Acute toxicity of chlorpyrifos and its metabolite 3,5,6-trichloro-2-pyridinol alone and in combination using a battery of bioassays

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Abstract
Acute toxicity of chlorpyrifos (CP) and its principal metabolite 3,5,6-trichloro-2-pyridinol (TCP) alone and in combination have been evaluated using a test battery comprising aquatic organisms from different trophic levels: luminescent marine bacteria Aliivibrio fischeri, freshwater unicellular alga Pseudokirchneriella subcapitata, and cladoceran Daphnia magna. As expected, D. magna was the more sensitive organism to the compounds tested, being CP more toxic than its metabolite. On the contrary, TCP was found to be more toxic than its parental compound to A. fischeri and P. subcapitata. In all cases, the mixture of CP and its metabolite was more toxic than the compounds tested separately, multiplying between 5 and 200 times CP toxicity level and up to 15 times TCP toxicity level. These results indicate that the co-existence of parent chemical and its degradation product in the environment can result in a synergic interaction involving high risk to the aquatic ecosystems.

Keywords Bioassays · Chlorpyrifos · 3,5,6-Trichloro-2-pyridinol · Acute · Toxicity · Aliivibrio fischeri · Pseudokirchneriella subcapitata · Daphnia magna · Interaction · Synergism

Introduction
Organophosphorus compounds are among the most widely used chemicals worldwide. Although their main use is as pesticides in agriculture, aquiculture, vector control, or domestic use (Roberts and Aaron 2007), it also can be used for plastic making (Yadav et al. 2017), flame retardant, or hydraulic fluids (Andresen et al. 2004). Due to their low specificity, they can cause severe and lasting effects on the population of non-target aquatic species, particularly invertebrates (SSchulz and Liess 1999, Fulton and Key 2001). Chlorpyrifos (CP) is a broad-spectrum chlorinated organophosphate [O,O-diethyl O-(3,5,6-trichlor-2-piridil) phosphorothioate] with insecticide, nematocide, and acaricide activity used on different crops as well as grass and ornamental plants (John and Shaike 2015). Despite CP has been withdrawn from domestic use in many countries, its production and consumption increase dramatically every year (John and Shaike 2015) because of its low economic cost, which has led to the contamination of both terrestrial and freshwater and marine ecosystems in many regions of the world (Wightwick and Allinson 2007; Gebremariam et al. 2012; Álvarez et al. 2013). As is known, the main mechanism of toxicity of CP is the inhibition of acetylcholinesterase, which is present in all vertebrates, affecting other non-target species, as honeybees, earthworms, or fishes (Jin et al. 2015). It is considered moderately to extremely toxic to birds (Moore et al. 2014). In addition to producing disorders in the nervous system, damages to the immune system and endocrine disruption have been documented (Li et al. 2013; Ventura et al. 2016).
It has also been detected in foods from urban and rural areas, indoor floor dust and indoor air, urine samples, etc., raising important human health concerns (Morgan et al. 2005). During the last years, the presence and dissipation of CP have been studied in vegetables like lettuce, rice, maize, or tomato detecting significant levels (Zhang et al. 2012; Gvozdenac et al. 2013; Han et al. 2013; Ge et al. 2015). According to EPA, CP is considered moderately toxic and classified in toxicity category II (USEPA 2011). However, after various studies, the European Food Safety Authority (EFSA) reconsidered the toxicity level concluding that CP is much more toxic than previously advised in the European Commission for Health (EFSA 2013).

According to the physicochemical characteristics of CP, this is strongly retained in the soil with half-lives that vary between 1 and 24 weeks (Eisler 2000). This variability may be attributable to organic carbon content, moisture, or microbial activity (Racke 1993). Its entry in salt or freshwater aquatic systems can be caused by runoff from contaminated soils or by erosion and transport of soil particles.

Studies on the environmental fate of CP and its degradation mechanisms revealed 3,5,6-trichloro-2-pyridinol (TCP) as the main degradation product (Xu et al. 2008) produced by hydrolytic and photolytic mechanisms (Baskaran et al. 2003). It is more mobile than the parent molecule due to its higher water solubility, thus causing widespread contamination of soil and aquatic environments.

Regarding ecotoxicity studies, CP was found as very highly toxic to birds such as common grackles, house sparrows, ring-necked pheasants, and common pigeons with LC50 values ranging from 1 to 20 mg/kg (Solomon et al. 2001; Christensen et al. 2009; Eng et al. 2017). In aquatic systems, it is considered very highly toxic to invertebrates, freshwater fish, and estuarine and marine organisms with LC50 values ranging from 1 to 50 μg/L (Barron and Woodburn 1995; Varó et al. 2002; Ali and Kumar 2012; Wang et al. 2014; Majumder and Kaviraj 2019). A certain degree of bioaccumulation has been found in a number of species including TCP and other metabolites (Thomas and Mansingh 2002; Ashauer et al. 2006; Katagi 2010).

While there is considerable information related to the ecotoxicity of CP and TCP chemicals, it should not be forgotten that they are rarely found individually. The fact is that chemicals appear mixed at contaminated sites, more often the original chemical is in combination with its breakdown products or other chemicals. This is the case of CP and TCP. Thus, the principal aim of the present study was to examine the interactive toxicity of CP and its principal degradation product as a mixture, based on the observed toxicity of the individual chemicals and their combination to standardized biological models as luminescent marine bacteria Alivibrio fischeri, freshwater unicellular alga Pseudokirchneriella subcapitata, and invertebrate Daphnia magna.

Material and methods

Chemicals and test organisms

Chlorpyrifos (CAS: 2921-88-2) (O,O-diethyl O-3,5,6-trichloropyridin-2-il phosphorothionate; CP) 99.7% analytical standard and its metabolite 3,5,6-trichloro-2-pyridinol (TCP) (CAS: 6515-38-4) 99.3%, analytical standard were obtained from Sigma-Aldrich and the commercial product (CP 44.5% - 480 g/L, solvent, emulsifier, and others 55.5%) was manufactured by Dow AgroSciences. The properties of the selected compounds used in this study are described in Table S1. Stock solutions of compounds and dilution series were prepared in distilled water or in the appropriate culture medium for each toxicity assay. The test organism Alivibrio fischeri (strain NRRL B-11177) was supplied by Fisher Scientific (Madrid, Spain); the alga Pseudokirchneriella subcapitata (Algaltoxkit, MicroBioTest Inc, Gent, Belgium) and the ephippias (dormant eggs) of crustacean Daphnia magna (Daphtoxkit, MicroBioTest Inc, Gent, Belgium) were supplied by ECOtest S.L. (Valencia, Spain). Water employed in the experiments was Milli-Q grade. All reagents used for bacteria, algae, and crustacean tests were of analytical grade. For all bioassays, routine sensitivity controls were performed using the salt K2Cr2O7 (Sigma-Aldrich, Analytical reagent).

Alivibrio fischeri acute toxicity test

The bacteria luminescence inhibition assay was conducted according to the guideline ISO 11348:3:2007 (ISO 2007) with modifications, described below. The bacteria were exposed to initial concentrations of 15 mg/L of CP, TCP, or commercial CP, for 30 min. The mixture CP:TCP (1:1) was assayed at initial concentration of 4 mg/L. Immediately before starting the assay, the lyophilized bacteria were reconstituted and then, a bacteria suspension was prepared with the medium described below. The bacteria were exposed to concentrations ranging from 1 to 50 μg/L. As a diluent, 2% NaCl in distilled water is used. White flat-bottom 96-well microplates (Costar, Corning Inc., New York, USA) were used for the assay. Each plate contained 8 replicates of controls (diluent and bacterial suspension): 4 replicates of each concentration of compounds assayed (pollutant, diluent and bacteria suspension) and 4 blanks (diluent). The total volume per well was 200 μL. The preparation of the assay was carried out as follows. Serial dilutions for each sample were prepared in 2% NaCl in one microplate with the injector module of the Tecan Infinite M200 microplate reader; this plate was kept at 15 °C for 1 h. Another microplate was filled manually with the bacteria suspension and kept at 15 °C for 15 min. After the incubation period, luminescence was monitored for both plates. Then, the content of the first plate was transferred to the second plate with a multichannel pipette, incubated at 15 °C for...
30 min and the luminescence measured. To assess bioluminescence reduction, values were processed according to the ISO protocol to obtain the percentages of luminescence inhibition.

**Pseudokirchneriella subcapitata acute toxicity test**

A miniaturized growth inhibition test with *P. subcapitata* was carried out according to the ISO 8692:2012 protocol (ISO 2012a). The alga was exposed to initial concentrations of 50, 2.1, 6.3, and 0.1 mg/L of CP, TCP, commercial CP, and mixture, respectively, for 72 h. *P. subcapitata* was taken from the commercial system Algal Toxkit F. The organisms, immobilized in algal beads, were de-immobilized and resuspended in Bold’s Basal Medium (Bishoff and Bold 1963). The resulting stock culture was kept in a 250-mL flask, shaken (150 rpm) under continuous white light (6000–10,000 lux), and incubated at 23 ± 2 °C in a heated chamber. A 3-day algal culture was used for inoculum in toxicity tests. Assays were carried out in 96-well transparent flat-bottom microplates (Costar, Corning Inc., New York, USA). Each test consisted of 16 replicates of control (culture medium and algae); 8 replicates of each concentration of compounds assayed (pollutant, culture medium, and algae) and 8 replicates of blank (culture medium). Serial dilutions were made with the culture medium by two from the stock solution of each compound. The microplates were filled automatically using the injector module of the Tecan Infinite M200 plate reader. The initial concentration of cells in each well was 10⁶ cells/mL and the final volume 300 μL. Microplates were introduced in transparent plastic bags filled with 2% CO₂ and incubated in the same conditions than stock cultures. Growth of cultures was measured every 24 h by the fluorescence of the in vivo content of chlorophyll a, at 430 nm and 663 nm as wavelengths of excitation and emission, respectively. All the fluorescence data are transformed into cell concentration values and the percentages of growth inhibition of the algae exposed to compounds and mixture were determined, according to the protocol.

**Daphnia magna acute toxicity test**

Toxicity assessment of chemicals CP, TCP, and commercial CP with *D. magna* was carried out according to the ISO 6341:2012 directive (ISO 2012b). Ephippias were washed with tap water, resuspended with reconstituted water (0.294 mg/L CaCl₂;2H₂O; 0.123 mg/L MgSO₄;7H₂O; 0.065 mg/L NaHCO₃; 0.0058 mg/L KCl; hardness 250 mg/L as CaCO₃; pH 7.8 ± 0.5), and incubated at 22 ± 1 °C under constant illumination (3000–4000 lux) in a climatic chamber. In the toxicity test, the neonates, which have less than 24 h from hatching, were exposed to serial dilutions (dilution factor 2) of CP, TCP, or commercial CP, prepared with reconstitution medium from a stock solution of 31.3, 250, and 3.9 μg/L, respectively. The mixture CP:TCP (1:1) was assayed at an initial concentration of 7.8 μg/L. The assays consisted of controls and test concentrations were conducted in Petri dishes (5.5-cm diameter). All solutions were evaluated by quadruplicate. Five individuals of *D. magna* were transferred to each Petri dish containing 10 mL of test solution or reconstitution medium (controls). Plates were incubated for 24 h in the dark at a constant temperature of 20 ± 2 °C in a climatic chamber, and the number of immobile and live individuals was counted to obtain the percentage of immobility.

**Combination index calculation**

In order to quantify synergism, additive, or antagonism effect of the binary combination of CP and TCP (1:1), combination index (CI) values were calculated at inhibition rate in the toxicity parameters of 50% (IC₅₀) using the equation described by Chou (2006) and Yang et al. (2017). The effects of the mixture CP:TCP (1:1) to organisms assayed were classified according to Chou (2006) who described a detailed classification of CI < 0.1, very strong synergism; 0.1–0.3, strong synergism; 0.3–0.7, synergism; 0.7–0.85, moderate synergism; 0.85–0.90, slight synergism; 0.90–1.10, nearly additive; 1.10–1.20, slight antagonism; 1.20–1.45, moderate antagonism; 1.45–3.3 antagonism; 3.3–10, strong antagonism; > 10, very strong antagonism.

**Statistical analysis**

Data were analyzed to verify if adjusted to a normal distribution and homogeneity of variance and analyzed by using a one-way ANOVA followed by a post hoc analysis using Fisher’s least significance difference (LSD) test to determine differences between means and Dunnett’s correction test in order to determine NOEC value. The IC₅₀ (concentration that inhibits the bacteria luminescence or the algae growth by 50%) and the LC₅₀ (concentration that inhibits the mobility of microcrustacean by 50%) were calculated using probit analysis. All statistical tests were performed with a 95% confidence interval and a significance level of *p* < 0.05 with Statgraphics program v.6.0 and SPSS Statistics program v.2.0

**Results and discussion**

**Toxicity test accuracy**

The precision of acute toxicity tests, expressed as coefficient of variation (CV%) for K₂Cr₂O₇ IC₅₀ values, was 33.3% for *A. fischeri*, 23.0% for *P. subcapitata*, and 36.1% for *D. magna*. Interlaboratory precision data from a study of acute toxicity test methods using the reference toxicant potassium...
dichromate shows IC$_{50}$ values of 18.71, 1.19, and 1.12 mg/L for *A. fischeri*, *P. subcapitata*, and *D. magna*, respectively, with CVs of 32.9, 23, and 50% (ISO 2007, 2012a, 2012b). K$_2$Cr$_2$O$_7$ IC$_{50}$ values in the directives are closer with values reported here (*A. fischeri*, IC$_{50}$ 13.7 mg/L; *P. subcapitata*, IC$_{50}$ 3.1 mg/L; and *D. magna*, IC$_{50}$ 1.9 mg/L).

**Acute toxicity of test compounds**

Experimental data with *A. fischeri*, *P. subcapitata*, and *D. magna* exposed to CP, TCP, commercial CP, and mixture CP:TCP (1:1) are presented as concentration–response curves (Figs. 1, 2, and 3). The NOEC and IC$_{50}$ values for each compound or mixture are presented in Table 1.

As observed in Fig. 1, the range of concentrations causing significant effects ($p < 0.05$) to *A. fischeri* was between 1.25 and 15 mg/L for CP and between 0.63 and 15 mg/L for commercial CP and TCP, being 0.31 to 4 mg/L for mixture, indicating higher toxicity for latter. In case of alga (Fig. 2), concentrations that caused significant effects ($p < 0.05$) with respect to control were higher to CP than to other compounds, between 2.1 and 50 mg/L; for commercial CP and TCP, the concentrations ranged from 0.52 to 6.3 mg/L and from 0.15 to 2.1 mg/L, respectively. Significant effect concentrations of the CP:TCP mixture were markedly lower, between 0.008 and 0.10 mg/L. At last, the range of concentrations causing significant effects ($p < 0.05$) on the *D. magna* mobility was markedly lower than algae and bacteria, between 0.008 and 31.3 μg/L for CP, between 3.9 and 250 μg/mL for TCP, and between 0.49 and 3.9 μg/L for commercial CP; for CP:TCP mixture, the concentrations ranged from 0.24 to 7.8 μg/L (Fig. 3).

TCP was, approximately, 4 and 17 times more toxic than the parent compound for the bacteria and alga, respectively.

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CP, and CP:TCP mixture (Table 1). D. magna, as expected, was more sensitive to compounds than P. subcapitata and A. fischeri, presenting microgram per liter order IC\textsubscript{50} values for all compounds. It is known that organophosphorus pesticides inhibit acetylcholinesterase activity (Fulton and Key 2001) which is not present in bacteria and algae organisms. Barron and Woodburn (1995) in their review of ecotoxicology of CP point to invertebrates as the most sensitive organisms compared with marine mollusks and algae. CP toxicity, based on LC\textsubscript{50} values, to fourteen freshwater crustacean’s species ranged from 0.06 to 700 \mu g/L being cladocerans the most sensitive. LC\textsubscript{20} values reported in the literature for several cladocers species vary from 0.015 to 340 \mu g/L (Kersting and Van Wijngaarden 1992; Van Wijngaarden et al. 1993; Bailey et al. 1996; Moore et al. 1998; Tomlin 2000; Guilhermino et al. 2000; Sherrard et al. 2002; Brock 2003; Zalizniak and Nugegoda 2006; Cáceres et al. 2007; Palma et al. 2008) however, the majority of toxicity values were between 0.06 and 1.7 \mu g/L. These values are similar to toxicity data obtained in this work for D. magna for both pure CP (1.2 \mu g/L) and commercial CP (0.72 \mu g/L). The application of CP in a microcosm assay by Daam et al. (2008) led to significant changes in freshwater biological communities being the cladoceran Moina micrura the most susceptible species with NOEC of 0.1 \mu g/L. Van Wijngaarden and Leeuwangh (1989) found a NOEC of 0.065 \mu g/L for Daphnia pulex in ponds. Laboratory test data of pure and commercial CP obtained for the cladoceran in this work was higher, with NOECs of 0.49 and 0.24 \mu g/L, respectively (Table 1). Characterization of acute toxicity of CP by Giddings et al. (2014) comparing HC\textsubscript{5} values (hazardous concentration for 5% of species) showed that crustaceans were most sensitive (HC\textsubscript{5} = 0.034 \mu g/L), followed by insects (HC\textsubscript{5} = 0.087 \mu g/L) being fish less sensitive (HC\textsubscript{5} = 0.812 \mu g/L).

In our work, A. fischeri has been the most tolerant organism to CP, TCP, and mixture in accordance with Palma et al. (2008) who indicated that bacteria are not as sensitive to insecticides and herbicides as other aquatic organisms. Ecotoxicological effects of the herbicide atrazine and the insecticides endosulfan sulphate and CP were evaluated by Palma et al. (2008) using a test battery showed a higher sensitivity of microcrustacean D. magna than bacteria A. fischeri. Minagh et al. (2009) also found A. fischeri model system less sensitive than the D. magna exposure assay. IC\textsubscript{50} values of pure and commercial CP obtained in this study for A. fischeri have been 3.7 and 2.9 mg/L, respectively. These values are similar to the IC\textsubscript{50} value of 2.8 mg/L obtained by Palma et al. (2008) but were lower than reported by Somasundaram et al. (1990) for the same bacteria (46 mg/L) assayed in the Microtox.

Data about the algae toxicity of CP in the literature are diverse. An ecotoxicological review carried out by Barron and Woodburn (1995) reports that CP concentrations above 100 \mu g/L produced toxicity to freshwater and saltwater algae, with differences between species at least two orders of magnitude, indicative of high algal toxicity. The NRA review of CP (2000) reported a 96-h EC\textsubscript{50} value for the green alga Scenedesmus subspicatus of 660 \mu g/L. De Lorenzo and Serrano (2003) found significant decreases in the alga marina Dunaliella tertiolecta population growth rate at CP concentrations higher than 400 \mu g/L and 50% growth inhibition at 769 \mu g/L. The 72-h EC\textsubscript{50} for P. subcapitata was 64 \mu g/L (NRA 2000). CP toxicity (EC\textsubscript{50}) to five species of algae reported by Nikolenko and Amirkhanov (1993) was much lower, ranged

| Compound         | A. fischeri (mg/L) | P. subcapitata (mg/L) | D. magna (\mu g/L) |
|------------------|--------------------|-----------------------|--------------------|
|                  | NOEC   | IC\textsubscript{50} | NOEC   | IC\textsubscript{50} | NOEC   | IC\textsubscript{50} |
| Pure CP          | 0.63   | 3.7 (2.5–6.0)       | < 2.1  | 4.9 (4.8–5.1)       | 0.49   | 1.21 (0.97–1.49)     |
| Commercial CP    | 0.31   | 2.9 (2.3–4.0)       | 0.26   | 1.11 (1.08–1.15)     | 0.24   | 0.72 (0.58–0.91)     |
| TCP              | 0.31   | 0.98 (0.73–1.35)    | 0.087  | 0.29 (0.28–0.32)     | 2.0    | 9.2 (7.3–11.4)       |
| CP:TCP (1:1)     | 0.16   | 0.78 (0.57–1.12)    | 0.0063 | 0.022 (0.021–0.023)  | 0.12   | 0.62 (0.48–0.78)     |

Fig. 4 Comparison of toxicity of tested compounds

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from 6 to 42 mg/L. In this study, EC\textsubscript{50} for \textit{P. subcapitata} was found at 4.9 and 1.11 mg/L for CP and commercial CP, respectively. \textit{D. tertiolecta} was more sensitive than the freshwater algal species \textit{P. subcapitata} and more sensitive than many fish and invertebrates to several pesticides (USEPA 1999). Toxicity of pure and commercial CP obtained on \textit{P. subcapitata} was higher than toxicity reported by Chen et al. (2016) for \textit{Chlorella pyrenoidosa} and \textit{Merismopedia} sp. with 72-h EC\textsubscript{50} values of 11.46 and 25.80 mg/L, respectively and then toxicity on cyanobacteria \textit{Spirulina platensis} (EC\textsubscript{50} of 33.65 mg/L) reported by Bhuvaneswari et al. (2018).

Seven-day NOEC values of CP to six freshwater algae species were between 10 and 100 \mu g/L (Anzec and Armeanz 2000), much lower than the NOEC values found for \textit{P. subcapitata} (p < 0.05) at pure CP concentration above 2.1 mg/L and at commercial CP concentration of 0.26 mg/L.

Regarding assays with fish and invertebrates, the metabolite TCP shows to be slightly to moderately toxic, consistent with its hydrophilic character (NRA 2000); however, like CP, a high degree of variability has been observed by several authors among \textit{Daphnia} species. TCP does not cause acetylcholinesterase inhibition and, generally, is less toxic to aquatic organisms than CP (USEPA 2008). However, TCP was found to be more toxic than its parent chemical CP to \textit{Daphnia carinata} survival in cladoceran culture medium by Cáceres et al. (2007), whereas TCP showed no toxicity to organism up to a concentration of 2 \mu g/L in natural water. The values of TCP found in the literature to \textit{D. magna} varied between 2.9 and 10.4 mg/L (PubChem Database, Gorzinski et al. 1991). TCP LC\textsubscript{50} of 9.2 \mu g/L reported in this study for \textit{D. magna} was closer to that found by Cáceres et al. (2007) for \textit{D. carinata} with an LC\textsubscript{50} of 0.20 \mu g/L. The most sensitive organism to metabolite TCP was \textit{Daphnia} followed by the alga with toxicity values (IC\textsubscript{50}) of 9.2 \mu g/L and 0.29 mg/L, respectively. Toxicity of TCP to \textit{D. carinata} (0.20 \mu g/L) reported by Cáceres et al. (2007) was also much higher than toxicity to \textit{P. subcapitata} reported in the database PubChem (EC\textsubscript{50} 0.61 mg/L) but the metabolite toxicity to \textit{D. magna} (LC\textsubscript{50} 10.4 mg/L) was much lower than was for the alga (USEPA 2008). In a Microtox bioassay, TCP had an LC\textsubscript{50} of 18.6 mg/L to \textit{A. fischeri}, indicating an effect only at a high environmental unrealistic concentration (Somasundaram et al. 1990), but for the same bacteria, we found much lower toxicity (LC\textsubscript{50} 0.98 mg/L).

### Mixture toxicity

For all organisms assayed, the toxicity of CP:TCP mixture was higher than the compounds separately, with IC\textsubscript{50} values of 0.79 mg/L, 0.022 mg/L, and 0.62 \mu g/L to \textit{A. fischeri}, \textit{P. subcapitata}, and \textit{D. magna}, respectively. These results suggest that the CP and its degradation product can interact synergistically. For quantifying this interaction of the compounds, a combination index (CI) was calculated and classified according to Chou (2006) at a 50\% effect of assayed organisms. The CI values obtained indicate a very strong synergism for \textit{P. subcapitata} (CI = 0.04), strong synergism for \textit{D. magna} (CI = 0.29), and synergism for \textit{A. fischeri} (CI = 0.51). Cáceres et al. (2007) reported similar results when they use cladoceran growth medium as assay medium obtaining similar LC\textsubscript{50} values, 0.24 \mu g/L and 0.20 \mu g/L for CP and TCP respectively, and an increase of toxicity was observed for the mixture (0.08 \mu g/L), indicating a strong synergism between chemicals.

### Environmental risk of CP and TCP

According to the toxicity categories based on IC\textsubscript{50} values (United Nations 2011), pure and commercial CP can be classified as toxic for \textit{A. fischeri} and \textit{P. subcapitata}. All compounds were very toxic to cladoceran \textit{D. magna} but for \textit{A. fischeri} and \textit{P. subcapitata}, only TCP and CP:TCP mixture were toxic. These results indicate a high environmental risk to aquatic ecosystems.

Environmental concentrations of CP reported by some authors in water bodies by direct application to water or indirect entry ranged from 0.5 to 700 \mu g/L (Wood and Stark 2002; USEPA 2002; Moore et al. 2002; Mazanti et al. 2003; Bonifacio et al. 2017). In our study, no effect level and first observable effect level on \textit{D. magna} were 0.24 and 0.49 \mu g/L for the commercial insecticide, respectively, and 0.12 and 0.24 \mu g/L for CP:TCP mixture, respectively. These concentrations are much lower than the concentrations typically found in the aquatic environments; thus, results obtained in the present work indicate that CP may act in aquatic ecosystems at levels that produce toxic effects to microcrustaceans. Palma et al. (2008) suggest a potential risk to aquatic animal species from acute exposures to CP which may influence the density of some crustacean populations.

The presence of CP and their metabolite TCP in aquatic ecosystems is a concern because their mixture can cause alterations in several aspects such as behavioral, neurological, and reproductive parameters on microcrustaceans which have a key position in the food chain (De Silva and Samayawardhena 2005; Özcan Oruç 2010). However, TCP showed no toxicity in natural water up to a concentration of 2 \mu g/L due to its microbial-mediated degradation (Cáceres et al. 2007).

Moreover, it takes into account that certain physical conditions like pH can modify the stability of CP in aquatic systems. It has been demonstrated an increase of hydrolysis rate with pH: the half-life in water can vary from 1.5 days at pH 8 to 100 days at pH 7 (Tomlin 2000). De Silva and Samayawardhena (2005) reported a CP half-life of 16–77 days in freshwater bodies according to the environmental conditions. Several researchers have examined the behavior of CP in natural water and sediments and generally observed much
shorter dissipation half-lives than for laboratory studies due to dissipative and degradative forces (e.g., volatilization, hydrolysis) in natural waters and sediments (Barron and Woodburn 1995).

Conclusions

Generally, the toxicity studies based on single contaminants are not enough to predict the real toxicity in the environment. It is known that chemicals are not alone in the ecosystems but occur as mixtures; parental compounds can be found beside their degradation products, can interact with each other, and cause an increase of toxic effects. Also, in the environment, there are many species of organisms, by that, it is necessary to carry on studies on a battery of organisms. Because of that, both single and binary mixture toxicity of CP and TCP to V. fischeri, P. subcapitata, and D. magna were elucidated in this study. Results show that the toxicity assay response to compounds studied is dependent on the sensitivity of the species used. Concerning the crustacean species, D. magna was the most sensitive organism for pure CP, commercial CP, TCP, and mixture CP:TCP (1:1). TCP was more toxic than the parent compound for the bacteria and algae; however, for the cladoceran TCP, it was less toxic. The most toxic compound, for all the studied biological models, was commercial CP. Toxicity due to the synergic effect of CP and their metabolite TCP could result in negative effects on primary producers and consumers at environmental concentrations lower than expected.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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