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Fish inhabiting rice fields: Bioaccumulation, oxidative stress and neurotoxic effects after pesticides application

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ABSTRACT

The present study aimed to evaluate the biological effects triggered by the application of a current-use mixture of pesticides (the herbicide glyphosate, the insecticide bifenthrin, BF, and the fungicides azoxystrobin, AZ, and cyproconazole, CYP) on two native fish (Markiana nigripinnis and Astyanax lacustris) inhabiting a rice field. We analyzed pesticide residues in water, sediment and fish samples 21 days before and after a fumigation event. Additionally, organismic indices, oxidative stress and neurotoxicity biomarkers in fish at both sampling periods were compared. After fumigation, glyphosate, BF, AZ and CYP were detected in water and sediment samples, being also bioaccumulated in both fish species. A decreasing condition factor in A. lacustris and a higher liver somatic index in M. nigripinnis were observed as well. Overall, results showed that, after the application of the pesticide mixture, antioxidant mechanisms failed to prevent oxidative damage in the liver and gills of M. nigripinnis. Meanwhile, *A. lacustris* showed a different response: an inhibition of the antioxidant defenses without tissue lipid oxidative damage. Furthermore, acetylcholinesterase activity after spraying was significantly reduced in brain and muscle tissues of *A. lacustris* and in the brain of *M. nigripinnis*. Our results show that current-use pesticides, like glyphosate, BF, AZ and CYP, pose health risks on native fish populations inhabiting rice fields.

1. Introduction

Feeding a growing global population of approximately 8 billion people is a challenge for agriculture. Currently, rice is one of the most important crops all over the world, with approximately 161 million hectares cultivated worldwide (FAOSTAT, October 2019). Rice fields sustain human populations and also provide food and shelter for a diverse assemblage of species (Edirisinghe and Bambaradeniya, 2006; Stenert et al., 2009; Machado and Maltchik, 2010). Rice cultivation in a dry-wet cycle consists of dry seeding and later flooding, phase in which numerous aquatic organisms colonize the paddy fields (Fernando, 1993; Che Salmah et al., 2017).

In this type of production system, a wide range of pesticides is used to control pests and to improve yields. Herbicides constitute one of the most common pesticides used to treat the land prior to the rice planting stage (Liu et al., 2015). Meanwhile, as during the flowering stage of the cereal fungus and insects are major threats to rice crops; insecticides and fungicides are extensivily used being directly sprayed on the paddy. Consequently, insecticides are among the most persistent and frequently detected pollutants in water bodies close to rice fields (Elfman et al., 2011) and the marked increment in the application of fungicides had led to categorized them as contaminants of emerging concern (Elskus and Hackley, 2012). Since the supply of water for flooding and irrigation comes from surrounding water bodies, non-target aquatic organisms as fish enter the rice field inhabiting this particular ambient and coming into contact with these pesticides and their metabolites (Edirisinghe and Bambaradeniya, 2006; Nguyen et al., 2015). In this sense, fish possess a recognized value as bioindicators because they accumulate chemicals via contact with water, sediment or by ingestion of food (Clasen et al., 2018; Ernst et al., 2018; Arisekar et al., 2019). Besides, biological responses (biomarkers) in fish can serve as an early warning sign of potential ecological risks (Cazenave et al., 2009; Pérez et al., 2018).

Recently, the presence of pesticides was determined in fish collected in the Uruguay River with sampling sites bordering on rice paddy areas of Argentina, the regional leader in rice cultivation (INTA, 2016). It was found that fungicides cover half of the pesticides detected (Ernst et al., 2018). The highest occurrence rates were for the strobilurin family,

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while the triazole family was occasionally found although in high concentrations. In addition, herbicides and residues of pyrethroids have been detected in water and sediment samples from streams and rivers near crops in Argentina (Jergentz et al., 2005; Marino and Ronco, 2005; Ronco et al., 2016).

In particular, the herbicide glyphosate, the pyrethroid insecticide bifenthrin (BF) and the fungicides azoxystrobin (AZ) and cyproconazole (CYP) are among the pesticides commonly sprayed in paddy fields in Argentina. Several laboratory assays have studied the toxicity of these pesticides in fish. Exposure to glyphosate induced gill and hepatic inflammatory response and histological damage (Ma et al, 2019; Bonifacio and Hued, 2019), while genotoxic and neurotoxic effects have also been observed (Moreno et al, 2014; Sobjak et al, 2017). BF is of particular concern among synthetic pyrethroids because of its greatest contributions to aquatic toxicity, as it is extremely toxic to fish and aquatic invertebrates (Yang et al, 2018). Induction of osmotic imbalance, DNA damage (Paravani et al., 2019) and endocrine disruptive effects with negative reproductive consequences (Eni et al, 2019) have been described for this family of insecticide. Among the adverse effects of the strobilurin fungicide AZ, endocrine disruption, altered immune system (Jiang et al., 2018) and reproductive toxicity (Cao et al., 2016) were described. Additionally, it has been reported as a ROS inducer in fish since strobilurin family compounds disturb the transfer of electrons in mitochondrial respiratory chain (Olsvik et al., 2010; Han et al., 2016). Finally, although scarce information is available about CYP toxicity in fish (Muncke et al., 2007), it is known that propiconazole, also member of the triazole family compounds as CYP, produces oxidative stress and induces hepatic carcinogenic effects (Li et al., 2010; Tu et al., 2016).

Even though some studies in rice fields ecosystems analyzed the toxic effects of pesticides in fish, they were mostly focused on the exposure to certain pesticide formulations applied in rice crop, as well as on only a few biological responses. Wijeyaratne & Pathiratne (2006) found neurotoxic effects and histological alterations in gills of a Sri Lanka native fish (*Rasbora caverii*) collected in a rice field where herbicide paraquat and insecticides fenthion and phentoate were sprayed. Similarly, an inhibition of acetylcholinesterase (AChE) activity along with oxidative stress was observed on carp exposed *in situ* to herbicide penoxulam (Cattaneo et al., 2011a) and fungicide tebuconazole (Toni et al., 2011). Even more, Tam et al. (2018) demonstrated the neurotoxic effect of two insecticide formulations (chlorpyrifos and fenobucarb) on climbing perch (*Anabas testudineus*). However, no information is available about the effects of the mixture glyphosate, BF, AZ and CYP on fish health in field studies. On the other hand, most of them used fish reared under controlled conditions and then exposed to pesticides in paddy fields, while studies using fish inhabiting rice field are scarce (Teng et al., 2013; Zhang et al., 2016). The use of *in-situ* assays with wild animals increase the ecological realism as involves genetic diversity of wild populations together with realistic exposures to pesticides mixtures.

The present study aimed to evaluate biological responses triggered by the application of a current-use mixture of pesticides (the herbicide glyphosate, the insecticide bifenthrin and the fungicides azoxystrobin and cyproconazole) on two native fish (*Markiana nigripinnis* and *Astyanax lacustris*) inhabiting a rice field. For that, pesticide residues in water, sediment and fish samples were analyzed, as well as, organismic indices, oxidative stress and neurotoxicity biomarkers on both fish species. This information could be helpful for estimating the potential health risk of realistic pesticide exposure for natural fish populations inhabiting rice fields.

2. Materials and methods

2.1. Field sampling

The study took place in a rice field of Santa Fe province, Argentina (30°21′12.8″S 60°04′20.5″W). Previous to rice sowing season (October-November 2017) there was a stage of land preparation when the herbicide glyphosate (Roundup®) was sprayed. In order to flood and irrigate field, water was pumped from the San Javier River, an anabranch of the Paraná River. In January 2018, during the flowering stage of the cereal, 20 cm^3/ha of the insecticide bifenthrin (BF) (Green Star®) and 400 cm^3/ha of a mixture formulation of fungicides azoxystrobin (AZ) and cyproconazole (CYP) (Azoxy Pro®) were applicated by aerial spray.

Sampling of water, sediment and fish was carried out 21 days before (December 2017) and 21 days after (February 2018) the application of the insecticide and fungicides. Water samples (n = 3) were collected in brown glass bottles from different locations within the rice field. They were kept, together with the sediment samples (n = 3) collected in plastic containers at the same places, on ice until storage in a −80 °C freezer. Additionally, sediment and water samples (n = 3 each) from the water stream used for flooding and irrigation were also collected and stored as previously described. Fish assemblage was previously studied and two of the most abundant and representative species were selected. Fifteen individuals of *Markiana nigripinnis* and 15 individuals of *Astyanax lacustris* were collected, at each sampling period, with seine and dip nets. Body weight (g; BW) and total length (cm; L) were recorded. Fish were killed by medullary section. Ten individuals per species were processed *in situ* and tissues (brain, gills, liver and muscle) were dissected for biomarker analysis. Liver weight (g; LW) was registered for each individual. Condition factor (CF) was calculated as $CF = BW/L^3 * 100$ and liver somatic somatic index (LSI) was calculated as: $LSI = LW/BW * 100$ (Goede and Barton, 1990). The rest ones (n = 5), because of their small size, were preserved whole for bioaccumulation analysis. Tissue and fish samples were immediately frozen in liquid nitrogen until their storage in a −80 °C freezer. This study was conducted in accordance with national and institutional guidelines for the protection of animal welfare (CONICET, 2005).

2.2. Water quality and pesticide analysis

Physicochemical parameters as dissolved oxygen, temperature, pH and conductivity were measured *in situ*, using a multiparameter water quality meter (HACH HQ40d). Hardness, nitrate and phosphorus were measured in laboratory according to APHA (1998). Because water intake for flooding and irrigation came from surrounding water bodies, a total of 125 pesticides were searched in water, sediments and fish samples (see Supplementary Table) in order to perform a screening of all pesticides potentially present. For pesticide analysis, water samples were subjected to a preparation and cleaning process by solid phase extraction, based on the method described by Min et al. (2008), while pesticides from sediments and fish samples were extracted following the QuEChERS (quick, easy, cheap, effective, rugged, and safe) method (Anastassiades et al., 2003). The addiction of herbicide glyphosate plus its metabolite aminomethylphosphonic acid (AMPA) was carried out in a single method determination as described by Demonte et al. (2018). The polar and moderately polar pesticide residues were analyzed by an Ultra Performance Liquid Chromatograph coupled to an ionization source by electrospray and triple quadrupole mass spectrometer (UHPLC-ESI-MS/MS). The analysis of nonpolar compounds was carried out using a Gas Chromatograph coupled to a triple quadrupole mass spectrometer with ionization by Electronic Impact (GC-EI-MS/MS). The methodologies in all cases were fully validated according with the European Commission guidance document on analytical quality control and method validation procedure for pesticide residues, SANTE/2017/11813.

2.3. Oxidative stress

Oxidative stress was assessed by both antioxidant defenses and oxidative damage to lipids. The antioxidant defenses were evaluated by measuring the enzyme activity of glutathione S-transferase (GST), catalase (CAT) and superoxide dismutase (SOD) in liver, brain, gills and muscle tissues. Briefly, tissues were homogenized in an ice-cold 0.1 M sodium phosphate buffer, pH 6.5 containing 20% (v/v) glycerol, 1 mM EDTA and 1.4 mM dithioerythritol (Bacchetta et al., 2014). Homogenates were centrifuged at 20,000 × g (4 ∘C) for 30 min and the supernatant (enzyme extract) was collected and stored at −80 ∘C until spectrophotometric enzyme activity measurement. The activity of GST was determined using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate (Habig et al., 1974). CAT activity was determined according to Beutler (1982) while SOD activity was determined by its ability to inhibit the epinephrine autoxidation (Misra & Fridovich, 1972). Each enzymatic assay was carried out in triplicate. Oxidative damage to lipids was assessed, in the same mentioned tissues, through the malondialdehyde (MDA) reaction with 2-thio-barbituric acid (TBA) (Yagi, 1976). Thiobarbituric reactive substances (TBARS) levels were expressed as nanomoles of MDA formed per hour per milligram of proteins (nmol TBARS mg $prot^{-1}$). Enzymatic activities and TBARS levels were calculated in terms of the protein content according to Bradford (1976) using serum bovine albumin as standard.

2.4. Acetylcholinesterase activity

As a neurotoxic biomarker, the acethylcholinesterase (AChE) activity was measured in brain and muscle according to Ellman et al. (1961) adapted to a microplate reader. Briefly, each well contained 200 µL of color reagent 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) 0.25 mM in 50 mM phosphate buffer pH 7.7. The reaction started by addition of 10 µL of S-butyrylthiocholine iodide. Samples were read by using a microplate photometer Biotek Synergy HTK and the variation in optical density was recorded at 405 nm at 25 °C for 1 min.

2.5. Data analysis

All data were expressed as mean ± standard error. T- Student test or Mann-Whitney *U* test, depending on the data, was applied for comparisons between means of treatments. Shapiro-Wilks and Levene tests were used to assess normality and homogeneity of variances, respectively. Statistical analyzes were performed using InfoStat (InfoStat 2018). The criterion for significance was $p < 0.05$.

3. Results

3.1. Physicochemical water quality and pesticide analysis

Physical and chemical parameters of the water collected in the rice field at both sampling periods are presented in Table 1. No statistical differences were observed between the different parameters measured at the two sampling periods. Table 2 shows the concentration of the pesticides detected in water and sediment samples collected in the rice field. Only glyphosate and its metabolite AMPA were detected above the limit of quantification in water and sediment samples collected before the application of the insecticide BF and the fungicides AZ and CYP. Twenty one days after the application of these pesticides, both fungicides were still detected and quantified in water as well as in sediment samples. The insecticide BF was detected below its limit of quantification only in sediment while it was not detected in water samples. Again, the herbicide glyphosate and AMPA were quantified in both, water and sediment samples.

Table 1
Physical and chemical parameters of water measured before and after a fumigation event in a rice field.

	Before	After
Dissolved Oxygen (mg L^{-1})	2.22 ± 0.64	2.24 ± 0.75
Temperature (°C)	27.6 ± 2.7	25.9 ± 2.0
pH	6.7 ± 0.1	6.9 ± 0.2
Conductivity (µS cm^{-1})	248.0 ± 37.6	236.6 ± 35.0
Hardness (mg $CaCO_3$ L^{-1})	1.20 ± 0.56	1.95 ± 0.83
Phosphorus (mg L^{-1})	0.58 ± 0.18	0.57 ± 0.13

Values are mean ± standard error.

Table 2
Pesticide concentration in water (µg·L^{-1}) and sediment (µg·kg^{-1}) samples collected in a rice field before and after a fumigation event.

	Before	After
Water		
Azoxystrobin	n.d.	3,1 ± 2,0
Cyproconazole	n.d.	1,2 ± 0,6
Bifenthrin	n.d.	n.d.
Glyphosate	2,1 ± 1,3	5,2 ± 3,2
AMPA	11,4 ± 5,1	14,3 ± 7,2
Sediment		
Azoxystrobin	n.d.	8,1 ± 4,1
Cyproconazole	n.d.	2,2 ± 1,4
Bifenthrin	n.d.	< LOQ
Glyphosate	70,2 ± 21,1	56,3 ± 18,1
AMPA	91,3 ± 27,2	68,4 ± 21,2

Mean ± standard error; n.d. = not detected; < LOQ = limit of quantification. LOQ water: 0.1 µg·L^{-1} azoxystrobin, cyproconazole and bifenthrin; 0.6 µg·L^{-1} glyphosate and AMPA.
LOQ sediment: 1 µg·kg^{-1} azoxystrobin; 0.5 µg·kg^{-1} cyproconazole; 5 µg·kg^{-1} bifenthrin; 2 µg·kg^{-1} glyphosate and AMPA. The complete list of pesticides residues analyzed (n = 125) are presented in Supplementary Table.

The pesticide analysis of samples collected at the water stream used for flooding and irrigation of the rice field revealed that only AMPA was present in water (0,8 ± 0,4 µg/L) and only glyphosate was detected in sediment (7,1 ± 4,3 µg/kg).

3.2. Pesticide bioaccumulation

Pesticide bioaccumulation in fish at both sampling periods is shown Table 3. Before the insecticide and fungicides application, only one individual of *M. nigripinnis* and one individual of *A. lacustris* showed evidences of glyphosate bioaccumulation. In addition, one individual of *M. nigripinnis* showed accumulation of the glyphosate metabolite, AMPA. None of the other pesticides tested were detected in fish at this sampling period, neither the fungicides AZ and CYP nor the insecticide BF. After the insecticide and fungicides application, all specimens of *M. nigripinnis* tested showed evidences of bioaccumulation of AZ, CYP and

Table 3
Pesticide bioaccumulation in fish (ng g^{-1}) collected in a rice field before and after a fumigation event.

	Before	After
Markiana nigripinnis		
Azoxystrobin	n.d.	4.1–6.3 (100%)
Cyproconazole	n.d.	1.1–3.0 (100%)
Bifenthrin	n.d.	5.0–10.1 (100%)
Glyphosate	< LOD- 15.1 (20%)	< LOD-55.2 (20%)
AMPA	< LOD-163.9 (20%)	177.0–197.1 (40%)
Astyanax lacustris		
Azoxystrobin	n.d.	1.4–2.0 (80%)
Cyproconazole	n.d.	0.5–1.4 (40%)
Bifenthrin	n.d.	12.0–86.0 (100%)
Glyphosate	< LOD-92.3 (20%)	< LOD-49.9 (20%)
AMPA	n.d.	n.d.

Concentrations quantified are expressed as values range (minimum and maximum) for the compounds detected plus the frequency of occurrence in parenthesis. n.d. = not detected; LOD (limit of detection); LOQ (limit of quantification): 1 ng.g^{-1} azoxystrobin; 0.5 ng.g^{-1} cyproconazole; 5 ng.g^{-1} bifenthrin; 10 ng.g^{-1} glyphosate and AMPA. The complete list of pesticides residues analyzed (n = 125) are presented in Supplementary Table.

Table 4
Biometric parameters and organismic indices for *Markiana nigripinnis* and *Astyanax lacustris* before and after a fumigation event in a rice field.

	Before	After
Markiana nigripinnis		
Body weight (g)	4.81 ± 1.31	6.54 ± 2.61
Total length (cm)	7.06 ± 0.79	7.76 ± 0.84
Liver weight (mg)	46 ± 19	109 ± 41*
Condition factor	1.32 ± 0.26	1.41 ± 0.12
Liver somatic index	1.03 ± 0.13	1.73 ± 0.41*
Astyanax lacustris		
Body weight (g)	1.05 ± 0.25	2.83 ± 0.84*
Total length (cm)	4.02 ± 0.56	5.88 ± 0.66*
Liver weight (mg)	48 ± 16	68 ± 7*
Condition factor	1.62 ± 0.11	1.33 ± 0.10*
Liver somatic index	3.15 ± 0.31	2.67 ± 0.54

Values are expressed as means ± SE. * indicates $p \leq 0.05$ before vs after the insecticide and fungicides application.

BF. Glyphosate accumulated in one individual and its metabolite AMPA was detected and quantified in two specimens. Regarding the fish species *A. lacustris*, AZ residues occurred in 4 individuals, CYP in 2 fish, and BF showed evidences of bioaccumulation in all individuals tested. Herbicide glyphosate was detected and quantified only in one individual while its metabolite, AMPA, was not detected in *A. lacustris*. No other pesticides than the target ones were detected in fish samples.

3.3. Fish biomarkers

3.3.1. Biometric parameters and organismic indices

Table 4 shows biometric parameters and organismic indices for *M. nigripinnis* and *A. lacustris* before and after the application of BF and the mixture formulation of AZ and CYP in the rice field. *M. nigripinnis* increased LW and LSI while *A. lacustris* showed increased BW, TL, LW and lower CF after the fumigation event.

3.3.2. Oxidative stress

Antioxidant enzyme activities and TBARS levels in the different fish tissues are shown in Fig. 1 (*M. nigripinnis*) and Fig. 2 (*A. lacustris*). The antioxidant defenses were not activated in *M. nigripinnis* and even a decreased CAT activity in brain and gills was observed ($p = 0.033$ and $p = 0.0159$, respectively). Lipid oxidative damage in liver and gills ($p = 0.004$ and $p = 0.016$, respectively) occurred after the application of BF and the fungicides AZ and CYP; as increased TBARS levels were quantified in these tissues (Fig. 1). Meanwhile, the application of the insecticide and fungicides caused inhibition of the antioxidant defenses in most tissues of *A. lacustris*: SOD activity decreased in liver ($p = 0.0047$), CAT and GST activity were lower in muscle tissue ($p = 0.016$ and $p = 0.044$ respectively) and an inhibition of all enzymes activities tested was observed in brain (CAT, $p = 0.0098$; GST, $p = 0.008$; SOD, $p = 0.022$). Only in gills an increased GST activity ($p = 0.046$) was observed. The application of the insecticide and fungicides did not cause lipid oxidative damage in any of the tissues tested for *A. lacustris*, and even lower TBARS levels were observed in liver ($p = 0.030$) and brain ($p = 0.016$) (Fig. 2).

3.3.3. Acetylcholinesterase activity

Fig. 3 shows AChE activity in muscle and brain tissues of *M. nigripinnis* and *A. lacustris*. After the fumigation event, both fish species showed inhibition of brain AChE activity ($p = 0.006$ for *A. lacustris* and $p = 0.0043$ for *M. nigripinnis*), while only *A. lacustris* showed lower muscular AChE activity ($p = 0.0159$) when compared to organisms sampled before the mixture of pesticides was applied.

4. Discussion

There is an increasing concern throughout the world over the problems of environmental contamination by pesticides which affect non-target organisms such as fish. The impact of pesticides used in rice cultivation on wild fish species has been scarcely studied (Wijeyaratne and Pathiratne, 2006; Teng et al., 2013; Zhang et al., 2016). The present study assessed, for the first time, the biological responses tiggered by the application of glyphosate, BF, AZ and CYP, of two native fish species inhabiting a rice field. Clearly, our results showed that this current-used mixture of pesticides poses a risk for fish health. These compounds were bioaccumulated, provoked oxidative stress and neurotoxic effects in *M. nigripinnis* and *A. lacustris*.

4.1. Physicochemical water quality and pesticides analysis

No significant differences were observed regarding physicochemical water quality between sampling periods. Meanwhile, pesticides analysis revealed the presence of the applied pesticides in the rice field. The herbicide glyphosate and its metabolite were present in water and sediment samples at both sampling periods. This is consistent with the fact that this herbicide was sprayed at the stage of land preparation. In addition, the presence of the herbicide in the rice field could have also been due its input from the river already contaminated. Ronco et al. (2016) revealed high levels of glyphosate and AMPA in the middle- and lower-course Paraná River tributaries in accordance with the intensive agricultural activity in those regions. Hence, this fact could have contributed to the presence of the herbicide in all collected samples, as a tributary of the Paraná River was used for the rice field flooding and maintenance of water level. Even though, based on the guidelines recommended for the protection of the aquatic biota (SRHN, 2004), the water concentration of glyphosate was below the maximum acceptable level (0.24 mg/L). On the other hand, the fate of compounds in environment compartments is recognized to depend on the physicochemical properties which in turn impact its degradation and transformation (Singh et al., 2013). Degenhardt et al. (2012) have observed in wetland environments that degradation and sorption are the main pathways for the dissipation of glyphosate from water. Our findings demonstrate a similar pattern in the partitioning of glyphosate and AMPA, as their levels were higher in sediment than in water samples collected from the same site.

BF is lipophilic and therefore insoluble in water (solubility in water at 20 °C: 0.001 mg L^{-1}). This physicochemical characteristic is consistent with our results in water samples, where it was not detected. This synthetic pyrethroid pesticide tends to be stable and persist in aquatic sediment with a long half-life (8–17 months) (Gan et al., 2005). Our results showed there were no remains of the insecticide from the previous rice productive cycle since it was only detected in sediment samples collected after the latest application of pesticides on the rice field. Meanwhile, fungicides AZ and CYP have a semi-polar nature, with solubility in water at 20 °C of 6.7 and 93 mg L^{-1} respectively. Additionally, AZ is classified as moderately persistent in soil (half-life: 78 days) while CYP is classified as persistent (half-life: 142 days) (IUPAC Footprint, 2017). The time period between the latest application of pesticides and the one studied in the present study was 365 days. Hence, the presence of both fungicides was expected only in samples collected after the fumigation event studied in the present study.

4.2. Bioaccumulation

Bioaccumulation is related with an indirect measurement of lipid solubility, the logarithmic octanol–water partition coefficient (log Kow), and with the environmental persistence of compounds (Ernst et al., 2018). Pesticides have a high potential to accumulate on aquatic biota if log Kow is greater than 3 and a soil half-life greater than 30 days (Andreu and Picó, 2012). Among the pesticides sprayed in the rice field

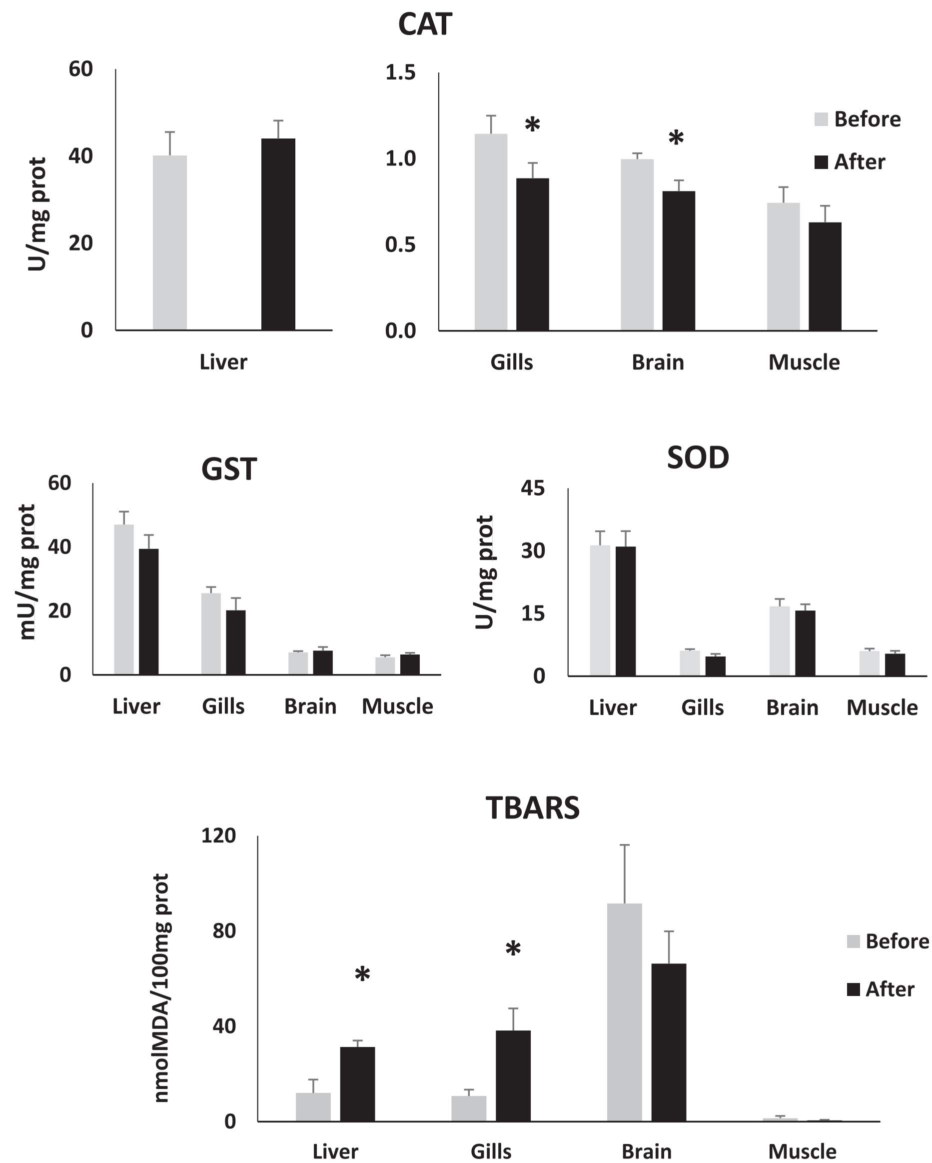

Fig. 1. Enzymatic activity (CAT, GST and SOD) and TBARS level in *M. nigripinnis* collected in a rice field before and after a fumigation event. Enzyme activities are expressed as mU mg $prot^{-1}$ (GST) or U mg $prot^{-1}$ (CAT, SOD). Bars represent the mean ± standard error. * indicates $p \leq 0.05$ before vs after the insecticide and fungicides application.

during the present study, the insecticide BF has the highest potential of being accumulated in fish as it is stable, highly persistent (half-life: 8–17 months) and lipophilic (log Kow = 6.6). It is followed by CYP (half-life: 142 days, log Kow = 3.1), AZ (half-life: 78 days, log Kow = 2.5) and glyphosate (half-life: 15 days, log Kow = -3.2) (IUPAC Footprint, 2017). Our results showed that the insecticide BF was bioaccumulated in both fish species with a 100% frequency of detection. It was followed by the fungicides AZ and CYP (90% and 70% frequency of detection respectively), while glyphosate and its metabolite AMPA showed the lowest frequency of detection (40%). As BF and the fungicides AZ and CYP were not detected in fishes collected before their application, these results indicate that the presence of pesticides in fish is due to the recent inputs of these compounds to the rice field environment. Although BF was detected below its limit of quantification within sediments samples, it was present in all analyzed fish. This high prevalence shows the readily bioavailable nature of this compound. Besides, bioaccumulation is also influenced by the ability of metabolization and elimination of compounds (Pérez-Parada et al., 2018). Pyrethroids detoxification involves a cytochrome P450s-mediated oxidative reaction followed by a hydrolytic reaction catalyzed by a carboxylesterase (Yang et al., 2018). Because aquatic species lack of carboxylesterase enzyme; metabolization and elimination of pyrethroids in fish are reduced. On this regard, accumulation of BF in zebrafish embryos was even much more rapid than other pyrethroids and with a quite slow depuration (Tu et al., 2014), as tissue concentration of BF in bluegills was only half-reduced 42 days after the exposure (Surprenant 1986).

On the other hand, there is few data regarding uptake and elimination rate for the fungicides from the strobirulin and triazole families in fish. Zhu et al. (2015) studied these routes for trifloxystrobin (a strobirulin as AZ) in *Gobiocypris rarus* embryos. The authors observed a progressive accumulation with no evidence of elimination until 6 days of exposure in water at sub μg L^{-1} levels. For their part, Konwich *et al.* (2006) observed that juvenile rainbow trout exposed to a mixture of nine triazole fungicides, including CYP, accumulated all of them rapidly during 8 day followed by a 95% elimination that ranged from 4.5 to 11.0 days. On the other hand, fish concentrate pollutants directly from water and diet, though their feeding behaviors also influence the profile

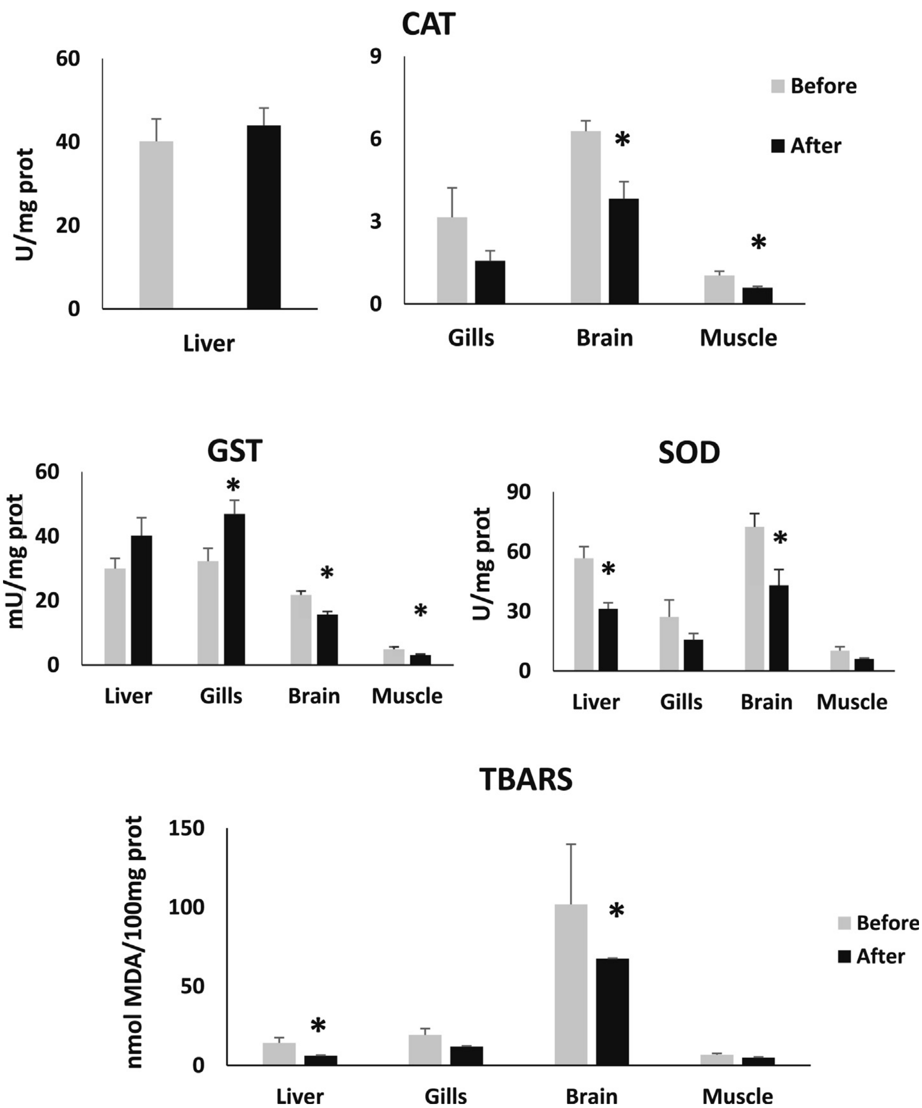

Fig. 2. Enzymatic activity (CAT, GST and SOD) and TBARS level in *A. lacustris* collected in a rice field before and after a fumigation event. Enzyme activities are expressed as mU mg prot^{-1} (GST) or U mg prot^{-1} (CAT, SOD). Bars represent the mean ± standard error. * indicates p ≤ 0.05 before vs after the insecticide and fungicides application.

of pesticides accumulated. In this sense, the presence of the herbicide glyphosate in *M. nigripinnis* and *A. lacustris* is consistent with their herbivorous feeding habits (Carrasco-Letelier et al., 2006), as accumulation of glyphosate in aquatic macrophytes has been described (Dey et al., 2016).

Rice field experiments evaluating bioaccumulation of pesticides in fish are scarce. The loach species *Misgurnus mohoity* and *Paramisgurnus dabryanus* living in paddy fields were found to have a high potential for persistent organic pollutants accumulation (as organochlorine pesticide and polychlorinated biphenyls) (Teng et al., 2013; Zhang et al., 2016) while the pyrethroid lambda-cyhalothrin and the fungicide tebuconazole were bioacumulated in *Cyprinus carpio* reared in an integrated rice–fish farming system (Clasen et al., 2018). Finally, glyphosate sprayed in crop plots accumulated in three omnivorous freshwater teleostean fishes (Dey et al., 2016).

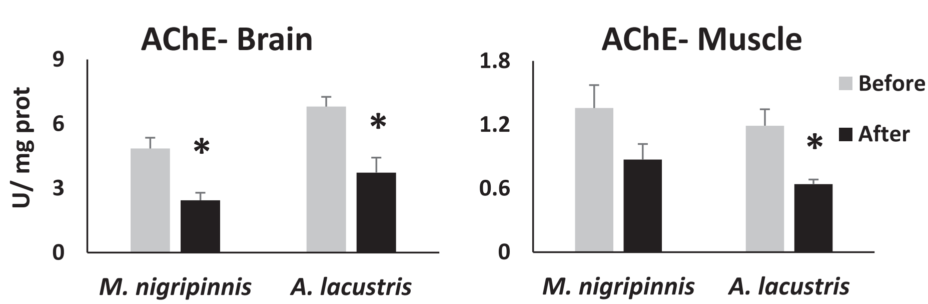

Fig. 3. Acetylcholinesterase (AChE) activity in muscle and brain of *M. nigripinnis* and *A. lacustris* collected in a rice field before and after a fumigation event. Enzyme activity is expressed as U mg prot^{-1}. Bars represent the mean ± standard error. * indicates p ≤ 0.05 before vs after the insecticide and fungicides application.

4.3. Biometric parameters and organismic indices

Our results showed a decreasing CF in *A. lacustris* and a higher LSI in *M. nigripinnis* after the fumigation. CF is used as an indicator of energy allocation and fish fitness, as energy reserves are related to growth, survival and reproduction among other processes of ecological relevance (Colin et al., 2016). Several factors may cause variation in CF values such as water quality and food availability. In line with our results, increased LSI has been observed in fish exposed to pesticides (Bacchetta et al., 2014; Samanta et al., 2018). The increments in liver weight could be due to alterations in the metabolic activity of the liver, consistent with its detoxification function (Adams, 1999; Campbell et al., 2003). Even though other factors could have also influenced these responses (Goede & Barton 1990), the present results suggest a link with the insecticide and fungicides application, as fish worsened their condition after the fumigation event.

4.4. Oxidative stress

Oxidative stress represents a possible mechanism underlying the adverse effects of pesticides in fish (Slaninova et al., 2009). Antioxidant mechanisms failed to prevent oxidative damage in *M. nigripinnis* after the fumigation event in the rice field. Oxidative damage to lipids significantly increased in liver tissue and gills while no significant changes in the enzyme activities tested were observed.

On the other hand, *A. lacustris* showed a different response, with a reduction in liver TBARS levels even though no significant enzymatic activation was observed. On the other hand, a lower hepatic SOD activity was detected after insecticide and fungicides application. Other non-tested antioxidant mechanism could have been operating in liver. For instances, damaged lipids in tissues can be converted to harmless products by degradation mechanisms, involving several enzymes as peroxidases and phospholipases (Kohen and Nyska, 2002). This mechanism could ultimately lead to the observed decreased TBARS levels. Moreover, the induction of GST activity in gills of exposed *A. lacustris* might indicate that phase II conjugation reactions were implicated, not only in the metabolism of pesticides but also of endogenous aldehyde products of lipid peroxidation, as some GST isoforms are highly reactive toward lipid peroxides (Regoli et al., 2011). This detoxifying action could have contributed to prevent lipid peroxidation in gills. Kubrak et al. (2012) and Husak et al. (2017) found similar results in studies with goldfish exposed to fungicides formulations, indicating the importance of this biotransformation pathway for pesticide detoxification in gills. The changes observed in liver and gills are consistent with the already known characteristics of these organs. Liver is a well-recognized target tissue of water pollution due to its central role in metabolism and biotransformation of pesticides (Van der Oost et al., 2003). Meanwhile, gills are the primary site for the absorption of pesticides since filaments and lamellae create a large surface area for direct contact with pollutants in water (Velmurugan et al., 2007).

The enzymatic defense system, tested in brain and muscle tissues, was down regulated in both fish species after the application of BF and the fungicides, although no changes in TBARS level were observed. *M. nigripinnis* only showed lower brain CAT activity and *A. lacustris* evidenced the greater antioxidant enzyme inhibition in these tissues: CAT, SOD and GST activities decreased in brain while CAT and GST activities decreased in muscle tissue. Brain counts with a comparatively low antioxidant defense system, in which the glutathione-dependent system is of great importance to counteract reactive oxygen species (ROS) production (Tabassum et al., 2016). Possibly, other components of this system (glutathione reductase, glutathione peroxidase) that were not evaluated in the present study, could have contributed to maintain the oxidative balance and avoid the oxidative damage to lipids. The lower GST activity observed in brain and muscle could have been due to the depletion of reduced glutathione and/or a disruption of its synthesis (Lu, 2009). Alternatively, an irreversible loss of activity because of covalent modification of the enzyme could have caused the inhibition (Cazenave et al., 2006). Finally, fluctuations of superoxide radicals could have caused the inhibition of CAT activity in these tissues (Wilhelm, 2007; Bagnyukova et al., 2006).

These results are consistent with the presence of pesticides in fish samples. Strobirulin family compounds as AZ produce oxidative stress in fish as they disturb the transfer of electrons in mitochondrial respiratory chain, enhancing ROS production (Olsvik et al., 2010; Han et al., 2016; Jiang et al., 2018). For their part, members of the triazole family of fungicides as CYP (Li et al., 2010; Toni et al., 2011; Zhu et al., 2014; Tu et al., 2016; Jia et al., 2018) and synthetic pyrethroids as BF (Jin et al., 2011; Paravani et al., 2019), were demonstrated to enhanced ROS generation and to cause altered antioxidant enzyme activities and transcription of genes related to oxidative stress. Additionally, herbicides as glyphosate may also exert their biological activity via induction of oxidative stress (Lushchak, 2011). However, among studies involving exposure of fish to single compounds, there are distinct and differential oxidative stress biomarkers responses, related with specific mechanisms of detoxification and antioxidant defense system of each tissue and fish species. AZ produced oxidative stress and altered antioxidant enzyme defense in Atlantic salmon (Olsvik et al., 2010), grass carp (Liu et al., 2013) and zebrafish (Han et al., 2016) (Jiang et al., 2018). For its part, BF induced the transcription of genes related to oxidative stress in exposed zebrafish embryos (Jin et al., 2013) and increased reactive oxygen species, lipid peroxidation, and the activities of antioxidant enzymes in different tissues of silver carp (Ullah et al., 2019a). Finally, glyphosate exposition caused transcriptional changes, oxidative stress and/or alteration of antioxidant defense in several fish species (Uren Webster and Santos, 2015; Lushchak et al., 2009; Glusczak et al., 2006; Glusczak et al., 2007; Cattaneo et al., 2011b; Guilherme et al., 2012; Modesto and Martinez, 2010; Dey et al., 2016). As far as we know, no studies concerning CYP impact on oxidative damage and antioxidant defenses in fish are available.

Scarce information is available about oxidative stress biomarkers in fish simultaneously exposed *in situ* to different family compounds. *Cyprinus carpio* reared in rice field and exposed to a mixture of insecticides (including the pyrethroid lambda-cyhalothrin) and fungicides (tebuconazole + trifloxystrobin), showed increased levels of TBARS and GST activity in gills, muscle and liver (Clasen et al., 2018).

4.5. Acetylcholinesterase activity

After spraying BF, AZ and CYP in the rice field, AChE activity was significantly reduced in brain and muscle tissues of *A. lacustris* and in the brain of *M. nigripinnis*. The observed enzyme inhibition could be due to the presence of the pesticides in both fish species. Although AChE is considered a specific neurotoxic biomarker of organophosphorus and carbamates exposition, several studies also describe an alteration of its activity by other pesticides in fish (de la Torre et al., 2002; Ren et al., 2016; Rodríguez-Fuentes et al., 2015), including fungicides from the triazole family (Tabassum et al., 2016; Altenhofen et al., 2017), synthetic pyrethroids (Singh et al., 2018; Yang et al., 2018; Ullah et al., 2019b; Legradi et al., 2018) and glyphosate (Sandrini et al., 2013; Cattaneo et al., 2011b; Glusczak et al., 2006, 2007). In particular, the presence of aromatic amino acids in the active site of AChE creates a hydrophobic region. Because of the lipophilic characteristic of pesticides from the pyrethroid family, they may interact with the active site causing inhibition of the enzymatic activity (Colović et al., 2013).

Similar to our results, *C. carpio* cultured in integrated rice–fish farming systems where several pesticides (including pyrethroid, strobirulin and triazole compounds) were applicated, showed lower brain AChE activity (Clasen et al., 2018). By contrast, the same fish species only exposed to tebuconazole for 7 days in a paddy field, showed increased brain AChE activity (Toni et al., 2011). These results reinforce the importance of evaluating pesticide in mixture in order to know organism's responses under realistic exposure scenarios.

Finally, the neurotoxic effects of these compounds on fish could lead to disturbed swimming behaviors (Liu et al., 2013; Zhu et al., 2015; Sani and Idris, 2006; Bridi et al., 2017) and feeding capacity; which consequently affect growth, survival and reproductive success (Werner and Moran, 2008). It is interesting to highlight that *A. lacustris* seems to have a greater sensitivity to the mixture of pesticides applicated in the rice field, although the frequency of occurrence of BF and fungicides was lower than in *M. nigripinnis*. Because of the important ecological role in the food web of *A. lacustris* and *M. nigripinnis*, as they represent an important food resource to piscivorous fish (Godoy 1975), these aspects are of great concern.

Our results, with ecological realism, represent a substantial contribution to the knowledge of biological effects of current-use rice field mixture of pesticide in native fish.

5. Conclusions

The present study adds to a growing body of evidence which highlights the environmental impact of pesticides on native fish population. Current-use rice field pesticides, as glyphosate, BF, AZ and CYP, are indeed being accumulated in fish and impacting their physiology, biochemistry and consequently their health. Our work reflects the threat to wild fish residing paddy field associated to water bodies.

Because the integration of fish rearing into rice farming provides a simultaneous and unique source of animal and vegetal protein, irrigated rice fields have a great potential to expand the pisciculture in rice producing countries (Frei et al., 2007). This aspect provides further evidence of the importance of our results, as fish bioaccumulation of this mixture of pesticides may also result in a potential risk to human health through food chain. More studies under real exposure scenarios like the present one are needed to fully understand the eco-toxicological effects, mechanisms and risks of using pesticide mixtures.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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