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TISSUE-SPECIFIC BIOCONCENTRATION AND BIOTRANSFORMATION OF CYPERMETHRIN AND CHLORPYRIFOS IN A NATIVE FISH (*JENYNYSIA MULTIDENTATA*) EXPOSED TO THESE INSECTICIDES SINGLY AND IN MIXTURESROCÍO INÉS BONANSEA,^{†‡} DAMIÁN J.G. MARINO,[§] LIDWINA BERTRAND,^{†‡} DANIEL A. WUNDERLIN,^{†||}
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Abstract: The aim of the present study was to evaluate the accumulation of cypermethrin and chlorpyrifos when the fish *Jenynsia multidentata* was exposed to these pesticides singly and in technical and commercial mixtures. Adult female fish were exposed over 96 h to 0.04 µg/L of cypermethrin; 0.4 µg/L of chlorpyrifos; 0.04 µg/L of cypermethrin + 0.4 µg/L of chlorpyrifos in a technical mixture; and 0.04 µg/L of cypermethrin + 0.4 µg/L of chlorpyrifos in a mixture of commercial products. Fish exposed to cypermethrin accumulated this compound only in muscle, probably because of the low biotransformation capacity of this organ and the induction of cytochrome P4501A1 (*CYP1A1*) expression in the liver. The accumulation of chlorpyrifos occurred in fish exposed to the insecticide (intestine > liver > gills) even when these fish had higher glutathione-S-transferase (GST) activity in gills and P-glycoprotein (*P-gp*) expression in the liver, compared with the control. Fish exposed to the technical mixture showed cypermethrin accumulation (liver > intestine > gills) with higher levels than those measured in fish after only cypermethrin exposure. Higher expression levels of *CYP1A1* in the liver were also observed compared with the Control. Fish exposed to the commercial mixture accumulated both insecticides (cypermethrin: intestine > gills and chlorpyrifos: liver > intestine > gills > muscle). In the organs where accumulation occurred, biotransformation enzymes were inhibited. Consequently, the commercial formulation exposure provoked the highest accumulation of cypermethrin and chlorpyrifos in *J. multidentata*, possibly associated with the biotransformation system inhibition. *Environ Toxicol Chem* 2017;36:1764–1774. © 2016 SETAC

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INTRODUCTION

Chlorpyrifos and cypermethrin concentrations were reported together in aquatic environments worldwide during the last 15 yr [1–4]. In Argentinean basins, the concentrations of these pesticides can range from less than 0.2 ng/L to 3.5 µg/L for cypermethrin and 0.2 ng/L to 17 µg/L for chlorpyrifos [4–7]. Cypermethrin and chlorpyrifos belong to pyrethroids and organophosphorus pesticides, respectively, and such a combination is common in pesticide applications both in households and in agricultural activities [8].

Mixed pesticides, compared with single pesticides, could cause significant synergistic effects of toxicity on target species, although they are also effective on nonpest species such as invertebrate or fish. Denton et al. [9] reported synergistic effects when mixtures of diazinon (organophosphorus insecticide) and esfenvalerate (pyrethroid insecticide) were tested using fish larvae fathead minnows (*Pimephales promelas*). Atrazine (herbicide of the triazine class) exposures in mixtures with chlorpyrifos significantly increase the inhibition of cholinesterase enzyme activity and chlorpyrifos-uptake kinetics in *Xenopus laevis* and *P. promelas* [10]. However, mixtures of carbofuran (carbamate insecticide) and methamidophos (organophosphorus

insecticide) exhibit a synergic or partial additive in the median lethal concentration values (LC50) after 96 h of exposure on trout larvae *Oncorhynchus mykiss* [11]. In addition, Brodeur et al. [12] observed synergistic effects in the value of LC50 in tadpoles (*Rhinella arenarum*) exposed to mixtures of glyphosate and cypermethrin for 96 h. Nevertheless, more studies discussing the direct effect of mixtures of pesticides on nontarget biota are needed, given that they are commonly applied concurrently and that this information is more environmentally relevant [13].

Once pesticides' uptake by biota occurs, they may be biotransformed for subsequent excretion, eliminated unmetabolized, or bioaccumulated. Bioaccumulation occurs when the rate of contaminant entry is significantly higher than metabolism and elimination [14]. Cypermethrin and chlorpyrifos are potentially bioaccumulative in biota because both have log octanol–water partition coefficients (log K_{OW}) above 3 (log K_{OW} cypermethrin = 6.6; log K_{OW} chlorpyrifos = 4.9 [15]).

In laboratory studies it is possible to calculate the bioconcentration factor (BCF), to express the degree in which bioconcentration occurs [16]. Bioconcentration is the process by which a chemical substance is absorbed by an organism from the environment only through its respiratory and dermal surface, without considering the diet exposure. Previous studies reported BCF values of cypermethrin between 372 L/kg and 821 L/kg in whole-body fish [17,18]. In addition, studies carried out with chlorpyrifos showed a wide range of BCF values between 745 L/kg and 2406 L/kg in whole-body fish [19,20]. Even

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though potential for accumulation of pesticides has been addressed, experimental bioconcentration test results in native biota are still scarce in the literature.

Metabolization reactions are catalyzed by enzymes present primarily in the liver, although significant activity has been reported in other tissues such as the brain and intestine [21]. In fish, the enzymes responsible for the phase I biotransformation of a large number of xenobiotics belong mainly to the cytochrome P450 family. In particular, the isoenzyme cytochrome P4501A (CYP1A), including the *CYP1A1* and *CYP1A2* genes, is a well-known biomarker of environmental pollution [14]. Moreover, the enzyme glutathione-S-transferase (GST) is involved in phase II biotransformation reactions catalyzing the conjugation of endogenous and exogenous compounds with the peptide glutathione. Finally, the P-glycoprotein (*P-gp*) is a carrier protein belonging to the system of multixenobiotic resistance that would be considered phase III biotransformation reaction and be involved in the excretion of compounds from the cell—either the parental structure or metabolites produced after phase I reactions [22].

Edwards and Millburn [23] and Carriquiriborde et al. [24] studied the metabolic profile in the rainbow trout *Salmo gairdneri* and in the South American fish *Odontesthes bonariensis* after oral and waterborne exposure, respectively, to cypermethrin. In both studies, residues of the pesticide or its metabolites were detected,

but the performance of phases I, II, and III biotransformation reactions was not evaluated. Probable biotransformation pathways for cypermethrin in fish are shown in Figure 1.

On the other hand, chlorpyrifos can also be accumulated in different tissues of fish after waterborne exposure. Barron et al. [25] studied the tissue distribution and metabolism of chlorpyrifos in channel catfish *Ictalurus punctatus* after 24-h exposure to 12 $\mu\text{g/L}$ of the pesticide. The authors found the highest concentration of chlorpyrifos in fat and the lowest in muscle, whereas in the whole fish the mean chlorpyrifos concentration was $612 \pm 64 \mu\text{g/kg}$. In addition, the metabolites of chlorpyrifos, trichloropyridinol, and trichloropyridinol glucuronide, were measured in blood, urine, and bile. Wang et al. [26] measured chlorpyrifos and its metabolite, chlorpyrifos-oxon, in both the spleen and the kidney of *Cyprinus carpio*, after a 40-d exposure to 1.16 $\mu\text{g/L}$ of chlorpyrifos and 1.13 $\mu\text{g/L}$ of atrazine and a chlorpyrifos mixture. In addition, accumulation of chlorpyrifos was measured in *Gambusia affinis* exposed to 60 $\mu\text{g/L}$ of this insecticide for 20 d, and reported the maximum amount of bioaccumulation on day 4. The maximum accumulation of chlorpyrifos was in viscera, followed by head, and body, with average BCF values of 109 L/kg, 9 L/kg, and 4 L/kg, respectively [27]. To the extent of our knowledge, there is only limited information concerning accumulation of cypermethrin and chlorpyrifos, particularly at environmentally

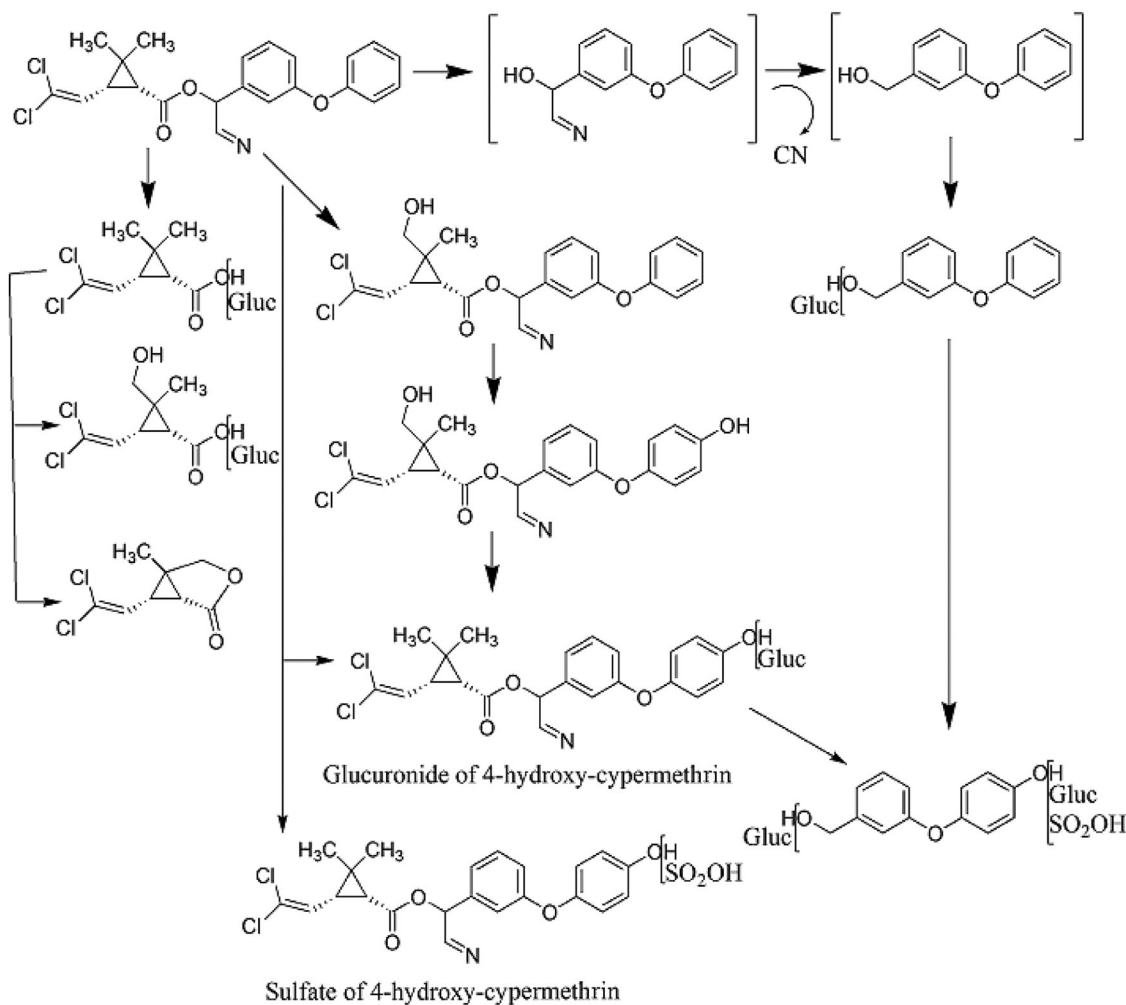


Figure 1. Pathways of biotransformation of cypermethrin proposed by Edwards and Millburn [23] and updated by Carriquiriborde et al. [24], according to the metabolites found in the bile of rainbow trout and pejerrey, respectively.

relevant concentrations. Moreover, bioaccumulation data of cypermethrin and chlorpyrifos after exposure to their mixtures has not been reported. Finally, Xing et al. [28,29] described that the herbicide atrazine and chlorpyrifos caused different responses in the activity of biotransformation enzymes when *C. carpio* was exposed to these compounds individually or in mixtures. The authors reported an increasing trend for the expression and activity of the biotransformation enzymes of phase I with accumulation of the pesticides and their metabolites in the liver when fish were exposed to 4.28 $\mu\text{g/L}$ of atrazine, 1.16 $\mu\text{g/L}$ of chlorpyrifos, and 1.13 $\mu\text{g/L}$ of their mixture [29]. Moreover, a significant decrease was observed in the GST activity in animals exposed to 428 $\mu\text{g/L}$ of atrazine, 116 $\mu\text{g/L}$ of chlorpyrifos, and 113 $\mu\text{g/L}$ of their mixture [28]. Proposed biotransformation pathways for chlorpyrifos in fish are shown in Figure 2.

The native fish *Jenynsia multidentata* (*Cyprinodontiformes*, *Anablepidae*) presents a wide distribution in South America [30]. It is a small (~ 4 cm in length), freshwater, viviparous species with external sexual dimorphism. It is also easy to transport and keep in laboratory conditions. This species is considered to be a good experimental model; it was used to evaluate the effects of lindane, glyphosate, endosulfan, cypermethrin, and chlorpyrifos on different biological processes [31].

Accordingly, the aim of the present study was to evaluate the bioaccumulation of cypermethrin and chlorpyrifos when the native fish *J. multidentata* was exposed to these pesticides individually or in mixtures of pure compounds (technical) and

commercial products at environmentally relevant levels. Biotransformation biomarkers were also measured to look for evidence of metabolic pathways of both pesticides in this native species.

MATERIALS AND METHODS

Fish

Individual *J. multidentata* were captured by backpack electrofisher equipment (LR-20B; Smith-Root) from an unpolluted area of the Yuspe River, Córdoba, Argentina [32]. Specimens of adult females were transported to the laboratory and acclimated to laboratory conditions for 2 wk before the experiments. They were maintained in a temperature-controlled room at $21 \pm 2^\circ\text{C}$ and in a 12:12-h light:dark photoperiod. Fish were of homogeneous size (mean standard length 4.2 ± 0.5 cm and mean body wt 0.8 ± 0.3 g). During acclimation and exposure periods, they were fed ad libitum once a day with commercial fish pellets (TetraMin) and the remainder of the food was removed after feeding.

Exposure conditions and design

After the acclimatization period, fish (mean standard length 4.2 ± 0.5 cm and mean body wt 0.8 ± 0.3 g) were exposed for 96 h to Control, 0.04 $\mu\text{g/L}$ of cypermethrin (99%; Sigma-Aldrich), and 0.4 $\mu\text{g/L}$ of chlorpyrifos (98%; Sigma-Aldrich) singly (cypermethrin or chlorpyrifos) and in a technical mixture (cypermethrin + chlorpyrifos; hereafter referred to as technical

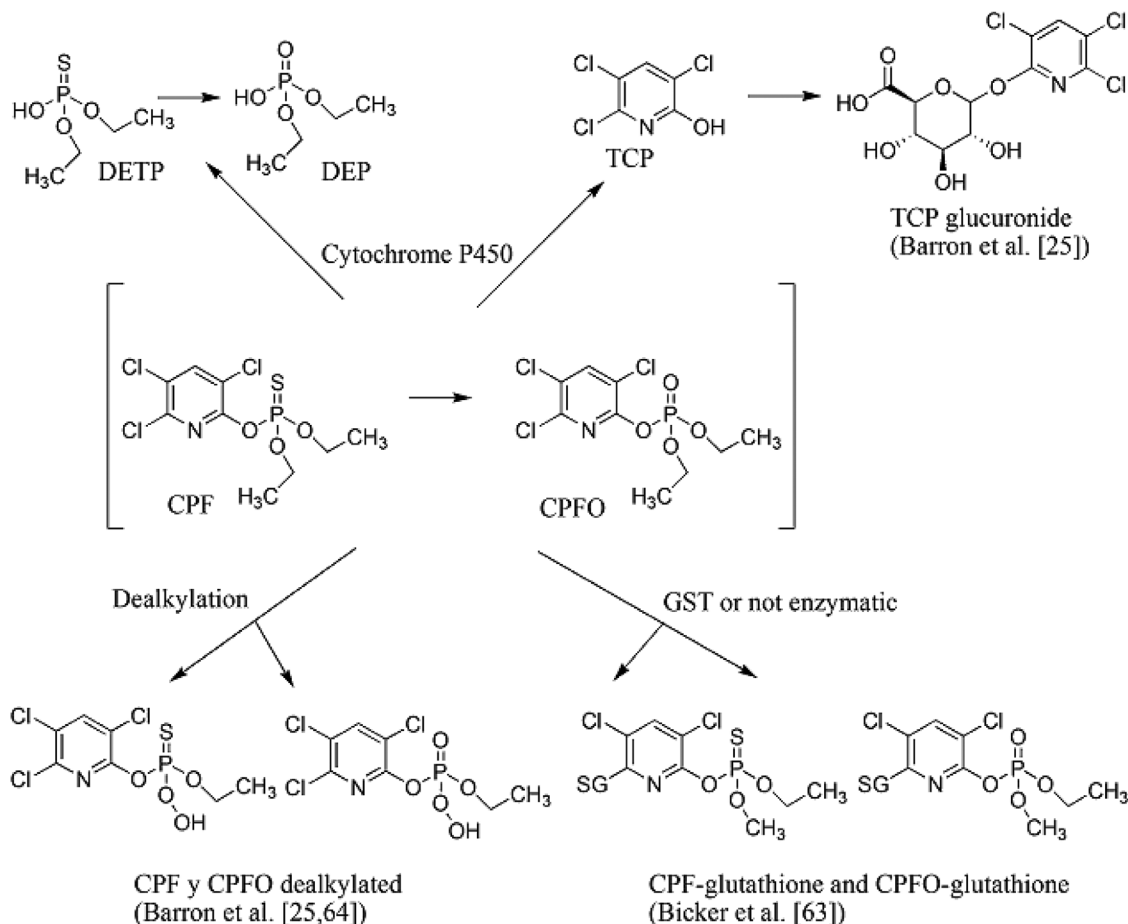


Figure 2. Pathway of biotransformation of chlorpyrifos proposed by Bicker et al. [63] and modified according to Barron et al. [25,64] and Hayes [21]. CPF: chlorpyrifos; CPFO: chlorpyrifos-oxon; TCP: 3,5,6 trichloropyridinol; DETP: diethyl thiophosphate; DEP: diethyl phosphate; GST: glutathione S-transferase.

mixture). In addition, the same mixtures, 0.04 $\mu\text{g/L}$ of cypermethrin (25% Glacoxan; Punch Química) and 0.4 $\mu\text{g/L}$ of chlorpyrifos (40% Clorfox; GLEBA) of a commercial product were tested (hereafter referred to as commercial product).

The exposure concentrations were selected taking into account 3 main criteria: 1) concentrations of cypermethrin and chlorpyrifos in natural freshwaters (cypermethrin = 0.2 ng/L–3.5 $\mu\text{g/L}$, chlorpyrifos = 0.2 ng/L–17 $\mu\text{g/L}$ [4–7]); 2) for 96-h medium lethal concentrations (96-h LC50) available for *Poecilia reticulata*, which belong to the same order as *J. multidentata* (Cyprinodontiformes, cypermethrin 96-h LC50 $\times 2009 = 21.4 \mu\text{g/L}$ [33], and chlorpyrifos 96-h LC50 = 176 $\mu\text{g/L}$ [34]), the sublethal concentrations 0.2% and 2% of the 96-h LC50 were chosen; and 3) exposure to equitoxic mixture as well as in a proportion usually used for agricultural purposes (0.002 and 0.02 toxic units according to 96-h LC50 for *P. reticulata* and a proportion of 5 cypermethrin, 50 chlorpyrifos [8]).

All experiments were conducted in 5-L glass aquariums (1 fish per liter) containing aquarium-prepared water (distilled water containing 100 mg/L of sea salt, 200 mg/L of CaCl_2 , and 103 mg/L of NaHCO_3 [35]). Pesticides dissolved in acetone were added to the aquarium water. Acetone was also added to the control condition. The final concentration of dissolvent was the same in all the treatments and was always lower than 0.05%.

Each treatment was carried out in 5 independent exposure tanks, meaning 25 specimens per condition and a total of 125 individuals for the present study.

Because of the unstable nature of cypermethrin and chlorpyrifos during the exposure conditions, a semistatic exposure system was used. New exposure tanks were prepared every 24 h. Fish were transferred daily to new tanks 10 min after the tank was prepared.

During the exposure period, aquarium water showed the following conditions: temperature $23.5 \pm 0.1 \text{ }^\circ\text{C}$, pH 7.92 ± 0.06 , dissolved oxygen $6.73 \pm 0.09 \text{ mg/L}$, and conductivity $948 \pm 33 \mu\text{S/cm}$.

After the exposure period, the animals were euthanized and the liver, intestine, gills, brain, and muscle were taken and stored at $-80 \text{ }^\circ\text{C}$ until pesticide and enzyme analysis. For *CYP1A1* and *P-gp* quantification, organs were snap-frozen in liquid nitrogen and stored in ribonucleic acid (RNA) later (QIAGEN) at $80 \text{ }^\circ\text{C}$ until analysis. From the 25 organisms exposed per treatment, 15 were used for measuring pesticide accumulation, 5 were utilized for measuring enzyme activity (GST), and 5 were used to evaluate expression of *CYP1A1* and *P-gp*.

Quantification of cypermethrin and chlorpyrifos

Aquarium water samples. The measurement of cypermethrin and chlorpyrifos in the exposure medium was performed by solid phase extraction–solid phase microextraction–gas chromatography coupled to mass spectrometry, according to Bonansea et al. [4].

The cypermethrin and chlorpyrifos concentrations were determined in water samples collected in the aquariums 30 min after the preparation of the tanks ($t = 0 \text{ h}$) and before the daily transference of fish to new tanks ($t = 24 \text{ h}$).

Fish tissue samples

Tissue sample preparation. Fish tissues were pooled to get enough quantity for analysis (3 pools of 5 individuals each taken randomly from 5 exposure tanks conducted for each treatment). Approximately 10 mg to 500 mg of pooled tissue samples were homogenized in a mortar with 3 g of anhydrous sodium sulfate.

Then 2 g of Florisil (60 mesh–100 mesh) were added and the samples were homogenized again. The mixture of sorbent material and fish tissue was packed into a 10-mL cartridge that was fitted with 3 g of alumina for sample clean-up. The elution was carried out with 10 mL of ethyl acetate. Subsequently, the elute was evaporated under nitrogen current until dry, redissolved with 0.5 mL of ethyl acetate, and transferred to an auto sampler vial for gas chromatography coupled with electron capture detector (Agilent) analysis. Polychlorinated biphenyl #103 was added to each vial as an internal standard. The extracts with cypermethrin or chlorpyrifos concentrations above the detection limit were afterward concentrated until dry, and redissolved in 0.3 mL of acetonitrile (ultrapure water, 70/30, v/v) to be confirmed by high-performance liquid chromatography coupled to mass spectrometry electrospray ionization source using a quadrupole time-of-flight analyzer (HPLC–ESI–qTOF) (Agilent–Bruker Daltonics).

Recovery percentages of cypermethrin and chlorpyrifos were previously evaluated from spiked samples at 6.7 μg of cypermethrin/kg or chlorpyrifos/kg and 667 μg of cypermethrin/kg or chlorpyrifos/kg wet weight, obtaining $71 \pm 3\%$ for cypermethrin and $84 \pm 4\%$ for chlorpyrifos.

Instrument and operational conditions. Analyses were performed on a gas chromatograph (Agilent 6890) equipped with a ^{63}Ni μ -electron capture detector (Agilent). One microliter of the sample was injected and separated on a (5%-phenyl)-methylpolysiloxane capillary column (HP-5; Agilent, 30 m \times 0.25 mm ID, 25- μm film thickness) with helium as carrier gas at a flow rate of 7 mL/min. The oven temperature was programmed starting at $160 \text{ }^\circ\text{C}$ and held for 1 min, followed by increases of $17 \text{ }^\circ\text{C/min}$ up to $200 \text{ }^\circ\text{C}$, $6 \text{ }^\circ\text{C/min}$ up to $270 \text{ }^\circ\text{C}$ and held for 4 min, with a total acquisition program of 19.02 min. The limits of detection (LODs) and limits of quantification (LOQs) of the method were experimentally estimated as the concentration of analyte giving a signal-to-noise ratio of 3 and 10, respectively [36]. The limits obtained in tissue samples for both insecticides were $\text{LOD} = 0.5 \mu\text{g/kg}$ and $\text{LOQ} = 2 \mu\text{g/kg}$.

The presence of cypermethrin and chlorpyrifos in tissues was confirmed by HPLC–ESI–qTOF (Agilent–Bruker Daltonics). The liquid chromatography separation was performed on a C-18 column (Kinetex; Phenomenex) at a flow rate of 0.3 mL/min. Binary mobile phases used were 0.1% formic acid in acetonitrile and 0.1% formic acid in water. Accurate mass spectra were recorded across the range 80 m/z to 800 m/z. An internal calibrant (20 mM of sodium formate solution) was delivered by an external syringe pump during 1 min in each run for post-run mass internal calibration. The instrument was operated in full-scan mode. Recorded data were processed with the Compass 1.3 software (Bruker Daltonics). Positive identifications of target compounds were based on: 1) accurate mass measurement of the base ion with a mass error $< 10 \text{ ppm}$ (molecular ions $[\text{M}+\text{H}]^+$ in PI mode); 2) accurate mass of 2 product ions (cypermethrin $\text{C}_{22}\text{H}_{19}\text{Cl}_2\text{NO}_3$ m/z: 416.084 and 418.081; chlorpyrifos $\text{C}_9\text{H}_{11}\text{Cl}_3\text{NO}_3\text{PS}$ m/z: 349.936 and 351.933); and 3) comparison of the retention time of the analyte in the sample with that corresponding to pure compounds ($\pm 2\%$). The LODs and LOQs obtained in tissue samples were for cypermethrin: $\text{LOD} = 5 \mu\text{g/kg}$ and $\text{LOQ} = 15 \mu\text{g/kg}$, and for chlorpyrifos: $\text{LOD} = 3 \mu\text{g/kg}$ and $\text{LOQ} = 10 \mu\text{g/kg}$. All measurements were performed in triplicate.

Enzyme analysis

Enzyme extracts were prepared from individual intestine, liver, gills, brain, and muscle (5 organs, not pooled, coming from 5 specimens taken randomly from 5 exposure tanks

conducted for each treatment) according to Monferrán et al. [37]. Thus cytosolic and microsomal protein fractions were obtained from each sample. Enzymatic activities were determined by spectrophotometry, using a microplate reader (Synergy BioTek). The activity of soluble GST and membrane-bound GST (EC 2.5.1.18) was determined using 1-Chloro-2,4-dinitrobenzene (CDNB) as substrate [38]. The enzymatic activity was calculated in terms of the protein content of the sample, measured at 595 nm by the Bradford method [39], and it is reported in nanokatal per milligram of protein (nkat mg/prot), where 1 kat is the conversion of 1 mol of substrate per second. Each enzymatic measurement was carried out in triplicate.

Expression of *CYP1A1* and *P-gp*

For *CYP1A1* and *P-gp* quantification, intestine, liver, gills, brain, and muscle were analyzed. The processed tissues came from 5 specimens taken randomly from 5 exposure tanks conducted for each treatment. Total RNA was extracted from tissues by the guanidine thiocyanate–phenol chloroform extraction method in accordance with Chomczynski and Sacchi [40]. Nonspecific reverse transcription was performed with an Oligo (dT)₁₅ primer (Biodynamics), and Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen) [41]. Quantitative polymerase chain reaction was performed with a Bio-Rad iQ cyclor and was used to amplify and measure the transcript abundance of *CYP1A1* and *P-gp* in all studied tissues. We have previously characterized the partial cDNA sequences for *CYP1A1* and *P-gp* of *J. multidentata* [41,42] (GenBank accession numbers are EF362746 and EF362745, respectively), and have designed specific *J. multidentata* primers for real-time polymerase chain reaction (*CYP1A1* forward: 5'-CTGGATCGAAC TCCTACTATCACTGA-3', reverse: 5'-GCAGTGTGGGATT-GTGAAAGGTA-3'; *P-gp* forward: 5'-CTGCACGCTAGCG-GAAAAC-3', reverse: 5'-CCTCTATCTCCTCCATGGTCA CA-3'). The primers were designed using Primer Express Software (Applied Biosystems), and obtained from Sigma-Aldrich. The use of *J. multidentata* β-actin [41] as a reference gene was tested. Relative gene expression was analyzed by using the standard curve method and was carried out in triplicate.

Bioconcentration factor

Bioconcentration factors (in L/kg) of cypermethrin and chlorpyrifos in *J. multidentata* tissues at each exposure treatment were estimated as the ratio between the concentration of the corresponding pesticide in a fish organ (μg/kg wet wt) divided by the measured pesticide concentration in exposure water samples (μg/L) [43].

The pesticide concentration in whole-fish body was estimated as the ratio between the addition of the measured quantities of the corresponding pesticide in each organ (μg) to the addition of the mean wet weight of each organ measured [44].

Statistical analysis

All values are expressed as mean ± standard deviation. Normal distribution of data was analyzed by the Shapiro–Wilk test, whereas Levene's test was used to test the homogeneity of variance. To evaluate the differences of enzymes' activity measured, as well as *CYP1A1* and *P-gp* expression, one-way analysis of variance followed by the Tukey test were performed to compare among treatments. When the data showed abnormal distribution, they were subjected to a nonparametric statistical

analysis (Kruskal–Wallis test) followed by Dunn's test. The InfoStat/P software [45] was employed in all cases. Significance was accepted at $p < 0.05$.

RESULTS AND DISCUSSION

Pesticide concentrations in water samples

Initial concentrations of insecticides in aquarium water were measured in all treatments. However, no significant differences between concentrations of chlorpyrifos or cypermethrin in different treatments were observed. Consequently, the averages obtained from all treatment samples are reported to simplify subsequent calculations. Cypermethrin and chlorpyrifos concentrations at $t = 0$ h were cypermethrin = 0.04 ± 0.01 μg/L and chlorpyrifos = 0.31 ± 0.03 μg/L. The decay in pesticide concentrations after 24 h ($t = 24$ h) was 49% and 81% for cypermethrin and chlorpyrifos, respectively, justifying the daily renewal of the exposure medium. Insecticide concentrations in control aquariums were below the LODs of the method (cypermethrin = 0.2 ng/L; chlorpyrifos = 0.2 ng/L).

Accumulation and biotransformation response

According to van de Oost et al. [14], bioaccumulation depends on the balance of uptake, biotransformation, and elimination. The accumulations of cypermethrin and chlorpyrifos were observed in the intestine, liver, gills, and muscle of *J. multidentata* (Table 1). In contrast, the insecticides were always below the LOD in the brain. Moreover, the distribution of the insecticides among tissues was different, whether the exposure was made with pesticides individually or in mixtures.

To contribute to the understanding of the mechanisms involved in the biotransformation of cypermethrin and chlorpyrifos in different organs of *J. multidentata*, enzymes of phases I, II, and III were evaluated as biomarkers of these processes; the results are shown in Table 2. Expression of *CYP1A1* and *P-gp* was not measured in muscle because low yields in RNA extractions were obtained.

Cypermethrin exposure. When *J. multidentata* was exposed to cypermethrin for 96 h, the insecticide was only detected in muscle (Table 1). Meanwhile, fish exposed to cypermethrin significantly increased the *CYP1A1* expression in the liver (1.9-fold change compared with control, Table 2). In addition, a significant decrease (0.4- to 0.6-fold change compared with control) was evidenced in the activity of GST microsomal (GSTm) and GST cytosolic (GSTc) in the intestine, brain, and muscle as well as GSTm in the liver. The expression of *P-gp* did not indicate significant differences in any of the organs studied compared with the control.

Edwards and Millburn [23] have reported the accumulation of cypermethrin in the rainbow trout *Salmo gairdneri* when it was exposed to the pesticide for 24 h. However, the pesticide levels were higher in viscera than in other parts of the fish body (including the muscle). The differences with our results could be the result of different exposure concentrations (Edwards and Millburn [23] = 5 and 10 μg/L; the present study = 0.04 μg/L) and a faster metabolism of cypermethrin in other organs than in muscle. In fish, the main route of cypermethrin biotransformation described by other authors is a hydroxylation followed by conjugation with sulfate or glucuronic acid [23,24]. This oxidation may be mediated by the cypermethrin enzyme system (Figure 1). Moreover, there is some evidence that cypermethrin is a good substrate for the *CYP1A1* isoform, which has been demonstrated for humans and rats [46]. Therefore, this mechanism may be occurring in the liver of *J. multidentata* and

Table 1. Levels of cypermethrin and chlorpyrifos expressed as µg/kg (wet wt) in the intestine, liver, gills, brain, and muscle of *Jenynsia multidentata* after exposure to sublethal concentrations of cypermethrin and chlorpyrifos singly and in mixtures for 96 h^a

Treatments	Intestine			Liver			Gills			Brain			Muscle		
	Cypermethrin	Chlorpyrifos	Product	Cypermethrin	Chlorpyrifos	Product	Cypermethrin	Chlorpyrifos	Product	Cypermethrin	Chlorpyrifos	Product	Cypermethrin	Chlorpyrifos	Product
Control	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
CYP	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
CPF	<LOD	67 ± 49B	<LOD	<LOD	58 ± 25	<LOD	<LOD	42 ± 31B	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
CYP + CPF	52 ± 30	<LOD	<LOD	156 ± 35A	<LOD	<LOD	8 ± 5	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Product	116 ± 67	50 ± 29AB	<LOD	<LOD	323 ± 187	<LOD	52 ± 30	11 ± 6AB	<LOD	<LOD	<LOD	<LOD	<LOD	8 ± 5	<LOD

^aData are expressed as mean ± 1 standard deviation (mean and standard deviation are calculated from triplicates, pooling 5 individuals each). Capital letters indicate significant differences among treatments, for each column and into the same organ ($p < 0.05$). Cypermethrin: limit of detection is 5 µg/kg; limit of quantification is 15 µg/kg. chlorpyrifos: limit of detection is 3 µg/kg; limit of quantification is 10 µg/kg. Cypermethrin 0.04 µg/L; chlorpyrifos 0.4 µg/L; Cypermethrin + chlorpyrifos 0.04 µg/L plus 0.4 µg/L in a technical mixture. Product: Cypermethrin 0.04 µg/L plus chlorpyrifos 0.4 µg/L in a mixture of commercial products. CYP = cypermethrin; CPF = chlorpyrifos; <LOD = below limit of detection; LOQ = limit of quantification.

would be sufficient to prevent the accumulation of the insecticide in the liver, as well as in other organs. The exposure of *J. multidentata* to 1 µM of β-naphthoflavon for 24 h has also markedly induced the expression of CYP1A in the gills and liver of this fish [47]. The occurrence of cypermethrin in muscle could be associated with a lower capacity of this organ to biotransform, when compared with other organs. For instance, the lower capacity of muscle to biotransform exogenous compounds has been reported in the neotropical silverside fish *O. bonariensis* exposed to microcystins [48].

Glutathione conjugation by GST has not been reported in previous studies of cypermethrin metabolism, both in mammals and fish [23,24,49]. Our results partially coincide with these reports because no increase was observed in the enzyme activity, but inhibition of GST was evidenced in various organs of *J. multidentata*. When inhibition takes place, the enzymatic activity is blocked. This could be explained by several hypotheses. Cypermethrin or its metabolites may be competing with GST substrates for the active sites on the GST enzyme [50]. Alternatively, a decrease in GST protein synthesis can occur as a result of damage generated in cells by increased reactive oxygen species (ROS) produced by cypermethrin [51]. Inhibition may also be caused by a covalent modification of the enzyme by the insecticide, leading to an irreversible loss of activity [52]. Similar results were observed in the brain of rats exposed to cypermethrin [51].

Chlorpyrifos exposure. Fish exposed to chlorpyrifos showed concentrations of this pesticide in the following descending order: intestine > liver > gills (Table 1). The distribution of chlorpyrifos in different tissues of *J. multidentata* presents a typical pattern of hydrophobic compounds. Moreover, a similar distribution was reported in the catfish *I. punctatus* and the mosquitofish *G. affinis* exposed to the same insecticide [25,27]. The occurrence of chlorpyrifos in the intestine and gills could mean 2 possible uptake routes for dissolved chlorpyrifos: by the gastrointestinal tract through drinking the exposure medium, or by the gills during ventilation. The adsorption of chlorpyrifos from fish food is less possible because fish were fed for a short period and in small quantities. This hypothesis agrees with that expressed by Bruggeman et al. [53].

On the other hand, the presence of chlorpyrifos in the intestine and gills, which are highly vascularized, could be a result of desorption from the blood stream for its excretion (by feces or during ventilation). However, according to Welling and de Vries [54], the elimination of unchanged chlorpyrifos is insignificant in comparison with metabolic degradation.

The liver is located in a strategic position within the organisms, receiving a large volume of blood that contributes to the distribution of chemical compounds and their metabolites to other organs. In addition, the liver has an abundant reserve of nonspecific enzymes, giving it the capacity to metabolize a broad spectrum of organic compounds [55].

In fish exposed to chlorpyrifos, no changes were evidenced in the expression of *CYP1A1* in any of the organs when compared with control. On the other hand, a significant decrease was observed for GSTc and GSTm activity in the intestine (0.5-fold and 0.6-fold change compared with control) and the brain (0.4-fold change compared with control), as well as for GSTm activity in the liver and muscle (1.8-fold and 2-fold change compared with control, respectively). In contrast, in the gills of *J. multidentata*, GSTm activity significantly increased (0.2-fold change compared with control), whereas only in the liver a

Table 2. Relative cytochrome P4501A1 and P-glycoprotein multidrug transporter protein gene expression, and glutathione-S-transferase cytosolic (GSTc) and glutathione-S-transferase microsomal (GSTm) activity in the intestine, liver, gills, brain, and muscle of *Jenynsia multidentata* after exposure to sublethal concentrations of cypermethrin and chlorpyrifos singly and in mixtures for 96h^a

Treatments	Intestine	Liver	Gills	Brain	Muscle
<i>CYP1A1/β-actin</i>					
Control	0.6 ± 0.1BC	0.7 ± 0.2B	1.7 ± 0.7B	0.8 ± 0.3A	ND
CYP	0.6 ± 0.2BC	2.0 ± 0.1D	1.4 ± 0.2AB	0.4 ± 0.1A	ND
CPF	0.42 ± 0.09AB	0.8 ± 0.2B	1.7 ± 0.5B	0.6 ± 0.2A	ND
CYP + CPF	0.61 ± 0.06C	1.3 ± 0.3C	0.9 ± 0.5A	0.6 ± 0.1A	ND
Product	0.24 ± 0.03A	0.25 ± 0.07A	2.0 ± 0.3B	1.4 ± 0.6A	ND
GSTc (nkat/mg protein)					
Control	4 ± 1C	3.8 ± 0.6	1.3 ± 0.2	5 ± 2B	0.4 ± 0.1C
CYP	2 ± 1A	3.6 ± 0.8	1.2 ± 0.2	3 ± 1A	0.3 ± 0.1A
CPF	2 ± 1A	3.4 ± 0.4	1.1 ± 0.2	3.1 ± 0.3A	0.3 ± 0.1BC
CYP + CPF	3 ± 1AB	4.1 ± 0.6	1.1 ± 0.2	3.2 ± 0.6A	0.3 ± 0.1AB
Product	3 ± 1BC	4.5 ± 0.8	1.0 ± 0.2	3.8 ± 0.5B	0.3 ± 0.1AB
GSTm (nkat/mg protein)					
Control	2.0 ± 0.5B	2.4 ± 0.3B	0.5 ± 0.1B	2.0 ± 0.5B	0.4 ± 0.1C
CYP	0.9 ± 0.2A	1.4 ± 0.7A	0.6 ± 0.1BC	1.1 ± 0.4A	0.2 ± 0.1AB
CPF	0.8 ± 0.2A	1.3 ± 0.2A	0.6 ± 0.1C	1.2 ± 0.1A	0.2 ± 0.1A
CYP + CPF	1.6 ± 0.4B	1.5 ± 0.3A	0.3 ± 0.1A	2.2 ± 0.7B	0.3 ± 0.1A
Product	1.0 ± 0.1A	2.2 ± 0.4B	0.3 ± 0.1A	1.8 ± 0.3B	0.10 ± 0.01AB
<i>P-gp/β-actin</i>					
Control	1.0 ± 0.2B	1.0 ± 0.4A	1.8 ± 0.5B	0.5 ± 0.1AB	ND
CYP	1.0 ± 0.2B	0.8 ± 0.2A	1.4 ± 0.4AB	0.6 ± 0.1BC	ND
CPF	0.9 ± 0.2AB	1.7 ± 0.6B	1.3 ± 0.2AB	0.57 ± 0.05BC	ND
CYP + CPF	1.0 ± 0.2B	0.9 ± 0.2A	0.9 ± 0.4A	0.41 ± 0.03A	ND
Product	0.6 ± 0.1A	0.9 ± 0.1A	1.8 ± 0.4B	0.64 ± 0.03C	ND

^aData are expressed as mean ± 1 standard deviation. Capital letters indicate significant differences among treatments, for each column and into the same organ ($p < 0.05$). Cypermethrin 0.04 μg/L; chlorpyrifos 0.4 μg/L; cypermethrin + chlorpyrifos 0.04 μg/L plus 0.4 μg/L in a technical mixture. Product: cypermethrin 0.04 μg/L plus chlorpyrifos 0.4 μg/L in a mixture of commercial products.

CYP = cypermethrin; CPF = chlorpyrifos; *CYP1A1* = cytochrome P4501A1 gene expression; *P-gp* = P-glycoprotein multidrug transporter protein gene expression; GST = glutathione-S-transferase; ND = no data.

significant increase in the expression of *P-gp* was observed (0.7-fold change compared with control).

Phase I enzyme activity would be the main pathway in the biotransformation of chlorpyrifos [56]. Wheelock et al. [57] observed a decrease in protein levels of CYP1A in the species *Oncorhynchus tshawytscha* exposed to chlorpyrifos. Conversely, the expression of *CYP1A1* and the activity of cypermethrin enzymes in the species *C. carpio* were induced [29]. Given that in *J. multidentata* the expression of only 1 isoform of the cypermethrin system was measured, it cannot be rejected that chlorpyrifos is suffering from other phase I reactions. To confirm this mechanism in this species, an alternative would be to measure ethoxyresorufin-O-deethylase activity. Phase II conjugation by GST enzymes has been reported as a major metabolic pathway of chlorpyrifos in fish [25]. In addition, the P-gp protein would play an additional role in the detoxification of this insecticide, transporting chlorpyrifos-oxon to the extracellular space [58]. Thus the increase of both GSTm activity in the gills and *P-gp* expression in the liver could be indicating that phases II and III detoxification mechanisms are occurring in *J. multidentata*. Nevertheless, this increase in biotransformation pathways was not sufficient to prevent accumulation, also favored by the inhibition of the activity of GSTc in the other organs tested. The inhibition of this enzyme caused by chlorpyrifos could be a result of the same mechanisms presented for cypermethrin (see section *Cypermethrin exposure*) because it is a general response found for hydrophobic compounds [52,55].

Technical mixture exposure. Unexpectedly, fish exposed to the technical mixture accumulated only cypermethrin in the following descending order: liver > intestine > gills (Table 1).

Thus 2 aspects of these results can be discussed: the differences observed in the distribution of cypermethrin singly if compared with the exposure to cypermethrin, and the concentrations of chlorpyrifos below the LOD. As was previously stated, the only detection of cypermethrin in the muscle could be attributed to a fast metabolism of the insecticide in the other organs. In the presence of chlorpyrifos, the capability of the liver and other organs to biotransform could be diminished and, as a consequence, cypermethrin could accumulate. According to Wheelock et al. [57], the exposure of the juvenile Chinook salmon (*O. tshawytscha*) to chlorpyrifos significantly inhibited liver carboxylesterase activity at 1.2-μg/L and 7.3-μg/L chlorpyrifos dose exposure (56% inhibition and 79% inhibition, respectively). These enzymes are, among other factors, responsible for cleavage of the ester linkage of cypermethrin, a necessary step for its detoxification. Thus inhibition in pesticide metabolism favors accumulation in its original unmetabolized chemical structure. On the contrary, chlorpyrifos below the LOD in all the studied organs could be caused by increased oxidation of the insecticide to form oxidized derivatives, not measured in the present study. This oxidation could be attributed to the presence of cypermethrin, which is known to be capable of favoring the production of ROS [59].

Fish exposed to the technical mixture of the pesticides showed a significant increase of *CYP1A1* expression in the liver (0.9-fold change compared with control) and a significant decrease of the expression of the same enzyme in the gills, with a 0.5-fold change in respect to control. Moreover, significant differences were evidenced with decreased activity of GSTm and GSTc in muscle (0.25-fold change compared with control, for both enzymes' activities), of GSTc in the intestine and the

brain (0.2-fold and 0.4-fold change compared with control, respectively), and of GSTm in the liver and gills (0.4-fold change compared with control, for both organs). A significant decrease in the expression of *P-gp* in the gills was also observed (0.5-fold change compared with control). Increased *CYP1A1* expression in the liver could be caused by an attempt by that organ to metabolize cypermethrin, which was insufficient because the insecticide was bioaccumulated in the liver, intestine, and gills. The inhibition of GSTc in the intestine and the inhibition of enzymes responsible for phases I, II, and III reactions in the gills could also favor accumulation in these organs, where direct contact with the parental compounds could occur. Widespread inhibition of GST could affect the metabolism of chlorpyrifos, as observed after chlorpyrifos exposure; however, this insecticide did not accumulate. Therefore, it could be assumed that the oxidation of chlorpyrifos caused by ROS is the most important route for biotransformation of this insecticide in *J. multidentata* exposed to the technical mixture.

Commercial mixture exposure. Fish exposed to the commercial mixture of cypermethrin and chlorpyrifos showed that both pesticides accumulated in various organs of *J. multidentata*. The levels of cypermethrin were found in the following decreasing order: intestine > gill (Table 1), showing the presence of the pesticide only in the possible uptake organs. In contrast to the results obtained for the technical mixture, no accumulation was observed in the liver. Regarding the bioaccumulation of chlorpyrifos, the concentration of this pesticide showed the following descending order: liver > intestine > gill > muscle (Table 1), revealing a wide distribution of the insecticide in different organs of *J. multidentata*. As in chlorpyrifos exposure, chlorpyrifos accumulated in major uptake organs of the pollutant (the intestine and gills), as well as in the liver of the fish. In addition, when *J. multidentata* was exposed to the commercial product, the fish bioaccumulated chlorpyrifos in muscle. This difference could be the result of a greater ability acquired by chlorpyrifos to penetrate the membranes when it is accompanied by adjuvant compounds present in commercial formulations.

Fish exposed to cypermethrin and chlorpyrifos showed a different response pattern in biotransformation biomarkers when compared with the technical mixture. Therefore, they showed significant differences with decreased expression of *CYP1A1* in the liver and intestine (0.6-fold change compared with control, for both organs), and a significant 0.8-fold change increased *CYP1A1* expression in the brain, when compared with control. Furthermore, a significant decrease in GSTm and GSTc activity was observed in the muscle (0.75- and 0.25-fold change compared with control, respectively) and the gills (0.4- and 0.2-fold change compared with control, respectively), as well as of GSTm in the intestine (0.5-fold change compared with control). Relative expression of *P-gp* decreased 0.4-fold in the intestine and increased 0.3-fold in the brain when compared with control. Cytochrome P4501A1 expression inhibition observed in the liver could be responsible for the accumulation of chlorpyrifos through a lower efficiency of biotransformation by this route. In addition, this response could be caused by a desulfurization process of chlorpyrifos mediated by cypermethrin enzymes producing chlorpyrifos-oxon. The liberated sulfur ion could suppress cypermethrin enzymes' activity through binding to the heme group of these proteins [57,60].

The inhibition of the biotransformation system (phases I, II, and III) in the intestine could be associated with the accumulation of the 2 insecticides. Conversely, in the gills,

where the 2 insecticides were accumulated, only the inhibition of GST was observed. This difference might be caused by greater accumulated concentrations of pesticides in the intestine than in the gills, or differential response of each tissue [52]. The decreased activity of GST in muscle might be diminishing the biotransformation of chlorpyrifos, thus causing the accumulation of the pesticide in this organ. The increased activity or expression of the biotransformation system (phases I and III) observed in the brain could be a result of the presence of the insecticides being metabolized effectively, preventing their accumulation in this organ. To summarize, fish exposed to the commercial product showed significant inhibition of the biotransformation system (phases I, II, and III) in the intestine, liver, muscle, and gills, causing the accumulation of 1 or 2 insecticides in these organs. Conversely, in the brain the accumulation of pesticides was avoided by increased activity or expression of the biotransformation system.

BCF

From the amounts of cypermethrin and chlorpyrifos quantified in each organ, the concentration of the insecticides present in the whole fish for each treatment was estimated (Figure 3). Moreover, the BCFs of cypermethrin and chlorpyrifos in different organs of *J. multidentata* were calculated and are shown in Table 3.

Fish exposed to the singular insecticides showed BCFs equaling 244 ± 136 L/kg in muscle for cypermethrin, and BCFs in a range from 133 L/kg to 212 L/kg in the intestine, liver, and gills for chlorpyrifos, indicating that the 2 compounds have the capacity to bioconcentrate in *J. multidentata*. This result agrees with $\log K_{OW}$ values of the insecticides, both >3 ($\log K_{OW}$ cypermethrin = 6.6; $\log K_{OW}$ chlorpyrifos = 4.9). Chemical compounds with $\log K_{OW}$ >3 are considered bioaccumulative in an organism [15]. Furthermore, in the fish exposed to the technical mixture of these insecticides, only cypermethrin accumulated, whereas chlorpyrifos was below the LOD. The BCFs obtained for cypermethrin in *J. multidentata* organs were higher than those found in fish exposed to cypermethrin individually. The behavior of the compounds together, as explained in the section *Technical mixture exposure*, might be caused by the decreased biotransformation processes of cypermethrin and augmented oxidation of chlorpyrifos to other chemical metabolites, which were unmeasured in the present

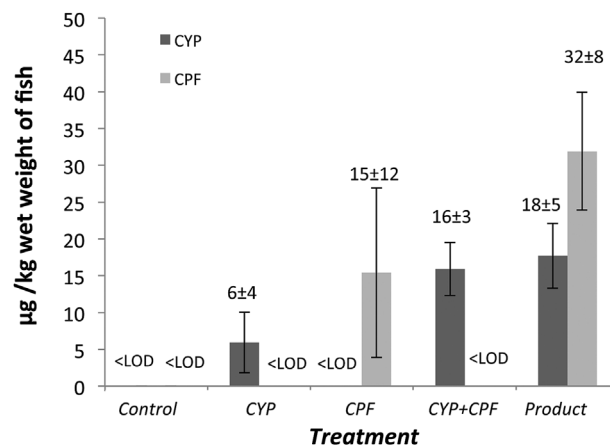


Figure 3. Estimated concentrations of cypermethrin (CYP) and chlorpyrifos (CPF) in whole fish for each treatment. Shown as average \pm standard deviation. LOD = limit of detection. Product = 0.04 μ g/L of cypermethrin + 0.4 μ g/L of chlorpyrifos commercial mixture.

Table 3. Bioconcentration factor of cypermethrin and chlorpyrifos expressed as L/kg (wet wt) in the intestine, liver, gills, brain, and muscle of *Jenynsia multidentata* after exposure to sublethal concentrations of cypermethrin and chlorpyrifos singly and in mixtures for 96 h^a

Treatments	Intestine		Liver		Gills		Brain		Muscle	
	Cypermethrin	Chlorpyrifos	Cypermethrin	Chlorpyrifos	Cypermethrin	Chlorpyrifos	Cypermethrin	Chlorpyrifos	Cypermethrin	Chlorpyrifos
Control	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Cypermethrin	nd	nd	nd	nd	nd	nd	nd	nd	244 ± 136	nd
Chlorpyrifos	nd	212 ± 155	nd	184 ± 79	nd	133 ± 98	nd	nd	nd	nd
Cypermethrin + Chlorpyrifos	1409 ± 813	nd	4228 ± 949	nd	217 ± 136	nd	nd	nd	nd	nd
Product	3144 ± 1816	158 ± 92	nd	1024 ± 593	1409 ± 813	35 ± 19	nd	nd	nd	25 ± 16

^aData are expressed as mean ± 1 standard deviation (mean and standard deviation are calculated from triplicates, pooling 5 individuals each). Cypermethrin 0.04 µg/L; chlorpyrifos 0.4 µg/L; cypermethrin + chlorpyrifos 0.04 µg/L plus 0.4 µg/L in a technical mixture. Product: cypermethrin 0.04 µg/L plus chlorpyrifos 0.4 µg/L in a mixture of commercial products. CYP = cypermethrin; CPF = chlorpyrifos; nd = not detectable.

study. *Jenynsia multidentata* exposed to the commercial product mixture showed BCFs for cypermethrin in the intestine and gills with higher values than those found in the exposure to the technical mixture. Bioconcentration factors for chlorpyrifos calculated for fish exposed to the commercial product varied from low values (25 ± 16 and 35 ± 19 in muscle and gills, respectively) to middle and high values (158 ± 92 and 1024 ± 593 for the intestine and liver, respectively). The BCF of chlorpyrifos in the liver of fish exposed to the commercial product was higher than the value obtained in fish exposed to chlorpyrifos individually (Table 3). Furthermore, in the exposure to the commercial product it was observed that *J. multidentata* bioconcentrated both compounds, unlike *J. multidentata* exposed to the mixture of pure insecticides (Figure 3). This could be associated with the presence of adjuvant compounds, substances that generally are solvents and emulsifiers, which are included in the formulations of commercial insecticides to increase their efficiency. No significant correlation was found between BCF and biotransformation biomarkers in the present study; however, further studies on dose–response tests could be interesting.

Other fish species of food interest, may bioconcentrate cypermethrin and chlorpyrifos in muscle similarly to *J. multidentata*, meaning a risk to human health. The World Health Organization jointly with the United Nations Food and Agriculture Organization established values of acceptable daily intake for cypermethrin = 20 mg/kg body weight per day [61] and for chlorpyrifos = 10 mg/kg body weight per day [62]. Considering the average value of cypermethrin and chlorpyrifos found in the muscle of the fish (Table 1), and assuming that a 70-kg adult ingests 100 g of fish per day, calculations reveal consumption of 0.01 mg/kg of body weight per day, for both cypermethrin and chlorpyrifos. As a result, the calculated daily intake indicates the lack of risk associated with eating fish exposed to these concentrations of pesticide. However, in this approach the biomagnification is not considered possibly occurring under environmental conditions. From these results, there arises the need for studies of cypermethrin and chlorpyrifos accumulation in organisms with importance for human consumption. Further studies on dose–response tests should be performed to better understand these responses.

CONCLUSIONS

The native fish *J. multidentata* accumulated cypermethrin and chlorpyrifos in various organs, with higher concentrations in the liver > intestine > gill being lower than the quantities measured in muscle, and below the LOD in the brain. The bioaccumulation of the insecticides was detected in all treatments showing higher accumulated concentrations and distribution in the organs of fish exposed to commercial mixtures. However, the BCFs calculated in these organs were low, when compared with other exposed organisms. This effect could be a consequence of the biotransformation system showing responses of phases I and II in fish exposed to cypermethrin, phases II and III in fish exposed to chlorpyrifos, and phases I, II, and III in fish exposed to the technical mixture and the commercial product. Nevertheless, the response of the biotransformation system was insufficient to prevent accumulation of these compounds in *J. multidentata*.

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Data availability—Data, associated metadata, and calculation tools are available from the corresponding author (vame@fcq.unc.edu.ar).

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