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Full Length Research Paper

Quantification of pharmaceutical residues in wastewater impacted surface waters and sewage sludge from Lagos, Nigeria

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Information on the occurrence of pharmaceuticals in the environment of Nigeria is limited, only a single publication previously on pharmaceutical occurrence in the environment of Nigeria, which measured general estrogen levels in Enugu, South-East Nigeria. In order to establish a first overview, surface water samples from six locations as well as ten sewage sludge samples from waste water treatment plants were analysed for a range of different pharmaceuticals, including antibiotics, estrogens, and lipid-lowering drugs. The results of this monitoring campaign were evaluated in comparison to published measured environmental concentrations in Africa and worldwide. In surface water samples, 12 of 37 pharmaceutical substances were detected at concentrations ranging from Limits of Detection (LOD) up to 8.84 µg/l. Four of these pharmaceuticals were found at concentrations exceeding ecotoxicological predicted no-effect concentrations (PNEC). In industrial, domestic, and hospital sewage sludge, nine different pharmaceutical substances were detected with the NSAID diclofenac present in all samples at concentrations of up to 1100 µg/kg dry weight, exceeding the highest measured concentration of 560 µg/kg reported in sludge samples worldwide. This study proves the presence of several pharmaceuticals at relevant concentrations in the environmental matrices studied. Further, more comprehensive monitoring campaigns, especially in locations with high population density and low dilution of treated or untreated waste water in receiving streams are recommended.

Key words: Nigeria, pharmaceuticals, liquid chromatography-tandem mass spectrometry (LC-MS/MS), water, sludge.

INTRODUCTION

The global presence of pharmaceuticals in the environment has been validated as a real and ongoing situation of concern by several studies. This has in part

led to the current initiative to have pharmaceuticals included in the list of environmentally and ecologically active chemical entities, under the global SUNEP (UBA,

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2014). Pharmaceuticals enter subterranean and surface water environments after human/veterinary use in trace amounts regardless of attenuating bio-physical processes, which include degradation, dilution, and sorption (Scheytt et al., 2001; WHO, 2012), primarily through excretion and sewage release as completely non-metabolised parent drugs, partly metabolized bio-active metabolites, and re-activatable pro-drugs (Bound and Voulvoulis, 2005; Jjemba, 2008). Pharmaceutically Active Compounds [PhACs] have also been demonstrated to be incompletely removed from waste waters by current sewage treatment regimes, with some entirely unchanged by such processes (Nakada et al., 2007; Miede et al., 2009).

Since human waste streams have been implicated as the most significant sources of environmental pharmaceutical loading (Bound and Voulvoulis, 2005) the degree of human waste impact on surface water bodies conceivably should determine the concentrations of detectable PhAC residues for individual locations. In developing country urban situations as studied here, waste water treatment is inadequate and sanitation poor. PhACs could potentially enter surface waters without any preliminary treatment, and the effects consequent on this fact, especially on non-target species such as fish and other aquatic fauna, could potentially be even more profound than conjectured at present.

Sewage Treatment Facility (STF) sludge could be a significant catchment matrix and source for environmental, especially water loading of pharmaceutical residues (Carballa et al., 2004; Ternes et al., 2004), especially where such post-treatment sludge is used as manure for crop production. Lagos, the commercial capital of Nigeria, with an estimated population of about 12,614,000 million people (UN, 2014), is one of the world's fastest growing metropolises, already gaining megacity status. High population density clusters in largely unplanned urban and peri-urban locations, poverty, and the overstretched public infrastructure and works, are largely descriptive of living conditions in Lagos.

The generally poor sanitary conditions in most of the city and suburbs, aggravated by the tropical climate characterized by seasonal heavy rains, and inadequate primary sewage treatment, make wastewater impact on surface waters a stark and ongoing reality. This study seeks to identify and quantify the levels of pharmaceutical residues in some surface waters and sewage sludge from locations within Lagos municipality, and a nearby state, and to compare detected concentrations with existing information from other studies for perspective.

MATERIALS AND METHODS

Sampling locations

Selection criteria for the locations surveyed included population

(numbers, density, commercial activities, etc.), proximity to hospitals, pharmaceutical manufacturing concerns, and waste disposal sites. The chosen locations were Amuwo-Odofin, Ajido via Badagry, Isolo, Liverpool/Apapa, Ojo, and from the river Owo, a tributary of River Ogun which receives treated and untreated effluents from the Agbara Industrial Estate in Lagos and municipalities in Ogun States, south west Nigeria which it flows down through (Figure 1).

Sewage sludge

Sewage sludge was sampled in four domestic wastewater treatment plants, five industrial effluent treatment plants and one hospital wastewater treatment plant from January to April 2012 at different locations in Lagos, Oyo and Ogun State, all in South West Nigeria (Sindik et al., 2013). Table 1 gives details of the sampled plants.

Water collection, processing, and analysis

Water samples were collected (between August and September 2013) in duplicate, in the morning from each of the six locations into 500 mL amber coloured PET capped bottles, ensuring that contamination of the collection equipment, and samples before, during and after collection was minimal. The "clean hands, dirty hands" protocol (USGS, 2013) for ensuring the latter was employed in all cases. All locations were flowing water sites. Water was collected using the dip sampling method, which involved de-capping the bottles and dipping them below the surface till full. A little of the contents were routinely poured away to ensure enough room for expansion during cold storage. Field measurements including temperature, DO, conductivity and pH were done immediately after collection using a handheld Henna instruments® model HI-98129 meter (Table 2). Following collection, water bottles were placed in Styrofoam coolers with ice packs and transferred to the laboratory where kept at -20°C until analysed.

Analyses of water samples were carried out by IWW Water Centre in Muelheim, Germany according to published guidelines or validated in-house methods.

Polar pharmaceutical substances

Sample aliquots were spiked with an internal standard (4-chlorobenzoic acid), and adjusted to pH 2 by HCl addition. SPE was done using a pre-conditioned SPE column (200 mg ENV+ adsorber material). The analytes were eluted in 9 mL of ethyl acetate, concentrated to a volume of 200 µL under a gentle stream of nitrogen, and derivatized with diazomethane following DIN EN ISO 15913 guidelines prior to GC-MS analysis (column: Phenomenex ZB 50 or J&W DB 5).

Non-polar pharmaceutical substances

Sample aliquots spiked with an internal standard (Fluazifo-butyl) and adjusted to pH 7 by NaOH addition were extracted using pre-conditioned SPE columns (200 mg ENV+) as before. Desired analytes were eluted in 6 mL of acetone and concentrated to a volume of 200 µL prior to GC-MS analysis.

All GC-MS analyses were done using an Agilent GC-MS system (6890 GC and 5973 MSD single quadrupole mass analyzer) equipped with an automatic sampler (MPS 2) and Cooled Injection System (CIS 4, Gerstel, Mülheim, Germany).

Antibiotics

Typical sample aliquots were adjusted to pH 3 by HCl addition and

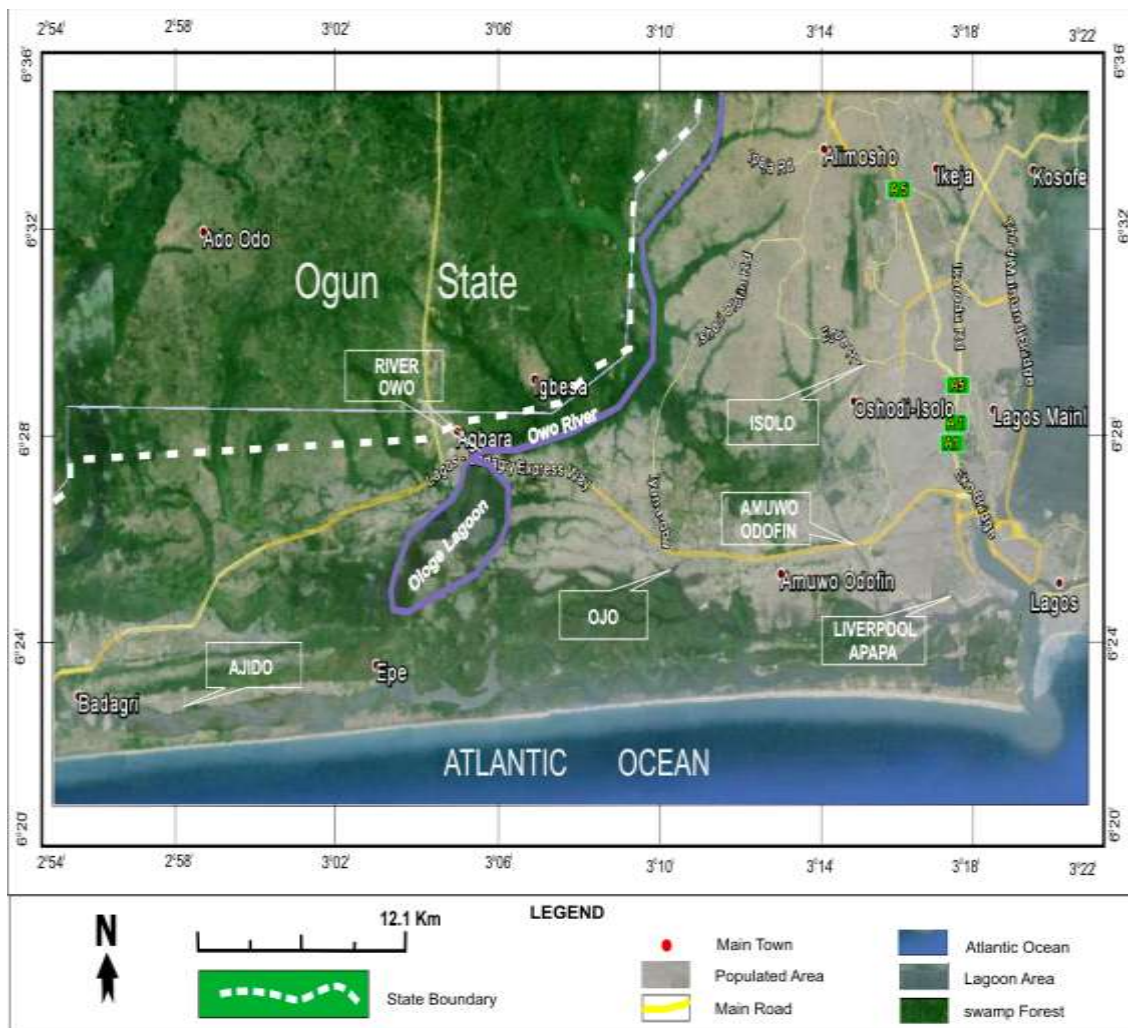


Figure 1. Showing water sampling locations.

Table 1. Characteristics of the wastewater treatment plants studied in Nigeria (Sindik et al., 2013).

Treatment plants	Location	Type of wastewater treated	Treatment type	Capacity [m ³ /h]
Brewery	Lagos	Industrial	Activated sludge system	126
WEMABOD	Lagos	Industrial	Aerated lagoon system	n.a.
Dairy	Ibadan, Oyo state	Industrial	Activated sludge system	n.a.
AGBARA	Ota, Ogun state	Industrial	Aerated lagoon system	n.a.
Food	Ota, Ogun state	Industrial	Activated sludge system	100
Ikeja	Lagos	Domestic	Oxidation pond system	60
Abesan	Lagos	Domestic	Activated sludge system	80-100
Iponri	Lagos	Domestic	Lagoon oxidation system	150
Oke Afa	Lagos	Domestic	Lagoon oxidation system	120
UCH	Ibadan, Oyo state	Hospital	Activated sludge system	n.a.

n.a. not available.

extracted on preconditioned SPE columns (500 mg ENV+). The analytes were eluted in 10 mL of methanol, concentrated to

dryness, and transferred into 1 mL of acetonitrile/water (v/v 60:40) solvent prior to analysis by LC-MS/MS (column: Nucleosil C18 HD

Table 2. Physicochemical data measurements on collection dates by location.

Parameter	Location					
	River Owo	Ojo	Liverpool	Ajido	Amuwo	Isolo
Conductivity	134	3999	3999	488	3999	492
pH	7.06	7.83	6.6	7.65	7.60	7.45
Temperature (°C)	26.4	31.4	34.7	29	28.2	27.7

solvent prior to analysis by LC MS/MS (column: Nucleosil C18 HD 250 x 3 mm, 5 µm).

Tetracyclines

The analysis of the tetracyclines was conducted without pre-concentration using SPE by LC-MS/MS (column: Discovery HS C18150 x 2.1 mm, 3 µm).

Hormones

SPE was done on sample aliquots adjusted to pH 2 by HCl addition using preconditioned SPE columns (1.0 g RP-C18-Polar-Plus-Material). The analytes were eluted four times in 3 mL of methanol, concentrated to a volume of 100 µL, and diluted to a final volume of 500 µL by addition of water/methanol solvent prior to analysis with LC-MS/MS (column: Synergie Polar 4 µm, 250 mm x 2 mm ID). Waters Acquity ultra performance liquid chromatography (UPLC-TQD/PDA and UPLC-TQS) coupled with an electro-spray ionization tandem mass spectrometric system (Milford, USA) was used to carry out LC-MS/MS analyses.

Data acquisition, processing, and evaluation were carried out using Agilent ChemStation software combined with Gerstel Maestro software package. The limit of detection (LOD) was defined by a signal to noise ratio of 3:1 for all applied methods.

Sewage sludge collection, processing, and analysis

Sludge samples were collected from wastewater treatment plants and the respective sewage stabilization ponds in amber glass flasks. Samples were transported to the laboratory on ice for further treatment and processing. Sludge samples had a water content of approximately 95%.

The samples were air-dried, ground, and homogenized by sieving through a stainless steel 2-mm sieve, and consequently stored in a freezer (Sindik et al., 2013). Analysis of the freeze-dried sewage sludge samples were carried out by the Institute of Energy and Environmental Technology (IUTA), Duisburg, Germany. Samples were extracted by accelerated solvent extraction (ASE 200, Dionex, Idstein, Germany) during a single extraction cycle of 15 min at temperature and pressure settings of 100°C and 100 bar respectively, using methanol as solvent. After evaporation, resolving and filtering, pharmaceuticals were analysed using an Agilent 1100 HPLC (Agilent Technologies, Böblingen, Germany), which was coupled to an API 3000 mass spectrometer (AB Sciex, Darmstadt, Germany).

The separation of the analytes was performed with a Synergi 4u Polar –RP 80A column (Phenomenex). Confirmation measurements were carried out on a Symbiosis™ Pico LC system (Spark Holland, Emmen, Netherlands) connected to a QTRAP™ 6500 mass spectrometer (AB SCIEX, Darmstadt, Germany). Analyst 1.6 software was used for data acquisition and processing. Limits of

detection were defined as a signal to noise ratio of 3:1 for the applied method. Samples were quantified with the method of standard addition according to DIN 32633. Hormone samples were analysed by GC-MS.

Therefore, extracted samples were filtered, dried until dryness, derivatized, and analysed using a Thermo Trace GC and a Thermo ISQ mass analyser (Thermo Fisher Scientific GmbH, Dreieich, Germany). Data acquisition, processing, and evaluation were carried out using Excalibur software.

RESULTS

Water

Preliminary results from analysis of water samples for the presence or absence of a wide variety of pharmaceuticals and their degradates yielded information about the presence of twelve pharmaceutical entities out of Thirty seven checked for, which comprised of six antibiotics, four non-steroidal anti-inflammatory analgesics (NSAIDs), one antilipidemic and, phytoestrogen each (Table 3).

The detected agents were found at concentrations exceeding the detection limit of used analytical methods. The highest individual pharmaceutical concentrations measured were detected in the sample taken from the Isolo sampling location for ibuprofen (8.84 µg/l), sulfamethoxazole (1.5 µg/l) and erythromycin (1.0 µg/l). Total locational concentrations for determined pharmaceuticals were highest for the Isolo location at 13.55 µg/l followed by Amuwo Odofin at which total concentrations were 2.13 µg/l. All compounds detected were found in the water sample from Amuwo Odofin, followed by Isolo where all but acetylsalicylic acid were detected (Table 2).

Betasitosterol was detected in all locations including Isolo where detected levels were highest (0.67 µg/l). Ajido recorded the fewest pharmaceutically active compounds with just two entities detected, betasitosterol and naproxen. Detection frequency of detected residues were in the order betasitosterol (100%) > ibuprofen (45.5%) > chloramphenicol, diclofenac and sulfamethoxazole (33.37%) > naproxen (27.27%) > erythromycin–A–dihydrate, erythromycin, sulfadiazine and trimetoprim (18%) > clofibrate (9%).

Table 4 displays maximum concentrations measured in the six water samples from Nigeria in comparison to measured concentrations in surface water or groundwater in other African countries, and in comparison

Table 3. Analyses of 6 water samples from Nigeria.

Pharmaceutical agent	Therapy group	Analytical methods			Water samples from Nigeria (µg/l)					
		Method	Lod	Ajido	River Owo	Amuwo-Odofin	Ojo	Liverpool	Isolo	
16-alpha-Hydroxyestrone	Estrogens	LC-MS/MS	0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	
17-alpha-Ethinylestradiol	Estrogens	LC-MS/MS	0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	
Acetylsalicylic acid	Analgesics	DIN EN ISO 15913	0.02	< 0.02	0.13	0.10	0.03	< 0.02	< 0.02	
Betasitosterol	Phytosterol	LC-MS/MS	0.02	0.47	0.06	0.37	0.24	0.18)	0.67)	
Bezafibrate	Lipid-lowering drugs	DIN EN ISO 15913	0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	
Carbamazepine	Antiepileptic drugs	LC-MS/MS	0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	
Chloramphenicol	Antibiotics	LC-MS/MS	0.01	< 0.01	< 0.01	0.03	0.03	0.03	0.36	
Chlortetracycline	Antibiotics	LC-MS/MS	0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	
Clarithromycin	Antibiotics	LC-MS/MS	0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	
Clofibric acid	Lipid-lowering drugs	DIN EN ISO 15913	0.02	< 0.02	< 0.02	< 0.02	0.04	< 0.02	< 0.02	
Erythromycin-A dihydrate	Antibiotics	LC-MS/MS	0.06	< 0.06	< 0.06	0.12	< 0.06	< 0.06	0.48	
Diazepam	Psychiatric medication	LC-MS/MS	0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	
Diclofenac	Analgesics	DIN EN ISO 15913	0.02	< 0.02	< 0.02	0.07	0.04	0.03	0.27	
Doxycycline	Antibiotics	LC-MS/MS	0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	
Erythromycin	Antibiotics	LC-MS/MS	0.06	< 0.06	< 0.06	0.06	< 0.06	< 0.06	1.00	
Estradiol	Estrogens	LC-MS/MS	0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	
Estriol	Estrogens	LC-MS/MS	0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	
Estrone	Estrogens	LC-MS/MS	0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	
Etofibrate	Lipid-lowering drugs	LC-MS/MS	0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	
Fenofibrate	Lipid-lowering drugs	LC-MS/MS	0.04	< 0.04	< 0.04	< 0.04	< 0.04	< 0.04	< 0.04	
Fenoprofen	Analgesics	DIN EN ISO 15913	0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	
Gemfibrozil	Lipid-lowering drugs	DIN EN ISO 15913	0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	
Ibuprofen	Analgesics	DIN EN ISO 15913	0.02	< 0.02	0.04	0.57	0.22	0.11	8.84	
Indometacin	Analgesics	DIN EN ISO 15913	0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	
Ketoprofen	Analgesics	DIN EN ISO 15913	0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	
Mestranol	Estrogens	LC-MS/MS	0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	
Naproxen	Analgesics	DIN EN ISO 15913	0.02	0.03	< 0.02	0.02	< 0.02	< 0.02	0.02	
Oxytetracycline	Antibiotics	LC-MS/MS	0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	
Pentoxifylline	Vasculardilatation	GC-MS	0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	
Phenacetin	Analgesics	GC-MS	0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	
Phenazone	Analgesics	GC-MS	0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	
Roxithromycin	Antibiotics	LC-MS/MS	0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	
Sulfadiazine	Antibiotics	LC-MS/MS	0.01	< 0.01	< 0.01	0.02	< 0.01	< 0.01	0.04	
Sulfadimidine	Antibiotics	LC-MS/MS	0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	
Sulfamethoxazole	Antibiotics	LC-MS/MS	0.01	< 0.01	< 0.01	0.65	0.14	0.09	1.50	
Tetracycline	Antibiotics	LC-MS/MS	0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	
Trimethoprim	Antibiotics	LC-MS/MS	0.01	< 0.01	< 0.01	0.12	< 0.01	< 0.01	0.40	

Table 4. The analyses of ten sewage sludge samples from Nigeria (*positive, but below LOQ; n/e = not evaluable due to high matrix interferences).

Pharmaceutical substance	Unit	Brewery	WEMABOD	Dairy	Agabara	Food	Ikeja	Abesan	Iponri	Oke AFA	UCH
		Lagos	Lagos	Ibadan	Ota	Ota	Lagos	Lagos	Lagos	Lagos	Ibadan
		I	I	I	I	I	D	D	D	D	H
17-alpha-Ethinylestradiol	µg/kg	<10	<30	<40	<10	n/e	<10	<10	<10	<15	<10
17-beta-Estradiol	µg/kg	<10	<10	<20	<10	n/e	<10	<10	<10	<10	<10
Azithromycin	µg/kg	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Bezafibrate	µg/kg	<10	<10	<10	<10	<10	11	<10	<10	<10	<10
Bisoprolol	µg/kg	<10	<10	<10	<10	<10	<10	<10	<10	<10	<80
Carbamazepine	µg/kg	<10	<10 *	49 *	23	24	39	<30 *	<10 *	<10 *	71
Clarithromycin	µg/kg	<10	<10	<10	<10	<10	<10	<10	<10	<10	43
Diclofenac	µg/kg	30	38	395	1100	169	635	119	<10 *	71	111
Erythromycin	µg/kg	<10	<10	<10	<10	<10	39	147	<10 *	38	49
Fenofibric acid	µg/kg	n/e	n/e	n/e	n/e	n/e	n/e	n/e	n/e	n/e	n/e
Ibuprofen	µg/kg	360	<100	<100	210	<100	<100	<100	<100	<100	<60
Ketoprofen	µg/kg	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Metoprolol	µg/kg	<10	<10 *	<10	<10	<10	<10	<10	<10	<10	<30
(Naproxen)	µg/kg	n/e	n/e	n/e	n/e	n/e	n/e	n/e	n/e	n/e	n/e
Propranolol	µg/kg	<10	<10	33	<10	<10	25	<10	<10	<10	<10
Roxithromycin	µg/kg	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Sotalol	µg/kg	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Sulfamethoxazole	µg/kg	<10	<10	<10	11	<10	<10	<10	<10	<10	<10
Trimethoprim	µg/kg	<10	<10	<10	31	<10	19	<10	<10	<10	38

Source: Sindiku et al. (2013). Key: I, industrial; D, domestic; H: Hospital: *1, positive, but below LOQ; *2, Positive using an optimised measurement method; *3, positive, corrected result.

to ecotoxicological effect concentrations (PNEC predicted no effect concentrations).

Sewage sludge

Nine of nineteen pharmaceutical substances were detected in at least one of the studied ten sludge samples (Table 4). Diclofenac was detected in each of the ten sludge samples in a concentration range of < 10 to 1140 µg/kg dry weight, followed by Carbamazepine with positive detection in nine

samples (< 10 to 70 µg/kg) and Erythromycin in five samples (< 10 to 147 µg/kg). Bezafibrate, Clarithromycin, Ibuprofen, Metoprolol, Propranolol, Sulfamethoxazole, and Trimethoprim were positively detected in one or two sludge samples only (concentration range < 10 to 360 µg/kg). Two substances (Fenofibric acid and Naproxen) could not be evaluated due to high matrix interferences. Tables 5 and 6 contain details of maximum concentrations measured in the ten sewage sludge samples from Nigeria in comparison to maximum concentrations in sewage

sludge samples measured in Africa and worldwide respectively.

DISCUSSION

There are several routes of entry of human and veterinary pharmaceuticals into environmental matrices including water. Earlier work has firmly established the presence of pharmaceutically active compounds, their derivatives and other forms of the active moieties in all environmental

Table 5. Maximum concentrations measured in the six water samples from Nigeria (this project) in comparison to measured concentrations in surface water or groundwater in other African countries and in comparison to ecotoxicological effect concentrations (PNEC predicted no effect concentrations).

Pharmaceutical substance	6 water samples from Nigeria (this project)		Measured Concentrations in Africa (IWW SAICM-Database)		Ecotoxicological no-effect concentration (Bergmann et al., 2011)
	Max. conc. [$\mu\text{g/l}$]	Max. conc. [$\mu\text{g/l}$]	Country	Literature	PNEC [$\mu\text{g/l}$]
16-alpha-Hydroxyestrone	< 0.01				n.d.
17-alpha-Ethinylestradiol	< 0.01	< 0.01	South Africa	de Jager et al. (2011)	1.0E-05
Acetylsalicylic acid	0.13				1.0E+01
Betasitosterol	0.67				n.d.
Bezafibrate	< 0.02				1.2E+00
Carbamazepine	< 0.01	4,5	Kenya	K'Oreje et al. (2012)	2.5E+00
Chloramphenicol	0.36				1.9E-02
Chlortetracycline	< 0.05				3.0E-02
Clarithromycin	< 0.05				2.0E-01
Clofibric acid	0.04				2.0E-01
Diazepam	< 0.01				2.7E+00
Diclofenac	0.27	1.52	Cote d'Ivoire	Kouadio et al. (2009)	1.0E-01
Doxycycline	< 0.05				5.4E-02
Erythromycin	1.00	0.141	South Africa	Tredoux et al. (2012)	2.1E-01
Erythromycin-A dihydrate	0.48				n.d.
Estradiol	< 0.01	2.15	South Africa	Aneck-Hahn et al. (2009)	2.0E-05
Estriol	< 0.01	< 0.01	South Africa	de Jager et al. (2011)	n.d.
Estrone	< 0.01	0.251	South Africa	Hendricks (2011)	1.0E-01
Etofibrate	< 0.02				n.d.
Fenofibrate	< 0.04				1.6E+00
Fenoprofen	< 0.02				n.d.
Gemfibrozil	< 0.02				3.8E+00
Ibuprofen	8.84	21	Kenya	K'Oreje et al. (2012)	6.0E+01
Indometacin	< 0.02				2.3E-01
Ketoprofen	< 0.02				3.1E+00
Mestranol	< 0.01				2.0E-02
Naproxen	0.03	0.074	Cote d'Ivoire	Kouadio et al. (2009)	3.3E+00
Oxytetracycline	< 0.05				1.1E+00
Pentoxifylline	< 0.01				2.0E+01
Phenacetin	< 0.01				n.d.
Phenazone	< 0.01				2.0E+01
Roxithromycin	< 0.02				2.0E-01
Sulfadiazine	0.04				1.4E+00
Sulfadimidine	< 0.01				1.5E-02
Sulfamethoxazole	1.50	2.93	Ghana	Takada et al. (2012)	5.9E-01
Tetracycline	< 0.05				2.5E-01
Trimethoprim	0.40	5.5	Kenya	K'Oreje et al. (2012)	2.0E+01

Measured concentrations exceeding PNEC values are listed in bold. (n.d. = not determined).

water compartments (Servos et al., 2007; Sanderson, 2011; Jongh et al., 2012). Potential hazards due to pharmaceutical release into the environment have become increasingly important to government regulators, the pharmaceutical industry and the lay public which has increasingly more access to information about the issue,

sometimes not from the most factual sources e.g. the popular media. It however cannot be said that the public's renewed interest in this matter is misplaced.

The increased detection capabilities using bio-analytical approaches are increasingly being enhanced as a result of improved analytical capabilities and the

Table 6. Maximum concentrations measured in the ten sewage sludge samples from Nigeria (this project) in comparison to maximum concentrations in sewage sludge samples measured worldwide.

Pharmaceutical substance	10 Sewage sludge samples from Nigeria (this project)	Measured concentrations worldwide (IWW SAICM-Database)		
	Max. Conc. ($\mu\text{g}/\text{kg}$)	Max. Conc. ($\mu\text{g}/\text{kg}$)	Country	Literature
17-alpha-Ethinylestradiol	< 40	6,800	Sweden	Andersson et al. (2006)
17-beta-Estradiol	< 20	463	USA	Andaluri et al. (2012)
Azithromycin	< 10	5,770	Croatia	Senta et al. (2013)
Bezafibrate	11	640	Germany	Ivashechkin (2005)
Bisoprolol	< 80	16	Germany	Ivashechkin (2005)
Carbamazepine	71	680	Germany	Ivashechkin (2005)
Clarithromycin	43	503	Japan	Okuda et al. (2009)
Diclofenac	1100	560	Sweden	Andersson et al. (2006)
Erythromycin	147	1,000	Sweden	Fick et al. (2011)
Fenofibrate	n/e	150	Germany	Ivashechkin (2005)
Ibuprofen	360	22,000	Sweden	Andersson et al. (2006)
Ketoprofen	< 10	1,500	Sweden	Andersson et al. (2006)
Metoprolol	< 30	410	Sweden	Fick et al. (2011)
(Naproxen)	n/e	14,000	Sweden	Andersson et al. (2006)
Propranolol	33	170	UK	Gardner et al. (2013)
Roxithromycin	< 10	1,800	Spain	Nieto et al. (2007)
Sotalol	< 10	40	Germany	Ivashechkin (2005)
Sulfamethoxazole	11	178	Spain	Nieto et al. (2007)
Trimethoprim	38	188	Croatia	Senta et al. (2013)

commissioning of focused field surveys (Daughton, 2001; Focazio et al., 2004).

There are arguments that the levels at which these are found are well below those deemed inimical to human health. However, other pertinent perspectives look farther than the latter and try to establish the effects of long term/chronic, sublethal effects on the environment and denizen flora and fauna, a perspective called pharmacoenvironmentology, a term still to catch on in the regular ecotoxicological lexicon, but which encapsulates the new thinking about the relevance of continual pharmaceutical loading of the environment and the need for constant surveillance of impacted environments.

Most work on Nigeria has focused on heavy metal pollution, and several papers exist documenting this phenomenon while others are being churned out on a regular basis. To our knowledge, there has been only a single publication previously on pharmaceutical occurrence in the environment of Nigeria, which measured general estrogen levels in Enugu, South-East Nigeria (Maduka et al., 2010).

There thus exists a need to deepen the focus of research into water, especially, micro-pollutant water pollution, as the probable effects on resident humans, denizen fisheries, and potable water quality could be inimical to the interests of surrounding populations, the generality of whom are poor and for who fish remains the most affordable source of protein. As noticed in other

countries where pharmaceutical pollution of ground, surface and drinking water have been well documented, and household and healthcare facility wastes and effluents incriminated in such pharmaceutical loading, it is conceivable that these sources could also be significant contributors in Nigeria.

In Nigeria, as is common elsewhere, purpose built STFs are of the activated sludge type. For the most part, wastewater treatment is ineffective or frankly lacking in Lagos, thus the attempts at improved water treatment to reduce quantities of active pharmaceutical ingredients by STF treatment before release and reuse are not applicable. In continuation, management and use practices in various parts of the world are incongruent as exposure pathways in one may be less important in another, and thus not directly extrapolatable between countries (Boxall et al., 2012).

The choice of water collection locations for pharmaceutical presence estimations in this study was deliberately done on the basis of human populations in these areas, relative to the total population of the city itself.

Since the major route for pharmaceuticals' entry to surface water is primarily through discharge of human wastewater into surface waters such as streams, rivers, and lakes, water samples from the selected locations (generally sewage impacted) were more likely than others to show detectable levels of these substances,

and measured concentrations conceivably should follow a trend along impactation severity and population. The exception in this deliberate choosing of sample locations was Ajido, a location with a relatively smaller population as compared to other locations. It was thus chosen as the reference location.

In the present study twelve pharmaceutical entities were determined and discovered at environmentally significant concentrations in water samples. Higher concentrations have been previously recorded for ten of eleven pharmaceuticals in other African countries than in the present study.

However, erythromycin has been found in higher concentrations in the Nigerian water samples than in any other African surface water investigation. Notably, four substances (Acetylsalicylic acid, Beta-Sitosterol, Clofibrac acid, and Sulfadiazine) detected in the present study have not previously been measured/not been reported in studies from other African countries. Four of the pharmaceuticals measured in Nigerian surface waters were found at concentrations exceeding ecotoxicological effect concentrations (Chloramphenicol, Diclofenac, Erythromycin, and Sulfamethoxazole). It therefore has to be expected that these pharmaceutical substances have adverse effects on ecosystem health in Nigerian surface waters at these locations though as yet undocumented. The frequency of detection of antibiotic and NSAID residues in samples from studied locations is an indication of their widespread use, as observed in other locations in countries worldwide. The especially high concentrations of erythromycin, sulfamethoxazole and Ibuprofen in the sample ISOLO could be attributable to the proximity of the water collection location to a midsized government (referral) hospital, and a large municipal landfill in which a significant quantity of solid wastes from the hospital ends up.

It is conceivable that the common denominator explaining the general presence of beta-sitosterol in study locations would be faecal contamination with coprosterols. No endogenous or synthetic animal estrogens were detected in this study. Considering the dense population clusters in most of the studied locations and the virtually uncontrolled entrees of wastewaters and domestic sewage effluents into the surface waters same, the opposite would have been expected.

This situation could be explained in part considering that untreated wastes which impact the studied waters are often heavily laden with sewage microorganisms. In sewage treatment procedures, heterotrophic bacteria are routinely utilised for the controlled biodegradation of pharmaceuticals in sewage, and though the removal of estrogens is not total following such treatment, the levels are significantly reduced. Benka-Coker and Ojior (1995) reported high levels of heterotrophic bacteria in a sewage impacted river in Nigeria, corroborating results from a similar study by Olayemi (1994) which also established higher heterotrophic bacterial counts during the rainy

season, probably due to increased sewage overflows into surrounding water bodies. Incidentally, our water collection was carried out during the rains. There are at the present time no functional STFs in all the studied locations, thus it is possible that the high levels of heterotrophs could be responsible for the increased mobilization and breakdown of endogenous human estrogens found in domestic sewage, and the dilution effect of higher water levels and flow during the rainy season could account for the null returns from analysis of sampled waters for these compounds.

At present, there are no verifiable figures for clofibrate prescription quantities in Nigeria. It is thus difficult to link concentrations detected and frequencies of detection to usage figures and statistics, information which could help explain the limited occurrence of clofibrac acid in the current study. Considering the well documented persistence of clofibrac acid in the environment, and specifically its resistance to photodegradation (most important here where concerted sewage treatment is poor), it would have been expected that clofibrac acid would be detectable at more locations than found, due to continuing use and the aforementioned inherent environmental persistence. In the absence of more comprehensive usage data, the low levels of detection and concentrations of clofibrac acid could be attributable to low prescription rates in the studied locales. It is also conceivable that later studies which would include more locations, some highbrow (as obesity and consequent hypercholesteremia in Nigeria, though increasing across socioeconomic divides, is still more of a condition linked to affluence), would yield more representative results.

Pharmaceuticals have been demonstrated to exist in sewage sludge at concentrations much higher than in STF influents and effluents. Generally, study data on pharmaceutical occurrences in sewage sludge are few compared to surface water and STF data. None exist for Nigeria, and indeed Africa except those reported here. The NSAIDs, diclofenac and ibuprofen were found the highest concentrations in sludge with diclofenac occurring in all samples. Carbamezapine, an anticonvulsant and mood stabilising agent, was also generally discovered in all locations in significant quantities.

The well documented biorecalcitrance and environmental persistence coupled with poor removal rates by treatment may account for the near ubiquitous presence and high concentrations of diclofenac and carbamezapine recorded in the current study.

In addition the especially high diclofenac concentrations in the locations Agbara and Ikeja, could be due to the presence of many pharmaceutical production facilities in both locations which are both industrial/residential layouts. In Nigeria the use of STF sludge as fertiliser is not widespread, but studies into the presence of pharmaceuticals in soil are needed to give a more balanced-overall picture of pharmaceutical micropollution of the environment and associated

ecological sequelae.

In summary, this study has confirmed the presence of pharmaceutically active compounds in waste water impacted surface water bodies and sewage sludge in Lagos, Nigeria. The findings from this investigation has also established that the concentrations of detected PHACs, and the locational frequency of detection are comparable to reports from other countries, especially from developing countries from where unfortunately, such reports are few, limiting further comparisons.

Based on the results, it was concluded that several pharmaceuticals occur at relevant concentrations in the environment of Nigeria. It is recommended that more comprehensive water monitoring campaigns be conducted, especially in locations with high population density and low dilution of treated or untreated wastewater in receiving streams. Furthermore, investigations of groundwater, tap water/drinking water, manure, soil, and sediments as additional matrices of concern needs to be done in the nearest future to help generate wholistic pictures of the spatial environmental presences and concentrations of PhACs in them. In doing this, representative sampling strategies, improved sample conservation and pre-treatment should be used.

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Conflict of Interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Sensitivity, ecotoxicity and histopathological effects on neotropical fish exposed to glyphosate alone and associated to surfactant

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The aim of this research was to evaluate neotropical fish sensitivity (*Piaractus mesopotamicus*, *Phallocerus caudimaculatus*, *Hyphessobrycon eques*, and *Brachydanio rerio*) to a reference substance (potassium chloride); to estimate the lethal concentration (LC₅₀; 96 h) for glyphosate, formulated as Rodeo[®] alone and in association with 0.5 and 1.0% Aterbane[®] BR surfactant and to evaluate the histopathology of the gills, liver, and kidney from the fish after acute exposure. *P. caudimaculatus* and *H. eques* are good bioindicators like *B. rerio* because they have similar sensitivity. The LC₅₀;96 h for glyphosate alone and in association with 0.5% Aterbane[®] BR was similar (>975.0 mg L⁻¹) for all the fish. Aterbane[®] BR alone was the most toxic substance to *P. caudimaculatus* (5.81 mg L⁻¹ LC₅₀;96 h) and glyphosate associated to 1.0% Aterbane[®] BR was more toxic to *H. eques* (411.91 mg L⁻¹ LC₅₀;96 h). The glyphosate alone and in association with Aterbane[®] BR was classified as practically non-toxic, whereas Aterbane[®] BR alone was considered moderately toxic for the tested organisms. The histopathological effects caused by glyphosate exposure on gills, liver, and kidneys are reversible, except for the liver necrosis on *P. caudimaculatus*. *H. eques*, *P. caudimaculatus*, and *P. mesopotamicus* present great potential to be used as standard organisms for herbicides monitoring and the use of glyphosate without surfactant addition is enough to cause histological alterations on *H. eques* and *P. caudimaculatus*, which makes them possible to be applied on environmental monitoring studies as biomarkers.

Key words: Bioindicator, pesticide, histology, biomarker, gill, herbicide.

INTRODUCTION

Glyphosate (GFT) is the most used herbicide for weed control on several crops, due to its wide action spectrum,

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Table 1. Concentrations of the substances used on the toxicity assays.

Substances	Fish			
	<i>P. mesopotamicus</i>	<i>P. caudimaculatus</i>	<i>H. eques</i>	<i>B. rerio</i>
KCL (g L ⁻¹)	1.0, 1.5, 2.0, 2.5	0.5, 1.0, 1.5, 2.0, 2.5	1.0, 1.5, 2.0, 2.5	1.0, 1.5, 2.0, 2.5, 3.0
Glyphosate (mg L ⁻¹)	900.0, 925.0, 9 _{50.0} , 975.0	900.0, 925.0, 9 _{50.0} , 975.0	900.0, 925.0, 9 _{50.0} , 975.0	900.0, 925.0, 9 _{50.0} , 975.0
Surfactant (mg L ⁻¹)	8.0, 9.0, 10.0, 11.0, 12.0	3.0, 4.0, 5.0, 6.0, 7.0, 8.0	4.0, 6.0, 8.0, 10.0, 12.0, 14.0	7.0, 8.0, 9.0, 10.0, 11.0
*GF+0.5% (mg L ⁻¹)	900.0, 925.0, 9 _{50.0} , 975.0	900.0, 925.0, 9 _{50.0} , 975.0	900.0, 925.0, 9 _{50.0} , 975.0	900.0, 925.0, 9 _{50.0} , 975.0
*GF+1.0% (mg L ⁻¹)	475.0, 500.0, 525.0, 5 _{50.0} , 575.0	900.0, 925.0, 9 _{50.0} , 975.0	400.0, 425.0, 4 _{50.0} , 475.0	900.0, 925.0, 9 _{50.0} , 975.0

*GFT+0.5% glyphosate + 0.5% surfactant; GFT+1.0% glyphosate + 1.0% surfactant

lower selectivity, post-emergence application, systemic action and does not represent environmental risks to the water bodies (Shiogiri et al., 2010), and has a large potential to control aquatic weeds. The macrophyte present countless benefits to the environment, however they grow in an accelerated rate under eutrophic conditions, causing negative impacts to the use of the water body, like fishing, recreation, and power generation.

The herbicide application on the aquatic environment needs a higher scientific base, due to the lack of knowledge about the molecules for non-target organisms, morph-physiological effects, and control effectiveness (Botelho et al., 2009) and residues on water. Another issue is the toxicity of the other formulation components, since the inert components, such as the surfactants, may be more toxic than the active ingredient (IA) for non-target organisms (Carraschi et al., 2011). For Amarante Jr et al. (2002), Tsui and Chui (2003), and Navarro and Martinez (2014), the surfactants from the glyphosate formulations are more toxic for fish than the molecule itself.

At this context, the acute and chronic toxicity assays may be performed to characterize the effects and to evaluate dangerousness and environmental risks of the formulations (Schmitt-

Jansen et al., 2008). Thus, the organism selection is based on a series of criteria, such as sensibility, short reproduction cycle, operation easiness, and optimization costs (Cruz et al., 2008).

The neotropical species may be used as bioindicators for xenobiotics presence at the aquatic environment. In Brazil, the study about the application of this method for environmental monitoring is scarce (Gluszczak et al., 2006; Shiogiri et al., 2012).

Thus, the aim of this study was to evaluate the sensitivity to a reference substance (potassium chloride) and the acute toxicity (LC₅₀;96 h) of the glyphosate and the Aterbane[®] BR surfactant (alkylphenolpolyglycol ether) alone and in association with glyphosate (GFT + 0.5 and 1.0% surfactant) for the neotropical fish *Piaractus mesopotamicus*, *Hyphessobrycon eques* and *Phallocerus caudimaculatus* and the comparison with the standard species *Brachydanio rerio* and to evaluate possible histological effects caused by the glyphosate on gills, liver and kidney after acute exposure.

MATERIALS AND METHODS

The potassium chloride with 99.9% purity was used as reference substance, the Rodeo[®] herbicide was the

glyphosate source (480.0 g L⁻¹) and Aterbane[®] BR was used as the surfactant alkylphenolpolyglycol ether source (466.0 g L⁻¹).

The 50% lethal concentration values were estimated by the Trimmed Spearman-Kärber (Hamilton et al., 1977).

Sensitivity and acute toxicity tests

To perform the tests, the fish (*P. mesopotamicus*, *H. eques*, *B. rerio* and *P. caudimaculatus*) were acclimatized during seven days, under bioassay room conditions (25.0 ± 2°C temperature and 12 h photoperiod).

The fish were exposed to potassium chloride, glyphosate, surfactant and GFT + surfactant associations (Table 1), with three repetitions per treatment, three fish per repetition, at maximum density of 1.0 g L⁻¹, with 96 h duration (ABNT, 2011).

The substances concentrations on the definitive assays were previously adjusted by preliminary tests, to find the concentrations interval which caused zero and 100% mortality.

Histological analysis after glyphosate exposure

After 96 h exposure, three representative animals from each treatment and each species (n=3) were euthanized through anesthesia (1.0 g L⁻¹ benzocaine). Liver, kidney, and gills were collected. The samples were immersed in buffered formaldehyde solution (0.1 m; pH 7.3) for 24 h. After the fixation, the pieces were dehydrated in alcohol, diaphanized on xylene and included in Histosec[®] (Merck).

Table 2. Potassium chloride lethal concentration (LC₅₀, 96 h) for fish.

Parameter	<i>P. mesopotamicus</i>	<i>H. eques</i>	<i>P. caudimaculatus</i>	<i>B. rerio</i>
Upper limit	2.26	1.22	0.91	1.89
LC ₅₀ , 96 h	1.86	1.98	0.55	1.58
Lower limit	1.72	1.49	0.34	1.33

Table 3. Lethal concentration (CL₅₀, 96 h) of the glyphosate, surfactant and their associations (mg L⁻¹) for fish.

Parameter	Glyphosate	Surfactant	Glyphosate + 0.5% Surfactante	Gly + 1.0% Aterbane®
<i>B. rerio</i>	>975.00	8.61	>975.00	>975.00
<i>P. mesopotamicus</i>	>975.00	9.45	929.69	528.50
<i>H. eques</i>	>975.00	8.21	922.62	411.91
<i>P. caudimaculatus</i>	>975.00	5.81	>975.00	>975.00

*Surfactant: alkylphenolpolyglycol ether (Aterbane®BR)

Then, the microtomy was carried out in an automatic microtome (RM2155 - Leica®), performing cuts ranging from 3.0 to 5.0 µm thickness. The pieces were placed in glass slides, which were colored with hematoxylin-eosin and PAS (Schiff's periodic acid) (Behmer et al., 1976).

The histological alterations were transformed into effect index, and then classified according to each tissue, as described in the results and discussion. For the gills, the following indexes were evaluated: 0 - similar to the control - normal; 1 - Hypertrophy and hyperplasia of primary and secondary lamellae; 2 - Epitelial lifting; 3 - Lamelar tip fusion; 4 - secondary lamella disarrangement; 5 - secondary lamella congestion; 6 - decrease of the interlamellar epithelium; and 7 - sub epithelial edema on the secondary lamella. For the liver, the indexes were as follows: 0 - similar to the control - normal; 1 - capillaries congestion; 2 - hepatocytes hypertrophy with nucleus displacement to the periphery; 3 - hepatocytes fusion; 4 - disarrangement of cordonal structure; 5 - picnotic nucleus; and 6 - necrosis. And the indexes for the liver were the following: 0 - similar to the control - normal; 1 - Bowman's capsule release; 2 - epithelium disarrangement from the proximal tubules; and 3 - light increase of the proximal and distal tubules.

RESULTS AND DISCUSSION

Fish sensitivity

The most sensitive fish to the potassium chloride (KCl) was *P. caudimaculatus* and the most tolerant was *H. eques*; however, the sensitivity between species was similar (Table 2). Thus, the potassium chloride was classified as moderately toxic for the fish ($1.0 < CL_{50} < 1.0$ mg L⁻¹), except for *P. caudimaculatus*, which was highly toxic ($0.1 < CL_{50} < 1.0$ mg L⁻¹) (Zucker, 1985).

Based upon the sensitivity and in comparison with the standard fish *B. rerio*, the tested species may be standardized for ecotoxicological assays in Brazil, except for *P. mesopotamicus*, because it does not fill the requirements for a test organism. This fish has low availability, because it is a rheophilic species, with only

one spawning per year. The other species have a higher availability, due to a short reproduction cycle, as shown by Cruz et al., (2008) on the sensitivity test for *H. eques* exposure to potassium dichromate.

Neotropical fish sensitivity is similar to *B. rerio*, with higher sensibility to potassium chloride than *Pimephales promelas* (7.62 g L⁻¹ LC₅₀;96 h) (USEPA, 2001). The fish sensibilities were similar as described for *H. eques* (1.5 g L⁻¹) (Fujimoto et al., 2012) and for *P. mesopotamicus* (1.33 g L⁻¹) (Carraschi et al., 2012).

According to USEPA (2002), the advantage on the use of potassium chloride over potassium dichromate is the presence of hexavalent chromium on the potassium dichromate composition, which is highly toxic for the environment and may cause severe morphological and functional effects on neotropical fish as *P. mesopotamicus* (Castro et al., 2014).

Acute toxicity for glyphosate, surfactant and the associations

The lethal concentration 50% (LC₅₀; 96 h) of glyphosate for the tested species was >975.0 mg L⁻¹ (Table 3). The mortality ranged from 10 to 40%, regardless of the tested concentration. The lethal concentration was not estimated precisely, due to a lack of pattern regarding the ratio concentration/mortality.

The most sensitive species to the surfactant exposure was *P. caudimaculatus*, although the LC₅₀;96 h was similar for all tested species (Table 2). No mortality occurred for *B. rerio* under 7.0 mg L⁻¹ exposure; however, 100% mortality occurred with 11.0 mg L⁻¹. For *P. mesopotamicus*, no mortality occurred with 8.0 mg L⁻¹, and 100% mortality occurred with 12.0 mg L⁻¹ surfactant exposure. For *H. eques*, no mortality occurred with 4.0 mg L⁻¹ and 100% mortality was achieved with 14.0 mg L⁻¹.

For *P. caudimaculatus* no mortality occurred at 3.0 mg L⁻¹ and 100% mortality was obtained with 8.0 mg L⁻¹ surfactant exposure.

The LC₅₀;96 h for the glyphosate + 0.5% surfactant association was > 975.0 mg L⁻¹ for *B. rerio* and *P. caudimaculatus*. For *P. mesopotamicus* and *H. eques* the LC₅₀;96 h was > 900.0 mg L⁻¹ (Table 3). For *P. mesopotamicus*, 22% mortality occurred with 875.0 mg L⁻¹ and 100% mortality was achieved with 975 mg L⁻¹. For *H. eques*, no mortality occurred at 875.0 mg L⁻¹ exposure and 100% mortality occurred at 975 mg L⁻¹. *B. rerio* and *P. caudimaculatus* displayed no mortality during association exposure.

The LC₅₀;96 h observed for the glyphosate + 1.0% surfactant association were as follows: *P. caudimaculatus* and *B. rerio* > 975.0 mg L⁻¹; *H. eques* > 400.0 mg L⁻¹; *P. mesopotamicus* > 500 mg L⁻¹ (Table 3). With 400.0 mg L⁻¹ exposure, 22.2% mortality occurred for *H. eques*; 100% mortality occurred with 475 mg L⁻¹ exposure. No *P. mesopotamicus* mortality occurred with 475.0 mg L⁻¹ exposure, whereas 100% mortality occurred with 575.0 mg L⁻¹. No mortality occurred for *B. rerio* and *P. caudimaculatus* during the association exposure.

The glyphosate (Rodeo®) is practically non toxic (LC₅₀ > 100.0 mg L⁻¹) for all studied species, different from found for *Prochilodus lineatus* exposed to Roundup® (LC₅₀;96 h = 13.69 mg L⁻¹) (Langiano and Martinez, 2008). Other authors also found different results for Roundup Ready® and *P. mesopotamicus* (LC₅₀;96 h = 3.74 mg L⁻¹) (Shiogiri et al., 2012); other glyphosate formulations and *Rhinella arenarum* (LC₅₀;96 h = 6.8 to 72.8 mg L⁻¹) (Brodeur et al., 2014); and Roundup Original® and *Pseudoplatystoma* species (LC₅₀;96 h = 15.0 mg L⁻¹) (Sinhorin et al., 2014).

The glyphosate toxicity observed in this study was similar to the values found for *Salmo gairdneri* and *Oncorhynchus tshawytscha* (LC₅₀;96 h = 1070.0 to 1440.0 mg L⁻¹) (Mitchell et al., 1987); *Hybognathus amarus* and *P. promelas* (LC₅₀;96 h > 1000.0 mg L⁻¹) (Beyers, 1995) and *P. promelas* and *Polonichthys macrolepidoyus* (LC₅₀;96 h = 1154.0 to 1132.0 mg L⁻¹) (Riley and Finlayson, 2004). The commercial formulations type (inert compounds and surfactant) has an important role for the glyphosate toxicity characterization. The lower toxicity of the formulation studied in this paper (Rodeo®) may be due to the absence of surfactants. Thus, the knowledge about the formulations toxicity is fundamental at the moment of decision making, concerning the monitoring of possible glyphosate environmental effects.

The fish evaluated for this study were more tolerant to the alkylphenolpolyglycol ether than *S. gairdneri*, *P. promelas*, *Ictalurus punctatus* and *Lepomis macrochirus* exposed to the MON0818 surfactant (amine ethoxylate) (LC₅₀ = 1.4 to 3.0 mg L⁻¹) (Wan et al., 1989). *Hypomesus transpacificus*, *P. promelas* and *P. macrolepidoy* were also less tolerant when exposed to the surfactants

alkylphenolpolyethoxylate and alcohol polyalkoxylate acid (LC₅₀ = 0.7 to 3.9 mg L⁻¹) (Riley and Finlayson, 2004). And the surfactant Ammoeng 130® was also classified as more toxic for *P. promelas* (LC₅₀;96 h = 0.51 to 1.80 mg L⁻¹) (Versteeg et al., 2006). However, the toxicity observed for *Onchorynchus mykiss* exposed to alkylphenolpolyethoxylate was similar to the toxicity observed in this study, with 5.18 to 6.57 mg L⁻¹ LC₅₀ (Curran et al., 2004).

The surfactant presence in any concentration has increased the formulation toxicity in about 50% for *H. eques* and *P. mesopotamicus*, indicating that the surfactant addition interfere on the glyphosate toxicity, regarding some non-target organisms. According to Giesy et al. (2000), the polyethoxylate amine surfactant (POEA) may be responsible for making the Roundup® formulation more toxic for aquatic organisms. Riley and Finlayson (2004) observed that the LC₅₀;96 h for *H. transpacificus* was 5.5 and 3.9 mg L⁻¹ for *P. promelas*, after the exposure to Rodeo® formulation with the addition of R-11® surfactant, while the LC₅₀ of the commercial formulation without the surfactant was 270.0 and 1132.0 mg L⁻¹ for the fish, respectively, showing the connection between the surfactant presence and glyphosate toxicity.

Based on the classification by Zucker (1985) and Giesy et al. (2000), the glyphosate and the associations with 0.5 and 1.0% surfactant (recommended doses for herbicide application) can be considered practically non-toxic and the alkyl phenolpolyglycol ether is classified as moderately nontoxic (1 > LC₅₀ < 10 mg L⁻¹).

Glyphosate histopathological effects for fish

Gills

The pacu (*P. mesopotamicus*) gills consist of four branchial arches, supported by two rows of primary lamellae, which are covered with stratified epithelium. The secondary lamellae consist of epithelial, lining, pillar, chloride, and mucous cells (Figure 1A), as described by Severi et al., (2000). The observed histological changes are described below according to each concentration (Figure 1), on Table 4.

The epithelium increase and the blood congestion at the secondary lamellae also occurred on *O. mykiss* exposed to methiocarb insecticide (Altonok et al., 2006) and on *Oreochromis niloticus* exposed to diquat herbicide (Henares et al., 2008). The epithelium increase works as a barrier to decrease the xenobiotics absorption (Mallatt, 1985), similar as described for *Cyprinus carpio* exposed to GFT (Neskovic et al., 1996).

The presence of sub epithelial edema and the disarrangement of the secondary lamellae was reported for *C. carpio* (Neskovic et al., 1996) and for *O. niloticus* exposed to GFT (Jiraungkoorskul et al., 2002). The blood

Table 4. Histological alterations on fish gills after glyphosate exposure.

Concentration (mg L ⁻¹)	<i>P. mesopotamicus</i>	<i>P. caudimaculatus</i>	<i>H. eques</i>
0.0	0	0	0
900.0	0	0	0
925.0	1	1 and 7	3, 4 and 8
950.0	1	1 and 7	5 and 6
975.0	2	-	5 and 6

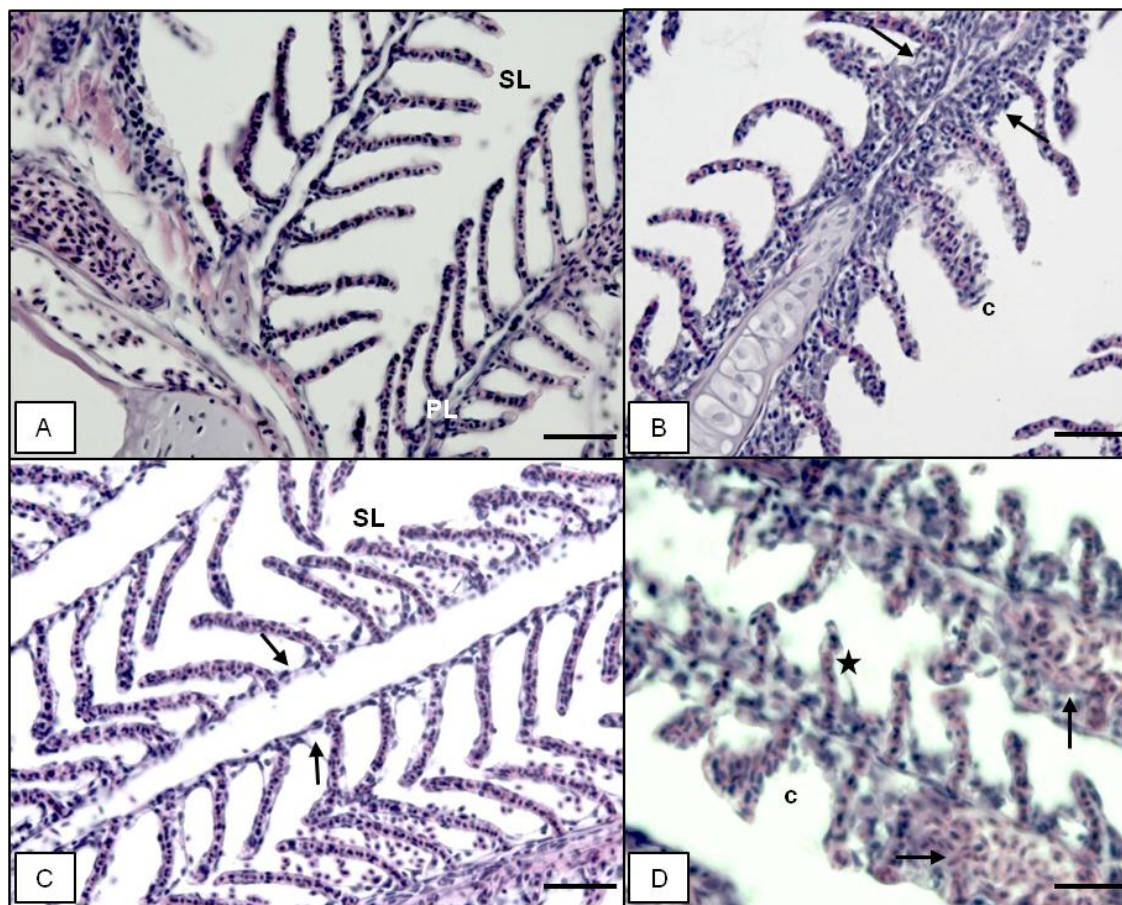


Figure 1. Gills photomicrography. A. Primary and secondary lamellae (PL and SL) from *P. mesopotamicus* with no exposure (control). B. Hypertrophy and hiperplasia of the primary lamellae (arrow) and congestion in the secondary lamellae (c) (925.0 mg L⁻¹) on *H. eques*. C. Decrease of the interlamellar epithelium height (arrow) (975.0 mg L⁻¹) on *H. eques*. D. Sub epithelial edema (star) and hypertrophy and hyperplasia of primary and secondary lamellae (arrow) (925 mg L⁻¹) on *P. caudimaculatus*. Bar =12.5 μm. Color HE.

congestion caused by the blood flow increase leads to the sub epithelial edema and the lamellae disarrangement. According to Shiogiri et al. (2012), *P. mesopotamicus* exposed under acute conditions to Roundup Ready® also presented pillar cells hyperplasia and lamellar epithelium hypertrophy. Although, such alterations are reversible (Henares et al., 2008; Shiogiri et al., 2012) and after some days of exposure, the tissue can remake its common histomorphology, different from

paraquat herbicide, which caused cells disarrangement, uncommon regeneration of the epithelial cells and deformation of the branchial cartilage cells from *Colossoma macropomum*, exposed to 10.0 mg L⁻¹ (Salazar-Lugo et al., 2011).

H. eques was the most sensitive bioindicator to GFT, with several histological changes at the gills. Despite the changes not being specific to the GFT toxicity, this kind of biomarkers indicates the presence of xenobiotics on the

Table 5. Histological alterations on the fish liver after glyphosate exposure.

Concentration (mg.L ⁻¹)	<i>P. mesopotamicus</i>	<i>P. caudimaculatus</i>	<i>H. eques</i>
0.0	0	0	0
900	0 and 1	2 and 4	2, 3, and 5
925	2	4 and 6	2 and 2
9 ₅₀	2, 3 and 4	4 and 6	2 and 3
975	2, 3 and 4	4 and 6	2 and 3

environment. This fact is important to environmental monitoring and identification of contaminants on the aquatic environment.

P. caudimaculatus displayed branchial structure similar as observed for *P. mesopotamicus* and *H. eques* on the control and under 900.0 mg L⁻¹ exposure. The sub epithelial edema on the secondary lamellae and the increase of the interlamellar epithelium was similar to a report with *O. niloticus* exposed to 36.0 mg L⁻¹ glyphosate as the Roundup[®] formulation (Jiraungkoorskul et al., 2002).

Liver

The evaluated fish liver (Control) showed cordonal organization of the hepatocytes in direct contact with the capillary sinusoids. The hepatocytes showed a hexagonal shape, with central nucleus, basophil, decondensed chromatin, a visible nucleolus and rosy cytoplasm, indicating high acidophilia (Figure 2A).

The main histological changes occurred after acute exposition were as follows: capillary sinusoids congestion (hyperemia), hypertrophy and fusion of the hepatocytes and cordonal structure disarrangement of the hepatocytes, with 900.0 and 925.0 mg L⁻¹ exposure (Table 5), followed by tissue necrosis with 925.0 to 975.0 mg L⁻¹ exposure for *P. caudimaculatus* (Table 5) (Figure 2C, D, E, F).

The cellular nucleus displacement to the periphery and the hepatocytes hypertrophy was also observed for *O. niloticus* exposed to GFT (Jiraungkoorskul et al., 2002). The displacement indicates the increase of the organelles and of the enzymes which are responsible for the xenobiotics metabolism, whereas the blood congestion may be a signal of the pressure decrease of the hepatic circulation, similar to *O. mykiss* exposed to 5.0 mg L⁻¹ GFT (Topal et al., 2015). The cordonal disarrangement also occurred with *Spaurus aurata* exposed to imazapyr, terbutrin and triasulfuron (Arufe et al., 2004) and with *Trichogaster trichopterus* exposed to paraquat (Banaee et al., 2013). According to Shiojiri et al. (2012), the *P. mesopotamicus* liver showed hepatocytes vacuolization and cells hypertrophy when exposed to 3.0 or 4.0 mg L⁻¹ glyphosate as Roundup Ready[®]. The vacuolization may work as storage of compounds which are difficult to be

metabolized.

The liver, used as GFT exposure biomarker, was the tissue which exhibited the highest histological index, reaching tissue necrosis during *P. caudimaculatus* exposure to Rodeo[®]. This alteration was also described by Ayoola (2008) on *C. gariepinus*, same for the fibrosis which occurred on *O. mykiss* exposed in a chronic manner to 5.0 or 10.0 mg L⁻¹ GFT (Topal et al., 2015). The necrosis probably occurred due to an excessive work performed by the hepatocytes, in attempt to eliminate the herbicide during the detoxification process.

Kidneys

The fish kidneys are formed by a renal corpuscle containing the glomerulus constituted by capillaries and the Bowman's capsule. Around the renal corpuscle are also found the hematopoietic tissue with basophilic cells, proximal and distal tubules, as observed on the control for all studied species on this research (Figure 3A).

The alterations were the shrinkage of the Bowman's capsule and disarrangement of the proximal tubules epithelium in all assessed concentration for *P. caudimaculatus* and *H. eques*, whereas did not occurred alterations of the tissue structure on *P. mesopotamicus* in any tested concentration (Table 6 and Figure 3B).

The glomerulus capsule release, the disarrangement of the proximal tubules epithelium and the proximal and distal tubule light increase was also observed on *O. mykiss* exposed to linuron herbicide (Oulmi et al., 1995) and on *O. niloticus* exposed to Roundup[®] (Jiraungkoorskul et al., 2002). The Bowman's capsule space increase, or the capsule release, and the disarrangement of the tubules cells was also observed on *C. gariepinus* exposed to GFT, which leads to the kidneys physiological functions loss (Ayoola, 2008).

Conclusions

The fish species *H. eques*, *P. caudimaculatus* and *P. mesopotamicus* present great potential to be used as standard organisms for ecotoxicological assays for herbicides monitoring, for they present an excellent answer to the tested reference substance. The addition of

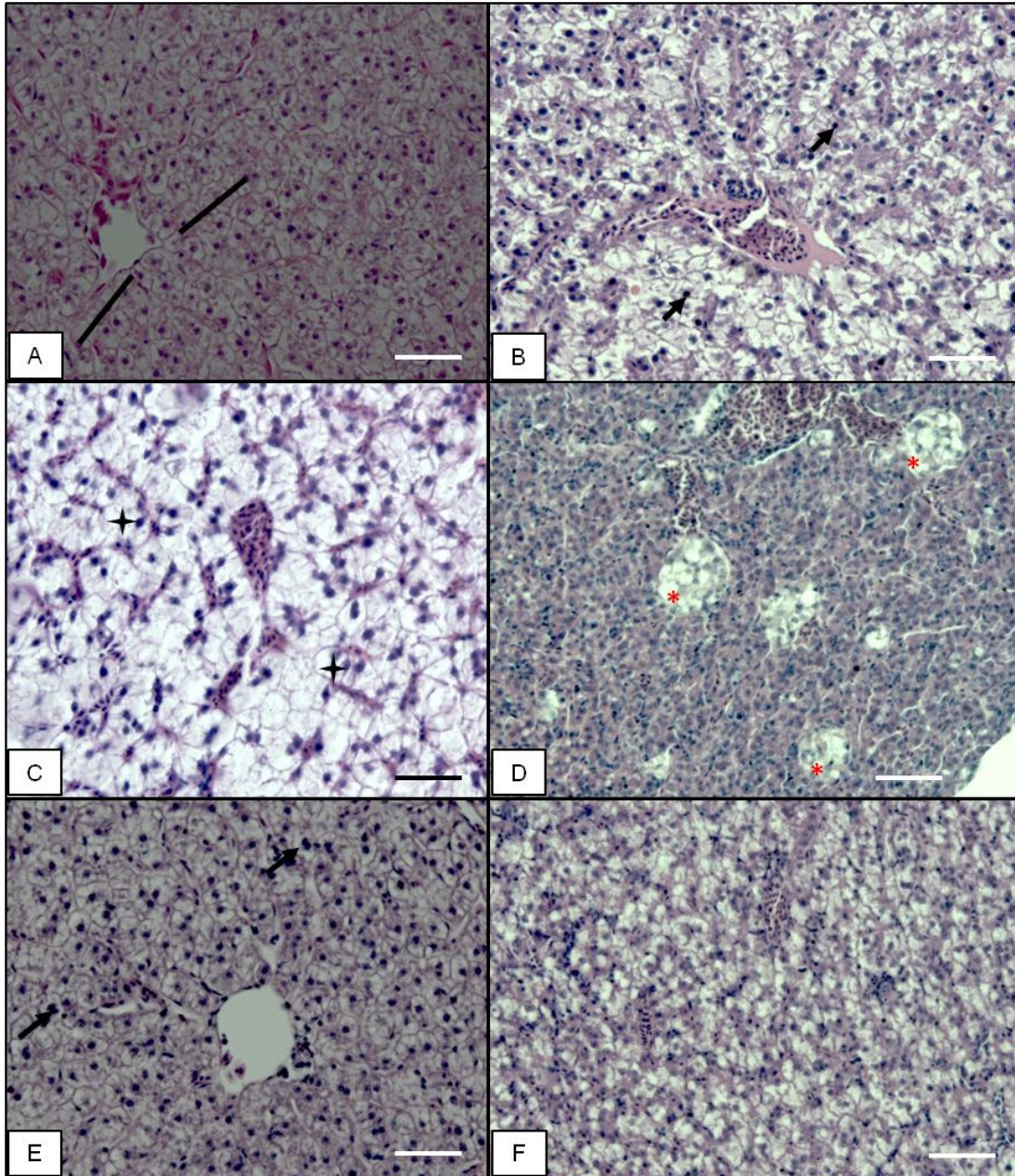


Figure 2. A. Cordonal organization of the hepatocytes (line) from *H. eques* (control). B. Picnotic nucleus (Arrow) (900.0 mg L^{-1}) on *H. eques*. C. Hepatocyte hypertrophy (star) (925.0 mg L^{-1}) on *H. eques*. D. Necrosis spots (asterisk) (975.0 mg L^{-1}) on *P. caudimaculatus*. E. Cell fusion (arrow) (950.0 mg L^{-1}) on *P. mesopotamicus*. F. Disarrangement of the hepatocytes cordonal structure (900 mg L^{-1}) on *P. caudimaculatus*. Bar = $12.5 \mu\text{m}$. Color HE.

Table 6. Histological alterations on fish kidneys after glyphosate exposure.

Concentration (mg.L^{-1})	<i>P. mesopotamicus</i>	<i>P. caudimaculatus</i>	<i>H. eques</i>
0.0	0	0	0
900	0	1, 2 and 3	1, 2 and 3
925	0	1, 2 and 3	1, 2 and 3
9 ₅₀	0	1, 2 and 3	1, 2 and 3
975	0	1, 2 and 3	1, 3 and 3

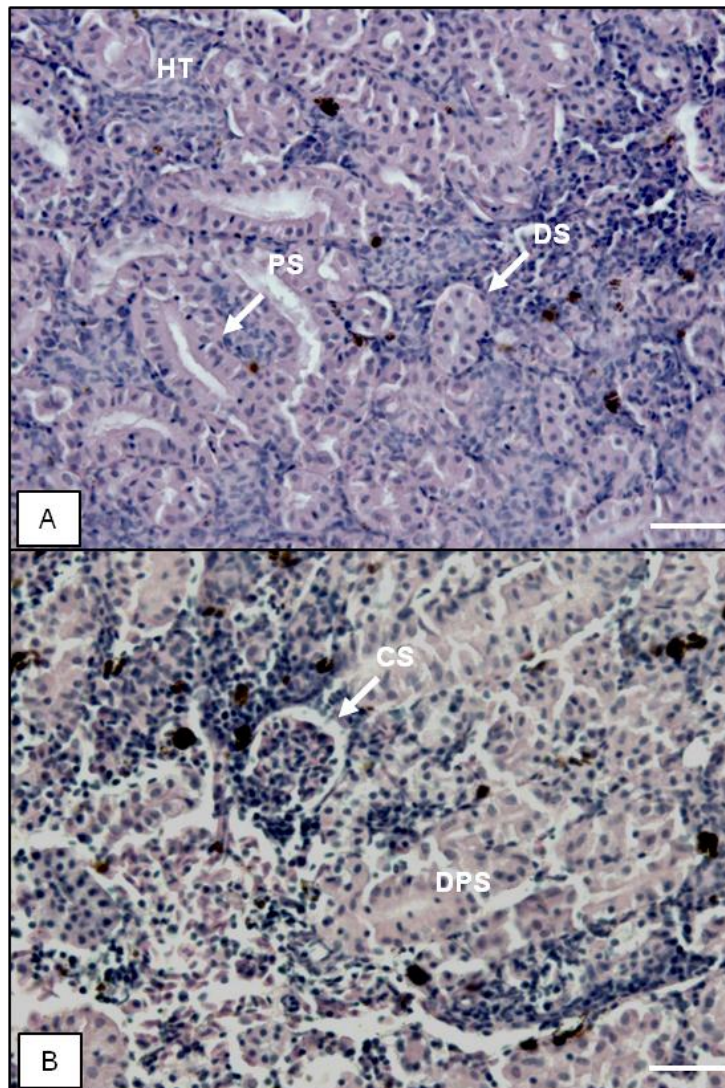


Figure 3. Kidneys photomicrography. A. Proximal segment (ps), distal segment (ds) (control), hematopoietic tissue (HT) on *P. mesopotamicus*. B. Shrinkage of the Bowman's capsule (cs) and disarrangement of the epithelium proximal segment (dps) on 975 mg L⁻¹ on *H. eques*, Bar: 12.5 μ m. Color HE.

surfactants to glyphosate formulations may change the herbicide acute toxicity pattern.

The use of glyphosate without surfactant addition is enough to cause histological alterations on *H. eques* and *P. caudimaculatus*, featuring specially the kidneys and liver as the xenobiotics presence biomarkers, which makes them possible to be applied on environmental monitoring studies.


Conflict of interests

The authors have not declared any conflict of interests.

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