected for pesticide analysis. Water samples from each site were also collected for pesticide analysis. Larval length and developmental stage were determined by measuring body length and head capsule width of larvae, respectively, using a stereomicroscope (Leica MS5, Leica Microsystems AG, Germany) fitted with a calibrated eye-piece micrometer. Growth (body length increase) of larvae was calculated by subtracting the average initial length from each individual final length. *C. riparius* larval instar was determined according to Watts and Pascoe (2000).

### 2.4. Laboratory bioassays

In all treatments, on day 0, field water (300 ml) was introduced into five chambers (glass flasks) with a conditioned sediment layer of 5 cm depth. Five third instar larvae were introduced into each of the chambers and 30 mg of Tetramin<sup>®</sup> in suspension, was added to each chamber. The chambers were provided with artificial aeration under a 14-h light: 10-h dark photoperiod with a temperature of ~20°C. At the end of the experiment (day 6), the surviving larvae were collected from the chambers, killed and preserved in Von Törne conservant (Gama, 1964) for later measurement. Measurement procedures were as described for in situ bioassays. At the same time, another chamber was also included in each treatment without the addition of larvae and its water and sediment were collected for pesticide analysis at the end of the experiment. The treatments used were: (1) water and conditioned sediment brought from each site and (2) ASTM hard water (ASTM, 2000), control water, and natural sediment from the Bestança river, used as a control treatment.

### 2.5. Physical and chemical analysis

In field and laboratory tests, hand-held field meters were used to measure water pH (pH 330/SEF-2, Weilheim, Germany), conductivity (Cond 330i/SET, Weilheim, Germany) and dissolved oxygen (DO) (Oxi 330/SET, Weilheim, Germany) and a maximum–minimum thermometer was used to determine the minimum and maximum temperature of water (880/847881, Electronic Temperature Instruments Ltd, UK) for day 0 and day 6. Water samples were collected on day 0 and on day 6 to determine nitrate, nitrite, ammonia and phosphate concentrations of the water with a direct reading spectrophotometer (DR/2000, Hach company, USA).

The sediment and water samples collected for pesticide analysis were placed in dark glass containers and preserved at 4°C in the dark. The analysis of endosulfan and molinate were carried out by organic solvent extraction with cartridge containing Oasis<sup>®</sup> hydrophilic–lipophilic balance sorbent (HLB), a reversed-phase sorbent for all compounds, followed by a concentration determination using liquid chromatography–mass spectrometry (LC-MS). Propanil analysis was carried out by solid phase extraction (SPE), and concentrations were determined by liquid chromatography–mass spectrometry (LC-MS). Since endosulfan is frequently used associated with deltamethrin by farmers in central Portugal, water and sediment samples collected to determine endosulfan concentrations were also used to determine deltamethrin concentrations, but the results of deltamethrin analysis were below the detection limits (0.002 µg L<sup>−1</sup>). All pesticide concentrations were determined by the accredited laboratory Innova-lab, Spain.

### 2.6. Statistical analysis

The statistical analysis comprised one-way analysis of variance (ANOVA) followed, when necessary, by Tukey HSD multiple comparison tests to test for significant differences in responses of biological endpoints (development and growth) between treatments and between laboratory and field exposures (Zar, 1996). One-way analysis of variance (ANOVA) followed by Dunnett tests was used to compare responses of biological endpoints between reference and contaminated (low- and high-) treatment (Zar, 1996). Responses were tested for normality using the Kolmogorov–Smirnov test. Linear regression analyses were used to investigate significant relationships between biological responses and minimum and maximum water temperature. Statistical analyses were performed using Minitab<sup>™</sup> Release 14.

### 3. Results

#### 3.1. Physical and chemical parameters

Physical and chemical conditions were more variable in the field than in the laboratory (Tables 1 and 2). Physical and chemical parameters of water quality in the in situ bioassays were similar in reference and low contaminated sites but differed between these sites and the highly contaminated sites for the three pesticides (Table 1). Higher values of maximum temperature were observed in the highly contaminated site during the exposure period. Dissolved oxygen and pH values were also higher in the highly contaminated site than in reference and low contamination sites, during all pesticide treatments. In the laboratory bioassays, physical and chemical parameters of water samples were similar in all pesticide treatments (Table 2).

#### 3.2. Pesticide concentrations

Molinate and propanil concentrations and total endosulfan concentrations, the sum of α and β isomers and endosulfan sulphate, in water and sediment during field and laboratory exposure are presented in Table 3. On day 0, the endosulfan concentration in water varied from 0.91–2.78 µg/L<sup>−1</sup>. In the sediment of the rice field this was 0.62 µg kg<sup>−1</sup>, while at the sites in the wetland channel endosulfan was not detected in the sediment. On day 0, the molinate concentration in the water column varied from 4.63 to 0.31 µg L<sup>−1</sup> and in sediment it ranged from 1.43 to 1.09 µg L<sup>−1</sup>. On day 6, the endosulfan and molinate concentrations in both water and sediment were higher in field than in laboratory. Propanil was not detected in sediment samples, however, it was detected in the water from the rice field on day 0 (2.58 µg L<sup>−1</sup>) and in the laboratory mesocosms on day 6 (0.14 µg L<sup>−1</sup>).
3.3 Biological responses

The herbicides molinate and propanil did not cause biological responses of *C. riparius* larvae (Fig. 2). No significant (*P* > 0.05) differences in endpoints between treatments were observed in laboratory and field exposures to herbicides, although a trend of decreasing larval growth was observed from the highest to the lowest (reference) contaminated sites during molinate exposure in field (Fig. 2).

The insecticide endosulfan had a negative effect on larval growth under field and laboratory exposure, but did not affect larval development. During endosulfan exposure, all larvae changed from the third to the fourth instar and no significant (*P* > 0.05) differences were observed in head capsule width of larvae between treatments, although lower values were observed in the rice field channel (Fig. 3).

In situ, larval growth was significantly lower in the rice field than in the reference and low contaminated sites in the wetland channel (Fig. 3), whilst no significant differences in larval growth were observed between reference and low contaminated sites (*P* > 0.05).

In the laboratory differences in larval growth were smaller but similarly significant between treatments, with lower growth observed in the treatment with water and

### Table 1
Physical and chemical parameters of water in day 6 and day 0 (in parenthesis) of in situ bioassays

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temp$_{\text{min}}$ (°C)</th>
<th>Temp$_{\text{max}}$ (°C)</th>
<th>pH</th>
<th>DO (mg L$^{-1}$)</th>
<th>Cond (ms cm$^{-1}$)</th>
<th>Nitrate (mg L$^{-1}$)</th>
<th>Nitrite (mg L$^{-1}$)</th>
<th>Amonia (mg L$^{-1}$)</th>
<th>Phosphate (mg L$^{-1}$)</th>
<th>Hardness (mg Ca CO$_3$ L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endosulfan</td>
<td>Ref</td>
<td>15.0</td>
<td>26.0</td>
<td>7.9 (7.9)</td>
<td>7.0 (6.8)</td>
<td>790 (730)</td>
<td>2.20 (2.13)</td>
<td>0.10 (0.05)</td>
<td>0.26 (0.59)</td>
<td>0.57 (0.37)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>14.0</td>
<td>29.0</td>
<td>7.8 (7.9)</td>
<td>7.3 (6.9)</td>
<td>810 (733)</td>
<td>2.12 (2.23)</td>
<td>0.12 (0.05)</td>
<td>0.27 (0.50)</td>
<td>0.50 (0.42)</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>12.5</td>
<td>40.0</td>
<td>8.8 (8.0)</td>
<td>9.7 (10.7)</td>
<td>667 (507)</td>
<td>0.95 (1.7)</td>
<td>0.02 (0.07)</td>
<td>0.20 (0.28)</td>
<td>0.01 (0.04)</td>
</tr>
<tr>
<td>Molinate</td>
<td>Ref</td>
<td>12.0</td>
<td>20.0</td>
<td>7.7 (6.8)</td>
<td>8.7 (8.5)</td>
<td>315 (389)</td>
<td>1.14 (1.32)</td>
<td>0.02 (0.02)</td>
<td>0.40 (1.14)</td>
<td>0.24 (0.15)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>13.0</td>
<td>29.0</td>
<td>7.8 (6.5)</td>
<td>9.2 (8.6)</td>
<td>442 (352)</td>
<td>0.72 (1.54)</td>
<td>0.005 (0.01)</td>
<td>0.93 (1.10)</td>
<td>0.04 (0.26)</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>7.0</td>
<td>32.0</td>
<td>7.2 (8.3)</td>
<td>10.2 (10.7)</td>
<td>397 (386)</td>
<td>0.43 (1.51)</td>
<td>0.002 (0.02)</td>
<td>0.38 (0.92)</td>
<td>0.02 (0.08)</td>
</tr>
<tr>
<td>Propanil</td>
<td>Ref</td>
<td>12.0</td>
<td>30.0</td>
<td>8.4 (7.9)</td>
<td>7.4 (7.8)</td>
<td>716 (751)</td>
<td>2.03 (2.20)</td>
<td>0.02 (0.03)</td>
<td>0.28 (0.49)</td>
<td>0.11 (0.08)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>17.0</td>
<td>31.0</td>
<td>8.2 (7.9)</td>
<td>(7.3) 8.02</td>
<td>713 (781)</td>
<td>1.93 (2.11)</td>
<td>0.02 (0.04)</td>
<td>0.26 (0.45)</td>
<td>0.10 (0.07)</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>18.0</td>
<td>47.0</td>
<td>8.9 (8.0)</td>
<td>10.5 (10.7)</td>
<td>636 (831)</td>
<td>0.12 (0.43)</td>
<td>0.02 (0.04)</td>
<td>0.51 (0.22)</td>
<td>0.03 (0.02)</td>
</tr>
</tbody>
</table>

Abbreviations: Temp: temperature; Cond: conductivity; DO: dissolved oxygen. *Ref = water from reference site and conditioned natural sediment, Low = water from low contaminated site and conditioned natural sediment, High = water from high contaminated site and conditioned natural sediment.*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temp min (°C)</th>
<th>pH</th>
<th>DO (mg L$^{-1}$)</th>
<th>Cond (ms cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endosulfan</td>
<td>Control</td>
<td>17.8</td>
<td>7.6 (7.7)</td>
<td>7.0 (7.5)</td>
</tr>
<tr>
<td></td>
<td>Ref</td>
<td>17.3</td>
<td>8.0 (7.7)</td>
<td>7.5 (7.2)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>17.3</td>
<td>8.2 (7.6)</td>
<td>7.3 (7.7)</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>17.4</td>
<td>8.3 (7.9)</td>
<td>7.2 (8.0)</td>
</tr>
<tr>
<td>Molinate</td>
<td>Control</td>
<td>19.1</td>
<td>8.4 (7.6)</td>
<td>8.1 (8.3)</td>
</tr>
<tr>
<td></td>
<td>Ref</td>
<td>19.3</td>
<td>8.3 (7.8)</td>
<td>8.3 (8.9)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>19.0</td>
<td>8.2 (7.9)</td>
<td>8.0 (8.9)</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>19.0</td>
<td>8.2 (7.6)</td>
<td>8.5 (8.7)</td>
</tr>
<tr>
<td>Propanil</td>
<td>Control</td>
<td>19.8</td>
<td>8.0 (7.7)</td>
<td>7.5 (8.0)</td>
</tr>
<tr>
<td></td>
<td>Ref</td>
<td>19.6</td>
<td>8.1 (7.9)</td>
<td>7.5 (7.3)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>19.4</td>
<td>8.2 (8.0)</td>
<td>7.2 (8.0)</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>19.7</td>
<td>8.0 (7.9)</td>
<td>7.9 (8.2)</td>
</tr>
</tbody>
</table>

Abbreviations: Temp: temperature; Cond: conductivity; DO: dissolved oxygen. *Control = ASTM and not conditioned natural sediment, Ref = water from reference site and conditioned natural sediment, Low = water from low contaminated site and conditioned natural sediment, High = water from high contaminated site and conditioned natural sediment.*
conditioned sediment from the rice field in comparison to treatments with water and conditioned sediment from reference and low contamination sites (Fig. 3). As was observed in situ, laboratory bioassay found no significant differences in growth between the treatments with water and conditioned sediment from reference and low contamination sites (\( P > 0.05 \)). Comparison with the control treatment revealed an inhibition of larval growth in high, low and reference treatments of the insecticide (Fig. 3). Regression analyses indicated that larval development was significantly negatively related (\( r^2 = 0.72, \ P < 0.01 \)) to maximum temperature during in situ exposure to pesticides.

### Table 3

Pesticide concentrations in water (\( \mu \text{g L}^{-1} \)) and sediment (\( \mu \text{g kg}^{-1} \)) treatments during in situ and laboratory bioassays

<table>
<thead>
<tr>
<th>Treatment (d.l.)</th>
<th>Water (( \mu \text{g L}^{-1} ))</th>
<th>Sediment (( \mu \text{g kg}^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 0</td>
<td>day 6 (field)</td>
</tr>
<tr>
<td>Endosulfan (0.002 ( \mu \text{g L}^{-1} ))</td>
<td>0.91</td>
<td>0.62</td>
</tr>
<tr>
<td>Reference</td>
<td>0.91</td>
<td>0.62</td>
</tr>
<tr>
<td>Low contaminated</td>
<td>1.18</td>
<td>0.81</td>
</tr>
<tr>
<td>High contaminated</td>
<td>2.78</td>
<td>1.82</td>
</tr>
<tr>
<td>Molate (0.03 ( \mu \text{g L}^{-1} ))</td>
<td>0.31</td>
<td>0.81</td>
</tr>
<tr>
<td>Reference</td>
<td>0.31</td>
<td>0.81</td>
</tr>
<tr>
<td>Low contaminated</td>
<td>1.48</td>
<td>0.97</td>
</tr>
<tr>
<td>High contaminated</td>
<td>4.63</td>
<td>1.31</td>
</tr>
<tr>
<td>Propanil (0.03 ( \mu \text{g L}^{-1} ))</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>High contaminated</td>
<td>2.58</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Abbreviations: d.l.: detection limit, lab: laboratory, n.d.: not detected.
4. Discussion

4.1. Pesticide concentrations

On day 0 of the in situ bioassay, 2 days after endosulfan and molinate spraying of rice fields, the wetland channel was contaminated due to the spray drift and/or runoff. The highest pesticide concentrations were found in the rice field, as expected, since this site was subject to direct application (Table 3). The lowest pesticide concentrations were found in the reference site, located in the wetland channel. Although pesticide concentrations in the reference site were lower than in the low contamination site, the differences in concentration between the two sites were lower than we expected, since the reference site and the low contamination site were located upstream and downstream, respectively, from the contaminated rice field effluent. This might be explained by the occurrence of a reflux of water between the two sites, which were in close proximity to each other in the same channel. This effect may be greater when water from the rice fields is discharged into the channel. Differences in the molinate and endosulfan concentrations in water and conditioned sediment between field and laboratory treatments on day 6 may have been caused by the input of additional endosulfan during in situ exposure period or they may have resulted from the storage and transport of water and sediment from field to the laboratory.

4.2. Biological responses

C. riparius larvae were not affected by herbicides, suggesting that this species is not a suitable to assess herbicide contamination. The decreasing trend of the growth response to molinate from the highest to the lowest (reference) levels of contamination observed in the in situ bioassay (Fig. 2) might be explained by differences in minimum and maximum temperature between treatments (Table 1). Although no significant relationships between larval growth and temperature were observed in this study, Péry and Garric (2006) found high significant relationships between the growth of C. riparius larvae not exposed to contamination and temperature.

In contrast to the herbicides, the insecticide endosulfan negatively affected growth of C. riparius larvae, conforming to expectations given that chironomids represent a target group of insecticide application. Growth was negatively affected by endosulfan contamination in both laboratory and in situ bioassays (Fig. 3). However, while the inhibition of growth in laboratory can be related to the presence of endosulfan concentrations, since physical and chemical conditions were similar between treatments, inhibition of growth in the rice field during in situ exposure may also have been influenced by differences in physical and chemical conditions, in particular the high values recorded for maximum temperature. This may have caused an additional stress to C. riparius larvae.
(1999) verified that toxicity of several organophosphate insecticides (e.g., azinphos methyl, disulfon, fensulfothion, terbufos) to *C. riparius* larvae varied with pH and temperature. Similarly, Lydy et al. (1999) found a positive correlation between temperature and toxicity of the organophosphate insecticides, chlorpyrifos and m-parathion to *Chironomus tentans*.

Contrary to growth, larval development was not affected by the insecticide. No significant differences were observed in larval head capsule width between treatments in laboratory or between different sites in the in situ exposures. The lowest head capsule widths were observed in the rice field, the highly contaminated site (Fig. 3), and also for chironomids subject to in situ exposure to propanil (Fig. 2) and appear to be related to higher maximum temperature at that site (40 and 47 °C) compared to the reference (26 and 30 °C) and the low contamination sites (29 and 31 °C) (Table 1), with regression analysis indicating a negative relationship between head capsule width and maximum temperature. Frouz et al.’s (2002) detailed study of the effects of temperature on the developmental rate of *Chironomus crassicaudatus* demonstrated that larval development increased rapidly with increasing temperature up to 20 °C, slowed between 20 and 27.5 °C, and decreased at temperatures higher than 27.5 °C.

### 4.3. Relevance of *C. riparius* larvae bioassay to assess ecological impact of pesticides

Head capsule with of *C. riparius* larvae appeared to be more influenced by physical and chemical conditions of exposure, especially by temperature, than by the insecticide endosulfan, suggesting that development is not a suitable measurement to assess endosulfan contamination. In contrast, larval growth was highly affected by endosulfan contamination for both in situ and laboratory exposures, indicating that bioassays with *C. riparius* larvae using growth as an endpoint is sensitive and a suitable tool to assess the impact of endosulfan contamination in freshwater ecosystems.

Growth impairment in chironomid larvae may lead to reduced fitness with a subsequent decrease in population abundance (Liber et al., 1996; Sibley et al., 1997). Because chironomids are typically one of the predominant macro-invertebrates in freshwater ecosystems (Vos, 2001) and are important prey items for birds and fish (Van de Bund, 1994; Prat and Rieradevall, 1995; Garcia-Berthou, 1999), the detrimental effects on the growth of chironomids larvae caused by a contaminant may have ramifying effects throughout the ecosystems. Organochlorine insecticides, such as endosulfan, are lipid-soluble and tend to accumulate in the fatty tissues of organisms. This problem can be further compounded by biomagnification, which results in increased concentrations of contaminants in higher trophic levels. Although endosulfan in the water column can be toxic to fish (Maier-Bode, 1968; Jonsson et al., 1993) and aquatic birds (National Library of Medicine, 1987), endosulfan entering organism through food ingestion may increase its toxic effects. Nagel and Loskill (1991) found that where endosulfan had been taken up by the prey items of small fish, the resultant body load in fish was five orders of magnitude higher than the concentrations in the ambient water. Bahner et al. (1977) similarly observed that significant quantities of the organochlorine insecticide chlordane were transferred from prey to predatory fish. In aquatic birds, concentrations of organochlorine contaminants can be up to 100 times greater in body tissue than in the surrounding water (Anderson and Hickey 1976; Norstrom et al., 1976).

The sublethal effects (growth inhibition) on *C. riparius* larvae caused by endosulfan contamination in the wetland channel of the Natural Reserve of Arzila Marsh, resulting from pesticide treatment, may be indicative of ecological impairment in this protected wetland channels and similarly indicate the ecological risk that the endosulfan spray drift and runoff from rice fields constitute to freshwater ecosystems.

### 4.4. Laboratory versus in situ bioassays

Higher growth of *C. riparius* larvae found during laboratory bioassays compared to in situ bioassays, has also been documented by other authors (e.g., Castro et al., 2003) and maybe be explained by the occurrence of high temperatures and the increased variability in physical and chemical conditions under condition of in situ exposure. Nevertheless, a similar biological response to pesticides, principally inhibition on larval growth, was found under both laboratory and field exposures to endosulfan, providing evidence for comparability of the respective experimental designs. Tucker and Burton (1999) also found significantly higher lethal effects on *C. tentans* larvae exposed to contaminated rivers surrounded by an agricultural area compared to larvae exposed to water and sediment from the same rivers but under laboratory conditions. These authors similarly attributed this difference to the variation in the parameters of physical and chemical water-quality, highlighting the potential for bias in extrapolating laboratory results to field-based scenarios.

In the laboratory bioassay it was also possible to detect toxicity effects in larval growth resulting from endosulfan contamination of the wetland channel, by comparing the growth of larvae in treatments with water and conditioned sediment from the wetland channel with the control treatment, while this was not possible in the in situ exposure due to absence of a control. Although, in situ bioassays have several advantages: (1) they eliminate biases associated with laboratory-to-field extrapolation; (2) they reduce sampling-related artefacts; (3) they allow stressor concentrations to fluctuate naturally (Tucker and Burton, 1999), laboratory bioassays can be important for the assessment of low levels of contamination by enabling the comparison under controlled conditions. This was the case of the study area because all wetland channels within the...
limits of Natural Reserve of Arzila Marsh receive inputs agriculture fields. Furthermore, laboratory bioassays with water and sediment collected in situ provide an effective way to discriminate effects caused by stress contamination from effects caused by stress of other physical and chemical conditions. Thus, when in situ toxicity testing is used in combination with laboratory toxicity testing and physico-chemical characterization, a more realistic assessment of pesticide effects to freshwater ecosystems can be made.

5. Conclusions

Impairment of larvae growth by endosulfan contamination in the wetland channel adjacent to rice fields, suggests that insecticide spray in rice fields may cause ecological impairment in adjacent freshwater ecosystems. These results also lends support to the use bioassay based on C. riparius larvae using growth as the endpoint to assess ecological impairment caused by insecticide applications to rice fields. The study also indicates that bioassays with C. riparius larvae conducted in the laboratory provide a valuable auxiliary tool to the interpretation of in situ bioassays based on this species.

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References


