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Research article

Liver biomarkers response of the neotropical fish *Aequidens metae* to environmental stressors associated with the oil industry

Wilson Corredor-Santamaría [a,b], Diego A. Mora-Solarte [a], Ziv Arbeli [b], José M. Navas [c], Yohana M. Velasco-Santamaría [a,*]

[a] *Grupo de Investigación en Biotecnología y Toxicología Acuática y Ambiental - BioTox, Facultad de Ciencias Agrícolas y Recursos Naturales, Universidad de los Llanos, km 12 vía Puerto López, vereda Barcelona, Villavicencio, Colombia*

[b] *Unidad de Saneamiento y Biotecnología Ambiental (USBA), Departamento de Biología, Facultad de Ciencias, Pontificia Universidad Javeriana, Cra. 7 N. 43-82, Bogotá, Colombia*

[c] *Departamento de Medio Ambiente, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA_CSIC), Ctra. De la Coruña Km 7.5, E-28040, Madrid, Spain*

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ABSTRACT

The Acacias River in Colombia receives large volumes of industrial effluents mostly derived from the oil industry. To contribute to the study of the possible effects of industrial wastewaters on the aquatic environment and particularly on fish populations, a native neotropical fish, *Aequidens metae* was used as a sentinel species. Wild specimens of *A. metae* were caught at three different places of the Acacias River taking as reference the point of discharge of an oil industry effluent; upstream, downstream, and at the vicinity of the discharge pipe. A fourth sampling site was chosen as a reference site away from urban settlements. Samplings were performed twice, during the rainy and dry seasons. After anesthesia animals were weighted and measured, and humanely sacrificed. Livers were extracted, frozen on site and transported to the laboratory. Condition indices were calculated. Total protein content and the detoxification 7-ethoxyresorufin-*O*-deethylase (EROD) enzyme activity were estimated. Histopathological alterations were also evaluated. Water quality was estimated through the measurement of several variables. Results obtained evidenced that the highest induction in EROD activity and the strongest histological alterations in liver of the monitored fish appeared during the dry seasons at the discharge site and downstream to this point.

1. Introduction

The Orinoquia region in Colombia shows a biological diversity and ecological functionality of great importance. This region is also the main contributor to the production of crude oil in the country. The effluents generated by the processes of extraction of petroleum are regularly discharged in rivers (Velasco-Santamaría et al., 2019) after primary treatment processes such as mechanical evaporation (Mesa et al., 2018). There is an enormous lack of knowledge about the effect of this wastewater, known as produced water (PW), on the health of aquatic biota. In Colombia, the Acacias River runs through a region with a very intense oil extraction activity and receives significant PW loads.

PW contains a plethora of natural compounds that appeared along millions of years during the process of oil formation. These substances include inorganic salts, metals, radioisotopes, and a wide variety of organic chemicals, mainly polycyclic aromatic hydrocarbons (PAHs), among others (Neff et al., 2011). PAHs are harmful persistent organic pollutants, of which the United States Environmental Protection Agency (USEPA) has included 16 as priority pollutants due to their potential carcinogenicity for humans (Keith, 2015). Exposure to PAHs can induce various toxic responses in fish (Cousin and Cachot, 2014). Many xenobiotic compounds are transformed by fish into water-soluble metabolites following the reaction routes of phases I and II (Ikenaka et al., 2013). These processes lead to a reduction of toxicity, but they can also result in the bioactivation of chemicals that are transformed into more toxic compounds (Incardona et al., 2006). These biotransformation processes take place in the liver, which is one of the main target organs of PAHs (Soltani et al., 2019). PW as a source of PAHs has been monitored in *in situ* studies using exposed aquatic organisms (Tornero and Hanke, 2016). However, information regarding the effect of wastewater from

* Corresponding author.
E-mail address: ymvelascos@unillanos.edu.co (Y.M. Velasco-Santamaría).

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continental exploitations of the oil industry on native fish species is scarce (Zabbey and Olsson, 2017). Dynamic ecosystems may respond differently in *in situ* studies compared to experimental conditions. Therefore, to understand the actual effects of exposure to PAHs, sampling of the ecosystem components (e.g., water and fish) is required.

To provide information on the impact of pollution on aquatic organisms, our research group has proposed the native neotropical cichlid fish *Aequidens metae* as a potential bioindicator, given its benthic and detritivorous habits, and its abundance and adaptability to laboratory conditions (Corredor-Santamaría et al., 2019; Corredor;Santamaría et al., 2016).

Polyaromatic compounds internalized by organisms are detoxified through oxidation and conjugation reactions catalyzed by enzymes such as the cytochrome P450 oxidase superfamily. Among these, cytochrome P4501A (CYP1A) has an important role in the metabolism of planar compounds such as some PAHs (Zanger and Schwab, 2013). The induction of CYP1A can be evidenced by the measurement of a related activity such as 7-ethoxy-resorufin-O-deethylase (EROD) activity (Petrulis et al., 2001).

Likewise, histological studies in wild fish exposed to contaminated water bodies provide complementary information about the pathological processes triggered by contaminated effluents on the tissue morphology. However, to understand histological findings in field studies, the observed changes must be related to results from other biomarkers (Liebel et al., 2013).

The aim of this study was to increase our knowledge about the impact of PW on the Acacias River biota, particularly on fish, by evaluating histopathological and biochemical biomarkers of exposure to petroleum hydrocarbons in a native cichlid fish species, *A. metae*.

2. Materials and methods

2.1. Sampling sites

A discharge pipe from an oil extraction station was localized at the Acacias River and used as a point of reference for fish and water samplings. The neotropical cichlid fish *A. metae* was selected for this study. Fish and water samples were collected at three different sites of the Acacias River in Meta, Colombia: site 1, upstream of the discharge pipe (3°57.490′ N 73°40.345′); site 2, in the vicinity of the discharging pipe (3°57.214′ N 73°40.100′); site 3, downstream of the discharge pipe (3°57.268′ N 73°39.832′). In addition, samplings were carried out at a fourth location known as Caño Cuncia (4°03.117′ N 73°43.780′) chosen as a reference site, considering that it is isolated from human activity and shows a reduced likelihood to receive pollutants.

In each sampling event, temperature and dissolved oxygen were measured with a HACH-5130 portable probe (Loveland, CO, USA). Alkalinity, pH, nitrates, nitrites, and phosphates were determined with an YSI 9300 photometer (YSI, Yellow Springs, OH, USA). Fish and water samples were collected from each sampling site (n = 3) at the Acacias River and from the reference site during November 2018 and February 2019 (dry season), and April and May 2019 (rainy season). At each sampling site, one water sample (500 mL) was collected and sent to the Water Laboratory at the Universidad Industrial de Santander (UIS) to establish the concentration of the 16 PAHs prioritized by the United States Environmental Protection Agency (USEPA, 1986).

2.2. Sample procedure

Based on previous studies with the sea fish Yellowtail scad (*Trachurus novaezelandiae*) and skipjack trevally (*Pseudocaranx wrighti*) and the freshwater fish Black Bream (*Acanthopagrus butcheri*) (Gagnon and Hodson, 2012; Gagnon and Rawson, 2016), twelve wild specimens of *A. metae*, were caught at each monitoring site according to Espírito-Santo et al. (2009). Immediately after capture, fish were anesthetized by immersion in a 300 mg/L solution of 2-phenoxyethanol (J. T. Baker, Phillisburg, USA). Subsequently, the fish were desensitized by medullary sectioning. Liver was removed and weighed (HOPEX Germany® digital balance). A portion was fixed in 10% buffered formaldehyde for histopathological analysis. The remaining tissue (~100 mg) was transported in nitrogen vapors (Doble11 2001® series) to the laboratory and stored at -70 °C until analysis of EROD activity. In addition, due to logistical aspects it was not possible to collect blood samples and therefore no hematological parameters were analyzed. The fish specimens were registered in the collection of the Museum of Natural History of Unillanos - Universidad de los Llanos. The number of male and female fish varied in each sampling event.

2.3. Condition indices

Hepatosomatic index (HSI) and condition factor (CF) were calculated according to the following equations: HSI = (liver weight/total body weight) × 100; CF = (total body weight/total length^3) ×100.

2.4. Histological evaluation

Tissues were fixed in buffered formaldehyde (pH 7.2) for five days at 4 °C. Subsequently, they were dehydrated using increasing concentrations of ethanol (70–100 %). The obtained tissues were mounted in paraffin (MERCK, melting point 52–54 °C) and sectioned at 3 μm using a CUT 5062 microtome (SLEE Medical GmbH, Germany). Finally, the samples were stained with hematoxylin and eosin (H&E). Five sections of each tissue from each fish were examined using an Eclipse-E100 optical microscope (Nikon, Japan) coupled to a Nikon camera.

The occurrence of liver histopathological alterations was determined semi-quantitatively, considering the degree of tissue change (DTC), which was based on the severity of the lesions. Histopathological alterations were classified as a score from 0 to 3, where 0 = no alteration, 1 = slight alteration, 2 = moderate alteration and 3 = severe alteration (Taheri et al., 2016). Slight, moderate, and severe alterations in liver were considered as follows according to Bernet et al. (1999). (I) Slight alteration, implies changes that do not cause damage to the hepatic tissue, considering that recovery of the liver can occur with the restoration of normal environmental conditions. These changes are limited to small, isolated pockets in the liver. (II) Moderate alteration implies changes that are more severe and produce negative effects that could affect the functioning of the organ. Although lesions are repairable, however, if damaged areas increase or if tissues are chronically exposed to pollution, they may derive into severe alterations. Moderate alterations can involve more than half of the liver tissue. (III) In the severe alterations the restoration of the architecture of the liver is not possible so that the observed damage is irreversible.

A DTC value was calculated for each animal using the following formula: DTC = (1X SI) + (10X SII) + (100X SIII), where SI, SII and SIII correspond to the sum of the number of alterations for each stage (n = 6 fish). DTC values between 0 and 10 indicate normal organ functioning; values between 11 and 20 indicate slight organ damage; between 21 and 50 indicate moderate organ changes; between 50 and 100 indicate severe organ damage; and values above 100 indicate irreparable organ damage (Poleksic and Mitrovic-Tutundzic, 1994).

2.5. Histometric evaluation

The diameters, area (μm) and number of hepatocyte nuclei (which correspond to the number of hepatocytes in the observed photographic field) were measured and counted in 30 photographic fields per fish liver (n = 6 per sampling event) using a region of interest (ROI) of 10,000 μm^2. Results are presented as the mean and standard error for each sampling site. ImageJ (NIH version 1.44, USA) was used for the measurements.

2.6. EROD activity assay

The procedure for the determination of EROD activity was selected according to multiple scientific literature supporting the effectiveness of the protocol (Lammel et al., 2015; Quesada-García et al., 2013, 2015; Valdehita et al., 2012) and its reproducibility was standardized and validated in preliminary assays with different PAHs and flavonoids such as benzo[a]pyrene, pyrene and beta-naphthoflavone. EROD activity in liver of *A. metae* was determined in a S9 fraction according to the method described by Valdehita et al. (2012) after standardization. Approximately 30 mg of liver was gently homogenized at 4 °C in 1 mL of homogenization buffer (0–1 M Tris-HCl pH 7,0–25 mM sucrose, 150 mM KCl, 1 mM EDTA, 1 mM DTT, 0–25 mM PMSF and 20% glycerol). The homogenized material was centrifuged at 6000 g for 10 min. After centrifugation, the pellet was discarded, and the resulting supernatant was centrifuged at 16 000 g for 60 min. The resulting pellet was suspended in 100 μL of homogenization buffer. Finally, EROD activity and protein content were immediately measured as described by Burke and Mayer (1974). Fluorescence measurements were made in a Cytation 3 spectrophotometer (BioTek®, Winooski, VT, USA). EROD activity was normalized to the protein content and measured with fluorescamine (Navas and Segner, 2000).

The biochemical and histological procedures were performed in the Toxicology and Biotechnology Laboratory at the School of Animal Science at the Universidad de los Llanos, Villavicencio, Meta, Colombia. All procedures involving handling of animals were performed in accordance with the standards and procedures for the use of laboratory animals approved by the Bioethical Committee from the Universidad de los Llanos.

2.7. Statistical analysis

Descriptive statistical analysis was performed expressing the data as mean ± standard error of the mean (SEM). Homogeneity of variance (Levene's and Bartlett) and normal distribution of data (Kolmogorov-Smirnov) tests were performed to verify these assumptions. To evaluate the effect of different monitoring points on response variables, a two-way analysis of variance (ANOVA) was performed followed by the Tukey test. When the data did not fulfill the assumptions of normality and homogeneity of variance, we proceeded to transform them (log, square-root, or arcsine transformations). If data did not meet in the end the assumptions, then they were ranked. In all cases, a value of $p < 0.05$ was used as the level to consider statistically significant differences. Statistical procedures were performed using SAS software for Windows version 9.02 (2002–2006, SAS Institute Inc., Cary, NC, USA) and GraphPad v 5.0.

3. Results and discussion

This is a pioneering study for the dissemination of data concerning the effect of pollution of the Acacias River on aquatic biota using a native fish species as sentinel.

3.1. Physicochemical parameters

The increase in alkalinity at the PW discharge site has been associated with the decarboxylation of some acids present in the effluent as acetate and other short-chain aliphatic acids, which are an important source of CO_2 in these waters (AlAnezi et al., 2018). Likewise, alkalinity of PW can be associated to the presence of dissolved bicarbonate and carbonate, which neutralize acids, in addition to carbon dioxide, contributing to reactions that modify the pH of natural waters (Neff et al., 2011). The high concentration of phosphates at the discharge site and in downstream waters (Table 1) at different times of the year evidence the dumping of high amounts of organic matter into the Acacias River, as reported in other natural water bodies exposed to industrial (Sengupta et al., 2015) and domestic (Trujillo-González et al., 2017) wastewater. The observed concentrations clearly exceed the recommended concentration of 0.02 mg/L for temperate continental waters. The mentioned levels for temperate waters cannot be directly comparable with those observed in tropical waters that naturally show a high content in phosphates (as it is the present case and it can be observed in waters upstream of the discharge site and in the reference site, Table 1). However, they can be contributing to eutrophication of rivers and lakes (Sengupta et al., 2015). Water eutrophication can lead to lesions in fish gills such as lamellar hyperplasia in a first attempt to increase gill surface area. Paradoxically, hyperplasia can provoke laminar fusion leading to a reduction of water space and of gas exchange capacity (Dalzochio et al., 2017; Hassaninezhad et al., 2014). It must also be considered that river

Table 1. Physicochemical parameters and PAHs concentrations in waters samples from the Acacias River and reference site, Meta Department, Colombia.

Water physicochemical parameters	Upstream discharge		Discharge		Downstream discharge		Reference	
	Dry	Rainy	Dry	Rainy	Dry	Rainy	Dry	Rainy
Temperature °C	27.40 ± 3.00	25.35 ± 0.25	28.60 ± 1.30	29.30 ± 0.50	31.00 ± 2.40	26.55 ± 0.55	27.75 ± 1.05	24.95 ± 1.05
pH	7.28 ± 0.27	6.88 ± 0.28	7.90 ± 0.10	7.40 ± 0.60	7.60 ± 0.40	6.83 ± 0.02	7.30 ± 0.70	6.68 ± 0.67
Dissolved oxygen (mg/L)	7.00 ± 1.30	8.42 ± 2.28	6.12 ± 0.82	6.46 ± 1.14	7.86 ± 1.47	8.89 ± 1.61	7.02 ± 1.52	7.88 ± 1.52
Alkalinity (mg/L)	32.50 ± 22.50	70.00 ± 30.0	525.0 ± 75.0#	550.0 ± 50.0#	82.50 ± 32.50	85.25 ± 0.25	15.00 ± 5.0	10.50 ± 55.00
Nitrites (mg/L)	0.33 ± 0.10	0.10 ± 0.04	0.52 ± 0.43	0.09 ± 0.00	0.50 ± 0.48	0.10 ± 0.05	0.02 ± 0.01	0.02 ± 0.01
Nitrates (mg/L)	2.99 ± 0.37	3.54 ± 0.94	3.74 ± 1.26	3.08 ± 1.32	3.59 ± 0.8s9	2.14 ± 0.22	2.02 ± 0.59	3.41 ± 0.39
Ammonia (mg/L)	0.00 ± 0.00	0.15 ± 0.03	1.07 ± 0.94	1.41 ± 0.60	0.56 ± 0.44	0.24 ± 0.21	0.00 ± 0.0	0.11 ± 0.01
Phosphates (mg/L)	9.30 ± 2.0	15.45 ± 7.65	15.65 ± 11.85	32.20 ± 7.10	15.15 ± 11.85	16.20 ± 7.80	8.30 ± 4.80	14.48 ± 2.70
Streamflow (m^3/s)	0.35 ± 0.25	1.75 ± 0.95	0.13 ± 0.03	0.80 ± 0.40	0.30 ± 0.10	1.00 ± 0.40	0.45 ± 0.15	0.90 ± 0.50
PAHs analysis (ng/L)								
Naphthalene	•	130	250	<50*	<50*	<50*	<50*	•
2-Methylnaphtalene	•	270	760	•	<50*	•	<50*	•
Acenaphthene	•	•	<50*	•	•	•	•	•
Fluorene	•	•	<50*	•	•	•	•	•
Phenanthrene	•	•	70	•	•	•	•	•
Pyrene	•	•	<50*	•	•	•	•	•
Benz[a]anthracene	•	•	<50*	•	•	•	•	•

Dry season (DS); rainy season (RS); # For alkalinity parameter a statistical difference was observed among the monitoring sites for the same season • Below detection limit * Detected below limit of quantification. Detectable limit (DL): 50 ng/L. m^3/s: cubic meters per second. Values for PAHs correspond to water samples taken at a depth of 50 cm in the water column.

water phosphate levels have shown a dramatic increase by a factor of more than 10 over the past 20 years, mainly due to anthropogenic inputs (Yang et al., 2008).

3.2. Water analyses

Colombian legislation regarding the discharge of wastewater from mining activities (Resolution 0631/2015, Decree 1594/84) does not establish maximum permitted levels of PAHs. Thus, the gap in environmental regulations can generate risks to the organisms inhabiting aquatic ecosystems.

The highest concentrations of naphthalene (250 ng/L), methylnaphthalene (760 ng/L), and phenanthrene (70 ng/L) appeared during the dry season at the discharge site (Table 1). High concentrations of naphthalene (130 ng/L) and 2-methylnaphthalene (270 ng/L) were also found upstream of the discharge site. They can be attributed to biomass burning (Etter et al., 2010). It should be noted that the PAHs levels found upstream may have contributed to the increased concentration at the discharge site. Several studies carried out in water bodies receiving PW showed much higher PAHs concentrations than those observed in the present study with values of 538.43 ng/L for naphthalene, 28.22 mg/L for methylnaphthalene and 28.42 mg/L for phenanthrene (Akinsanya et al., 2018; Okpashi et al., 2017).

Concentrations detected in the present work may cause alterations on the exposed fish. For instance, intraperitoneal injection of 50 μg/g of naphthalene in rainbow trout (*Oncorhynchus mykiss*) provoked a decrease in serotonin metabolism in hypothalamus, preoptic region, pituitary, and brain stem (Gesto et al., 2006). In guppy (*Poecilia vivipara*), water exposure to phenanthrene (200 μg/L) induced behavioral alterations such as reduced swimming resistance and speed that led to reduced prey capture rates (Torreiro-Melo et al., 2015). Intraperitoneal injection of 10 μg/g of phenanthrene in the Neotropical characidae *Astyanax bimaculatus*, generated genotoxic effects such as induction of micronuclei and other nuclear abnormalities in peripheral erythrocytes (Corredor-santamaría et al., 2012). Similarly, in seabream (*Acanthopagrus latus*) intraperitoneal exposure to 20 and 40 μg/g of phenanthrene caused after 4 days immunosuppression with significant decreases in plasma levels of immunoglobulin M, phagocytic activity, and respiratory burst. This immunosuppressive effect was accompanied by an increase in the frequency of micronuclei in erythrocytes and induced pathological alterations in the spleen, including increased melanomacrophage centers (MMC), destroyed red blood cells, and bleeding (Shirmohammadi et al., 2017).

Regarding acenaphthene, fluorene, pyrene, and benzo[a]anthracene concentrations, they were below the limit of quantification, i.e., close to the concentration of 50 ng/L. For the other PAHs, concentrations were below the limit of detection (Table 1).

3.3. Condition indices

Liver average weight was 182.35 ± 17.49 mg. Responses to environmental stress were reflected in the HSI (Table 2). The highest values of HSI correspond to fish captured at the discharge site during the dry season. They are similar to those reported by Araújo et al. (2017), who observed in the freshwater catfish *Pimelodus maculatus* higher HSI values related with higher EROD induction at contaminated sites in comparison with a reference site. High values of HSI could have resulted from exposure to PAHs and other pollutants, which cause liver hypertrophy.

Condition factor values are shown in Table 2. The highest CF values were observed in fish caught upstream of the discharge site in the dry season. The CF is a parameter describing the health status of the fish, considering that the heaviest fish of a given length are in a better physiological condition (Jisr et al., 2018). Likewise, the CF evidence recent physical and biological alterations since it is strongly influenced by biotic and abiotic environmental factors. CF changes in response to the interaction between eating habits, parasitic load, and physiological condition of fish (Maceda-Veiga et al., 2017). It can be used to compare the welfare of fish from similar or different habitats and serves as an index to assess the status of the aquatic ecosystems in which fish are present (Hook et al., 2014).

3.4. Histopathological analysis

Liver architecture of *A. metae* exhibited polygonal hepatocytes with granular cytoplasm, central rounded nuclei, and evident nucleoli (Figure 1A). In this species, hepatocytes did not show the characteristic disposition in filaments bordering the sinusoids as observed in other fish species. Vacuolization and displaced nuclei were observed in the periphery of hepatocytes (Figure 1D, E and F) of the liver of fish caught in the discharge and downstream of the discharge sites (Figure 1D, E and F).

Calculated values for DTC appear in Table 3. Stage I DTC is characterized by reversible changes such as deformation of the nuclear contour, nuclear displacement to the cell periphery, vacuolated cytoplasm, irregular shape of the hepatocytes and nuclei, and lateral position of the nucleus. Stage I DTC were observed at all sites in the Acacias River, with greater intensity in the dry season, Livers under Stage II DTC exhibited nuclear degeneration (Figure 1D) that was higher in fish captured at the discharge place and downstream in both seasons. -Stage III DTC features (necrosis), constituting an irreparable tissue injury, were rarely observed, and only appeared in fish caught at the discharge site and downstream in the dry season. The observed liver lesions are probably related with the key role played by this organ in the metabolism and excretion of toxic substances, so that morphological changes associated to detoxification processes occur normally and are exacerbated by increases in the concentration of pollutants.

An increased frequency of histological alterations can indicate dysfunctions induced by toxic agents with a reduction of metabolically active areas, leading to a possible reduction in the overall functioning of this organ (Kostić et al., 2017; Van der Oost et al., 2003)., DTC values observed in livers of *A. metae* were below 50 corresponding to Stages I and II, being classified as repairable (Table 3). Other studies evaluating the impact of wastewater discharges on native fish (*Astyanax bimaculatus*, *Capoeta capoeta*, *Carassius auratus*, *Zacco platypus*, and *Zacco koreanus*) living in contaminated rivers (Ocoa-Colombia, Karasu-Turkey, Eungcheon, Mihocheon, and Busocheon-Korea, respectively) (Dane and Şişman, 2015; Samanta et al., 2018; Velasco-Santamaría et al., 2019) reported similar effects.

Table 2. Hepatosomatic index and condition factor (mean ± SEM) of wild *A. metae* caught at three places of the Acacias River and in a reference water body in dry and rainy seasons.

Monitoring Site	Hepatosomatic Index (%)		Condition Factor	
	Dry	Rainy	Dry	Rainy
Upstream discharge	$2,06 \pm 0,15^{def}$	$1,36 \pm 0,13^{ab}$	$2,60 \pm 0,08^{de}$	$2,20 \pm 0,05^{abc}$
Discharge	$3,83 \pm 0,21^{g}$	$1,80 \pm 0,14^{bde}$	$2,06 \pm 0,09^{ab}$	$2,24 \pm 0,06^{abcd}$
Downstream discharge	$2,39 \pm 0,23^{ef}$	$1,11 \pm 0,15^{a}$	$2,03 \pm 0,10^{a}$	$2,33 \pm 0,07^{abcd}$
Reference	$1,54 \pm 0,15^{abcd}$	$0,96 \pm 0,10^{abc}$	$2,11 \pm 0,07^{abc}$	$2,27 \pm 0,12^{abcd}$

^{abcdef} Significant comparisons between seasons (rainy and dry) and among monitoring sites are shown with different superscript letters (Tukey's test; $p < 0.05$).

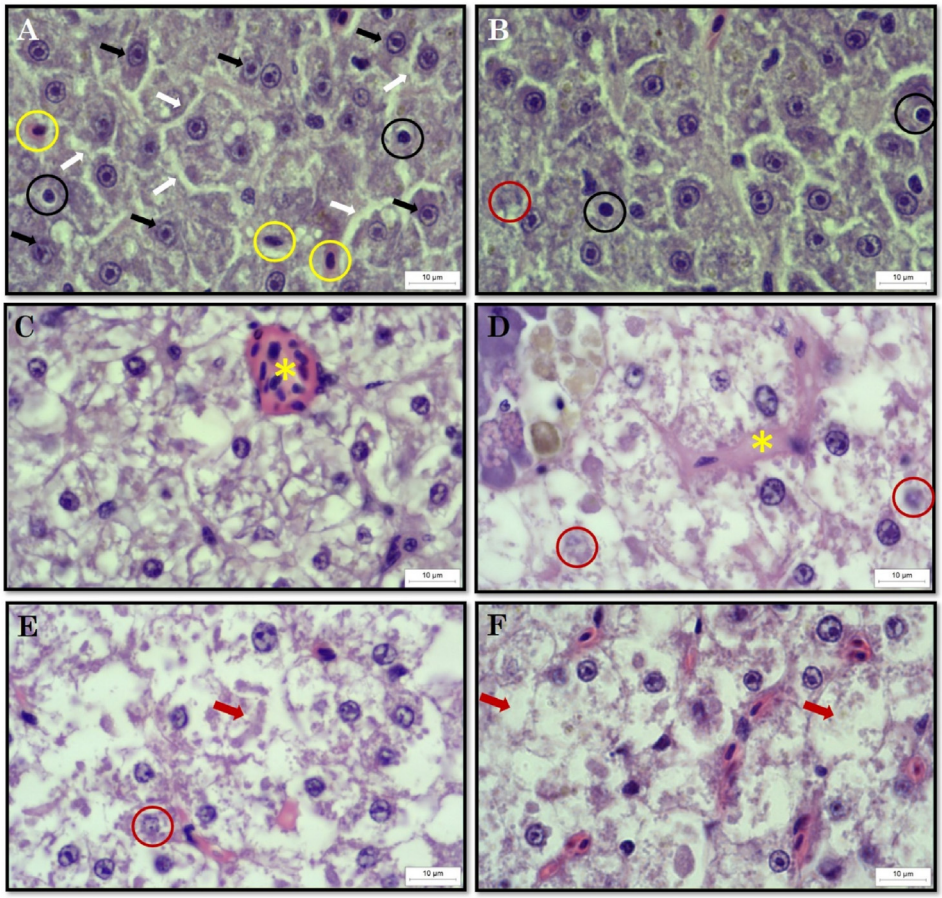

Figure 1. Alterations in hepatic tissue of *A. metae* caught at the Acacias River and in a reference water body in different precipitation seasons. A. Liver of reference site captured fish showing normal morphology. Hepatocytes exhibit central regular size nuclei and concentric nucleolus (black arrow). Erythrocytes (yellow circle)-Endothelial cells (black circle). Sinusoid (white arrow). B. Liver from fish captured upstream of the discharge site. C-D. Liver from fish captured at the discharge site. E- F. Downstream to the discharge site. Hyperemia (yellow asterisk), nuclei degeneration (red circle), cytoplasmic degeneration (red arrow), can be observed in B to F. Note in C–F the hepatocellular and nuclear pleomorphism, with enlarged hepatocytes exhibiting loose granular cytoplasm. Hematoxylin and eosin staining.

Slight morphological alterations in livers of fish captured upstream of the discharge site and in the reference, site was found as physiologically tolerable although they were indicative of exposure to xenobiotics as it has been reported in a study previously published by our group (Velasco-Santamaría et al., 2019). In turn, although alterations evidenced on the hepatic architecture of fish captured at the discharge site and downstream are classified as reversible, they may have a notable impact on detoxification of PAHs and water pollutants (Camargo and Martinez, 2007).

A. metae specimens caught in the Acacias River showed cytoplasmic and nuclear degeneration and slight areas of focal necrosis, which could be associated with energy depletion, microtubule separation, and inhibition of protein synthesis, which could induce fragmentation of cytoplasmic and nuclear material (Van Dyk et al., 2007). Consequently, necrotic foci derived from hypoxia could be evidenced in the presence of hyperemia (Maharajan et al., 2016).

Regarding histometric evaluation, nuclei diameter and hepatocyte area were higher in *A. metae* caught at the discharge site and downstream ($p < 0.05$) than in fish caught upstream and at the reference site in both seasons (Table 3). Variations in hepatocyte nuclei size are associated with a response to a stressful stimulus and can point out to the activation of liver function as an outcome of increased metabolic activity against adverse conditions (Figueiredo-Fernandes et al., 2007; Wolf and Wheeler, 2018). Similarly, the hypertrophy of hepatocytes found in *A. metae* caught at the discharge site and downstream t can be associated with structural changes related to increased metabolic activity.

A higher number of hepatocyte nuclei were observed in *A. metae* caught in all sites of Acacias River compared to fish captured in reference site ($p < 0.05$) (Table 3).

In the present study, the increase of hepatocyte nuclei (i.e., the increase of cells in the observed microscope fields) and the presence of necrosis in the parenchyma could be associated to the release of substances that induce cell proliferation to replace dead cells and restore liver structural and functional architecture (Wolf and Wolfe, 2005). This signal reflects the induction of the cell cycle that takes place in parallel to alterations caused on liver function by PAHs during detoxification processes (Chramostová et al., 2004; Figueiredo-Fernandes et al., 2007).

Table 3. Degree of tissue change (DTC) in hepatic tissue, area, diameter, and number of hepatocytes in *Aequidens metae* caught at the Acacias River and reference site, Meta, Colombia, during the dry and rainy seasons. (n = 6).

Monitoring Site	DTC		Number of nuclei		Area (μm^2)		Diameter (μm)	
	Dry	Rainy	Dry	Rainy	Dry	Rainy	Dry	Rainy
Upstream discharge	$12,78 \pm 0,42^{abc}$	$13,85 \pm 0,57^{bc}$	$23,60 \pm 0,84^{bc}$	$23,19 \pm 0,75^{b}$	$17,64 \pm 0,16^{bc}$	$17,62 \pm 0,15^{bc}$	$5,19 \pm 0,02^{bcd}$	$5,18 \pm 0,02^{bcd}$
Discharge	$34,83 \pm 0,69^{def}$	$33,58 \pm 1,23^{de}$	$29,78 \pm 0,97^{ef}$	$31,99 \pm 1,09^{efg}$	$23,90 \pm 0,20^{d}$	$20,25 \pm 0,14^{d}$	$5,98 \pm 0,02^{e}$	$5,35 \pm 0,02^{e}$
Downstream discharge	$32,04 \pm 0,65^{d}$	$33,94 \pm 0,84^{def}$	$24,84 \pm 0,70^{bcd}$	$34,53 \pm 0,76^{g}$	$17,31 \pm 0,08^{b}$	$18,81 \pm 0,10^{d}$	$5,14 \pm 0,01^{bc}$	$5,13 \pm 0,01^{b}$
Reference	$11,93 \pm 0,53^{a}$	$12,24 \pm 0,61^{ab}$	$27,00 \pm 0,64^{de}$	$18,68 \pm 0,48^{a}$	$16,21 \pm 0,12^{a}$	$20,41 \pm 0,13^{d}$	$4,91 \pm 0,02^{a}$	$5,45 \pm 0,03^{e}$

^{abcdef} Significant comparisons between seasons (rainy and dry) and among monitoring sites are shown with different superscript letters (Tukey's test; $p < 0.05$).

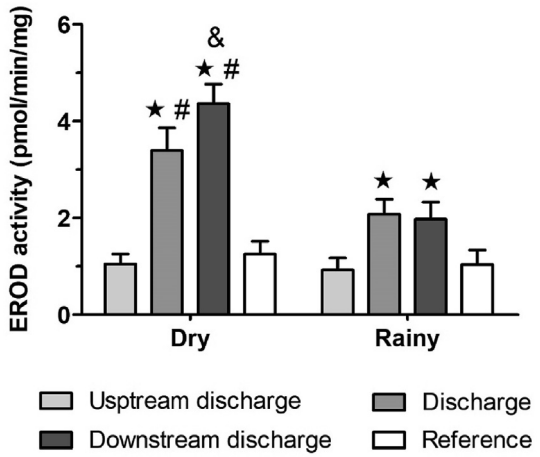

Figure 2. Hepatic EROD activity ±standard error of the mean (n = 12) in livers of wild *A. metae* captured on two seasons (dry and rainy) in three different places (discharge, downstream and upstream of the discharge site) of the Acacias River, Colombia, and in a reference water body with lower probability of pollution. * Indicates significant differences with respect to the site upstream of the discharge place, for the same season. # Indicates significant differences with respect to the reference site, for the same season. & Indicates significant differences with respect to the same site at different season. Two-way ANOVA and Tukey's post hoc test ($p < 0.05$).

3.5. EROD activity and total protein concentration

The higher EROD activity observed in fish caught at the discharge site and downstream (Figure 2), suggests that the Acacias River waters can have high levels of xenobiotics. This effect could be linked to the presence of PAHs and other pollutants that are also inducers of this activity. Increased EROD activity has been reported in fish exposed to a wide range of pollutants, such as PAHs, polychlorinated biphenyls, and tetrachlorodibenzo-p-dioxin (Basu et al., 2004; Gagnon and Rawson, 2016; Lindberg et al., 2017). In a series of in vitro studies, an increase of EROD activity has been described and characterized in a rainbow trout liver derived cell line exposed to the water accommodated fraction (WAF) of oil samples collected from an oil spill (Prestige oil spill, in the North-West of Spain) (Navas et al., 2006). Such results suggest that this enzyme activity is an appropriate biomarker for detecting effects of oil spills or oil derivatives in wild fish. Among different biomarkers evaluated in several studies, EROD activity appears as the most specific for PAHs exposure. Strong responses occur in PAHs exposed fish regardless of habitat or route of exposure, indicating that EROD activity is a robust biomarker suitable for a variety of fish species and experimental scenarios (Santana et al., 2018). However, information on differences in EROD activities in wild fish exposed to pollutants from PW is limited in the Colombian Orinoco region.

Regarding the influence of environmental variables, it has been reported that high water temperature can contribute to the induction of CYP1A (EROD) liver activity in juvenile Atlantic cod (*Gadus morhua*) exposed to the WAF and the chemically enhanced (or dispersed) WAF of crude oil (Lyons et al., 2011). Similarly, in the present study the highest EROD activity was seen in fish caught at the sites that recorded the highest temperatures.

Regarding the effect of pH, it has been observed that exposure of *Oreochromis mossambicus* to low concentrations of naphthalene (6 mg/L), led to an increase in EROD activity with increasing water pH (Amutha and Subramanian, 2010). In the present work, higher EROD activity was observed in liver of fish captured in sampling sites with higher pH values.

In this study, the dry season had a more noticeable effect on HSI, EROD activity and on the presence of significant morphological changes, which could be associated with a higher concentration of pollutants in the water column, such as naphthalene, 2-methylnaphthalene, and phenanthrene. A similar effect was reported in other *in situ* studies. For instance, in the Bay of Paranaguá in southern Brazil, which has been affected by urban, industrial, agricultural, port activities, and oil spill accidents, chemical analysis of the bile of the neotropical native fish *Atherinella brasiliensis* showed a continuous bioavailability of PAHs. Simultaneously, a considerable incidence of severe histopathological alterations in the liver and gills were observed, which were associated to biochemical alterations and genetic damage with greater prevalence during spring (Ribeiro et al., 2013). Similarly, Corredor-Santamaría et al. (2019), found higher incidence of liver morphological alterations and inflammatory lesions in specimens of the native fish *A. metae* and *A. bimaculatus*, caught during the rainy season in places of the Ocoa River (Colombia) with higher domestic and industrial wastewater discharge. In environmental monitoring studies evaluating the impact of xenobiotics on aquatic organisms, the influence of the dynamics of water bodies on the availability of oxygen and pollutants must be considered. In the present study, the Acacias River flow is reduced at the wastewater discharge site so that there is a higher exposure of fish to PAHs and a lower oxygen availability, while the increase in phosphate concentration may indicate increased organic matter degradation (Table 1).

Regarding total protein concentration in the liver of *A. metae*, no significant statistical differences were found. This allows inferring that the protein concentration did not influence the result of the EROD activity.

Finally, the evaluation of histological and EROD activity biomarkers in the native fish *A. metae* were found to be useful as biomonitoring tools for assessing the in situ effects of environmental pollutants.

4. Conclusion

Based on the results of increased EROD activity and the DTC analysis, the discharge site and places downstream of the discharge of PW showed evident signs of environmental quality degradation that could lead to an ecological threat. Additionally, considering the low concentrations of PAHs found in the water column, it can be inferred those other pollutants alongside with PAHs, including other hydrocarbons not considered in our work (see for instance González-Doncel et al., 2008), may exert negative effects on the organisms monitored at these sites in the Acacias River. Therefore, additional studies are necessary for an appropriate and accurate assessment of the minimum concentrations that should be allowed for the discharge of wastewater from oil activities by environmental legislation in Colombia. A better knowledge of the effects induced by PAHs pollution in freshwater will allow environmental managers and policy makers to implement appropriate legislative actions.

Declarations

Author contribution statement

Wilson Corredor-Santamaría: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Diego A. Mora-Solarte: Performed the experiments.

Ziv Arbeli & José M. Navas: Conceived and designed the experiments.

Yohana M. Velasco-Santamaría: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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