Revista Ambiente & Água
ISSN: 1980-993X
Instituto de Pesquisas Ambientais em Bacias Hidrográficas

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Revista Ambiente & Água, vol. 13, no. 6, 2018
Instituto de Pesquisas Ambientais em Bacias Hidrográficas

DOI: 10.4136/ambi-agua.2085

Ambiente & Água - An Interdisciplinary Journal of Applied Science
ISSN 1980-993X – doi:10.4136/1980-993X
www.ambi-agua.net
E-mail: ambi.agua@gmail.com

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ARTICLES doi:10.4136/ambi-agua.2085

Received: 20 Mar. 2017; Accepted: 09 Sep. 2018

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ABSTRACT

Aquatic environmental pollution due to negative human activities remains a major problem. Bioindicators that primarily describe the total concentration of the respective pollutant are very useful tools to aid in the chemical analysis of water in order to obtain knowledge about the levels of pollutants in the environment. This study therefore used Electron Spin Resonance Spectroscopy (ESR) to detect the presence of transition metals (copper, iron and manganese) and possible radicals present in samples of *Neoechinorhynchus curemai* and its host tissues *Prochilodus lineatus*, as well in water and sediment of the Batalha River, at the same sample site where fish were collected. Spectral analysis of samples showed the presence of three metals (Cu^{2+}, Fe^{3+} and Mn^{2+}), in addition to nitric oxide (NO) and humic acid (HA). Quantification of the elements in the samples was possible only for Cu detected in the spectrum of parasites, which was equivalent to 2 ppm. ESR proved to be efficient in the detection of transition-metal ions (Cu^{2+}, Fe^{3+} and Mn^{2+}), in addition to NO and HA. However, the low concentration values of these compounds in *P. lineatus* tissues (liver, muscle and intestine) and in the water and sediment samples collected did not allow their quantification, as they were below the limit of detection. It can be concluded that *N. curemai* had the capacity to accumulate these ions, especially copper.

Keywords: environmental monitoring, free radicals, *Neoechinorhynchus curemai*, *Prochilodus lineatus*, transition metals.

Ressonância do Spin Eletrônico (ESR) na detecção de poluição aquática através da relação parasito-hospedeiro

RESUMO

A poluição de ambientes aquáticos em decorrência de atividades antrópicas negativas permanece sendo um grande problema. Para se obter conhecimento acerca dos níveis biológicos de poluentes disponíveis no meio, os bioindicadores tornam-se uma ferramenta muito útil no

auxílio das análises químicas da água que primariamente descrevem a total concentração do respectivo poluente. Nesse contexto, o objetivo desse trabalho foi o de empregar a técnica de Espectroscopia de Ressonância do Spin Eletrônico (ESR) para detectar a presença de poluentes, diretamente em espécimes de *Neoechinorhynchus curemai* e nos tecidos do seu hospedeiro *Prochilodus lineatus*, assim também como na água e sedimento do rio Batalha, no mesmo ponto onde os peixes foram coletados. A análise do espectro das amostras identificou a presença de três metais (Cu^{2+}, Fe^{3+} e Mn^{2+}), além do óxido nítrico (NO) e do ácido húmico (AH). A quantificação dos elementos nas amostras só foi possível para o Cu, detectado no espectro dos parasitos, que foi de 2ppm. A ESR se mostrou eficiente na detecção de íons metálicos de transição (Cu^{2+}, Fe^{3+} e Mn^{2+}), além do NO e do AH. Entretanto, a baixa concentração desses compostos nos tecidos (fígado, músculo e intestino) de *P. lineatus.* não possibilitaram sua quantificação, já que as concentrações dos mesmos estão abaixo do limite de detecção do aparelho. É possível concluir que *N. curemai* teve a capacidade de acumular esses íons, especialmente cobre.

Palavras-chave: metais de transição, monitoramento ambiental, *Neoechinorhynchus curemai*, *Prochilodus lineatus*, radicais livres.

1. INTRODUCTION

Pollution of aquatic ecosystems as a result of anthropogenic activities remains a major problem in these environments, causing disastrous effects on the health of living organisms and becoming subject of many discussions (Khan and Thulin, 1991; Sures, 2008). In freshwater ecosystems, both organic and inorganic pollution (especially metals) are global issues and deserve attention since they directly and indirectly affect organisms, causing them irreparable damage (Wen et al., 2017; Schmeller et al., 2018; Väänänen et al., 2018).

Bioindicators that primarily describe the total concentration of a respective pollutant are very useful tools in the chemical analyses of water in order to obtain knowledge about levels of pollutants in the environment. Recent studies have shown that, in addition to already established indicators, certain parasites and their hosts can be used as biomonitoring tools, since the pollution also effects their health and, consequently, their occurrence and distribution. Another important point to consider is that, just like pollutants, parasites affect the health of organisms, and some of these organisms' responses to pollutants and parasites are very similar (Lafferty, 2008; Lafferty and Kuris, 2005; Sures, 2008; Thielen et al., 2004).

Studies evaluating the role of parasites as accumulators and pollution indicators have been carried out for decades, using a vast host-parasite combination (Sures et al., 1999; 2017) where fish are the most frequently used hosts (Vidal-Martínez and Wunderlich, 2017). In addition, the high potential of parasites for metal accumulation (especially heavy metals) and their high effectiveness in assessing aquatic pollution compared to free-living organisms (such as mussels, for example) has been clearly demonstrated (Sures et al., 1997; 1999;).

Different fish endoparasites have been suggested for use in the detection of pollution in aquatic environments. Among them, acanthocephalans are highlighted as presenting a high capacity for heavy metal accumulation (Vidal-Martínez et al., 2009; Nachev et al., 2013). Laboratory studies have shown that fish parasitized by acanthocephalans have lower levels of heavy metal contamination in their tissues when compared to non-parasitized specimens. However, the results of the analyses are difficult to predict, and there is a need for further studies in this field to contextualize information already obtained in different types of environments with different levels of degradation (Thielen et al., 2004; Sures, 2008).

Usually bioaccumulation studies in the host-parasite relationship are made using inductively coupled plasma mass spectrometry (ICP-MS) (Brázová et al., 2012; 2015; Leite et

al., 2017; Nachev et al., 2010; 2013; Thielen et al., 2004) or Atomic absorption spectrometry (AAS) (Baruš et al., 2001; Bayoumy et al., 2015; Baruš et al., 2007; Dural et al., 2011; Morsy et al., 2012). Eletron Spin Resonance (ESR) is a spectroscopy designed to detect non-zero electronic spin systems, such as free radicals and some transition metals. In biological systems, it can be used to detect the presence of some ions, including copper, manganese and iron, directly in tissues without biochemical manipulations, thus reducing the risk of structural disturbances. This makes the technique a powerful tool in research involving biological and environmental systems (Wertz and Bolton, 2012).

ESR has already been used to detect pollutants (both organic and inorganic) in several systems. In aquatic ecosystems, ESR has been used to detect Reactive Oxygen Species (ROS) in amphibians (D'Errico et al., 2018) and to detect and quantify Mn^{2+}in fish otoliths (Di Beneditto and Franco, 2018). In both cases the technique proved to be efficient, and could be used to aid other spectrometric techniques. In the host-parasite system, however, there are still no published studies where ESR was used. In this context, the objective of this study was to use the ESR technique to detect transition metals (copper, iron and manganese) and possible radicals present in parasites and host tissues (muscle, liver and intestine).

2. MATERIAL AND METHODS

2.1. STUDY AREA

This study was conducted in a stretch of the Batalha River, the Tietê-Batalha River Basin, located in the municipality of Reginópolis, State of São Paulo (21º53'17" S and 49º13'31" W). It is an area with severe anthropic influences and predominantly lotic characteristics. Limnologic and structure variables of the stretch are plotted in Table 1.

The river area in this stretch, even though surrounded by native riparian forest for most of its extension, is used predominantly for agricultural and livestock purposes, with emphasis on cattle raising, sugar cane, corn and eucalyptus plantations, increasing the amount of organic matter and leachate pollutants. In addition, the municipality does not treat sewage, and so sewage is thrown *in natura* into a tributary of the river (São Paulo, 2010; Santos and Heubel, 2008; Sistema Nacional De Informações Sobre Saneamento, 2016).

Table 1. Mean values of physicochemical parameters: pH, temperature, dissolved oxygen (DO) and conductivity (CD); and structural parameters: width of the stretch, and width of the permanent preservation area (APP) surrounding the sampling point, located in the municipality of Reginópolis.

Parameter	Value ± SD
pH	7.7 ± 0.3
Temperature (ºC)	26.5 ± 1.5
DO (mg/L)	4.9 ± 1.6
CD (µS/cm)	68 ± 14.7
Width (m)	13.2 ± 1.7
APP width (m)	83.1 ± 7.9

2.2. SAMPLING AND PROCESSING OF PARASITES AND HOSTS

Thirty-nine specimens of *Prochilodus lineatus* (Valenciennes, 1837) (Characiformes: Prochilodontidae) were collected between May 2015 and May 2016 on the Batalha River. Fish were necropsied in the laboratory, and the internal organs were analyzed separately using a

stereomicroscope to collect parasites. In this study, only fish specimens that showed positive results for *Neoechinorhynchus curemai* Noronha, 1973 (Acanthocephala: Neoechinorhynchidae) parasitism were used, totaling 19 *P. lineatus* specimens analyzed.

After collection, the parasites were placed in a recepticle containing distilled water and then washed with a vortex mixer. Subsequently, they were placed in glass flasks and frozen at -20ºC until the ESR analysis. Samples of muscle, liver and intestine were also taken from hosts. The washing and storage of the tissue samples followed the same methodology used for parasites.

2.3. WATER AND SEDIMENT SAMPLING

Water and sediment samples were collected from the Batalha River at the same point of the fish collection. The sampling followed the procedures recommended by Gomes and Filizola (2006) for sediment and Pires et al. (2006) for water. The samples were stored in 20 mL plastic tubes and frozen at -20ºC until ESR analysis.

2.4. ESR ANALYSIS

Samples of parasites and host tissues were externally cleaned with ultrapure water (Milli Q) and then dehydrated in absolute alcohol by immersion for 10 minutes. These materials and sediment were then oven-dried at 40ºC (Table 2). After drying, the samples were carefully crushed with agate mortar and pestle for homogenization under low impact, in order to avoid induction of other radicals by mechanical action. Subsequently, an aliquot of each sample was transferred to an ESR quartz tube, with a 3mm internal diameter. The mass used to record the spectrum was determined (Table 2). The water sample was placed in a capillary tube and sealed prior to insertion in the quartz tube. The tube containing the sample was inserted into a quartz dewar for freezing with liquid nitrogen for spectrum recording (77K).

A JEOL Band-X spectrometer was used to record the spectra, some of them with a scan width of 500mT and others with 80mT, centered on the g~2 spectral region. Some spectra were recorded simultaneously with a Mn^{2+} standard, present in the spectrometer, that consists of MgO crystal doped with Mn^{2+}. A 0.5 mM solution of $CuSO_4$ complexed with $C_3H_7NO_2$ was used for the quantification of copper (Cu^{2+}) present in the sample. The spectra of the parasite and the copper solution were acquired under the same spectrometer conditions of microwave power, modulation and gain. Copper can be complexed with several compounds producing different spectral symmetries. Alanine was used as a ligand due to the same symmetry of the complex found in the parasites samples. The volume of the standard solution used was 20µL.

Table 2. Mass values (mg) of parasites, host tissues and sediment samples before and after drying, and the amount used in ESR analysis.

Mass	Parasite	Intestine	Liver	Muscle	Sediment
Wet	102 mg	189.5 mg	690 mg	674.4 mg	--
Dry	17.1 mg	20.21 mg	77.44 mg	114 mg	--
ESR	5.68 mg	6.08 mg	24.45 mg	5.8 mg	77.6 mg

3. RESULTS AND DISCUSSION

The specimens of *P. lineatus* showed mean standard length and weight of 27.44 ± 6.37 cm and 568.38 ± 318.14 g, respectively. The specimens of *N. curemai* were all adults and had mean abundance of 2.83 ± 0.12 parasites per fish and mean intensity of 5.52 ± 0.13 parasites.

The analysis of the parasite samples' spectrum in the 500mT scan was performed under two conditions: only parasite, and parasite with Mn^{2+} as a secondary standard. The analysis of

the obtained signals showed that there is no Mn^{2+} in the parasite samples (Figure 1A). In the spectrum of the central region g~2 it was possible to notice characteristic lines of copper (Figure 1B). To confirm the presence of Cu^{2+} in the sample, spectral simulation of the Cu^{2+} present in the parasites was carried out using Simfonia-Bruker software. Through the simulation, it was possible to observe the agreement between the simulated Cu^{2+} signal and the radical present in the parasites. Double integration of the Cu^{2+} simulated signal was compared to element pattern spectrum. The Cu^{2+} mass of the total mass of the parasite was obtained from the atomic mass of the ion (63.6g), resulting in a concentration of 2 ppm of Cu^{2+} in the parasites (Figure 1C).

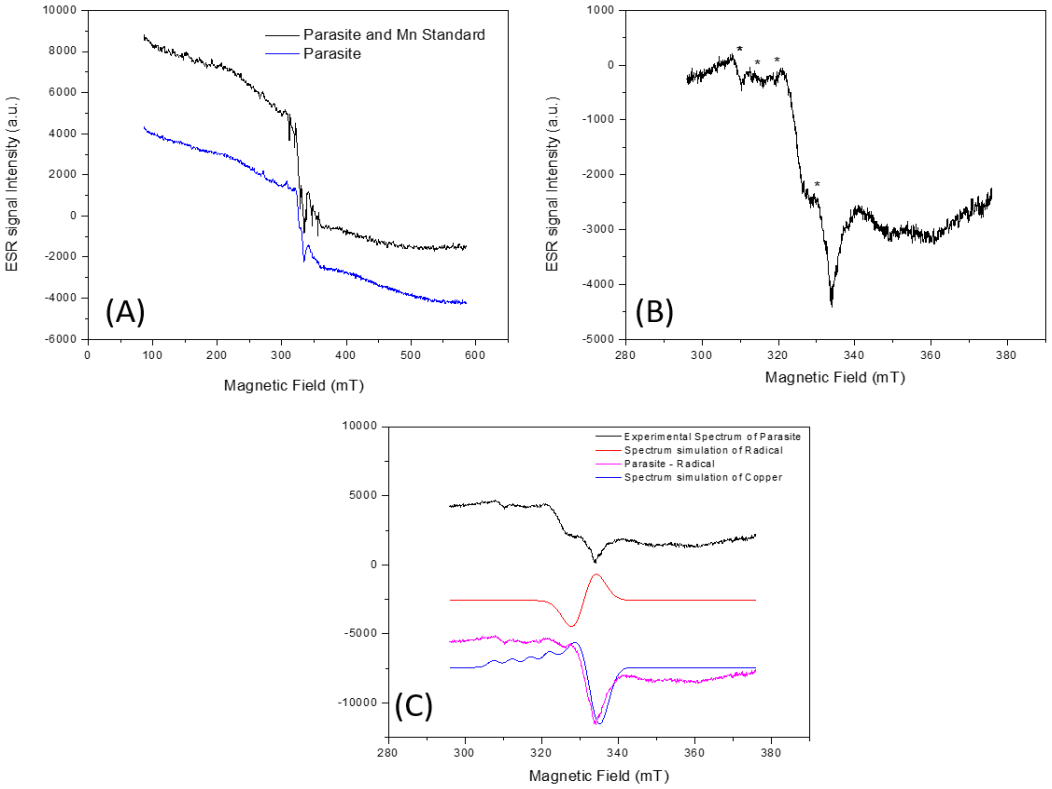

Figure 1. ESR spectrum of *Neoechinorhynchus curemai* specimens with and without the simultaneous register of manganese (A), centered on the g~2 region, where the signals indicated by asterisks represent the lines belonging to the copper (B)($g_{\perp}$: 2.0032 ± 0.0001; $g_{//}$: 2.1160 ± 0.0001; $A_{\perp}$: 1.1 ± 0.1 mT; $A_{//}$: 4.8 ± 0.1 mT) and spectral simulation of the radical (g-factor: 2.0084 ± 0.0001) (C). Acquisition parameters are: Center Field 336 mT, Modulation Frequency 100 kHz, Modulation amplitude 0.1 mT, gain 2000, microwave frequency 9131.4 MHz, Microwave Power 5 mW. In (A) sweep width 500mT and (B) 80 mT.

The liver sample spectrum was recorded with 500mT scanning width. The spectrum was dominated by iron, as shown in the simulation (Figure 2A). In the g~2 region of the spectrum it was possible to identify other radicals. By performing a more detailed recording of the spectrum in the g~2 region it was possible to observe a structure of lines. The spectrum was recorded with the manganese pattern for investigation, where very weak signals of this ion in this region can be noted (Figure 2B), indicating a small concentration of Mn^{2+} in the tissue. A more detailed scan at the g~2 region showed that the lines' structure in the central region corresponds to nitric oxide (NO), bound to Fe^{3+}, leading to the three-line structure observed. Figure 2C shows the spectrum of the liver sample and the simulation of the NO radical spectrum.

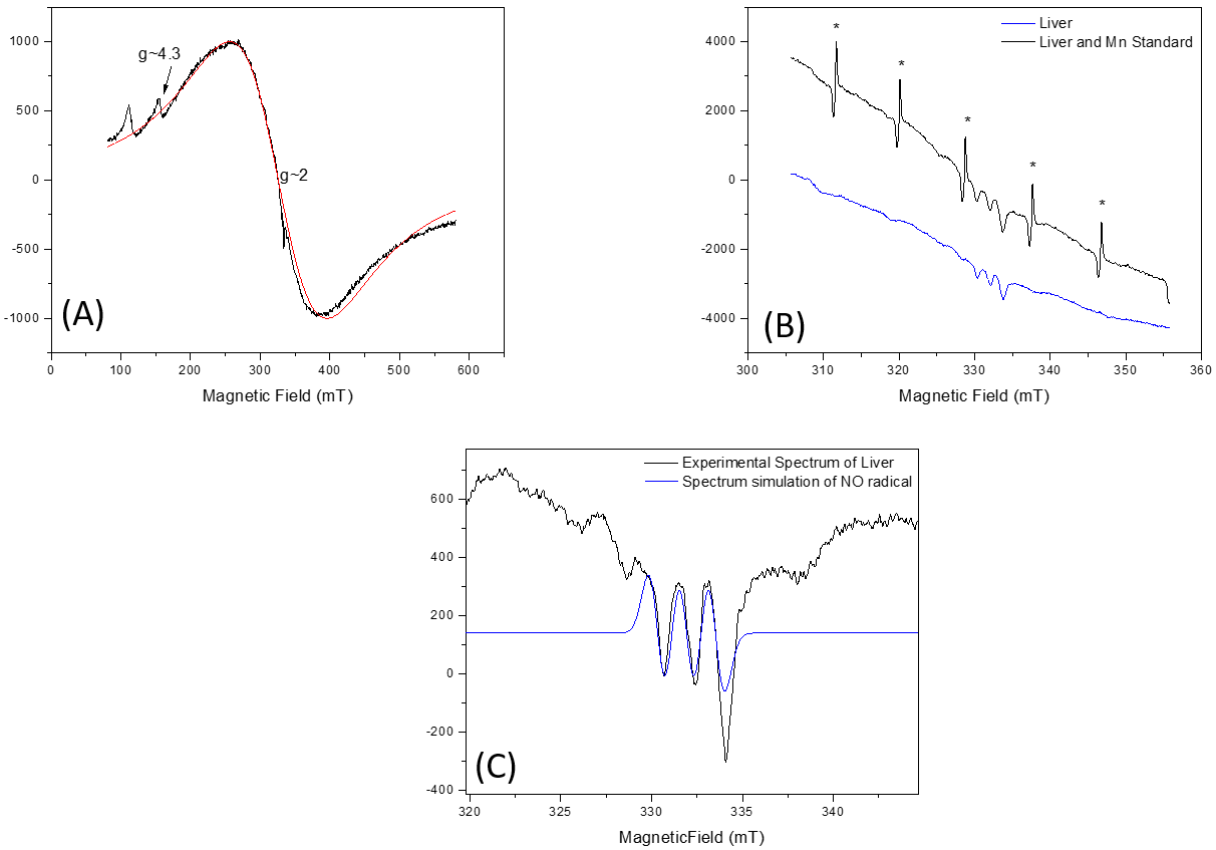

Figure 2. ESR spectrum in the 500mT scan of the fish liver samples and iron spectral simulation in red (A) (g-factor = 2.0453± 0.0001). The spectrum centered on the g~2 region with and without the manganese (*) pattern (B). Spectrum of the sample also centered on the g~2 region and the simulation of the NO (g-factor = 2.0040 ± 0.0001, A=1.82 ± 0.01mT) present in the sample (C). Acquisition parameters are: Center Field 330 mT, Modulation Frequency 100 kHz, Modulation amplitude 0.1 mT, gain 300, microwave frequency 9133.4 MHz, Microwave Power 5 mW. In (A) sweep width 500mT and (B) 100 mT.

In the spectrum of fish intestine samples, it was possible to notice the presence of Fe^{3+} and Mn^{2+} (Figure 3A). The spectrum was recorded with Mn^{2+}standard to confirm the presence of this element in the sample (Figure 3B). Incorporation of manganese ions in different matrices have been studied with multifrequency ESR, and this can give more information about the complex formed; but these techniques are not easily found, and these studies have corroborated the experiments done at X band (Murzakhanov et al., 2017).

In the fish muscle spectrum, it was also possible to notice the presence of the Fe^{3+} element and Mn^{2+} traces (Figure 4), suggesting that this element, although present in the sample, was in low concentration.

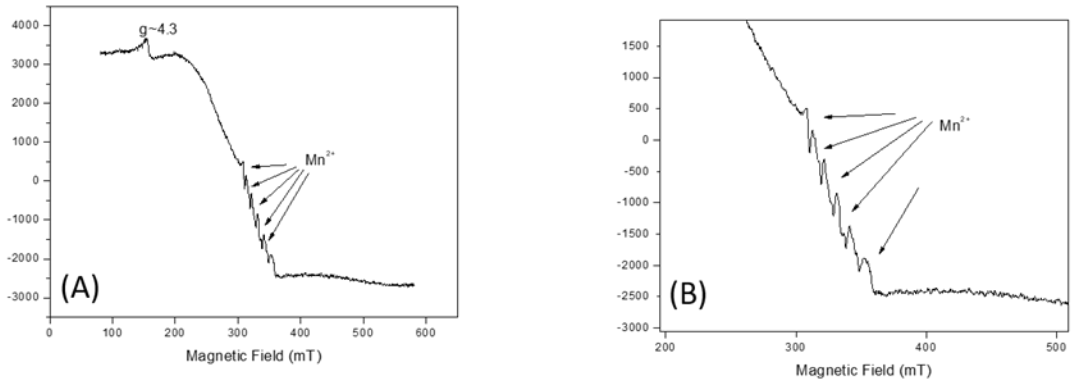

Figure 3. ESR spectrum of the fish intestine samples centered in the g~2 region where the presence of iron is noted, with a peak at g~4.3 and manganese, as indicated (A). Spectrum in the Mn^{2+} region, recorded with manganese standard, for confirmation (B). Acquisition parameters are: Center Field 330 mT, Modulation Frequency 100 kHz, Modulation amplitude 0.1 mT, gain 300, microwave frequency 9133.4 MHz, Microwave Power 5 mW, sweep width 500mT.

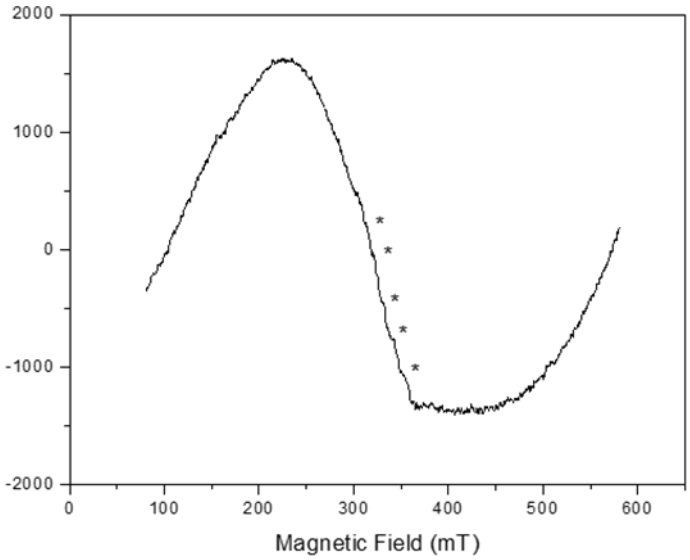

Figure 4. ESR spectrum of the fish muscle samples, where the presence of iron is observed and traces suggesting the presence of manganese (*). Acquisition parameters are: Center Field 330 mT, Modulation Frequency 100 kHz, Modulation amplitude 0.1 mT, gain 300, microwave frequency 9133.4 MHz, Microwave Power 5 mW, sweep width 500mT.

In the spectrum of the sediment samples analyzed, the presence of Fe^{3+} and Mn^{2+} can be observed. In addition to these elements, another central radical was recorded in the g~2 region, attributed to humic acid (AH) (Figure 5B). In the water sample, the spectrum showed only the presence of Fe^{3+} (Figure 5A).

Low concentrations of elements considered essential, that is, those necessary for the development of organisms and that occur naturally in the environment (Merian et al., 2004), are expected in acanthocephalans fish parasites, which are more susceptible to accumulate high concentrations of toxic elements (Nachev et al., 2013). Although copper is considered essential and is involved in several metabolic processes of living organisms (Momčilović, 2004), it is a heavy metal and at higher concentrations can bring potential risks to the health of animals, including humans (Papagiannis et al., 2004). The impacts of copper on the aquatic environment

depend on water physico-chemical characteristics, such as alkalinity, hardness and pH (Carvalho and Fernandes, 2006). Generally, concentrations of this element in water are lower than those found in fish and parasites (Porto and Ethur, 2009; Brázová et al., 2012), and the maximum concentration of copper allowed for Class 2 waters, such as the Batalha River, is 0.009 mg/L (Conama, 2005). In this case, as the element was not detected in fish-tissue spectrums nor in the water or sediment collected from the river, it was assumed that the concentrations are below the ESR detection limit.

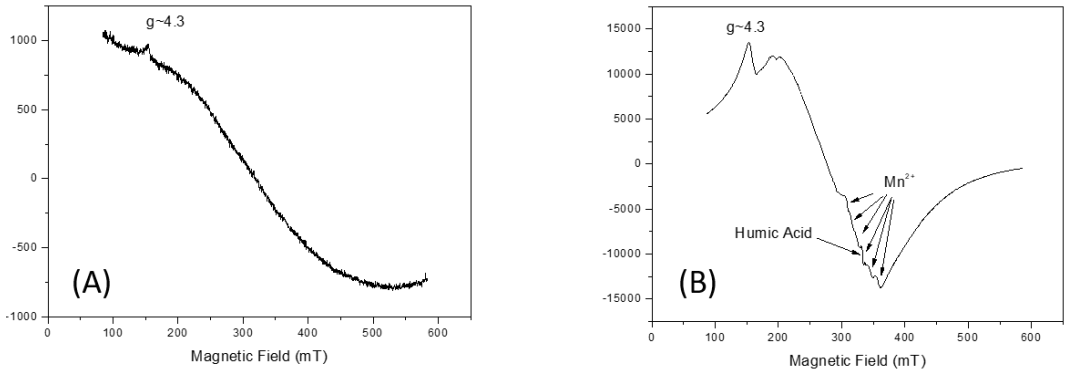

Figure 5. ESR spectrum of the water sample, where the presence of iron (A) is noted. The ESR spectrum of sediment sample shows the presence of iron, traces of manganese, as indicated, and the presence of humic acid in the g~2 region (g-factor = 1.9976 ± 0.0001) (B). Acquisition parameters are: Center Field 330 mT, Modulation Frequency 100 kHz, Modulation amplitude 0.1 mT, gain 300, microwave frequency 9133.4 MHz, Microwave Power 5 mW, sweep width 500mT.

The presence of iron and manganese ions in the spectrum of the tissues (liver, muscle and intestine) of *P. lineatus* and also in the samples of water and sediment of the Batalha River was already expected due to the high natural occurrence of these elements, which are the two most abundant metals in the environment (Förstner and Wittmann, 1983). Both elements in water and sediment are considered essential for both flora and fauna (Howe et al., 2004; Schümann and Elsenhans, 2004). Iron in fish acts mainly in oxidation-reduction and oxygen-transport processes, occurring as a component of the respiratory pigment (heme), as hemoglobin and myoglobin, as well as the heme enzymes (peroxidase, catalase and cytochromes) being absorbed from the water through the gills (Lim et al., 2001). Manganese plays a key role in the metabolism of amino acids, lipids, proteins and carbohydrates, besides being involved in functions of the immune system, blood glucose regulation, reproduction and also in defense mechanisms against free radicals (Keen et al., 1999).

In aquatic vertebrates, iron excess can cause several effects such as breathing problems, reduced growth, feeding difficulties, high mortality and histopathological changes in liver cells (Bury et al., 2003; Lim et al., 2001), in addition to the production of oxygen free radicals, which may be toxic to cells (Bury et al., 2003). Negative effects of excess Mn on fish include anemia, leukocytosis, disruption of sodium balance, impaired calcium absorption and impacts on metabolism (Agrawal and Srivastava, 1980; Barnhoorn et al., 1999; Gonzalez et al., 1990; Nath and Kumar, 1987; Reader et al., 1988).

The presence of NO detected in the spectrum of fish liver samples may also be associated with Fe^{3+} domain in the spectrum of the same tissue, since the molecule exerts its physiological functions through the binding with Fe^{3+} present in heme (Denninger and Marletta, 1999). In fish, NO has important roles in cardiovascular homeostasis, neurotransmission, immune defenses, vasodilation, muscle performance and embryonic development (Eddy, 2005; Jensen, 2009; Moncada and Higgs, 2006; Rudnick et al., 2004), and may be potentially toxic depending

on tissue concentration or clearance (Hansen and Jensen, 2010). In addition, NO production may be directly associated with hypoxias situations, where the increase in NO causes fish and other aquatic organisms to become more resistant to situations of low oxygen availability in water (Jensen, 2009; McNeill and Perry, 2006). Hansen and Jensen (2010), evaluating the production of NO in goldenfish (*Carassius auratus* Linnaeus, 1758), observed that in situations of severe hypoxia, NO production by fish was increased as well as its resistance capacity, since the vasodilatation caused by NO increased the oxygen delivery rate in the organs. Thus, it is possible to verify that presence of NO in the tissues of *P. lineatus* can be an indicative of the lack of oxygen in the water, which in the study site of the Batalha River is around 4.9 mg/L, below the established limit by CONAMA for Class 2 waters (Conama, 2005). However, it would be necessary to quantify NO in the samples in order to obtain safer and more accurate results. Another factor that should be considered is the fact that NO production is also directly associated with production of nitrosamines, which are potentially carcinogenic organic compounds (Al Bulushi et al., 2009). In humans, the highest rate of exposure to this compound occurs through food, which includes water and fish (Dutra et al., 2007).

Humic acids (HA), derived from humic substances (HS), which are part of organic matter, belong to the class of natural products most abundant in the biosphere, being precursors of fossil fuels. They are the most-studied fraction of HS, since they can contain voids of different sizes in their molecular structures, and can accommodate a wide range of pollutants (Mangrich and Vugman, 1988). HA act as binders for the complexation of ions, mainly Mn^{2+}, Fe^{3+} and Cu^{2+}, where the formation and transport of these complexes, and their deposition, can be important mechanisms in the accumulation of metals in the sedimentary deposits (Saab, 1999). It is known that in HA the content of certain ions, such as Fe^{3+}, in concentrate domain sites increases with decreases in the degree of environmental pollution (Silva, 2001). However, the mere presence of HA in the sediment spectrum cannot be considered an indicator of pollution, and a more robust analysis of its structure is necessary to establish relationships with environmental impacts such as forest-pasture conversion (Araújo et al., 2011) or pollution by heavy metals, pesticides and other substances of anthropogenic origin (Toscano, 1999).

4. CONCLUSION

In this study, the Electron Spin Resonance technique proved to be efficient in detecting transition metals (manganese, iron and copper) and nitric oxide and humic acid. However, low concentration values of these compounds in *P. lineatus* tissues (liver, muscle and intestine) and in water and sediment samples collected did not allow their quantification, as the concentration of metals in the tissues is at a level below the limit of detection. It can be concluded that *N. curemai* had the capacity to accumulate these ions, especially copper.

5. ACKNOWLEDGMENTS

We thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (process nº 2014/12682-0 and 07/06720-4) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, for the financial support.

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