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Bioassessment of Ecological Risk of Amazonian Ichthyofauna to Mercury

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Methylmercury (MeHg) is a well known human neurotoxin (Clarkson 1994). The general population is primarily exposed to MeHg through fish consumption (WHO 1990, Clarkson 1994, USEPA 2001). It has been demonstrated that Hg usually accumulates in fish tissues as MeHg, from inorganic mercury (Hg) sources (Huckabee et al. 1979). Whereas potential human health effects from Hg exposure have received considerable attention, relatively few studies have explored effects of realistic environmental Hg concentrations on fish or have attempted to use wild fish collected from polluted site where interactions among factors including diet, water chemistry and other variables could be important. Physiological biomarkers may identify effects at a tissue/organ before they are apparent at a clinical/pathological level. Some studies have been shown that MeHg has affinity for red blood cells (WHO 1990). So, hematology might be an important diagnostic test related to Hg exposure. It is possible to classify and evaluate fish anemias and this can be particularly important in fish, since clinical signs of anemia are often masked until quite late in the pathogenesis of the disease (Stoskopf 1993). Additionally, experimental Hg poisoning in fish showed marked hematological anomalies (Gill and Pant 1984). However, in order to evaluate the contaminant's adverse effects on ichthyofauna and other wildlife, one should establish the normal or reference values, overall to Amazonian species (Almosny and Santos 2001), since the information is limited.

Likewise, there are some studies that suggest that Hg exposure could induce chromosomal damage. This toxic effect may also be indirect assessed by micronuclei incidence in erythrocytes. The micronuclei (MN) are indicators of clastogenic or aneugenic effects. The micronuclei test in fish erythrocytes has increasingly been used to detect the genotoxic effects of environmental mutagens and its frequency is considered to reflect the genotoxic damage to cells, mainly the chromosomes. There are some evidences that Hg can induce genotoxic effects in experimental fish (Nepomuceno et al. 1997).

The objectives of this work are: (i) to establish and compare the hematological parameters and investigate their relationships for Hg accumulation by the Amazonian piscivorous fish Tucunaré (*Cichla*), from a contaminated area and

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from a reference site; (ii) to establish and compare the dose-response relationship for Hg accumulation by Tucunarés from a contaminated and reference area; (iii) to conduct a temporal analysis of the magnitude of mercury contamination in Tucunarés during a decade (1991-2001) by using DRAC ("Dose-Resposta para Avaliação da Contaminação", which means Dose-Response as a tool for contamination assessment) methodology; (iv) to test the DRAC methodology to proceed field sampling of fish for Hg contamination assessment and; (v) to conduct a field study of the genotoxicity of the MeHg in a fish species from the Amazonian region.

MATERIALS AND METHODS

Fish samples were collected from two areas: at the contaminated gold mining area and at a reference area. The contaminated area is located in the Tapajós river between the cities of Jacareacanga and Itaituba (04°15'23"S-55°54'33"W), where the gold mining sites are distributed alongside the tributaries of the Tapajós river. The reference site is located in a fluvial lacustrine system near Santarém city (02°25'11"S-54°42'16"W), more than 250 km downstream from the contaminated site. It is not as contaminated as the site influenced by the goldmining, but has the same basic environmental characteristics.

We sampled and analyzed 111 specimens of Tucunaré; 56 specimens (ΔLt= 190-520mm) from the contaminated site and 55 specimens (ΔLt= 201-410mm) from the reference site. Each specimen was weighed (Wt), and its length (Lt) was measured at the time of collection and put in polyethylene bags and frozen. Hg was analyzed in the fish muscle through Atomic Absorption Spectrophotometer (KK.Sanso SS) using a Vapor Generation Accessory-VGA (CVAAS). For the analysis of Hg-total, approximately 0.5g of tissue was weighed in 50-ml-vol flasks, to which was added 2 ml of HNO_3-$HClO_4$ (1:1), 5 ml of H_2SO4, and 1 ml of H_2O (Hg free), and heated on a hot plate to 230-250°C for 20 minutes. After cooling, the digested sample solution was made up to 50 ml with water. An aliquot (5 ml) of digested sample solution was introduced in the Automatic Mercury Analyzer Hg 3500. Reference standard DORM-1 fish muscle tissue with a certified Hg concentration of 0.798±0.13 μg.g-1 was also analyzed, giving a value of 0.793±0.06 μg.g-1 (n=8).

The fish sampling was oriented by DRAC methodology, previously described (Castilhos et al. 2001), which consists, briefly, in considering the growth of fish as an interaction between the specimen and the environment and as assumed as inference to exposure time. The relationship between length and age (or exposure time) of fish can be expressed by the von Bertalanffy (1957) mathematical equation. Some methods are used to calculate dose-response relationship and, among them, there is the "probit" method (American Public Health Association 1985, Ross and Gilman 1985). The potential exposure times were inferred from estimated age and were transformed in their logarithms. The frequency of responses were transformed in "probit" units. The AD_{50} for accumulation of Hg

by fish (accumulation dose 50) indicates the exposure time necessary to attain those tissue concentration levels by half of the exposed individuals. This resulting time can be related to response, as follows: $t_{exposure} * C = constant$ (adapted from Dämgen and Grünhage 1998); in which a certain response (K, constant) can be achieved from a exposure time ($t_{exposure}$) and the concentration in the aquatic environment C; such concentration will result as a potential dose or daily uptake rate (DUR), expressed in $\mu g.kg^{-1}.d^{-1}$. From these results one could estimate the exposure time to reach 500 $\mu g.kg^{-1}$ (or other level) and compare the contamination magnitude (or bioavailability) among different aquatic ecosystems.

After fish were caught, still alive, the blood was drawn by caudal or heart puncture with an EDTA containing syringe. In the field, the manual methods for counting erythrocytes were performed at the same time as the total leukocyte count, as recommended by Almosny et al.(1993) and Almosny and Santos (2001). A specific dilution of the blood was made with a diluent Gowers and Giemsa staining solutions, and the Newbauer$^{(Improved)}$ cell counting chamber was flooded. Smears, two slides per individual, were prepared without anticoagulant substances from fresh blood, air dried, fixed in methanol, and stained with Giemsa's solution. Blood corpuscules were examined by immersion microscopy and photographed. The hematocrit (or globular volume) was performed by microhematocrit method, using small capillary tubes, which were filled approximately two-thirds full with anticoagulated blood and centrifuged for five minutes at 14,000G. The percentage of packed cells to total volume was determined by direct measurement. The mean corpuscular volume (MCV), a Wintrobe erythrocyte index, was calculated as follows:

$$MCV = [(\text{hematocrit}) \times 100] / \text{Total erythrocyte count}$$

For MN examination, blood samples were smeared on clean glass slides, dried, fixed with methanol (20 min), and stained with Giemsa (6 min). Then slides were rinsed with distilled water and air-dried. Slides were observed under a light microscope with immersion oil. Two thousand blood erythrocytes per slide were scored. Only non-refractary particles with the same color of erythrocyte nuclei were interpreted as micronuclei.

The Lilleforts test, based on a Kolmogorov Smirnov test, was used to determine where data were normally distributed. Statistical differences among Hg concentrations, physical, hematological parameters and MN between different sites were tested using parametric Student's T-test after Levene's test for equality of variance, or, if the underlying assumptions for parametric testing are not met, a nonparametric test of significance, the Mann-Whitney U-test was employed. The significant level considered was the probability level ≤ 0.05. Correlations were determined with both the Pearson correlation coefficient on log transformed data and the Spearman rank correlation coefficient on the original data. Significance of the correlations was determined with a two-tailed Student's *t* test. One-way ANOVA followed by Duncan pos-hoc were performed when appropriate.

RESULTS AND DISCUSSION

Studies on the Hg concentrations of the fish fauna and their potential impact to humans have been performed in a contaminated and a reference area (Bidone et al. 1997, Castilhos et al. 1998) in the Tapajós River region. The transference factors of Hg through the food-chain involving Amazonian ichthyofauna suggested that biomagnification may occur in both the contaminated and reference areas (Castilhos and Bidone, 2000). Many factors have been considered important in the bioaccumulation and biomagnification of Hg in fish and spacial and/or temporal comparisons have been normalized by several means (Johnels et al. 1967, Häkanson 1991, Post et al. 1996, Scruton et al. 1994, Watras et al. 1998). We suggest using the DRAC methodology (Castilhos et al. 2000) besides statistical tests. This methodology was used as a tool for assessing mercury contamination magnitude of several aquatic ecosystems considering different fish species (Castilhos and Lima 2002).

The Tucunaré species was chosen for many reasons, including the fact that they have been considering as a good bioindicator of Hg accumulation in the Amazonian ecosystem, specially because of its time-integration capacity and their food habit (they are top-predatory, carnivorous ichthyophagous), they are the most sedentary and present a territorial behavior and their density does not change strongly during the year. Spawning season is long and it is not necessarily at the beginning of the flood time. Their preferred habitat is lentic (slow moving) water. The influence of Amazonian seasonality (well characterized by two hydrological periods: a low water level and a high water level period) on Hg accumulation in fish was studied and the results showed that carnivorous fish, including tucunaré, were not affected by seasonality (Castilhos, 1999). Their fine taste and abundance in native habitat have made it an important commercial specie (Ruffino and Isaac, 1995). The scientific identification of fish was performed by Emílio Goeldi Museum. The predominant species was *Cichla monoculus*, whereas in the contaminated area *Cichla* sp (43%) was also found. The fish sampling was oriented by DRAC methodology and the length and number of the specimens were shown in Table 1.

Both the level of Hg in tissue and the hematological responses were measured in each specimen. The results of total Hg in fish muscles and fish blood parameters were used as effects biomarkers. The K-S (Lilliefors) test showed that physical parameters (weigh and length) and Hg levels were non-normal distribution data, whereas the hematological parameters showed normal distribution. For comparison between *Cichla monoculus* and *Cichla* sp from contaminated area and *Cichla monoculus* from reference area, the ANOVA-one way with Duncan pos-hoc test were performed. The results showed that *Cichla* sp was higher ($p>0.01$; 325.7mm±59.9; n=25) and heavier ($p>0.05$; 830.8g±668.7; n=25) than *Cichla monoculus* (285.7mm± 38.3; n=26 and 520.7g±171.7; n=27), both from the contaminated area, but no statistical differences were shown when compared to *Cichla monoculus* (301.9mm± 38.7; n=55 and 638.2g± 262.4; n=54) from reference area. For other parameters, the *Cichla* species from contaminated area

did not show significant statistical differences. Based on these results, we decided to analyze the total fish collected from contaminated area and the summary results are shown in Table 2. For comparison between areas, the Student t-Test and Mann-Whitney U-test were performed, as appropriate. When the results were not different one each other, the parametric results are shown. The results showed no significant differences in length and weight between areas, which was expected by employing DRAC. Significant differences in muscles Hg levels and hematological parameters between reference and contaminated areas (Student's t-Test) were found.

Table 1. Length and number of specimens collected from reference site and mercury contaminated area.

Length (mm)	Specimens number	
	Reference area	Contaminated area
< 240	3	5
241 – 320	38	37
321 – 400	14	14

Table 2. Results of biometrics, total Hg in fish muscles (wet weight) and fish blood parameters as effects biomarkers (*Student t-Test) from reference and contaminated area

Parameters	Site	N	x±sd
Length (mm)	Reference	55	301,9± 38,7
	Contaminated	56	300,1± 67,3
Weight (g)	Reference	54	638,2± 262,4
	Contaminated	56	669,1± 493,0
Hg ($ug.kg^{-1}$)	Reference	53	237,5± 109,8***
	Contaminated	55	714,6± 218,6
Erythrocyte count	Reference	34	2,560,600± 635,500***
	Contaminated	53	2,000,200± 422,600
Hematocrit	Reference	35	44.4± 8.1**
	Contaminated	51	40.3± 5.6
MCV	Reference	35	183.1± 36.7*
	Contaminated	49	211.5± 41.4
Leukocyte count	Reference	31	53,161.3± 11,165.7***
	Contaminated	49	36,224.5± 9,973. 4

Student-T-test: *** $p<0.0001$, **$p<0.005$, * $p<0.01$

The Hg levels in muscles and MCV are higher in fish from contaminated area, whereas erythrocyte count, hematocrit and leukocyte count are lower than background area. These results showed that the specimens from the contaminated area have reduced numbers of erythrocytes and higher MCV values than

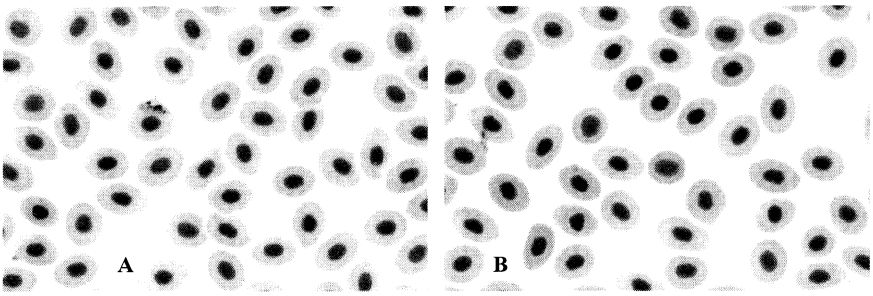

Figure 1. Blood smears of Tucunaré from A) the contaminated and B) the reference areas (x 500).

Tucunarés from the background area, suggesting that their erythrocytes should be larger than normal ones. This hypothesis was confirmed by microscopic investigation, as show in Figure 1. The erythrocytes' increased size has been associated with electrolytic imbalance, related to sodium, potassium, calcium, magnesium, chlorite, and intracellular water. It may also be related to abnormal erythrocyte metabolism. These larger erythrocytes may be related to non-regenerative anemia, which may be a fish mercury adverse effects. Additionally, the reduced erythrocyte number can cause deficits in the tissue oxygen levels and metabolism. The leukocyte numbers in blood of *Cichla* from the contaminated area, which were significantly lower than those from the background area, can cause immune deficiency.

One could suggest that a decreased number of erythrocytes and leukocytes could be also due to renal tissue damage, one of the most frequently described non-neural effects of mercury exposure, associated with the affinity of Hg for the kidneys (Sweet and Zelikoff, 2001). Additionally, the decreased leukocyte number, which may cause a deficit in animal immune defense capacity, was correlated to mercury tissue concentration. However, when interpreting correlation coefficients, it should not be assumed that correlation automatically implies causation; several effects could show a positive correlation with exposure but a causal-effect relationship is a difficult task to access.

No differences were found on the frequency of MN between the reference area (0.56± 0.88; n=32) and the contaminated area (0.50 ± 0.86; n= 43), by using Student T-test. It has been widely shown that frequency of MN is induced by Hg (Galan et al. 1999, Linde et al. 2001) in fish species both in vitro and in vivo. The micronuclei assay performed in the field with this fish species does not show any genotoxic effect caused by the pollution of methylmercury in that area. Future studies may be carried out to evaluate the potency of MeHg as a clastogenic agent in this fish species and to gain a deeper insight into its potential effects on the ecosystem under study.

In order to conduct a temporal analysis of magnitude of mercury contamination in fish during the last decade (1992-2001), the dose-response relationship for Hg

accumulation by Tucunaré for the contaminated and reference areas was performed by using the DRAC methodology. The results are shown in Table 3. The results showed that the estimated daily uptake rate by Tucunaré from contaminated area is about 3.5-4 times higher than reference area in both 1992 and 2001. The fish Hg contamination in the reference and contaminated areas increased close to 2 times during the last decade. Considering that almost 100% of total Hg is MeHg in carnivorous fish, these results suggest that that has been increasing the MeHg bioavailability in both areas. In addition, the MeHg bioavailability in the contaminated area remains 4 times higher than in reference area.

Some studies suggest the occurrence of some adaptation or compensatory mechanisms in chronically polluted aquatic organisms. Fish and other aquatic organisms are capable of biochemical and physiological responses to counteract pollutant effects. However, considering the present data, one may suggest that such mechanisms could be exceeded, resulting in observed hematological effects. One could suggest that hematologic profile investigation, a non-destructive technique, may uphold the aquatic mercury exposure bioassessment.

Table 3. The summary of temporal and spatial analysis of Hg level in Tucunaré from the contaminated (Itatituba/Jacareacanga) and the reference area (Santarém) in the Tapajós River.

Date	Site	n	Hg (x±dp)	AD ($\mu g.kg^{-1}$)	TE50 (years)	C ($\mu g.kg^{-1}$.daily)
1992	Itaituba/Jacareac.	33	421.4±193.3	300	1.7	0.8
	Santarém	28	115.5±52.0	100	6.8	0.2
2001	Itaituba	55	714.6±218.6	700	1.0	1.4
	Santarém	53	237.5±109.8	230	3.6	0.38

The results of arithmetic mean and standard deviation (x±dp) of total mercury (wet weight) in muscle of Tucunaré, the number of specimens (n), the accumulation dose (AD), the time of exposure (TE50) and the estimated daily uptake rate (C) are shown.

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