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MESTRADO EM RECURSOS AQUÁTICOS E PESCA

DÉBORA BATISTA PINHEIRO SOUSA

UM MODELO PREDITIVO BASEADO EM BIOMARCADORES EM PEIXES APLICADO A UMA ÁREA PROTEGIDA DO MARANHÃO

São Luís – MA

2015

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Dissertação apresentada em cumprimento às exigências do Programa de Pós-Graduação em Recursos Aquáticos e Pesca da Universidade Estadual do Maranhão, para obtenção do grau de Mestre.

Orientadora: Profa Dra Raimunda Nonata Fortes Carvalho Neta

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There will come a time when you believe everything is finished. That will be the beginning. (Louis L`Amour)

Quem quiser ser o primeiro, esteja em último lugar.
(São Marcos)

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RESUMO

Neste estudo, objetivou-se obter um modelo preditivo de impactos baseado em biomarcadores em tambaqui (*Colossoma macropomum*) da Área de Proteção Ambiental do Maracanã, São Luís-MA. Os peixes foram amostrados em dois locais da Área de Proteção Ambiental do Maracanã: Lagoa Serena (A1) e Rio Ambude (A2), durante seis (6 coletas) no período chuvoso e de estiagem. Amostras de água foram coletadas para análise dos parâmetros abióticos. O sangue foi retirado de cada exemplar e, posteriormente lâminas foram confeccionadas para análise de micronúcleo e anormalidades eritrocíticas com giemsa e laranja de acridina (LA). As brânquias de cada exemplar foram desidratadas em uma série crescente de álcoois e incluídas em parafina. Cortes de 5 μm foram corados com hematoxilina e eosina. O grau de severidade das lesões histopatológicas foi baseado de acordo com Bernet et al., (1999). A construção do modelo preditivo para o efeito dos impactos ambientais foi estabelecido através de um ajuste de uma superficie aos dados mensurados que estão distribuídos no espaço em três dimensões (necrose, anormalidades e micronúcleo) para A2 no período seco e chuvoso. Os dados de comprimento total e furcal foram sempre maiores para os peixes de A1 quando comparados com A2. Foram identificadas anormalidades eritrocíticas apenas nos peixes coletados em A2. Os micronúcleos foram encontrados em A1 e A2. Nos eritrócitos corados com LA, foram encontradas anormalidades eritrocíticas do tipo: eritrócitos maduros (monocromáticos), micronúcleo, núcleo entalhado, núcleo vaculolizado, núcleo binucleado. Os dados com LA nos permitiram encontrar mais alterações celulares sendo considerada uma metodologia válida. Em relação aos parâmetros hematológicos, a média dos cálculos para o volume corpuscular médio, hemoglobina corpuscular média e concentração de hemoglobina corpuscular foram menores em A2 em relação aos peixes coletados em A1. Provavelmente, esses peixes podem estar sofrendo algum tipo de estresse, seja ele causado pelos metais encontrados nas análises químicas ou pela sazonalidade, especialmente em A2. A necrose é caracterizada como o mais alto grau de severidade histopatológica nos peixes e só é possivel ser constatada pelo exame histopatológico. Nossos resultados sugerem que o modelo preditivo baseado em parâmetros sanguíneos e morfológicos podem subsidiar programas de biomonitoramento em áreas protegidas e estabelecer valores de condição fisiológica e patológica para o tambaqui, apenas com a retirada de sangue, sem a necessidade de eutanásia do animal.

Palavras-chave: Área de Proteção Ambiental do Maracanã, modelo matemático, tambaqui.

ABSTRACT

This study aimed to obtain a predictive model of impacts based on biomarkers in tambaqui species in the Maracanã Protected Area, São Luís-MA. Fish were sampled from two locations within the protected area, Serena Lagoon (A1) and Ambude River (A2), on six occasions (dry and rainy season). Water samples were collected for physical-chemistry parameters.. Blood samples were examined for micronuclei changes stained with giemsa and Acridine Orange (AL). The gills of each fish were dehydrated in a progressive series of ethanol dilutions and embedded in paraffin. Sections were stained with hematoxylin and counterstained with alcoholic eosin. Histopathological lesions score were classified according Bernet et al., (1999). The construction of the predictive model for environmental impact was determined through an adjustment to a measured surface data that is distributed in space in three dimensions was accomplished (micronucleus, abnormalities and necrosis) for A2 in the dry and rainy season. The total length of data and furcal were always higher for fish in A1 compared with A2. Erythrocytic abnormalities were identified just only in the collected fish in A2. The micronucleus found in A1 and A2. In the erythrocytes analyzed with AL, erythrocytic abnormalities type were found: mature erythrocytes (monochrome), micronucleus, notched nucleus, vacuolated nucleos, binucleated núcleos. Data with AL have allowed us to find more cellular changes being considered a valid methodology. Regarding the hematological parameters, the average of the calculations for the mean corpuscular volume, mean corpuscular hemoglobin and corpuscular hemoglobin concentration were lower in A2 in relation to the breeding of fish in A1. Probably, these fish may be suffering some form of stress, whether caused by metals found in the chemical analysis or by seasonality, especially in A2. Necrosis is characterized as the highest degree of histopathologic severity in fish and it is only possible to be observed by histopathological examination. Our results suggest that the predictive model based on blood and morphological parameters can support biomonitoring programs in protected areas and establishing physiological and pathological condition values for tambaqui, with just only blood withdrawal, without the need of the animal euthanasia.

Keywords: Maracanã Protected Area, mathematical model, tambaqui.

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SUMÁRIO

1 INTRODUÇÃO

A Área de Proteção Ambiental do Maracanã (São Luís-MA) foi criada em 1991, sendo caracterizada por uma Unidade de Conservação de Uso Sustentável (MARANHÃO, 1991) e, após 23 anos do seu decreto de criação, foi empossado, em 2014 o primeiro Conselho Consultivo da APA da Região do Maracanã (MARANHÃO, 1991). Contudo, de acordo com Farias-Filho et al. (2015), o Conselho Consultivo da APA do Maracanã terá muitos desafios, tais como: a) estruturar um regimento interno para estabelecer as regras a serem seguidas pelos seus próprios integrantes e b) exercer o controle social na gestão do patrimônio natural e cultural da região, acompanhando de forma participativa a criação e a implementação do plano de manejo da APA.

A APA do Maracanã localiza-se estrategicamente próximo ao parque industrial de São Luís, onde estão instaladas as principais empresas de minério que movimentam a economia local (FARIAS-FILHO et al., 2015) e, além disso, há muitos registros de que essa região possuía várias empresas que trabalhavam com o curtume de couro de gado e combustão de materiais (CURVERO-MATOS, 2014). Atualmente, é crescente programas de construção civil do governo federal nessa Unidade de Conservação, que indiretamente prejudica a flora e a fauna local (FARIAS-FILHO et al., 2015). De acordo com Reis (2012), tais atividades podem acarretar a degradação do solo e da vegetação, prejudicando os recursos hídricos da região, já que nessa região encontram-se várias nascentes de rios, tais como Ambude, e Maracanã, que abastecem a represa do Batatã.

Nesse contexto, fazem-se necessário o desenvolvimento e a padronização de metodologias capazes de predizer os efeitos da contaminação dos organismos aquáticos da região. Entre essas metodologias, o uso de biomarcadores de contaminação aquática em peixes é particularmente importante porque mostra respostas biológicas iniciais, podendo ser útil para subsidiar ações de monitoramento e de gestão ambiental (PINHEIRO-SOUSA et al., 2015). Os biomarcadores são respostas biológicas ao estresse provocado pelos poluentes e/ou estressores físicos e, podem ser utilizados para identificar sinais iniciais de danos nos organismos aquáticos (HINTON et al. 1992).

A continuidade dos estudos baseados em biomarcadores e a geração dos dados sobre genotoxicidade é de grande importância para a atuação dos órgãos ambientais

estaduais e federais no que diz respeito a políticas de manejo e monitoramento em áreas distintas da APA do Maracanã. Assim, no presente trabalho, será avaliado parâmetros hematológicos, genotóxicos e histológicos como biomarcadores, em uma espécie de peixe dulcícola (*Colossoma macropomum*) de rios e de tanques de cultivo, a fim de subsidiar programas de biomonitoramento e de gestão para a APA do Maracanã.

O trabalho desenvolvido será aqui apresentado da seguinte forma: objetivos, fundamentação teórica sobre Unidades de Conservação, biomarcadores em peixes e a utilização de modelos matemáticos para o monitoramento de ambientes aquáticos e uma metodologia geral do trabalho desenvolvido. Os resultados serão discutidos na forma de dois artigos, sendo: a) o capítulo I, que abordará uma discussão sobre os parâmetros hematológicos e genotóxicos em *Colossoma macropomum*, que podem ser utilizados como indicadores de saúde ambiental e subsidiar programas de monitoramento de baixo custo em áreas protegidas e, b) o capítulo II, que discutirá a construção de um modelo preditivo baseado em biomarcadores de exposição (micronúcleo, anormalidades eritrocitárias e necrose).

2 OBJETIVOS

2.1 Objetivo Geral

Obter um modelo preditivo de impactos ambientais, baseado em biomarcadores, em tambaqui (*Colossoma macropomum*), na Área de Proteção Ambiental do Maracanã, São Luís-MA.

2.2 Objetivos Específicos

- Quantificar micronúcleo písceo, anormalidades eritrocíticas e lesões branquiais como biomarcadores em *Colossoma macropomum*;
- Comparar metodologias de identificação de micronúcleo e anormalidades eritrocíticas em tambaqui;

- Indicar qual variável morfológica (lesão branquial) pode ser predita a partir dos parâmetros sanguíneos (micronúcleos e anormalidades eritrocíticas);
- Obter um modelo (matemático) preditivo dos efeitos dos impactos ambientais a partir dos biomarcadores avaliados.

3 FUNDAMENTAÇÃO TEÓRICA

3.1 Monitoramento ambiental em Unidades de Conservação

As áreas legalmente protegidas foram criadas com o propósito de equilibrar a conservação da diversidade biológica com o desenvolvimento sustentável (BRASIL, 2000). O Sistema Nacional de Unidades de Conservação (SNUC) define Área de Proteção Ambiental (APA) como sendo "uma área em geral extensa, com um certo grau de ocupação humana, dotada de atributos abióticos, bióticos, estéticos ou culturais especialmente importantes para a qualidade de vida e o bem-estar das populações humanas, e tem como objetivos básicos proteger a diversidade biológica, disciplinar o processo de ocupação e assegurar a sustentabilidade dos recursos naturais" (BRASIL, 2000).

Segundo o SNUC, as Unidades de Conservação podem ser federais, estaduais e municipais, podendo ser divididas em dois grupos: Unidade de proteção integral e Unidades de uso sustentável. As APAs são enquadradas nesta última divisão e seguem as normas de órgãos ambientais no âmbito de sua criação (federal, estadual ou municipal) (BRASIL, 2000). No caso da APA do Maracanã (que é uma Unidade de Conservação Estadual), o órgão responsável pela sua gestão é a SEMA (Secretaria de Estado de Meio Ambiente e Recursos Naturais do Maranhão) (MARANHÃO, 1991).

A APA da região do Maracanã foi criada pelo Decreto Estadual n. 12.103 de 1991 e ocupa uma área de 1.831 hectares (MARANHÃO, 1991). Essa Unidade de Conservação estadual, localizada na capital maranhense, limita-se ao Norte com o Parque Estadual do Bacanga e ao sul com o rio Grande, englobando as localidades do Maracanã, Alegria, Bacanguinha, Ferventa, Alto Alegre, parte da Vila Maranhão, Vila Sarney, Vila Esperança e Rio Grande (ESPÍRITO-SANTO, 2006).

A APA do Maracanã foi criada considerando a necessidade de preservação da área não só pelo seu aspecto paisagístico mas, principalmente, pela necessidade de proteção dos Recursos Hídricos que ali afloram (MARANHÃO, 1991). Atualmente, essa Unidade de Conservação têm funcionado como zona de amortecimento de impactos do Parque Estadual do Bacanga (CARVALHO-NETA, 2010), possuindo, ainda, uma extensa área de juçarais, que protegem naturalmente os cursos d'água associados a essas microrregiões. Dentre as principais atividades da região, destacam-se o extrativismo e a piscicultura (CARVALHO-NETA, 2010; SILVA et al., 2011).

A cobertura vegetal das margens dos rios citados é, em geral, caracterizada pela presença de juçarais/buritizais (vegetação típica de várzea), ou, no caso de sua substituição quando da construção de residências, matas de sítios (CARVALHO-NETA, 2010). Nesse contexto, o monitoramento ambiental da APA do Maracanã é de extrema importância para a conservação dos principais corpos hídricos da região, visto que a região encontra-se inserida na microbacia do rio Maracanã que abriga as principais nascentes do Rio Bacanga (ABRANTES, 2013).

Todavia, os tanques de piscicultura existentes na APA do Maracanã que cultivam espécies exóticas (tilápia e carpa) e o tambaqui, são localizados, normalmente, nas regiões próximas às margens ou nascentes desses rios, especialmente, dos rios Maracanã e Ambude, onde a espécie íctica mais frequente é o tambaqui (*C. macropomum).* Por se tratar de uma espécie atualmente muito cultivada na região, esse táxon foi introduzido nos rios e torna-se frequente nas pescarias do Rio Ambude. Assim, a utilização do tambaqui em estudos de biomonitoramento, pode torná-lo um potencial modelo biológico para indicar contaminação ambiental nos ecossistemas aquáticos dessa Unidade de Conservação.

3.1.1 Peixes como bioindicadores de contaminação ambiental

Os corpos d'água doce da APA do Maracanã estão entre os ecossistemas que mais têm sofrido agressões no processo de expansão urbana da cidade de São Luís. Apesar desses recursos hídricos serem importantes para a qualidade ambiental e a manutenção da própria Unidade de Conservação e estarem "protegidos" legalmente, diversas têm sido as intervenções humanas que têm causado modificações profundas na

sua dinâmica e nas espécies de peixes de importância econômica da região (CARVALHO-NETA, 2010).

Nesse contexto, a seleção de espécies que possam refletir a situação ambiental da APA do Maracanã, torna-se de grande relevância para monitorar as interferências que essa Unidade de Conservação vem sofrendo ao longo dos anos. Os peixes são excelentes bioindicadores por estarem no topo da cadeia trófica e refletem a médio ou a longo prazo os impactos em um determinado ecossistema através de suas funções vitais normais e/ou da sua composição orgânica (ARIAS et al., 2007).

Um bioindicador adequado, deve possuir características específicas que revele a sua ausência e/ou presença em um ambiente aquático, sendo que a espécie e/ou grupo deve refletir o mais prontamente possível o impacto das mudanças ambientais sobre um habitat, comunidade ou ecossistema, sendo indicativo da perda e/ou aumento da diversidade dentro de uma área (GERHARDT, 2009).

De acordo com Gerhardt (2009) existem três categorias aplicáveis de bioindicadores para estudos de biomonitormanto de ambientes aquáticos e terrestres: 1) bioindicador a nível ambiental é aquele em que a espécie e/ou grupo de espécies respondem previsivelmente à perturbação ambiental e/ou alteração ambiental, tais como organismos sentinelas, acumuladores, e aqueles que são utilizados em bioensaios; 2) bioindicador a nível ecológico é aquele em que os indivíduos são conhecidos pela sensibilidade a locais poluídos e/ou contaminados, o que pode ocasionar a fragmentação do habitat; 3) bioindicador a nível de biodiversidade é um tipo que leva em consideração a riqueza de um táxon que é utilizado como indicador da riqueza de espécies de uma comunidade.

No presente trabalho iremos enfocar os peixes como bioindicadores a nível ambiental, por se tratar de um estudo que utiliza biomarcadores de contaminação aquática e outros parâmetros ambientais para compreender a saúde dos indivíduos provenientes de ecossistemas naturais da APA do Maracanã (São Luís, Maranhão).

3.2 Biomarcadores em organismos aquáticos

A metodologia baseada em biomarcadores de contaminação aquática em peixes tem sido considerada de grande importância porque apresenta muitas vantagens, tais

como alta sensibilidade, rapidez e baixo custo (AELION, 2009; CARVALHO-NETA, 2014a; PINHEIRO-SOUSA et al., 2013; CASTRO et al., 2014). Essa metodologia têm sido bastante eficaz, pois com o aumento de pesquisas no Brasil que comprovam a presença de agentes contaminantes na biota dos diferentes ecossistemas de Unidades de Conservação, várias são as demandas no sentido de se padronizar metodologias capazes de diagnosticar sinais iniciais de danos aos organismos aquáticos.

O maior desafio do pesquisador na área da ecotoxicologia aquática é identificar qual o biomarcador apropriado para o melhor diagnóstico do ecossistema em que se pretende investigar, devendo-se levar em consideração o contaminante a ser estudado e o melhor modelo biológico para a avaliação de impacto ambiental (JESUS; CARVALHO, 2008). Para tanto, existem várias classificações acerca dos biomarcadores e sua aplicabilidade (AELION, 2009).

A divisão clássica da ecotoxicologia aquática, e a mais utilizada pelos pesquisadores, subdivide os biomarcadores em nível de exposição, efeito e de suscetibilidade (NRC, 1987; WORLD HEALTH ORGANIZATION, 2001; AMORIM, 2003; JESUS; CARVALHO, 2008). Por outro lado, os biomarcadores são categorizados de acordo com indicadores e/ou marcadores específicos presentes nos indivíduos, tais como: enzimas de biotransformação e stresse oxidativo, proteínas reguladoras, parâmetros hematológicos, imunológicos, reprodutivos, genotóxicos, neuromusculares, fisiológicos e a nível morfológico ou histológico (VAN DER OOST et al., 2003).

Neste trabalho, iremos utilizar a classificação de Van der Oost et al. (2003) que leva em consideração a utilização de marcadores biológicos histológicos (epitélio branquial) e genético (micronúcleo e alterações nucleares sanguíneas).

Os biomarcadores histológicos (celulares e teciduais) mais utilizados em estudos de contaminação aquática são as alterações nas brânquias, fígados e rins de peixes, as quais são consideradas biomarcadores de exposição a estressores ambientais, sinalizando os efeitos e/ou respostas resultantes da exposição a um ou mais agentes tóxicos (HINTON et al., 1992; CARVALHO-NETA et al., 2014a). Essas categorias de biomarcadores têm a vantagem de permitir o exame de órgãos-alvo e células específicas em animais expostos a poluentes tanto em condições de laboratório como no campo, sendo de relativo baixo custo, visto que não exigem reagentes caros nem equipamentos sofisticados para a sua realização (HINTON et al., 1992).

O material genético de células eucarióticas das espécies ícticas também pode ser alterado mediante exposição a substâncias químicas dissolvidas na água, resultando na formação de micronúcleos, os quais podem ser utilizados como biomarcadores para avaliar o grau de contaminação no meio ambiente (SILVA et al., 2008; MAZZEO; MARIN-MORALES, 2015). Os micronúcleos são provenientes de fragmentos cromossômicos resultantes de quebras que não são incorporados no núcleo principal das células filhas após a mitose em decorrência de danos introduzidos nas células parentais (AL-SABTI; METCALFE, 1995) Testes de micronúcleos já foram realizados nos peixes da APA do Maracanã (PINHEIRO-SOUSA et al., 2015) e poderão servir para obter indícios mais completos de contaminantes que podem estar induzindo a formação dessas estruturas irregulares nos eritrócitos dos organismos. Todavia, estudos sobre a biologia e a ecologia de espécies nativas nos rios da APA do Maracanã (associados a análises físico-químicas da água e do sedimento dos ambientes) ainda são escassos, e não permitem uma avaliação mais detalhada sobre os efeitos dos poluentes nos processos fisiológicos desses táxons. Em associação, também não se conhece todas as espécies, os métodos de manejo e os problemas da piscicultura local. Desse modo, a investigação acerca das lesões branquiais e micronúcleo em *C. macropomum* (tambaqui) da APA do Maracanã será de grande importância para a seleção de metodologias inovadoras, a exemplo da construção de modelos matemáticos baseados em biomarcadores, para melhor subsidiar programas de biomonitoramento na região, já que esses parâmetros podem oferecer um diagnóstico seguro sobre a saúde dos peixes da região.

3.3 Modelos matemáticos para biomonitoramento de ambientes aquáticos

A modelagem matemática tem sido amplamente empregada para avaliação quantitativa e qualitativa de questões ambientais e agrícolas (ALLMAN et al., 2003). Contudo, a modelagem aplicada a sistemas biológicos resume-se consideravelmente a estudos de fisiologia dos sistemas (ALLMAN et al., 2003), e, essa metodologia pode ganhar um novo enfoque com a utilização de dados sobre biomarcadores em peixes da Área de Proteção Ambiental do Maracanã para subsidiar o Plano de Manejo dos recursos naturais.

Na literatura tem sido relatado o desenvolvimento de vários modelos matemáticos para severidade de lesões histopatológicas em brânquias, fígado e rins de peixes (SCHWAIGER et al., 1997; BERNET et al., 1999), mas nenhum desses índices leva em consideração a severidade individual de cada lesão e outros tipos de parâmetros biológicos, tais como os hematológicos e genotóxicos de peixes. Além disso, não existem modelos matemáticos para *C. macropomum*, especialmente em unidades de conservação maranhenses, o que aumenta a possibilidade para novos estudos a partir dos dados biológicos da espécie e a criação de novas linhas de pesquisa dentro da ecotoxicologia aquática e da matemática aplicada.

Alguns modelos matemáticos de dados biológicos estão sendo amplamente utilizados para a correlação de biomarcadores bioquímicos e histopatológicos e fornecem uma análise mais realística sobre o estresse ocasionado pelos contaminantes da região portuária de São Luís (CARVALHO-NETA et al., 2014b), podendo ser utilizados como um instrumento promissor para a interpretação da situação ambiental em unidades de conservação do Estado do Maranhão. Esses modelos poderão fornecer subsídios para a criação de novos bancos de dados de outras espécies da APA do Maracanã e a construção de modelos matemáticos usando o tambaqui como referência para futuros estudos de manejo dos recursos ícticos nativos da região.

3.4 *Colossoma macropomum* como um modelo biológico para avaliação de impactos na Área de Proteção Ambiental do Maracanã

A espécie *Colossoma macropomum* (Curvier, 1818) (Fig. 2), popularmente conhecido como tambaqui, é um peixe nativo da bacia amazônica explorado comercialmente pelas pescarias locais da APA do Maracanã (GOULDING; CARVALHO, 1982; GROFF, 2010; CARVALHO-NETA, 2010).

Taxonomicamente o tambaqui pertence a família Serrasalmidae, apresentando corpo romboidal, opérculos alongados, nadadeira dorsal curta com raios na extremidade, ausência de dentes na maxila e rastros branquiais numerosos (ALBUQUERQUE et al., 2007). Indivíduos dessa espécie são de fácil manejo e possuem hábito alimentar onívoro, na fase jovem e frúgivoro, na fase adulta (SAINT-PAUL, 1984), podendo atingir até um metro de comprimento e 30 kg em peso (VALENTIN et al., 2000)

Figura 2 – Exemplar de *Colossoma macropomum* (Cuvier, 1818) coletado na APA do Maracanã, São Luís-MA.

Estudos sobre a biologia da espécie têm sido realizados por vários autores, tais como: Golding; Carvalho (1982); Saint-Paul (1984); Sagri (1990). Esses estudos mostram dados pretéritos sobre a reprodução e acondicionamento dessa espécie em taques de cultivo. No entanto, dados sobre a ecologia e comportamento do tambaqui ainda são pouco conhecidas para a APA do Maracanã, sendo a espécie frequentemente registrada nos rios e tanques de cultivo dessa região (CARVALHO-NETA; FARIAS-FILHO, 2010).

Dados sobre a genotoxicidade realizados por Groff (2010) colocam o tambaqui (*C. macropomum)* como potencial modelo experimental para o monitoramento dos corpos aquáticos na região amazônica. No entanto, no Estado do Maranhão, essa é uma espécie alóctone e introduzida nos rios da APA do Maracanã e, ainda não existe estudos mais aprofundados sobre o grau de sanidade dessa espécie.

Assim, a criação de um modelo preditivo baseado em biomarcadores subsidiará resultados capazes de assessorar politicas públicas em Unidades de Conservação do Estado do Maranhão.

4 MATERIAL E MÉTODOS

4.1 Área de estudo

De acordo com o decreto de criação da APA da região do Maracanã (MARANHÃO, 1991), esta Unidade de Conservação (Fig. 1) está delimitada pelos seguintes pontos: Ponto 1 – Cruzamento da Rua da Vitória com a REFSA – São Luís – Teresina, daí segue em linha reta (perpendicular) para a BR-135 até o início de Rio Grande; Ponto 2 – Lat. 2°09’39”S e Long. 44°17’11”W, segue pela estrada até o Ponto 3; Ponto 3 – Lat. 2°09’42”S e Long. 44°17’45”W – limite do Distrito Industrial cruzando com a REFSA São Luís – Teresina; Ponto 4 – Lat. 2°08’40”S e Long. 44°18’14”W; Ponto 5 – Lat. 2°07’00”S e Long. 44°18’32”W; Ponto 6 – Lat. 2°06’35,6”S e Long. 44°18’19”W; Ponto 7 – Lat. 2°06’24”S e Long. 44°18’04”W, deste Ponto segue pelo limite Sul do Parque Estadual do Bacanga, ou seja, Rio Bacanga até a sua confluência com o Rio Maracanã; o Rio Maracanã da foz às cabeceiras. A Leste, uma linha partindo da nascente do Rio Maracanã às cabeceiras do Rio Batata e, daí, até a Ferrovia São Luís – Teresina, ponto do qual seguirá a mesma Ferrovia até o ponto 1 desta APA

POINTS	COORDINATES	
	S	W
AMBUDE RIVER BORN	2° 38.225'	44° 17.887'
MIDLE OF RIVER	2° 37.553'	44° 17.620
DOWNSTREAM OF AMBUDE RIVER	2° 37.750'	44° 17.748′
SERENE LAGON	2° 37.955'	44° 17.874'

Figura 1 – Localização da APA do Maracanã, Sao Luís-MA. Pontos de coleta dos peixes: Rio Ambude e Lagoa Serena.

4.2 Determinação das áreas amostrais na APA do Maracanã e coleta dos peixes

Os peixes foram coletados na APA do Maracanã no Rio Ambude (montante - 2^{o}38.225’S, 44^{o} 17.887W; meio -2^{o}37.553’S, 44^{o}17.620W; jusante 2^{o}37.750’S, 44^{o}17.748W) e no criatório de peixes Lagoa Serena (2^{o}37.955’S, 44^{o}17.874W),

trimestralmente, no período de estiagem e no período chuvoso em 2012, 2013 e 2014. As estações foram georreferenciadas por GPS (Global Position System).

Os peixes foram capturados com tarrafas ou redes de emalhar e, imediatamente identificados com chaves de identificação. Posteriormente, os pexies foram colocados em uma recipiente plástico (10L) com água do próprio local de coleta, e, anestesiados com solução hidroalcólica de bezocaína á 5%. Os peixes de cultivo foram comprados dos piscicultores locais nas mesmas épocas de coleta dos peixes dos rios.

4.3 Análise dos parâmetros abióticos

Durante a captura dos peixes, foram obtidos *in situ* dados das seguintes variáveis abióticas: temperatura, pH e oxigênio dissolvido foram analisados no multiparâmetro (HI 9829 – Hanna). Também foram coletadas amostras de água em frascos esterilizados, colocados em isopor com gelo, e transportados para o laboratório de Química dos Solos, da Universidade Estadual do Maranhão para realização da análise química. Em laboratório, todas as amostras foram filtradas e acidificadas até a mensuração dos parâmetros inorgânicos (Cobre, Zinco, Chumbo, Ferro, Cromo, Níquel, Manganês, Cadmio, Mercúrio, Manganês, Molibdênio, Cobalto, Cálcio, Potássio e Fósforo). Todas as análises foram feitas em tréplicas, e, a concentração de metais foi analisada através da espectofotometria de emissão (óptica) de plasma acoplado indutivo (ICP- Varian 720-ES) (TYLER, 1991).

4.4 Micronúcleo písceo, anormalidades eritrocitárias e registro de dados biométricos dos peixes

Após a captura e anestesia dos peixes, o sangue foi retirado via punção caudal, com auxílio de seringas descartáveis e heparinizadas. Uma gota de sangue de cada espécime foi inserida em uma lâmina microscópica para a realização do esfregaço. Após a secagem as lâminas foram fixadas em etanol absoluto durante 30 minutos. Posteiromente, as lâminas foram coradas com giemsa e laranja de acridina e analisadas em microscopia óptica (ZEISS) e microscópio de fluorescência (Leica DMLP). Em cada lâmina, foram analisados mínimo de 2000 células. A análise dos

eritrócitos dos peixes foi realizado de acordo Ditmar et al. (2010) para a determinação da frequência de micronúcleos e de alterações morfológicas nucleares (AMN).

Em laboratório, para cada exemplar de peixe do qual foram retirados o sangue e as brânquias foram registrados os dados de comprimento total (Lt), comprimento padrão (LP) e em cm, peso total (Wt) e o peso das gônadas (Wg) em g. Depois de pesados e medidos, procedeu-se à classificação macroscópica das gônadas, considerando-se a seguinte escala de estágios de desenvolvimento gonadal dada por Vazoller (1996) e modificada por Carvalho-Neta; Castro (2008): EG1 (imaturo), EG2 (em maturação ou repouso), EG3 (maduro) e EG4 (esgotado).

4.5 Análises histopatológicas

As brânquias foram fixadas em formol a 10%. Em seguida, foram lavadas e mantidas em álcool 70% até o processamento histológico. O segundo arco branquial direito de cada espécime foi desidratado em série crescente de álcoois, diafanizado em xilol, impregnado e incluído em parafina. Cortes transversais, de aproximadamente 5 μm de espessura, foram corados com Hematoxilina e Eosina (HE). Foram analisados quatro cortes para cada órgão de cada animal. A leitura da lâminas foi realizada em microscópio óptico (ZEISS) e as lesões encontradas foram fotomicrogafadas em fotomicroscopio AXIOSKOP – ZEISS.

Foram considerados 11 tipos de lesões histopatológicas e o score para cada lesão encontrada com base na abordagem utilizada por Bernet et al (1999). De acordo com esse autor, cada tipo de alreração histopatológica apresenta um fator de importância (w) que é mensurado de 1 a 3, sendo: fator de importância 1 = importância patológica mínima (a lesão é facilmente reversível); 2 = importância patológica moderada (lesão é reversível na maioria dos casos); 3 = importância patológica grave (a lesão é irreversível geralmente) (Bernet et al., 1999).

4.6 Tratamento estatístico dos dados

Os dados biométricos (Lt, Lf, Wt e Wg), teste do micronúcleo, anormalidades eritrocitárias e lesões branquiais dos peixes do Rio Ambude e lagoa Serena foram submetidos ao teste de normalidade e as médias para machos e fêmeas nas diferentes épocas do ano foram comparadas entre si pelo teste-t de Student.

4.7 Modelo matemático preditivo

O modelo preditivo relativo aos efeitos dos impactos ambientais para a APA do maracanã foi estruturado através de ajustes de dados mensurados que estão distribuídos em duas dimensões (necrose e micronúcleo) e em três dimensões (micronúcleo, anormalidades eritrocíticas e necrose), representados pelas eaquações da reta 1.1 e do plano 1.2:

$$\mathbf{z = \beta + \alpha x}$$ (Equação 1.1)

$$\mathbf{z = \beta + \alpha_1 x + \alpha_2 y + \alpha_3 xy}$$ (Equação 1.2)

Onde:

z é a variável dependente (necrose);

xy são variáveis independentes (micronúcleo e anormalidades eritrocíticas);

β o coeficiente linear e;

α o coeficiente angular.

Todas as curvas e ajustes da equação da reta e do plano foram realizadas no software MATLAB para a Lagoa Serena e o Rio Ambude, durante o período de estiagem e chuvoso.

5 RESULTADOS

Os resultados desta dissertação serão apresentados em dois artigos. No primeiro artigo, pretendemos mostrar como os parâmetros hematológicos e genotóxicos em *Colossoma macropomum* podem ser utilizados como indicadores de diagnóstico ambiental e subsidiar programas de monitoramento de baixo custo em Áreas Protegidas Maranhenses. No segundo artigo, iremos mostrar o modelo preditivo baseado em biomarcadores de exposição e compreender como uma variável morfológica (necrose) pode ser predita através de parâmetros sanguíneos (micronúcleo e anormalidades eritrocitárias).

5.1 Genotoxic and hematological parameters as biomarkers in *Colossoma macropomum* (Pisces, Serrasalmidae) for environmental impact assessment in a Protected Area in Northeastern Brazil[1]*

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Genotoxic and hematological parameters as biomarkers in *Colossoma macropomum* (Pisces, Serrasalmidae) for Environmental Impact Assessment in a Protected Area in Northeastern Brazil

[1] Parte desse capítulo foi utilizado no resumo:

Micronucleus frequency and hematologic index in *Colossoma macropomum* (Pisces, Ariidae) for environmental impact assessment at a protected area in Brazil. Apresentado (comunicação oral) na 10TH International Conference of Computational Methods in Sciences and Engineering (ICCMSE2014), 2014, Grécia - Athens.

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Abstract

This study used genotoxic and hematological parameters as biomarkers in the freshwater fish *Colossoma macropomum* to assess environmental impacts in Maracanã Protected Area, Maranhão State, Brazil. Fish were sampled from two locations within the protected area, Serena Lagoon (reference site) and Ambude River, on four occasions (dry and rainy season). Biometric data (length and weight) and an aliquot of blood were collected from each fish for analysis. Erythrocyte indices, including mean corpuscular volume, average corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were calculated. Blood samples were examined for micronuclei and nuclear morphological changes stained with Giemsa and Acridine Orange. Micronuclei were found in fish from both locations, although the frequency was significantly higher in fish from Ambude River and nuclear morphological changes were identified only in fish collected from Ambude River. Several different nuclear morphological changes and a large number of micronuclei were found in erythrocytes stained with Acridine Orange compared to those stained with Giemsa. On average, erythrocyte indices were lower in fish collected from Ambude River than in those from Serena Lagoon. Our results confirm that genotoxic and hematological parameters can be used in *C. macropomum* as indicators of environmental health, and can be important tools for monitoring environmental conditions in protected areas.

Keywords: Freshwater fish; Micronucleus; Nuclear abnormalities; Giemsa; Acridine Orange; Tambaqui; Erythrocyte.

Introduction

When aquatic ecosystems are polluted with organic and inorganic contaminants, the fish will inevitably be contaminated (Streit 1998; Amado et al. 2006; Sousa et al. 2013). The effects of contaminants in fish can be assessed using different biomarkers. These markers are defined as biological responses to the effects of pollutants that can be used to identify early signs of damage to organisms (Martinez and Collús 2002). Biomarkers can be measured at the molecular, cellular or even organism level, and may be specific to certain pollutants (Livingstone 1993; Carvalho-Neta et al. 2012; Carvalho-Neta and Abreu-Silva 2013).

The use of hematological and genotoxic parameters as biomarkers in fish allows us to evaluate the health of organisms and the quality of aquatic ecosystems. Furthermore, these tools show the effects of pollutants and identify biological changes caused by toxic xenobiotics (Jesus and Carvalho 2008). Some biomarkers of exposure, such as histopathological changes and markers of genotoxicity, were among the most relevant for identifying the impacts of xenobiotics on aquatic organisms (Sousa et al. 2013; Gagnon and Bakhtyar 2013).

The term genotoxicity refers to changes in the overall structure or arrangement of chromosomes (clastogenicity) and the sequences of base pairs of DNA (mutagenicity) by exposure to toxic agents (Al-Sabti and Metcalfe 1995). The interaction of genotoxic compounds (such as polycyclic aromatic hydrocarbons and heavy metals) with DNA may cause structural changes in the DNA molecule. Among these changes, the most cited in the literature are those of a covalent binding of the compound to DNA bases, as well as DNA strand breaks (Flammarion et al. 2002).

As the main city of Maranhão (Brazil) expands, Protected Areas and its rivers are under threat (Silva et al. 2013; Carvalho-Neta and Farias-Filho 2010). Despite water resources being lawfully protected, many human interventions have negatively altered the ecosystem dynamics of conservation areas, e.g. Maracanã Protected Area (São Luís, Maranhão). Riverbank construction, overfishing, (e. g. tambaqui), the introduction of exotic species, riparian vegetation reduction, and pollution by the most important harbor in Brazil have major impacts on the area (Carvalho-Neta et al. 2014). Fish are considered to be the most feasible biological models for pollution biomonitoring in protected areas because of their function as a carrier of energy from lower to higher trophic levels (Van der Oost et al. 2003). The freshwater fish tambaqui used as bioindicator in this study, *Colossoma macropomum* (native species)

was particularly suitable for this research because of its commercial importance in São Luís. In this study the hematological parameters, frequencies of micronuclei and morphological nuclear abnormalities in erythrocytes in the peripheral blood of tambaqui (Colossoma macropomum), in two different sites in a protected area (São Luís, Maranhão, Brazil), were analyzed.

Methods and Materials

Sampling Sites

Maracanã Protected Area is an important natural reserve on the north of Brazil (São Luís-MA). Two sampling sites were chosen (Fig. 1), a potential contaminated site (Ambude River) located near the port facilities (2°37′ 57″ S, 44° 17′ 43″W) and a reference site (Serena Lagoon) located at an upper part of the reserve (2° 36′ 57″ S, 44° 17′ 58″W).

Fig. 1 Map showing Maracanã Protected Area, São Luís, Maranhão, Brazil

License and Ethics Declaration

The capture of fish was authorized by a permit issued by the State Department of Natural Resources and Environment - SEMA (001/2012). The protocol was approved by the Ethics Committee of Maranhão State University (04/2012 CRMV-MA) and met the guidelines of the Brazilian College for Animal Experimentation (COBEA, 2015).

Sampling of *Colossoma macropomum*

Tambaqui (*Colossoma macropomum*) specimens were collected from two locations within Maracanã Protected Area: Serena Lagoon (40 specimens) and Ambude River (40 specimens) (fig. 1). Sampling was conducted on two occasions in 2012 (September and December) during the dry season and twice in 2013 (February and April) during the rainy season. Fish were collected with a gill net and transferred to a vat (1 m × 1 m). The tambaquis were anesthetized with alcoholic solution of benzocaine 5%.

Biometric data

Biometric data including the total length (TL), fork length (FL), standard length (SL) and total weight (TW) of each specimen of *C. macropomum* were recorded. All fish were juveniles and gonads were

staged macroscopically into only two classes in accordance with Vazoller (1996): immature (GS1) or maturing (GS2).

Microhematocrit determination

Blood was collected from fish, anticoagulated with ethylenediaminetetraacetic acid (EDTA) and homogenized. Capillary tubes (75 mm × 1 mm) were 2/3 filled with blood and the ends sealed using a Bunsen burner flame. The tubes were centrifuged at 13460 for 3 minutes. A microhematocrit reading device was used to determine the volume occupied by erythrocytes as a percentage of the total volume. The following erythrocyte indices were calculated in accordance with Tavares-Dias et al. (2000):

Mean corpuscular volume (MCV) = (Hematocrit X 100)/(Number of erythrocytes),

Mean corpuscular hemoglobin (MCH) = (Hemoglobin X 10)/(Number of erythrocytes),

MCH concentration (MCHC) = (Mean corpuscular hemoglobin)/(Mean corpuscular volume).

Micronucleus and nuclear abnormalities

Blood of *C. macropomum* was collected from blood vessels of the gills using heparinized syringes. A drop of blood from each sample was placed on a microscope slide and smeared. The slides were left to dry at room temperature for 24 hours, and then fixed in absolute ethanol for 30 minutes. For each specimen were stained two slides, one for Giemsa and another for Acridine Orange. Microscope slides (n = 80) were stained with 10% Giemsa diluted in phosphate buffer (pH 6.8), and analyzed using a light microscope. The remaining slides (n = 80) were stained with Acridine Orange (10 μm mL^{-1} distilled water) and analyzed using a fluorescence microscope. On each slide, 2000 cells were analyzed. Micronuclei and nuclear morphological changes in erythrocytes were classified as indicative of genotoxicity (Ayllón and Garcia-Vazquez 2001).

Results

Biometric data

The results of biometric data for males and females of *C. macropomum* during the dry and rainy seasons in the two sites (Serena Lagoon and Ambude River) in Maracanã Protected Area can be seen in table 2 and 3 respectively. The average and standard deviation F\fish caught at Ambude River were

smaller (total length and fork length, $P < 0.05$) than fish from Serena Lagoon. All fish were immature (GS1) or were in early-maturing (GS2).

Table 1 Biometric data for fish collected from Serena Lagoon and Ambude River, from Maracanã Protected Area, São Luis, Brazil, during dry season

Parameters	Average ± Standard deviation			
	Serena Lagoon (Dry Season)		Ambude River (Dry Season)	
	Females	Males	Females	Males
TL (cm)	26.49±6.10	21.48±4.2	22.27±2.86	20.45±3.56
FL (cm)	23.5±5.70	18.87±2.62	19.06±1.46	16.06±1.46
TW (g)	150.20±43.10	251.7±60.19	90.49±41.59	85.49±39.59

Number of females: Serena Lagoon = 10, Ambude River = 7. Number of males: Serena Lagoon = 12, Ambude River = 11. Biometrics: total length (TL), fork length (FL), and total weight (TW).

Table 2 Biometric data for fish collected from Serena Lagoon and Ambude River, from Maracanã Protected Area, São Luis, Brazil, rainy season

Parameters	Average ± Standard deviation			
	Serena Lagoon (Rainy Season)		Ambude River (Rainy Season)	
	Females	Males	Females	Males
TL (cm)	24.43±2.4	20.46±6.87	20.43±4.41	20.43±4.41
FL (cm)	29.86±2.04	20.28±3.99	16.43±4.40	16.43±4.40
TW (g)	113.64±36.10	122.35±14.17	27.67±2.17	27.67±2.17

Number of females: Serena Lagoon = 10, Ambude River = 15. Number of males: Serena Lagoon = 8, Ambude River = 7. Biometrics: total length (TL), fork length (FL) and total weight (TW).

Microhematocrit determination

Throughout the frequency of *C. macropomum* erythrocytes, a different hematological profile was observed among fish from the two locations (tables 4 and 5).

Table 3 Hematological parameters in *C. macropomum* collected during dry season at Serena Lagoon and Ambude River locations in Maracanã Protected Area, Maranhão-Brazil

Parameters	Average ± Standard deviation			
	Serena Lagoon		Ambude River	
	Females	Males	Females	Males
Hematocrit (%)	21.2 ± 4.9	20.2 ± 2.3	17.1 ± 4.2	15.0 ± 2.1
MCV[a] (fL)	15.6 ± 2.8	12.3 ± 3.1	20.1 ± 1.2	17.7 ± 2.7
MCH[b] (pg)	14.1 ± 2.9	17.5 ± 2.2	21.8 ± 2.8	14.5 ± 2.3
MCHC[c] (g/dL)	16.5 ± 3.2	14.3 ± 4.7	19.3 ± 1.1	11.0 ± 1.8

Number of females: Serena Lagoon = 10, Ambude River = 7. Number of males: Serena Lagoon = 12, Ambude River = 11. [a] Mean corpuscular volume, [b] Mean corpuscular hemoglobin, [c] Mean corpuscular hemoglobin concentration.

Table 4 Hematological parameters in *C. macropomum* collected during rainy season at Serena Lagoon and Ambude River locations in Maracanã Protected Area, Maranhão-Brazil

Parameters	Average± Standard deviation			
	Serena Lagoon		Ambude River	
	Females	Males	Females	Males
Hematocrit (%)	19.7 ± 3.7	19.2 ± 2.9	20.2 ± 3.3	17.1 ± 2.6
MCV[a] (fL)	13.5 ± 2.4	10.4 ± 2.6	19.4 ± 2.9	18.2 ± 2.4
MCH[b] (pg)	11.6 ± 1.8	16.8 ± 2.9	20.9 ± 3.8	13.6 ± 3.3
MCHC[c] (g/dL)	15.2 ± 2.6	12.9 ± 3.2	20.7 ± 3.6	12.3 ± 2.9

Number of females: Serena Lagoon = 10, Ambude River = 15. Number of males: Serena Lagoon = 8, Ambude River = 7. [a] Mean corpuscular volume, [b] Mean corpuscular hemoglobin, [c] Mean corpuscular hemoglobin concentration.

Micronucleus and nuclear abnormalities

The data indicate that the rate of nuclear morphological changes observed in Ambude River fish were lower compared with the incidence of micronuclei in the same region (Fig. 2). There was a low incidence of micronuclei (MN) and an absence of nuclear morphological changes (NMC) in fish from Serena Lagoon (Fig. 2). The frequency of micronuclei was significantly high in the dry season (Fig. 2).

Fig. 2 Frequency of micronuclei and nuclear morphological changes in *C. macropomum* from Serena Lagoon and Ambude River locations in Maracanã Protected Area, Maranhão-Brazil, during the sampling period. Stain: Giemsa

We find different MN percentages in the same fish, depending on the stain. A small number of MN (10%) and NMC (5%) were found in the erythrocytes of *C. macropomum* collected from Ambude River (fig. 3 and 4) stained with Giemsa. Both MN and NMC were present in the erythrocytes of fish at this location, during each collection period. A larger range of MN (75%) and NMC (50%) were identified in erythrocytes stained with acridine orange including: micronuclei, notched nuclei, binucleate nuclei and vacuolated nuclei (Fig. 5).

Fig. 3 Micrograph of erythrocytes of *C. macropomum* collected at Serena Lagoon, in Maracanã Protected Area, Maranhão-Brazil: a) detail of erythrocytes showing normal cells (arrow), b) detail showing the erythrocyte micronucleus (arrow). Giemsa stain, × 1000

Fig. 4 Micrograph of erythrocytes of *C. macropomum* collected at Ambude River, Maracanã Protected Area, Maranhão-Brazil: a) detail of erythrocytes showing normal cells (arrow), b) detail of erythrocytes showing a binucleate nucleus (arrow). Giemsa stain, × 1000

Fig. 5 Micrograph of erythrocytes of *C. macropomum* collected at Serena Lagoon and Ambude River, Maracanã Protected Area, Maranhão-Brazil: A) Detail of normal erythrocytes (monochrome) and micronucleus (arrow) – Serena Lagoon, B) notched nucleus (arrow) – Ambude River, C) vacuolated

nucleus – Ambude River and D) binucleate nucleus (arrow) - Ambude River. Acridine Orange stain, × 1000

Discussion

Our data presented here confirms *Colossoma macropomum* as a potential model, and the use of hematological and genotoxic biomarkers as an effective tool for environmental impact assessment in the study area. Several studies have demonstrated the potential use of micronucleus as biomarkers and fish as sentinel organisms (e.g. Ferraro et al. 2004; Andrade et al. 2004; Souza and Fontanetti 2006). Additionally, some studies on genotoxicity indicate that tambaqui (*Colossoma macropomum*) has potential as an experimental model for monitoring aquatic ecosystems (Chapadense et al. 2009; Groff et al. 2010; Rocha et al. 2011).

Counts of micronuclei and nuclear morphological changes are effective tools for analyzing the health status of fish (Webb and Gagnon 2002; Noga 2000). The tambaquis from Ambude River showed a range of nuclear morphological changes (NMC) and a higher frequency of micronuclei (MN) compared with fish from Serena Lagoon. The increased frequency of micronucleated cells is a marker of genotoxic effects and may reflect exposure to agents with clastogenic or aneugenic modes of action (Bombail et al. 2001). Micronuclei can develop by nondisjunction as a result of exposure to a "spindle toxin" or may be induced by toxic chemicals (Heddle 1973; Webb and Gagnon 2002). The end result is the production of small cell bodies that resemble micronuclei (Heddle 1973; Carrasco et al. 1990). Although the origins of nuclear morphological changes have not been identified, several studies support the hypothesis that these are due to genotoxic events (Ferraro et al. 2004). The number and type of nuclear morphological alterations should be considered as complementary to records of other alterations resulting from cytogenotoxic agents (Ayllón and Garcia-Vazquez 2001; Gravato and Santos 2002).

The frequency of occurrence of micronuclei and nuclear changes may be related to changes in seasons (Andrade et al. 2004; Guilherme et al. 2008). According to Matsumoto et al. (2006), the mutagenic effect in genotoxicity studies was registered in greater quantity during the dry period. This happens due to low water flow, which results in higher concentrations of organic compounds, metals, and industrial effluents. This relationship was observed for the Ambude River, where the largest variation in the

environmental parameters measured and the increased frequency of micronuclei and nuclei morphologically altered was higher in the dry period.

It is important to highlight in this study that the micronucleus test using Acridine Orange was more sensitive than the tests that used Giemsa. Acridine Orange stain has been related as the most sensitive method for analysis of MN and NMC, and conventional Giemsa staining can lead to false positives as a result of artifacts. As Acridine Orange dye is selective for nucleic acids (Polard et al. 2011; Seriane et al. 2012), this reagent is particularly useful for viewing MN in organisms that have small chromosomes (Udroi 2009).

The decrease in hematocrit values and the fish size may be considered a stress response caused by poor water quality (Oba et al. 2009). Lower hematocrit values associated with genotoxic effects may be associated with damage caused by hydrocarbons, metals, industrial effluents, agricultural runoff, municipal waste water, pH, water temperature and the level of damaged cells (Stahl 1991; Sousa and Fontanetti 2012).

The map presented in Fig. 1 shows that the Maracanã area is close to the harbor of an industrial area (metallurgy). Some portions of the Ambude River are close to industrial areas, such as roads with high traffic. Considering the relief of the region, the pollutants from cited pollution sources can be moved by wind and rainwater into the right tributary of the Ambude River. A study conducted by Carvalho-Neta (2010) found that in addition to experiencing the impacts of the metallurgy industry, the Maracanã area is undergoing a long process of urbanization, with housing construction and recreational activities occurring in the vicinity of rivers. A few decades ago, there were several tannery (animal skin processing) companies that discarded waste directly into regional rivers (Carvalho-Neta 2010). In addition, other studies conducted by Carvalho-Neta et al. (2012) in the port region, detected the presence of the heavy metals in an area that was directly influenced by companies located near Maracanã Protected Area. It is important to note that all values of metal and inorganic compounds were found above the limits allowed by resolution 357 of the Brazilian Environmental Council (Conama 2005). However we can not say that contaminant levels measured by Brazilian law is safe. It would be necessary to carry out ecotoxicological tests for each type of metal and establish maximum limits for tambaqui.

This data likely explains why fish from Ambude River present strong biological responses related to the influence of environmental stressors: the hematocrit values were below the average considered

normal for fish (Tavares-Dias et al. 2003; Lazzari et al. 2011), the fish present high frequency of micronuclei and nuclear changes and are smaller than fish from Serena Lagoon. Further studies focusing on hematological characteristics of tambaqui under different environmental conditions are needed to establish background values for this species within the region. This will contribute to biomonitoring programs in the state of Maranhão, located in Northeastern Brazil.

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Conflict of Interest

The authors declare that they have no competing interests.

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FIGURES LEGEND

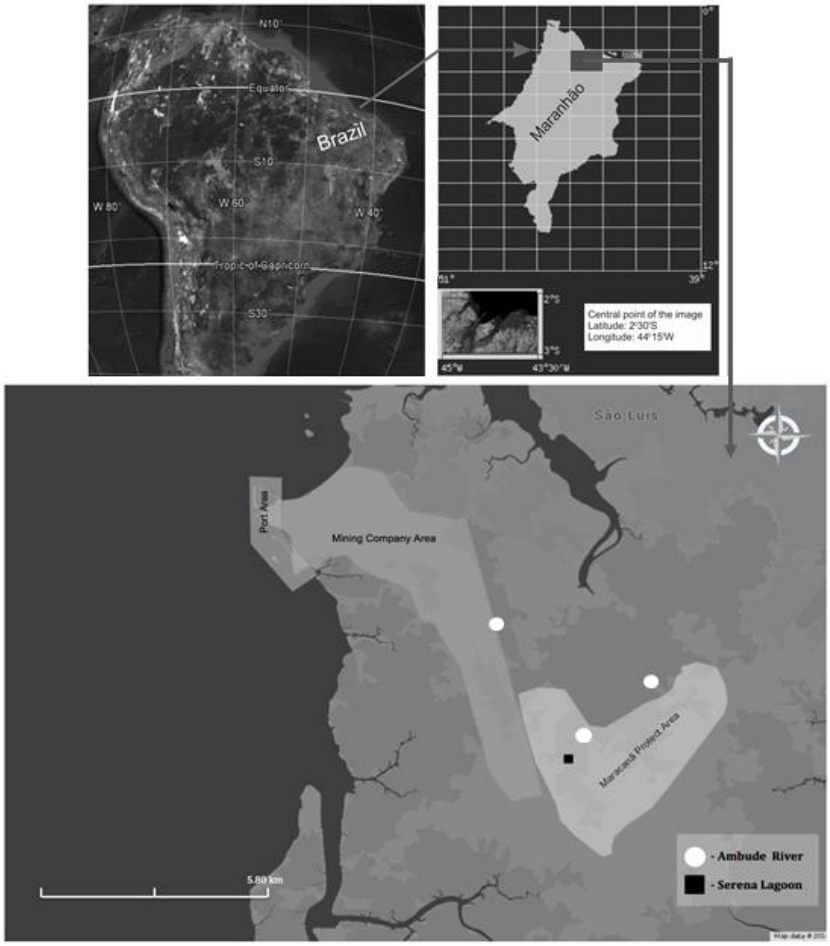

Fig. 1 Map showing Maracanã Protected Area, São Luís, Maranhão, Brazil

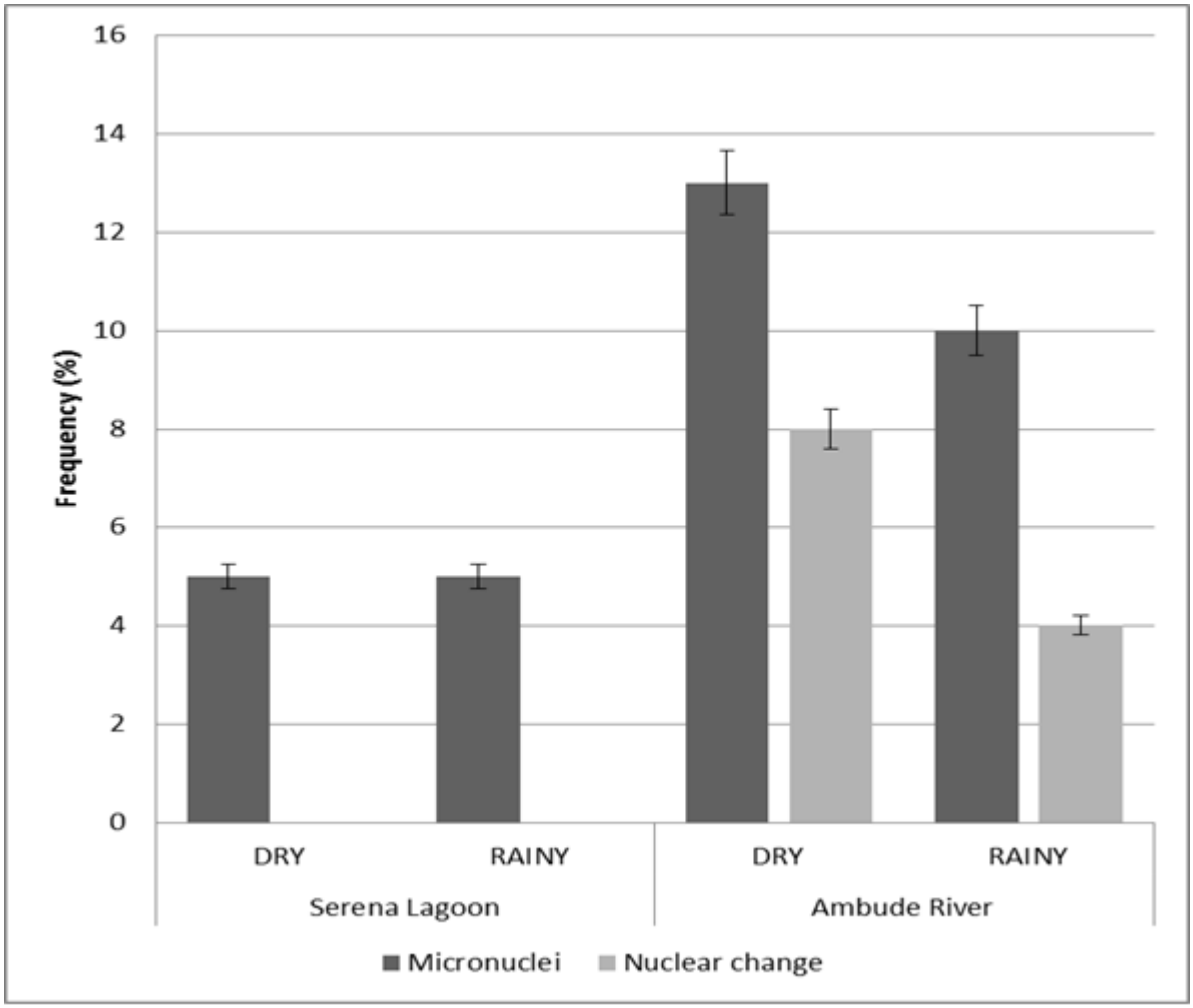

Fig. 2 Frequency of micronuclei and nuclear morphological changes in *C. macropomum* from Serena Lagoon and Ambude River located in Maracanã Protected Area, Maranhão-Brazil, during the sampling period. Stain: Giemsa

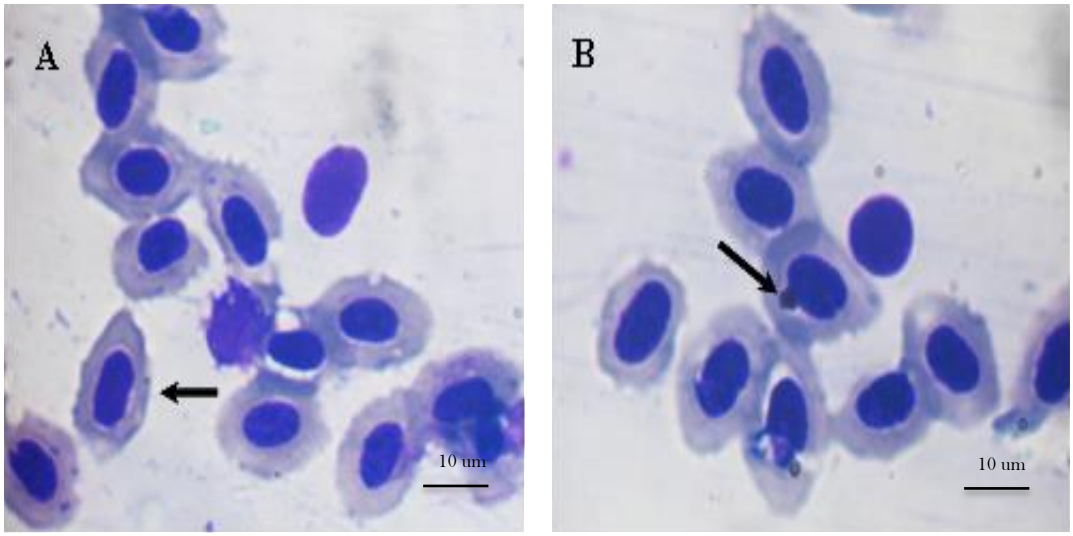

Fig. 3 Micrograph of erythrocytes of *C. macropomum* collected at Serena Lagoon, in Maracanã Protected Area, Maranhão-Brazil: a) detail of erythrocytes showing normal cells (arrow), b) detail showing the erythrocyte micronucleus (arrow). Giemsa stain, × 1000

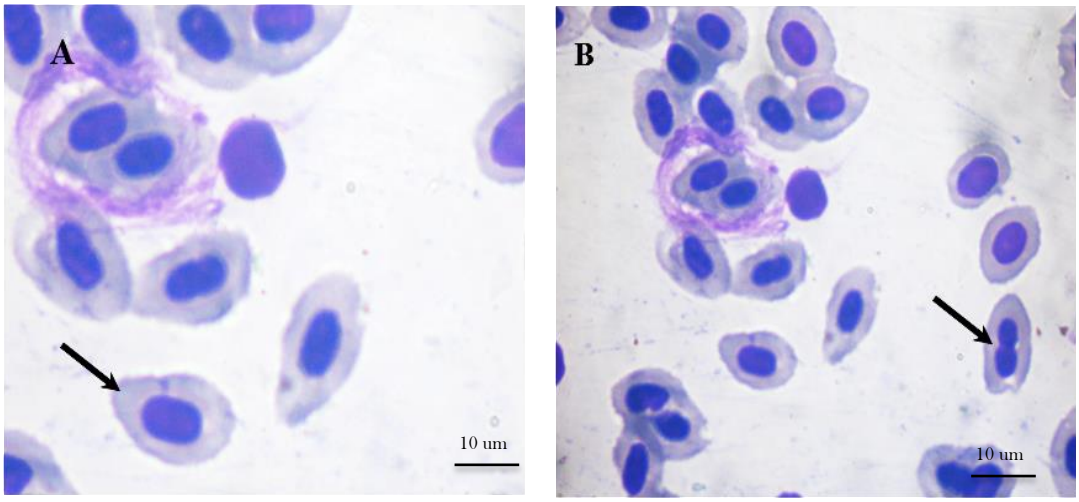

Fig. 4 Micrograph of erythrocytes of *C. macropomum* collected at Ambude River, Maracanã Protected Area, Maranhão-Brazil: a) detail of erythrocytes showing normal cells (arrow), b) detail of erythrocytes showing a binucleate nucleus (arrow). Giemsa stain, × 1000

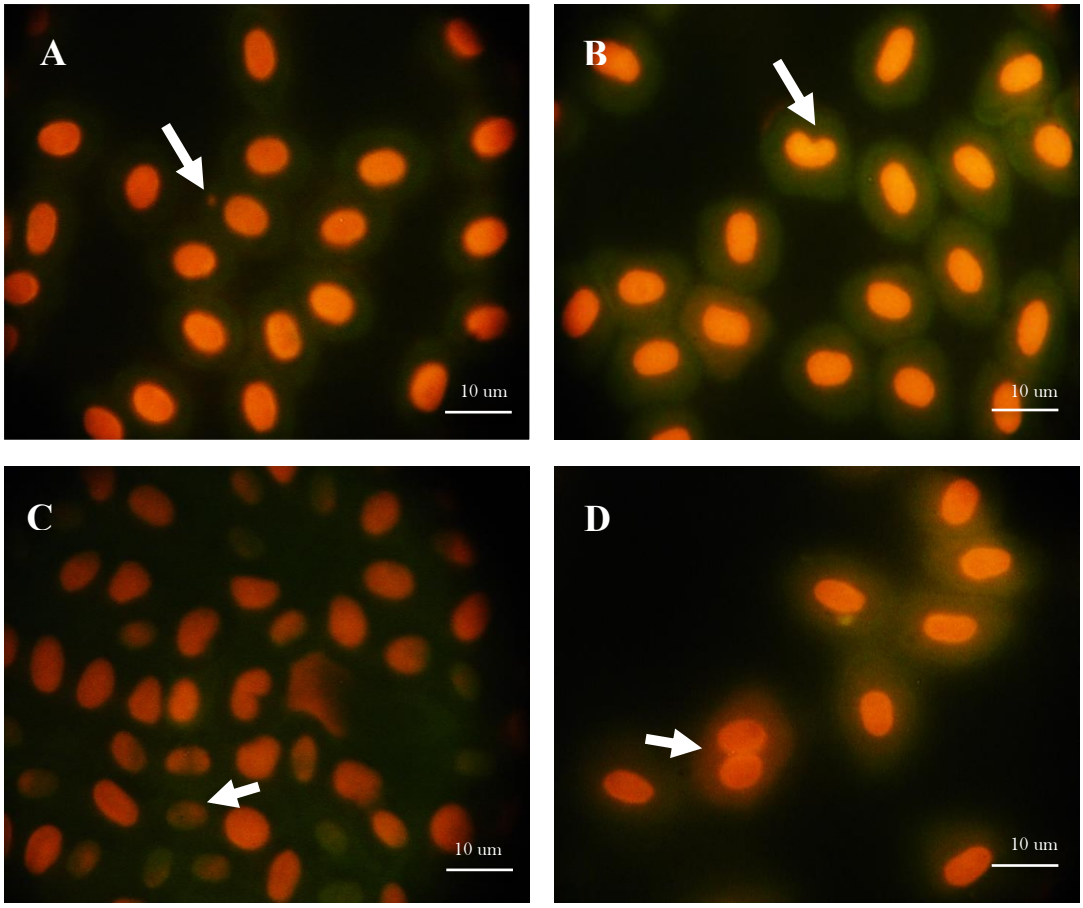

Fig. 5 Micrograph of erythrocytes of *C. macropomum* collected at Serena Lagoon and Ambude River, Maracanã Protected Area, Maranhão-Brazil: a) Detail of normal erythrocytes (monochrome) and micronucleus (arrow) – Serena Lagoon, b) notched nucleus (arrow) – Ambude River, c) vacuolated nucleus – Ambude River and d) binucleate nucleus (arrow) - Ambude River. Acridine Orange stain, × 10

5.2 Building a predictive model to evaluate environmental impacts on Maracanã Protected Area in the northeast of Brazil, based on tambaqui species (*Colossoma macropomum*) as exposure biomarker [2]*

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Abstract

The present work describes a predictive model based on exposure biomarkers, using tambaqui species to assess environmental impacts on Maracanã Protected Area in northeast of Brasil. Fish were collected from two sites along the protected area, Serena Lagoon (A1) and Ambude River (A2), in six periods throughout the course of dry and rainy seasons. Water samples were also collected for physic-chemistry parameters. Blood samples of all fishes were examined for micronuclei changes after stained with Acridine Orange. Gills of each fish were dehydrated in a progressive series of ethanol dilutions and embedded in paraffin. Sections were stained with hematoxylin and counterstained with alcoholic eosin for structural analysis of gills. Histopathological lesions score were classified according to an attributed factor of importance (w) ranging from 1 to 3. The building of the predictive model for evaluate the environmental impact was expressed by an adjustment of a measured surface data three dimensionally distributed which was accomplished comprising micronucleus, erythrocyte

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abnormalities and necrosis, for A2 in dry and rainy seasons. Necrosis features the highest degree of histopathologic severity in fish and it was verified by micoscopic examination. In our proposed model it is possible to predict the probability of the occurrence of necrosis by simply withdrawing blood from the fish without sacrifice the animal. Our findings suggest that building a predictive model based on biomarkers exposure can be of great value in future monitoring programs of environment protected areas.

Keywords: environmental protection , *Colossoma, macropomum,* tambaqui, micronuclei, necrosis,

Introduction

Various studies has been reported about the development of mathematical models to evaluate the severity of histopathological lesions in gills, liver and kidneys of fish (Schwaiger et al. 1997; Bernet et al. 1999). None of them however takes into consideration the individual severity of each lesion and important biometric parameters as total length, total weight, sex, stage of gonadal maturation besides the seasonality. Furthermore, there are no mathematical models for tambaqui species, which increases the possibility of further studies based on biological data of this species, providing clues for new researchs in aquatic ecotoxicology and applied mathematics in such route.

Mathematical models for biological data are being widely used for the correlation of biochemical and histopathological biomarkers and supporting a more realistic analysis of the stress caused by contaminants in the Sao Luis port complex (Carvalho-Neta et al. 2014). These models can be used as a promising tool for the interpretation of environmental disasters and provide the creation of new databases using the tambaqui as a biomonitor for future management studies at the Maracana Protected Area.

The selected species*, Colossoma macropomum* (Curvier, 1818) is native from Amazon River basin, and lives both in natural environments as well as in fish farming, at the Maracanã Protected Area (Goulding and Carvalho 1982; Groff 2010). Nevertheless, there are strong features that makes this species a suitable biomarker for biological models: a) generally tambaqui reaches 30 kg, which allows sufficient tissue collection for various types of analysis; b) the species is easy to cultivation, which facilitates its use in control groups; c) as tambaqui is the most widely cultivated species in the region, there is a natural demand for studies about its health status in environment, and d) there are already

other studies using biological parameters (genotoxic and morphological data) as biomarkers for tambaqui, indicating that the species could be promising for monitoring purposes.

Relevant laboratory studies utilized biomarkers in tambaqui and chemistry analyses provided more detailed data about the effects of pollutants from industrial district of São Luís/Brazil. Studies conducted by Kim et al. (2001) emphasized that histopathological changes in fish tissues have been regarded as important biomarkers of exposure to toxic substances, which reflect changes in biochemical functions. In genotoxic pollution of freshwater, toxicants are mostly introduced into the water bodies through anthropogenic actions such as industrial, agricultural, domestic, and urban activities (Obiakor et al. 2012). Thus, papers reports that micronuclei test can be applicable to monitoring studies of freshwater (Baršienė et al. 2015; Hayashi et al. 1998) and that health of the biota depends on its contact with hazardous chemicals capable of damaging the DNA and perpetuating the irreversible effects evidenced by micronuclei formations (Obiakor et al. 2012).

In addition, the creation of a predictive model using biological parameters can be taken into account when designing biomonitoring programs (Carvalho-Neta et al. 2014). In this sense, we aim in this study to build a predictive model for environmental impacts using exposure biomarkers (micronucleus, erythrocyte abnormalities and necrosis).

Method and Materials

Sampling Sites

Maracanã Protected Area is an important protected area of sustainable use on the Northeast of Brazil (São Luís-MA). Two sampling sites were chosen (Fig. 1), a potentially contaminated area, Ambude River, A2, located close to the industrial district of São Luís, and a reference area, Serena Lagoon, A1, located at an upper part of the reserve.

Fig. 1 Map showing the location of sampling sites in Maracanã Protected Area, São Luís/Brazil. A1 Serena Lagoon, A2 Ambude River.

License and Ethics Declaration

The capture of fish was authorized by the collection permit number SEMA (001/2014-2015), issued by the State Department of Natural Resources and Environment. The protocol was approved by the Ethics Committee of Maranhão State University under number 04/2014-2015 CRMV-MA which meets the guidelines of the Brazilian College for Animal Experimentation (SBCAL/COBEA, 2015).

Sampling of tambaqui and gonadal stage

Tambaqui specimens were collected from two locations within Maracanã Protected Area: Serena Lagoon –A1 (50 specimens) and Ambude River – A2 (50 specimens) (fig. 1). Sampling were grouped throughout three years (2012, 2013 and 2014) during the dry and wet season. Fish were collected with a gill net and transferred to a plastic container (1 m × 1 m). Tambaquis were anesthetized for 15 minutes with hydroalcoholic solution of benzocaine 5%. The macroscopic classification of the gonadal stage (GS) was also undertaken: immature (GS1), early maturing or at rest (GS2), mature (GS3), following the scale by Vazzoler 1996 and modified by Carvalho-Neta and Castro 2008.

Physical-chemistry parameters

Physic-chemistry parameters, temperature, pH and dissolved oxygen, from each site, of dry and rainy seasons, were analyzed in the meter multiparameter HI 9829 – Hanna. Water samples were collected from the Serena Lagoon (A1) and the Ambude River (A2) for quantification of contaminant levels. Samples were analyzed for a suite of chemicals: metals include copper, zinc, lead, iron, nickel, manganese, cadmium, mercury, manganese, molybdenum, cobalt, calcium, potassium and phosphorus. The concentrations of metals in water were determined using Inductively Coupled Plasma Emission Spectrometry, ICP- Varian 720-ES (Tyler, 1991).

Micronuclei test

Blood samples of tambaqui species were obtained by caudal vein puncture using a heparinized syringe. A drop of blood from each sample was placed on a microscope slide and smeared. Slides were left to dry at room temperature for 24 hours and then fixed in absolute ethanol for 30 minutes. For each specimen one slide were stained with Acridine Orange, and a total of 100 microscope slides (n = 100) were analyzed using a fluorescence microscope (LEICA DMLP). On each slide, minimum of 2000 cells were analyzed.

Histopathological analisys

Fish were dissected and their gills were immediately fixed in a 10% formalin solution. The gills of each individual were dehydrated in a progressive series of ethanol dilutions and embedded in paraffin. Sections were stained with hematoxylin and counterstained with alcoholic eosin for structural analysis of gills and liver. Four tissue sections from each fish were examined by a Zeiss light photomicroscope. Histopathological lesions score were classified according to an attributed factor of importance (w) ranging from 1 to 3 based on the scale of Bernet et al. 1999.

Mathematical predictive model

The building of the predictive model for evaluate the environmental impact was expressed by an adjustment of a measured surface data, three dimensionally distributed, which was accomplished comprising micronucleus, erythrocyte abnormalities and necrosis, for A2 in dry and rainy seasons, represented by the equation:

$$z = \beta + \alpha 1x + + \alpha 2y\ \alpha 3xy,$$

where z is related to the necrosis number (dependent variable); x refers to erythrocyte abnormalities (independent variables); y refers to micronucleus (independent variables); β is the linear coefficient and α is the angular coefficient.

All curves, plane equation adjustments and the correlation coefficient (R^2) were performed to Rio Ambude site, during the dry and rainy seasons, using the MATLAB software.

Results

Physic-chemistry parameters

Table 1 Physic-chemical data from water samples collected at Lagoon Serena and Ambude River

Parameters	Dry season				Wet season				LOD (mg/L)	LOQ (mg/L)	[a]Recommended values
	A1	A2			A1	A2					
		P_1	P_2	P_3		P_1	P_2	P_3			
Dissolved O_2 mg/L O_2	5.9	3.8	4.1	4.2	6	3.9	4	4.1			>5 mg/ mg/L O_2
pH	4.5	3.5	3.3	3.5	5	3.5	3.6	3.5			6-8
Temperature (°C)	29	28.9	29	28	29.1	28.9	28.8	28			28–32°C
Copper (mg / L)	ND	0.0160	0.0151	0.0150	ND	0.0159	0.0150	0.0155	0.012	0.04	≤0,009 mg/L Cu
Zinc (mg / L)	ND	0.0170	0.0170	0.0169	ND	0.0170	0.0169	0.0169	0.104	0.35	≤0,18 mg/L Zn
Lead (mg / L)	ND	0.0317	0.0315	0.0315	ND	0.0314	0.0310	0.0310	0.04	0.13	≤0,01 mg/L Pb
Iron (mg / L)	ND	2.59	2.55	2.54	ND	2.55	2.54	2.54	0.010	0.03	≤0,3 mg/L Fe
Chrome (mg / L)	ND	0.225	0.225	0.224	ND	0.225	0.224	0.224	0.0013	0.04	≤0,05 mg/ L Cr
Nickel (mg / L)	ND	0.001	0.001	0.001	ND	0.001	ND	ND	0.07	0.006	≤0,025 mg/L Ni
Manganese (mg / L)	ND	0.002	0.002	0.002	ND	0.002	ND	0.002	0.014	0.05	≤0,1 mg/L Mn
Cadmium (mg / L)	ND	0.008	0.008	0.008	ND	0.008	0.008	ND	0.0015	0.005	≤0,001 mg/L Cd
Mercury (mg / L)	ND	0.0223	0.0221	0.0222	ND	0.0255	0.0256	0.0250	0.0002	0.02	0,0002 mg/L Hg
Magnesium (mg / L)	ND	0.695	0.690	0.690	ND	0.690	0.690	0.690	0.009	0.002	Gabriel et al., 2013
Cobalt (mg / L)	ND	0.060	0.061	0.061	ND	0.060	0.061	0.061	0.0006	0.006	≤0,05 mg/L Co
Molybdenum (mg / L)	ND	0.012	0.012	0.011	ND	0.011	0.011	0.011	0.023	0.006	≤0,01 mg/L Mo

[a]All values are average from measurements along 2012, 2013 and 2014 years;
A1 = Serena Lagoon; A2 = Ambude River;
ND, not detected; LOQ = Quantification limits; LOD = Detectation limits.
P_1 - river upstream; P_2 – river middle; P_3 – river donwstream.
[a]Recommended values based on the Resolution Numbers 357 and 430 of Brazil's National Environmental Council, CONAMA 2005 and 2011 respectively; Birceanu et al. (2008); Duarte et al. (2010); Gabriel et al. (2013); Gonzalez et al. (1998); Matsuo et al. (2005); Winter et al. (2012).

Mathematical predictive model

From the proposed model (fig. 2), we can see that observational data distributed in the plan equation show that necrosis can be predicted through the erythrocytic and micronucleus abnormalities. The correlation coefficient (R^2) = 0.78 suggests that variables can be appropriately correlated. Necrosis features the highest degree of histopathologic severity in fish and it was verified by micoscopic examination. The proposed model allows to predict the probability of the occurrence of necrosis by simply withdrawing blood from fishs without sacrifice the animals.

Figure 2 Mathematical model, based on adjustment of measured data to a surface, three dimentially distributted (necrosis, erythrocyte abnormalities and micronuclei) in tambaqui species from the Ambude River, during dry and wet seasons. R^2=0.78; R = 0.89.

Moreover, micronucleus and erythrocyte abnormalities strongly correlates with most serious branchial lesions (necrosis), indicating the irreversibility of the process in fish from Ambude River (A2). Using values for the minimal and moderate branchial lesions, scored by the index based on Bernet et al. (1999), and the occurrence of micronucleus and erythrocyte abnormalities, it was not possible to observe a correlation between the variables.

Dicussion

The present predictive model, based on biomarkers, indicates that the occurrence of necrosis can be predicted by observing the incidence of micronuclei and erythrocyte abnormalities in *C. macropomum*, sampled at a polluted area of Ambude River at Maracanã Protected Area. Factors such as water pollution can be associated to the occurrence of micronucleus, erythrocyte abnormalities and branchial lesions in tambaqui. Concentrations of all measured elements (Cu, Zn, Pb, Fe, Ni, Mg, Cd, Hg, Mn, Mo, Co, Ca, K and P) at A2 site were higher than that acceptable limits defined by the national standards (Conama 2005; Conama 2011) and ecotoxicological assay performed for tambaqui species (Birceanu et al., 2008; Duarte et al., 2010; Gabriel et al., 2013; Gonzalez et al., 1998; Matsuo et al., 2005; Winter et al., 2012).

Water temperature was shown to have a direct effect on the mitotic rate and consequently on the

formation of micronuclei (Barsiene 2006). Furthermore, metal toxicity in fish is expected to be most severe in soft waters because of the low availability of cations (particularly Ca^{2+}) to out-compete the metal forms for binding sites on the gills (Matsuo et al. 2005). Assays carried out in tambaqui showed waterborne Cd toxicity can be the major threat to tambaqui living in soft waters because of the low availability of Ca^{2+} to protect against Cd toxicity.

Copper may be extremely toxic to fish and causes tissue damages in gills (Tavares-Dias et al. 2003). A reduction in chloride, sodium and potassium levels in freshwater affect fishes leading to damage in gill cells by compromising the osmotic regulation (Nussey et al. 1995). Gill lesions are used as sensitive biomarkers to environmental impacts on fish (Stentiford et al. 2003) and histopathological examination has been recognized by many researchers as a valuable tool for assessment of environmental impacts on fish populations (Teh et al. 1997). Those morphologic alterations certainly occur because gills of fishes are in permanent contact with the environment (Heath, 1995). The detection of early warning signals as through branchial lesions, besides ecologically relevant, has economic and quickness advantages, to be used as a biomarker (Pinheiro-Sousa et al. 2013).

Various assays have show that peripheral erythrocytes of fishes have high incidence of micronuclei after exposure to different contaminants under field and laboratory conditions (Cajaraville et al. 2003). Al-Sabti (1994) observed that, mercury and a mixture of others metals induce micronuclei formation under laboratory conditions in the bi-nucleated erythrocytes of *Carassius auratus gibelio* (Prussian carp). Mercury, probably residual from old tannery companies located inside the Maracanã Protected Area, was found to be above the allowed conditions for the Al-Sabti test (1994), indicating that this pollutant affects the health of tambaqui. This element requires more accurate laboratorial studies to establish the maximum value for the induction of micronuclei and other nuclear morphological changes in tambaqui species.

The formation of micronuclei (MN) can result from several processes (Heddle 1973), as some induced by clastogen chemicals, causing structural chromosome changes, which can be morphologically distinguished from those induced by aneugens, which induces chromosome numerical changes, because they are smaller and due to the frequency in which centromeres are present (Cajaraville et al. 2003). It might be due to oxidative damages to DNA, that shears off a chunk of the DNA from chromosomes and once without the centromere, these chunk are left behind and form an extra-nuclear

nuclei, which we term as MN (Al-Sabti and Metcalfe 1995; Heddle 1973).

In another mechanism, it has been proposed that spindle motor protein dysfunction might cause a lagging segregation and prior to karyokinesis, the lagging strand fails to incorporate the nucleic material within the main nucleus, thus forming an extra-nuclear inclusion which we find as micronuclei (Bolognesi 2011). Therefore, changes in chromosome number evidenced by micronucleus formations may affect the gene activity or gene transmission by altering the position, order, or number of certain genes in a cell and genome of an organism (Okonkwo et al. 2011). Such changes often, but not always, can cause a genetic imbalance which is harmful to the body and/or for future generations (Hartwell et al. 2000) or that micronuclear content can be degraded without inducing an immediate cell cycle arrest or causing the cell to enter apoptosis (Terradas et al. 2010).

Our model showed that the occurrence of necrosis can be predicted through a correlation between micronucleus and erythrocyte abnormalities. It is noteworthy that highly correlated variables do not necessarily present any cause and effect relationship (Helsel and Hirsch, 2002). The correlation is simply the tendency of variables present their combined variation (Helsel and Hirsch, 2002). Therefore, we believe that the same external environmental factor could influence the occurrence of severe changes, necrosis, micronucleus and erythrocyte abnormalities.

Spontaneous miconuclei frequencies in erythrocytes (micornuclei/1000 cells) from different fish species, under laboratory condition and in the field, have shown that each species boasts its baseline. For *Oreochromis niloticus* the lowest baseline varied 0.006 (Hoshina et al. 2008). We believe that the basal level of miconuclei frequencies in erythrocytes for tambaqui is low (10/2000 cells = 0.005), because from this value we have already observed a positive correlation between the occurrence of micronucleus and branchial lesions. Laboratory assays to establish baselines of micronuclei at random and those induced by some toxic agent should be carried out to establish the background level for disease and blood profile of tambaqui species.

Concluding, we can state that our findings suggest that the predictive model we developed could be used as a suitable methodology in futures bio-monitoring programs in Protected Areas. We can emphasize that this methodology could predict physiological and pathological condition values for tambaqui, following very fast and low cost protocol, within an expressive advantage of avoid any

animal damage.

Acknowledgements

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Conflict of Interest

The authors declare they have no competing or any other conflict of interest.

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FIGURES AND LEGEND

POINTS	COORDINATES	
	S	W
AMBUDE RIVER BORN	2° 38.225'	44° 17.887'
MIDLE OF RIVER	2° 37.553'	44° 17.620
DOWNSTREAM OF AMBUDE RIVER	2° 37.750'	44° 17.748′
SERENE LAGON	2° 37.955'	44° 17.874'

Fig. 1 Map showing the location of the sampling site in Maracanã Protected Area, São Luís, Maranhão, Brazil. A1 Serena Lagoon, A2 Ambude River.

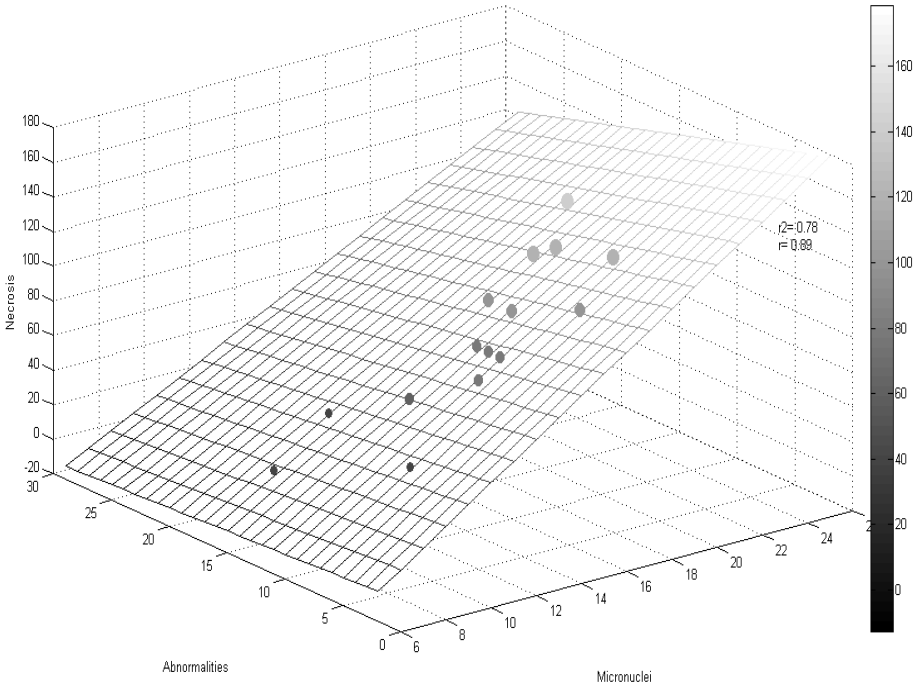

Figure 2 Mathematical model based on a adjustment of measured data to a surface who are distributed in the space in three dimensions (necrosis, abnormalities and micronuclei) in tambaqui species from the Ambude River, during dry and wet seasons. R^2=0.78; R = 0.89.

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Cite references in the text by name and year in parentheses. Some examples:

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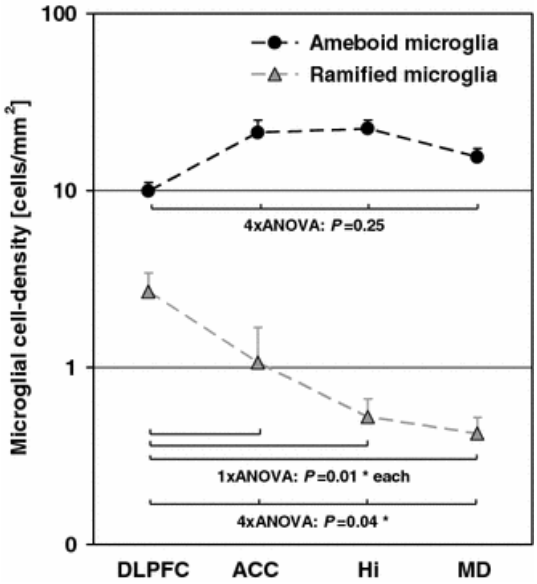

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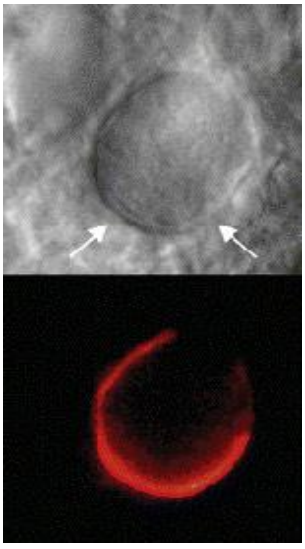

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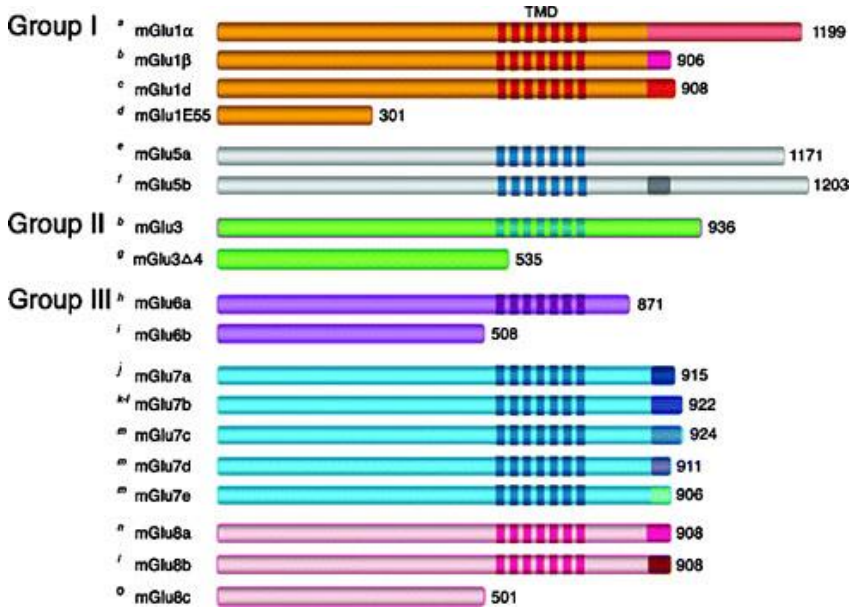

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CONCLUSÕES

Os dados obtidos no presente trabalho para a construção do modelo preditivo baseado em biomarcadores em peixes da Área de Proteção Ambiental do Maracanã, permitiu concluir que:

- O tambaqui (*C. Macropomum*) comportou-se como uma potencial espécie para avaliação da qualidade ambiental utilizado biomarcadores de exposição para a APA do Maracanã;

- No rio Ambude, foram encontrados uma maior frequência de micronúcleo, alterações eritrocíticas e lesões brânquias, sugerindo que os peixes amostrados no período encontravam-se com uma saúde comprometida, provavelmente, em função dos poluentes encontrados na análise química;

- As metodologias de análises de micronúcleos e anormalidades eritrocíticas com os corantes Giemsa e Laranja de Acridina mostraram respostas diferenciadas, sendo que o laranja de acridina foi mais sensível para identificar danos genotóxicos em tambaqui;

- Nosso modelo preditivo é capaz de prever a probabilidade da presença de necrose (histopatologia) a partir do conhecimento do número de micronúcleos e anormalidades eritrocíticas, sem a necessidade de eutanásia do animal.

Estudos futuros sobre características hematológicas de *C. macropomum*, principalmente de outras espécies de condições de cultivo, são necessários para estabelecer valores basais provendo, assim, padrões para investigações fisiológicas ou patológicas. Além disso, ensaios laboratoriais expondo o tambaqui a diferentes concentrações metais, tonará possível ajustar esse modelo preditivo em relação ao tempo e identificar valores mensuráveis de dose-respota para a espécie.

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