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The problem with implementing fish farms in agricultural regions: A trial in a pampean pond highlights potential risks to both human and fish health

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HIGHLIGHTS

- Atrazine, Glyphosate and AMPA were detected in the water of the pond.
- Atrazine and malathion were detected in fish muscle.
- Exposure to pesticides caused DNA damage in the fish.
- Exposure to pesticides inhibited GST activity in the liver.
- Exposure to pesticides induced cholinesterase activity in both liver and plasma.

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ABSTRACT

The safety of creating fish farms in agricultural settings was evaluated by growing *Piaractus mesopotamicus* in a pond, while crops where cultivated in a nearby field under a pesticide application regime typical of the Pampa region. Atrazine, glyphosate and its metabolite, aminomethylphosphonic acid (AMPA), were detected in the water of the pond at concentrations ranging between 92 and 118 μg/L for atrazine, 12 and 221 μg/L for glyphosate and 21 and 117 μg/L for AMPA. Atrazine and malathion were detected in fish muscles at concentrations ranging between 70 and 105 μg/kg for atrazine and 8.6 and 23.7 μg/kg for malathion. Compared to fish raised in a pisciculture, fish from the agricultural pond presented reduced values of pack cell volume, hemoglobin, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration, together with significantly greater cholinesterase activity in both plasma and liver and reduced glutathione-S-transferase activity in the liver. A comet assay also demonstrated that *P. mesopotamicus* from the agricultural pond presented a significantly greater level of DNA damage in both erythrocytes and gill cells. Overall, the present study demonstrates that pisciculture ponds established in an agricultural setting may receive pesticides applied to nearby cultures and that these pesticides may be taken up by the fish and affect their physiology and health. The accumulation of

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pesticides residues in fish flesh may also present a risk to human consumers and should be closely controlled.

1. Introduction

The South American pacú (*Piaractus mesopotamicus*) is a ray-finned freshwater fish native from the "La Plata" river basin, which comprises rivers from Paraguay, Brazil, Uruguay and Argentina. Captured for sport and commercial fishing for many years, the species has now practically disappeared from the La Plata River and the lower Parana and Uruguay Rivers (Quiros, 1990). Overfishing, contamination and water flow regulation through dams are believed to have caused its disappearance (Wicki et al., 2008, 2009). The notable diminution in the number of captures achieved by fisheries was compensated in Argentina by a sustained increase in the production of cultivated individuals. Indeed, *P. mesopotamicus* aquaculture has grown steadily over the last ten years, especially in Northeastern Argentina (Luchini and Wicki, 2003). Famous for its delicate and tasty flesh, *Piaractus mesopotamicus* is also extensively cultivated in Brazil and is one of the most important food species farmed in the Pantanal wetland areas of the Parana–Paraguay basin (Oliveira et al., 2004; Urbinati and Gonçalves, 2005).

In Argentina, *P. mesopotamicus* aquaculture has markedly grown from 1996, surpassing trout aquaculture in 2011 and exceeding 2000 metric tons per year in 2014 (Wicki and Wiltchiensky, 2017). *P. mesopotamicus* is often produced by ranchers and farmers aiming to diversify their production (Luchini and Wicki, 2003; Wicki et al., 2008). Although the approach is interesting from a productive and economical point of view, concerns exits about the safety of growing fish in the vicinity of field lots treated with pesticides. The establishment of aquaculture ponds near agricultural fields may, indeed, represent a risk to the health of farmed fish and the safety of the fish meat produced, as crop protection products employed on nearby fields may enter the ponds through diffuse entry pathways such as spray drift, runoff and percolation (Shafer et al., 2011). The presence of pesticides in surface waters of the agricultural Pampa region of Argentina has been well documented (Peruzzo et al., 2008; Aparicio et al., 2013; Bonansea et al., 2013; De Gerónimo et al., 2014; Lupi et al., 2015; Hunt et al., 2016; Ronco et al., 2016; Etchegoyen et al., 2017; Pérez et al., 2017; Primost et al., 2017; Castro Berman et al., 2018).

As is the case for wild specimens, fish farmed in contaminated lakes and pond will be constantly exposed to pesticides either through breathing, through the food they eat or from the medium they live in (Stanley and Preetah, 2016). Under such conditions, the uptake, and the possible accumulation of pesticides residues into fish tissues and organs is a likely eventuality. Indeed, although modern families of pesticides are designed to present a lower bioaccumulation potential than their antecessors, evidence shows that fish living in chronically contaminated freshwaters commonly present various pesticide residues in their tissues and organs. This fact has been demonstrated in various regions of the world (Corcellas et al., 2015; Reindl et al., 2015; Pico et al., 2019; Akan et al., 2019; Basopo and Muzvidziwa, 2020), including the wide agricultural extensions of South America (Oliveira et al., 2015; Ernst et al., 2018; Jonsson et al., 2019) and the Argentine Pampa (Brodeur et al., 2017).

In this context, the aim of the present study was to evaluate the safety of creating fish farms in agricultural settings by assessing the health and pesticide contents of *P. mesopotamicus* grown in a pond while crops typical to the Pampa region where cultivated under a traditional pesticide application regime in a nearby field.

2. Materials and methods

2.1. Test species

The South American *Piaractus mesopotamicus* is a robust laterally-flattened fish with an ovoid shape. It is a grey to silver fish with a white belly and a yellow breast with yellow to orange fins lined with a black border (Ringuelet et al., 1967). It is an omnivorous species with a strong herbivorous tendency that eats mainly fruits, seeds, vegetables and invertebrates such as crustaceans, molluscs and insects (Machado-Allison, 1980). For this reason, production costs of cultured specimens can be considerably reduced by feeding the animals with a low content of fish meal or even without fish meal (Wicki et al., 2012). A fast growing warm water fish, *P. mesopotamicus* is especially adapted to subtropical climates. It is found in the temperature range of 15–35 °C, although the optimal range for farming the species lies within 20 and 28 °C (Milstein et al., 2000). *P. mesopotamicus* may reach up to 8 kg in its natural environment but cultured specimens are normally sold at an average weight of 1.5 kg (Wicki et al., 2008).

2.2. Study site

The study took place in a 1-ha surface area pond sited at the downhill edge of a 16-ha experimental agricultural field (31°50′18S; 60°31′47W). The pond is situated within the experimental station of the "Instituto Nacional de Tecnología Agropecuaria", which is located on the outskirts of the city of Paraná, province of Entre Ríos, Argentina (Fig. 1). No-till agriculture has been taking place in the agricultural field adjacent to the pond for at least 20-years. The pond receives runoff from this agricultural field because the field is crossed by a water canal that opens up into the pond. The land on both sides of the canal present a slope of 4 and 5% on the western and eastern side, respectively. A maize crop was cultivated in the field during the experimental period, which lasted from March 2015 to June 2016. Pesticide applications performed and water sampled taken over this period are indicated in Table 1.

2.3. Fish used and holding conditions

Juvenile *P. mesopotamicus* were maintained in a mesh enclosure placed in the agricultural pond during one year and 3 months while normal agricultural activities took place in the nearby field. The enclosure was 2 m diameter and 1.45 m high (4.5 m^3 volume) and was made with an iron frame and 11 mm plastic mesh. The fish originated from a nearby pisciculture and were three months old when introduced into the enclosure. One hundred twenty juvenile fish were introduced in the enclosure. Fish were fed daily with a floating extruded fishmeal containing 30% protein. Minimum and maximum temperatures in the pond were respectively 9 and 38 °C over the course of the experiment. Water pH varied between 6.9 and 7.5.

For comparison purposes, a control group of *P. mesopotamicus*

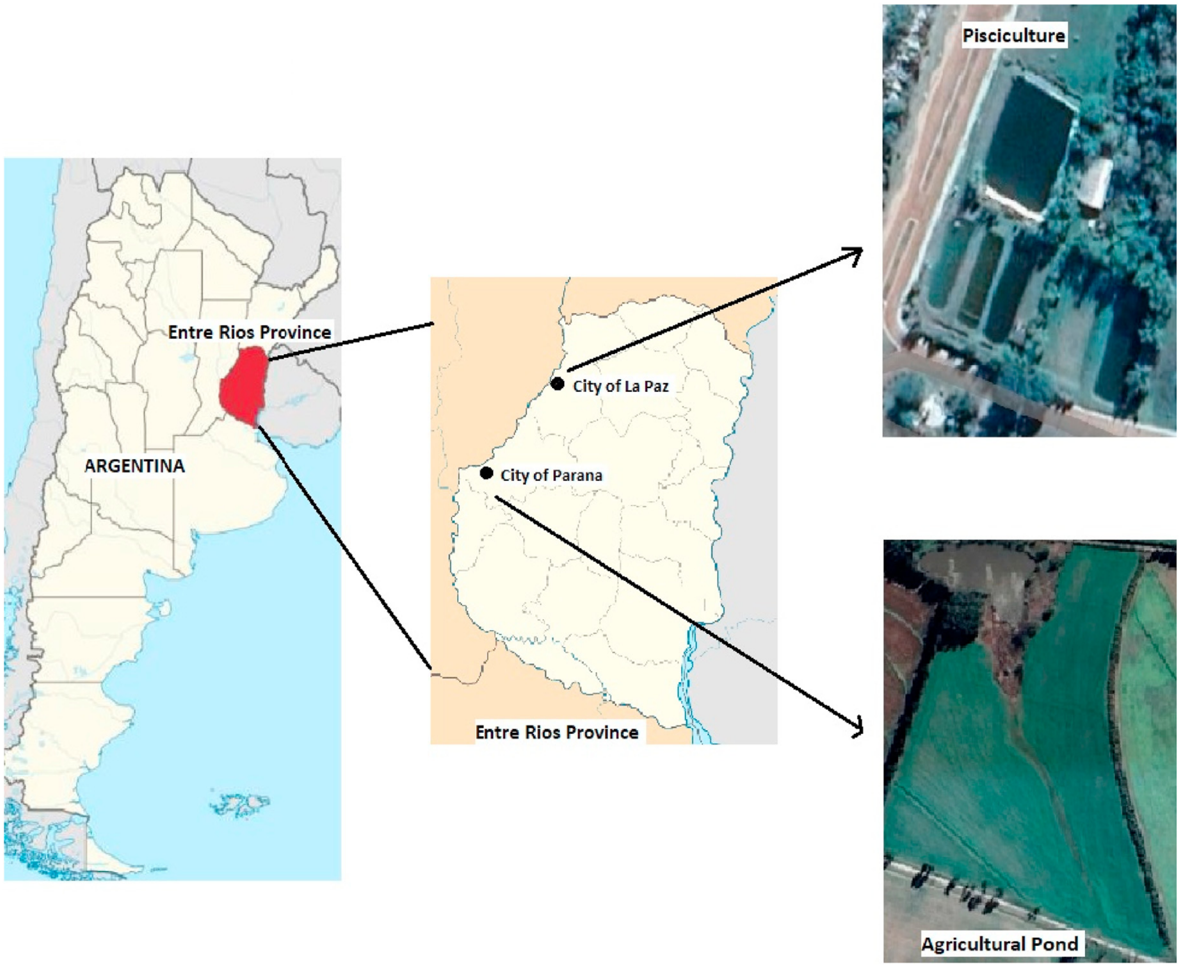

Fig. 1. Geographic location of the pisciculture and the agricultural pond.

Table 1
Timeline of events in the studied pond and adjacent agricultural field.

Date	Event	Pesticides Applied to the Field*	Pesticide Detections in Pond Water (μg/L)			
			Atrazine	2,4-D	Glyphosate	AMPA
11/03/2015	Fish introduced into the pond					
24/07/2015	Pesticide determinations in water		ND	ND	ND	ND
18/08/2015	Application of pesticides to the field	Glyphosate: 1.08 kg/ha Atrazine: 1 kg/ha Dicamba: 116 g/ha				
12/09/2015	Direct sowing of corn and fertilization with phosphorous and nitrogen					
13/09/2015	Application of pesticides to the field	Atrazine: 1.44 kg/ha Metolachlor: 1.1 kg/ha				
16/10/2015	Application of pesticides to the field	Imazapic: 60 g/ha Imazapyr: 20 g/ha				
06/11/2015	Pesticide determinations in water		118	ND	221	117
06/05/2016	Pesticide determinations in water		110	ND	31	21
18/05/2016	Application of pesticides to the field	Glyphosate: 1.08 kg/ha Dicamba 116 g/ha Metsulfuron: 4.2 g/ha				
04/06/2015	Pesticide determinations in water		109	ND	590	320
23/06/2016	Pesticide determinations in water		92	ND	12	52
23/06/2016	Fish sampled					
23/06/2016	Pesticide determinations in water from the pisciculture**		ND	6	5	2

* Application rates are expressed in terms of active ingredients. ** Pesticide concentrations determined in the water from the pisciculture are given for comparison.

was sampled from a pisciculture located in the city of La Paz, province of Entre Ríos, Argentina (30° 75′05S, 59° 65′45W). Fish from the pisciculture were raised in an open-air 10 × 30 × 1.60 m pond (width x length x depth) excavated from the ground and filled with groundwater. Fish from the pisciculture were fed the same feed as those in the experimental pond. Minimum and maximum temperatures in the pisciculture pond were respectively 12 and 32 °C over the course of the experiment. Water pH varied between

7.1 and 7.4.

2.4. Fish samplings

Fish were removed from the water with dip nets and immediately insensibilized by administering an accurate blow to the head and cutting the neural cord behind the brain. Eleven fish from the agricultural pond and ten fish from the pisciculture were sampled with only one-day of interval. Body mass and standard length were recorded and a blood sample was immediately taken from the caudal vasculature with a heparinized syringe. Collected blood was used for hematology and genotoxicity determinations as described below. The remaining blood was centrifuged at 10 000 g for 5 min, and plasma was removed and immediately frozen in liquid nitrogen for further biochemical analyses. Fish were sacrificed by cutting the neural cord behind the brain and both gill arches were removed to determine DNA breaks. A longitudinal cut was made in the abdomen and the liver was excised. Finally, a 1 cm^3-piece of white muscle was sampled from the left side. Tissue samples were stored at −80 °C until biochemical analyses were performed. All procedures involving live fish were conducted according to the reference guidelines for research with laboratory, farm and wild animals from the National Scientific and Technical Research Council of Argentina (CONICET, 2005). Furthermore, all animal manipulations were performed under approval of a local institutional ethics committee for the care and use of laboratory animals (CICUAL Protocol 006-26-17, National University of La Plata).

2.5. Pesticide residues in water and fish tissues

One-liter water samples were taken on the dates indicated in Table 1. Water samples were taken in brown colored acid-washed glass bottle and immediately frozen at −20 °C until analysis were performed. In the laboratory, pesticides determination was conducted by solid phase extraction (SPE) using GCB, C18 and PSA (Furlong et al., 2001; Zaugg et al., 1995). Obtained extracts were analyzed for the 42 pesticide molecules listed in Table 2 as described below. In the case of glyphosate and AMPA, the QuPPe method without derivatization was employed (Anastassiades et al., 2015). Most pesticides molecules examined are current-use pesticides commonly employed in extensive cultures of the Pampa Region, but some organophosphates and carbamates are legacy pesticides which use is currently prohibited.

For their part, muscle tissues were extracted by the QuEChERS (Quick, Easy, Cheap, Effective, Rugged & Safe) technique before being analyzed for the 42 pesticide molecules listed in Table 2. Briefly, muscle tissues were thawed, dried with a piece of paper towel, and homogenized in 1 mL of acetonitrile containing 3% magnesium sulfate, 3% sodium acetate and 1.5% sodium chloride using a Teflon-glass Potter-Elvehjem homogenizer. Homogenates were sonicated for 10 min before being centrifuged at 10 000 g for 5 min to remove cell debris. The resulting supernatant was analyzed for pesticides through gas chromatography/mass spectrometry or liquid chromatography/mass spectrometry as previously described by Brodeur et al. (2017). The bioconcentration factor (BCF) or every pesticide detected in the muscle tissue of the fish was calculated by dividing the concentration of pesticide determined in the fish by the concentration detected in the water for the same pesticide.

2.6. Hematology

Red blood cells (RBC) were counted in duplicate in an improved Neubauer chamber, after a 1:200 dilution of entire blood in Natt Herrick's Solution (Natt and Herrick, 1952). Hemoglobin (Hb) concentrations were determined by the cyanmethemoglobin method using a 1:200 dilution of entire blood in Drabkin's solution (Drabkin and Austin, 1935). To estimate packed cells volume (PCV), blood was collected in heparinized capillary tubes that were centrifuged at 10 000 g for 5 min. Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated as described by Brodeur et al. (2020).

2.7. Biochemical parameters

Enzymatic activities of cholinesterase (ChE), catalase, 7-ethoxyresorufin-O-deethylase (EROD), 7-benzyloxyresorufin-O-dealkylase (BROD) and glutathione-S-transferase (GST) were measured in liver, whereas activities of ChE and GST were measured in plasma. Hepatic tissues were homogenized in ice-cold 50 mM tris(hydroxymethyl)aminomethane buffer (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid and 0.25 M of sucrose using a Teflon-glass Potter-Elvehjem homogenizer. Homogenates were centrifuged at 10 000 g for 5 min at 4 °C to remove debris and the resulting supernatant was used for enzymatic determinations.

ChE, catalase and GST activity were determined as described by Brodeur et al. (2017). EROD activity was fluorometrically assessed following the method of Quabius et al. (2002) using black microtiter plates (Microfluor®). The procedure was conducted adding to each well 10 μl of sample homogenates and 290 μl of reaction buffer (50 mM Tris, 25 mM $MgCl_2$, pH 7.5, 47 μM NADPH, and 0.5 μM 7-ethoxyresorufin). The amount of produced resorufin was measured against an external five-points calibration curve of resorufin standard included in each microplate. The BROD activity was measured as the EROD activity but adding 0.5 μM 7-benzyloxyresorufin instead 7-ethoxyresorufin to the reaction buffer. Protein concentrations were measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

2.8. Genotoxicity: comet assay and micronucleous test

The alkaline comet assay (CA) and the micronucleus frequency test (FMN) were performed to assess genotoxicity. To determine FMN, two blood smears were stained with acridine orange for each individual, and FMN was manually scored under a fluorescent microscope at 400X by analyzing 1000 erythrocytes per animal (Poletta et al., 2008).

For its part, CA was performed with both erythrocytes and gill cells as previously described by Poletta et al. (2008, 2013). To extract epithelial gill cells, gill filaments were sliced from the arches, and washed two times with Ca^{2+} and Mg^{2+} -free PBS to remove blood cells. Samples were then dipped in 1 mL of PBS, softly dissociated for approximately 2–3 min, and 50 μL of the cell suspension was diluted with 950 μL of RPMI-1640 medium. For their part, blood samples were diluted 1:19 (v/v) with RPMI-1640 medium. In both cases, cell viability was determined by fluorescent DNA-binding dyes. To achieve this, 100 μl of cell suspension was mixed with 4 μl of working solution of a dye-mix (acridine orange and ethidium bromide) and examined under a fluorescent microscope (Mikoba S350) equipped with a U-RFLT 50 excitation filter at 100X. A total of 100 cells were counted per sample and the percent of viable cells was obtained (Mercille and Massie, 1994). All samples presented more than 90% of viability.

CA was performed by mixing cell dilutions with 200 μl of low melting point agarose 1%. Two slides were prepared for each sample. To lyse the cellular and nuclear membranes of the embedded cells, the key-coded slides were immediately immersed in freshly-prepared ice-cold lysis solution (2.5 M NaCl, 100 mM

Table 2
Chromatographic conditions of pesticide analyses. Retention time and abundance of the confirmation ion (Ion C) relative to that of the quantification ion (Ion Q) were used as identification criteria. In all cases, the detection limit was 1 μg/L and the quantification limit was 4 μg/L. GC = gas chromatography, LC = liquid chromatography, EI = electron impact, ESI = electrospray ionization.

	GC	LC			
	EI	ESI (+)	Ion Q (m/z)	Ion C (m/z)	Chemical Group
INSECTICIDES					
Dichlorvos	X	–	109	220	Organophosphate
Chlorpyrifos-methyl	X	–	125	286	Organophosphate
Pirimiphos-methyl	X	–	290	276	Organophosphate
Fenitrothion	X	–	277	125	Organophosphate
Malathion	X	–	173	125	Organophosphate
Chlorpyrifos	X	–	197	314	Organophosphate
Diazinon	X	–	137	179	Organophosphate
Dimethoate		X	199	125	Organophosphate
Methidathione		X	145	303	Organophosphate
Monocrotophos		X	127	224	Organophosphate
Azinphos-methyl		X	132	160	Organophosphate
Methyl-parathion		X	109	263	Organophosphate
Permethrin	X	–	183	165	Pyrethroid
Cypermethrin	X	–	163	181	Pyrethroid
Fenvalerate	X	–	167	181	Pyrethroid
Deltamethrin	X	–	181	253	Pyrethroid
Cyfluthrin	X	–	163	206	Pyrethroid
lambda-cyhalothrin	X	–	181	197	Pyrethroid
Bifenthrin	X	–	181	165	Pyrethroid
Carbofuran	X	–	164	149	Carbamate
Carbaryl		X	202	145	Carbamate
Aldicarb		X	116	208	Carbamate
Triflumuron		X	156	359	Benzoilurea
Methoxyfenocide		X			Diacylhydrazine
Thiametoxam		X	211	292	Neonicotinoid
HERBICIDES					
Atrazine	X	–	173	215	Triazine
Acetochlor	X	–	146	223	Chloroacetamide
2,4 – D			105	163	Alkylchlorophenoxy
Metsulfuron-methyl		X	167	382	Sulfonylurea
Glyphosate*		X (ESI-)	168	150	Phosponoglycine
AMPA*#		X (ESI-)	110	79	
FUNGICIDES					
Metalaxil	X	–	206	249	Acylalanine
Azoxystrobin	X	–	344	388	Strobilurin
Kresoxim-methyl	X	–	116	206	Strobilurin
Pyraclostrobin	–	X	194	388	Strobilurin
Trifloxystrobin		X	186	409	Strobilurin
Thiophanate		X	151	343	Benzymidazole
Cyproconazole	–	X	125	292	Triazole
Epoxiconazole	–	X	121	330	Triazole
Prothioconazole	–	X	326	344	Triazole
Propiconazol		X	191	259	Triazole
Tebuconazole	–	X	125	308	Triazole
Fludioxonil	–	X	247	169	Phenylpyrrole
Imazalil		X	159	297	Imidazole

#AMPA = Aminomethylphosphonic acid. AMPA is one of the primary degradation product of glyphosate. * Glyphosate and AMPA were only determined in water samples. They were not determined in fish muscle.

Na_2EDTA, 10 mM trizma base, 1% Triton X-100 and DMSO 10%; pH 10) and left at 4 °C for 1 h. After lysis, 50 μl of endonuclease III (Endo III) enzyme solution (or buffer alone in the case of controls) was placed on the gel and covered with a cover slip. Endo III presents N-glycosylase activity specific for a number of oxidized pyrimidine residues (Kow and Wallace, 1987; Dizdaroglu et al., 1993). It allows to recognize oxidized pyrimidine bases in DNA by converting them to strand breaks, which can be detected by microscopy. Slides were placed in a moist box to prevent desiccation and incubated at 37 °C for 30 min (Collins et al., 1997). The slides were then immersed in freshly prepared alkaline electrophoresis solution (300 mM NaOH and 1 mM Na_2EDTA; pH > 13), first for unwinding (10 min) and then for electrophoresis (0.7–1 V cm^{-1}, 300 mA, 10 min at 4 °C). All of these steps were carried out in darkness. All samples were coded for blind analysis; the slides were stained with acridine orange and comet images were analyzed under the fluorescent microscope. For each slide, 100 randomly selected nucleoids were visually classified into five classes according to tail size and intensity. A single DNA damage score (damage index = n1+2 n2+3 n3+4 n4) and Endo III-sensitive site index (damage with Endo III − damage with buffer) was calculated for each animal in each tissue.

2.9. Dose of pesticide contained in an average-size meal of *P. mesopotamicus* flesh

According to the Environmental Protection Agency of the United States (USEPA), a 70 kg adult consumes on average 227 g of cooked fish per meal (USEPA, 1997). Given that the weight loss associated with cooking a fish fillet is approximately 20% (Jacobs et al., 1998), this value represents about 275 g of uncooked fish meat. The dose of pesticide ingested by a 70 kg adult consuming a meal of *P. mesopotamicus* meat was therefore estimated as follows:

1 The amount of pesticide present in a 275 g meal (uncooked) of *P. mesopotamicus* meat was calculated based on the pesticide concentration detected in the muscle tissue.
2 The amount of pesticide was divided by 70 kg (average adult weight) to calculate the dose in terms of mg/kg/meal.

2.10. Statistical analysis

Statistical differences between the two experimental groups were evaluated through a T-test when the data presented normality and equal variance. Groups were compared by a Mann-Whitney Rank Sum test when these assumptions could not be achieved.

3. Results

3.1. Pesticide residues in water and fish tissues

The herbicides atrazine and glyphosate were repeatedly detected in the water of the agricultural pond (Table 1). AMPA, one of the primary degradation product of glyphosate was also detected. Water concentrations of atrazine were stable between 92 and 118 μg/L, whereas concentrations of glyphosate and AMPA were more variable, respectively ranging between 12 and 221 μg/L and 21 and 117 μg/L (Table 1). A decreasing trend in glyphosate and AMPA could be observed as the time since the last application increased (Table 1). In contrast to the agricultural pond, only traces of 2,4-D, glyphosate and AMPA were detected in the water from the pisciculture, the concentration of these pesticides being very low and ranging between 2 and 6 μg/L (Table 1). All other pesticides examined were below detection levels in both sampling sites.

In line with the results obtained in the water samples, detection of pesticide residues in fish muscle demonstrated the presence of atrazine in all ten individuals sampled from the agricultural pond (Table 3). Muscle concentrations of atrazine detected ranged between 70 and 105 μg/kg, representing BCF of about 1 with respect to the concentrations detected in the water. Furthermore, malathion concentrations of 8.6 and 23.7 μg/kg were detected in the muscle tissue of two P. mesopotamicus s from the agricultural pond, even though malathion was not detected in water, nor was applied to the field lot during the course of the experiment (Table 3). Although concentrations and frequency of detection were lower, atrazine was also detected in the muscle tissue of three *P. mesopotamicus* from the pisciculture. Concentrations detected in *P. mesopotamicus* from the pisciculture ranged between 16 and 25 μg/kg.

Table 3
Concentrations (μg/kg) of atrazine and malathion detected in South American *Piaractus mesopotamicus* muscle tissues. N.D. = Below detection limit of 1.00 μg/kg. MLC = Below quantification limit of 4 μg/kg.

Site	Fish number	Atrazine	Malathion
Agricultural Pond	1	73.22	23.68
	2	105.60	MLC
	3	83.78	ND
	4	93.63	ND
	5	82.37	ND
	6	83.07	ND
	7	94.34	ND
	8	73.22	8.61
	9	102.08	ND
	10	70.40	ND
Pisciculture	1	ND	ND
	2	25.32	ND
	3	ND	ND
	4	ND	ND
	5	ND	ND
	6	16.88	ND
	7	ND	ND
	8	ND	ND
	9	24.26	ND
	10	ND	ND

3.2. Hematology

Body length and weight of *P. mesopotamicus* from the pisciculture were respectively 36.3 ± 2.8 cm and 1.8 ± 0.4 kg (mean ± S.D.), whereas the length and weight of fish from the agricultural pond were 23.8 ± 1.7 cm and 0.6 ± 0.1 kg. *P. mesopotamicus* from the agricultural pond presented PCV and Hb values that were respectively 17.6 and 29.1% lower than those of the fish from the pisciculture (Table 4). MCH and MCHC were also lower in the fish from the agricultural pond, the levels presented by these fish being respectively 34.6 and 15.9% lower than those of pisciculture individuals. RBC and MCV did not significantly differ amongst the two groups (Table 4).

3.3. Biochemical parameters

Fish from the agricultural pond presented significantly greater cholinesterase activity in both the plasma and liver compared to fish from the pisciculture (Table 5). For its part, the liver activity of GST was significantly lower in *P. mesopotamicus* from the agricultural pond than in fish from the pisciculture, whereas plasma GST activity did not significantly differ amongst the two groups (Table 5). EROD activity was too low to be measured in both groups of fish, but BROD activity was greater and clearly detectable. No significant difference was observed between the two groups of fish with respect to catalase and BROD activity (Table 5).

3.4. Genotoxicity

Results from the CA demonstrated that *P. mesopotamicus* from the agricultural pond presented a significantly greater level of DNA damage than those from the pisciculture (Table 6). This difference was observed both in erythrocytes (127.73 ± 21.10 vs 113.22 ± 22.82; $p = 0.031$) and in gill cells (221.91 ± 13.68 vs. 167.44 ± 32.15; $p < 0.001$) (Table 6). However, results from the modified CA with the addition of Endo III did not allow to associate the genetic damage to pyrimidine base oxidation, as no significance

Table 4
Hematological parameters (mean ± S.E.) of South American *Piaractus mesopotamicus* sampled in a pisciculture and an agricultural pond. The number of animals sampled is indicated in brackets and the coefficient of variation is indicated in italics. * = Significantly different from fish sampled in a pisciculture ($p < 0.05$).

	Pisciculture (10)	Agricultural Pond (11)
RBC (10^{12} cells/L)	0.9 ± 0.2	0.92 ± 0.09
	22.2%	*9.8%*
PCV (%)	39.8 ± 7.3	32.8 ± 3.8*
	18%	*11.6%*
Hb (g/dL)	7.9 ± 1.4	5.6 ± 1.0*
	17.7%	*17.9%*
MCV (fL)	466.4 ± 133.3	358.9 ± 47.1
	28.5%	*13.1%*
MCH (pg)	92.8 ± 22.3	60.7 ± 10.8*
	24%	*17.8%*
MCHC (g/dL)	20.1 ± 2.9	16.9 ± 2.4*
	14.4%	*14.2%*

RBC = Red Blood Cells. PCV = Packed Cell volume. Hb = Hemoglobin. MCV = Mean Corpuscular Volume. MCH = Mean Corpuscular Hemoglobin. MCHC = Mean Corpuscular Hemoglobin Concentration.

Table 5
Enzymatic biomarkers values (mean ± S.E.) of South American *Piaractus mesopotamicus* sampled in a pisciculture and an agricultural pond. Enzyme activity is expressed in μmol/min/mg protein, except for BROD (7-benzyloxyresorufin-O-dealkylase) which is expressed in picomol/min/mg protein. The number of animals sampled is indicated in brackets. * = Significantly different from fish sampled in a pisciculture ($p < 0.05$).

	Pisciculture (10)	Agricultural Pond (11)
Catalase		
Liver	54.6 ± 14.3	59.8 ± 14.5
BROD		
Liver	1.07 ± 0.08	1.18 ± 0.09
Cholinesterases		
Liver	22.3 ± 8.8	40.2 ± 10.1*
Plasma	2.5 ± 0.8	5.7 ± 3.5*
Glutathion-S-Transferase		
Liver	2.4 ± 0.6	2.0 ± 0.5*
Plasma	0.22 ± 0.07	0.26 ± 0.05

differences in Endo III-sensitive sites was detected between the two groups in any of the two tissues ($p > 0.05$, Mann Whitney *U* test). Comparing the sensitivity of the two tissues, epithelial gill cells showed a higher DNA damage than erythrocytes in *P. mesopotamicus* from the agricultural pond (221.91 ± 13.68 vs. 127.73 ± 21.10; $p < 0.001$) as well as in those from the pisciculture (167.44 ± 32.15 vs. 113.22 ± 22.82; $p = 0.001$). The FMN of the two groups of fish was not significantly different ($p = 0.131$) (Table 6).

3.5. Dose of pesticide contained in an average-size meal of *P. mesopotamicus* meat

As mentioned above, atrazine and malathion were detected in the muscle tissues of fish grown in the pond. Table 7 shows the dose of both pesticides that would ingested by a 70 kg adult eating a 275 g meal (uncooked) of meat from these fish. In the case of malathion, the amount of pesticide ingested is 300–1000 times inferior to the acceptable daily intake (ADI) of 30 μg/kg recommended in Europe (EFSA, 2014). The ADI recommended by the joint evaluation of the Food and Agriculture Organization (FAO) and World Health Organization (WHO) of the United Nations (FAO/WHO, 2016) is even higher at 300 μg/kg (Table 7). For atrazine, the amount of pesticide theoretically ingested by a 70 kg adult ranges between 0.2 and 0.41 μg/kg, which is 20 times lower than the 20 μg/kg ADI recommended by FAO/WHO (2007) (Table 7).

4. Discussion

The safety of creating *P. mesopotamicus* fish farms in the vicinity of agricultural field lots in the Pampa region of Argentina was examined by determining pesticide concentrations in the water of a pond where fish were grown while typical crops where cultivated under a traditional crop protection regime in a nearby field. After one year and three months of presence in the pond, pesticide residues were determined in the flesh of the fish, and compared to their respective ADI to evaluate the risks to consumer's health. In parallel, the health of farmed *P. mesopotamicus* was assessed by evaluating a series of hematological, biochemical, and genetic biomarkers. Results obtained demonstrated that the herbicides atrazine and glyphosate were the main pesticides detected in the pond. Water concentrations of atrazine were stable over time, whereas concentrations of glyphosate and AMPA (the degradation product of glyphosate) decreased in between applications. In agreement with the detections made in the water of the pond, atrazine residues were detected in the muscle tissue of all ten fish sampled from the agricultural pond. These results demonstrate that atrazine applied to nearby fields reaches the pond and is taken up by farmed fish. The method for analyzing glyphosate and AMPA residues in fish tissues is different from the one used for the other pesticides and was not compatible to the one performed in the laboratory, so it was not possible to provide information on the uptake of these compounds.

The capacity of fish to uptake and accumulate atrazine from the water has been demonstrated many times before, both in the laboratory (Gunkel and Streit, 1980; Gorge and Nagel, 1990; DuPreez and Van Vuren, 1992; Shunlong et al., 2011; Wang et al., 2013; Xing et al., 2015) and in the field (Akan et al., 2019; Basopo and Muzvidziwa, 2020). The BCF, which relates the concentration of a contaminant in an aquatic animal to its concentration in the water at steady state, is normally quite low for atrazine. Indeed, values for the muscle tissue usually round between one or two (Gunkel and Streit, 1980; DuPreez and Van Vuren, 1992), which is similar to the value of about one that was obtained in the current study. A BCF of one basically means that the concentration of atrazine in the fish is in equilibrium with the concentration in the water. Atrazine is quickly eliminated from the fish and no biomagnification occurs, but the constant presence of atrazine in the water of the pond results in the constant presence of atrazine in the fish.

In this context, the next step is to evaluate whether this constant presence of atrazine in the fish is a problem for both the normal growth and the health of the fish, and/or for the health of the humans consuming the fish. With respect to the risk for consumers, it was shown that the amount of atrazine theoretically ingested by a 70 kg adult eating an average portion of fish grown in the pond is 20 times lower than the 20 μg/kg ADI recommended by the FAO/WHO. As the amount of malathion, the other pesticide detected in the fish, is also many times lower than the recommended ADI, the farmed *P. mesopotamicus* would appear to be safe for human consumption based on the data obtained. Nevertheless, it has to be reminded that glyphosate was also detected in the water of the pond, so the potential accumulation of glyphosate residues should also be evaluated in future studies. Furthermore, although the pesticide residues detected in the fish from this specific case study were lower than the recommended ADI, the simple fact that pesticides residues were detected in both the pond water and the flesh of the fish, indicates that care should be taken when undertaking pisciculture activities in an agricultural region. Indeed, locally driven agronomic and environmental conditions are likely to have a major influence on the nature and the level of the pesticide contaminants encountered is the fish, and so every situation requires a specific

Table 6
Results of genotoxicity evaluation in South American *Piaractus mesopotamicus* sampled in a pisciculture and an agricultural pond (mean ± S.D.). The genetic damage index and the number of Endo III sensitive sites were determined by the comet assay. FMN = frequency of micronucleus. The number of animals sampled is indicated in brackets. * = Significantly different from fish sampled in a pisciculture ($p < 0.05$).

		Damage Index	Endo III sites	FMN
Pisciculture (10)	**Erythrocytes**	113.22 ± 22.82	30.11 ± 37.78	0.67 ± 0.86
	Gill cells	167.44 ± 32.15	6.33 ± 5.56	–
Agricultural pond (11)	**Erythrocytes**	127.73 ± 21.10*	3.00 ± 6.66	1.45 ± 1.13
	Gill cells	221.91 ± 13.68*	10.73 ± 13.94	–

Table 7
Dose of atrazine and malathion ingested by a 70 kg adult eating a 275 g meal (uncooked) of flesh of *P. mesopotamicus* from the pond.

	Atrazine	Malathion
Estimated ingested dose	0.2–0.41 μg/kg	0.034–0.093 μg/kg
Acceptable Daily Intake (ADI)	20 μg/kg (FAO/WHO[a])	300 μg/kg (FAO/WHO[b]) 30 μg/kg (Europe[c])

[a] FAO/WHO (2007).
[b] FAO/WHO (2016).
[c] EFSA (2014).

evaluation. The use of mitigation strategies such as wetlands and riparian belts construction would also be highly recommended (Reichenberger et al., 2007).

Together with the quality of the fish fillets produced, the second critical issue in a fish farm is to insure optimal and healthy life conditions to the growing fish. In the present study, *P. mesopotamicus* from the agricultural pond presented statistically significant levels of DNA damage, together with an alteration in blood parameters and enzyme activities. Altogether, these alterations indicate that the health and the physiology of *P. mesopotamicus* grown in the agricultural pond were altered by the pesticide residues present in the water. In a commercial operation, poor fish health would traduce itself in reduced yields and productivity, together with an increased risk of disease (Brodeur et al., 1997).

The presence of higher DNA damage in the fish grown in the pond is consistent with a number of previous studies which have demonstrated that both atrazine and glyphosate, the two main pesticides detected in the water, are genotoxic to fish at environmentally-relevant concentrations (De Campos Ventura et al., 2008; Cavas, 2011; Santos and Martinez, 2012; Guilherme et al., 2012; De Castilhos Ghisi and Margarete Cestari, 2013; Botelho et al., 2015). The genotoxic response to glyphosate of blood, gill and liver cells of *P. mesopotamicus* was also previously demonstrated by Leveroni et al. (2017). Accumulation of genetic damage, such as demonstrated in this study with *P. mesopotamicus*, can cause altered gene expression, abnormal cell growth, disruption of normal cell function, genomic instability, mutation, which may eventually result in cancer (Kang et al., 2013).

For their part, hematological parameters measured in the current study were, overall, similar to values previously reported for *P. mesopotamicus*, although some slight deviations could be observed. Namely, RBC values measured in both study groups from the current study were slightly lower (0.9 10^{12} cells/L vs 1.6 to 1.9 10^{12} cells/L) than those previously reported for pisciculture *P. mesopotamicus*. These lower RBC also resulted in somewhat greater VCM and MCH values compared to those previously reported in the literature (Ranzani-Paiva et al., 1999; Tavares-Dias and Mataqueiro, 2004; Garcia et al., 2007; de Almeida Bicudo et al., 2009: Sado et al., 2014). Concerning inter-site differences, fish from the agricultural pond presented systematically lower values of PCV, Hb, MCH and MCHC than animals from the pisciculture (Table 4). Although these differences may illustrate a physiological consequence of the greater pesticide contamination levels experienced by fish from the agricultural pond, they may also originate from differences in water chemistries.

Concerning the biochemical determinations performed, the possibility to detect BROD but not EROD in liver homogenates of both groups of fish highlights the existence of a clear difference in the expression of the different isoforms of cytochrome P450 in *P. mesopotamicus.* The pesticide contamination present in the agricultural pond did not induce BROD activity. It is possible that EROD activity was somewhat induced, but the methodology employed in the current study did not allow to detect such an eventuality because post-mitochondrial homogenate fractions were used. Future studies should employ more concentrated post-microsomal homogenate fractions in order to detect EROD activity in *P. mesopotamicus.*

GST activity was significantly decreased in the liver of fish from the agricultural pond. GST plays a critical role in the second phase of biotransformation by conjugating xenobiotics with reduced glutathione to facilitate dissolution in the aqueous cellular and extracellular media toward the subsequent elimination (Hayes and Pulford, 1995). The observed decrease in GST activity therefore indicates that pesticide elimination mechanisms were reduced in *P. mesopotamicus* from the agricultural pond. Similar results have been previously observed in fish exposed to atrazine (Santos and Martinez, 2012; Blahova et al., 2013), although GST induction by both glyphosate (Modesto and Martinez, 2010; Murussi et al., 2016; Sobjak et al., 2017) and atrazine (Shunlong et al., 2011) have also previously been observed. Overall, these results indicate that atrazine and glyphosate contamination have the potential to alter GST activity, the amplitude and direction of this variation being most likely related to the concentration of herbicide present and the duration of the exposure.

Cholinesterase activities were significantly increased in both the liver and plasma of the fish from the agricultural pond. Vertebrates present two types of enzymes with cholinesterase activity, which are classified according to their substratum: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). AchE plays a key role in the regulation of cholinergic nervous transmission and is present in the cholinergic synapses and motor end plates where it is responsible for the hydrolytic degradation of acetylcholine (Ach), which is the primary neurotransmitter in the sensory and neuromuscular systems in most animal species. BChE is synthesized by the liver and released into the plasma. Although the function of BChE is less clear, it has been found to have an auxiliary role in synaptic transmission and act as a scavenger; binding cholinergic toxins such as the organophosphorus insecticides before they reach the nervous system (Thompson, 1999; Sanchez-Hernandez, 2007; Cerasoli et al., 2020), and scavenging Ach unsplit with AChE (Chuiko et al., 2003). Although the above-described dichotomy between AChE and BChE is generally clear in birds and mammals, the two enzymes often more closely resemble one another functionally in fish and considerable cross-species and cross-family differences exist (Sturm et al., 2000; Chuiko et al., 2003; Pezzementi et al., 2011; Pereira et al., 2019). The Ach substrate employed in the current study allowed to simultaneously measure both types of cholinergic activities (BChE and AChE), as BChE can hydrolyze both Ach and butyrylcholine, whereas AChE hydrolyzes only Ach (Sanchez-Hernandez, 2007).

It is interesting to note that, although the cholinesterase-inhibiting organophosphate insecticide malathion was detected in fish from the agricultural pond, this was not the main element affecting ChE activity, as increases, and not decreases in activity were observed. Although cholinesterases have been historically used to monitor carbamate- and organophosphate-induced inhibitions, a number of new studies have demonstrated the capacity of various pesticides, including glyphosate (Salbego et al., 2010; Cattaneo et al., 2011; Sobjak et al., 2017) and atrazine (Aparecido

Dos Santos et al., 2015) to stimulate cholinesterase activity (Dos Santos Miron et al., 2005; Moraes et al., 2011), such as was observed in the current study. Alternatively, the inhibition of cholinesterases by both glyphosate and atrazine has also been reported (Glusczak et al., 2007; Modesto and Martinez, 2010; Xing et al., 2010, 2013). The mechanisms through which these pesticides may elevate cholinesterase activity remains unknown. The activation of AChE activity may cause an increase hydrolysis of the neurotransmitter Ach, with a consequent decreased activation of nicotinic and muscarinic receptors, resulting in deleterious neurotoxic effects.

In conclusion, the present study demonstrated that pisciculture ponds established in an agricultural setting may receive pesticides applied to nearby crops and that these pesticides may be taken up by the fish and affect their physiology and health. The detection of pesticides residues in the fish meat highlights the need to control that fish products are safe to consumers.

Credit authors statement

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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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