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RESEARCH ARTICLE

Mucociliary transport, differential white blood cells, and cyto-genotoxicity in peripheral erythrocytes in fish from a polluted urban pond

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Abstract The present study evaluated the water quality of a polluted pond through the analysis of in vitro mucociliary transport, hematological parameters, and biomarkers of cyto-genotoxicity in the Nile tilapia (*Oreochromis niloticus*). Blood and mucus samples were collected from ten specimens from the polluted pond and from ten specimens from a control area. The fish were anesthetized with 3% benzocaine, mucus was collected directly from the gills, and blood was drawn from the caudal artery. Blood smears were stained using the May-Grünwald Giemsa process for the differential leukocyte counts and to determine the frequency of leukocytes, thrombocytes, erythroblasts, micronuclei, and nuclear abnormalities. The results revealed low transportability in vitro, a high percentage of monocytes and eosinophils, and increased frequency of leukocytes and nuclear abnormalities in fish from the polluted pond. However, the frequency of thrombocytes and erythroblasts and the percentage of lymphocytes and neutrophils were significantly lower. It is possible to conclude that changes in fish are due to poor water quality and that these non-destructive biomarkers can be used for the biomonitoring of aquatic environments vulnerable to contamination.

Keywords Aquatic pollution · Biomarkers · Mucus · Hematology · Cytotoxicity · *Oreochromis niloticus*

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Introduction

The growth of the world's human population and the consequently higher demands for food and manufactured products have both led to increased waste on the planet. Domestic sewage, industrial effluents, urban drainage, and agricultural leachates represent important sources of contamination in water bodies. Reduced water quality is one of the causes of water shortages worldwide; thus, modern societies have recognized the importance and preciousness of water to human health, agricultural and industrial productivity, and an ecological balance (Morris et al. 2016; Flores-Ramirez et al. 2017).

In urban areas, aquatic ecosystems are more susceptible to degradation, and many vertebrates and invertebrates have been used to monitor environmental quality. Changes in functional enzymes (Bonnail et al. 2016; Vieira et al. 2017; Sadauskas-Henrique et al. 2017), effects of cyto-genotoxicity (Prado, et al. 2014, de Almeida Duarte et al. 2016), mutagenicity (Araújo al. 2014; Weldetinsae et al. 2017), and hematological, immunological, and histopathological changes (da Cruz et al., 2015; Corrêa et al. 2017; Thummabancha et al. 2016; Hedayati and Darabitabar 2017) all represent well-documented biomarkers used in environmental monitoring.

Several fish species have been frequently used to detect and assess the biological effects of contaminants released into water bodies, as they may exhibit lethal and sublethal responses to chemicals. These organisms are considered appropriate for use in pollution monitoring in aquatic systems because they occupy different positions within the food web and may transfer energy and biomass between trophic levels. Furthermore, the presence of pollutants in the environment can cause stress by changing molecular, physiological, histological, or behavioral responses.

In Brazilian legislation, the quality of environmental surface waters is monitored using only physicochemical and microbiological parameters (e.g., fecal coliforms), which are compared to the national limits established for the respective variables and water classes. Good-quality waters must not present variables exceeding the national guidelines for environmental contamination; however, for a proper understanding of ecological risks to fauna, specific information on bioavailability or potential toxicological effects on the biota must be gathered.

The aim of this study was to determine whether fish from a polluted artificial urban pond that receives urban effluents exhibited alterations in mucociliary transport in vitro, cyto-genotoxicity in peripheral erythrocytes, and immunological responses, as measured by differential leukocyte counts and frequency of total leukocytes, thrombocytes, erythroblasts, micronuclei, and nuclear abnormalities as non-destructive biomarkers for aquatic monitoring, toxicological studies, and aquaculture.

Methods

Study area

The study area was a polluted artificial urban pond in the city of Santo André, in the state of São Paulo, Brazil (23° 37′ 53″ S, 46° 31′ 58.73″ W). Previous studies had reported that the pond receives domestic effluent and storm water discharge, processes which periodically result in algal blooms and/or episodes of massive fish mortality (Seriani et al. 2012a). The water quality is poor, with high concentrations of ammonia, as well as high conductivity and hardness; the pond's volume is low and its maintenance depends exclusively on rainfall precipitation (Seriani et al. 2012b).

In vitro mucociliary transport

Mucociliary transport was measured in vitro according to the method used by Seriani et al. (2015a). This technique is an adaptation of the protocol previously described by King (1986) and Macchione et al. (1995) and Trindade et al. (2018). For the mucus sampling (20 μL), the gills were carefully scraped with a brush and placed in Eppendorf® microtubes, each of which contained 1 mL of mineral oil to prevent dehydration and was stored at − 20 °C until processing.

In the laboratory, the in vitro mucociliary transport rates were evaluated using an American bullfrog (*Lithobates catesbeianus*) palate preparation (King 1986; Macchione et al. 1995). The experiments were performed at room temperature (20 °C), and five measurements were recorded for each mucus sample. The results are expressed as the relative speed of mucus along the palate, which was calculated by dividing the distance traveled (6 mm) by the elapsed time (seconds). After measuring the elapsed time, the value of the sample was divided by that of the autologous mucus (from the frog) to establish the relative speed (sample vs. frog; Saldiva 1990; Macchione et al. 1995). It is important to note that this procedure was incorporated into other research projects conducted in our laboratory and that the mucociliary transport analysis was therefore performed only when frogs needed to be euthanized for other scientific purposes; no animals were killed solely to produce data for the present investigation.

Test organisms and hematological analyses

The experimental protocol was approved by the Research Ethics Committee of São Paulo State University (UNESP). The animals were handled according to the ethical standards of animal experimentation, and at the end of the procedures, the surviving individuals were acclimatized with the same water for recovery and reinserted into their environment.

Twenty Nile tilapia (*Oreochromis niloticus*) specimens were collected for this study (ten from the polluted pond and ten from a private fish farm used as a control site). The mean weight of the collected fish was 30.0 ± 1.1 g, and the mean length was 10.1 ± 0.2 cm. All specimens were placed in tanks containing water from the respective collection sites and were acclimatized for 60 min to minimize the stress of collection (Corrêa et al. 2017). Next, the fish were anesthetized with clove oil according to Simões et al. (2012) and to Delbon and Paiva (2012). Blood was drawn from the caudal artery, a procedure which was performed using heparin-treated syringes. Blood smears were then prepared on glass slides and stained using the May-Grünwald Giemsa process (Rosenfeld 1947; Seriani et al. 2011). Two thousand cells were analyzed per slide/animal under an optical microscope (× 1,000), and the frequencies of total leukocytes, thrombocytes, and erythroblasts were identified and counted. Additional blood smears were used for differential (percent) leukocyte counts (lymphocytes, neutrophils, basophils, monocytes, and eosinophils).

Cyto-genotoxicity analysis

Two thousand cells were analyzed per slide/animal under a microscope (× 1,000). Micronuclei, which were defined as two completely separated nuclei within a single erythrocyte's cytoplasm, were identified, and their frequencies were recorded. Nuclear abnormalities are understood as precursors of further DNA damage, and they include invaginations of the nuclear envelope of different sizes, noticeable depressions in the center in which nuclear material was otherwise absent, as well as other morphological abnormalities that did not fit into the previous categories (Bucker et al. 2012; Prado et al. 2014; Seriani et al. 2015b; Thomé et al. 2016).

Water samples

Surface water samples were collected using glass flasks. They were frozen and stored until the use in the physicochemical analyses, which considered the measurements recommended by the APHA (2005): conductivity (μS/cm), temperature (°C), pH, hardness (mg/$CaCo_3$), and dissolved oxygen content (mg/L). Total ammonia concentrations (NH_3–NH_4) were measured using the colorimetric method (Koroleff 1970).

Statistical analysis

Descriptive analyses for quantitative data with normal distribution are presented as means and their respective standard deviations. The distribution of data (normality) and the homogeneities of variances were confirmed using the Shapiro-Wilks test and the Bartlett test, respectively. The results were then compared to their respective controls using Student's *t* test for independent samples in order to determine any significant differences. The level of significance was set to 5%, and statistical analyses were performed using the SPSS software, version 15.0.

Results

Mucociliary transport

The mucociliary transport capability exhibited by the fish from the polluted pond (0.3 ± 0.1) was significantly lower ($p < 0.05$) than that observed in fish from the control site (1.01 ± 0.1; Table 2).

Water

Water temperatures and pH levels were similar at both sites. However, conductivity, and ammonia concentrations, and water hardness presented high values in the polluted pond. In addition, dissolved oxygen was also lower in the waters form artificial pond. The physicochemical variables of the water are presented in Table 1.

Hematology and cyto-genotoxicity

When the hematological parameters of the fish from both sites were compared, the fish from the polluted pond presented significantly higher quantities of monocytes and eosinophils and significantly lower numbers of lymphocytes and neutrophils ($p < 0.05$). Leukocytes and nuclear abnormalities were more frequent in fish from the polluted pond. Meanwhile, the number of erythroblasts was significantly lower in fish from the polluted pond. Basophil frequency did not differ significantly between the groups. Rates of micronuclei were similar between the fish from the two sites, but nuclear abnormalities were significantly more frequent in fish from the polluted pond. The results are compiled in Table 2.

Discussion

This study revealed associations between water quality, hematological changes, nuclear abnormalities, and mucociliary transport. The mucociliary transport capabilities exhibited by fish from the polluted pond were significantly lower than those of the controls. Mucus analysis is a promising tool for biomonitoring, because secretion plays a critical role in animal defense. It serves as a natural, semipermeable, chemical, and biological barrier with immunological functions. In fish populations, a link has long been demonstrated between environmental pollution and disease due to the impairment of the innate immune system (Arkoosh et al. 2000; Guardiola et al. 2015; Seriani et al., 2015a; Guardiola et al., 2016).

Changes in fish mucus have been reported by Beamish (1972), Zieske and Bernstein (1982), and Northcott and Beveridge (1988). Lichtenfels et al. (1996) reported that the viscosity of fish mucus may increase the production of acidic glycoprotein (acid mucus), thus enhancing particle retention. Our results are consistent with these studies and suggest that low transportability and high density in vitro is a response of mucus acidification and an effect of the pollutants observed (Lemos et al. 1994; Pires-Neto et al. 2006; Yoshizaki et al. 2010; Seriani et al. 2015c; 2015d; Yoshizaki et al. 2017).

The data also showed that the fish from the polluted pond may be not healthy, since they presented increased numbers of monocytes, eosinophils, total leukocytes, and nuclear abnormalities, lower numbers of lymphocytes, neutrophils, erythroblasts, and thrombocytes, and a lower relative speed of mucociliary transport.

Changes in hematological parameters have been reported in fish exposed both to mixtures of pollutants and to single substances (Bacchett et al. 2011; Seriani et al. 2013; Fujimoto et al. 2012; Carraschi et al. 2016; Cruz et al. 2015; Thummabancha et al. 2016; Kumar et al. 2017). Low frequencies of erythroblasts and thrombocytes could be explained by water contaminants generating intrasplenic and intrahepatic

Table 1 Physicochemical variables of water samples collected from the polluted urban pond and the control site

Variable	Control	Polluted pond	Brazilian guidelines CONAMA 357/2005
Temperature (°C)	24 ± 0.1	24 ± 0.2	–
Dissolved oxygen (mg/L)	7.4 ± 0.1	6.1 ± 0.7	> 6
pH	7.0 ± 0.3	7.1 ± 0.6	6–9
Hardness ($CaCO_3$)	54.3 ± 2.5	130.1 ± 10	–
Conductivity (μS/cm)	227.1 ± 20.2	420.1 ± 20	–
Total ammonia (mg/L)	< 0.1	3.5 ± 1.1	3.7
Unionized ammonia (mg/L)	< 0.01	0.5 ± 0.2	–

damage, thus decreasing hematopoiesis. Çavaş (2008) and Corredor-Santamaría et al. (2016) described erythrocyte abnormalities that resulted from the presence of high levels of ammonia and toxic metals. Moreover, Jeney et al. (1992) showed that ammonia could reduce numbered blood cell counts and distorted red blood cell shapes. The experimental fish in this study exhibited lower erythroblast counts.

Leukocytes exhibit high phagocytic activity, and thrombocytes are involved in both blood clotting and the organism's defense system (Passantino et al. 2005). Fish from the polluted urban pond exhibited a higher frequency of total leukocytes, a result previously observed by other authors in studies on fish exposed to contaminants (Zutshi et al. 2010; Gupta et al. 2013; Seriani et al. 2013). An increased amount of leukocytes suggests the occurrence of an adaptive response to chronic stress induced by xenobiotics or pathogens. Prolonged exposure to higher concentrations of toxicants was found to cause failures in leukocyte production; however, increased leukocyte production could be a protective mechanism against pollutants (Svobodova et al. 1994). Leukocytosis in fish may therefore

Table 2 Biological parameters of *O. niloticus* from control waters and a polluted pond. Speed of mucociliary transport (mm/min), frequency of total leukocytes, thrombocytes, micronuclei, nuclear abnormalities, erythroblasts, and percentage of lymphocytes, neutrophils, basophils, monocytes, and eosinophils. Asterisks show significant difference ($p < 0.05$) between groups

Parameters	Control	Polluted pond
Speed of mucociliary transport (mm/min)	1.0 ± 0.1	0.4 ± 0.1*
LC (%o)	11.1 ± 4.8	26.4 ± 6.4 *
TC (%o)	32.3 ± 11.9	10.9 ± 5.9 *
Micronuclei (%o)	0.6 ± 0.4	0.8 ± 0.5
Nuclear aAbnormalities (%o)	9.3 ± 0.5	17.5 ± 4.8 *
Erythroblasts (%o)	20.0 ± 3.2	12.1 ± 5.7 *
Lymphocytes (%)	91.7 ± 5.8	86.1 ± 2.4 *
Neutrophils (%)	9.2 ± 3.7	6.9 ± 1.7
Basophils (%)	0.4 ± 0.6	0.5 ± 0.6 *
Monocytes (%)	4.0 ± 2.3	7.3 ± 1.7 *
Eosinophils (%)	0.3 ± 0.6	2.0 ± 0.6 *

be the consequence of the direct stimulation of their immunological defense systems.

According to Rowley (1988), monocytes normally occur in low numbers in differential white blood cell counts, but they are actively phagocytic cells in piscine fish. They are also involved in the removal of cellular debris from necrotized tissues as part of inflammatory response and other degenerative processes, so much so that monocytosis could be suggestive of an inflammatory response in teleost fish. These blood cells, considered to be the most important in the immune system (Clauss et al. 2008), could migrate to sites that may have been damaged as a result of toxic effects (such as apoptosis or tissue damage). Therefore, if leukopoietic centers were lesioned, as observed previously by Lemly (2002) and by Sorensen et al. (1982), (Sorensen and Bauer 1984), the release of these cells would be inhibited or even impeded. Thus, the presence of these cells in the present study is a clear indicator of internal lesions in fish from the polluted pond.

Some reports indicate that eosinophils are also involved in inflammatory responses, despite their limited phagocytic capability (Rowley 1988). Rothenberg and Hogan (2006) showed that eosinophils are pleiotropic multifunctional leukocytes involved in the initiation and propagation of diverse inflammatory responses, as well as modulators of innate and adaptive immunity. This cell type is rarely observed in the blood of fish, and the high number of eosinophils in fish from the polluted site was surprising: these results suggest that the increase in this type of cell may be associated with inflammatory processes due to either parasite infestation or chemical compounds present in effluents (Corrêa et al. 2017).

On the other hand, there were lower counts of some cell types, including lymphocytes and neutrophils. The frequency of the thrombocytes was also lower. According to Sorensen (1991), lower neutrophils counts may indicate a disruption of phagocytic capacity and, consequently, a reduction in the fish's resistance to pathogens. Lymphocyte frequency has been associated with resistance to disease. Nicholson et al. (1990) reported that, at low to moderate levels of stress, this kind of reduction may be not caused by direct suppression of antibody production. Thus, reduced lymphocyte counts in fish from the polluted pond considered herein were due to lymphocytopoiesis and not to mechanisms involving cortisol.

The thrombocytopenia observed in our study could have devastating effects on fish, because these cells are responsible not only for blood clotting, but also for controlling fluid loss from surface wounds in fish (Clauss et al. 2008). The decrease in the number of these cells in peripheral blood may suggest migration to hemorrhagic foci, as observed by Mazon et al. (2002) and Pereira et al. (2013) in studies on the genus *Prochilodus*.

Cyto-genotoxicity biomarkers, such as frequency of nuclear abnormalities and micronuclei, were measured, but only nuclear abnormalities appeared to be significantly higher in fish from the polluted pond. Measurements of nuclear abnormalities seemed to be more sensitive than those of micronuclei. These results suggest the occurrence of genotoxicity in *O. niloticus* specimens (Kirschbaum et al. 2009; Çavas and Ergene-Gözükara 2005). Similar effects have been reported in studies on fish collected from other polluted sites. García-Medina et al. (2011) investigated fish collected from sites along the Ocoa River receiving untreated domestic sewage and observed an increased incidence of binucleate erythrocytes; the authors attributed the genotoxic effects to the pollutants found in the river waters. In our study, nuclear abnormalities were found to be more frequent than micronuclei.

The water from the polluted pond seemed to exhibit some evidence of changes in quality, since conductivity and hardness were higher than those of the control site. Ammonia levels were higher in the polluted pond but were still below the limits established by the National Environmental Council of Brazil (CONAMA 2005) and the São Paulo State Sanitation Technology Company (CETESB 2009). On the other hand, electric conductivity data provide information on ion concentrations and aid in the detection of pollutant sources in aquatic ecosystems (Esteves 2011). Conductivity and ion concentrations in particular indicate changes in the chemical composition of the water; however, these data do not provide any indication of the specific concentrations of each ion (CETESB 2009). Moreover, Esteves (2011) also stated that the water hardness may be influenced by rainfall regime, geological characteristics of the catchment or basin, and inputs of anthropic origin. In this sense, despite the lack of legal standards for hardness and conductivity in Brazilian waters, the higher values found in the polluted pond may represent degradation resulting from the proximity of residential areas. It is also important to note that this body of water receives domestic effluent discharge, urban drainage, and storm waters, and that water renewal is very low and depends exclusively on rainfall rates.

Hardness is associated with concentrations of calcium and magnesium (carbonates and bicarbonates), which can modify the solubility of some metals or the behavior of organic compounds in the aqueous medium. Hardness has been found to be associated with increases in metal toxicity in studies on rainbow trout (Sinley et al. 1974), *Poecilia reticulata* (Gianotti 1986), and *Clarias gariepinus* (Molokwu and Okpokwasili 2002), whereas other studies have found that the increase in water hardness lowers metal toxicity (Javid et al. 2007; Kim et al. 2001; Pyle et al. 2002).

Conductivity represents the number of suspended particles in the water, particles which may be anything from organic matter to microbeads and other pollutants. The ideal value of conductivity for fish health lies between 20 and 100 μS/cm (Zimmermann et al. 2001). Moreover, conductivity provides important data about aquatic metabolism and helps to identify the presence of pollutants: high values could be indicative of elevated levels of decomposition, and lower values may point to primary productivity. According to Felipe and Súarez (2010), high values of hardness increase the abundance of species tolerant to losses of water quality.

Changes in ammonia, hardness, and conductivity levels have been frequently associated with the input of sewage and/or urban storm water runoff, sources which are considered complex mixtures containing many toxic compounds, such as organic substances, pharmaceuticals, personal care products, metals, hydrocarbons, detergents, pesticides, drugs, and other toxics (Sibanda et al. 2015; Pereira et al. 2016; Silva et al. 2017).

In conclusion, the fish from the polluted pond exhibited several signs of effects on blood cell composition, as well as more frequent nuclear abnormalities and changes to mucus transportability. These findings suggest that the polluted pond's waters are not suitable for the aquatic organisms. The biomarkers used in this study can be considered good indicators of pond water quality, and they are advantageous as tools because they do not require euthanasia. The causes of the increase in leukocyte (monocyte and eosinophil) quantities remain unclear and require further investigation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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