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Efeitos antrópicos sobre a comunidade de peixes e a integridade biótica de riachos: monoculturas, cinzas e espécies não-nativas

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Tese apresentada ao Programa de Pós-Graduação em Biologia Comparada do Centro de Ciências Biológicas da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Doutor em Biologia das Interações Orgânicas.

Orientadora: Profa. Dra. Evanilde Benedito
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(APÓSTOLO PEDRO - 1 Pedro 3:15)

Efeitos antrópicos sobre a comunidade de peixes e a integridade biótica de riachos: monoculturas, cinzas e espécies não-nativas

RESUMO

O desmatamento da vegetação ripária das bacias hidrográficas e a consequente conversão em áreas de cultivo são impactos antrópicos relevantes em ecossistemas tropicais. No Brasil, a expansão da área de cultivo para o plantio de cana-de-açúcar tem sido associada à perda de habitat e à contaminação química. Além disso, a prática da colheita de cana é realizada com uso do fogo e apresenta prejuízos ao ecossistema, entre eles, a liberação de cinzas da cana-de-açúcar (CCA), que apresentam diferentes hidrocarbonetos policíclicos aromáticos (HPA) e outros compostos tóxicos. O fogo tem sido comumente utilizado em práticas agrícolas há muito tempo, e por isso o problema das cinzas no ambiente (oriundas da cana ou não) pode passar de uma escala regional para uma escala global, se considerarmos todos os países que sofrem com queimadas de biomassa vegetal. A Península Ibérica, por exemplo, apresenta sérios problemas com fogos florestais. As cinzas são nocivas para o ambiente, e consideradas um problema de saúde pública. Entretanto, pouco se sabe sobre seus efeitos prejudiciais sobre a biota aquática, que em períodos de queimadas, pode sofrer intensamente com o incremento dos compostos tóxicos que as compõem. Este foi o primeiro estudo experimental a avaliar os efeitos negativos de cinzas de origens distintas (i.e. florestal e da cana-de-açúcar) sobre diferentes espécies de peixes, e em diferentes níveis organizacionais, desde o genético individual até o ecológico populacional. Nossos resultados confirmaram o potencial tóxico das cinzas florestais sobre o comportamento e a condição hepática do peixe ibérico *Luciobarbus bocagei*, por meio da observação da diminuição dos padrões nas variáveis *searching*, *boldness* e *shoaling cohesion*, e do aumento do índice hepatossomático (IHS) dos indivíduos expostos em comparação aos do controle. Além disso, cinzas originadas da queima da palha de cana-de-açúcar também foram tóxicas ao núcleo celular de eritrócitos do peixe neotropical nativo *Astyanax lacustris* comparado ao não-nativo *Oreochromis niloticus*, indicando que este poluente pode interferir na dinâmica de estabelecimento de espécies em novos ambientes. Estas informações, ainda incipientes, são fundamentais para o estabelecimento de políticas de proteção à biodiversidade aquática. Além da abordagem experimental presentes nos dois primeiros capítulos, integrou-se um terceiro, sendo um estudo de campo com a elaboração de uma ferramenta de avaliação da qualidade ambiental de riachos Neotropicais de solo arenoso, um índice de integridade biótica (N3S-IBI), composto de 6 métricas. Dada a degradação que os recursos aquáticos estão sofrendo na região Sul do Brasil com o aumento das áreas de cultivo nas últimas décadas, principalmente com plantações de cana e seus impactos nas bacias hidrográficas, desenvolver ferramentas para avaliar seu status ecológico é um grande desafio, necessário para o manejo de ecossistemas de água doce. Assim, esta tese é composta por três capítulos. Os dois primeiros são bioensaios que avaliaram os impactos de cinzas (em Portugal e no Brasil) em diferentes espécies de peixes, e o terceiro capítulo é a elaboração de um IBI baseado em peixes, para avaliação de riachos neotropicais de solo arenoso. Por fim, identificou-se neste trabalho que, direta ou indiretamente, culturas que sofrem queimadas causam efeitos letais e subletais em peixes de espécies nativas e não nativas, afetando a comunidade ictiológica.

Palavras-chave: Cana-de-açúcar. HPA. IBI. Tilápia. *Poecilia*.

Anthropic effects on the fish community and the biotic integrity of streams: monocultures, ashes and non-native species

ABSTRACT

Deforestation of riparian vegetation in river basins and consequent conversion to cultivated areas are relevant anthropic impacts on tropical ecosystems. The expansion of the crop area for sugarcane planting has been associated with habitat loss and chemical contamination in Brazil. In addition, sugarcane harvesting is carried out using fire and presents damage to the ecosystem, including the release of sugarcane ash (CCA), which have different polycyclic aromatic hydrocarbons (PAH) and other toxic compounds. The fire has been commonly used in agricultural practices for a long time, and so the problem of ash in the environment (whether from sugarcane or not) can shift from regional to a global scale if we consider all countries suffering fires from plant biomass. The Iberian Peninsula, for example, presents serious problems with forest fires. Ashes are harmful to the environment and are considered a public health problem. The aquatic ecosystem may suffer an increase of the toxic compounds of the ashes in periods of wildfire, which arrive by runoff. However, its damaging effects on aquatic biota is poorly known. This was the first experimental study to evaluate the negative effects of ash of distinct origins (i.e., forest and sugar cane) on different species of fish, and at different organizational levels, from the individual genetic to the ecological population. Our results confirm the toxic potential of forest ash on the behavior and liver status of the Iberian fish *Luciobarbus bocagei*, by observing the decrease of the patterns in the searching, boldness and shoaling cohesion variables, and the increase in the hepatosomatic index (IHS) of the individuals compared to those in the control group. In addition, ash from the burning of sugarcane straw was also toxic to the cell nucleus of erythrocytes of the native neotropical fish *Astyanax lacustris* compared to non-native *Oreochromis niloticus*, indicating that this pollutant can interfere in the dynamics of the establishment of species in new environments. This information incipient yet is fundamental for the establishment of policies to protect aquatic biodiversity. In addition to the experimental approach present in the first two chapters, a third one was integrated as a field study was carried out with the elaboration of an environmental quality assessment tool for the headwaters of the Northwest of the State of Paraná, an index of biotic integrity (N3S-IBI), composed by 6 metrics. Given the degradation that aquatic resources are suffering in southern Brazil, due to an increase in cultivated areas in the last decades, mainly with sugarcane plantations and their impacts in the river basins, the development of tools to evaluate their ecological status is a challenge for the management of freshwater ecosystems. In this way, this thesis is composed of three chapters. The first two are bioassays that evaluated the impacts of wildfire ashes (in Portugal) and sugarcane (in Brazil) on different species of fish, and the third chapter is the elaboration of an IBI based on fish, for evaluation of Neotropical sandy soil streams. Finally, it was identified in this work that, directly or indirectly, crops that suffer burnings cause lethal and sublethal effects in fish of native and non-native species, affecting the fish community.

Keywords: Sugarcane. PAH. IBI. Tilapia. *Poecilia*.

SUMÁRIO

INTRODUÇÃO GERAL

Os ecossistemas de água doce ocupam menos de 1% da área total do planeta e abrigam aproximadamente 10% das espécies animais descritas, que corresponde a mais de 15.000 espécies de peixes (45% do total) (STRAYER; DUDGEON, 2010; CARPENTER et al., 2011). No entanto, estes ecossistemas estão entre os mais perturbados e, nas últimas décadas, sofreram taxas mais rápidas de declínio na biodiversidade do que as observadas em ecossistemas terrestres (SAUNDERS et al., 2002; CARPENTER et al., 2011). Os principais motivos relacionados à perda de biodiversidade são a fragmentação e destruição de habitat, a poluição ambiental e a disseminação de espécies não-nativas (EHRLICH, 1997; WILCOVE et al., 1998; CZECH et al., 2000; PIMENTEL et al., 2005; HUGHES et al., 2011; STRAYER, 2012; PAOLUCCI et al., 2013; THOMAZ et al., 2015). O desmatamento e a consequente conversão em áreas de cultivo são considerados os maiores impactos antrópicos em ecossistemas tropicais (WINEMILLER et al., 2008; BARLETTA et al., 2010). Especificamente nas regiões sudeste e sul do Brasil, sendo a última o principal local deste estudo, a expansão da área de cultivo para atender às necessidades crescentes de alimentos e combustíveis, com ênfase no plantio de cana-de-açúcar, tem sido associada à perda de habitat e à contaminação química (CONAB, 2017; FAOSTAT, 2018).

A cana-de-açúcar destaca-se como uma das principais culturas agrícolas de escala global (FAOSTAT, 2018). Além da fragmentação e destruição de habitat, a prática da colheita de cana é realizada com uso do fogo e apresenta prejuízos ao ecossistema (ECHAVARRIA, 1996; MENDOZA et al., 2002), entre eles, a liberação das cinzas da cana-de-açúcar (CCA), que apresentam diferentes hidrocarbonetos policíclicos aromáticos (HPA) e outros compostos tóxicos (DARLEY; LERMAN, 1975; ZAMPERLINI et al., 1997; FERREIRA et al., 2014; NIH, 2017). O fogo tem sido comumente utilizado em práticas agrícolas há muito tempo (RIBEIRO; MARTINS, 2014), e por isso o problema das cinzas no ambiente (oriundas da cana ou não) já atinge uma escala global se considerarmos todos os países que sofrem com queimadas de biomassa vegetal. O cerrado brasileiro, por exemplo, que é um bioma de savana, é conhecido como o mais suscetível a incêndios devido às suas características (van der Werf et al., 2017). Se pensarmos em locais do hemisfério norte, a Península Ibérica, que não é produtora de cana, apresenta sérios problemas com fogos florestais (MOREIRA et al., 2011; MONAGHAN et al., 2016). As cinzas são nocivas para organismos terrestres e, quando liberada na atmosfera, apresentam-se como um problema de saúde pública (ROSEIRO; TAKAYANAGUI, 2004; ARBEX et al., 2007; FRANÇA et al., 2012). Entretanto, pouco se sabe sobre os efeitos prejudiciais deste poluente sobre a biota aquática, que em períodos de queimadas, pode sofrer intensamente com o incremento dos compostos tóxicos que compõem as cinzas (NUNES et al., 2017).

Além de se conhecer os efeitos causados pelas cinzas no ambiente aquático, é importante conhecer a qualidade destes corpos de água presentes em áreas afligidas pelo uso do fogo e outros impactos antrópicos. A região Sul do Brasil sofreu um aumento das áreas de cultivo nas últimas décadas, principalmente com plantações de cana e seus impactos nas bacias hidrográficas (HEPP; SANTOS, 2009). Dada a degradação que os recursos aquáticos estão promovendo nesta região, o desenvolvimento de ferramentas para avaliar seu *status* ecológico é um grande desafio, necessário para o manejo de ecossistemas de água doce. Um dos métodos mais utilizados para avaliar a integridade ecológicas de rios e riachos é o Índice de Integridade Biótica (IBI), apresentado primeiramente por Karr (1981) e já foi adaptado aos diversos ambientes ao redor do globo (LI et al., 2015; HERMOSO et al., 2010; van OOSTERHOUT and van der VELDE, 2015).

Desta forma, propõe-se este, que é o primeiro estudo a avaliar os efeitos negativos de cinzas de origens distintas (i.e. florestal e da cana-de-açúcar) sobre diferentes espécies de peixes. Estes efeitos foram analisados experimentalmente em diversos níveis organizacionais, sendo nível *i)* genético (genotoxicidade); *ii)* morfofisiológico (índice hepatossomático); *iii)* de organismo individual (mortalidade, tolerância, comportamento, *instant activity*, *boldness*); *iv)* de espécie; *v)* ecológica (nativa e não-nativa) e *vi)* populacional (índice de cardume). Ademais, foram consideradas quatro esferas de abordagem: *atmosfera*, pela origem aérea das cinzas; *hidrosfera*, quando as cinzas atingem os corpos aquáticos; *biosfera*, quando este poluente é capaz de afetar negativamente os peixes; e *antroposfera*, quando se reconhece a atividade antrópica como origem deste impacto. Estas informações, ainda incipientes, são fundamentais para o estabelecimento de políticas de gestão e proteção à biodiversidade aquática. Além da abordagem experimental, integrou-se um estudo de campo com a elaboração de uma ferramenta de avaliação da qualidade ambiental de riachos Neotropicais de solo arenoso, um índice de integridade biótica.

Diante do exposto, esta tese é composta por três trabalhos. Os dois primeiros capítulos compreendem bioensaios que avaliaram os impactos de cinzas *i)* florestais (em Portugal) e *ii)* de cana-de-açúcar (no Brasil) sobre diferentes espécies de peixes, e o terceiro, consistiu na elaboração de um IBI baseado em peixes, para avaliação de riachos Neotropicais de solo arenoso. Ao final, foi elaborado um capítulo integrador com as considerações referentes a todo o trabalho da tese. Pretendeu-se, portanto, fornecer informações oportunas acerca dos efeitos negativos do uso do fogo à sociedade, à comunidade científica, aos órgãos de fiscalização e aos tomadores de decisão, a fim de contribuir acadêmica e efetivamente no campo da gestão ambiental.

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CAPÍTULO 1

Short-term effects of wildfire ash exposure on behaviour and hepatosomatic condition of a potamodromous cyprinid fish, the Iberian barbel *Luciobarbus bocagei* (Steindachner, 1864)

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Short-term effects of wildfire ash exposure on behaviour and hepatosomatic condition of the cyprinid fish, the Iberian barbel *Luciobarbus bocagei* (Steindachner, 1864)

HIGHLITGHS

1. Wildfire ash runoff is an important disturbance for freshwater fish in Med rivers.
2. Barbel behaviour and hepatosomatic condition were addressed after exposure to ash concentrations.
3. Tested parameters included routine activity, boldness, shoaling cohesion and HSI.
4. Differences on fish behaviour and hepatosomatic condition were found between treatments.
5. Maintenance of unfragmented rivers is fundamental for species recovery from fire.

GRAPHICAL ABSTRACT

ABSTRACT

Wildfires are a common phenomenon in Mediterranean regions that is becoming increasingly frequent and severe, causing several environmental concerns, of which ash runoff represents an important source of disturbance for aquatic organisms, in particular for fishes. Studies on the behavioural response of fishes to wildfire ash runoff are scarce and seldom include cyprinid species. The goal of this study was to investigate in a 3-artificial flume channel mesocosm, the behavioural and hepatosomatic condition responses of a native widespread potamodromous fish, the Iberian barbel (*Luciobarbus bocagei*), previously exposed for 24 h to different concentrations of wildfire ashes: 0.0 g/L (the control, no ash), 1.0 g/L (low concentration) and 2.0 g/L (high concentration). Behavioural parameters included i) routine activity, ii) boldness and iii) shoaling cohesion. The hepatosomatic index (HSI) was further determined to assess the health condition of fish. Significant differences on fish behaviour parameters were detected between the control and the high concentration of ash. Accordingly, i) an increasing proportion of fish were found on resting activity (56.2% vs 30.6% in the control), whereas the proportion of fish on searching behaviour (58.4% in the control) decreased (41.5%); ii) the proportion of bolder individuals was found to decrease (42.5% in the control vs. 29.4%) and iii) the same trend was detected for shoaling cohesion (61.3% in the control to 33.8%, of all fish within a body length of each other). Such differences were paralleled by an increase in the HSI from 1.62% (control) to 2.40% (high concentration). The present study shows that even short duration exposure to ash-loaded runoff can alter fish behaviour and hepatosomatic condition and highlights the need to maintain an unfragmented river network, or, when this is not possible, to prioritize the removal or retrofitting of barriers to increase movement dispersal and provide conditions for species recovery from fire-disturbances

Keywords: Ash concentration; Activity; Boldness; Shoaling cohesion; Hepatosomatic Index; Impact

1. INTRODUCTION

Mediterranean ecosystems are considered as one of the most fire-prone in the world, with an yearly average of approximately half a million hectares of land burned by wildfires in the countries of southern Europe, causing large ecological and socio-economic impacts (Monaghan et al., 2016; Moreira et al., 2011). Rivers ecosystems in such regions have been historically impacted by wildfires (Dunham et al., 2003; Monaghan et al., 2016). These rivers often originate in forested watersheds, which experience recurrent fires and are therefore vulnerable to post-fire contamination (Nunes et al., 2018). Freshwater fish inhabiting Mediterranean rivers are particularly susceptible to wildfires, as not only they have to deal with maximum natural stress associated with seasonal low flows, increased water temperatures, and hypoxia (Magalhães et al., 2007; Monaghan et al., 2016; Oliva-Paterna et al., 2003), as coinciding with such seasonal environmental disturbances, they have to cope with the effects of fire acting on river habitats. One of the most important source of disturbance of wildfires on Mediterranean stream fishes, is the occurrence of ash flows which are associated with increased runoff and erosion from severely burned areas during storms, particularly the first intense rains (Dunham et al., 2003; Nunes et al., 2018; Whitney et al., 2015). Post-fire ash flows often lead to an increase in water turbidity, pH, conductivity and dissolved oxygen depletion (Earl and Blinn, 2003; Silva et al., 2016) and are particularly common in Mediterranean rivers, where the first intense rains (November-December) directly follow the fire season (June-September; Gasith and Resh, 1999). As the current climate change scenarios are predicting an increased wildfire risk along a higher frequency of extreme events, in particular of heat waves which will further exacerbate the exploitation of freshwater resources, followed by heavy rainfall events (IPCC WG II, 2014), it is of crucial importance the assessment of fire-related disturbances, such as ash flows, on freshwater fish in order to better inform managers and guide river restoration actions.

There has been a limited understanding of the effects of ash flows on fish populations (Dunham et al., 2003; Rieman and Clayton, 1997). This uncertainty is related to the diverse and complex effects that such fire-related disturbances can have on aquatic ecosystems (Bisson et al., 2003), which will depend on fire severity, timing, area, location, intensity, as well as the characteristics of the aquatic ecosystem itself (and associated terrestrial environment) and the species present therein (Gresswell, 1999; McMahon and DeCalesta, 1990). Direct effects include mortality or displacement of individuals extending for several kilometres, though they are often patchy within and among the rivers influenced by fire (e.g. Gresswell, 1999; Rieman et al., 2012). Indirect effects may include reductions in abundance, spatial distributions, and even local extirpations, which are mainly related to decreased water quality associated with postfire ash flows, loss of habitat connectivity during periods of natural reduced flow, postfire flooding, and loss of habitat and food resources (Gresswell, 1999; Howell, 2006; Rieman et al., 2012; Rinne and Carter 2008). The few studies that addressed the response of fish to ash flows refer mostly to salmonid species (e.g., Detenbeck et al., 1992; Gresswell, 1999; Howell, 2006; Rieman and Clayton, 1997). More recently, Monaghan et al., (2016) studied the effects of wildfire on the persistence of fish assemblages of Atlantic-Mediterranean streams in northern Portugal, by employing a chrono-sequence survey covering an 18-year gradient of impact-recovery from major fire events, and found that cyprinids, in contrary to salmonids (trout) showed resistance to fire, as shown by occurrence of abundant populations at both burnt and non-burnt sites. However, to the best of our knowledge, no information exists on the effects of wildfire ash runoff on fish behaviour and hepatosomatic condition, particularly of potamodromous cyprinid fish, that need to seasonally undergo reproductive migration within rivers. Such studies, preferentially performed under controlled conditions where variables of interest can be manipulated while controlling for confound effects, offer an excellent opportunity to improve knowledge of fire-related disturbance on fish and better inform managers for future risk assessment (Auer et al., 2017).

The goal of this study was to assess, in an experimental mesocosm, the effect of previous ash-exposure on behaviour and hepatosomatic condition of widespread potamodromous cyprinid, the Iberian barbel *Luciobarbus bocagei* (Steindachner, 1864). Behaviour parameters included, routine activity, boldness and shoaling cohesion. Routine activity is a commonly assessed behavioural trait in fish (e.g. Brownscombe et al., 2014). From the ecological point of view, activity is relevant because increased activity has been shown to increase feeding and growth rates (Laubenstein et al., 2018; Werner and Anholt, 1993). Boldness, or the propensity to take risks, is another commonly measured behavioural trait in fishes (Ariyomo and Watt, 2012; Wilson et al., 1994). As with increased activity, increased boldness in fishes has been linked to a search for more favourable areas for spawning, feeding and growing and to find refuge from disturbed locations (Biro et al., 2003). Shoaling cohesion is also a common behavioural strategy assessed in fishes (Smith et al., 2018). It is a relevant metric as it may confer increased survivorship to fish living in groups, by improving threat detection, reducing stress, individual risk (“dilution effect”, Foster and Treherne, 1981) and physiological cost of movement, although it may cost increased competition for space and food. Fish condition was measured through the assessment of the Hepatosomatic Index (HSI), which provides information about the condition of liver and body and also about the impact of stressors on fish (Traven et al., 2013). Due to the acute toxicity of ashes (Campos et al., 2012; Earl and Blinn, 2003), it is expected that the liver, the primary detoxifying organ, should reflect such changes. Specifically, this study asked the following question: does Iberian barbel exhibit behavioural and hepatosomatic condition differences upon previous exposure to wildfire ash? We hypothesised that previous exposure to increasing levels of ash concentration in the water would (1) reduce fish searching activity, while increasing resting (or holding position) of individuals, (2) decrease the proportion of bolder individuals and (3) decrease shoaling cohesion. We would also expect a decrease in HSI in response to previous exposure to increasing ash concentration in the water. The findings obtained from this study will be used to inform river restoration practices to be considered in fire-prone Mediterranean landscapes.

2. MATERIALS AND METHODS

2.1. Ash collection

Wildfire ashes were collected from a recently burnt catchment in central Portugal, in two locations at the Figueiró dos Vinhos municipality (N 39 ° 55’16.3”, W 8 ° 15’56.9” and N 39 ° 55’40.9”, W 8 ° 17’09.0"). Samples were collected with a brush and shovel seven days after the end of one of the most severe fire in Portugal (late June 2017), that consumed a vast area (ca. 30.000 ha burned, San-Miguel-Ayanz et al., 2018) of eucalyptus and pine tree. Ashes were then sifted with a 5 mm sieve, mixed to homogenize and brought to the School of Agriculture campus, University of Lisbon, where they were stored at -4 °C until the further biological experimentation.

2.2. Fish collection

Juvenile Iberian barbel used in the experiments (n= 48; mean standard length (SL) ± standard deviation (SD) = 77.9 ± 15.3 mm) were captured in the Lizandro River, a small Atlantic coastal river, West Portugal in late-summer (September 2017). Sampling was performed by wadeable electrofishing (Hans Grassl IG-200) according to the protocol adopted by the European Committee for Standardization (CEN, 2003). Two electrofishing events were performed (one event per week), collecting 24 fish per event. Fish were transported to the campus of the School of Agriculture, University of Lisbon, in a fish transport box (Hans Grassl, 190 L) filled with river water and featured with a portable aeration device (ELITE, Germany) to minimize transportation stress. At the campus, fish were maintained for a maximum period of 7 days in two filtered and aerated acclimation tanks (1000 L tanks; High Performance Canister Filter FX5, Fluval, Quebec, Canada) each featured with two U-shape ceramic roof tiles (45 cm long x 25 cm wide x 10 cm high) to provide shelter (Stammler and Corkum, 2005). During that period, fish were fed with Tetra Pond flakes (Tetra, Germany) up to 24 h before the beginning of experiments (Amaral et al., 2016; Silva et al., 2009). No fish died following transportation and maintenance in the acclimation tanks. Water quality in the acclimation tanks (temperature = 19.2 ± 1.6 ºC; pH = 8.36 ± 0.1 and conductivity = 875 ± 7.07 μS/cm) was checked every two days with a multiparametric probe (HANNA, HI 9812-5). Feeding stopped 24h prior to the experiments.

2.3. Mesocosm facility

The effects of wildfire ashes on fish behaviour and on metabolic activity were tested at a mesocosm facility located at the School of Agriculture campus, University of Lisbon, Portugal. Mesocosm are outdoor experimental systems that examine the natural environment under controlled conditions, to incorporate natural variations (e.g. photoperiod, air temperature). In this way they provide a link between field surveys and highly controlled laboratory experiments. The present mesocosm consists of a set of 3 stainless-steel-lined outdoor artificial flume channels (each 0.4 m wide x 4 m long x 0.2 m deep), to where water is supplied after being stored in 3000 L central tank (Fig. 1). The source of water is an in situ natural spring considered to have good quality (Temperature: 19.1 °C; pH = 8.06; Conductivity = 865 μS/cm; Dissolved oxygen (DO) = 9 mg/L). Water is then distributed to head containers (70 L) located in the uppermost section of each channel. Each channel, delimited downstream by a fixed mesh panel, runs to a downstream tank (70 L) connected to a pump (Kripsol OK-71 B, 0.56 kW) operating in a recirculation flow system to the head container which allows upholding water conditions independently of the source tank. Water distribution and recirculation was ensured with a PEAD pipe system. To identify each fish position during the experiments, each channel was divided in four equally-sized sections (1 m x 0.4 m = 0.4m2), from A (lowermost section) to D (uppermost section).

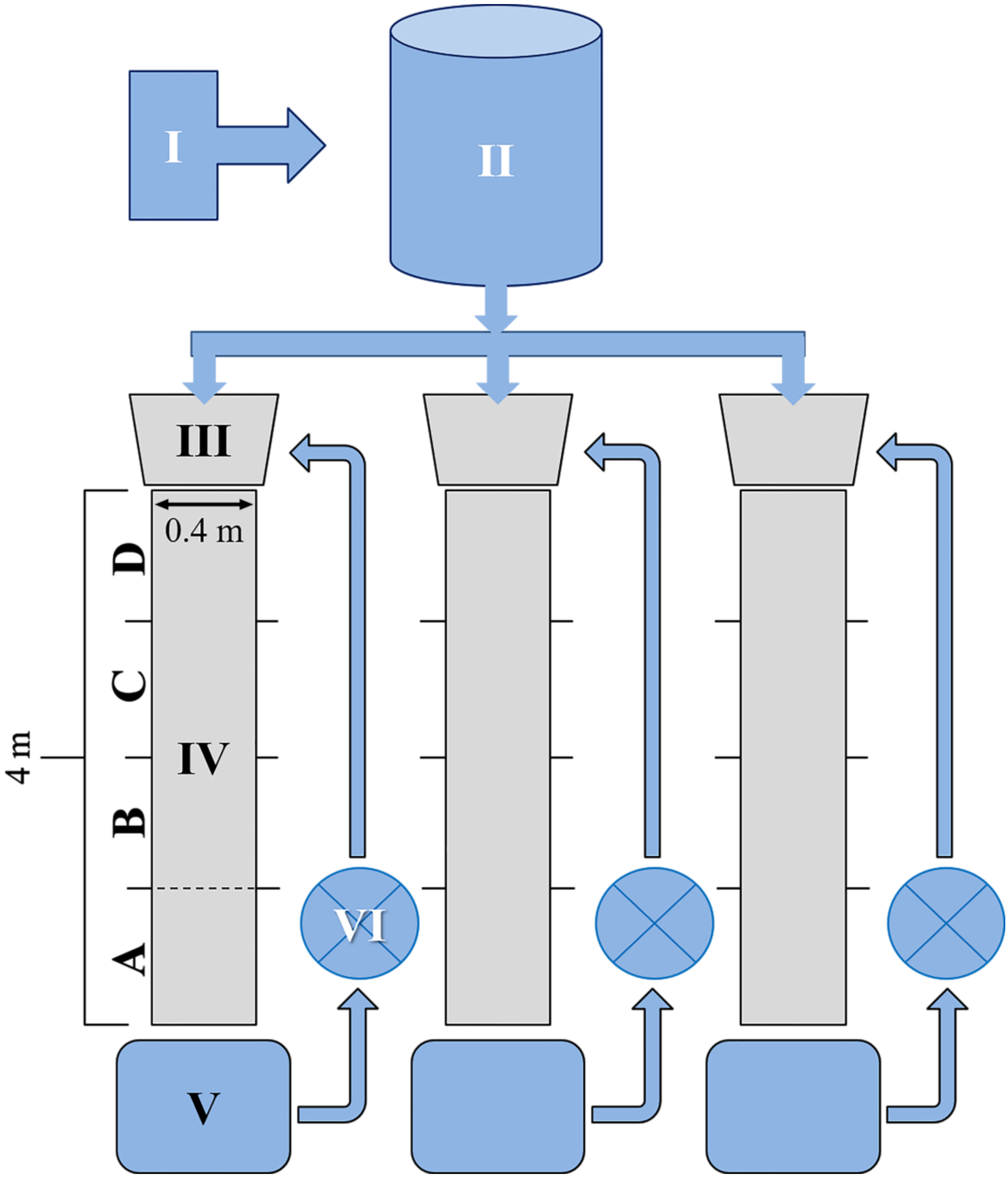

Figure 1. Schematic overview of the mesocosm system, encompassing 3 artificial flume-channels, each with an individual water recirculating system. I –water source inlet from a spring, II –central source tank with distributing pipe system, III –head containers, IV –experimental area with the four equally-sized sections (A-D), with indication of the removable mesh panel by a dotted line, V –downstream tank, VI-pump.

2.4. Experimental setup

Fish experiments were carried out in compliance with ethical provisions on the welfare of experimental animals enforced by the European Union, and were coordinated by JM Santos who holds FELASA level C certification (www.felasa.eu) to direct animal experiments.

Experiments took place on early autumn (October 2017), resembling the time after the typical fire season, when the first rains start to fall, carrying out the accumulated fire ashes into the streams (Cid et al., 2017). Fish (n = 3 schools of 4 fish) were first exposed for 24 h, under natural photoperiod, to three treatments of wildfire ashes concentration - 0.0 g/L (control, no ash); 1.0 g/L, (low concentration – [Low]) and 2.0 g/L (high concentration – [High]) - in 50 L tanks using the same spring water. Each of these tanks included an external oxygen supplier (ELITE, Germany) and one ceramic roof tile for fish shelter (the same type that was used for fish acclimation). The variability of ash concentrations in burned areas depends on several factors relating to the severity of the fire and the characteristics of the surrounding landscape and flow regime (i.e. magnitude, frequency and duration), and making it very difficult to find reference values on the literature. Therefore, in order to provide a framework for the present experimental ash concentration in the water, the extreme was previously selected on the basis of the fact that 2.0 g/L reflected the value at which ash concentration started to become stressful for populations of a benthopelagic characiform fish (*Moenkhausia forestii*) (Gonino et al., 2019), being 1.0 g/L, selected as the midpoint (and half of the extreme value) of the gradient, and the 0.0 g/L as the control (no ash). After the 24-h period, water quality parameters (temperature, conductivity, pH and DO) were measured for each of the treatments with multiparametric water probes (HANNA, model HI 9812-5 and HI98193). Even though fish were left starved 24h before ash exposure, minimizing the chance of having potential toxic ammonium and nitrites from organic wastes, an aquarium test strip (Tetra, Germany) was nonetheless dipped in each of the tanks, after the exposure period, to check if water conditions within each one were below stressful thresholds (ammonium: < 0.5 ppm; nitrites: < 0.15 mg/l) for fish (all were, thus minimizing any potential interaction of these variables with the ash effects). Fish were then transferred to each of the 3 mesocosm channels which were ran with the natural spring water, to assess their behaviour and hepatosomatic effects after previous ash exposure. Therefore, each of the 3 channels contained i) fish not previously exposed to ash (control), ii) fish previously exposed to low concentration of ash and iii) fish previously exposed to high concentration of ash. Fish were first acclimatized to channel conditions for 10 minutes prior to the beginning of experiments by maintaining a water column of 12 cm with an average velocity of 9 cm/s, which corresponds to the average velocity this is available for barbel in Mediterranean rivers during early summer (Santos et al., 2018). Acclimation took place in the lowermost section of each channel (section A) which was delimited upstream by a removable mesh panel.

At the start of each trial, the upstream mesh panel was removed to enable fish access to the whole channel, whereby fish were allowed to move on their own volition. Each of the three experimental treatments (i.e., no ash, [Low] and [high]) was repeated 4 times, giving a total of 12 replicates (3 [ash] x 4 replicates). Each replicate lasted for 60 minutes and consisted in the instantaneous sampling, every 3 minutes, of behaviour parameters for each of the 4 barbel individuals that composed a school. To avoid pseudo-replication issues, at the beginning of each of the 4 replicates, the flumes that received the schools subjected to each treatment were randomized (i.e. the same treatment was not always housed by the same flume and the treatments relative position within the mesocosm were randomly changed at each replicate) as well as the observer (i.e. fish schools were monitored by a different observer in each of the 4 replicates). Parameters included i) routine activity, classified as resting (i.e. holding position), searching, fleeing or directional changes (i.e. a 90◦ alteration in bearing by the fish) (Brownscombe et al., 2014), ii) boldness, assessed by how fish used space within a flume channel, based on the idea that a novel open field is considered dangerous, and that venturing into the uppermost section represents boldness, or the willingness to undertake risk (Laubenstein et al., 2018). Therefore, boldness was quantified as the location occupied by the fish in each of the four equally-sized sections in the channel, A-lowermost section (denoting shyer individuals) to D-uppermost section (denoting bolder individuals) and iii) shoaling cohesion, classified as 1, no fish within a body length of each other; 2, two fish within a body length of each other; 3, three fish within a body length of each other; 4, all fish within a body length of each other (Manek et al. 2014).

Fish behaviour was continuously monitored by visual observations. Observations were made at 0.50m distance below from each channel, and therefore with full view of it, allowing to have a snapshot status of fish behaviour every 3 minutes. The operators (1 for each channel/treatment) stood still during the observations, approaching or leaving discreetly the observation points, respectively before and after each replicate. This procedure was tested before with an independent set of fish to determine if the presence of the observer incited sudden changes in fish behaviour. Since it did not, this procedure was considered adequate.

After each experiment, fish were removed from the channel, anesthetized and sacrificed by placing them in an ice bath (Magalhães, 1993), and then fixed in 4% formalin for 72 hours (AVMA, 2013; Summerfelt et al., 1990), for later removal and weighing of the liver. This procedure was chosen to avoid measurement errors due to the size of the animals and their respective internal organs. As fresh liver of juvenile (small) fish is extremely delicate, individuals were then left to dry at ambient temperature (20°) for 3 hours to prevent the loss of hepatic tissue. Fish were then measured (SL, to the nearest mm) and weighed (to the nearest g), before their livers being carefully excised, removed and weighed.

2.5. Data analysis

Significant differences on physico-chemical parameters of water where fish were previously 24h exposed to the different ash concentration treatments (control, low and high concentration) were searched. For this, non-parametric Kruskal-Wallis (K-W) ANOVAs were performed, as previous tests of normality (Shapiro-Wilk) revealed data to be not normally distributed.

Chi-square tests of proportions were employed to test for significant differences in routine activity, boldness and shoaling cohesion within the channel, between the control, low and high ash concentration treatments.

The Hepatosomatic index (HSI) of fish was also determined to assess the hepatosomatic condition under the different ash concentration treatments. Changes in HSI have been detected in fish species after a similar time of exposure to other stressors: Almeida et al. (2005) reported significant differences in HSI of streaked prochilod (*Prochilodus lineatus*) after 24h of exposure to sediment from sites with different urban contamination conditions. We therefore believe that ash contamination in water can cause changes in HSI even after 24h, and hence, provide important information concerning the effect of ashes on the liver. It was determined according to Vazzoler (1996) and calculated as follows: HSI = (liver mass/body mass) × 100. A non-parametric K-W ANOVA was further used to assess the effect of ash concentration on the HSI, as a previous Shapiro-Wilk test revealed that data were not normally distributed.

The Chi-square proportions tests were performed in the MedCalc software (MedCalc Software bvba), and the K-W ANOVA was run in the package STATISTICA (STATSOFT, 2000). A critical alpha level of 0.05 was used for all tests.

3. RESULTS

3.1. Experimental ash exposure conditions

Water quality monitored after the 24 h period of ash exposure, displayed on Table 1, showed overall significant differences in the mean values of all parameters (Kruskal-Wallis ANOVAs (K-W), $P<0.05$). Differences were specifically noted (K-W, $P< 0.05$) between the control and the high ash concentration treatment for all tested parameters: temperature (°C) (control = 19.2; [high] = 18.5), conductivity (µS/cm) (control= 861, [high]= 925), pH (control= 8.06, [high]= 8.26) and dissolved oxygen (mg/L) (control = 8.99, [high]= 8.34).

3.2. Fish behaviour

Significant differences were found on fish's routine activity between the different treatments (Fig. 2). Accordingly, an increasing proportion of fish were found on resting activity between the control (30.6 %) and the high (56.2 %) concentration treatment ($\chi^2 = 15.51$, $P<0.001$). Differences were also found between this latter one and the low concentration treatment (42.5 %, $\chi^2=5.3$, $P<0.05$). An opposite trend was detected for fish searching across the channel, i.e. for these fish, a lower proportion was found on the high concentration treatment (41.5 %) relatively to the control one (58.4 %, $\chi^2 = 8.115$, $P<0.01$). No significant differences ($P>0.05$) were detected between the different treatments for the other types of fish activity (directional changes and fleeing).

Boldness, as shown by the fish position within the flume channels varied according to treatment. Fish subjected to both the control and low ash concentration treatments were mainly found to occupy the uppermost section (D) (42.5 % and 45.6 % of occupation, respectively), whereas the proportion of fish occupying such area under the high concentration treatment was significantly lower (29.4 %, $\chi^2 = 3.462$; $P<0.1$; $\chi^2 = 5.503$; $P<0.05$). Conversely, fish proportion occupying the lowermost section of the channels was significantly higher under such conditions (40.6 %) relatively to the control (20.9 %) and to the low concentration (20.9 %, $\chi^2 = 6.74$; $P<0.001$) treatments.

Significant differences (K-W ANOVA, $H=17.64$, $P<0.001$) were found on the shoaling cohesion, in response to the previous exposure to ash concentrations, as the proportion of all fish within a body length of each other under the control conditions (61.3 %), was found to decrease when fish were subjected to the high concentration treatment (33.8 %, $\chi^2 = 4.227$; $P<0.05$).

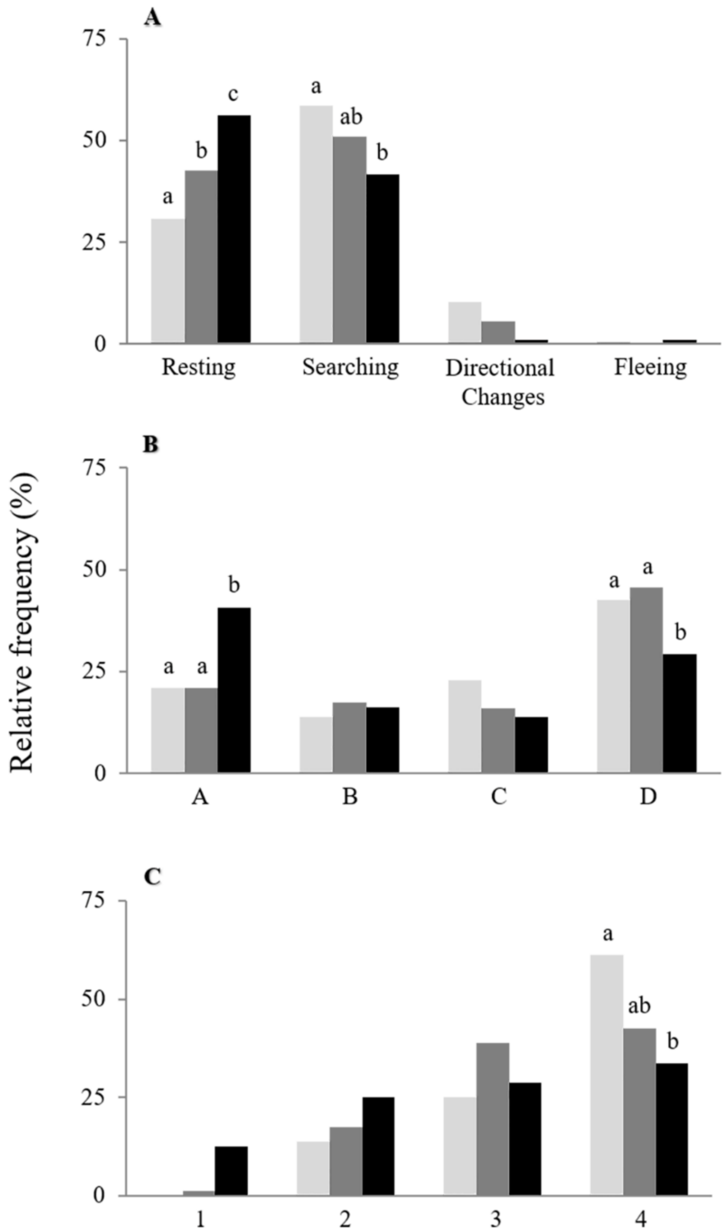

Figure 2 The effect of fish exposure to different concentrations of wildfire ashes (light grey- control 0.0 g/L dark grey- low concentration 1.0 g/L; and black- high concentration 2.0 g/L) on subsequent: (A) routine activity, classified as resting (i.e. holding position), searching, directional changes or fleeing; (B) boldness, quantified as the location occupied by the fish in each of the four equally-sized sections in the channel (A-lowermost to D-uppermost); and (C) shoaling cohesion, classified as 1, no fish within a body length of each other; 2, two fish within a body length of each other; 3, three fish within a body length of each other; 4, all fish within a body length of each other. Significant differences ($p < 0.05$) between proportions (Chi-square tests) are represented by different letters.

3.3. Fish Hepatosomatic Index

Changes in the liver condition of fish were detected (K-W ANOVA, $H = 15.52$; $n = 48$; $P < 0.01$), as the HSI values were shown to significantly increase from the control (1.62 %) to the high concentration treatment (2.4 %) (Fig. 3).

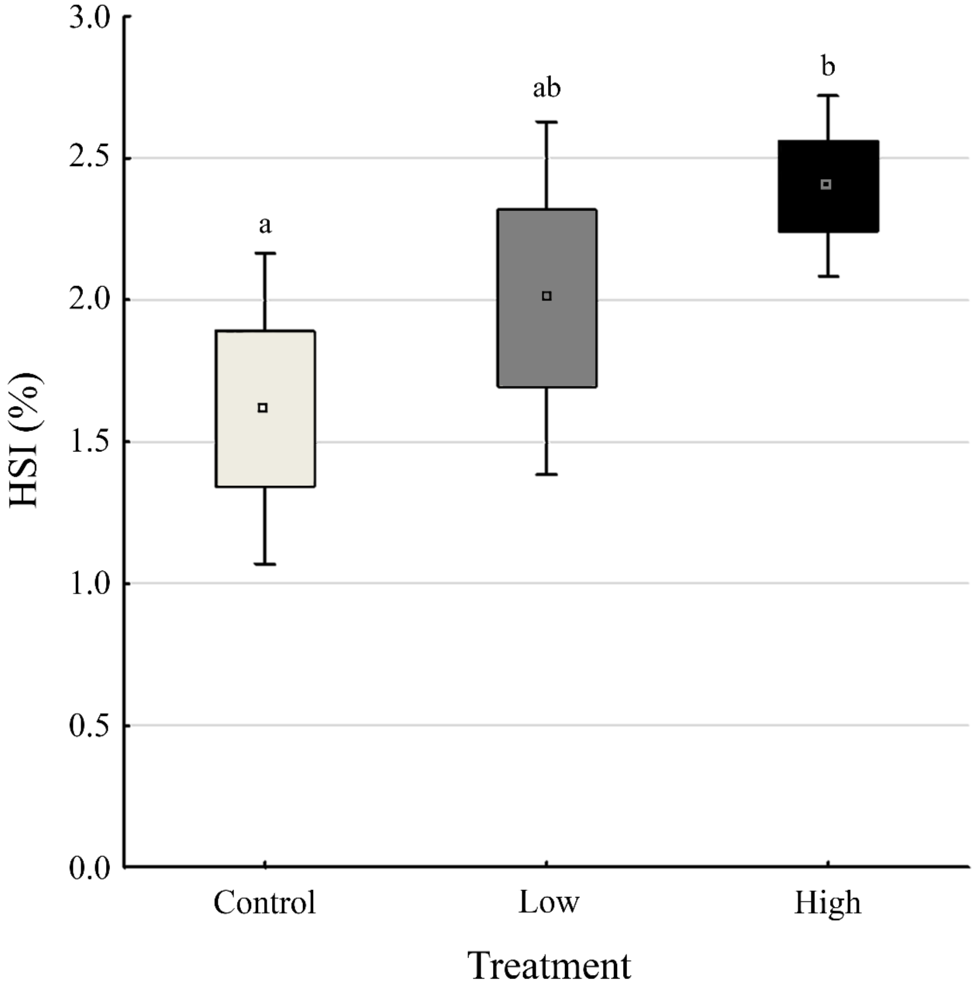

Figure 3. The effect of exposure to different concentrations of wildfire ashes (light grey- control 0.0 g/L; dark grey- low concentration 1.0 g/L; and black- high concentration 2.0 g/L) on subsequent fish conditions, as shown by the hepatosomatic index (HSI). The mean (central point), standard error (box), and standard deviation (whiskers) are shown in the plot. Significant differences (Kruskal-Wallis test, $p < 0.05$) are represented by different letters.

4. DISCUSSION

The assessment of the effects of fire-related disturbances, such as ash flows, on freshwater fish is of crucial importance to better inform managers and plan river restoration practices. However, studies that document the response of fish to such disturbances are rare and are mainly limited to salmonid species (Dunham et al., 2003; Howell, 2006; Monaghan et al., 2016; Whitney et al., 2015). Of these, to the knowledge of the authors, none has so far focused on the effects on fish behaviour, particularly on potamodromous cyprinids. The limited understanding about the short-term effects of fire on fish is related to the diverse and complex array of physical and biological factors that interact in nature. Physical factors include the nature of the fire and postfire events (e.g., floods, landslides and debris flow and their severity, extent, patchiness and timing), past management (e.g., roads, fish passage, fire suppression; Rieman et al., 2003), and the quantity, quality, and connectivity of the habitat (Dunham et al., 2003). Biological factors include proximity of other populations, life history diversity (particularly migratory traits), and presence of non-native species (Dunham et al., 2003). It is therefore quite difficult to separate the effect of each component in situ, suggesting that fire effects need to be considered in the context of several potential confounding effects (Howell, 2006). The present study was carried out in an experimental mesocosm, isolating the effect of ash-exposure on behaviour of a potamodromous cyprinid.

Results show that both behaviour and hepatosomatic condition of a potamodromous cyprinid species, changed according to previous short-time exposure to treatments of wildfire ash concentration in the water. Behaviour parameters included routine activity, boldness and shoaling cohesion. With regard to routine activity, results showed that fish significantly reduced their searching activity, while adopting a resting (i.e. holding position) behaviour in response to an increase ash concentration in the water. One would be tempted to argue that such decrease in activity could be due to alterations to water quality parameters (e.g., higher pH, conductivity and lower dissolved oxygen) caused by the complex chemical composition of ash input, which create a stressing scenario, mainly hypoxia (Whitney et al., 2015), that can ultimately results in fish mortality (e.g. Charette and Prepas, 2003; Earl and Blinn, 2003; Minshall et al., 2001; Rand and Schuler, 2009; Rieman et al., 2012; Silva et al., 2016). However, though our DO values in the most stressing scenario (8.34 mg/L) were significantly lower than the ones recorded during the control (8.99 mg/L), they were much higher than the ones that configure a lethal or sub-lethal scenario to fish (c. 2 mg/L) (Bohlen and Jörg, 2003; Rao et al., 2014). During an in-situ ash experiment conduct in a first order stream to monitor on-site physico-chemical responses, Earl and Blinn (2003), found immediate alterations in water quality parameters (increased turbidity, conductivity, pH and decreased DO), though they were generally short-lived, as most parameters returned to pre-experiment levels after 24-h of ash input. These effects are thus, in general, of a short duration and limited to smaller streams, as increasing water volume should buffer changes in water quality (Rieman and McIntyre, 1995; Rieman et al., 2012). The present results are however similar with the ones obtained from Branco et al. (2016) who analysed the interactive effect of hypoxia (c. 1.5 mg/L) and reduced connectivity on the movements of this species under experimental conditions and inferred decreased activity in response to an increasing oxygen deficit as would be expected (Domenici et al., 2013). Therefore, we cannot exclude that other unmeasured parameters, such as inorganic trace elements on the ashes (metals, metalloids and non-metallic elements) or particularly that other direct effects resultant from the ash exposure, for example, the clogging of fish gills, (Earl and Blinn, 2003; Newcombe and Macdonald, 1991), may have affected fish activity, highlighting the need for further research on this issue.

Boldness, as measured by how fish used space within the flume channel and that venturing into the uppermost section represents boldness, varied according to the treatment. Fish under the control and the low ash concentration were found to mainly occupy the uppermost section of the flume (i.e. bolder individuals) relative to the fish previously subjected to the high concentration ash treatment, which were predominantly found at the lowermost one (i.e. shyer individuals). This link between organism movement and boldness has been outlined in several studies, particularly in an important behaviour such as dispersal, where bolder individuals disperse farther than shy individuals (Chapman et al. 2011). As with routine activity, it appears that juvenile barbel decreased their bolder character and become shyer upon previous exposure to a higher ash concentration in the water. Again, it is unlikely that such behaviour could have resulted from the lower DO values, which should not pose a stressful scenario to fish, but possibly from the same unmeasured factors (inorganic trace elements, clogging of fish gills during previous exposure) that affected routine activity, therefore preventing fish from adopting risky behaviours and increase their survivorship in adverse environments (Biro and Dingemanse, 2009). The mechanism for this interaction is not currently clear and could represent an avenue for future research.

As with routine activity and boldness, shoaling cohesion was also found to change between the control and the high concentration ash treatment, where the proportion of all fish within a body length of each other, decreased significantly (from 61.3% to 33.8%). Despite we found no previous studies that dealt with the effects of ash runoff on fish shoaling cohesion, similar results to ours (i.e. a decrease in cohesion) were found by other authors who analysed experimental barbel school integrity (a proxy of shoaling cohesion), in response to other stressor, i.e. hypoxia (1.5 mg/L), resembling the effects of the discharge of organic wastes in rivers (Hayes et al., 2017). Once again, as hypoxia was excluded to drive fish behaviour, we hypothesize that the previous presence of a higher ash concentration in the water could have been responsible for the subsequent decreased shoaling cohesion in our experiments. It is thus possible that after ash input in the streams, individual movements may override shoal movements, as the former may risk leaving shoal protection to find more suitable (i.e. with less ash) areas further away, thus reducing cohesion by splitting the shoal into two or more groups. This result implies that under a high ash runoff, fish may face several predation risks, such as i) an increase in the individual risk of being caught by a predator as group size diminishes (Foster and Treherne, 1981); ii) a decrease in the chances of detecting a predator more rapidly (i.e., a decrease in the "many-eyes" effect; Godin et al., 1988; Ward et al., 2011); and iii) the lower group capacity to counter an attack by means of synchronized evasion manoeuvres (i.e., Hall et al., 1986; Pitcher and Wyche, 1983). A decrease in shoaling cohesion may also mean that individuals may spend more time searching for food than shoals (Magurran et al., 1985; Olla and Samet, 1974), therefore spending less time eating and more time being vigilant (Magurran et al., 1985). Another disadvantage is that fish, and particularly this species (Romão et al., 2018), may lose hydrodynamic efficiency, that naturally benefit cohesive shoals by taking advantage of the flows and vortices created by the fish in front of them (Liao et al., 2003).

Fish hepatosomatic condition, as reported by the HSI values, was shown to differ from the control to the high concentration ash treatment, where, contrarily to our expectations, a significant increase has been noted. Several studies in the literature have come to contradicting effects concerning the behaviour of HSI in response to stressors (see review by Goede and Barton, 1990). For example, some studies have reported HSI to decrease after fish exposure to water pollution, considering that higher HSI values mean that fishes are growing rapidly and have a good aquatic environment, and that a decrease in HSI means that fish are not growing well and are thus facing unhealthy environmental problems (Norris et al., 2000). Others, however, reported an increase in HSI after exposure to stressors (Porter and Janz, 2003; Toft et al., 2003, 2004), and sometimes distinct responses to the same stressors: Akerman et al. (2003) found a decrease in HSI values after nine weeks in rainbow trout (*Onchorhynchus mykiss*), subjected to a widely used herbicide, the paraquat; on the contrary, Figueiredo-Fernandes et al. (2006) found an HSI increase in tilapia (*Oreochromis niloticus*) after exposure to the same stressor. Therefore, exposure to some stressors can results in an increase in HSI of fish, although exposure to others (and even the same ones) can reduce the HSI relative to the controls. Other factors, however, such as physiological development, food availability and parasites can also influence HSI (Sanchez et al., 2008). Taken together, these facts urge to the need of conducting long-term (weeks to months) future studies to disentangle to effects of ash exposure on fish condition, as measured though the HSI.

5. CONCLUSION

Results from the present study evidenced that fish behaviour and hepatosomatic condition were affected by previous short-term exposure to a gradient of ash concentration in the water, suggesting that ash flows associated with wildfires can represent an important source of disturbance for fish populations. Though cyprinids are expected to recover relatively quicker than salmonids (Dunham et al., 2003) due to traits (e.g. habitat generalists, omnivorous diet) that appear to facilitate their resistance after fire-disturbance events (Monaghan et al., 2016), the fact that even a short-duration exposure (24 hours) to ash runoff as in the present study, was sufficient to alter both behaviour and hepatosomatic condition of a medium-tolerant species as the barbel (Segurado et al., 2011), highlighting the need to conduct similar studies via long-term monitoring to better understand resilience of native species. The detected physiological alteration may promote deterioration of the fitness of the population, thus extending the short-term effect of exposure to ash to a long-term effect at the population level.

Future studies should also take into account that the effects on fish behaviour could become more evident with multiple stressors in addition to increasing ash concentration in the water, because in a natural setting ash runoff as a stressor, most likely, co-occurs with other stressors (e.g. increased nutrient availability, increased solar radiation and altered water temperature regimes) that may interact synergistically, augmenting the effect of ash runoff acting in isolation (Piggott et al., 2015). For this and other potamodromous fish species that need to seasonally perform upstream migrations for spawning purposes, maintaining an unfragmented river network will help ensure movement dispersal among different habitats and thus providing conditions for species recovery from fire-disturbance (Dunham et al., 2003; Pander et al., 2018; Whitney et al., 2016). When this is not possible, and funds are limited, prioritization of barriers to be removed (Branco et al., 2014) or retrofitted with fish transfer devices (i.e. fishways, Santos et al., 2012) before fire-related disturbance (pre-fire management), are likely to provide considerable benefits for the maintenance of fish populations. Nonetheless, in view of riverscape theory and limited empirical evidence on fish response to fire-related disturbances, management of river ecosystems will be most effective if a catchment (i.e. broad-scale) approach is explicitly addressed.

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SUPPLEMENTARY MATERIAL

Table 1SM - Physico-chemical parameters (mean ± SD) of water, measured after the 24-h exposition period of fish to the different ash concentration treatments (control= 0.0 g/L; low= 1.0 g/L and high= 2.0 g/L). Significant differences (Kruskal-Wallis test, $P < 0.05$) between treatments for each parameter are represented by different letters.

Parameters	Control	Low	High
Temperature (°C)	19.2 ± 0.1^{a}	18.9 ± 0.1^{ab}	18.5 ± 0.2^{b}
Conductivity (μS/cm)	861 ± 12^{a}	890 ± 14^{ab}	925 ± 11^{b}
pH	8.06 ± 0.07^{a}	8.17 ± 0.06^{ab}	8.26 ± 0.06^{b}
Dissolved oxygen (mg/L)	8.99 ± 0.04^{a}	8.78 ± 0.09^{ab}	8.34 ± 0.07^{b}

CAPÍTULO 2

Perspectiva ecotoxicológica das cinzas de cana-de-açúcar sobre peixes nativos e não-nativos

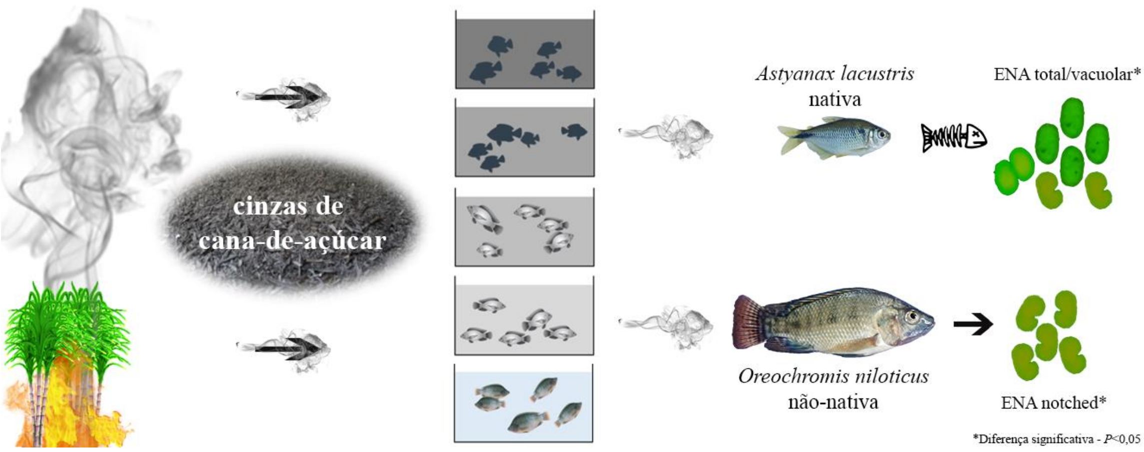
Artigo elaborado e formatado conforme as normas para publicação científica no periódico *Science of the Total Environment*.

Perspectiva ecotoxicológica das cinzas de cana-de-açúcar sobre peixes nativos e não-nativos

HIGHLITGHS

1. Cinza de cana-de-açúcar (CCA) constituem perturbação para peixes de água doce;
2. A exposição à CCA resultou em morte apenas de indivíduos nativos;
3. 24 h de exposição não induziu a formação significativa de micronúcleo (MN);
4. Espécie nativa mostrou mais alterações nucleares (ENA) que a não-nativa;
5. CCA foi mais tóxica para a espécie nativa do que a não-nativa.;

GRAPHICAL ABSTRACT



Perspectiva ecotoxicológica das cinzas de cana-de-açúcar sobre peixes nativos e não-nativos

RESUMO

A cana-de-açúcar é uma das principais culturas agrícolas de escala global, cultivada principalmente para a produção de sacarose e biocombustível. A prática da colheita de cana com uso do fogo pode apresentar prejuízos ao ambiente, entre eles, a liberação das cinzas da cana-de-açúcar (CCA), que apresentam diferentes hidrocarbonetos policíclicos aromáticos (HPA) e outros compostos tóxicos em sua composição. A CCA é nociva para o ambiente aéreo, apresentando-se como um problema de saúde pública. Entretanto, pouco se sabe sobre seus efeitos prejudiciais sobre a biota aquática, em especial sobre a dinâmica da tolerância de espécies nativas e não-nativas aos seus efeitos. Assim, o objetivo deste estudo foi avaliar e comparar a toxicidade aguda de diferentes concentrações de CCA em duas espécies de peixes, a nativa *Astyanax lacustris* (fam. Characidae) e a não-nativa *Oreochromis niloticus* (fam. Cichlidae), por meio dos parâmetros genéticos como teste do micronúcleo (MN) e alterações nucleares em eritrócitos (ENA). Ambas as espécies foram expostas durante um período de 24 h, a cinco concentrações de CCA diluídas em água (0, 1000, 1500, 2000 e 2500 mg/L). A exposição de *A. lacustris* e *O. niloticus* a diferentes concentrações de CCA resultou em morte apenas de indivíduos nativos e em ENA significativas nos núcleos de eritrócitos de ambas as espécies, com maior efeito prejudicial para a espécie nativa. A concentração de 1500 mg/L apresentou os maiores valores de ENA para ambas espécies. Para além do indivíduo, a presença de MN e ENA indicam efeitos de ecotoxicidade que podem refletir negativamente na ecologia das espécies, e consequentes alterações na composição e estrutura populacionais. Este trabalho contribui para o estudo dos impactos causados pela monocultura de cana sobre o ambiente aquático, em particular em espécies nativas e exóticas, e deve ser visto como um ponto de partida para maiores esclarecimentos dos efeitos deste poluente *in situ*.

Palavras-chave: Micronúcleo; Alterações nucleares; Mortalidade; Tilápia; *Oreochromis niloticus*; *Astyanax lacustris*;

An ecotoxicological perspective of sugarcane ash on native and non-native fish

ABSTRACT

Sugarcane is one of the main agricultural crops of global scale, mainly cultivated to produce sucrose and biofuel. The use of fire as a practice of sugarcane harvesting can damage the environment, including the releasing of sugarcane ash (CCA). CCA present different polycyclic aromatic hydrocarbons (HPA) and other toxic compounds in their composition. CCA is harmful to the air environment, considered a public health problem. However, its negative effects are poorly known on aquatic biota, especially their effects on the dynamics of the tolerance of native and non-native species. Thus, we aimed to evaluate and compare the acute toxicity of different concentrations of CCA in two species of fish, the native *Astyanax lacustris* (fam. Characidae) and the non-native *Oreochromis niloticus* (fam. Cichlidae), through the parameters such as micronucleus (MN) and nuclear alterations in erythrocytes (ENA). Both species were exposed to five water-diluted CCA concentrations (0, 1000, 1500, 2000 and 2500 mg/L) during 24 h. The exposure caused death to native species only and results in significant ENA values in both species, with a greater detrimental effect on the native species. In addition to the individual, the presence of MN and ENA indicate effects of ecotoxicity that may reflect negatively on the ecology of the species and consequent changes in population composition and structure. This work contributes to the study of the impacts caused by sugarcane crops to the aquatic environment, on native and non-native species, and should be a starting point for the further explanation of the CCA effects *in situ*.

Keywords: Micronuclei test; Nuclear alterations; Mortality; Tilapia; *Oreochromis niloticus*; *Astyanax lacustris*;

1. INTRODUÇÃO

A importância das atividades agroflorestais para a economia mundial compreende desde a produção de alimento (i.e. soja, trigo, arroz) até os bens de consumo, como o papel e a madeira (Mazoyer and Roudart, 2010), porém sua prática é dependente da destruição e fragmentação de habitat (Czech et al., 2000; Kerr and Deguise, 2004). A cana-de-açúcar (*Saccharum* sp; doravante cana) é uma das principais culturas agrícolas de escala global e também no Brasil (CONAB, 2017; FAOSTAT, 2018), cultivada principalmente para a produção de sacarose e biocombustível, este último como alternativa aos combustíveis fósseis (Goldemberg, 2007; Obama, 2017; Szuromi et al., 2007). Além da fragmentação de habitats nos países produtores e exportadores (Bernard et al., 2011; Costa et al., 2016), o cultivo de cana é caracterizado pela prática da queima das folhas secas para o processo de colheita.

Justifica-se o uso da queimada da cana com a facilidade e aumento de rendimento da colheita (Andreae, 1991). Apesar dos benefícios para a colheita, a prática da queima de cana pode apresentar prejuízos ao ambiente (Echavarria, 1996; Mendoza et al., 2002). As cinzas da cana-de-açúcar (CCA) apresentam diferentes hidrocarbonetos policíclicos aromáticos (HPA) pirogênicos em sua composição (e.g. antraceno, benzopireno e fluoranteno), além de outros compostos tóxicos (Darley and Lerman, 1975; Ferreira et al., 2014; NIH, 2017; Zamperlini et al., 1997). A CCA é reconhecida como nociva para o ambiente aéreo quando liberada na atmosfera, apresentando-se como um problema de saúde pública (Arbex et al., 2007; França et al., 2012; Roseiro and Takayanagui, 2004). Martínez-Valenzuela et al., (2015), por exemplo, registraram danos genotóxicos significativos por meio da análise de micronúcleo e alterações nucleares em células bucais de cortadores de cana do México. A CCA (incluindo os HPAs) podem permanecer no solo ou ser transportadas para os ecossistemas aquáticos por escoamento de água durante eventos de precipitação (Gasith and Resh, 1999; Nunes et al., 2017; Rose and Rippey, 2002). Entretanto, pouco se sabe sobre os efeitos prejudiciais da CCA sobre a biota aquática.

Diversas substâncias químicas podem induzir o desenvolvimento de micronúcleo (MN) e alterações nucleares em eritrócitos (ENA) de peixes, e por isso estes são reconhecidos como importantes ferramentas de avaliação do potencial genotóxico de poluentes do ambiente aquático (Al-Sabti and Metcalfe, 1995; Lohner et al., 2001; Santos and Pacheco, 1996; Sayed et al., 2018; Shahjahan et al., 2018; Talapatra and Banerjee, 2007; Walia et al., 2013). Eritrócitos de peixes são preferidos para estudos desta natureza pois a frequência de alterações em seu núcleo pode indicar diferentes níveis toxicológicos (Gomes et al., 2015; Sayed et al., 2017). Existem diferentes tipos de alterações nucleares em eritrócitos e consequentemente diversas explicações para elas (Alberts et al., 2002; Hussain et al., 2012; Seriani et al., 2011; Shimizu et al., 1998). Contudo, apesar das diferentes explicações, existem danos mais relacionados com a apoptose do que outros, como a ENA do tipo vacuolar (Dini et al., 1996; Ghisi et al., 2014), podendo ser considerado mais prejudicial. Estudos que enfatizam a aplicação de biomarcadores em espécies de peixes nativas como ferramentas para avaliar a qualidade da água ainda são escassos no Brasil (Akaishi et al., 2004; Vieira et al., 2017; Wilhelm Filho et al., 2001).

Entretanto, há uma lacuna ainda maior no emprego destes biomarcadores no entendimento da tolerância de espécies nativas e não-nativas ao ambiente modificado. A CCA, por exemplo, além de causar a morte de organismos (Gonino et al., *no prelo*), pode aumentar a suscetibilidade dos ecossistemas aquáticos à invasão de espécies não-nativas se comprovado que apresenta danos subletais em peixes. Espécies não-nativas com potencial para serem invasivas têm maior tolerância a estressores ambientais (Fedorenkova et al., 2013; Karatayev et al., 2009; Leuven et al., 2011). Por este motivo, as espécies comparadas neste trabalho foram a nativa *Astyanax lacustris* (Lütken, 1875) (fam. Characidae) e a não-nativa, *Oreochromis niloticus* (Linnaeus, 1758) (fam. Cichlidae). Estas espécies foram selecionadas com base em sua ampla distribuição na bacia do rio Paraná (Brasil) e em sua importância econômica e ecológica, respectivamente (Costa et al., 2008; Lamboj, 2004). Espécies do gênero *Astyanax* são úteis como modelo animal para ensaios por serem de fácil coleta, terem tamanho adequado para experimentação, e se adaptarem bem às condições laboratoriais. Além disso, *A. lacustris* é onívora oportunista (Ghisi et al., 2014; Viana et al., 2018, 2013). *O. niloticus*, por sua vez, também onívora, apresenta fácil adaptação a novos ambientes, tolerância a xenobióticos, ampla distribuição (Gomes et al., 2015; Linde-Arias et al., 2008; Vijayan et al., 1996). Além disso, ambas as espécies têm importância na alimentação humana.

Propõe-se aqui o primeiro estudo a examinar a capacidade da CCA em induzir alterações genéticas em peixes e estabelecer comparações entre espécies nativas e não-nativas. Estas informações, ainda inéditas, são fundamentais para o estabelecimento de políticas de proteção à biodiversidade aquática. Desta forma, o objetivo deste estudo foi avaliar e comparar a toxicidade aguda de diferentes concentrações de CCA em duas espécies de peixes, sendo uma nativa (*Astyanax lacustris*) e outra não-nativa (*Oreochromis niloticus*) à bacia do rio Paraná, por meio de parâmetros genéticos. Hipotetiza-se que a espécie nativa seja menos tolerante ao potencial tóxico de CCA do que a espécie não-nativa, seja em número e/ou tipo de alterações nucleares. Espera-se que a exposição às diferentes concentrações de CCA promova danos nucleares mais graves na espécie nativa.

2. MATERIAL E MÉTODOS

2.1. Cinzas de cana-de-açúcar

As cinzas de cana utilizadas neste trabalho foram obtidas a partir da queima de folhas secas, coletadas de uma única área agrícola de monocultura extensiva de cana, próxima de riachos (23° 20' 29.9" Sul e 51° 57' 59.3" Oeste). As folhas foram submetidas a combustão controlada, simulando a queima no processo de colheita da cana. Após a queima e o resfriamento natural, as cinzas foram crivadas em malha 0,5 cm, homogeneizadas, acondicionadas em frascos plásticos e congeladas, ao abrigo de luz direta, para evitar possível degradação dos HPAs, até o momento da utilização nos ensaios (Korfmacher et al., 1980).

2.2. Espécimes de peixes

Foram utilizados juvenis da espécie nativa *Astyanax lacustris* (n= 15; comprimento total médio (TL) ± desvio padrão (SD) = 1,84 ± 0,47 cm) e da não-nativa *Oreochromis niloticus* (n= 9; TL ± SD = 38,17 ± 7,05 cm) obtidos de criadores autorizados. Os peixes foram mantidos em tanques de 1.000 litros com água de distribuição desclorada, no período mínimo de 48 horas, e receberam tratamento preventivo contra fungos e bactérias (Selosse and Rowland, 1990) através da adição de sal. Os indivíduos receberam alimentação *ad libitum* com ração comercial em *pellets* durante o período nestes tanques, até o momento da aclimatação nos microcosmos.

2.3. Desenho experimental

Seguiu-se o desenho experimental de acordo com Gonino et al. (*no prelo*), com cinco concentrações de CCA diluídas em água (0, 1000, 1500, 2000 e 2500 mg/L – Fig. 1). Cada concentração de CCA foi repetida 3 vezes, totalizando 15 microcosmos individuais (aquários de vidro), contendo 25 L de água de distribuição previamente desclorada com tiossulfato de sódio (temperatura 24,5 ± 1,3 °C, fotoperíodo natural e aeração constante). Os indivíduos foram adicionados para aclimatação às condições do aquário 24 h antes do início do ensaio, onde permaneceram sem alimentação. Devido à diferença de tamanho dos juvenis de cada espécie, o número de indivíduos por aquário foi de 5 para *A. lacustris* e 3 para *O. niloticus*, executados separadamente. O início do ensaio deu-se com a diluição de CCA nos aquários, com posterior exposição dos espécimes durante um período de 24 horas. Determinou-se este tempo devido à degradação natural de HPA presentes nas cinzas por foto-oxidação, cujas propriedades podem ser alteradas com o tempo (Cerniglia, 1992). Os ensaios foram realizados separadamente por espécie (agosto de 2016) e os valores das propriedades físicas e químicas da água foram registrados ao longo de cada ensaio, nomeadamente temperatura (°C - termômetro analógico), oxigênio dissolvido (mg/L – oxímetro YSI® 550A), pH (potenciômetro Digimed®), condutividade (μS/cm – condutivímetro DM3 Digimed®) e turbidez (UNT - turbidímetro LaMote®).

Figura 1. Esquema do desenho experimental de uma réplica, no qual juvenis de *Astyanax lacustris* e *Oreochromis niloticus* foram submetidos a cinco concentrações de cinzas de cana-de-açúcar (0, 1000, 1500, 2000 e 2500 mg/L) durante 24 horas. Cada concentração foi repetida em 3 aquários. Adaptado de Gonino et al. (*no prelo*).

2.4. Amostragem de sangue e testes de ecotoxicidade

Após 24 h de exposição os peixes foram retirados dos aquários e anestesiados em solução alcoólica de benzocaína 5%. Foram coletadas amostras de sangue por punção da veia caudal de cada indivíduo, com auxílio de seringas e agulhas previamente heparinizadas, para confecção de duas lâminas de esfregaço sanguíneo, colocadas para secar em temperatura ambiente *overnight*. Os esfregaços foram fixados com metanol 100% por 10 minutos e corados com laranja de acridina (0,03%) no momento da análise. As lâminas foram observadas com auxílio de microscópio óptico de fluorescência (aumento de 1000 x). Foram analisadas 1.000 células por indivíduo (Heddle et al., 1991) para registro da abundância de MN e de ENA. Foram calculadas as médias da quantidade de MN, de ENA Total (i.e., a soma de todos os tipos de alterações nucleares nos eritrócitos, exceto MN) e dos diferentes tipos de ENA de cada concentração. Chamou-se MN o material genético separado, com tamanho menor que 1/3 e com a mesma coloração do núcleo principal, não refrativo; e ENA os núcleos que apresentaram morfologia do tipo: vacuolados (*vacuoled*), em forma de rim (*notched*), lobulado *(lobed*), segmentado (*blebbed*), com broto (*bud*), em forma de oito (*eight shaped*), irregular e células binucleadas, seguindo o proposto por Carrasco et al. (1990) e Furnus et al. (2014). Todos os procedimentos foram aprovados pelo Comitê de Ética no Uso de Animais da Universidade Estadual de Maringá (CEUA nº 8945150316).

2.5. Análise de dados

Foram testados os pressupostos de normalidade e homoscedasticidade dos dados de MN e ENA. Para testar as premissas de danos genotóxicos mais graves na espécie nativa comparou-se os valores médios dos danos apresentados pelas duas espécies frente às diferentes concentrações de CCA. Utilizou-se o teste não-paramétrico de Kruskal-Wallis para a comparação das diferenças entre os grupos (espécies e concentrações) e de Dunn, como *post-hoc*. As análises estatísticas e gráficas foram realizadas com o software R (R Core Team, 2018), com nível de significância de 0,05.

3. RESULTADOS

A temperatura manteve-se próxima de 24,4 (±0,60) °C para todos os aquários, e assim como o oxigênio dissolvido, com média de 6,38 (±0,18) mg/L, não diferiu significativamente em relação ao controle. Entretanto, os valores médios de turbidez (168,84 ±95,91 NTU), condutividade (259,38 ±66,60 µS/cm^3) e pH (8,77 ±0,67), foram diferentes.

Registrou-se letalidade de aproximadamente 20 e 50 % de indivíduos da espécie nativa nas duas maiores concentrações de CCA, 2000 e 2500 mg/L respectivamente, ao contrário do observado para a espécie não-nativa, em que não houveram mortes (Fig. 2).

Foram registrados, além dos micronúcleos, sete tipos de alterações nucleares em eritrócitos de *A. lacustris* e *O. niloticus* (Fig. 3).

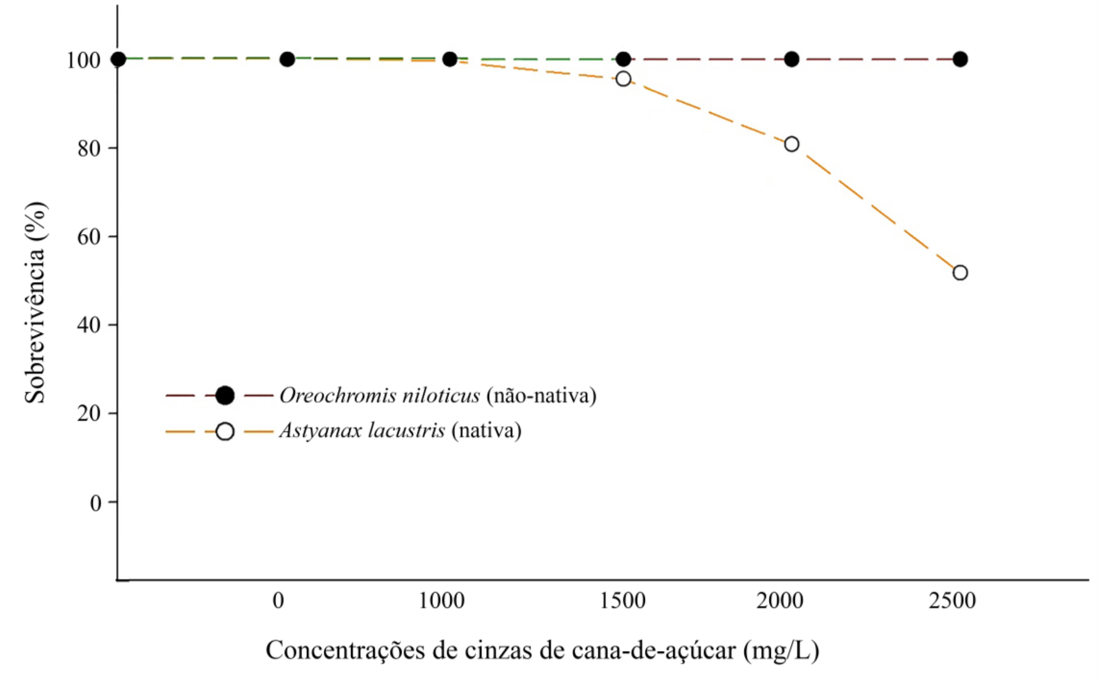


Figura 2. Mortalidade de peixes nativos e não-nativos expostos a cinco diferentes concentrações de cinzas de cana-de-açúcar por 24 horas.

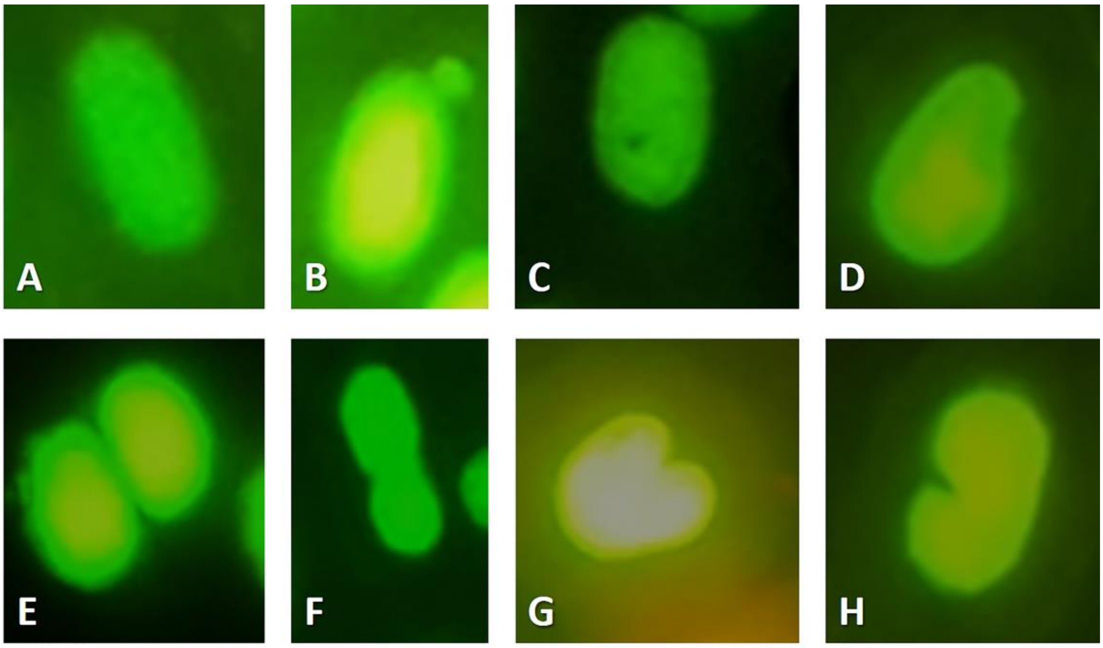


Figura 3. Eritrócitos com anormalidades nucleares observados em *Astyanax lacustris* (A, B, C e D) e *Oreochromis niloticus* (E, F, G e H) expostos a cinzas de cana-de-açúcar. A – eritrócito normal. B – micronúcleo. C – núcleo com vacúolo (*vacuoled*). D – Núcleo com broto superior (*bud*). E – célula binucleada. F – núcleo em formato de 8 (*eight shaped*). G e H– núcleo riniforme ou talhado (*notched*). Coloração: laranja de acridina. Escala: 1000 µm.

3.1. Teste do Micronúcleo (MN)

O número de micronúcleos foi baixo para as duas espécies de peixes testadas. Este número foi ligeiramente maior na espécie nativa (*A. lacustris*) em relação à espécie não-nativa (*O. niloticus*), porém, não houveram diferenças significativas (estatística do teste *P*> 0,05). (Fig. 4)

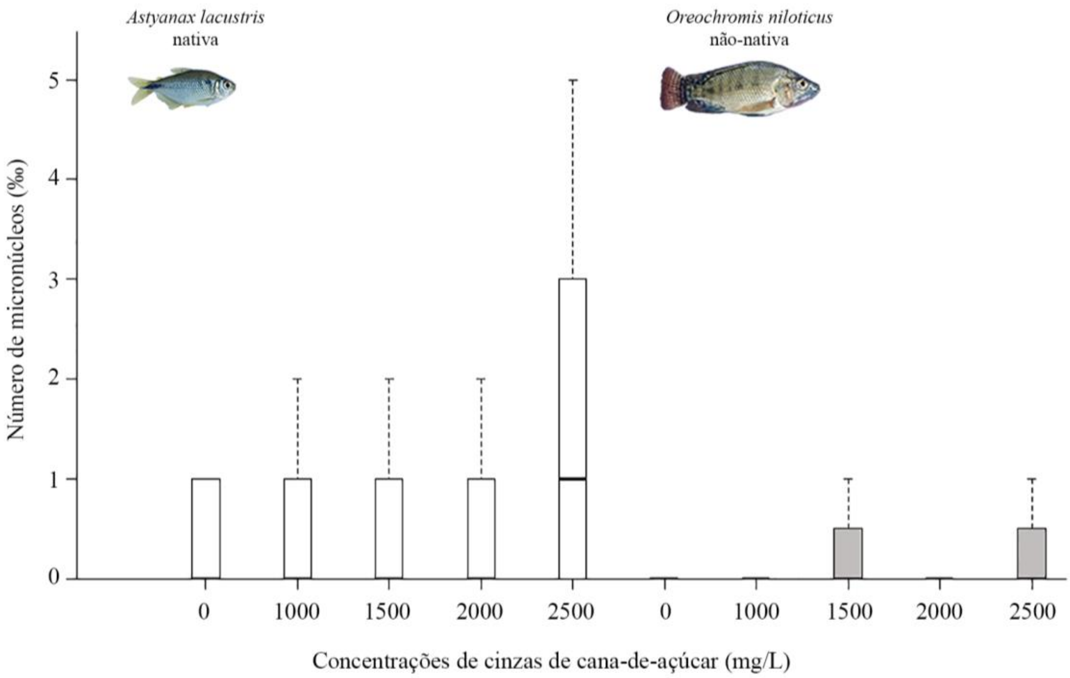


Figura 4. Frequência de Micronúcleos em *Astyanax lacustris* (nativa - branco) e *Oreochromis niloticus* (não-nativa - cinza) expostos a cinco diferentes concentrações de cinzas de cana-de-açúcar (0, 1000, 1500, 2000 e 2500 mg/L), durante 24h. As linhas verticais representam o intervalo, as caixas são os quartis e a linha horizontal, a mediana.

3.2. Alterações Nucleares Eritrocíticas (ENA)

3.2.1. ENA Total

Os valores de ENA Total diferiram significativamente entre as concentrações de CCA para a espécie nativa (H = 11,478, df = 4, *P* = 0,02) mas o mesmo não ocorreu para a espécie não-nativa (H = 9,3345, df = 4, *P* > 0,05) (Fig. 5). A concentração de 1500 mg/L apresentou os maiores valores de ENA Total, diferindo significativamente do controle (*P* = 0,01) e da concentração de 1000 mg/L (*P* = 0,02).

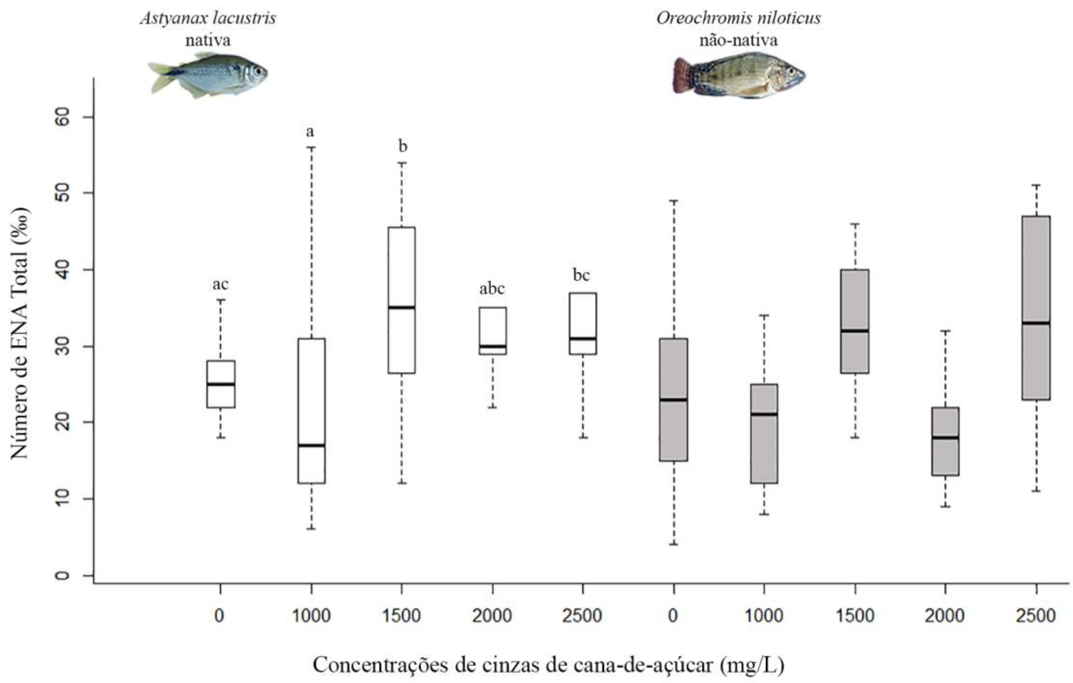


Figura 5. Número de alterações nucleares totais em eritrócitos de *Astyanax lacustris* (nativa) e *Oreochromis niloticus* (não-nativa) expostos a cinco concentrações de cinzas de cana-de-açúcar (0, 1000, 1500, 2000 e 2500 mg/L), durante 24h. As linhas verticais representam o intervalo, as caixas são os quartis e a linha horizontal, a mediana. Letras diferentes indicam diferenças significativas ($P < 0.05$) apenas no ensaio com a nativa.

3.2.2. ENA do tipo vacuolar e *notched*

Não foram registradas diferenças significativas para ENA do tipo *blebbed*, *bud*, *eight shaped*, irregular e binucleadas para ambas as espécies. Entretanto, foram encontradas diferenças significativas nos valores de ENA dos tipos vacuolar (*A. lacustris* - H =12,773, df = 4, *P* = 0,01) e *notched* (*O. niloticus* - H = 10,796, df = 4, *P* = 0,03). As alterações do tipo vacuolar foram as mais encontradas na espécie nativa (Fig. 6A) enquanto as do tipo *notched* ocorreram com mais frequência na espécie não-nativa (Fig. 6B). No ensaio com a nativa *A. lacustris* houveram diferenças significativas de ENA vacuolar entre as concentrações 1500 mg/L em relação ao controle (*P* = 0,002) e à concentração 1000 mg/L (*P* = 0,006), e da concentração 2500 mg/L com o controle (*P* = 0,01). Para *O. niloticus*, registrou-se diferença significativa de ENA do tipo *notched* (H = 10,796, df = 4, *P* = 0,03) da concentração 1500 em relação às concentrações 1000 e 2000 mg/L (*P* = 0,01 e 0,004, respectivamente), e entre 2000 e 2500 mg/L (*P* = 0,01) porém nenhum tratamento diferiu significativamente do controle.

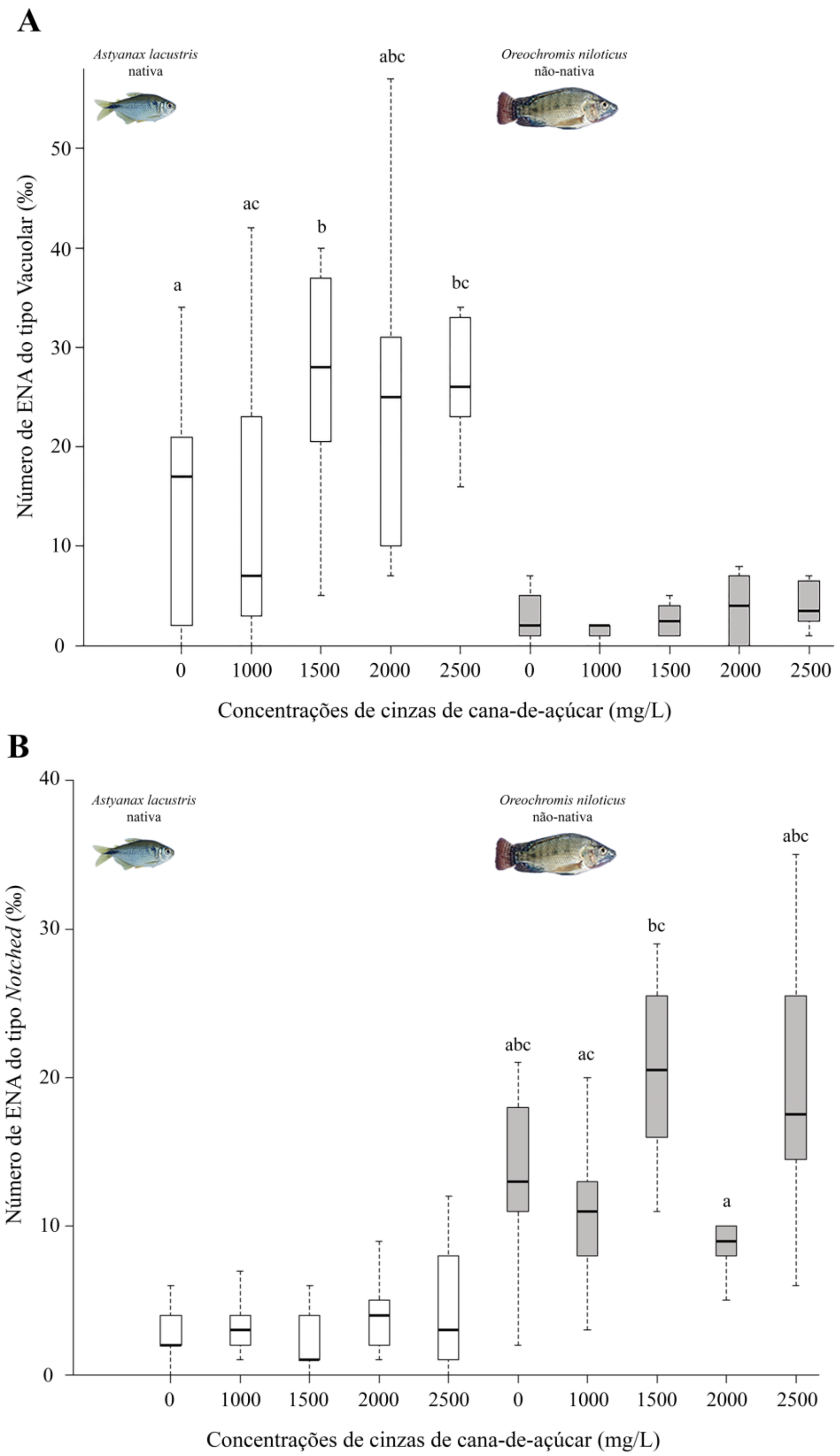


Figura 6. Número de alterações nucleares do tipo vacuolar **(A)** e do tipo *notched* **(B)** em eritrócitos de *Astyanax lacustris* (branco) e *Oreochromis niloticus* (cinza), expostos a cinco concentrações de cinzas de cana-de-açúcar (0, 1000, 1500, 2000 e 2500 mg/L), durante 24h. Letras diferentes, quando presentes, indicam diferenças significativas (P < 0.05). As linhas verticais representam o intervalo, as caixas são os quartis e a linha horizontal, a mediana.

4. DISCUSSÃO

Nosso objetivo de avaliar e comparar os efeitos negativos da CCA sobre biomarcadores genéticos de duas espécies de peixes, sendo uma nativa e outra não-nativa, foi alcançado. A hipótese de que a espécie nativa é menos tolerante ao potencial tóxico de CCA do que a espécie não-nativa não foi rejeitada pelo nosso estudo. A exposição de *A. lacustris* e *O. niloticus* a diferentes concentrações de CCA resultou em morte apenas de indivíduos nativos (nas concentrações 2000 e 2500 mg/L) e alterações morfológicas significativas nos núcleos de eritrócitos de ambas as espécies, com maior efeito prejudicial para a espécie nativa, conforme esperado. Os efeitos tóxicos de cinzas de diferentes espécies vegetais têm sido estudados com auxílio de diversos modelos animais, e seus resultados sugerem que as variações nos efeitos podem estar relacionadas também à origem vegetal dessas cinzas (Nunes et al. 2017; Brito et al. 2017; Gonino et al. *no prelo*).

4.1. Teste do Micronúcleo (MN)

A presença de MN em eritrócitos é considerado um indicador de danos genéticos (Samanta and Dey, 2012). A frequência de MN espontâneos (i.e., frequência basal) é mais comum em peixes de águas poluídas (Obiakor et al., 2014), porém sua indução é útil para avaliar danos genotóxicos causados por agentes mutagênicos (Heddle et al., 1983). A ausência de diferenças significativas no teste do MN para ambas as espécies expostas a CCA pode ser explicada pelo fato da frequência basal de MN ser naturalmente baixa em peixes (Ferraro et al., 2004). Apesar de ser considerado um excelente biomarcador para o monitoramento da poluição em ambientes aquáticos (Minissi et al., 1996; Obiakor et al., 2014), as frequências baixas de MN dos peixes expostos a CCA assemelha-se ao apresentado para *O. niloticus* (Bolognesi and Hayashi, 2011) e para *A. altiparanae* (Vieira et al., 2017). Esta baixa frequência pode estar relacionada, entre outras coisas, ao tamanho dos peixes (Carrasco et al., 1990), à intensidade e tempo da exposição aguda (Heddle et al., 1991), ou ao tipo de tecido utilizado, pois o MN se origina do ciclo celular completo (Fenech, 2007) e é dependente da proporção de células que entram em divisão (Rocha et al., 2009; Salvadori et al., 2003; Vanzella et al., 2007). Ensaios com *A. lacustris* expostos a diferentes concentrações do HPA Naftaleno por 96h, não detectaram a presença de MN, e atribuíram este resultado ao tempo de exposição (Disner et al., 2017). Também podem ser explicações para a baixa quantidade de MN nos ensaios aqui apresentados, o grau de genotoxicidade da CCA e a tolerância das espécies, que exibem diferentes graus de sensibilidade ao dano genético, como encontrado por Kligerman (1982) e Obiakor et al. (2014).

4.2. Alterações Nucleares em Eritrócitos (ENA)

O aumento significativo no número de ENA Total apenas para a espécie nativa confirma a premissa de que a exposição às diferentes concentrações de CCA promoveria danos genotóxicos mais graves a essa espécie. Diversos estudos mostram a relação positiva entre as frequências de ENA e MN, e suas vantagens como poderosa ferramenta para o estudo de danos genotóxicos e citotóxicos em eucariotos, encorajando seu emprego para este tipo de estudo em peixes (Al-Sabti and Metcalfe, 1995; Botelho et al., 2018; Ergene et al., 2007; Ferraro et al., 2004; Sayed et al., 2017). Em ensaio conduzido por Disner et al. (2017), *A. lacustris* não desenvolveu ENA mesmo após 96 h de exposição ao HPA Naftaleno. Desta forma, entende-se que o tempo de exposição de 24 h foi suficiente para gerar um incremento no número de ENA Total nas duas espécies testadas, confirmando a genotoxicidade da CCA, mas não o foi para aumentar significativamente a indução de MN pelas substâncias tóxicas que a compõem. Ensaios de toxicidade crônica ajudarão a elucidar se a CCA é capaz de gerar danos genéticos irreparáveis.

As alterações nucleares nos eritrócitos dos peixes do presente estudo podem dever-se aos efeitos genotóxicos da CCA. Existem diferentes explicações para a formação de ENA, nomeadamente danos à lâmina do citoesqueleto, processo de reparo e eliminação de dano cromatínico, estresse oxidativo, divergências cromossômicas, aneuploidias e processos apoptóticos (Alberts et al., 2002; Hussain et al., 2012; Seriani et al., 2011; Shimizu et al., 1998). Portanto, é aceitável supor que os diferentes tipos de ENA acarretam diferentes tipos de danos às células. Dini et al. (1996) relacionaram os diversos caminhos de fragmentação nuclear que levam à apoptose com o tipo de estímulo indutor, neste caso, o poluente CCA. Assim, o aumento e predominância significativos de ENA vacuolar em *A. lacustris*, em relação ao controle, e ENA do tipo *notched* apenas entre as concentrações de *O. niloticus*, é um indicativo de diferença na tolerância destas espécies contra CCA. Vieira et al. (2017) mostraram que *A. altiparanae* expostos a diferentes contaminantes agrícolas têm predomínio de ENA vacuolar. Ao contrário de Ventura et al. (2008), que encontraram este mesmo tipo de ENA vacuolar como a anormalidade nuclear mais frequente em *O. niloticus*, expostos ao herbicida atrazina. (Ghisi et al., 2014) aproximaram o processo de vacuolização causado por substâncias tóxicas com apoptose dos eritrócitos. Se considerarmos a letalidade observada das espécies testadas, é tentador supor que o tipo vacuolar sofrido pela espécie nativa reflita um dano mais grave que o tipo *notched*, mais frequente na não-nativa, fato que reforçaria a premissa deste estudo. A CCA contém diversos tipos de HPA e outros compostos tóxicos em sua composição (Zamperlini et al., 1997), e isso pode explicar a formação significativa de ENA numa exposição de 24 h nas duas espécies testadas.

4.3. Potencial tóxico da CCA

Diversos contaminantes como metais, compostos orgânicos halogenados, pesticidas e HPAs podem causar micronúcleos e outros danos genéticos (Disner et al., 2017; Dourado et al., 2016; Viana et al., 2018). Há registro de diferentes HPAs indutores de ENA em peixes (Botelho et al., 2018; Disner et al., 2017). Entretanto, entende-se a CCA como uma mistura complexa, contendo mais de 30 HPAs identificados, sendo 16 deles reconhecidos como mutagênicos e carcinogênicos (EPA, 1998; Zamperlini et al., 1997). Esta pode ser uma explicação para a exposição de 24 h realizada neste trabalho ter sido capaz de promover letalidade e indução significativa de ENA nos peixes, ao contrário de exposições mais longas a HPAs isolados, como mostraram Disner et al. (2017). Destaca-se o fato da concentração de 1500 mg/L de CCA ter apresentado padrão inesperado para um gradiente de concentração, uma vez que resultou em efeitos mais pronunciados que as concentrações mais elevadas. Acredita-se que este padrão se deve ao ponto de saturação aquosa de CCA estar próximo do valor 1500 mg/L, e o aumento da concentração não acarretar maiores quantidades de HPAs presentes na água (i.e. solução saturada/hipersaturada). Por isso a concentração 1500 mg/L ter sido a mais danosa nos resultados significativos nas espécies nativa e exótica. Fato semelhante foi registrado por Silva et al. (2012) onde a planta *Tradescantia pallida* apresentou maiores valores de MN para a menor concentração exposta a cinzas de bagaço de cana. Tal como eles, espera-se que níveis significativos de ENA e MN somente sejam encontrados em níveis tóxicos ou quase tóxicos. Sendo assim, estudos sugerem que concentrações mais baixas podem conter mais agregados de partículas e a fração ultrafina pode favorecer a adsorção nas células de componentes mutagênicos, como HPAs (Claxton et al., 2004; Silva et al., 2012).

4.4. Implicações para a gestão

Para além do indivíduo, a presença de MN e ENA indicam efeitos de genotoxicidade que podem resultar em consequentes alterações na composição (ou seja, número de indivíduos, frequência ou abundância) e estrutura (e.g. alterações ao nível das classes etárias, estágios de vida, balanço juvenis/adultos) populacionais (Bickham and Smolen, 1994; van der Oost et al., 2003; Vanzella et al., 2007). Desta forma, a CCA pode refletir na ecologia e dinâmica da ictiofauna em ecossistemas aquáticos de água doce, seja por causar a mortalidade direta das espécies nativas ou por efeitos genéticos subletais que resultarão em novos processos biológicos (e.g. enfraquecimento e diminuição de populações, invasão de espécies). A própria cadeia trófica, por exemplo, pode influenciar e ser influenciada pela frequência espontânea de MN e ENA como uma consequência do processo de bioacumulação (Porto et al., 2005; Vanzella et al., 2007). A CCA pode afetar mais significativamente espécies nativas de hábito alimentar onívoro, como as do gênero *Astyanax*, que constituem um importante componente das comunidades ictiológica em riachos. Além disso, essas espécies podem ingerir resíduos carbonizados de palha de cana, aumentando os níveis de HPA em seu corpo, e consequentemente aumentar os riscos de eventuais danos genéticos. Estes efeitos podem ser intensificados em ambientes que recebem CCA na época de queimada de cana para a colheita quando a disponibilidade deste agente tóxico será maior (de Andrade et al., 2010). Possíveis medidas para diminuir o impacto passam pela substituição do processo de queima até a introdução de faixas de vegetação junto às linhas de água, com espécies florestais resilientes ao fogo e que retenham as cinzas.

4.5. Implicações para as invasões

Do ponto de vista das invasões biológicas, a hipótese de tolerância biótica sugere que ecossistemas com maior diversidade de espécies nativas são mais tolerantes ao processo de invasão (Elton, 1958). Nossos resultados indicam o potencial efeito deletério da CCA para espécies nativas, o que resultaria em redução da diversidade nativa e consequentemente redução na tolerância biótica aos processos de invasão. Ainda, espécies não-nativas são consideradas uma das mudanças globais (Sala et al., 2000). Um dos impactos negativos das invasões é a homogeneização biótica causada pelo aumento da competição entre espécies nativas e não-nativas (Petsch, 2016). Dessa forma, ambientes que recebem a CCA poderiam estar mais susceptíveis à invasão (redução da tolerância biótica), e, em tendo sucesso, a espécie não-nativa poderia intensificar a redução das espécies nativas (tolerância da espécies não-nativa à CCA e competição entre espécies nativas e não-nativas) devido à interação positiva entre os efeitos da CCA e da invasão sobre as espécies nativas.

4.6. Implicações para a saúde pública

Devido aos seus potenciais efeitos prejudiciais sobre os organismos vivos, incluindo seres humanos, os HPAs são registrados em listas europeias e americanas de poluentes prioritários que precisam ser monitorados no ambiente (USEPA, 2008). Entre as formas mais importantes de exposição humana aos HPAs estão a inalação de ar poluído e a ingestão de alimentos ou de água contaminada (IPCS, 1998). Mansilha et al. (2014) encontraram 15 de 16 HPAs em amostras de água subterrânea coletadas em áreas que sofreram queimadas florestais, em Portugal. Alguns HPAs presentes nas cinzas (independentemente de sua origem vegetal) podem persistir no meio aquoso devido a características que dificultam sua biodegradação (Mueller et al., 1991), e assim contaminar a cadeia alimentar por bioacumulação em espécies aquáticas como peixes (Van der Oost et al. 1991). Desta forma, pode-se considerar que corpos aquáticos inseridos em microbacias onde há queima constante de palha de cana, esteja recebendo aportes constantes de HPAs, aumentando o fluxo e biocumulação destes poluentes na biota local, com possibilidade crescente de chegar aos seres humanos. Por exemplo, Akpan et al. (1994) calcularam a quantidade do HPA Benzo(a)pireno ingerida a partir do consumo diário de peixes provenientes de águas contaminadas em três cidades nigerianas. Muitos experimentos com HPAs demonstraram sua carcinogenicidade em animais, que nos leva a crer que estes podem impactar significativamente como causa de vários tipos de câncer na população humana (Phillips, 1999). Desta forma, há poucas dúvidas sobre o impacto antrópico causado pela queima da cana sobre a saúde pública, não somente por meio do ambiente aéreo, como exposto por Arbex et al. (2007), mas também pela via aquática quando ocorrem em riachos após queimadas (Smith et al. 2011).

5. CONSIDERAÇÕES FINAIS

Por fim, os resultados aqui apresentados concordam com os estudos que apontam a queima da cana-de-açúcar como causadora de grande impacto ao meio ambiente (Arbex et al., 2007; Mendoza et al., 2002) e à saúde humana (Akpan et al. 1994; USEPA, 2008). Este trabalho contribui para o estudo dos impactos causados pela monocultura de cana sobre o ambiente aquático de água doce e deve ser visto como um ponto de partida para o esclarecimento das ações ecotóxicas relacionadas à CCA na hidrosfera. Sugerem-se ensaios voltados especificamente à tolerância fisiológica das espécies nativas e não-nativas, a fim de elucidar se as últimas são naturalmente mais tolerantes ou apresentam melhorias em seus mecanismos de defesas (e.g., eficiência no sistema de reparo de DNA e a dinâmica de remoção celular) envolvidos na variação do número de ENA e MN identificados.

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CAPÍTULO 3

A fish-based index of biotic integrity for Neotropical rainforest sandy soil streams – Southern Brazil

Artigo elaborado e formatado conforme as normas para publicação científica no periódico *Science of the Total Environment*.

A fish-based index of biotic integrity for Neotropical rainforest sandy soil streams – Southern Brazil

HIGHLITGHS

1. We developed a fish-based IBI for Neotropical Sandy Soil Streams (N3S-IBI).
2. Six metrics were approved in all tests to compose the N3S-IBI.
3. N3S-IBI showed an excellent performance to separate least and most disturbed sites.
4. Non-native Cyprinodontiformes are related to lower values to N3S-IBI.
5. IBI and RAP integration was beneficial for assessing sandy soil streams.

GRAPHICAL ABSTRACT

N3S - IBI

Metrics	Type
Simpson's dominance	Composition
Number of Characiformes individuals	Abundance
Percent of Characidae species	Richness
Percent of insectivorous less tolerant	Trophic
Number of non-native individuals	Alien
Percent of tolerant species	Tolerance

A fish-based index of biotic integrity for Neotropical rainforest sandy soil streams – Southern Brazil

ABSTRACT

Multimetric indices are considered a low-cost and rapid means of assessing ecological integrity in streams. The present study aimed to develop a fish-based Index of Biotic Integrity for Neotropical Sandy Soil Streams (N3S-IBI) in an agricultural dominated region originated from the Atlantic rainforest. Our hypothesis is that the predominance of Characiformes and Siluriformes, and the presence and/or absence of Cyprinodontiformes are driven by the low biotic quality of the streams (i.e. lower value of IBI) also in our study area, and we did not reject this. Using a Rapid Assessment Protocol (RAP) especially designed for the study region habitat we sampled 23 stream sites, where 7 of them were the reference areas (least disturbed – LD), 9 were intermediary and 7 were the most disturbed sites (MD). N3S-IBI result in a list of 6 metrics, being Simpson's dominance (composition), number of Characiformes individuals (abundance), percent of characidae species (richness), percent of insectivorous less tolerant (trophic), number of non-native individuals (alien) and percent of tolerant species (tolerance). It showed an excellent performance to separate least and most disturbed sites through fish-based biotic integrity values in our study area. The number of 6 metrics helps to make a quick and easy biomonitoring tool. Our study deals with this gap in one of the most impacted regions by monocultures, especially sugarcane, providing more information about this anthropogenic action impacts. In this way, although it needs to be validated, N3S-IBI was a well-developed tool to freshwater systems in a region of approximately 3 million ha. Tools like N3S-IBI may become important that are more accessible to facilitate the engagement of people inside and outside the control bodies and the academic environment. In practice, this would be well utilized by directing restoration actions to MD sites and strengthening the preservation of LD sites.

KEYWORDS: IBI; Environmental quality; Characiformes; Cyprinodontiformes; *Poecilia;* Sugarcane.

1. INTRODUCTION

Surface freshwaters are among the most disturbed ecosystems on the planet, and in recent decades have suffered faster rates of decline in biodiversity than those observed in terrestrial ecosystems (Carpenter et al., 2011; Saunders et al., 2002). The conversion of natural landscapes to human use are among the main drivers of the impact of these systems (Allan, 2004; Sabater and Elosegi, 2013). In fact, agricultural practices are the most widespread cause of stream degradation, modifying natural hydrological patterns, increasing chemical inputs and sediments, and altering natural habitats (Allan, 2004; Carpenter et al., 2011; Henley et al., 2000; Wang et al., 1997). Many studies have shown that agricultural land may strongly influence the integrity of fish communities, affecting ecological traits related to feeding and reproduction and promoting the abundance increasing of non-native and tolerant species (Bramblett et al., 2005; Feld 2013; Meador and Goldstein, 2003; Santos and Esteves, 2015; Wang et al., 1997).

The deforestation of the watersheds and the consequent conversion to crops is the largest anthropic impact on tropical stream ecosystems (Barletta et al., 2010; Winemiller et al., 2008). The southern region of Brazil has also experienced an increase of crop areas in last decades, mainly sugarcane, corn, wheat and soybean plantations, with obvious impacts on watersheds, including loss of riparian habitats, decreasing bank stability, increasing bed sedimentation and contamination with pesticides, heavy metals and herbicides (Hepp and Santos, 2009; Santos et al., 2015). Common endpoints are the replacement of habitat and feeding of specialist fish species by widespread generalists and the increasing of species that are tolerant to a wide range of physicochemical conditions (Bozzetti and Schulz, 2004; Esteves and Alexandre, 2011; Santos and Esteves, 2015). The predominance of Characiformes and Siluriformes species in preserved streams of the neotropical region is well known (Castro et al., 2004, 2003; Cetra et al., 2016; Lowe-McConnell, 1987), but in disturbed conditions, there may be changes in the initial composition, with the dominance of more tolerant species of Perciformes and Cyprinodontiformes (Alves et al., 2016; Caetano et al., 2016; Casatti et al., 2009).

Given the degradation that aquatic resources in these regions are experiencing, the development of tools to assess their ecological status is a major challenge in the management of freshwater ecosystems. One of the most used methods for assessing the ecological integrity of streams and rivers is the Index of Biotic Integrity (IBI). Firstly, it was presented by Karr (1981) and adapted for a wide range of conditions worldwide like Temperate (Li et al., 2015; Pont et al., 2006), Mediterranean (Hermoso et al., 2010; Magalhães et al., 2007) and Neotropical streams (Ruaro and Gubiani, 2013; Santos and Esteves, 2015; Terra et al., 2013; van Oosterhout and van der Velde, 2015). The IBI was based on the principle that biological communities respond to human changes in aquatic ecosystems in a predictable and quantifiable manner, representing an integration of the physical, chemical, and biological conditions of the system (Karr et al., 1986). This type of tools is composed by a set of metrics related to the species composition and functional attributes of fish assemblages, such as taxa richness, trophic and habitat niche, and abundance. Application of this multimetric approach often involves the adaptation of metrics for each river type to the specific biota and environmental conditions under study, and the subsequent use of empirical methods for their selection (Whittier et al., 2007). The selected metrics should be sensitive to many types of degradation and respond in a predictive way to the pressure gradient. The value of the index at a site is compared with the expected value on the same river type representing the least disturbed conditions (reference conditions), thus giving this deviation an assessment of the biotic integrity of the stream.

Giving the least relevance of biotic indicators in environmental regulations in Brazil (Carvalho et al., 2017) and the increasing pressure of agricultural activities in the southern region, there is an urgent need to provide managers and decision makers with user-friendly tools based on the biotic component of the aquatic systems. In this paper, our goal was to develop a fish-based IBI for an agricultural dominated Neotropical region originating from the Atlantic rainforest. In this context, almost always the percentage of the ichthyofauna of lotic neotropical headwater streams shows the predominance of Characiformes and Siluriformes, constantly near to 80% (Cetra et al., 2016; Ferreira, 2007; Lowe-McConnell, 1987; Silva et al., 2013). However, the great representativeness of native (*P. harpagos*) and non-native (*P. reticulata*) Cyprinodontiformes seems to be common in first-order streams of the upper Paraná river basin (Casatti, 2005, 2004; Cetra et al., 2012; Cionek et al., 2012). Our hypothesis is that the predominance of Characiformes and Siluriformes, and the presence and/or absence of Cyprinodontiformes are driven by the low biotic quality of the streams (i.e. lower value of IBI) also in our study area. Therefore, we expect biotic integrity to be greater in places where there is the predominance of Characiformes and Siluriformes. We also predict that the presence of Cyprinodontiformes, especially non-native, is related to the lower biotic integrity of neotropical streams.

2. MATERIALS AND METHODS

2.1. Study area

We sampled streams in four sub-basins of the Paraná river basin, which is the 2nd largest river in South America (Dias et al., 2017), totalizing 30,000 km^2 of area (Fig 1) (Fidalski, 1997; IPARDES, 2004; SEMA, 2010). This is an area mainly comprised of sandy soils with homogeneous grain sizes, originated from Caiuá Sandstone geological formation, characterized by dystrophic dark red alic latosol and eutrophic red-yellow podsol, under the domains of the semi-deciduous forest, originating from the Atlantic rainforest neotropical biome (Campos et al., 2000; EMBRAPA, 1984; Fernandes and Magalhães-Ribeiro, 2015; Torres, 2003).

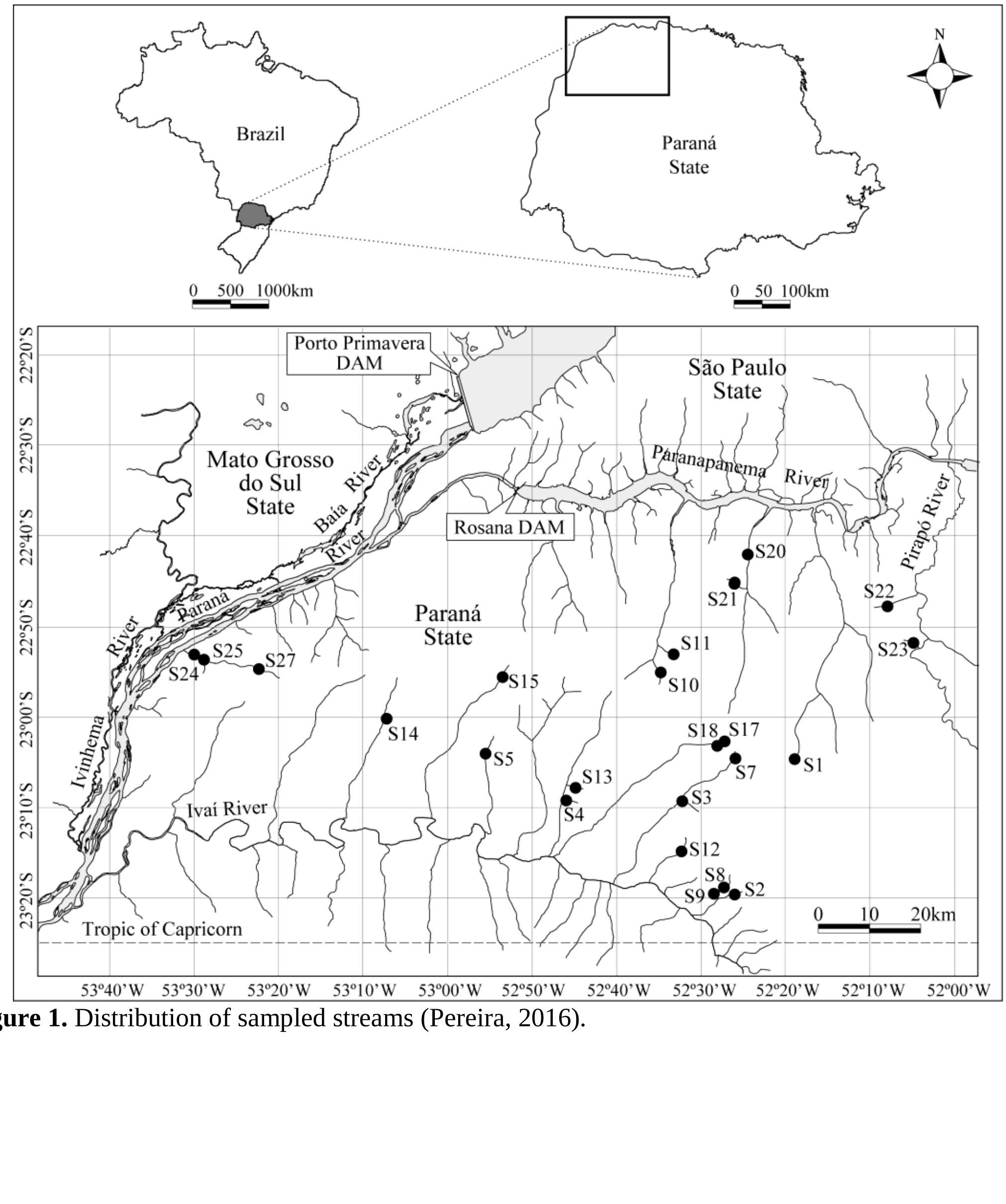

Figure 1. Distribution of sampled streams (Pereira, 2016).

The primary selection of the study sites followed Cionek (2016) using calculated land-use percentage area and secondarily the accessibility and landowner permission criteria to sample 23 streams (Ferreira and Casatti, 2006; Zeni et al., 2017). A Rapid Assessment Protocol (RAP) especially designed for the study region (Cionek et al., 2011) was used to quantify anthropogenic disturbance at the sites during each sampling visit. A score between 0 and 20 was assigned to each of 9 different variables, with 0 indicating high and 20 indicating low anthropogenic influence. The variables included: (a) underwater substrate, (b) underwater habitat complexity, (c) velocity and depth variety, (d) channel sinuosity, (e) water level amplitude, (f) channel integrity, (g) bank stability, (h) bank riparian protection, and (i) vegetation conservation on the surrounding environment.

2.2. Reference conditions and sites

We defined the reference conditions has been the least disturbed sites according to Whittier et al. (2007). The least disturbed sites were determined by the percentage of agriculture (less than 35%, including sugarcane crop) and the non-urbanized area from watersheds. We also used the results of RAP to classify environmental quality sites as least (LD), intermediary (ID) and most (MD) disturbed sites. The employment of land-use criteria and RAP permits a multiscale analysis for this work.

2.3. Fish Sampling

Fish assemblages were sampled in April/2015 that is the beginning of the drought period, to minimize the effect of rainfall on the habitat structure. These rivers do not dry out completely in the low water season. We used daytime electrofishing where the sampling effort was characterized by three consecutive passages applied to each 80-meter stretches site, seine-blocked to prevent fishes from escaping (Mazzoni and Lobón-Cerviá, 2008). Voltage was adjusted according to water conductivity to maximize capture efficiency and minimize mortality of aquatic organisms (Kimmel and Argent, 2006). All individuals were counted and screened on the field, anesthetized in benzocaine (AVMA, 2013) and fixed in 10% formalin. From each fish we obtained total weight (g), standard length (cm) and confirmed them with the specific literature in the laboratory (Ota et al., 2018). All species had specimens deposited in the Ichthyology Collection of Nupélia (Nucleus of Research in Limnology, Ichthyology and Aquaculture) from the State University of Maringá.

2.5. Data analysis

2.5.1. Fish assemblage composition

Biologic characteristics of the fish species were determined from the literature, according to Pereira (2016). Diversity was calculated with the Shannon–Wiener Index and Simpson's dominance (Krebs, 1989). Distribution of the Characiformes, Siluriformes and Cyprinodontiformes (native and non-native individuals) was analyzed to verify if it followed the one proposed for the lotic environments of the neotropical region (Britski, 1992; Langeani et al., 2007).

2.5.2. Metrics evaluation and IBI construction

Metrics were calculated using standardized measures of richness, proportion of taxa, proportion of individuals, and the absolute number of individuals for 1000 m^2 for every candidate metric. The Index of Biotic Integrity for Neotropical Sandy Soil Streams (N3S-IBI) metrics were developed following Whittier et al. (2007) and Krause et al. (2013), and result in a list of candidate metrics for possible inclusion, organized into the following groups: habitat, tolerance, trophic, reproductive, composition, richness, life history, biological traits, alien, and abundance. The candidate metrics that passed by four criteria were used as selected metrics: *a) range of homogeneity test*, that it is the distribution of metric values across all of the available data, whose function is identify metrics that have very small ranges to eliminate them; *b) responsiveness to disturbances*, by Kruskal-Wallis test ($p < 0.1$) to verify the capacity of metrics to distinguish between the least and most disturbed sites; *c) redundancy*, by Spearman correlation coefficients to choose metrics that did not contain redundant information with others selected metrics, and *d) range test for metric values*, to check if the majority of sites wouldn't have the same metric score.

For metric scoring and calculation of final N3S-IBI we used the 75th percentile of the LD sites distribution of values for each positive metric (i.e., those that are highest in the least disturbed sites) as the scoring ceiling and the 5th percentile of the distribution of values at all sites as the scoring floor (score = 0). Metric scores between the 5th and 95th percentile was interpolated linearly (Whittier et al., 2007). For negative metrics we reverse the floor and ceiling values. To reduce subjectivity and noise the scored metrics were summed and divided by the number of metrics, and the index was scaled to a range of 0 to 1, where 0 corresponds to the worst and 1 to the best quality of each stream (Hering et al., 2006). The quality is expressed within one of five quality classes that facilitate the communication of results to non-specialists.

In addition, we checked the correlation between final index scores of the N3S-IBI and RAP for all sites using Spearman's test. A comparison between the disturbance categories of sites was made using the Kruskal-Wallis test to verify the ability of IBI to classify environmental quality. We use R software for all statistical analysis (R Core Team, 2018).

3. RESULTS

3.1. Reference conditions and fish assemblage

The chosen methodology to the reference conditions returned to us 7 LD, 9 ID and 7 MD sites where we collected 5,166 individuals comprised of 32 fish species, 13 families, and 6 orders (Table 1SM - Supplementary material). Cyprinodontiformes was the most abundant order on the total abundance, followed by Siluriformes and Characiformes respectively (Table 1). The Cyprinodontiformes non-native species *Poecilia reticulata* (fam. Poeciliidae) was the most abundant species, especially in MD sites, while the native *Phalloceros harpagos* (fam. Poeciliidae) was the second most abundant, especially in LD sites. The Siluriformes *Hypostomus ancistroides* (fam. Loricariidae) was the highest frequency, captured in 16 of the 23 streams.

Table 1. Abundance of sampled fish orders (%) according to the disturbance sites. MD = Most disturbed sites, LD = least disturbed sites and ID = intermediary sites.

Order	Abundance at sites (%)			
	LD	ID	MD	TOTAL
Siluriformes	30.2	26.4	26.4	26.7
Characiformes	39.0	45.8	0.34	8.7
Cyprinodontiformes	27.5	20	73.1	63.3
Native *(P. harpagos)*	27.0	8.0	2.30	5.4
Non-native *(P. reticulata)*	0.57	12	70.7	58.0

3.2. N3S-IBI data development

A list of 370 candidate metrics was elaborated using the literature (Table 2SM), and of these, only 6 metrics were approved in all tests for metric scores to compose the final N3S-IBI (Table 2). Figure 2 shows the result of the Kruskal-Wallis test of the six metrics approved to compose the final N3S-IBI. The chosen metrics were Simpson's dominance (composition), number of Characiformes individuals (abundance), percent of characidae species (richness), percent of insectivorous less tolerant (trophic), number of non-native individuals (alien) and percent of tolerant species (tolerance).

Table 2. The number of remaining metrics after each metric evaluation test by metric class.

Metric Class	Start	Metric evaluation test			
		Range	Responsiveness	Redundancy	Range Metrics
Composition	92	32	8	6	1
Tolerance	24	20	6	3	1
Trophic	41	41	8	2	1
Reproductive	15	15	6	2	0
Habitat	24	24	5	3	0
Life History	9	9	0	0	0
Richness	9	9	9	4	1
Functional traits	48	48	1	1	0
Abundance	102	102	15	3	1
Alien	6	6	4	2	1
TOTAL	370	306	62	26	6

Final scores of the N3S-IBI and RAP values and their categories are shown in table 3. Positive Spearman's correlation between N3S-IBI and RAP for all sites was marginally significant ($\rho = 0.41$; $p = 0.055$) but ecologically interesting.

Regarding the disturbance gradient, there was a significant difference in N3S-IBI between the proposed conditions (Kruskal–Wallis test: $H = 9.52$; $P = 0.009$; $n = 23$), especially between LD and MD sites (Fig. 3).

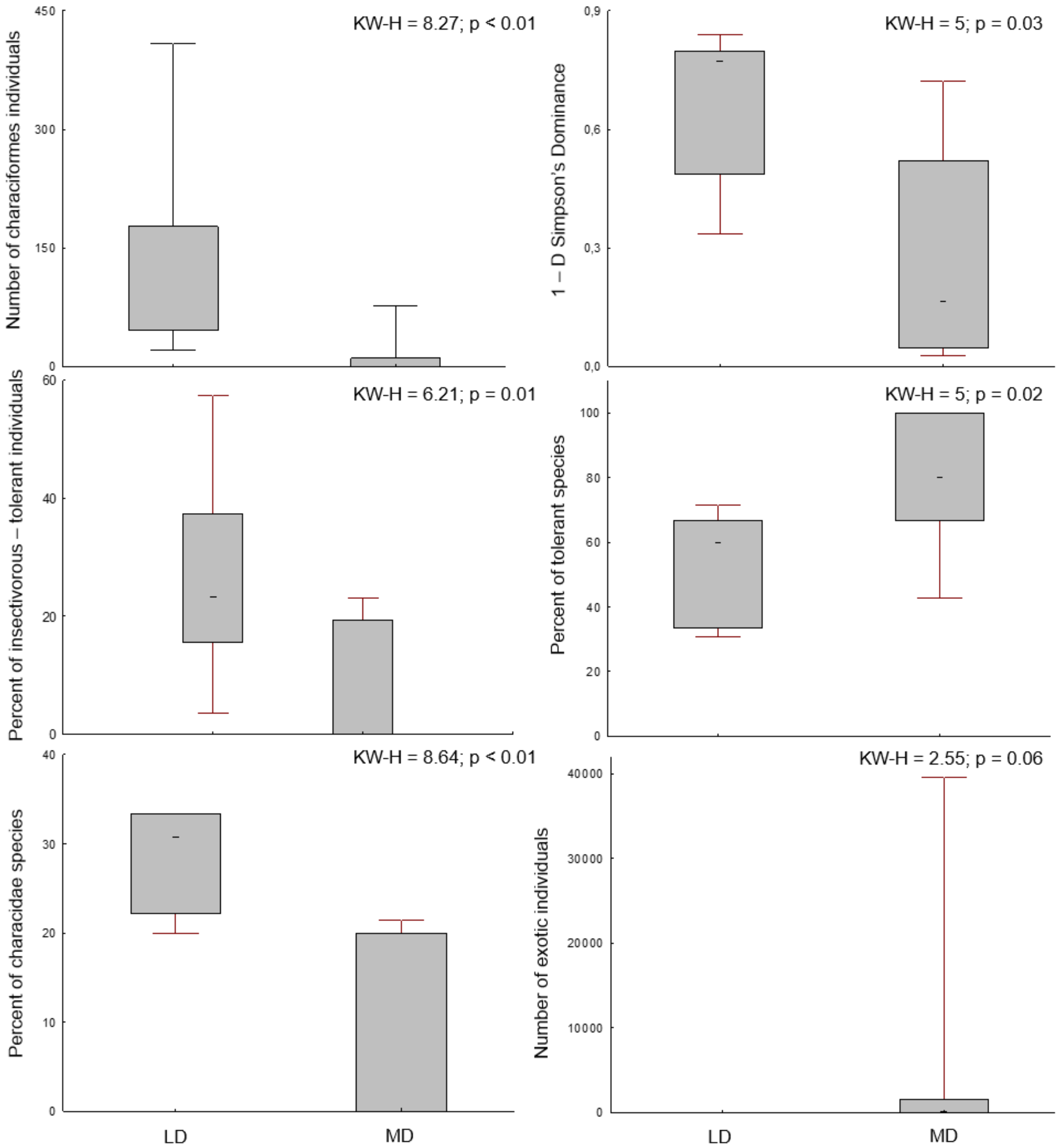

Figure 2. Difference of medians from the six metrics approved in the range test for metric scores that composed the final N3S-IBI for least (LD) and most (MD) disturbed sites, and results from the Kruskal-Wallis test (n = 14; *P* < 0.05).

Table 3. N3S-IBI metric scores ordered and obtained for twenty-three Neotropical sandy soil streams of Northwest of the state of Paraná, Brazil. Condition is least disturbed (LD), most disturbed (MD) and intermediary (ID) sites.

Streams	IBI-value	IBI-Result	RAP-value	RAP-Result	Disturbance
s20	1.00	Excellent	160	Excellent	LD
s04	0.99	Excellent	150	Excellent	ID
s23	0.97	Excellent	91	Good	ID
s15	0.84	Excellent	86	Good	LD

s08	0.83	Excellent	97	Good	ID
s27	0.82	Excellent	117	Good	LD
s25	0.76	Good	86	Good	LD
s05	0.74	Good	108	Good	LD
s02	0.72	Good	135	Excellent	ID
s24	0.70	Good	70	Regular	LD
s14	0.54	Regular	115	Good	LD
s03	0.54	Regular	61	Regular	MD
s13	0.54	Regular	148	Excellent	ID
s09	0.40	Poor	37	Regular	MD
s22	0.36	Poor	116	Good	ID
s11	0.36	Poor	79	Regular	MD
s10	0.30	Poor	115	Good	ID
s17	0.27	Poor	51	Regular	MD
s12	0.17	Very Poor	58	Regular	MD
s21	0.17	Very Poor	162	Excellent	ID
s01	0.16	Very Poor	94	Good	ID
s07	0.09	Very Poor	76	Regular	MD
s18	0.00	Very Poor	60	Regular	MD

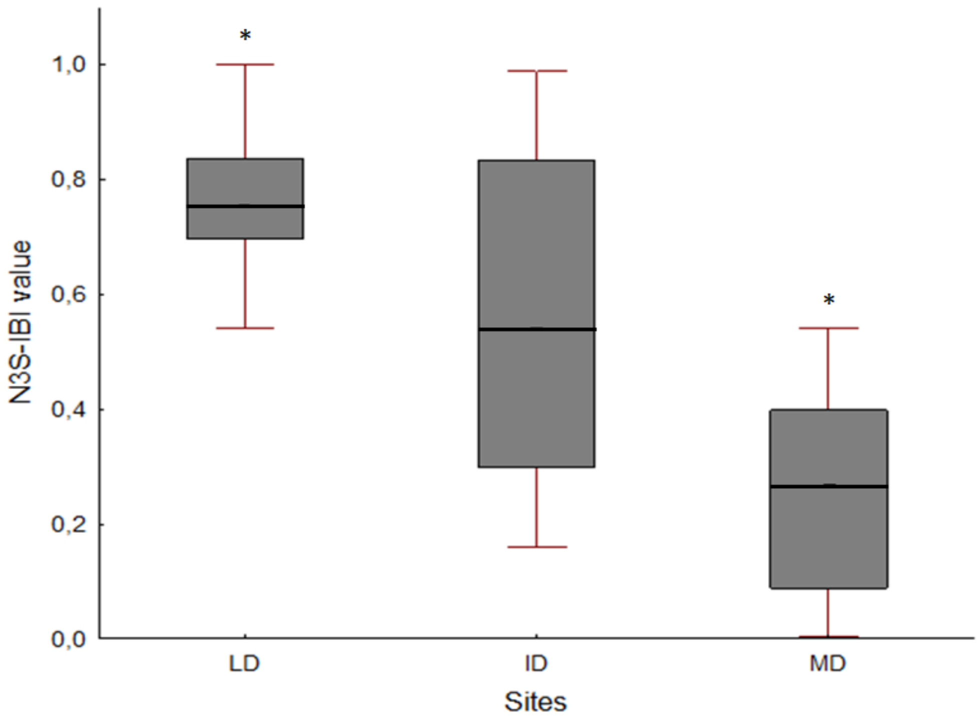

Figure 3. Medians of N3S-IBI results from least (LD), intermediary (ID) and most disturbed (MD) sites of sandy soil streams. *represents significant difference – (Kruskal–Wallis: H = 9.52; *P* = 0.009; n = 23).

4. DISCUSSION

The IBI for Neotropical sandy soil streams was composed by six metrics: Simpson's dominance, number of Characiformes individuals, percent of Characidae species, percent of insectivorous less tolerant individuals, number of exotic individuals and percent of tolerant species. Our hypothesis was not rejected and the N3S-IBI showed an excellent performance to separate least and most disturbed sites through its values in our study area. The low occurrence, especially of Characiforms, was determinant for the contrast with the standard to Neotropical assemblages of fish, even being bigger in the streams with greater biotic integrity, confirming our first prediction. In addition, the great representativeness of native (*P. harpagos*) and non-native (*P. reticulata*) Cyprinodontiformes, common in first-order streams of the upper Paraná river basin (Casatti, 2005, 2004; Cetra et al., 2012; Cionek et al., 2012), can be explained by oviviviparity as a reproductive strategy, characteristic common only to these two species in the sampled set (Aranha and Caramaschi, 1999; Cionek et al., 2012; Langeani et al., 2007) or due to a variety of feeding habits (Casatti et al., 2009; Rocha et al., 2009). However, it was the non-native species of Cyprinodontiformes that most negatively influenced the biotic integrity values of the sampled sites, also confirming the second prediction. According to the reference sites classification, *P. reticulata* reinforces the choice of the *number of non-native individuals* as a metric of negative impact because it is clear their dominance at the MD sites.

We agree with Karr and Chu (2000) that the selection of appropriate metrics is a major step in the proper functioning of this type of index, and we believe that robust statistical criteria are essential to the choice of metrics. However, many papers select metrics from the literature, without employing judicious statistical tests for this (Araújo, 1998; Bozzetti and Schulz, 2004; Ferreira and Casatti, 2006; Machado et al., 2011; Pinto et al., 2006). Some characteristics of our statistical-based metrics were: *i) Simpson's dominance*: is a common metric in IBI, in which disturbed streams tend to show dominance by few species and present low overall species richness, whereas preserved streams exhibit few dominant species and many rare species (Ferreira and Casatti, 2006; Hughes and Oberdorff, 1999; Prudente et al., 2018; Santos and Esteves, 2015); *ii) Number of Characiformes individuals*: the abundance of this order is a metric that tends to decrease in disturbed streams because it is usually replaced by more disturbance-tolerant orders, such as Cyprinodontiformes or Perciformes (Ferreira and

Casatti, 2006); this same pattern may also be expected for the *iii) percent of Characidae species*, by the taxonomic level perhaps, however, it was not redundant with the last metric; *iv) the percentage of insectivorous less tolerant individuals*: who tends to be replaced by more generalist and tolerant ones in disturbed sites, is an unprecedented metric, results of our speculative statistic tests; *v*) *percentage of tolerant species*: tolerance is a frequent metric for biomonitoring tools, with some variations between them (Bozzetti and Schulz, 2004; Casatti et al., 2009; Ferreira and Casatti, 2006). It is also common the use of metrics with species demonstrating some kind of tolerance (Santos and Esteves, 2015); *vi) the number of exotic individuals*: we also considered using *P. reticulata* percentage for this metric, as did Ferreira and Casatti (2006), or the percentage of Cyprinodontiformes, since all three presented the same result. However, we choose the number of non-native individuals due to the data on native and non-native species of this basin that were tolerant to the negative effects of sugarcane crop (Gonino et al, in press) widely diffused in the study area. In addition, *P. harpagos* was used as an indicator of negative impact on IBI metrics for Neotropical streams of Santos and Esteves (2015), but in our study it was not statistically significant for this approach. Thus, this is one example of the importance of developing IBIs of regional scale in multi-diverse regions.

The number of 6 metrics helps to make a quick and easy biomonitoring tool. So as Ruaro and Gubiani (2013), we also believe that a simple and fast index is efficient for monitoring aquatic environments. It is practically impossible to apply a single monitoring tool to an entire country when it has continental proportions and heterogeneity of biomes, such as Brazil. However, we support proposals such as that of Stoddard et al. (2008), with the development of local and regional tools, interconnected in a single monitoring system. Although there is no standardization in the collection methodologies for studies of IBIs based on fish in Brazil, there are some papers using this tool in many states, especially in south-central of country (Carvalho et al., 2017; Casatti et al., 2009; Costa and Schulz, 2010; Pinto et al., 2006; Polaz et al., 2017). However, we suggest the adoption of an electrofishing protocol as an initial step for a single regional tool that would facilitate the use of fish data collected by colleagues throughout all localities, similar to proposed by Oliveira et al. (2009) in Portugal. Ruaro et al. (2018) proposed an MMI (Multimetric Index) to another Neotropical basin and analyzed the influence of non-native species on the loss of ecological integrity.

However, there was no study with a judicious method of choosing metrics on the biotic integrity of headwater streams for these 30,000 km^2 of northwestern Paraná, from Caiuá Sandstone geological formation. Our study deals with this gap in one of the most impacted regions by monocultures, especially sugarcane, providing more information about this anthropogenic action impacts.

The establishment of reference sites is one of the first steps in assessing the ecological status of streams using IBI and (Hughes, 1995) defined as being that with least anthropic influence. Our reference sites did not overlap in the results (Table 3). As expected, the MD and LD sites were totally separated by the IBI values, and the ID sites were equally distributed among them. We understand that the same did not occur with the RAP classification because it is a specific habitat tool, however, it was useful to determine our reference sites. Land-use in the studied sub-basins is comprised of agriculture and integration between crop and livestock in more than 70% of its area, dominated by corn, soybean, and sugarcane (IPARDES, 2013). N3S-IBI was significantly affected by land-use and proved to be a useful tool to assess changes in this environment, clearly identifying the degree of disturbance in sampled streams. Nevertheless, some authors did not find relation between the types of land-use and the biotic integrity (Li et al., 2015; Machado et al., 2011). We find that the sites with poor and very poor N3S-IBI values are located further east of the study area. This is a sign of greater anthropogenic action in this area, and needs more attention from the decision makers. Thus, our results showed that the chosen of the land-use and the RAP was an efficient methodology to establish reference sites for this work.

Often public management bodies collect information restricted only to monitoring fluviometric levels, physics and chemical parameters and quantification of specific bacteria (IPARDES, 2013; SEMA, 2010), possibly due to the volume of work and costs. Water is a vital resource for all living beings, so we understand that their monitoring must go beyond these parameters and include the biotic quality of their community. Initiatives to conserve freshwater systems in a large country such as Brazil go through the development of regional tools. In this way, although it needs to be validated, N3S-IBI is a well-developed tool to freshwater systems in a region of approximately 3 million ha (SEMA, 2010). The contamination by agricultural run-off, urban and industrial sewage is a reality anywhere that presents these three anthropic components. To search for improvements is necessary in the monitoring system, and actions of public institutions or non-governmental groups that help collect data of the biological integrity of aquatic systems are welcome. Since environmental researchers in Brazil have been neglected the political role of their scientific contributions (Loyola and Bini, 2015), tools like N3S-IBI may become important that are more accessible to facilitate the engagement of people inside and outside the control bodies and the academic environment. In practice, this would be well utilized by directing restoration actions to MD sites and strengthening the preservation of LD sites.

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SUPPLEMENTARY MATERIAL

Table 1 MS. Classification of sampled species according to their biological characteristics related to trophic group, tolerance and their origin.

CLASSIFICATION	TROPHIC GROUP	TOLERANCE	ORIGIN
CHARACIFORMES			
Characidae			
Astyanax aff. *fasciatus*	Insectivore[1,8]	Intolerant[13]	Native[15]
Astyanax aff. *paranae*	Insectivore[1,3]	Intolerant[13]	Native[15]
Astyanax lacustris	Omnivore[1,2]	Intolerant[13]	Native[15]
Astyanax bockmanni	Herbivore[4,7]	Intolerant[13]	Native[15]
Moenkhausia forestii	Insectivore[3]	Intolerant[13*]	Native[15]
Oligosarcus paranensis	Insectivore[10]	Intolerant[17,*]	Native[15]
Oligosarcus pintoi	Insectivore[2,11]	Intolerant[17,*]	Native[15]
Cheirodontinae			
Serrapinnus notomelas	Detrictivore[2]	Tolerant[13]	Native[15]
Stevardiinae			
Bryconamericus exodon	Insectivore[5]	Intolerant[14]	Native[15]
Knodus moenkhausii	Insectivore[2,3]	Tolerant[13]	Native[15]
Piabarchus stramineus	Omnivore[1]	Intolerant[14]	Native[15]
Piabina argentea	Insectivore[2,4]	intolerant[13]	Native[15]
Crenuchidae			
Characidium aff. *zebra*	Insectivore[1,2]	Intolerant[17]	Native[15]
Erythrinidae			
Hoplias sp3.	Piscivore[1,8]	Tolerant[17]	Native[15]
Parodontidae			
Parodon nasus	Detrictivore[1]	Intolerant[13]	Native[15]
CICHLIFORMES			
Cichlidae			
Crenicichla britskii	Insectivore[2]	Intolerant[13]	Native[15]
CYPRINODONTIFORMES			
Poeciliidae			
Phalloceros harpagos	Omnivore[3,6]	Tolerant[17]	Native[15,16]
Poecilia reticulata	Detrictivore[2,3]	Tolerant[13]	Non-native[15,16]

GYMNOTIFORMES			
Gymnotidae			
Gymnotus inaequilabiatus	Insectivore[3,4]	Tolerant[13]	Native[15]
Gymnotus sylvius	Insectivore[2]	Tolerant[13*]	Native[15]
Sternopygidae			
Sternopygus macrurus	Insectivore[9]	Intolerant[16]	Native[15]
SILURIFORMES			
Callichthyidae			
Callichthys callichthys	Detrictivore[6]	Tolerant[17]	Native[15]
Corydoras aeneus	Insectivore[2,3]	Tolerant[12]	Native[15]
Heptapteridae			
Cetopsorhamdia iheringi	Insectivore[4,7]	Intolerant[14]	Native[15]
Imparfinis schubarti	Insectivore[2,3]	Intolerant[13]	Native[15]
Phenacorhamdia tenebrosa	Insectivore[4,8]	Intolerant[14]	Native[15]
Rhamdia quelen	Insectivore[2,3]	Tolerant[13,17]	Native[15]
Loricariidae			
Hypostominae			
Hypostomus ancistroides	Detrictivore[2,7]	Tolerant[13,17]	Native[15]
Hisonotus francirochai	Detrictivore[2]	Intolerant[13]	Native[15]
Rineloricaria pentamaculata	Insectivore[7]	Tolerant[17,*]	Native[15]
Trichomycteridae			
Trichomycterus davisi	Insectivore[3,6]	Intolerant[17,*]	Native[15]
SYNBRANCHIFORMES			
Synbranchidae			
Synbranchus marmoratus	Insectivore[3]	Tolerant[17]	Native[15]

Cavalli et al., 2018; [1]Smith et al., 2013; [2]Zeni & Casatti 2014; [3]Cionek, 2016; [4]Silva, 2013; [5]Novakowski et al., 2008; [6]Abilhoa et al., 2008; [7]Silva et al., 2012; [8]Ferreira 2007; [9]Luz-Agostinho et al., 2006; [10]Esteves et al., 2008; [11]Casatti, 2002; [12]Araújo & Garutti, 2003; [13]Casatti et al., 2009; [14]Casatti et al., 2012; [15]Graça & Pavanelli, 2007; [16]Petry et al., 2013; [17]Bozzetti & Schulz, 2004. *The classification of these species followed its genus pattern.

Table 2 MS. Total candidate metrics (370) for the N3S-IBI.

MEASURE	METRIC	GROUP
	Richness of species	Richness
	Shannon index	
	Simpson's dominance	
Number, percentage and biomass of individuals	Biomass	Abundance
	Density	
	A. aff. *fasciatus*	
	A. aff. *paranae*	
	A. bockmanni	
	A. lacustris	
	B. exodon	
	C. aeneus	
	C. britskii	
	C. callichthys	
	C. iheringi	
	C. aff. *zebra*	
	G. inaequilabiatus	
	G. sylvius	
	H. ancistroides	
	H. francirochai	
	Hoplias sp3	
	I. schubarti	
	K. moenkhausii	
	M. forestii	
	O. paranensis	
	O. pintoi	
	P. argentea	
	P. harpagos	
	P. nasus	
	P. reticulata	
	P. stramineus	
	P. tenebrosa	
	R. pentamaculata	
	R. quelen	
	S. macrurus	
	S. marmoratus	
	S. notomelas	
	T. davisi	
	non-native	Alien
	native	
	Characiformes less tolerant	Composition
	Characiformes + Siluriformes	
	Characiformes + Siluriformes less tolerant	
	Characiformes	
	Cyprinodontiformes	
	Gymnotiformes	
	Cichliformes	
	Siluriformes less tolerant	
	Siluriformes	

Synbranchiformes	
Characidae	
Loricariidae	
Heptapteridae	
Benthic	Habitat
Bentopelagic	
Demersal	
Estuarine demersal	
Estuarine pelagic	
Nektonic	
Benthic less tolerant	
Nektonic less tolerant	
With parental care	Reproduction
Without parental care	
External reproduction	
Internal reproduction	
Without parental care less tolerant	
Intolerant	Tolerance
Tolerant	
Low vulnerability	
Moderate vulnerability	
High vulnerability	
Very high vulnerability	
Richness of intolerant	
Richness of tolerant	
detrictivore	Trophism
herbivore	
insectivore	
omnivore	
piscivore	
detrictivore less tolerant	
insectivore less tolerant	
omnivore less tolerant	
trophic level 2-3	
trophic level 3-4	
trophic level 4-5	
Phylogenetic diversity index 1	Life history
Phylogenetic diversity index 2	
Phylogenetic diversity index 3	
head height 1	Functional traits
head height 2	
head height 3	
head width 1	
head width 2	
head width 3	
relative height of mouth 1	
relative height of mouth 2	
relative height of mouth 3	
subdorsal mouth	
subventral mouth	
terminal mouth	
ventral mouth	

	mouth width 1 mouth width 2 mouth width 3	
Number and percentage of species	Characiformes Characiformes + Siluriformes Characiformes less tolerant Characiformes + Siluriformes less tolerant Siluriformes Siluriformes less tolerant	Composition
	detrictivores omnivores insectivores insectivores less tolerant	Trophism

Table 3 MS. Groups of metrics and their description.

CLASS	DESCRIPTION	METRICS
Habitat	Preferred habitat for each vertebrate species (e.g., benthic, water column, or hider)	Water column position (bentopelagic, demersal, nektonic, benthonic, estuarine)
Tolerance	General tolerance to common anthropogenic, physical, and chemical stressors (sensitive, intermediate, tolerant, or very tolerant)	Tolerant or intolerant; low, medium or high vulnerability
Trophic	Primary source of nutrition for each vertebrate species as an adult (herbivore, invertivore, invertivore–piscivore, piscivore, or omnivore)	Herbivore, insectivore, detrictivore, omnivore or piscivore
Reproductive	Reproductive habit for each vertebrate species (e.g., lithophil, nest builder, or crevice spawner)	Internal or external fertilization, parental care behaviour
Composition	The representation of different taxonomic groups (e.g., family) in the assemblage	Order, family
Richness	The number of different kinds of taxa	Richness, Simpson's dominance, Shannon index
Life history	The general life history strategy for each vertebrate species (e.g., migrating [vagile], long-lived, etc.)	Functional traits
Alien	Whether each vertebrate species is native or introduced in the region where it was collected	Native or non-native
Abundance	The number of individuals of an assemblage, taxonomic group, or guild collected	Density, biomass, species

CONSIDERAÇÕES FINAIS

O material particulado originado da combustão da matéria vegetal (i.e. as cinzas) confirmou-se um importante poluente do ambiente aquático. As hipóteses propostas a partir da toxicidade dos compostos químicos, como HPA, de que as cinzas afetam negativamente a performance de peixes não foram rejeitadas para os ensaios que avaliaram o comportamento, condição hepática e alterações nucleares. Nossos resultados confirmaram o potencial tóxico das cinzas florestais sobre o comportamento e a condição hepática do peixe potamódromo ibérico *Luciobarbus bocagei,* que mesmo a exposição de curta duração foi capaz de alterar o comportamento dos peixes e a sua condição hepatossomática. Neste capítulo, destacamos a necessidade de manter uma rede fluvial não-fragmentada ou, quando isso não for possível, priorizar a remoção ou adaptação de barreiras para aumentar dispersão do movimento e fornecer condições para a recuperação de espécies frente às perturbações dos incêndios.

No segundo capítulo, destacamos o efeito antrópico da queimada da cana-de-açúcar ao nível celular de peixes, uma vez que a exposição de curta duração à CCA resultou em maior toxicidade ao núcleo de eritrócitos do peixe neotropical nativo *Astyanax lacustris* comparado ao não-nativo *Oreochromis niloticus*. Esta diferença significativa de tolerância tem implicações ecológicas, como a interferência na dinâmica de estabelecimento de espécies não-nativas em novos ambientes, com potencial efeito deletério para a comunidade nativa. Existem também as implicações para a saúde púbica, uma vez que alguns HPAs presentes nas cinzas podem persistir na água e contaminar a cadeia alimentar por bioacumulação em espécies aquáticas como peixes, e atingir os seres humanos. Assim, possíveis medidas para diminuir o impacto da CCA em época de queimada passam pela substituição do processo de queima até a introdução de faixas de vegetação junto às linhas de água, com espécies florestais resilientes ao fogo e que retenham estas cinzas.

Portanto, ficou claro que os incêndios em monoculturas (seja de cana-de-açúcar ou reflorestamento) causam efeitos letais e subletais em peixes de espécies nativas e não nativas, afetando a comunidade ictiológica.

Por fim, no último capítulo, como forma aplicada de retornar o conhecimento adquirido com os bioensaios, propusemos uma ferramenta de avaliação da integridade biótica para o noroeste do estado do Paraná, uma área de 3 milhões de hectares, no noroeste do estado do Paraná. Esta área, caracterizada pela formação geológica Arenito Caiuá, sofre intensos impactos antrópicos relacionados com a agricultura, em especial a cultura da cana-de-açúcar. Foi possível elaborar um índice de integridade biótica baseado em peixes para os riachos de solo arenoso (N3S-IBI), composto de 6 métricas com base em suas características ictiológicas. É uma ferramenta fácil de ser aplicada, cujas respostas ajudam a direcionar os esforços de preservação das áreas menos degradadas e recuperação das mais impactadas.

ANEXO 1

Artigos publicados durante o período do doutorado

Gonino, G.M.R., Figueiredo, B.R.S., Manetta, G.I., Zaia Alves, G.H., Benedito, E. 2019. Fire increases the productivity of sugarcane, but it also generates ashes that negatively affect native fish species in aquatic systems. **Sci. Tot. Env.** Vol. 664, 215-221. DOI: 10.1016/j.scitotenv.2019.02.022.

Gonino, G., Branco, P., Benedito, E., Ferreira, M.T., Santos, J.M. 2019. Short-term effects of wildfire ash exposure on behaviour and hepatosomatic condition of a potamodromous cyprinid fish, the Iberian barbel *Luciobarbus bocagei* (Steindachner, 1864). **Sci. Tot. Env.** Vol. 665, 226-234. DOI: 10.1016/j.scitotenv.2019.02.108.

ANEXO 2

GUIDE FOR AUTHORS of Science of the Total Environment

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Science of the Total Environment is an international journal for publication of original research on the total environment, which includes the atmosphere, hydrosphere, biosphere, lithosphere, and anthroposphere.

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The total environment is characterized where these five spheres overlap. Studies that focus on at least two or three of these will be given primary consideration. Papers reporting results from only one sphere will not be considered. Field studies are given priority over laboratory studies. The total environment is studied when data are collected and described from these five spheres. By definition total environment studies must be multidisciplinary.

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• Agriculture, forestry, land use and management
• Air pollution quality and human health
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• Ecotoxicology and risk assessment
• Emerging fields including global change and contaminants
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• Nanomaterials in the environment
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The editors discourage submission of papers which describe results from routine surveys or monitoring programs, studies which are local in scope, laboratory experiments, hydroponic or pot studies measuring biochemical/physiological endpoints, food science studies, screening of new plant species for phytoremediation, testing known chemicals in another setting, and experimental studies lacking a testable hypothesis.

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ANEXO 3

CAPÍTULO X - DAS DISSERTAÇÕES, TESES E CONCESSÃO DE GRAU

Art. 49. Todo candidato ao grau de mestre ou de doutor, deve preparar e defender uma dissertação ou tese e nela ser aprovado.

Parágrafo único. A tese de doutorado deverá basear-se em trabalho de pesquisa original, que represente real contribuição ao conhecimento científico do tema.

Art. 50. Para apresentação da dissertação ou tese, o pós-graduando deve ter integralizado os créditos exigidos em disciplinas e ter obtido aprovação no exame de conhecimento de língua inglesa.

Parágrafo único. Para apresentação da tese, além das exigências dispostas no *caput* deste Artigo, o candidato ao grau de doutor deve ter obtido aprovação no Exame de Qualificação.

Art. 51. A dissertação ou tese deve ser redigida em língua portuguesa, com resumo em língua portuguesa e inglesa.

§ 1°. Pode fazer parte da dissertação ou tese, um ou mais trabalhos submetidos, no prelo ou publicados, na língua exigida pelo periódico, produzidos durante o período do curso.

§ 2°. Para atender à exigência disposta no *caput* deste Artigo, a dissertação ou tese deve conter, obrigatoriamente, um trabalho inédito em português, ainda não encaminhado para publicação.

Art. 52. As normas quanto ao formato da dissertação e da tese devem ser determinadas pelo Conselho Acadêmico do Programa.

Art. 53. O julgamento da dissertação ou tese deverá ser requerido pelo candidato e pelo orientador ao Conselho Acadêmico do Programa.

§ 1°. O requerimento de julgamento deverá ser acompanhado por 5 (cinco) exemplares da dissertação ou 7 (sete) exemplares da tese.

§ 2°. O orientador encaminhará os exemplares da dissertação ou tese, com seu parecer, ao Conselho Acadêmico do Programa.

Art. 54. A dissertação ou tese será defendida perante uma banca composta de, no mínimo, 3 (três) e 5 (cinco) membros, respectivamente, para o mestrado e doutorado, dos quais um será o orientador, cabendo a ele, a presidência da sessão.

§ 1°. Os membros da banca examinadora, propostos pelo orientador, serão designados pelo Conselho Acadêmico do Programa.

§ 2°. Na falta ou impedimento do orientador, o Conselho Acadêmico do Programa designará um substituto.

§ 3°. Os membros das bancas examinadoras devem ser portadores do grau de doutor.

§ 4°. Nas bancas examinadoras deve haver pelo menos um membro titular de outra Instituição.

§ 5°. As bancas examinadoras devem ter dois suplentes, sendo pelo menos um, de outra Instituição.

Art. 55. A defesa da dissertação ou tese deve ser pública, em local, data e horário previamente divulgados.

Parágrafo único. No caso de trabalho que poderá resultar em pedido de depósito de patente pode ser admitida a sessão fechada.

Art. 56. A Banca Examinadora, em decisão por maioria de seus membros, anteriormente à defesa, poderá rejeitar *in limine* a dissertação ou tese.

Parágrafo único. Nestes casos, a dissertação ou tese não será admitida à defesa.

Art. 57. Após a defesa, a banca examinadora avaliará reservadamente, expressando seu julgamento, por meio de uma das seguintes alternativas:

I – aprovação;

II – reprovação;

III – reformulação.

§ 1°. Nos casos de reprovação, não será admitida a reapresentação do mesmo trabalho, mesmo que reformulado, caso o candidato reingresse no programa.

§ 2°. Nos casos de reformulação, o candidato deverá submetê-lo novamente à mesma banca examinadora, no prazo máximo de 90 (noventa) dias, a qual emitirá parecer por escrito aprovando ou reprovando as reformulações apresentadas.

§ 3°. Concluído o julgamento, a banca examinadora elaborará uma ata e o resultado será encaminhado ao Conselho Acadêmico do Programa para homologação.

§ 4°. Não caberá recurso em nenhuma instância, da decisão final sobre o resultado do julgamento da dissertação ou tese.

Art. 58. O mestrando ou doutorando que tenha satisfeito todas as exigências deste regulamento, acrescidas daquelas relativas à entrega dos exemplares corrigidos e submissão a periódico indexado, do trabalho resultante dos dados obtidos em sua dissertação ou tese, conforme as normas estabelecidas pelo Conselho Acadêmico do Programa, fará jus ao respectivo diploma.

§ 1°. Em caso de pedido de depósito de patente, o artigo poderá ser substituído pelo requerimento a Pró-Reitoria de Pesquisa e Pós-graduação/divisão de propriedade intelectual da UEM.

§ 2°. O grau de mestre ou de doutor será qualificado pela área de concentração do Programa – Biologia das Interações Orgânicas.

Art. 59. Os alunos regulares do PGB que não pleitearem o título de mestre por meio de defesa pública de dissertação, poderão requerer certificado de Especialização, caso tenham concluído todos os créditos exigidos em disciplinas do Programa.