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Assessing lethal and sub-lethal effects of trichlorfon on different trophic levels

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ABSTRACT

Trichlorfon (TCF) is one of the most used veterinary pharmaceuticals not only to fight infestations but also as a preventive measure worldwide. The high concentrations used generate concerns about environmental and human health. In this work we assessed the acute toxicity of this compound to non-target organisms belonging to different trophic levels: *Danio rerio* (early life stages and adults), *Daphnia magna* and algae (*Pseudokirchneriella subcapitata* and *Chlorella vulgaris*), and studied the potential of the biomarkers cholinesterase (ChE), glutathione-S-transferase (GST), lactate dehydrogenase (LDH) and catalase (CAT) to assess sub-lethal effects of trichlorfon in zebrafish and daphnids. The fish embryo test followed the OECD draft guideline FET and was based on the exposure of newly fertilized eggs to 0, 2.5, 5.0, 10, 20, 40, 80 and 160 mg/L of TCF for 5 days; the fish acute test followed the OECD guideline 203 and was based on the exposure of adult fish to 0, 2.5, 5, 10, 20, 40, 60 and 80 mg/L of TCF for 4 days; *Daphnia* sp. immobilization assay followed the OECD guideline 202 and was based on the exposure of juvenile daphnids to 0, 0.1, 0.3, 0.5, 0.7, 0.9, 1 and 2 μg/L of TCF for 2 days and the algae growth inhibition assay followed the OECD guideline 201 and was based on the exposure of the two species to 0, 1, 3.2, 10, 32, 100 and 300 mg/L of TCF for 4 days. Biomarker levels were measured after 96 h exposure to TCF in zebrafish early life stages and adults and after 48 h exposure in *D. magna*. Tested organisms seem to have dissimilar sensitivities towards TCF exposure. *D. magna* (48 h-LC_{50} = 0.29 μg/L) was the most sensitive organism, followed by early life stages and adults of zebrafish (96 h-LC_{50} = 25.4 and 28.8 mg/L, respectively) and finally by the algae *P. subcapitata* (96 h-LC_{50} = 274.5 mg/L) and *C. vulgaris* (no effect observed). As daphnids are a source of food for organisms of higher trophic levels, the impairment on its population is prone to have consequences in the entire ecosystem. The biomarker activities measured in daphnids and fish seemed to be useful tools in the assessment of trichlorfon effects, especially ChE activity which was the most sensitive biomarker tested for all organisms. Trichlorfon was teratogenic for zebrafish embryos leading to anomalies in the absorption of the yolk sac, spine bending and pericardial oedemas. The present research suggests that further work is urgently needed in order to monitor environmental concentrations of trichlorfon and to test the long term effects of environmentally realistic concentrations of this compound.

1. Introduction

In the last three decades, aquaculture has expanded worldwide, playing an important role in the economy of many countries, especially the less developed ones. However, many issues, particularly in the context of health protection and environmental impact have to be dealt with (Focardi et al., 2005). In commercial aquaculture, the use of chemicals to treat diseases, to prevent infections and promote growth (such as food supplements, antimicrobial agents and antiparasitic drugs) is essential for a successful production (Subasinghe et al., 1996) and has become a part of management strategies (Tonguthai, 1996). However, the overuse of these substances and inappropriate treatment of wastewaters pose risks for human and environmental health.

Trichlorfon (TCF) is a selective organophosphate insecticide used as a treatment for various parasitic infestations. The compound is mainly used to act against arthropods. It is an acetylcholinesterase inhibitor (EPA, 1997). The widespread use of TCF in aquaculture for a long time is generating concerns about the impact on public (Tonguthai, 1996) and environmental health (Graslund and Bengtssona, 2001). In Brazil TCF is used by fish farmers to control two common ectoparasites (*Lernae* and *Argulus*) but is very

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often used indiscriminately, repeatedly, at high concentrations and without specialized technical orientation (Guimarães and Calil, 2008). In many south-east Asian countries TCF is used in shrimp farming to kill disease vectors such as crabs and small shrimps but there is an almost complete lack of information about the quantities of chemicals used (Graslund and Bengtssona, 2001). The absence of information about the use and fate of TCF makes it very difficult to assess its impact to the environment.

As effects of environmental concentrations very rarely cause lethality, the risk assessment of TCF and other long term pollutants in aquatic ecosystems is better achieved if a battery of sublethal endpoints is used in ecotoxicity testing. In this context, biomarkers are useful tools that have been used as early indicators of environmental pollution due to their ability to detect effects at very low concentrations. Moreover, due to the specific modes of action of chemicals, organisms are affected differently according to their trophic levels. Thus it is important to include organisms from different levels in the study to allow a better understanding of effects in the entire ecosystem.

This work aims at (1) evaluating the acute toxicity of TCF using organisms of different trophic levels: the algae *Pseudokirchneriella subcapitata* and *Chlorella vulgaris*, the crustacean *Daphnia magna* and young and adult stages of the fish *Danio rerio* and (2) assessing the usefulness of using biomarkers in the detection of sub-lethal effects of TCF in daphnids and fish (young and adult stages). The biomarkers selected for this study include cholinesterase (EC 3.1.1.8, ChE), an important enzyme in the maintenance of normal nerve function (Olsen et al., 2001); glutathione-S-transferase (EC 2.5.1.18, GST), a family of enzymes with a key role in the general biotransformation of xenobiotics and endogenous substances (Hyne and Maher, 2003), lactate dehydrogenase (EC 1.1.1.27, LDH) which is involved in the carbohydrate metabolism (Diamantino et al., 2001) and catalase (EC 1.11.1.6, CAT) which is an antioxidant enzyme protecting organisms against hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS) generated in many situations of stress (Oruc and Uner, 2000). Zebrafish, *D. rerio*, is a small freshwater fish belonging to the family Cyprinidae and has been selected for this study because it is an important model vertebrate in a diversity of disciplines (Hill et al., 2005) and has been used in studies of developmental biology, molecular genetics, physiology and toxicology (Oliveira et al., 2009). *D. magna* was also selected for this study because it is very sensitive to changes in the chemical composition of aquatic environments and plays an important role in aquatic food webs placed between primary producers and fish. The green algae *P. subcapitata* and *C. vulgaris* are important species in aquatic trophic chains as primary producers and also as food for invertebrates and fish. Changes in the abundance and functions of any of these species can cause community or ecosystem- level responses (Barbosa et al., 2008; Perez et al., 2010).

This work is an attempt to elucidate how TCF toxicity varies across trophic levels and consequently which level will be more prone to be in risk due to long term TCF usage. Moreover, we evaluate TCF effects at sub lethal level in daphnids and fish by analysing different biochemical markers. A selection of the most sensitive species and the most sensitive biochemical marker is done for further use of this biomarker in studies of assessment of long term effects.

2. Materials and methods

2.1. Test chemical

TCF ($C_4H_8Cl_3O_4P$), purity 97% (m/v), (PESTANAL, Sigma–Aldrich, Taufkirchen, Germany) was used in all the performed assays.

2.2. Zebrafish assays

The zebrafish (*D. rerio*) facility established at the Department of Biology, University of Aveiro (Portugal) provided all organisms (zebrafish eggs and adults) used in the present study. In the zebrafish facility, organisms are maintained in carbon-filtered water complemented with salt "Instant Ocean Synthetic Sea Salt" (Spectrum Brands, USA), at 27.0 ± 1 °C and under a 16:8 h light:dark photoperiod cycle (conductivity: 750 ± 50 μS, pH 7.5 ± 0.5 and dissolved oxygen > 95% air saturation). This water was used as dilution water in the preparation of test solutions in all assays performed with fish. Temperature and photoperiod conditions mentioned above were used in all assays. Adult fish are fed twice daily with commercially available artificial diet (ZM 400 Granular, ZMsystems, Hampshire, UK) and brine shrimp.

2.2.1. Early life-stages assay

The assay was based on the OECD draft guideline on Fish Embryo Toxicity (FET) Test (OECD, 2006a) and is described in detail in Domingues et al. (2010). The test started with newly fertilized eggs exposed to the nominal concentrations of 0, 2.5, 5.0, 10, 20, 40, 80, 160 mg/L of TCF and run for 5 days. Forty-eight eggs per treatment (6 replicates) were selected and distributed in 24-well microplates. Embryos and larvae were daily observed under a stereomicroscope (Stereoscopic ZoomMicroscope-SMZ 1500, Nikon Corporation, Japan) (magnification used for observations was 70× for eggs and 40× for larvae). In the embryo phase the following parameters were evaluated: egg coagulation, eye and body pigmentation, somite formation, tail circulation, detachment of the tail-bud from the yolk sac, absorption of the yolk sac and hatching. After hatching, oedemas, tail malformation and mortality were observed and reported. A second test was performed for collection of larvae for biomarker analyses. Concentrations used were the same except for the last 3 concentrations that were skipped due to high mortality rates previously observed. Test ended at day 4 and 10 clusters of eight larvae per treatment were snap-frozen in microtubes (Eppendorf, Hamburg, Germany) for biomarker analyses.

2.2.2. Adult fish assay

The assay using adult fish followed the OECD guideline 203 (OECD, 1992) in semi static test conditions. Adult zebrafish of similar length and age (2 ± 1 cm, 6 months old) were selected for the test. In each treatment 12 fish were equally distributed in 4 aquaria (each one containing 1 L of test solution). The nominal concentrations of TCF used were: 0, 2.5, 5, 10, 20, 40, 60 and 80 mg/L and test solution was replaced every second day. Test run for 96 h. Fish were not fed during the test period and their mortality and behavioural changes were recorded daily. A second test using a similar design was run to allow the use of organs for biomarker analysis (12 fish per treatment were used). Concentrations used were the same except for the last 3 concentrations that were skipped due to high mortality rates previously observed. At the end of the test the living fish were sacrificed on ice by decapitation. Heads, muscles, liver and gills were isolated and snap-frozen in microtubes.

2.3. Daphnid assays

A culture of *D. magna* Straus clone K6 was established in the lab under a photoperiod of 16 h light:8 h dark, light intensity of 100–1000 lx and at 23 ± 1 °C in ASTM (1980) hardwater enriched with an organic extract (Marinure, Glenside Group, UK). The culture media has a total hardness of 175.41 ± 5.53 mg/L $CaCO_3$, pH of 8.15 ± 0.27 and a conductivity of 577.63 ± 9.01 μS/cm. Medium is renewed three times per week and Daphnids are fed daily with *C. vulgaris* algae at a concentration of 3.0×10^5 cells/mL. Newly

released *D. magna* neonates (<24 h old) from the third to fifth broods were used in the assay. Experimental conditions were similar to culture conditions.

2.3.1. Acute immobilization assay

The immobilization test was performed in accordance with the OECD guideline 202 (OECD, 2004). Neonates were exposed to control medium and TCF at the nominal concentrations of 0.1, 0.3, 0.5, 0.7, 0.9, 1 and 2 μg/L. In each treatment 15 juveniles of *D. magna* were equally distributed in 3 glass beakers (each one containing 100 mL of test solution). No food was provided during the assay. The number of immobilized daphnids was recorded at 24 and 48 h of exposure (immobilization was defined as the inability to swim or move within 15 s of gentle agitation, and was taken to indicate lethality).

2.3.2. Sampling for biomarker analysis

Daphnids were maintained and collected for biomarker analysis using a similar design. However, larger beakers containing 1 L of test solution and 50 daphnids in triplicate were used. Nominal concentrations tested were 0, 0.01, 0.025, 0.05, 0.1 and 0.25 μg/L of TCF. At the end of the test daphnids were rinsed with phosphate buffer, 0.1 M, pH 7.4 and frozen in pools of seven in microtubes.

2.4. Algae assay

The microalgae *P. subcapitata* and *C. vulgaris* which have been currently recommended as standard species for algal toxicity tests (OECD, 2006b) were obtained from nonaxenic batch cultures of Woods Hole MBL medium at 20 ± 2 °C and with a 16:8 h light/dark photoperiod. For the maintenance of the laboratory cultures and the start of new cultures, algae were harvested while still in the exponential growth phase (5–7 days old) and inoculated in fresh media.

2.4.1. Algae growth inhibition test

The algae growth inhibition test was used to evaluate the toxicity of TCF and was based on the OECD guideline 201 (OECD, 2006b). *P. subcapitata* and *C. vulgaris* were exposed in two independent experiments to the nominal concentrations of 0, 1, 3.2, 10, 32, 100 and 300 mg/L. Exposure conditions and analysis of endpoints were as described in Perez et al. (2010).

2.5. Biomarker determinations

ChE, GST, CAT and LDH activities on larvae and adults of *D. rerio* and *D. magna* were determined. All collected samples were immediately frozen at −80 °C in adequate buffer (phosphate buffer, 0.1 M, pH 7.2 for ChE and CAT; phosphate buffer, 0.1 M, pH 6.5 for GST and Tris–NaCl buffer, 0.1 M, pH 7.2 for LDH) until analysis. On the day of enzyme analysis, samples were defrosted on ice and homogenised (Ystral GmbH D-7801, Dottingen, Germany).

ChE activity was analysed from the heads and muscle of adult zebrafish, and whole organisms in the case of zebrafish embryos and daphnids in clusters of 8 larvae or 7 daphnids. LDH activity was analysed from the muscle of adult zebrafish, and whole organisms in the case of zebrafish embryos and daphnids in clusters of 8 larvae or 7 daphnids. GST activity was analysed in the heads, liver, gills and muscle of adult zebrafish, and whole organisms in the case of zebrafish embryos and daphnids in clusters of 8 larvae or 7 daphnids. The procedure for ChE, LDH and GST activity determinations is described in Domingues et al. (2010). Briefly, ChE activity was determined using acetylthiocholine as substrate and measuring at 414 nm the conjugation product between thiocoline (a product of the degradation of acetylthiocholine) and 5,5-dithiobis-2-nitrobenzoic acid (absorbance increase) according to the method of Ellman et al. (1961). GST activity assay was based on the measurement of the conjugation product between the 1-chloro-2,4-dinitrobenzene (substrate) and glutathione at 340 nm (absorbance increase) according to the method of Habig and Jakoby (1981). Determination of LDH activity was based in the decrease of absorvance due to the oxidation of NADH measured at 340 nm according to the method of Vassault (1983).

CAT activity was analysed in the liver and muscle of adult zebrafish, and whole organisms in the case of zebrafish embryos and daphnids in clusters of 8 larvae or 7 daphnids using the homogenates prepared for ChE analysis. CAT activity was determined based on the method described by Clairborne (1985). Fifty μL of homogenate were mixed with 500 μL H_2O_2 0.030 M, and 950 μL K-phosphate 0.05 M (pH 7.0) in spectrophotometer quartz cell and decomposition of the substrate (H_2O_2) measured at 240 nm.

Enzyme activities were determined in quadruplicate and expressed as nanomoles of substrate hydrolysed per minute per mg of protein. Protein concentration in samples was determined in quadruplicate by the Bradford method (Bradford, 1976), at 595 nm, using γ-globulin as standard. A Labsystem Multiskan EX microplate (Labsystems Inc., Franklin, MA, USA) reader was used for all biochemical determinations except CAT for which a Jenway 6505 UV/vis spectrophotometer (Bibby Scientific Limited, Staffordshire, UK) was used.

2.6. Statistical analysis

Sigma Stat 3.1 statistical package was used for statistical analyses (SPSS, 2004). A one-way ANOVA was used to detect the significant differences between the groups for normally distributed data sets. When data did not pass the Kolmogorov–Smirnov normality test and the homogeneity of variance test, a Kruskal–Wallis test was used. If significant results were found, the Dunnett or Dunn's test (depending on the nature of the test, parametric or non-parametric, respectively) was used to verify differences between the tested concentrations and control. EC_{50} for binary responses (zebrafish and daphnia survival) were calculated with the PriProbit software package (Sakuma, 1998) while EC_{50} for continuous responses (algae growth) were calculated with Sigma Stat software package using a non-linear allosteric decay function (SPSS, 2004). All statistical analyses based on 0.05 significance level.

3. Results

3.1. Zebrafish early life-stages assay

Organisms from the control group presented a normal embryo development as described by Kimmel et al. (1995). TCF proved to be embryotoxic with LC_{50} values of 25.4 (18.2–32.6) and 45.13 (27.5–62.7 mg/L) for 96 and 72 h, respectively (95% confidence interval (CI) in between brackets) (Fig. 1a). In the first day of exposure, effects were observed in the eye and body pigmentation and in the detachment of the tail at the highest concentration tested (160 mg/L) (Figs. 2, 3a and b) causing the death of 89.6% of these organisms by the second day.

On day 2, frequency of embryos with weak body pigmentation, delayed yolk sac absorption and pericardial oedemas was significantly increased at 80 mg/L (Figs. 2, 3c and d). Embryos exposed to 0, 2.5 and 5 mg/L of TCF started to hatch.

On day 3 all living embryos had hatched except at the concentration of 40 mg/L where a significant delay was observed with only 23.75% of hatching (Kruskal–Wallis; $H = 28.029$, $p < 0.001$). Incidence of oedemas and delayed absorption of the yolk sac was verified in larvae exposed to 20 and 40 mg/L (Figs. 2, 3e and f).

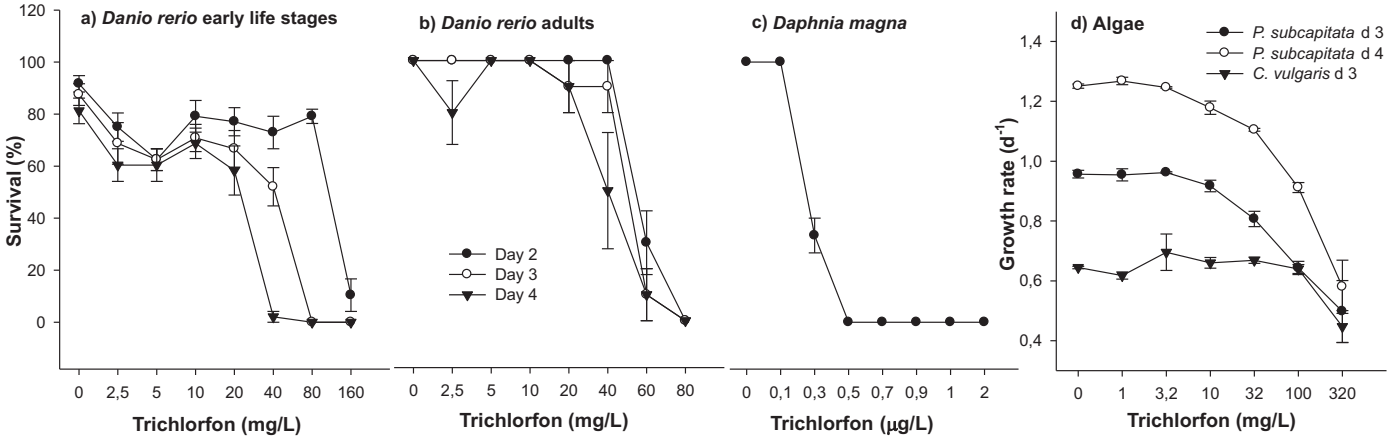

Fig. 1. Effects of trichlorfon in the tested species. (a)–(c) Percentage of survival (mean ± standard error) for *D. rerio* early life stages, adults and *D. magna*, respectively. (d) Algae growth rate (mean ± standard error). "d" stands for "day".

On day 4, 97.9% of the embryos exposed to 40 mg/L died while embryos exposed to 20 mg/L still presented a high incidence of oedemas and abnormal yolk sac absorption (Figs. 2, 3g and h). EC and LOEC (lowest observed effect concentration) values of developmental parameters are presented in Table 1. Biomarkers were also analysed at day 4 and were all responsive (Fig. 4e–h): GST activity increased at 20 mg/L, ChE showed to be dose dependent inhibited (IC_{50} = 2.47 ± 0.46 mg/L); LDH activity was inhibited at 20 mg/L and CAT activity inhibited at the two highest concentrations tested.

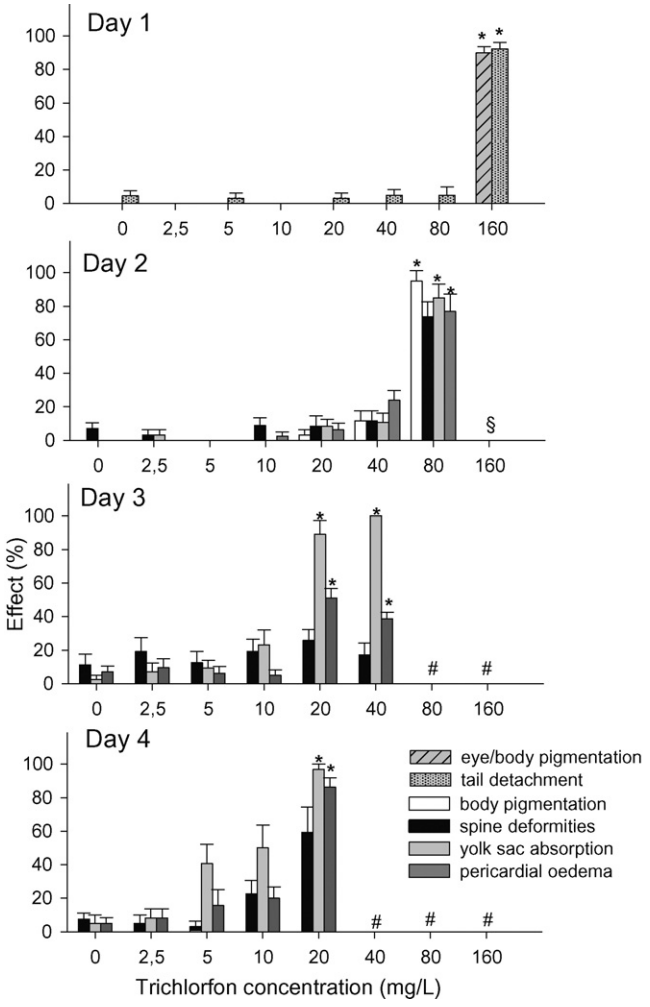

Fig. 2. Incidence of anomalies (mean ± standard error) during four days of development of embryos exposed to trichlorfon. "*" above the bars shows a result statistically different from the respective control (Dunn's method, $p < 0.05$ after Kruskal–Wallis). "§" indicates that number of surviving organisms was too low to evaluate developmental endpoints. "#" indicates no survival.

3.2. Adult zebrafish assay

The following LC_{50} values were calculated for 48, 72 and 96 h respectively: 58.4 (56.0–60.9), 43.8 (34.5–53.9) and 28.8 (14.2–74.0) (95% CI between brackets) (Fig. 1b). Behavioural alterations of fish were observed during the assay including erratic swimming, slow movements and long periods of staying still in the bottom of the flask, especially at the highest concentration. However, no detailed record of this information is available.GST activity was analysed in four types of zebrafish tissue: liver, head, gills and muscle (Fig. 4i). GST activity measured in the liver and head was a very sensitive parameter, responding with an increment at the lowest concentration tested (2.5 mg/L). However, while GST in the liver maintained increased activities at 5 and 10 mg/L, GST in the head was inhibited at 5, 10 and 20 mg/L. GST activity measured in the muscle and in the gills was not affected by TCF. ChE activity was inhibited concentration-dependently in the head and in the muscles (IC_{50} = 2.05 ± 0.23 mg/L). LDH activity measured in the muscle was inhibited at 10 and 20 mg/L. CAT activity measured in the muscle was not affected by TCF while CAT activity measured in the liver showed a steady inhibition at the last three concentration (5, 10 and 20 mg/L) (Fig. 4i–l).

Table 1
Effects of different concentrations of trichlorfon on developmental parameters of zebrafish embryos and larvae.

Endpoint	Day 1	Day 2	Day 3	Day 4
Eye pigmentation	160	n.e.	–	–
Body pigmentation	160	**56.6** (3.4)	–	–
Somite/Otolith formation	n.e.	–	–	–
Tail detachment	160	–	–	–
Pericardial oedema	160	**42.2** (4.3)	**12.3** (29.0)	**11.9** (2.2)
Yolk sac absorption	–	**61.8** (3.1)	**13.7** (1.1)	**7.4** (1.7)
Spine deformities	–	**65.8** (3.9)	n.e.	**10.6** (1.9)
Tail blood circulation	–	n.e.	n.e.	n.e.

Values are LOECs (lowest observed effect concentration). Bold values are EC_{50} (in mg/L) of dose responsive endpoints followed by the standard error in between brackets. "n.e." means no effect on the endpoint analysed.

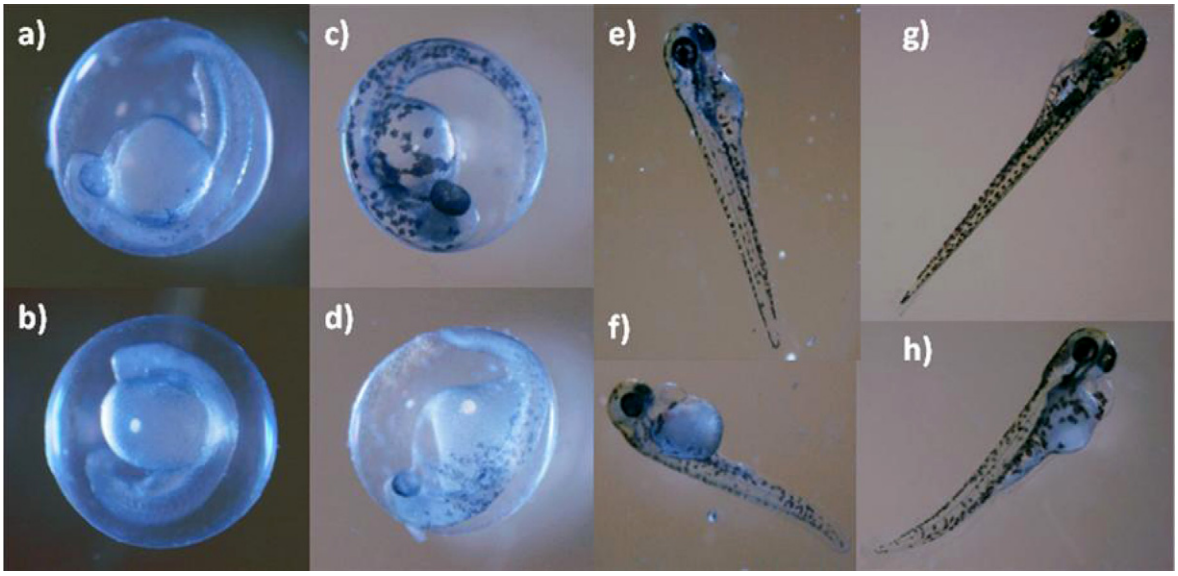

Fig. 3. Embryo and larval development anomalies of zebrafish exposed to trichlorfon. (a), (c), (e) and (g) Control organisms with normal development after 1, 2, 3 and 4 days, respectively; (b) a 1-day-old embryo exposed to 160 mg/L of trichlorfon with weak eye and body pigmentation; (d) a 2-day-old embryo exposed to 80 mg/L with weak body pigmentation, pericardial oedema and delay in the absorption of the yolk sac; (f) a 3-day-old larvae exposed to 40 mg/L with pericardial oedema, spine bending and delay in the absorption of the yolk sac; (h) a 4-day-old larvae exposed to 20 mg/L with spine bending, delayed yolk sac absorption and pericardial oedema.

3.3. *D. magna* assay

D. magna proved to be very sensitive to TCF with a 48 h-LC_{50} of 0.29 (95% CI: 0.27–0.31 μg/L) (Fig. 1c). GST activity increased only at concentration of 0.05 μg/L, while ChE showed a concentration dependent inhibition ($IC_{50} = 0.052 \pm 0.01$ μg/L). LDH was not affected by TCF exposure and CAT was induced at 0.05 and 0.1 μg/L (Fig. 4a–d).

3.4. *P. subcapitata* and *C. vulgaris* growth inhibition test

P. subcapitata proved to be very resistant to TCF exposure with a 96 h-EC_{50} (for growth rate) of 274.5 (95% CI: 216.6–332.4 mg/L) (Fig. 1d). *C. vulgaris* growth rate was not affected by exposure to TCF (Kruskal–Wallis; $H = 10.11$, $p = 0.072$; Fig. 1d).

4. Discussion

4.1. Acute toxicity

Based on the LC_{50} values calculated, the crustacean *D. magna* (48 h-LC_{50} = 0.29 μg/L) is the most sensitive organism tested, followed by early life stages and adults of zebrafish (with similar 96 h-LC_{50} of 25.4 and 28.8 mg/L, respectively) and finally by the algae *P. subcapitata* (96 h-LC_{50} = 274.5 mg/L) and *C. vulgaris*. Fig. 5 clearly shows the substantial difference in the sensitivities of the different organisms studied. The higher sensitivity of *D. magna* was the expected result given that TCF targets species of arthropod parasites. LC_{50} value agrees with values found in literature (0.26 μg/L in the work of Yoshimura and Endoh, 2005, and 0.21 μg/L in the work of Ren et al., 2007) for this crustacean. The relative insensitivity of algae compared to other trophic levels is already reported in literature (e.g. Papst and Boyer, 1980). Moreover, some works also suggest algal ability for organophosphorous (OP) pesticide degradation (Caceres et al., 2008).

4.2. Biomarkers sensitivity

Biomarkers levels were measured after exposure of zebrafish early life stages and adults and *D. magna* to TCF.

Induction of GST activity has been used as a biomarker of exposure to xenobiotics with electrophilic centers. In this work, a transient induction of GST was observed in *D. magna* (only verified at 0.05 μg/L). Few works are available in literature dealing with effects of OP pesticides in daphnid GST. However, absence of response to fenitrothion was observed in the work of Damasio et al. (2007) suggesting that GST is not a very important biomarker in the assessment of OP effects in daphnids. GST measured in adult zebrafish varied between different organs. While no response was observed in the muscle and gills, and an irregular response was observed in the head (only observed at 2.5 mg/L) a steady induction (except for the last concentration) was observed in the liver, indicating that this organ is the most suitable to evaluate TCF effects. In fact, the liver has an important role in degradation and bioactivation of pesticides (Oruc and Uner, 2000); induction of hepatic GST was also observed in *Oreochromis niloticus* exposed to diazinon (Uner et al., 2007) and in *O. mossambicus* exposed to monocrotophos (Rao, 2006a) indicating ongoing detoxification mechanisms. This tissue-dependent response of GST in zebrafish adults was also observed in works such as that of Kavitha and Rao (2009) with *O. mossambicus* exposed to profenofos and it is possibly the result of different isoforms of the enzyme present in the tissues. In zebrafish early life stages GST was not a very sensitive endpoint as a response (induction) was only observed in the last concentration (20 mg/L) which is close to the LC_{50} value.

ChEs are responsible for the degradation of the neurotransmitter acetylcholine in the cholinergic synapses. A concentration dependent inhibition of ChE was the expected response for all organisms tested given that TCF, as all Ops, has an anticholinergic action. ChE inhibition under OP exposure is already described in literature for *D. magna* (Barata et al., 2004; Printes and Callaghan, 2004), zebrafish early life stages (Kuster and Altenburger, 2006), and adults (Frasco and Guilhermino, 2002; Rao, 2006b; Kavitha and Rao, 2007). In this work, ChE activity proved to be, for all organisms tested, the most sensitive endpoint tested, with IC_{50} values for ChE inhibition of 19.7 (head of adult fish), 7.4 (fish early life stages) and 5.4 (*D. magna*) times lower than the respective LC_{50} values. ChE activity in *D. magna* was affected at concentrations as low as 0.025 μg/L.

LDH is a key enzyme in the anaerobic pathway of energy production, responsible for the catalysis of the interconversion of pyruvate

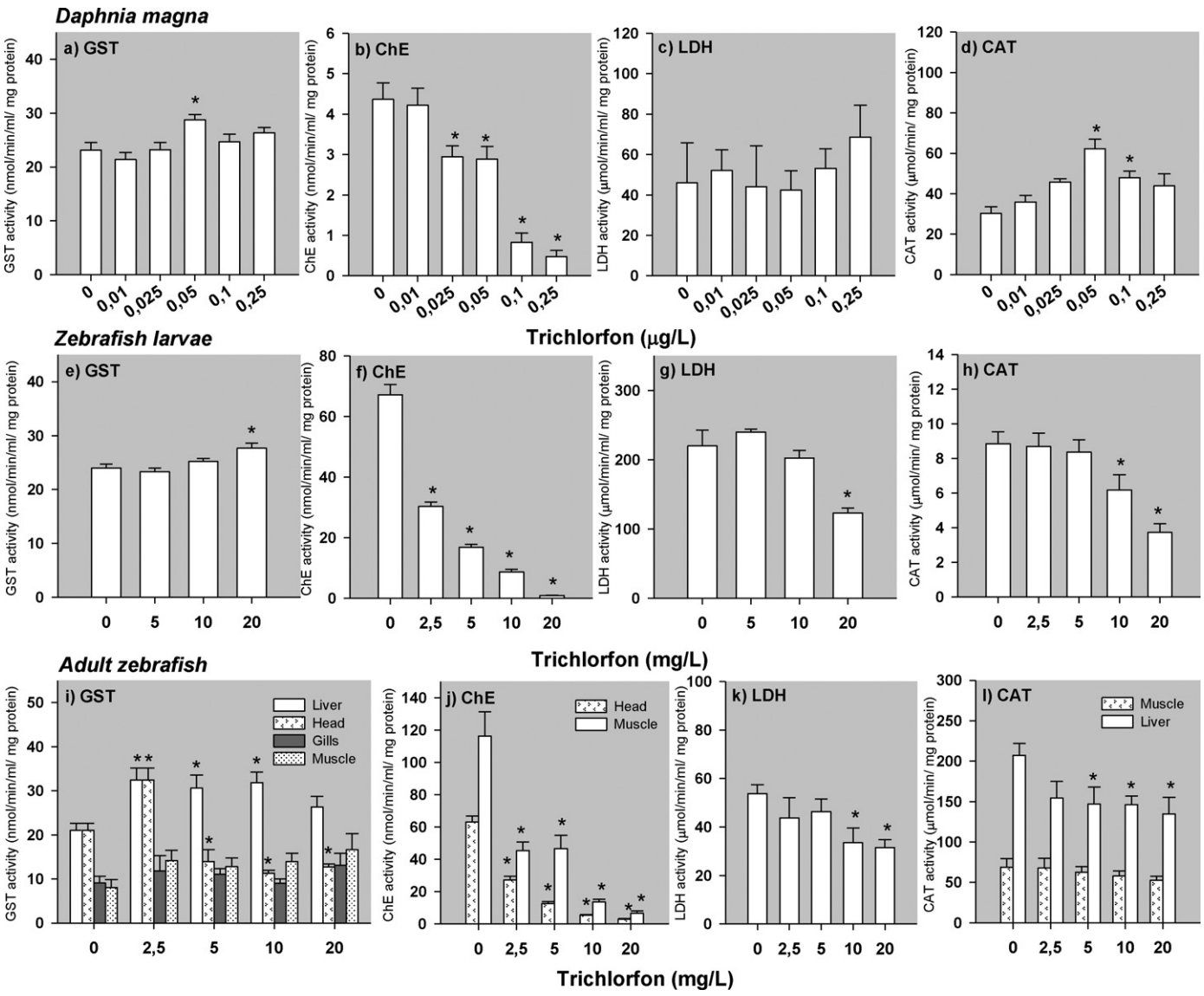

Fig. 4. Variation of biomarker activities (mean value ± standard error) on *Daphnia magna* and zebrafish larvae and adults after exposure to trichlorfon. Asterisks mean significantly different from the respective control treatment (Dunett's test, $p < 0.05$ after one-way ANOVA).

to lactate in glycolysis and has been used as general biomarkers of stress in fish (Almeida et al., 2001; Osman et al., 2007; Vieira et al., 2008). In this work LDH did not prove to be a useful biomarker for TCF exposure as it did not respond (*D. magna*) or responded only at very high concentrations (zebrafish adults and early life stages). Several works in which fish have been exposed to OP pesticides found an induction of LDH (indicating the use of the anaerobic pathway of energy production based on pyruvate) as it is the case of *P. reticulate* exposed to dimethoate (Frasco and Guilhermino, 2002) and *O. mossambicus* (gill and brain) exposed to monocrotophos (Rao, 2006a). However, in the same work of Rao (2006a), LDH activity decreased in liver and muscle similarly to what happened in this work, indicating, in these cases, possible tissue damage and muscular harm.

Several studies suggest that non-specific toxicity of OPs can be caused by the production of ROS and consequent oxidative stress (Oruc and Uner, 2000; Pena-Llopis et al., 2003). CAT belongs to the first line of defense against oxidative stress and occurs in peroxisomes, detoxifying H_2O_2 to O_2 and H_2O. In this work the response of CAT varied between daphnids and fish: while in *D. magna* a transient induction was observed at 0.05 and 0.1 µg/L suggesting the activation of the antioxidant defense mechanism; in zebrafish early life stages and adults (liver) an inhibition was observed at the highest concentrations. TCF has been reported to induce oxidative stress in fish (Demael et al., 1990; Hai et al., 1997; Pena-Llopis et al., 2003; Thomaz et al., 2009). Although an inhibition (or no effect when measured in the muscle) of CAT in zebrafish was found in this work, oxidative damage is not excluded as suggested by works such as Feng et al. (2008) in which *T. nilotica* CAT was not affected by TCF but oxidative stress was shown to occur (by the depletion of GSH which indicates that ROS could be involved in the toxic effects). Other works with fish species suggest that besides their anticholinergic action, OP pesticides may also lead to oxidative stress, such is the case of *O. mossambicus* exposed to profenofos, *G. affinis* exposed to chlorpyrifos and monocrotophos, *Sparus aurata* exposed to malathion and *O. niloticus* exposed to azinphosmethyl (Kavitha and Rao, 2007, 2008, 2009; Rosety et al., 2005; Oruc and Uner, 2000). However, in these works CAT is not always induced, meaning that this enzyme may or not have an active role in the antioxidant defense mechanism and may be even inhibited due to the excessive generation of ROS as it happened in this present work. In future works, the activity of other enzymes of oxidative stress

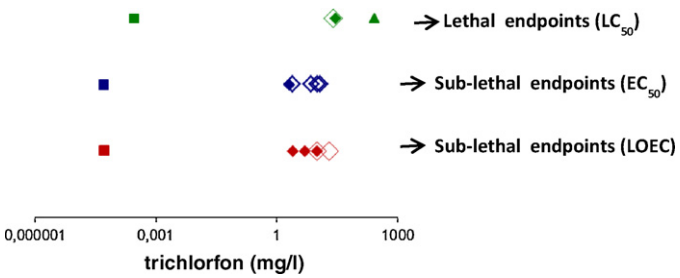

Fig. 5. Comparative sensitivity of the endpoints tested in different organisms: (■) Daphnid data; (♦) zebrafish adults; (◊) zebrafish embryos; (▲) algae (*P. subcapitata*).

and indicators of oxidative damage (such as lipid peroxidation) should be included in order to elucidate this mechanism.

4.3. Zebrafish early life stage assay

Fish embryo testing has been suggested as an alternative to fish testing (because fish embryos are not subject to the Directive 86/609/EEC, which regulates the use of animals in scientific experiments) (Braunbeck and Lammer, 2006). Fish embryos are also excellent models for the understanding of toxic mechanisms and long-term effects of pollutants (Scholz et al., 2008). In the case of zebrafish embryo assay a strong correlation in acute toxicity between embryonic stages and adults has already been verified (Nagel, 2002; Lammer et al., 2009). Moreover, it has been observed that very often biochemical parameters (biomarkers) are more sensitive in early life stages (Oliveira et al., 2009; Domingues et al., 2010) and that important information is provided by effects at embryo development level, which has no equivalent in the adult fish test. In this work TCF proved to be embryotoxic: the most important endpoints of embryo development at 24 h were the anomalies in the pigmentation of eye and body and the lack of tail detachment which proved to be lethal as embryos died the next day. Between 48 and 96 h, hatching delay, pericardial oedema, delay in the absorption of the yolk sac and spine bending were the most important effects observed. These effects in the embryo development are likely to be general responses of zebrafish embryos to organic pollutants and were also observed in *O. niloticus* embryos exposed to TCF (Guimarães et al., 2007) and zebrafish embryos exposed to other OP pesticides such as diazinon (Osterauer and Kohler, 2008).

96 h-EC_{50} values calculated for the concentration responsive endpoints analysed in this test indicate that the most sensitive endpoint was ChE activity (2.47 mg/L), followed by the absorption of the yolk sac (7.4 mg/L), spine bending (10.6 mg/L) and pericardial oedema (11.9 mg/L) proving that these are useful endpoints in the detection of sublethal effects of TCF (Fig. 5). A further link between these effects and future fitness impairment of zebrafish should be investigated to evaluate their ecological relevance.

Comparing biomarker responses in the early life stages assay with the adult assay, except for ChE activity that was equally sensitive, all biomarkers were more sensitive in the adult assay. This was not the expected since in previous works with different types of toxicants, the sensitivity of biomarkers in early life stages seemed to be higher than in adults (Oliveira et al., 2009).

4.4. Environmental relevance

Based on the results obtained for *D. magna* and as previously described on literature, the main concern for TCF is its effects on non-target crustaceans (Graslund and Bengtssona, 2001). The IC_{50} value of 0.052 μg/L calculated for ChE inhibition is significantly below values measured in the environment such as 10 μg/L found in several wells in Georgia (EPA, 1997) and has unknown consequences at population level. In the environment, an eventual reduction of the daphnid population under exposure to TCF would have direct effects on both upper and lower levels of the trophic chain. Organisms such as fish that feed on these crustaceans would face feeding constrains while algae due to the lack of predation and due to the natural lower sensitivity to TCF would have their populations enlarged (Daam et al., 2008) with pernicious consequences to the ecosystem such as water eutrophication.

Acute toxicity data for fish species ranges from highly toxic to practically non-toxic depending on the species (EPA, 1997). Most of the sub-lethal endpoints analysed in zebrafish (enzymes and embryo development parameters) were affected at concentrations between 2 and 10 mg/L. The lack of information about aquaculture usage (frequency and amounts), the efficacy of waste water treatments and the concentrations of effluents actually reaching aquatic ecosystems is of special concern because of two factors that can potentiate TCF effects on the environment. The first is the hydrolysis of TCF into the more toxic compound dichlorvos (Guimarães et al., 2007). This hydrolysis highly depends on pH and temperature, making a careful planning of treatments taking into account these factors necessary (Roth et al., 1993). The risk of unexpected fish kills is highly increased when concentrations are not respected. Second, the potential for combined toxicity of the several types of chemicals used simultaneously in aquacultures (fertilizers, pesticides, disinfectants, antibiotics, probiotics, vaccines) (Graslund and Bengtssona, 2001). The possibility of interactions between different types of chemicals resulting in synergistic effects highly increases ecological risk and was already verified, for instance, between TCF and the detergent sodium dodecyl sulfate (Feng et al., 2008).

5. Conclusions

Organisms from the different trophic levels tested had varying levels of sensitivity towards TCF exposure. *D. magna* (48 h-LC_{50} = 0.29 μg/L) showed highest sensitivity among organisms tested, followed by zebrafish early life stages (96 h-LC_{50} = 25.4 mg/L), zebrafish adults (96 h-LC_{50} = 28.8 mg/L) and finally by the algae *P. subcapitata* (96 h-LC_{50} = 274.5 mg/L) and *C. vulgaris*. The impairment of daphnid populations is prone to have consequences to the entire ecosystem given that it would affect organisms of higher levels (such as fish) that feed on them and it would increase populations of organisms of lower levels (algae) on which daphnids feed, disturbing the equilibrium within the ecosystem. The biomarker activities measured in daphnids and fish seemed to be useful tools in the assessment of TCF effects, especially ChE activity which was the most sensitive biomarker tested for all organisms, being a suitable tool for assessment of OP exposure in fish and daphnids. Moreover, other biomarkers tested proved to be very sensitive; namely, CAT in *D. magna* and CAT and GST in the liver of adult zebrafish. TCF is teratogenic for zebrafish embryos, and anomalies in the absorption of the yolk sac, spine bending and pericardial oedemas are observed. Given the potential environmental risk posed by TCF use and based on data from the present work, further work should be planned in order to monitor environmental concentrations of TCF and its degradation product, dichlorvos and test long term effects of normally used doses of these compounds.

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