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Responses of Aquatic Nontarget Organisms in Experiments Simulating a Scenario of Contamination by Imidacloprid in a Freshwater Environment

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Abstract

Several studies have indicated the presence of the neonicotinoid insecticide imidacloprid (IMI) in aquatic ecosystems in concentrations up to 320.0 µg L^{-1}. In the present study, we evaluated the effects of the highest IMI concentration detected in surface water (320.0 µg L^{-1}) on the survival of *Chironomus sancticaroli*, *Daphnia similis,* and *Danio rerio* in three different scenarios of water contamination. The enzymatic activities of glutathione S-transferase (GST), catalase (CAT), and ascorbate peroxidase (APX) in *D. rerio* also were determined. For this evaluation, we have simulated a lotic environment using an indoor system of artificial channels developed for the present study. In this system, three scenarios of contamination by IMI (320.0 µg L^{-1}) were reproduced: one using reconstituted water (RW) and the other two using water samples collected in unpolluted (UW) and polluted (DW) areas of a river. The results indicated that the tested concentration was not able to cause mortality in *D. similis* and *D. rerio* in any proposed treatment (RW, UW, and DW). However, *C. sancticaroli* showed 100% of mortality in the presence of IMI in the three proposed treatments, demonstrating its potential to impact the community of aquatic nontarget insects negatively. Low IMI concentrations did not offer risks to *D. rerio* survival. However, we observed alterations in GST, CAT, and APX activities in treatments that used IMI and water with no evidence of pollution (i.e., RW and UW). These last results demonstrated that fish are more susceptible to the effects of IMI in unpolluted environments.

The intensive use of pesticides contributes to the growth of agricultural productivity. These chemicals are used to eliminate pests that can cause injuries to crops, increasing the production and quality of the harvest. It is estimated that agriculture annually uses 2.5 million tons of active ingredients of pesticides worldwide (Chen et al. 2018; Fenner et al. 2013).

Neonicotinoids are the most important, effective, and best-selling class of new synthetic pesticides used to control insects in crops (Xia et al. 2016). These compounds were recently developed from the (*S*)-(-)-nicotine molecule, after isolation as an alkaloid from *Nicotiana* sp. (tobacco) (Jeschke et al. 2013). The neonicotinoids act as agonists of nicotine acetylcholine receptors (nAChR) in the nervous system of insects. The binding of neonicotinoids to nAChRs prevents the binding of acetylcholine. The enzyme acetylcholinesterase cannot degrade these pesticides, resulting in continuous stimulation of the receptors. The neural overstimulation results in tremors, paralysis, and death (Matsuda et al. 2001; Stara et al. 2019; Vignet et al. 2019; Yamamoto and Casida 1999).

The commercial success of this group of pesticides is due to their high efficiency against pests, long-term control, harvest guarantee, suitable for application in a wide range of crops, and the ability to act on a large number of insect species (Jeschke et al. 2011). Furthermore, these compounds

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show lower toxicity to vertebrates compared with carbamate and organophosphates insecticides (Jeschke et al. 2011; Anderson et al. 2015; Simon-Delso et al. 2015). The selective toxicity of these substances to invertebrates can result in unintentional adverse effects on nontarget species like bees. This fact led the European Union to adopt restrictions on the use of thiamethoxam, clothianidin, and imidacloprid (Domenica et al. 2017; EFSA 2018).

Imidacloprid (IMI) (1-(6-chloro-3-pyridylmethyl)-*N*-nitro-imidazolidin-2-ylideneamine) (Fig. 1) is considered the main molecule of the first generation of neonicotinoids developed in the early 1990s (Kagabu 2011). Currently, IMI it is widely used to protect several crops, such as rice, cotton, sugarcane, coffee, corn, beans, soy, and wheat (Xia et al. 2016). However, the IMI can move into adjacent areas after application and can further migrate into aquatic environments via several environmental fate processes (e.g., runoff, leaching, etc.) (Wood and Goulson 2017; Yadav and Watanabe 2018).

Recently, IMI was found in water bodies in several countries in concentrations up to 320.0 µg L^{-1} (Kreuger et al. 2010; Lamers et al. 2011; Starner and Goh 2012; Hayasaka et al. 2012a; Van Dijk et al. 2013; Morrissey et al. 2015). The presence of IMI in aquatic ecosystems is facilitated by its physical and chemical characteristics. IMI is highly soluble in water and displays a half-life time that varies from 0.24 to 2.22 days in aquatic environments (Lu et al. 2015). IMI reaches water bodies through runoff after rainfall events (Gupta et al. 2002), atmospheric deposition, and transport of contaminated dust to aquatic ecosystems adjacent to agricultural areas (Pisa et al. 2015). Additionally, IMI is hardly biodegraded (Van Dijk et al. 2013).

In aquatic ecosystems, IMI may be harmful to the ecosystem structure and function of the aquatic life. Several studies have reported the impacts of IMI on different nontarget aquatic species, including insects, crustaceans, and fish. Stonefly *Pteronarcys comstocki* exposed to IMI had its eating habit affected, as reported by Pestana et al. (2009). The authors observed a decrease in the leaf litter decomposition and feeding rates after exposure to 17.6 µg L^{-1}. IMI also can affect the reproduction of aquatic crustaceans (Böttger et al. 2013). Female individuals of *Gammarus roeseli* had smaller broods after repeated low-level and short-term exposure to IMI (12.0 µg L^{-1}), which

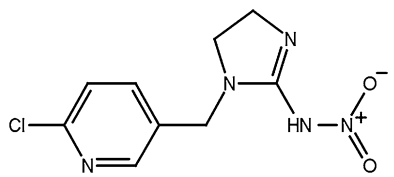

Fig. 1 Molecule of imidacloprid

demonstrated the potential of this insecticide to affect the individual's reproduction and to cause long-term effects on the population size (Böttger et al. 2013). IMI caused toxic effects on *Hyalella azteca* survival (LC_{50} = 230.0 µg L^{-1}) and the growth was reduced (Bartlett et al. 2019). Also, in sublethal concentrations, the IMI affects the nervous system and promotes the drift of macrozoobenthos (*Baetis rhodani* and *Gammarus pulex*) to downstream areas after 2-h exposure, as reported by Beketov and Liess (2008). Hong et al. (2018) observed genetic damage, a decrease in immune response, and changes in nuclei of erythrocytes in Chinese rare minnows *Gobiocypris rarus* after chronic exposure to IMI (0.1 to 2.0 mg L^{-1}). Oxidative stress caused by IMI also has been noticed in aquatic organisms (Iturburu et al. 2018; Qi et al. 2018; Vieira et al. 2018; Hong et al. 2020; Shan et al. 2020). Enzymes associated with the line defense in the antioxidant system, such as glutathione S-transferase (GST), catalase (CAT), and ascorbate peroxidase (APX), are essential for the detoxification of pollutants in aerobic organisms (Slaninova et al. 2009; Zhang et al. 2015). GST shows a rapid enzymatic response when exposed to azole compounds similar to imidazoline, which is a heterocyclic compound produced in the process of degradation of the IMI (Giraudo et al. 2017; Vieira et al. 2019). CAT and APX are important antioxidant enzymes that regulate intracellular H_2O_2 produced in the detoxification process (Gebicka and Krych-Madej 2019). Likewise, CAT and APX are good biomarkers to compounds that exhibit the formation of nitrogen compounds (Sinha et al. 2015), such as IMI (Simon-Delso et al. 2015).

Due to the immediate importance to understand the effects of this agent in aquatic systems, we aimed to further study this pesticide. In the present report, we simulated different scenarios of environmental contamination using water from different sources and IMI concentrations close to the highest concentration detected in an aquatic environment (320.0 µg L^{-1}). For this reason, we proposed a lotic indoor channels system to evaluate the IMI toxicity using water from different sources to represent three scenarios of water quality (water of known quality—reconstituted, natural water collected in an unpolluted area, and natural water collected in a polluted area). The high IMI concentration (320.0 µg L^{-1}) was used to validate the indoor system of artificial channels proposed in the present study. We performed a multispecific evaluation using simultaneously three freshwater organisms: *Chironomus sancticaroli*, *Daphnia similis*, and *Danio rerio*. We evaluated the effects on the survival of the three organisms and enzymatic activity (GST, CAT, and APX) in *D. rerio*. These assays allowed us to evaluate the possible synergistic effects between IMI and different levels of contamination.

Material and Methods

Water Samples

We performed the assays in the present study using three types of water. A1 was reconstituted water (RW) from *D. similis* culturing. Water A2 and A3 was collected in the Piquete River (Piquete—Sao Paulo, Brazil) at two different points: upstream (UW) in Piquete river in a preserved area close to the spring (22.35′ 39.7″ S; 45.13′ 35.5″ W) and downstream (DW) of an urbanized area through which the Piquete River flows (22° 37′ 22.5″ S; 45° 09′ 40.8″ W) (Online resource 1). The use of natural water from different sources aimed to evaluate a possible synergistic effect between IMI and other pollutants present in the water column of the river.

Parameters that were determined in situ included: pH, electrical conductivity (µS cm^{-1}), and temperature (°C) using a multiparameter probe YSI model ProDSS. We stored the collected samples in a polyethylene containers and sent them to the laboratory where turbidity (UNT), by turbidimetry (Tecnopon model TB-1000), dissolved oxygen (mg L^{-1}) and biochemical oxygen demand (BOD) (mg L^{-1}), by Winkler's method (Barnett 1939), and total phosphorus concentrations (µg L^{-1}), by the ascorbic acid method (APHA, 2005), were determined.

Chemicals

The commercial formulation Galeão® (Helm do Brasil Mercantil LTDA, São Paulo, SP—Brazil) (imidacloprid/inert ingredients (70:30, m/m), 460.0 µg Galeão® L^{-1}) was used as the source of the active ingredient to reach the concentration of 320.0 µg IMI L^{-1}. We used the highest IMI concentration (320.0 µg L^{-1}) detected in freshwater to validate the ecotoxicological assessment system proposed in the present study, facilitating the quantification of this pesticide. Because the main source of IMI in agricultural practice is the formulated product (Anderson et al. 2015), we chose to work with the final product instead of the pure active ingredient (imidacloprid) to approach the conditions found in the field.

The analytical standard Imidacloprid Pestanal® was purchased from Sigma-Aldrich (St. Louis, MO). The pesticide Galeão® (70% w/w of imidacloprid) was purchased from HELM DO BRASIL MERCANTIL LTDA (São Paulo, Brazil). Methanol and acetonitrile were analytical grade and were obtained from Merck KGaA (Darmstadt, Germany) and Honeywell (Muskegon, USA), respectively. Water was purified by a Milli-Q system (Millipore).

Summary of Experimental System and Treatments

Assays A1, A2, and A3 were performed to evaluate the toxicity of IMI (320.0 µg L^{-1}) in three different scenarios of water quality (RW, UW, and DW) using two indoor artificial channels systems (CS): control (without IMI) and treatment with IMI. We performed each CS assay twice.

Each system consisted of a glass channel (80 cm × 12 cm × 12 cm) and a reservoir containing a submerged pump (380 L h^{-1}) to promote the oxygenation and circulation of water between compartments. Channel and reservoir were connected by hoses. The capacity of the system was 20 L with the principal channel having a volume of 8 L and the reservoir a volume of 12 L (Fig. 2a).

Initially, we filled the systems with water (RW, UW, or DW). Then, we added the commercial product Galeão® in the experimental channels (final IMI concentration ≅ 320.0 µg L^{-1}). The systems remained circulating for 1 h to homogenize the compound before the introduction of any organism. After this period, we inserted the organisms

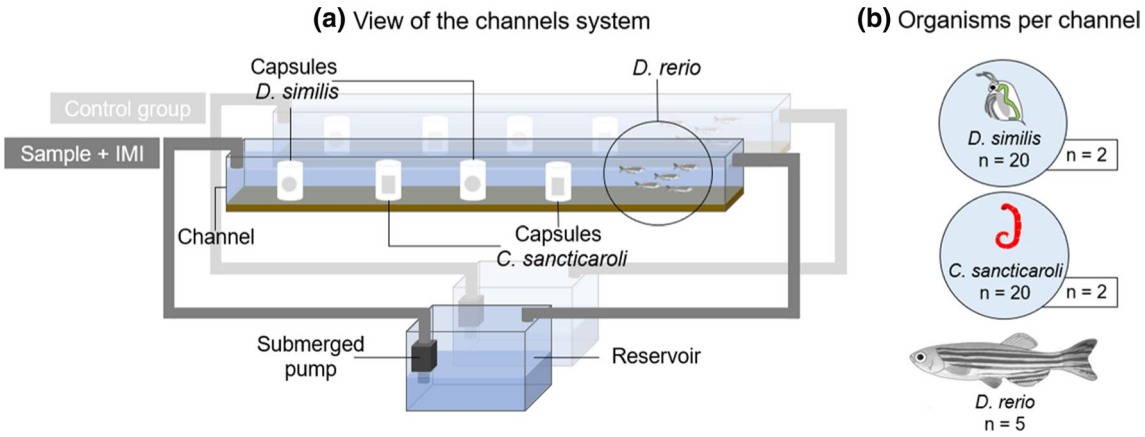

Fig. 2 Lotic indoor channels system. **a** Artificial channels used. **b** Assay performed in each channel of the system

Daphnia similis (neonates < 24 h old), *Chironomus sancticaroli* (first-instar larvae), and *Danio rerio* (adults) at the same time in both systems. To prevent predation by fish, we put *D. similis* and *C. sancticaroli* individuals in distinct PVC capsules with openings coated with a plankton net. In each channel, we used 20 neonates of *D. similis* per capsule ($n = 2$), 20 larvae of *C. sancticaroli* per capsule ($n = 2$), and 5 adults *D. rerio* (0.59 ± 0.06 g) (Fig. 2b). Each capsule was considered a replicate and we positioned them randomly inside the channel. The number of replicates was based on the standard NBR 12713:2016 (ABNT 2016), which defines two replicates as a minimum number in an ecotoxicological evaluation using *Daphnia* sp. The *C. sancticaroli* capsules contained quartz sand (0.6 mm) employed as a substrate for these organisms. The quartz sand was treated at 500 °C for 1 h to remove organic matter and volatile substances.

The exposure period adopted in this experiment was the same as that used to evaluate the acute toxic effect: 48 h for *D. similis* and *C. sancticaroli,* and 96 h for *D. rerio*. We performed the assays at room temperature. Previous experiments in the channels system demonstrated that the IMI concentration was maintained stable during the proposed period of the assays (96 h). The final IMI concentration did not display a statistically significant difference ($p < 0.05$) compared to the initial test concentration (Online resource 2).

During the CS assays, organisms and water parameters pH, conductivity (μS cm^{-1}), temperature (°C), and dissolved oxygen (mg L^{-1}) were monitored daily. After 48 h, capsules containing *D. similis* and *C. sancticaroli* were removed from the channels to verify the mortality rate.

According to the literature, the IMI displays high LC_{50} values for fish. Thus, in the present study we did not focus to understand the survival of this organism, but the physiological effects caused by the exposure to IMI instead. After 96 h, we removed the fish from the system and analyzed the enzymatic activity of glutathione S-transferase (GST), catalase (CAT), and ascorbate peroxidase (APX). The activity of these enzymes was determined for the fish from all three assays (A1, A2, and A3).

Standard acute tests using the water from the channels were simultaneously performed for each channel system experiment. The standard tests were performed in controlled conditions following ISO 6341 (2012) and OECD 235 (2011) for *D. similis* and *C. sancticaroli*, respectively, and Douglas et al. (1986) for *D. rerio*. The results obtained in the standard tests were compared with the results obtained in the channels system.

The adopted channels system simulated the natural conditions of an aquatic ecosystem. Thus, we expected to evaluate possible synergistic effects between IMI and water with different quality levels. Although there are studies that have evaluated the toxicity of IMI on Chironomidae (Kobashi et al. 2017; Chandran et al. 2018) and daphnids (Qi et al. 2018; Raby et al. 2018b; Rico et al. 2018), no work using *C. sancticaroli* and *Daphnia similis* was found in the literature so far. Moreover, *C. sancticaroli* is an insect found in tropical water bodies. Thus, the use of this organism allows to understand the effects of the IMI in aquatic ecosystems from these regions (Fonseca and Rocha 2004). Therefore, we used these organisms to obtain preliminary information about the effects of IMI on these species. Also, we decided to use *D. rerio* in the present study, because one of the reasons for the commercial success of IMI is the lower toxicity to vertebrates. Although *D. rerio* displays high LC_{50} values, minor effects could be evidenced by enzyme biomarkers. The Ethics Committee on Animals Use of the Biosciences Institute of the University of São Paulo (CEUA no. 310/2018) approved the experimental procedures adopted in the present study.

Sample Preparation and Chromatographic Analyses

The IMI concentration in the system was determined at the beginning of the CS assays. The water samples were filtered in a glass fiber prefilter (47 mm) using a Millipore System (Swinnex-47) and a 3-mL syringe. The samples (40 mL per Falcon® tube) were frozen and lyophilized. The dried samples were resuspended in 500 μL of methanol: water (1:1). The samples were diluted to adjust the concentrations to the linear interval of the standard curve. Aliquots (20 μL) of each sample were injected into the high-pressure liquid chromatograph (HPLC). The concentration of the active ingredient in the samples was expressed as μg of imidacloprid per L.

Quantification of the active ingredient (imidacloprid) in the samples containing the pesticide Galeão® was performed using a Shimadzu Prominence HPLC (Shimadzu, Kyoto, Japan) equipped with a photodiode array detector (PDA), SPD-M20A. The chromatographic separation was achieved using a Kinetex EVO C18 column (150 mm × 4.6 mm, 5 μm, Phenomenex) maintained at room temperature. All ultraviolet–visible spectra were recorded from 200 to 600 nm. For quantitative analyses, chromatograms were integrated at 270 nm. The volume injected was 20 μL. The mobile phases were: (A) Milli-Q water and (B) acetonitrile, and the flow rate was set at 1.5 mL min^{-1}. The gradient used for separation was 20% B at the start of the run, 90% B at 2.1 min, which was held until 3.5 min, followed by a 4.5 min equilibration at 20% B before the next injection. The identification of the peak was performed by comparing the chromatographic retention time with the standard and evaluating the characteristics of the electronic absorption spectra.

A stock solution (1 mg 1 mL^{-1}) was prepared with the analytical standard Imidacloprid Pestanal®. Calibration was performed using dilutions of the stock solution (0.39, 0.78, 1.56, 3.125, 6.25, 12.50, 25.00, and 50.00 μg L^{-1}). The

respective peak areas obtained in the PDA (270 nm) were plotted vs. the quantity (ng) of analyte in the samples. Standard curve displayed in Online Resource 3.

Enzymatic Activities on *Danio rerio*

After 96-h exposure to IMI, two individuals from each channel were randomly selected from which the homogenates were prepared (1:9, *w*/*v*) by maceration of the whole fish in cold potassium phosphate buffer (pH 7.4). The homogenates were centrifuged at 2000*g* for 20 min at 4 °C, and the supernatants were collected to determine the enzymatic activity of glutathione S-transferase (GST), catalase (CAT), and ascorbate peroxidase (APX). The same procedure was followed for fish from the acute toxicity testing in the standard tests.

The protein contents were determined by the Bradford method using bovine serum albumin (BSA) as a standard (Bradford 1976). Each enzyme activity was determined three times using the same homogenate of the original two individuals and the results used to calculate the specific activity of GST, CAT, and APX.

Glutathione S-transferase

Glutathione S-transferase (GST) activity was adapted from the method proposed by Habig et al. (1974). Tests were conducted in triplicate using 100 mM of potassium phosphate buffer (pH 6.5), 1.0 mM of EDTA, 9.5 mM of reduced glutathione (GSH), 1.0 mM 1-chloro-2,4-dinitrobenzene (CDNB), and 10.0 µL of homogenate. CDNB was used as a substrate for the reaction of converting GSH to thiolate anion of glutathione (GS^-), through the enzyme GST. The formation of conjugate S-(2,4-dinitrophenyl) glutathione was monitored for increased absorbance at 340 nm for 5 min in the UV–VIS spectrometer. The molar extinction coefficient of CDNB was 9.6 mM^{-1} cm^{-1}.

Catalase

Catalase (CAT) activity was determined following the method described by Aebi (1984). The tests were conducted using 100 mM of potassium phosphate buffer (7.0), 20.0 mM of H_2O_2, and 10.0 µL of homogenate. The activity was monitored by the consumption of H_2O_2 resulting in the decline of absorbance at 240 nm for 3 min in the UV–VIS spectrometer. The molar extinction coefficient to H_2O_2 was 40.0 $mM^{-1}.cm^{-1}$. Enzymatic activity was expressed from the consumption of 1 mmol of H_2O_2 min^{-1} mg $protein^{-1}$.

Ascorbate Peroxidase

Ascorbate peroxidase (APX) activity was determined from an adapted method by Nakano and Asada (1981). The tests were conducted using potassium phosphate buffer 50 mM (pH 7.0), ascorbic acid 0.5 mM, H_2O_2 0.1 mM, and 20.0 µL of homogenate. The activity was determined by the decrease in absorbance values at 290 nm caused by the consumption of ascorbate for 2 min in the UV–VIS spectrometer. Molar extinction coefficient was 2.9 mM^{-1} cm^{-1}. The activity was expressed in terms of the consumption of 1 mmol of H_2O_2 min^{-1} mg $protein^{-1}$.

Statistical Analysis

All values were expressed as the mean value ± standard deviation (SD). The normality of data was assessed using the Kolmogorov–Smirnov test, and homogeneity of variance was tested by Bartlett's test. The mortality was assessed by Fisher's test. The enzymatic activity was assessed by one-way analysis of variance (ANOVA) followed by Dunnett's test to determine the significant difference among IMI treatment and control group. MINITAB 19® software was employed for statistical evaluation with a level of significance set as $p < 0.05$.

Results

Water Parameters

UW and DW samples showed physical and chemical characteristics expected for unpolluted and polluted areas, respectively. Water collected downstream (DW) of the urban area in the Piquete river presented the highest values for turbidity, conductivity, BOD, and total phosphorus, which indicate the occurrence of anthropogenic pollution (Le Moal et al. 2019). Physicochemical parameters of reconstituted water (RW) and the water collected upstream (UW) and the downstream (DW) of the Piquete river are presented in Table 1.

Table 1 Physical and chemical variables of water used in the treatments

Variables	RW	UW	DW
pH	6.9	7.85	7.32
Turbidity (UNT)	0.01	0.40	0.65
Conductivity (µS cm^{-1})	214.0	37.5	318.0
Dissolved oxygen (mg L^{-1})	7.26	4.0	3.5
Biochemical oxygen demand (BOD) (mg L^{-1})	0.31	0.5	2.2
Total phosphorus (µg L^{-1})	0.29	1.36	22.43

RW reconstituted water, *UW* upstream water, *DW* downstream water

Test Solution Parameters

The pH variation at the beginning and the end of the *D. similis* and *C. sancticaroli* standard tests did not exceed 0.5 units in any treatment, and it did not present significant change ($p < 0.05$). Mean values of water parameters, pH, temperature (°C), conductivity (µS cm^{-1}), and IMI concentration (µg L^{-1}) measured in assays are shown in Table 2. The DO was maintained at 7.31 ± 0.25 mg L^{-1} by the oxygenation system during all experiments in the channels. The variation of the temperature between A3 and the other assays is related to the room temperature during the period that the experiments were performed.

As the test solutions were prepared using the commercial product targeting the highest concentration, we observed variations in the IMI concentrations, such as displayed in Table 2. The mean concentration between the CS assays was 287.60 ± 32.06 µg L^{-1}.

Toxicity

The survival rate was $100 \pm 0.0\%$ for all individuals in the control water (i.e. all water without IMI) except for the mortality of a number of daphnids in the DW water of the channels system (A3) (Table 3). However, the observed mortality rate did not indicate an acute toxic effect for *D. similis* (Fisher's test, $p < 0.05$), i.e., the observed mortality remained within natural variability. Differently, *C. sancticaroli* showed $100\% \pm 0.0\%$ mortality in all treatments that contained IMI; in both the channels system (CS) and standard tests (ST). IMI did not cause mortality of *D. rerio* in any treatment (Table 3).

Enzymatic Activity

Activities of GST, CAT, and APX were assessed to investigate the detoxification capacity of *D. rerio* to IMI. In the presence of IMI (320.0 µg L^{-1}), GST activity was reduced in the A1 (one-way ANOVA, $p < 0.05$), using reconstituted water (RW + IMI) (Fig. 3a). We observed a reduction of CAT activity in A2 (UW + IMI) in both systems, standard tests, and channels system (one-way ANOVA, $p < 0.05$; Fig. 3b). APX activity increased only in A2 (UW + IMI) (one-way ANOVA, $p < 0.05$) in the standard tests (Fig. 3c).

Table 2 Physical and chemical variables obtained from ecotoxicological assays performed in the channels system and IMI concentrations

Assay	Treatment	IMI (µg L^{-1})	Temperature (°C)	pH	Conductivity (µS cm^{-1})
A1	RW	0.00 ± 0.00	22.43 ± 0.51	7.80 ± 0.27	219.63 ± 4.75
	RW + IMI	278.80 ± 56.25	22.65 ± 0.31	7.57 ± 0.15	250.25 ± 4.65
A2	UW	0.00 ± 0.00	23.40 ± 0.37	7.80 ± 0.07	35.22 ± 8.26
	UW + IMI	335.69 ± 16.80	23.56 ± 0.67	7.76 ± 0.06	27.42 ± 7.38
A3	DW	0.00 ± 0.00	19.50 ± 0.14	7.80 ± 0.00	323.40 ± 7.60
	DW + IMI	248.32 ± 19.52	19.60 ± 0.07	7.70 ± 0.00	328.10 ± 5.80

Values expressed as mean value ± standard deviation

RW reconstituted water, *UW* upstream water, *DW* downstream water

Table 3 Mortality (%) of *Daphnia similis*, *Chironomus sancticaroli,* and *Danio rerio* observed at the end of standard tests (ST) and channels system (CS) tests with imidacloprid (IMI)

Assay	Treatment	*Daphnia similis*		*Chironomus sancticaroli*		*Danio rerio*	
		ST	CS	ST	CS	ST	CS
A1	RW	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	RW + IMI	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0*	100.0 ± 0.0*	0.0 ± 0.0	0.0 ± 0.0
A2	RW	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	UW	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	UW + IMI	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0*	100.0 ± 0.0*	0.0 ± 0.0	0.0 ± 0.0
A3	RW	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	DW	10.0 ± 0.0	12.5 ± 3.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	DW + IMI	0.0 ± 0.0	7.5 ± 3.5	100.0 ± 0.0*	100.0 ± 0.0*	0.0 ± 0.0	0.0 ± 0.0

Values expressed as mean value ± standard deviation

*Statistically significant difference ($p < 0.05$) according to Fisher's test

RW reconstituted water; *UW* upstream water; *DW* downstream water

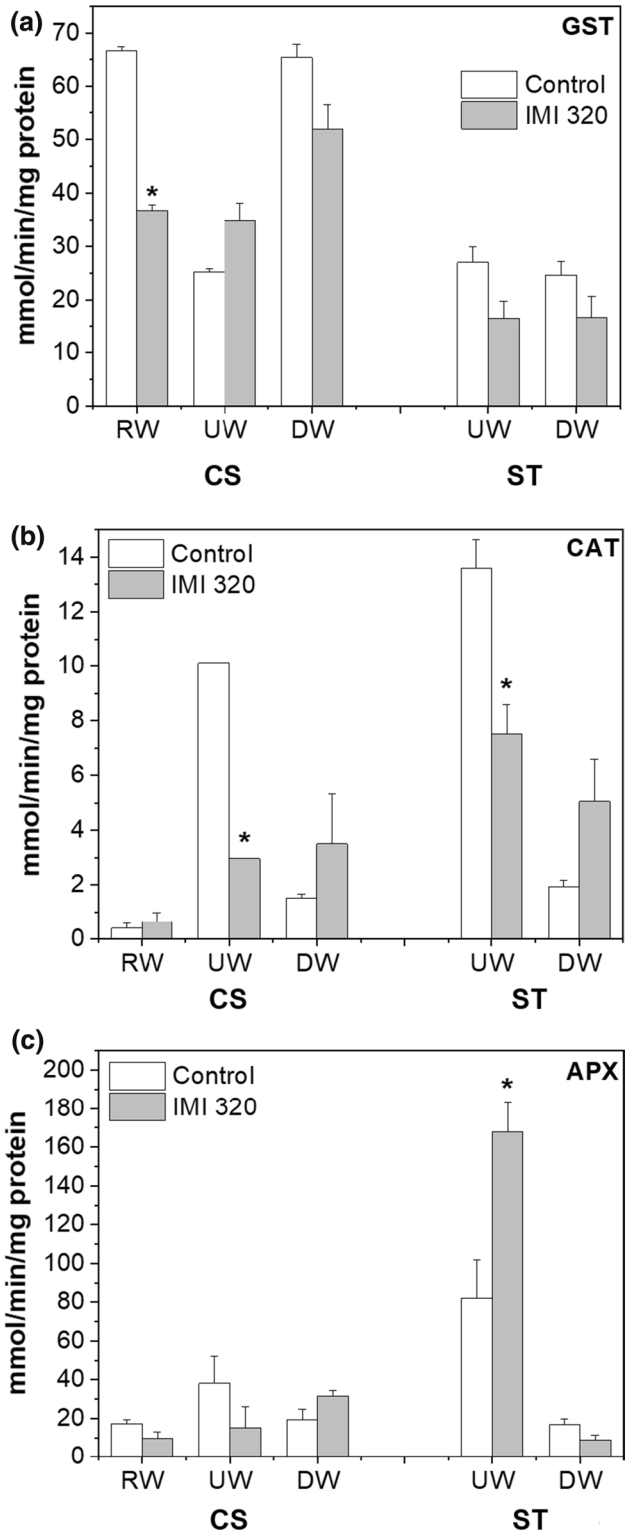

Fig. 3 GST, CAT, and APX activity in *Danio rerio* after exposure to imidacloprid (IMI) in experiments using reconstituted water (RW) and water collected upstream (UW) and downstream (DW) of Piquete river carried out in standard tests (ST) and channels system (CS). Values expressed as mean value ± standard deviation (SD). Errors bars represent the SD. *Significant differences between IMI and control groups (Dunnett's test, $p < 0.05$)

Discussion

According to Morrissey et al. (2015) review, imidacloprid (IMI) concentrations detected in worldwide surface waters varied from 0.001 to 320.0 µg L^{-1}. In the present study, we selected 320.0 µg L^{-1} as a reference to carry out the ecotoxicological evaluation. Although most of the reports indicate that the lower IMI concentrations are detected more frequently in natural conditions, we chose the highest concentration considering that this concentration can occur again in similar environmental contamination circumstances. Additionally, in a typical aquatic ecosystem, IMI is rapidly dissipated and the loss of this compound occur through different pathways including dilution, infiltration in the soil, photolysis, microbial degradation, and sorption to soil and sediment (La et al. 2014). Therefore, these sources of concentration reduction can mask the results of experiments using lowered concentrations, especially in polluted water as used herein. It also is important to notice that studies that quantify the IMI in water bodies report values from point samples, i.e., samples collected in a specific place and moment. This type of sampling does not consider the loss pathways of the compound in the aquatic environment and so often underestimates peak concentrations by 1–3 orders of magnitude and average concentrations by 50% (Xing et al. 2013). Thus, high IMI concentrations can be observed before this pesticide is diluted in the aquatic environment, mainly close to the agricultural areas. Moreover, another variable that increases the concentrations of IMI in the water column is the rainfall, which can intensify the runoff of the pesticide to the aquatic environment. However, other factors should be considered to understand the transport of the IMI to the streams, such as its increasing use observed each year and the information about the watershed (Hladik et al. 2014). Therefore, apart from the real possibility to find such a high concentration of IMI in water bodies, we chose to perform our assays using the high IMI concentration for the sake of simplicity and to maintain and monitor the target concentration in our indoor system of artificial channels. This also enables the validation of our system for further studies at lower concentrations of neonicotinoids.

The limnological parameters of the UW and DW sites fell in between the recommended standards for the protection of aquatic biota under Resolution CONAMA 357/2005 (BRASIL, 2005). However, DW high values for total phosphorus, conductivity, BOD, and turbidity (Table 1) indicate contamination by organic effluents from the urban area. Aquatic environments located close to urbanized areas are vulnerable to pollution by several contaminants that affect the ecosystem in the short and long term (do Amaral et al. 2018). Effluents released in

water bodies have high loads of organic matter, dissolved phosphorus, and nitrogen compounds (Mor et al. 2019), which explain the high values of physical and chemical parameters observed in DW water (Table 1). These nutrients occur naturally in aquatic ecosystems and are important components of the main energy pathways of lotic ecosystems. However, a significant increase in its concentrations can indicate a eutrophication process that results in an ecological imbalance in hydrobiocenosis (Milošević et al. 2018). According to the trophic index proposed by Cunha et al. 2013, we classified the trophic level of UW and DW as ultraoligotrophic (≤ 15.9 µg L^{-1}) and oligotrophic (16.0–23.8 µg L^{-1}), respectively.

Under the conditions of the present study, the tested IMI concentration (320.0 µg L^{-1}) did not cause mortality in *D. similis* and *D. rerio* (Table 3). Other studies evaluated the effects of IMI in different species of *Daphnia* sp. and found EC_{50} values between 16.5 and 56.6 mg L^{-1} (Tišler et al. 2009; Hayasaka et al. 2012b; Qi et al. 2018). Wu et al. (2018) determined LC_{50} of IMI for different development stages of *D. rerio*, the concentrations varied from 26.39 to 128.6 mg L^{-1}. In both cases, EC/LC_{50} values were relatively high compared with the concentration used in the present study. Moreover, no toxicity was observed in the water collected in the Piquete river used in the control group in A2 and A3 (Table 3). The absence of toxicity in A2 can be associated with the quality of the water collected upstream (UW) of the Piquete river. This sampling point consisted of a preserved area without human interference, i.e., without evidence of pollution (Table 1). On the other hand, the water used in A3 was collected downstream of this river. Although the river receives wastewater from the urban areas, its capacity for dilution and self-purification could explain the absence of toxicity observed in this assay.

Considering other studies, IMI can cause mortality in species of *Chironomus* sp. in low concentrations of the commercial product and analytical grade chemicals. The LC_{50} values vary from 1.7 to 31.5 µg L^{-1} (Stoughton et al. 2008; Pestana et al. 2009; Raby et al. 2018a; Chandran et al. 2018). As mentioned, there is a lack of studies evaluating the toxicity of IMI in *C. sancticaroli*. In the present study, we observed $100 \pm 0.0\%$ of mortality in *C. sancticaroli* individuals after exposure of 48 h to IMI (320.0 µg L^{-1}) in both systems, standard tests, and channels system (Table 3). The results indicated that low IMI concentrations detected in water bodies offer risks to the survival of populations of the genus *Chironomus* sp. This is especially true if we consider that IMI can remain in water bodies for long periods when not exposed to light, such as in sediment, where these benthonic organisms live part of their life (Sumon et al. 2018). From an ecological viewpoint, it is important to note that if benthic organisms are negatively impacted the entire ecosystem may suffer because they play a key role in the food web and nutrient cycling in water bodies (Silva et al. 2019). Aquatic invertebrates are important components of these ecosystems, acting as detritivore, herbivore, parasite, and predator. Furthermore, they provide food to vertebrates associated with these systems (Pisa et al. 2015).

The mortality rate in standard tests and channels system showed similar results. The use of the channels system did not affect the survival of the tested organisms. Thus, the channels system can be an alternative to evaluate the effects of environmental contaminants in a multispecific approach. Some adaptations, such as removing the capsules, could promote interactions between the tested species and bring greater ecological complexity to the analysis. Thus, these adaptations in the channels system would allow the evaluation of other variables that could not be obtained in a standard test, such as the predation index and behavior changes. In short, the channels system proposed in the present study demonstrated to be a promising method for the simultaneous assessment of different effects of the contaminants on the aquatic ecosystem.

In addition to mortality, aquatic contaminants cause physiological and biochemistry imbalances, increase the susceptibility to disease, and change the reproductive system. Bioaccumulation of these contaminants can induce oxidative stress characterized by an imbalance of the redox system, between oxidant and antioxidant mechanisms, which result in cell damage. The aquatic organisms can live in contaminated environments due to defense mechanisms that allow detoxification, antioxidant protection, excretion, and stress response of xenobiotics (Hook et al. 2014; Narra et al. 2017; Stara et al. 2019). In fish, oxidative stress can occur as a secondary aspect of hypoxia, presenting histopathological and biochemical alterations in the gills, as well as the nuclear abnormalities of the erythrocytes (Dantzger et al. 2018). Several studies have indicated the potential of IMI to change the enzymatic activity in different fish species. For instance, Topal et al. (2017) evaluated the IMI neurotoxicity in *Oncorhynchus mykiss* at concentrations of 5.0, 10.0, and 20.0 mg L^{-1} for 21 days. An increase in the activity of CAT, superoxide dismutase (SOD), glutathione peroxidase (GPx), malondialdehyde (MDA), and 8-hydroxy-2-deoxyguanosine (8-OHdG) was observed, but the activity of acetylcholinesterase (AChE) enzyme decreased. Wu et al. (2018) exposed *D. rerio* embryos to 0.38, 1.52, and 6.08 mg L^{-1} of IMI active ingredient for 96 h. The activity of GST, SOD, and CYP450 increased. On the other hand, the activities of carboxylesterase (CarE) and CAT decreased. Xia et al. (2016) observed a decrease in the glutamic-pyruvic transaminase (GPT) and glutamic-oxalacetic transaminase (GOT) activities of the *Misgurnus anguilicaudatus* after 96-h exposure to IMI in all tested concentrations (43.0, 67.0, 91.0, and 115.0 mg L^{-1}).

In the present study, we examined the oxidative stress caused by the tested IMI concentration (320.0 µg L^{-1}), which is lower compared with the reported ones (Xia et al. 2016; Topal et al. 2017; Wu et al. 2018). Thus, we measured the enzymatic activities of GST, CAT, and APX of *D. rerio* (Fig. 3). GST activity showed a statistically significant decrease ($p < 0.05$) in the presence of IMI in the treatment RW + IMI (Fig. 3a). In the treatment UW + IMI, we also observed a decrease in CAT activity in both experiments: standard tests and channels system (Fig. 3b). Differently, APX activity increased only in the treatment UW + IMI performed in standard tests (Fig. 3c). According to Hook et al. (2014), variations in the activity of enzymes responsible for the detoxification process demonstrate that the physiological system is capable of detecting these pollutants and identifying them as stressors agents, which must be excreted from the body.

GST has a key role in IMI elimination and its metabolites. IMI metabolism involves glucuronidation and methylation in the imidazole ring and glutathione (GSH) binding in chloropyridinyl groups. These processes lead to the formation of the metabolites *N*-acetylcysteine and *S*-methyl at the end of the detoxification process, which will be excreted by the body (Wang et al. 2018; Stara et al. 2019). However, in some cases, GST activity does not show significant changes or even displays lower activity values compared with the control group, as observed herein in the RW + IMI treatment. The decline or absence of GST activity in *D. rerio* also has been reported by Ge et al. (2015) even during a long exposure period to IMI concentrations that varied between 0.3 and 5.0 mg mL^{-1}.

The absence of GST activity is possibly associated with enzymes that failed to convert xenobiotics to adequate levels to activate GST during the initial stages of the detoxification process (Uguz et al. 2003). The decline of GST activity may be related to the excessive consumption of GSH as a substrate and the change in GST composition triggered by intermediate metabolites or associated with competitive inhibition between GST and its substrate (Egaas et al. 1999). Another possibility is that glutathione levels could decrease due to the excretion of its oxidized form during the exposure period to xenobiotic (DeLeve and Kaplowitz 1991).

Also, the GST activity can vary between the organs evaluated. Vieira et al. (2018) used the commercial product Nortox® to evaluate the changes in the enzymatic activity of *Prochilodus lineatus* at low IMI concentrations (1.25 to 1250.0 µg L^{-1}). After 5-d exposure, GST activity increased in the brain in concentrations from 125.0 µg L^{-1}. In contrast, a reduction in gills and kidneys was observed from 12.5 and 1250.0 µg L^{-1}, respectively. At low IMI concentrations, an analysis that considers organs separately brings more specific information about the effects of IMI in the physiological system of fish.

CAT and APX are enzymes that act as removing the toxic form of H_2O_2 by converting it into H_2O and O_2 molecules (Sellaththurai et al. 2019). The standard tests that used UW + IMI showed an increase in APX activity and a decrease in CAT activity (Fig. 3). Although CAT reduces H_2O_2 (Van der Oost et al. 2003), at high concentrations of superoxide anion and hydrogen peroxide, it can be inactivated (Lushchak et al. 2009; Semchyshyn and Lozinska 2012). Therefore, other peroxide detoxifying enzymes would need to be active, which increased APX activity. Inhibition of CAT has been reported by several studies evaluating the effects of different pesticides on fish, as in the present study (Coelho et al. 2011; Husak et al. 2014; Dantzger et al. 2018).

Although DW displayed the highest nutrient concentration and other parameters associated with the presence of high organic load (Table 1), the results of enzymatic activity demonstrated that the effect of IMI was significant only in samples without signs of pollution (RW and UW) (Fig. 3). The organisms could be subject to the effects of possible contaminants present in the collected water. Moreover, a synergic effect between pollutants and IMI could be observed increasing the toxicity in the tested organisms. However, we did not observe this synergism was not observed in the present study.

Some studies have demonstrated that IMI can interact with the sediment and nutrients, considered as contaminants, mitigating the impacts of this pesticide in macroinvertebrate communities (Alexander et al. 2016; Chará-Serna et al. 2019). That is, IMI appears to be more harmful to organisms that live in pristine and unpolluted water bodies could be more susceptive to suffer impacts from IMI. The pollutants can keep the detoxification system active, thus, when exposed to IMI, the organism can metabolize it more easily.

The use of biomarkers is important to obtain an integrative analysis of the impact of the pollutants on ecosystems. In the present study, we did not report acute toxic effects in *D. rerio* after exposure to IMI (Table 3), but we could observe changes in GST, CAT, and APX activities (Fig. 3), which indicate that the physiological system can be impaired. Physiological changes affect the behavior, health, and eating habits of individuals and, consequently, affecting the aquatic community.

Conclusions

The results obtained in the present study allow us to further understand the effects of IMI on different nontarget aquatic organisms from freshwater environments. We have simulated natural conditions using a lotic channels system to evaluate the effect of the highest IMI concentration detected in surface water. The mean value of IMI in the three CS assays was 287.60 ± 32.06 µg L^{-1}. The results

demonstrated that this concentration was able to affect the survival and the physiological balance of aquatic organisms. The simple system adopted in our studies allowed the evaluation of the IMI effects in a multispecific level using three different nontarget organisms simultaneously and offers a new way to assess the impacts in the aquatic community. Also, further experiments using this system can be developed without the use of capsules to promote greater ecological complexity and obtain data that standard tests cannot offer, such as interactions between the evaluated organisms.

The data obtained suggest that *Chironomus* sp. could suffer a populational decrease in the presence of the IMI concentration tested in the present study (320.0 µg L^{-1}) in natural conditions. Also, a high mortality rate could be observed even at low concentrations, if we consider the EC_{50} values for *Chironomus* sp. reported in the literature. Moreover, this genus can be used as a bioindicator in polluted areas.

In the present study, we did not observe the mortality in daphnids and fish exposed to IMI (320.0 µg L^{-1}). However, the oxidative stress analysis demonstrated that the tested IMI concentration caused physiological changes in *D. rerio*. The fish were more susceptible to oxidative stress in unpolluted environments.

The IMI concentration tested herein is high compared to most of the values detected in surface waters. However, considering losses pathways of the IMI in the aquatic environment, higher concentrations can be expected if we regard the proximity of the agricultural lands, rainfall events, and the variations of the persistence of the IMI in different compartments of the ecosystem. Besides that, we believe that the information obtained can support future experiments using a channels system to test lower concentrations more frequently detected in surface waters.

The channels system adopted in the present study along with the data obtained can contribute to the comprehension of the effects of IMI in different environmental conditions, supporting the monitoring, management, and establishment of future conservation policies about the use of IMI and similar active ingredients used as pesticides.

Author Contributions LGQ conceived the presented idea, designed and performed ecotoxicological experiments, created and built the indoor system of artificial channels, analyzed the data, elaborated tables, and figures and wrote the article; CCAP performed the activity enzymatic experiments and helped in the analysis of these results; ECA, FAD, and EP performed the chromatographic analysis and helped in the analysis of these results; FTS responsible for Laboratory of Ecotoxicology; TCBP supervised the project.

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Availability of Data and Material The raw data are stored in a data repository at the University of São Paulo and can be made available upon request to the author (lucasgoncalvesqueiroz@gmail.com).

Compliance with Ethical Standards

Conflict of interest The opinions presented in the present study are those of the authors. There was no financial support that could have influenced its outcome, and there are no known conflicts of interest related to this publication. All named authors approved the manuscript and the order of authors listed.

Ethics Approval Experimental procedures were previously approved by the Ethics Committee on Animals Use of the Biosciences Institute of the University of São Paulo (CEUA no. 310/2018).

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